Regulation of *Cyp2a5* transcription in mouse primary hepatocytes: roles of hepatocyte nuclear factor 4 and nuclear factor I

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The cytochrome P450_{2a5} (*Cyp2a5*) gene is expressed principally in liver and olfactory mucosa. In the present study, the transcriptional mechanisms of hepatocyte-specific expression of *Cyp2a5* were studied in mouse primary hepatocytes. The *Cyp2a5* 5'-flanking region – 3033 to + 10 was cloned in front of a luciferase reporter gene and transfected into hepatocytes. Deletion analysis revealed two major activating promoter regions localized at proximal 271 bp and at a more distal area from – 3033 to – 2014 bp. The proximal activation region was characterized further by DNase I footprinting, and a single clear footprint was detected in the studied area centred over a sequence similar to the NF-I (nuclear factor I)-binding site. The binding of NF-I was confirmed using an EMSA (electrophoretic mobility-shift assay). A putative HNF-4 (hepatocyte nuclear factor 4)-binding site was localized at the proximal promoter by computer analysis of the sequence,

and HNF-4 α was shown to interact with the site using an EMSA. The functional significance of HNF-4 and NF-I binding to the *Cyp2a5* promoter was evaluated by site-directed mutagenesis of the binding motifs in reporter constructs. Both mutations strongly decreased transcriptional activation by the *Cyp2a5* promoter in primary hepatocytes, and double mutation almost completely abolished transcriptional activity. Also, the functionality of the distal activation region was found to be dependent on the intact HNF-4 and NF-I sites at the proximal promoter. In conclusion, these results indicate that HNF-4 and NF-I play major roles in the constitutive regulation of hepatic expression of *Cyp2a5*.

Key words: cytochrome P450, hepatocyte nuclear factor 4 (HNF-4), liver, nuclear factor I (NF-I), transcription.

INTRODUCTION

Cytochrome P450 (CYP) enzymes constitute a superfamily of haemoproteins that play a major role in the detoxification of xenobiotics [1]. Xenobiotic-metabolizing CYP enzymes are expressed in a tissue-selective manner, and the main site of detoxification is the liver. The CYP2A5 enzyme is the major catalyst of coumarin 7-hydroxylation in mouse liver [2,3]. As far as its regulation is concerned, *Cyp2a5* seems to differ from most other CYP enzymes: it can be induced by structurally unrelated compounds and also by several chemicals that usually repress other CYP forms. *Cyp2a5* is induced by classical inducers, such as phenobarbital [4], and by various hepatotoxic agents, such as cocaine, pyrazole, solvents, heavy metals, hexachlorobutadiene and carbon tetrachloride [5–8]. *Cyp2a5* is also up-regulated by cAMP [9]. Elevated CYP2A5/CYP2A6 protein levels in hepatocytes are associated with the development of liver tumours in mice [10] and in humans [11] respectively. In addition, inflammation of the liver caused by hepatitis B virus induces *Cyp2a5* [12]. In the human liver, the orthologous form CYP2A6 increases in conjunction with liver cirrhosis [13]. It has been proposed that CYP2A5 may be one of the major xenobiotic-metabolizing CYP forms in a damaged liver [14].

The mouse *Cyp2a5* and the human CYP2A6 genes are both predominantly expressed in hepatocytes, but they are also present in some extrahepatic tissues, mainly olfactory mucosa [15,16].

On the other hand, the orthologous rat isoform CYP2A3 is not expressed in liver, and CYP2A3 has been detected only in olfactory mucosa and lung [15,16]. The mechanisms of liver-selective expression of *Cyp2a5* are poorly understood. *Cyp2a5* displays circadian expression in mouse liver, with circadian accumulation of CYP2A5 protein in mouse liver microsomes [9,17]. This circadian expression is regulated by the PAR family transcription factor DBP (D-binding protein) [17]. Otherwise, there is no information on the factors that mediate *Cyp2a5* activation in liver.

In the present study, we studied the transcriptional mechanisms of liver-selective expression of *Cyp2a5* in a mouse primary hepatocyte model. We localized the main regulatory regions and showed that the transcription factors HNF (hepatocyte nuclear factor)-4 and NF-I (nuclear factor I) play a central role in the activation of *Cyp2a5* transcription in hepatocytes.

