Hepatocyte Nuclear Factor 4α Regulates Expression of the Mouse Female-Specific Cyp3a41 Gene in the Liver

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ABSTRACT:

CYP3A41 is a female-specific cytochrome P450 in mouse liver. A putative hepatocyte nuclear factor 4α (HNF4α)-binding site was found at −99/−87 in the promoter of Cyp3a41 by reporter assays performed in the hepatocytes of female mice. Cotransfection of an HNF4α expression plasmid significantly increased transcription of the reporter gene. Although electrophoretic mobility shift assays with liver nuclear extracts did not show a sex-related difference, chromatin immunoprecipitation (ChIP) assays showed that larger amounts of HNF4α bound to Cyp3a41 in female than in male mice. A relation between the amount of HNF4α on the Cyp3a41 gene and mRNA expression was observed in hepatic tissue sets, which differ in mRNA expression depending on the sex, age, or endocrine status of mice. The degree of histone-3-lysine-4 dimethylation and histone-3-lysine-27 trimethylation around the HNF4α-binding site was higher in females and males, respectively. Moreover, the ChIP assay indicated greater acetylation of histone-4-lysine-8 of the Cyp3a41 chromatin in females than in males. HNF4α plays an important role in the transcriptional activation of the Cyp3a41 gene, and a sex difference in chromatin structure may contribute to the female-specific expression of Cyp3a41 in the livers of mice.

Introduction

Cytochrome P450s (P450s) are heme-containing enzymes responsible for the oxidative metabolism of various endogenous steroids, bile acids, hormones, and fatty acids, as well as foreign compounds, including environmental chemicals and a large number of active drugs (Guengerich, 1991). The CYP3A subfamily represents the most abundant forms of P450 in the adult human liver, constituting approximately 30% of the total P450 content. This subfamily comprises CYP3A4, CYP3A5, CYP3A7, and CYP3A43. CYP3A4 is the most prevalent and important isoform in adults, accounting for 95% of the CYP3A mRNA pool in the liver (Koch et al., 2002) and is involved in the metabolism of approximately half of all currently marketed drugs that undergo oxidative biotransformation (Williams et al., 2004).

Sex differences in drug metabolism are well established for some human P450 isoforms. Recent studies have suggested that CYP2B6, CYP2A6, and CYP3A have greater levels of activity in women than in men, and CYP2E1 and CYP1A2 have slightly higher activity in men, although significant levels of activity/protein/mRNA expression of these isoforms are detectable in both sexes. (Harris et al., 1993; Lamba et al., 2003; Anderson, 2005; Cotreau et al., 2005; Nakajima et al., 2006; Scandlyn et al., 2008). In contrast with those of humans, the expressions of many isoforms of P450s in rodents are markedly different between male and female animals (Kato and Yamazoe, 1993); for example, rat CYP2C12 (MacGeoch et al., 1984; Kamataki et al., 1985) and CYP3A9 (Kawai et al., 2000) and mouse Cyp3a11 and Cyp3a44 (Sakuma et al., 2002) all show female specificity in the adult liver. Sexually dimorphic plasma profiles of growth hormone (GH) have been reported to contribute to the sex-dependent regulation of P450 enzymes (MacGeoch et al., 1984). The intracellular signaling networks that establish and maintain the sex-dependent patterns of liver gene expression are probably complex and may involve the integrated actions of an array of liver transcription factors. These transcription factors are termed hepatocyte-enriched nuclear factors and include HNF1α, HNF3, HNF4α, HNF6, and several CCAT/enhancer binding proteins. The expression of most hepatocyte-enriched nuclear factors is regulated by GH (Wiwi and Waxman, 2004) and contributes to hepatic Cyp expression (Akiyama and Gonzalez, 2003).

HNF4α (NR2A1), a highly conserved hepatocyte-enriched nuclear factor, is required for the hepatic expression of several genes showing sex-specific expression in the liver, notably genes of the Cyp superfamily (Wiwi and Waxman, 2004). Male HNF4α knockout mice showed decreased expression of several male-specific Cyp genes and increased expression of some female-specific Cyp genes. In contrast, HNF4α was disclosed to play a dominant, positive role in the regulation of female-specific liver Cyp genes, with the down-regulation of these genes including Cyp3a41 in female HNF4α knockout mice (Wiwi et al., 2004; Holloway et al., 2006).