EXPERIMENTAL

Isolation and culturing of hepatocytes

Mouse primary hepatocytes were isolated from DBA/2 mouse liver as described previously [18]. The mouse liver was perfused with collagenase solution (Worthington Biochemical, Freehold, NJ, U.S.A.), and liver cells were collected. After filtration and centrifugation, the isolated hepatocytes were dispersed in William's medium E (Sigma, St. Louis, MO, U.S.A.) containing

Abbreviations used: CTF, CAAT box transcription factor; CYP, cytochrome P450; DBP, D-binding protein; EMSA, electrophoretic mobility-shift assay; HNF, hepatocyte nuclear factor; ITS, insulin/transferrin/sodium selenate; NF-I, nuclear factor I; NPTA, nasal predominant transcriptional activating element.

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The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank[®] and GSDB Nucleotide Sequence Databases under the accession number AY321510.

20 ng/ml dexamethasone (Sigma), ITS (5 mg/l insulin/5 mg/l transferrin/5 μ g/l sodium selenate; Sigma), 10 μ g/ml gentamicin (Invitrogen, Carlsbad, CA, U.S.A.) and 10 % (v/v) foetal bovine serum (Invitrogen) at a density of 300 000 cells/well in a twelve-well plate or 5×10^6 cells/90-mm-diameter dish. The cultures were maintained at 37 °C in a humidified incubator for 2–3 h, after which non-attached cells were discarded by aspiration, and the medium was replaced by serum-free William's medium E. The cultures were maintained for additional 24 h before transient transfection or nuclear extract preparation.

Cell lines

HepG2 cells and COS-1 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10 % (v/v) foetal bovine serum, 100 units/ml penicillin and 100 μ g/ml streptomycin (Invitrogen).

Cloning of *Cyp2a5* 5'-flanking region

Genomic DNA was prepared from male DBA/2J mouse spleens. DNA was partially digested with *Mbo*I, treated with calf intestinal phosphatase and ligated to the arms of the lambda EMBL 3 vector. The library was screened with a 32 P-labelled 2 kb fragment of the *Cyp2a5* gene (clone 29) [19], and 17 positive clones were obtained. The clone X1412-1 contained a 16.7 kb insert and was the longest among the *Cyp2a5*-containing clones. The identity of the insert was verified with the presence of a *Hind*III site in exon 4. The clone contained the *Cyp2a5* exons 1 to 4 and more than 13 kb of the 5'-flanking region. A part of the insert was subcloned and sequenced.

Plasmids

Reporter plasmid: the *Cyp2a5* 5' – 3033 to +10 (from the transcriptional start site) fragment was prepared by PCR amplification from DBA/2 mouse genomic DNA and cloned into the pGL3-basic vector (Promega, Madison, WI, U.S.A.) in front of the luciferase reporter gene. This construct was designated as *Cyp2a5* – 3033-Luc. In addition, several shorter 5' deletion constructs were prepared by PCR using the *Cyp2a5* – 3033-Luc plasmid as a template and subcloning the PCR products. The correct identity of the constructs was verified by sequencing. The rat HNF4 α 1 expression plasmid [20] was kindly provided by Dr Mary C. Weiss (Unité de Génétique de la Différenciation, Département de Biologie de Développement, Institut Pasteur, Paris Cedex 15, France).

Site-directed mutagenesis

Site-directed mutagenesis was performed with the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, U.S.A.), according to the manufacturer's instructions. Briefly, the mutations were introduced into *Cyp2a5* – 3033-Luc and *Cyp2a5* – 271-Luc plasmids using mutated oligonucleotides (NF-I mutation, – 141 5'-CCTCCTCCTCCAGTGTAAACAAT-GTCAAAACTTGGTGCAC-3' – 101; HNF-4 mutation, – 73 5'-GTTGCATAATCAAGACCAAAGTAAGTCCTTCTGTCT-CTGGATG-3' – 31; the mutated nucleotides are indicated in bold) and *PfuTurbo* DNA polymerase followed by selective digestion of the original template plasmid with the restriction enzyme *Dpn*I. Correct assembly of the mutations was confirmed by sequencing.