With respect to the female-specific mouse Cyp3a41 gene, we reported that the sex-specific pattern of GH secretion is a critical determinant of sexually dimorphic expression (Sakuma et al., 2002) and the expression of Cyp3a41 is under the cooperative control of GH.
and glucocorticoid hormone (Sakuma et al., 2004, 2008). Furthermore, in preliminary experiments with a series of reporter constructs containing deletions of the 5'-flanking region of the Cyp3a41 gene, we had found several enhancer regions, two of which included putative HNF4α-binding sites. Nonetheless, although the nuclear factor HNF4α was suggested to participate in the regulation of female-specific expression of Cyp3a41, the precise mechanism by which HNF4α regulates the sexually dimorphic expression of the gene had not been elucidated. This observation prompted us to investigate the role of HNF4α in the regulation of female-specific Cyp3a41 expression.

The present study suggests that sex differences in the chromatin structure including the modification of histones of the Cyp3a41 gene contribute to the sex specificity of Cyp3a41 expression by controlling access of the liver-specific transcription factors including HNF4α to the DNA.

Materials and Methods

Materials. Materials for the isolation and culturing of hepatocytes were purchased from Wako Pure Chemicals (Osaka, Japan), Invitrogen (Carlsbad, CA), and Sigma-Aldrich (St. Louis, MO). Percoll was obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK). The Takara RNa PCR Kit (AMV), version 3.0, was obtained from TAKARA Shuzo (Kyoto, Japan). The TaqMan MGB Gene Expression Detection Kit was a product of Applied Biosystems (Foster City, CA). TransIT-D1 Transfection Reagent was from New England Biolabs (Ipswich, MA). Trans IT-EE hydrodynamic delivery reagent was supplied by Japan SLC (Shizuoka, Japan). Japan SLC also supplied hypophosphite (Sigma-Aldrich) and were of the highest grade available. The Reticulocyte Lysate System was from Promega (Madison, WI). The antibodies against dimethyl-histone H3 (Lys4) (H3K4me2) and trimethyl-H3 (Lys7) (H3K4me3) were obtained from Millipore Corporation (Billerica, MA). [γ-32P]ATP was purchased from BM Bioproductions (Irvine, CA). All primers and oligonucleotides were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). Other chemicals were purchased from Wako Pure Chemicals or Sigma-Aldrich and were of the highest grade commercially available.

Animals. ddY mice (6 weeks old) of both sexes, weighing 25 to 30 g, were supplied by Japan SLC (Shizuoka, Japan). Japan SLC also supplied hypophosytoscerin or sham-operated 6-week-old female ddY mice. At 7 weeks of age, mice received an osmotic infusion (1.5 µg/kg) of recombinant human GH for 7 days. The osmotic minipump (Alzet 1007), implanted into the back of the mice, was designed to release its contents at a rate of 0.5 µl/h. Verification of the surgery was confirmed by a lack of weight gain and the absence of pituitary tumors. Luciferase activity was generated by replacing the DNA fragment between the HindIII and XbaI sites containing the coding sequence of Renilla luciferase of pRL-SV40 (Promega) with a 1425-bp cDNA fragment including the entire coding region of mouse HNF4α (pMNH4-4 (accession no. D29015) (Hata et al., 1995). The DNA sequences of all plasmids were determined using a dye terminator cycle sequencing FS Ready Reaction Cycle Kit with an ABI 310 genetic analyzer (Applied Biosystems).

Preparation of Primary Hepatocyte Culture. The livers of ddY mice were perfused with collagenase-containing Hanks’ solution as described previously (Nemoto and Sakurai, 1995). The cells were dispersed in Waymouth MB 752/1 medium containing bovine serum albumin (2 g/l), transferrin (0.5 mg/l), and selenium (0.5 µg/l) and seeded in dishes at a density of 5 × 10^6 cells/ml/35-mm dish. The Waymouth medium did not contain phenol red, a pH indicator, to exclude estrogen-like action. The culture dishes were maintained at 37°C in a CO2-humidified incubator. The medium was renewed 24 h after seeding.

Transfection of the Reporter Construct and HNF4α Expression Plasmid into Hepatocytes in Culture. Mouse hepatocytes were cultured for 24 h in Waymouth medium and then transfected using Transpass D1 Transfection Reagent (New England Biosciences). The transfection mixture consisted of Waymouth medium, the reporter construct, pRL-SV40, empty plasmid, or the HNF4α expression plasmid if required and Transpass D1 at 0.6 ml, 1.8 µg, 0.3 µg, 5.0 µg, and 1.5 µl, respectively. Transfection continued for 3 h, and then the medium was changed. After a further 48 h of incubation, a luciferase assay was performed. For the analysis of the effects of GH treatment on CYP3A41 mRNA expression in hepatocytes, GH was added 3 h after transfection of the HNF4α expression plasmid at a final concentration of 71 ng/ml, and the cells were cultured a further 24 h. The cells were harvested and total RNA was prepared.

Hydrodynamic Infusion of the Reporter Construct. Six-week-old ddY mice were given a rapid (5-s) tail vein injection of the pGL3-basic vector (10 µg), Cyp3a41 reporter construct (−163/+61-HNF4α mut-Luc, 10 µg), or Cyp3a41/HNF4α mutated reporter construct (−163/+61-HNF4α mut-Luc, 10 µg) and pRL-SV40 vector (1.5 µg), an internal standard, dissolved in transfection reagent in a volume equal to 10% of body weight. After 24 h, the mice were sacrificed, and the livers were homogenized in a 5-fold volume of the lysis buffer (0.1 M Tris-HCl, 2 mM EDTA, and 0.1% Triton X-100). The homogenates were then centrifuged (15,000 g, 4°C, 10 min). An aliquot of the supernatant was diluted 60 fold with 1 M HEPES (pH 7.5), and luciferase activity was determined.

 Luciferase Assay. The luciferase assay was performed with the dual-luciferase reporter assay system (Promega) as recommended, and luminescence was determined with a TD-20/20 luminometer (Promega). Values of firefly luciferase activity were normalized to those of Renilla luciferase activity in individual experiments.

Western Blot Analysis. Amounts of HNF4α protein in nuclear extracts was performed from the livers of male and female mice were examined by Western blotting. The same procedures were also analyzed by EMSA. Nuclear extracts were prepared from the livers of male and female ddY mice as reported (Gorski et al., 1986). Then, 20 µg of protein from the nuclear extracts was separated on an 8% SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and incubated for 1 h with a polyclonal rabbit antibody against mouse HNF4α at a dilution 1:1000. After incubation with a biotinyl-
ated anti-rabbit IgG secondary antibody and a streptavidin-biotinylated horse-radish peroxidase complex, bands were detected by chemiluminescence (ECL Plus; GE Healthcare) and visualized using LAS-1000 plus (Fujifilm, Tokyo, Japan).

**Electrophoretic Mobility Shift Assay.** In vitro transcribed/translated mouse HNF4α protein was synthesized using the TNT T7 Coupled Reticulocyte Lysate System (Promega) or the nuclear extract prepared from liver of male and female ddY mice according to a method reported previously (Gorski et al., 1986) was used. The control lysate for in vitro-transcribed/translated mouse HNF4α protein was prepared using nuclear-free water as a template. Double-stranded oligonucleotides were labeled with [γ-32P] ATP using T4 polynucleotide kinase and purified with MicroSpin G-25 Columns (GE Healthcare). The binding reaction was performed in a 20-μl volume containing 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 2.5 mM dithiothreitol, 2.5 mM EDTA, 5 mM MgCl2, 20% glycerol, 0.25 mg/ml poly(dI-dC), and 5 μl of synthesized HNF4α or 5 μl of the nuclear extract prepared from mouse liver. Reaction mixtures were preincubated at 25°C for 10 min before the addition of the [γ-32P] ATP-labeled probe. A 50- or 100-fold excess of unlabeled oligonucleotide-containing reaction mixture was also preincubated before addition of the radiolabeled probe. Samples were kept at 25°C for an additional 20 min. In supershift experiments, 2 μg of the anti-HNF4α polyclonal antibody was added to the binding reaction mixture at 25°C before addition of the probe, and then all samples were separated on a 4% polyacrylamide gel in 0.5 M Tris borate-EDTA buffer at 200 V for 90 min. The gel was dried and exposed to an imaging plate to detect DNA-protein complexes with a Bio-imaging analyzer 5000 (Fujifilm, Tokyo, Japan).