Transient transfection

After 24 h in culture, the mouse primary hepatocytes were washed with William's medium E and transiently transfected with

experimental *Cyp2a5*-Luc plasmid (0.5 μ g/well) using Tfx™-20 lipid-reagent (Promega) and OptiMEM I medium (Invitrogen). The pRL-TK plasmid (0.1 μ g/well) (Promega) containing the *Renilla* luciferase reporter gene was co-transfected with the experimental *Cyp2a5*-Luc constructs to provide an internal control for transfection efficiency. After a transfection period of 1 h, 1 ml of William's medium E containing dexamethasone, ITS and gentamicin was added. The cells were maintained for an additional 48 h, after which they were lysed and their luciferase activity was assayed with the Dual-Luciferase Reporter Assay System (Promega).

The COS-1 cells were seeded on 24-well plates 1 day before transfection. Cells were transiently transfected with 0.65 μ g of reporter plasmid, 50 ng of pRL-TK control plasmid and 50 ng of expression vector per well using Tfx™-20 lipid-reagent and OptiMEM I medium. The cells were maintained for an additional 24 h, after which they were lysed and their luciferase activity was measured with the Dual-Luciferase Reporter Assay System.

Preparation of nuclear extracts

Nuclear extracts were prepared according to Schreiber et al. [21]. Cultured mouse primary hepatocytes, or HepG2 cells, (90-mm-diameter dish) were first washed with 10 ml of PBS, then scraped into another 10 ml of PBS, and pelleted by centrifugation at 110 g for 5 min, suspended in 1 ml of PBS and centrifuged at 660 g for 15 s. The cell pellet was resuspended in cold hypotonic buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol and 0.5 mM PMSF), and the cells were allowed to swell on ice for 15 min. The cell membranes were lysed by adding 10 % solution of Tergitol (type Nonidet P40) (Sigma) to a final percentage of 0.6 %. The homogenate was centrifuged at 660 g for 30 s, and the nuclear pellet was resuspended in cold buffer C (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and 1 mM PMSF). The nuclear proteins were extracted by incubation at 4 °C for 15 min on a shaking platform. The samples were centrifuged at 15 000 g for 5 min, and the supernatant fractions containing the nuclear proteins were collected. The protein content of the nuclear extract was determined using the Bradford protein analysis method [22].

Nuclear extracts from DBA/2 mouse liver were prepared similarly, except that the tissue was first homogenized in lysis buffer [20 mM Tris/HCl, pH 7.5, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA and Complete™ Mini protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany)] (4 ml/g of tissue).

DNase I footprinting

The – 271 to +10 fragment of the *Cyp2a5* 5'-flanking region was PCR-amplified from the plasmid *Cyp2a5* – 271-Luc. The primers were designed to include 12 and 39 nucleotides of the vector sequence from the pGL3-basic at its 5' and 3' end respectively. The PCR product was then subcloned to the pCR 2.1-TOPO vector (Invitrogen). The DNA probe for footprinting was then prepared from the *Cyp2a5* 5' – 271 to +10 in pCR 2.1-TOPO by digestion with *Eco*RI. The fragment was treated with alkaline phosphatase and end-labelled with T4 polynucleotide kinase using [γ - 32 P]ATP. To produce a probe labelled at one end only, one of the labelled ends was cleaved either with *Hind*III or with *Kpn*I. The probe was finally purified using the QIAquick gel extraction kit (Qiagen, Venlo, The Netherlands).

DNase I footprinting analyses were performed with the Core Footprinting System (Promega), essentially according the manufacturer's instructions. Labelled probe (3.5 ng of 20 000 c.p.m.), 0.4 μ g of poly(dI-dC) · (dI-dC), 5 μ g of mouse hepatocyte nuclear

Table 1 Sequence of the double-stranded oligonucleotides used in EMSA

The core sequence is underlined and the mutations are indicated in bold.