**ChIP Assay.** The ChIP assay was performed using a kit purchased from Millipore Corporation according to the manufacturer’s protocol with some modifications. In brief, frozen liver tissues were fixed with 1% formaldehyde in phosphate-buffered saline at room temperature for 15 min and quenched with 0.125 M glycine for another 5 min. Liver tissues were washed twice with ice-cold phosphate-buffered saline and then homogenized by a Dounce homogenizer. After centrifugation, the cell pellets were resuspended in cell lysis buffer supplemented in the kit and incubated for 20 min, and the lysates were sonicated to obtain DNA fragments 600 bp in average length. Samples were then treated as indicated by the manufacturer’s protocol. The amount of antibodies used in the experiment was 3 μg each. After reversion of DNA protein cross-linking by incubation with NaCl, the remaining proteins were digested by addition of proteinase K (final concentration, 36 μg/ml) and incubation at 45°C for 2 h. The DNA was recovered using the Wizard SV Gel and PCR Clean-Up System (Promega), and 2 μl of product was used as a template for quantitative PCR. The primers for amplification of the Cyp3a41 gene were 3’-GCGTACATGCAGCTGACTGG-5’ (sense) and 5’-AAGGGCAATACCTTCTCAGTCTG-3’ (antisense). ApoCII promoter was used as the negative control gene, which is regulated by HNF4α but shows no sex-related difference in mRNA expression. The primers for amplification of the ApoCII gene were -336/+34 fragment were 5’-AGGAGGCGGTGACAGGCG-3’ (sense) and 5’-GTAGACTGCTGCTTCTAGG-3’ (antisense). Chromosomal DNA fragments of both genes were amplified by PrimeSTAR HS DNA polymerase (TaKaRa Shuzo, Kyoto, Japan) and detected with SYBR Green reagent. PCR conditions for the two genes were as follows: 40 cycles of denaturation at 98°C for 10 s and annealing/extension at 68°C for 30 s. The amount of immunoprecipitated chromosomal DNA of the respective gene was normalized to that inputted and expressed as a percentage of input. Amplification and detection were performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) with ABI Prism 7000 SDS software. A dissociation curve was obtained after PCR to verify the specificity of the amplification. The specificity of the amplification was also confirmed by direct sequencing of the PCR products of some samples.

**Total RNA Preparation and Quantitative RT-PCR Analysis.** Total RNA was prepared from primary mouse hepatocytes or hepatic tissues using guanidine thiocyanate as described previously (Nemoto and Sakurai, 1995). Quantitative real-time RT-PCR was performed using a TaKaRa RNA PCR Kit (AMV), version 3.0, in combination with a gene-specific TaqMan MGB Gene Expression Detection Kit or SYBR Green reagent. The forward primer, reverse primer, and TaqMan MGB probe of the TaqMan MGB Gene Expression Detection Kit for CYP3A41, designed by us with assistance from Primer Express software, were 5’-GCGACAAAGAGATTATAGAGGACTGAC-3’, 5’-GGTGTCAGGATTGAAAAAGTACA-3’, and 5’-FAM-ATCTTTGCTGTTTCTCGAT-5’ respectively. GAPDH cDNA was detected with SYBR Green reagent and a gene-specific primer set. The forward and reverse primers were 5’-TCCACTCTAGCGAAAATGTCAACG-3’ and 5’-TAGACTCTCCAGCATACTGTAACG-3’, respectively. PCR conditions were as follows: initial denaturation at 95°C for 4 min, then for 33 cycles of denaturation at 95°C for 5 s, annealing/extension at 60°C for 10 s, and for GAPDH, 40 cycles of denaturation at 95°C for 15 s, annealing/extension at 64°C for 1 min, and at 72°C for 1 min. The mRNA levels of CYP3A41 were normalized to that of GAPDH and expressed as the fold change with the control mRNA level as 1. Amplification and detection were performed using the ABI PRISM 7000 Sequence Detection System with ABI Prism 7000 SDS software.

**Statistical Analysis.** Data are presented as the mean ± S.D. Statistically significant differences among groups were identified by ANOVA (Tukey post hoc test). Significance was established at p < 0.05.

**Results**

With use of a series of Cyp3a41 luciferase reporter constructs, -3669/+61-Luc, -2396/+61-Luc, -1633/+61-Luc, -844/+61-Luc, -670/+61-Luc, -596/+61-Luc, and -163/+61-Luc, designed on the basis of their deletion end points relative to the Cyp3a41 transcription start site, reporter gene assays were performed in primary cultures of hepatocytes from female mice (Fig. 1). The construct -163/+61-Luc showed significant luciferase activity, the highest level among the constructs examined. Luciferase activity was decreased to approximately 60% of that of -163/+61-Luc by extension to -596. A further decrease was observed by extension from -1633 to -844. This decrease was partially recovered by extension up to -3669. These results suggest the existence of regions involved in transcriptional activation of the Cyp3a41 gene between -163 and +61 and between -3669 and -2396 and negative regulatory elements between -596 and -163, and between -1633 and -844.