Oligonucleotide	Sequence	Location
NF-I EMSA		
Cyp2a5	5'-CCTCCTCCAGTGT <u>TTGGCAATGTCCAACTTGGTGCAC</u> -3'	– 138 to – 101
Cyp2a5 mutation	5'-CCTCCTCCAGTGT <u>TTAACAATGTCA</u> AAACTTGGTGCAC-3'	– 138 to – 101
CYP2A3	5'-CCTCCTTGAGTGT <u>TTGGCTATGTCCCAACTAGG</u> -3'	– 138 to – 106
NF-I consensus	5'-GGCACCTGTTCAAT <u>TTGGCAGGAGCC</u> AACAG-3'	
NF-I consensus mutation	5'-GGCACCTGTTCAAT <u>TTGTTACGGAGTT</u> AACAG-3'	
HNF-4 EMSA		
Cyp2a5	5'-GTTGCATAATCAAGACCAAGTCCGTCCTTGTCTCTGGATG-3'	– 73 to – 31
Cyp2a5 mutation	5'-GTTGCATAATCAAGACCAAGTAAAGTCCTTGTCTCTGGATG-3'	– 73 to – 31

extract and 25 μ l of binding buffer [50 mM Tris/HCl, pH 8.0, 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA, 20 % (v/v) glycerol and 1 mM dithiothreitol] were incubated at room temperature (22 °C) for 30 min in a final volume of 50 μ l. DNase reaction buffer (containing 5 mM $CaCl_2$ and 10 mM $MgCl_2$) (50 μ l) was added, and the reaction mixtures were incubated at room temperature for an additional 1 min. RQ1 RNase-free DNase (0.15 unit) was then added, and the DNase digestion was allowed to proceed for 1 min at room temperature. The reactions were terminated by adding 90 μ l of the DNase stop solution [200 mM NaCl, 30 mM EDTA, 1 % (w/v) SDS and 100 μ g/ml yeast RNA]. DNA was recovered from the reaction mixtures by phenol/chloroform/isopentanol extraction and ethanol precipitation. The samples were analysed by electrophoresis through 6 % (w/v) polyacrylamide DNA sequencing gel and detected by autoradiography. The region protected from DNase I cleavage was localized by using a chemical A + G sequencing reaction [23].

EMSA (electrophoretic mobility-shift assay)

Single-stranded oligonucleotides were purchased from Sigma Genosys. Double-stranded oligonucleotides were prepared by annealing the desired sense and antisense oligonucleotides (Table 1). Double-stranded oligonucleotides were 5'-end-labelled with [γ - ^{32}P]ATP and T4 polynucleotide kinase and then purified using the QIAquick nucleotide removal kit.

EMSAs for NF-I were performed with the Nushift™ kit (Geneka Biotechnology, Montreal, Canada), essentially according to the manufacturer's instructions. Extract pre-mixtures containing 14 μ g of mouse liver nuclear extract, binding buffer B1 and stabilizing solution D were pre-incubated at 4 °C for 20–45 min in a final volume of 16 μ l. Probe pre-mixtures containing 0.08 pmol (16000 c.p.m.) of labelled double-stranded Cyp2a5 oligonucleotide, binding buffer C1, stabilizing solution D and, for competition experiments, unlabelled competitor oligonucleotide (at 5–100-fold excess) in a final volume of 8 μ l, were added to the extract pre-mixtures and incubated at 4 °C for an additional 20 min. For supershift experiments, 3 μ l of rabbit anti-NF-I polyclonal antibody [α -CTF (CAAT box transcription factor) antiserum] (a gift from Dr Naoko Tanese, Department of Microbiology, New York University School of Medicine, New York, NY, U.S.A.) was added to the extract pre-mixture before pre-incubation. The samples were separated by electrophoresis on a 6 % (w/v) polyacrylamide gel, and the retarded complexes were detected by autoradiography.

The HNF-4 EMSAs were performed using 10–20 μ g of mouse liver nuclear extract or HepG2 nuclear extract, binding buffer [25 mM Hepes, pH 7.9, 10 % (v/v) glycerol, 50 mM KCl, 0.5 mM

EDTA and Complete™ Mini protease inhibitor cocktail], 1 μ g/ μ l ssDNA and 0.04 pmol (30000 c.p.m.) of labelled double-stranded Cyp2a5 oligonucleotide. The reaction mixtures were incubated at room temperature for 30 min in a final volume of 15 μ l. For competition experiments, unlabelled competitor oligonucleotides (at 10–100-fold excess) were included in the mixtures. For supershift experiments, nuclear extracts were pre-incubated with 0.2 μ g of goat anti-HNF-4 α polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) on ice for 20 min. The samples were separated by electrophoresis on a 6 % (w/v) polyacrylamide gel, and the retarded complexes were detected by autoradiography.