**Fig. 1.** Transcriptional activity of the 5'-flanking region of the Cyp3a41 gene in primary cultured hepatocytes from female mice. A series of Cyp3a41 luciferase reporter gene constructs were prepared as described under Materials and Methods and are shown on the left. Numbers indicate the positions relative to the transcription start site. These reporter constructs were introduced into primary cultured hepatocytes of female mice at 24 h after perfusion. After a further 48 h of incubation, the cells were harvested. Cell extracts were assayed for firefly luciferase activity, which was normalized to Renilla luciferase activity. Each bar represents the mean ± S.D. of six determinations from a single experiment. Values are shown relative to that for -163/+61-Luc-transfected hepatocytes (= 100%). The data are representative of two independent experiments. *, p < 0.01, significantly different from cells transfected with the pGL3 basic vector; #, p < 0.05, significantly different from cells transfected with -163/+61-Luc; $, p < 0.01, significantly different from cells transfected with -844/+61-Luc; & p < 0.01, significantly different from cells transfected with the -2396/+61-Luc reporter construct. Significance was examined using one-way ANOVA followed by the Tukey test.
Searching the sequence between −163 and +61 using the Web-based program TFSEARCH revealed a binding site similar to the sequences containing direct repeats of the hexamer AGGTCA separated by one base (DR1), reported as the HNF4α-binding element. HNF4α, known as a member of the hepatocyte-enriched nuclear factor family, has been reported to act as a regulating factor in the hepatic expression of sex-specific genes in the liver (Wiwi and Waxman, 2004). We termed the possible regulatory element a putative HNF4α-binding site (Fig. 2A). To identify this putative HNF4α-binding site necessary for the expression of Cyp3a41 and to investigate the role of HNF4α, we performed a luciferase assay using a construct with a mutated HNF4α-binding site and mouse HNF4α expression plasmid in primary cultured hepatocytes from female mice. As shown in Fig. 2B, mutation of the putative HNF4α-binding site significantly decreased the transcriptional activity to nearly that of the pGL3 basic vector. Transcriptional activity of the wild-type construct was significantly increased by transfection of the HNF4α expression plasmid. In contrast, transfection of the HNF4α mutant expression plasmid had no effect on the reporter gene activity of the construct containing the mutated HNF4α-binding site. This result suggests that HNF4α is involved in the transcriptional activation of Cyp3a41 at the putative HNF4α-binding site.

To examine the ability of the putative HNF4α-binding site to bind HNF4α protein in vitro, an EMSA was performed with HNF4α protein synthesized in vitro (Fig. 3). A shift of the probe-protein complex was observed. The specificity of the binding was investigated with competition assays. The complex was completely competed out by a 100-fold excess of unlabeled HNF4α probe but not by the mutated HNF4α probe or nonspecific SP1 probe (Fig. 3B). The result of this EMSA indicates that the putative HNF4α-binding site of Cyp3a41 can bind to HNF4α in vitro.

Considering that HNF4α is essential for a large number of liver-expressed genes including sex-specific genes, we wondered whether it participates in the sex-specific expression of Cyp3a41. We anticipated that if this were the case, one possible mechanism might be a difference in cellular HNF4α activity. An EMSA using nuclear extracts prepared from the livers of male and female mice was then performed (Fig. 4A). When nuclear extracts were incubated with the radiolabeled putative HNF4α-binding site probe, two shifted bands were observed. The lower band was efficiently competed out by an unlabeled putative HNF4α-binding site probe and an unlabeled nonspecific SP1 probe. Supershift of the lower complex by the addition of anti-HNF4α antibodies was observed. These results indicate that the lower complex corresponds to that containing HNF4α. Unexpectedly, the shifts of the lower complexes observed with nuclear extracts were not significantly different between males and females. When the radiolabeled mutated HNF4α site probe was used, no shifted band was observed. This result indicates that the two-nucleotide substitution, which is the same substitution introduced into the reporter construct −163/+61-HNF4α mut-Luc (Fig. 2), caused the loss of HNF4α binding and that there were no new, artificial specific DNA/protein interactions with the mutated probe. A Western blot analysis revealed similar levels of HNF4α protein in males and females (Fig. 4B). These results suggest that cellular HNF4α activity is not a key determinant of female-specific Cyp3a41 expression.