RESULTS

Cloning of the *Cyp2a5* 5'-flanking region

The DBA/2J mouse genomic DNA library was screened with the *Cyp2a5* genomic fragment 29 [19], and a clone containing at least 13 kb of the *Cyp2a5* 5'-flanking region was obtained. A part of the insert was subcloned, and 3033 bp upstream from the transcription start site of the 5'-flanking region was sequenced. With the NCBI Blast program (<http://www.ncbi.nlm.nih.gov>), the *Cyp2a5* 5' – 3033 to – 1 sequence was found to be 85 % similar to the rat *CYP2A3* gene (GenBank® accession number M33190) 5'-flanking region.

Localization of the upstream activation regions of the *Cyp2a5* gene

To identify the sequences responsible for the transcriptional activation of the mouse *Cyp2a5* gene in primary hepatocytes, a series of 5'-truncated mouse *Cyp2a5* promoter–luciferase reporter plasmids were constructed and transfected into mouse primary hepatocytes (Figure 1). The highest luciferase activity was produced by the longest *Cyp2a5* – 3033-Luc construct. Deletion of the sequence from – 3033 to – 2014 decreased the transcriptional activity by 86 %, indicating the presence of activating enhancer elements in this region. Removal of the sequence from – 2013 to – 1021 did not markedly affect luciferase activity, but deletion of the sequence from – 1020 to – 272 increased this activity > 3-fold relative to the *Cyp2a5* – 1020-Luc construct, implying the presence of repressing sequence elements in the region of – 1020 to – 272. A 5' deletion from – 271 to – 64 decreased transcriptional activity by 90 %, bringing it close to the basal level of the promoterless control plasmid pGL3-basic. Thus the proximal promoter region from – 64 to – 271 contains activating *cis*-elements and, in addition to the distal enhancer at – 3033 to – 2014, forms a second activation region for the *Cyp2a5* gene.

Figure 1 Transcriptional activity of *Cyp2a5* 5'-luciferase constructs transfected to mouse primary hepatocytes

The cells were harvested and luciferase activities were measured 48 h after transfection. The activities produced by the studied constructs were normalized against co-transfected control plasmid (pRL-TK) activities. The values represent means + S.D. of four individual samples. The experiment was repeated twice with similar results.

Figure 2 DNase I footprinting of the *Cyp2a5* proximal promoter

A – 271 to +10 *Cyp2a5* probe was labelled and incubated with hepatocyte nuclear proteins, after which the reaction mixture was treated with DNase I. The resulting fragments were separated by PAGE and detected by autoradiography. The footprint was localized by chemical A + G sequencing reaction (results not shown). The footprint was identified to be similar to the NF-I motif with the MatInspector program using TRANSFAC 4.0 matrices.

DNase I footprinting analysis of the proximal promoter

DNase I footprinting was used to locate the protein-binding sites within the proximal activation region. A – 271 to +10 *Cyp2a5* probe was labelled and incubated with hepatocyte nuclear proteins, after which the reaction mixture was treated with DNase I. A single clear, protected footprint was detected in the non-coding strand and located by sequence analysis at –132 to –107 (Figure 2). Analysis of the coding strand produced a similar result (results not shown). A search with the MatInspector professional program (<http://www.gene-regulation.com/pub/>

Figure 3 Characterization of NF-I binding to the *Cyp2a5* promoter by EMSA

(A) Interaction of mouse liver nuclear proteins with the *Cyp2a5* 5' – 138 to – 101 sequence in EMSA. Lane 1 represents a binding reaction with no protein and lane 2 is a control reaction with no competing oligonucleotides. The other lanes represent competition reactions with different unlabelled oligonucleotides as indicated. The sequences of the oligonucleotides are presented in Table 1. (B) Supershift analysis of *Cyp2a5* promoter – 138 to – 101 binding proteins with anti-NF-I antibody. The mouse liver nuclear extract was pre-incubated with anti-NF-I antibody before the addition of 5'-end-labelled DNA oligonucleotide as described in the Experimental section. The effect of the antibody was compared with a control reaction with no antibody. The retarded protein–DNA complex competed by excess of unlabelled NF-I consensus oligonucleotides is indicated with an arrow on the left-hand side of the Figure. The anti-NF-I antibody supershifted complex is indicated with an arrow on the right-hand side of the Figure.

databases.html#transfac) revealed this region to be centred over a sequence similar to the consensus binding site of transcription factor NF-I.