To further explore whether HNF4α participated in the sex-related difference in Cyp3a41 gene expression, we next performed a ChIP assay. With this system, one can detect HNF4α bound to the HNF4α-binding site of the Cyp3a41 gene in the chromatin structure in situ. As shown in Fig. 5A, approximately 4-fold more HNF4α was detected on the Cyp3a41 gene in hepatic tissues of female mice than male mice. On the other hand, ApoCIII, known to be regulated by HNF4α but showing no sex-related difference in mRNA expression (Wiwi et al., 2004), bound to HNF4α at similar levels in both sexes (Fig. 5B). This finding suggests that differences in the histone modification of Cyp3a41 between female and male mice resulted in the different levels of HNF4α present at the HNF4α-binding site of the Cyp3a41 gene.

To examine whether the amount of HNF4α on Cyp3a41 is linked with the level of CYP3A41 mRNA, the amounts of HNF4α bound to the gene and mRNA expression were compared in two sets of hepatic tissue samples. CYP3A41 mRNA expression was similar between male and female livers at 3 weeks of age, whereas females specifically expressed the gene at 7 weeks of age (Fig. 6B). Consistent with the mRNA levels, amounts of HNF4α bound to the putative HNF4α element were similar between males and females at 3 weeks of age but were higher in females at 7 weeks of age (Fig. 6A). Figure 6, C and D, shows the results for hypophysectomized mice. Cyp3a41 expression is under the control of GH, the female-type secretion of which is the key determinant (Sakuma et al., 2002). Expression of CYP3A41 mRNA completely disappeared after hypophysectomy and was partially recovered by continuous administration of GH by an osmotic infusion pump, which mimics female-type secretion (Fig. 6D). In accordance with mRNA levels, amounts of HNF4α binding decreased to 40% of the control after hypophysectomy (Fig. 6C). These results indicating that the direction of the effect is consistent with HNF4α binding to the putative element are consistent with a role in regulating Cyp3a41 sex-specific expression.
The epigenetic code (e.g., DNA methylation and histone modification) is implicated in the regulation of gene expression. Methylation at histone-3-lysine-4 (H3K4) is linked to activation of gene transcription, whereas methylation at histone-3-lysine-27 (H3K27) is associated with repression of gene transcription and the maintenance of heterochromatin (Heintzman et al., 2007; Wang et al., 2008). Therefore, the degree of histone-3-lysine-4 dimethylation (H3K4me2) and histone-3-lysine-27 trimethylation (H3K27me3) around the HNF4α-binding site of the Cyp3a41 gene was investigated using a ChIP assay. As shown in Fig. 7, more H3K4me2 was found in adult females than adult males. The opposite was the case for H3K27me3. Histone acetylation results in chromatin relaxation (higher fluidity), allowing for greater accessibility of transcription factors to the recognition sites in nucleosomal DNA. The degree of histone H4 acetylation in the Cyp3a41 promoter was much higher in females than in males.

Fig. 3. Specific binding of HNF4α to the putative HNF4α-binding site of Cyp3a41 in vitro. A, nucleotide sequences of the oligonucleotides used for the EMSA are shown. The putative HNF4α-binding site of the Cyp3a41 gene is shown in uppercase. Mutated nucleotides are underlined. B, EMSAs were performed with a 32P-radiolabeled Cyp3a41 probe containing the putative HNF4α-binding site. Incubation was carried out with HNF4α synthesized in vitro as described under Materials and Methods. *, the second lane from the left was loaded with a sample consisting of radiolabeled probe and in vitro transcription/translation reaction mixture incubated with nuclease-free water as the template (negative control). Competition assays were performed with a 100-fold excess of unlabeled Cyp3a41 probe containing the putative HNF4α-binding site, or Cyp3a41 mutated competitor containing mutated HNF4α-binding site, or a 100-fold excess of nonspecific SP1 probe. The HNF4α-probe complex, nonspecific bands, and free probe are indicated.