Characterization of the protein binding to the – 132 to – 107 element

The proteins binding to the – 132 to – 107 footprinted sequence were elucidated further by EMSA. A 38 bp double-stranded, end-labelled oligonucleotide from position – 138 to – 101 of the *Cyp2a5* promoter was incubated with nuclear extract from mouse liver, and the resulting DNA–protein complexes were separated by gel electrophoresis. Two major retarded complexes were detected (Figure 3). The upper complex was competed by a 10–100-fold excess of unlabelled probe itself and also by a 5–50-fold excess of oligonucleotide-containing consensus binding site for the transcription factor NF-I (Table 1). Mutation of the nucleotides known to be critical for NF-I binding abolished competition by the NF-I consensus oligonucleotide. Furthermore, mutation of the NF-I core sequence in the *Cyp2a5* oligonucleotide also abolished competition. Thus the competition reactions of the EMSA assay suggest that the detected footprint results from interactions with an NF-I-like transcription factor or factors. The lower band was not competed by the NF-I consensus oligonucleotide and was not affected by the mutation of the *Cyp2a5* oligonucleotide, suggesting that this retarded complex does not involve NF-I-like proteins.

Involvement of NF-I in the formation of the detected DNA–protein complex was verified further by using the anti-NF-I antibody. A clear supershifted band was detected after incubation

Figure 4 Characterization of HNF-4 binding to the *Cyp2a5* promoter by EMSA

(A, B) Interaction of mouse liver (A) and HepG2 (B) nuclear proteins with the *Cyp2a5* 5' – 73 to – 31 sequence in EMSA. The retarded protein–DNA complexes are indicated with arrows. Lane 1 represents a binding reaction with no protein, and lane 2 is a control reaction with no competing oligonucleotides. The other lanes represent competition reactions with different unlabelled oligonucleotides as indicated. The sequences of the oligonucleotides are presented in Table 1. (C) Supershift analysis of the *Cyp2a5* promoter – 73 to – 31 binding proteins with anti-HNF-4 α antibody. The mouse liver and HepG2 nuclear extracts were pre-incubated with anti-HNF-4 α antibody before the addition of 5'-end-labelled DNA oligonucleotide as described in the Experimental section. The effect of the antibody was compared with a control reaction with no antibody. The retarded protein–DNA complexes formed in the control reaction are indicated with arrows on the left-hand side of the figure. The anti-HNF-4 antibody supershifted complex is indicated with an arrow on the right-hand side of the figure. (D) Comparison of HNF-4 binding to the *Cyp2a5* promoter HNF-4-binding element using nuclear extracts from mouse primary hepatocytes, mouse liver and HepG2 cells. Extract protein (20 μ g) was used for the experiment.

with the anti-NF-I antibody, and the upper complex was shifted (Figure 3). Thus the supershifting assay confirmed that an NF-I protein really binds to the *Cyp2a5* promoter region detected by DNase I footprinting.

Identification of the HNF-4 binding site in the *Cyp2a5* proximal promoter

Computer analysis (MatInspector) of the *Cyp2a5* gene revealed a putative binding site for the transcription factor HNF-4 α in the proximal promoter at position – 63 to – 47. We were unable to detect any footprint at that position in our DNase I footprinting analysis of the proximal promoter. However, HNF-4 is involved in transcriptional activation of several CYP genes, including the closely related *Cyp2a4* [24], and we therefore examined further whether HNF-4 plays any role in the regulation of *Cyp2a5*.