Fig. 4. Capability of nuclear HNF4α to bind the putative HNF4α-binding site of Cyp3a41 in vitro. A, nuclear extracts prepared from the livers of male and female mice were subjected to EMSA. A 32P-radiolabeled Cyp3a41 probe containing the putative HNF4α-binding site (HNF4α wild) or two-nucleotide substituted HNF4α-binding site (HNF4α mutated) was incubated with nuclear extracts and electrophoresed on a 4% polyacrylamide gel as described under Materials and Methods. Competition experiments comprised a radiolabeled probe incubated with nuclear extracts and a 100-fold excess of unlabeled HNF4α wild probe (W), or 100-fold excess of unlabeled HNF4α wild probe (W), or 100-fold excess of nonspecific SP1 probe (N). Supershift analyses were performed with 2 μg of antibody against HNF4α protein. *, in vitro translated mouse HNF4α protein was added as a positive control. The HNF4α-probe complex, supershifted HNF4α-probe complex, nonspecific bands, and free probe are indicated. NE, nuclear extracts. B, Western blot analyses of HNF4α expression in nuclear extracts prepared from the livers of male and female mice. Western blotting was performed in duplicate using antibodies against HNF4α as described under Materials and Methods.
We further examined the role of the different chromatin structures by conducting an in vivo reporter gene assay using the hydrodynamic method. In this assay, reporter gene activity does not reflect chromatin structure because a naked plasmid was transfected. We hypothesized that if the sex-related expression of Cyp3a41 is regulated by the difference in HNF4α activity between males and females, a sex difference in Cyp3a41 gene transcription would be observed. Meanwhile, if the sex-related expression of Cyp3a41 is regulated by the chromatin structure, no difference would be seen. As shown in Fig. 8, significant Cyp3a41 transcriptional activity in the liver was observed not only in females but also in males when the mice were transfected with the wild-type construct. The transcriptional activity was decreased approximately 50% by mutation of the putative HNF4α-binding site in both sexes. This result supports our hypothesis and also suggests that HNF4α activity in hepatocytes might not differ drastically between the sexes. This possibility is consistent with the results of EMSA using liver nuclear extracts and Western blotting of HNF4α protein shown in Fig. 4. With regard to the findings above, we next examined the effect of the HNF4α expression plasmid on the mRNA expression of Cyp3a41 in hepatocytes of female and male mice. We hypothesized that if causes other than the cellular amount of HNF4α, such as chromatin structure, determine the sex-specific expression of Cyp3a41 in the livers of female mice. Next, we examined the effect of GH treatment on HNF4α-induced CYP3A41 mRNA expression to see whether these factors cooperate in the regulation of the Cyp3a41 gene. As shown in Fig. 9, the response observed in female hepatocytes with both GH and HNF4α was additive.

### Discussion

The expression of some hepatic P450 genes in both rodents and humans shows sex differences. The regulation of sex-specific P450 genes which can be seen in vivo in the liver appears to be controlled by sex hormones and induction. In vivo experiments in mice have provided evidence that sex hormones control sex differences in the expression of Cyp3a41, Cyp3a5, and Cyp4a11 genes. In particular, Cyp3a41 mRNA expression was increased by administration of estradiol and androgens in vivo. However, the mechanisms that control the sex-specific expression of Cyp3a41 in the livers of female mice are not understood. We have observed that if causes other than the cellular amount of HNF4α, such as chromatin structure, determine the sex-specific expression of Cyp3a41 in the livers of female mice.

### FIG. 5. Quantitative ChIP analysis of HNF4α binding to the putative HNF4α-binding site of the Cyp3a41 gene in the livers of adult mice. Chromatin was extracted from the hepatic tissues of mice at 7 weeks of age. ChIP assays were performed using antibodies against HNF4α or control rabbit IgG. PCR was performed on chromatin templates with primer pairs that amplify ~207/+18 of the Cyp3a41 gene (A) or ~336/+34 of the ApoCIII gene (B). The region amplified by the primer pair for Cyp3a41 covers one HNF4α-binding site (~139/+127 and ~336/+318). PCR products of HNF4α-enriched chromatin were quantified and are shown as a percentage of input. Bars represent the mean ± S.D. from three individual samples. Significance was examined using one-way ANOVA followed by the Tukey test: *, p < 0.05, significantly different from male mice. □, male; ■, female.

### FIG. 6. Association between HNF4α binding on the promoter and transcription levels of the Cyp3a41 gene. Levels of HNF4α binding and mRNA expression were analyzed in hepatic tissue sets prepared from both sexes of mice at 3 and 7 weeks of age (A and B) or from sham-operated, hypophysectomized or human GH-infused hypophysectomized female mice at 7 weeks of age (C and D). A and C, chromatin was extracted from the hepatic tissues. ChIP assays were performed using an antibody against HNF4α or control rabbit IgG. PCR was performed on chromatin templates with a primer pair that amplifies ~207/+18 of the Cyp3a41 gene. PCR products were quantified and are shown as a percentage of input. B and D, mRNA expression of the Cyp3a41 gene was quantified by TaqMan real-time RT-PCR and then normalized to the level of GAPDH and are shown relative to that in the 3-week-old female (B) or sham-operated (D) group. Bars represent the mean ± S.D. from three individual samples. Significance was examined using one-way ANOVA followed by the Tukey test: *, p < 0.05; **, p < 0.01. □, male; ■, female. sham, sham-operated; hypox, hypophysectomized; GH (i), continuous infusion of human GH using an osmotic minipump.

### FIG. 7. Different chromatin modifications of the mouse Cyp3a41 gene (~207/+18) between males and females examined by the ChIP assay. Chromatin was extracted from the hepatic tissues of mice at 7 weeks of age. ChIP assays were performed using antibodies against H3K4me2, H3K27me3, and AcH4K8 or control rabbit IgG. PCR was performed on chromatin templates with a primer pair that amplifies ~207/+18 of the Cyp3a41 gene. PCR products were quantified and are shown as a percentage of input. Bars represent the mean ± S.D. from three individual samples. Significance was examined using one-way ANOVA followed by the Tukey test: *, p < 0.05, significantly different from male mice; **, p < 0.01, significantly different from male mice.
HNF4α-overexpressing hepatocytes (−163′/+61-Luc) and mutated (−163′/+61-HNF4α mut-Luc) reporter gene constructs are shown on the left. These constructs were introduced into mice by intravenous administration as described under Materials and Methods. After 24 h, the mice were sacrificed, and luciferase activity was determined. Relative luciferase activity is expressed as the firefly luciferase activity normalized to Renilla luciferase activity and is shown relative to that of −163′/+61-Luc in female livers (100%). Data are expressed as the mean ± S.D. of three to five determinations from a single experiment. The data are representative of two independent experiments. Significance was examined using one-way ANOVA followed by the Tukey test. * p < 0.05, significantly different from the pGL3-transfected hepatocytes of male mice; # p < 0.05, significantly different from the pGL3-transfected hepatocytes of female mice.

FIG. 8. Transcriptional activity of the 5′-flanking region of the Cyp3a41 gene and functional analysis of the putative HNF4α-binding site in male and female mice determined by the in vivo reporter assay. Schematic representations of the wild-type (−163′/+61-Luc) and mutated (−163′/+61-HNF4α mut-Luc) reporter gene constructs are shown on the left. These constructs were introduced into mice by intravenous administraton as described under Materials and Methods. After 24 h, the mice were sacrificed, and luciferase activity was determined. Relative luciferase activity is expressed as the firefly luciferase activity normalized to Renilla luciferase activity and is shown relative to that of −163′/+61-Luc in female livers (100%). Data are expressed as the mean ± S.D. of three to five determinations from a single experiment. The data are representative of two independent experiments. Significance was examined using one-way ANOVA followed by the Tukey test. * p < 0.05, significantly different from the pGL3-transfected hepatocytes of male mice; # p < 0.05, significantly different from the pGL3-transfected hepatocytes of female mice.

FIG. 9. Effects of transfection of the HNF4α expression plasmid and GH treatment on the expression of CYP3A41 mRNA in primary cultured hepatocytes of male and female mice. Hepatocytes isolated from male and female dLy mice were cultured for 48 h and transfected with empty plasmid or the mouse HNF4α expression plasmid. Three hours after transfection, GH was added at a final concentration of 71 ng/ml, and the cells were cultured for a further 24 h. The cells were harvested, and CYP3A41 mRNA expression was evaluated by quantitative real-time RT-PCR. The expression of each mRNA was normalized to the level of GAPDH mRNA and is shown relative to that in the empty plasmid-transfected male hepatocytes. Each column represents the mean ± S.D. of four determinations from a single experiment. The data are representative of two independent experiments. * p < 0.01, significance was examined by one-way ANOVA followed by the Tukey test.
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References


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