The putative *Cyp2a5* HNF-4 DNA element was subjected to EMSA analysis using nuclear extracts from mouse liver and HepG2 cells (that constitutively express HNF-4) (Figure 4). Strong retarded complexes were formed with both liver and HepG2 extracts. The bands were abolished by mutation of the nucleotides known to be critical for HNF-4 binding [24]. The bands were supershifted by HNF-4-specific antibody confirming that HNF-4 is able to bind to the *Cyp2a5* HNF-4-binding element. We next compared the protein binding to the *Cyp2a5* HNF-4 element using nuclear extracts from primary hepatocytes, liver and HepG2 cells. The strongest binding was detected with the HepG2 extract followed by the liver extract. The primary hepatocyte extract produced a very faint band (clearly visible only upon extended exposure) compared with liver or HepG2, indicating that low levels of HNF-4 are present in primary hepatocytes.

HNF-4 activates *Cyp2a5* transcription

We next studied whether or not HNF-4 is able to *trans*-activate *Cyp2a5*. The *Cyp2a5* promoter construct *Cyp2a5* – 271-Luc

Figure 5 Effect of HNF-4 co-transfection on *Cyp2a5* promoter activity in COS-1 cells

The expression vector for HNF-4 α was co-transfected with the *Cyp2a5* – 271-Luc reporter plasmid or with the reporter plasmid containing a mutated HNF-4-binding site (*Cyp2a5* – 271-Luc HNF-4 mut) into COS-1 cells. The cells were harvested and luciferase activities were measured 24 h after transfection. The activities produced by the studied constructs were normalized against co-transfected control plasmid (pRL-TK) activities. The results are presented as fold activation of the control experiment with no expression vector. The values represent means $\pm$ S.D. of four individual samples. *** P < 0.001 compared with the control without HNF-4 α expression vector co-transfection (independent-samples t test). The experiment was repeated twice with similar results.

was transfected together with the HNF-4 α expression plasmid to HNF-4-deficient COS-1 cells. HNF-4 activated this construct 5.2-fold compared with the control without HNF-4 expression. Furthermore, mutation of the *Cyp2a5* – 63 to – 47 HNF-4-binding site abolished activation (Figure 5).

Figure 6 Effect of NF-I- and HNF-4-binding sites on *Cyp2a5* transcription in mouse primary hepatocytes

The *Cyp2a5* 5'-Luc plasmids *Cyp2a5* - 3033-Luc (**A**) and *Cyp2a5* - 271-Luc (**B**) with mutated NF-I- or/and HNF-4-binding sites were prepared and transfected into mouse primary hepatocytes. The activities produced by the studied constructs were normalized against co-transfected control plasmid (pRL-TK) activities. The luciferase activities produced by the mutant constructs were compared with the activities produced by the construct with intact NF-I- and HNF-4-binding sites. The values are presented as percentages of control (unmutated construct) and represent the means $\pm$ S.D. of four individual samples. *** $P < 0.001$ compared with the unmutated construct (one-way ANOVA, followed by least-significant difference post-hoc test). The experiment was repeated twice with similar results.

Regulation of *Cyp2a5* transcription in primary hepatocytes by NF-I and HNF-4

The contribution of NF-I and HNF-4 to the transcriptional regulation of *Cyp2a5* in primary hepatocytes was examined using mutations of the *Cyp2a5* promoter-luciferase reporter plasmids. Mutation of the HNF-4 site strongly decreased the transcription of the *Cyp2a5* - 3033-Luc and *Cyp2a5* - 271-Luc constructs to only 1.6% and 4.5% of the control activities respectively. NF-I site mutation also very efficiently decreased the transcription of *Cyp2a5* - 271-Luc to 14% of the control and that of *Cyp2a5* - 3033-Luc to 15% of the control. The combined effect of HNF-4 and NF-I mutations was studied using double mutation of the *Cyp2a5* - 271-Luc construct. Mutation of both transcription-factor-binding sites almost completely prevented transcription (Figure 6).

DISCUSSION

Many genes display tissue-specific regulation or are expressed at variable levels in different cell types. Tissue-specific gene expression is regulated primarily by tissue-enriched transcription factors. This tissue-specific regulation has been most extensively studied in liver. Six families of liver-enriched transcription factors [HNF-1, HNF-3, HNF-4, HNF-6, C/EBP (CCAAT/enhancer-binding protein) and DBP] play an important role in liver-specific gene regulation [25]. However, the functional analysis of these transcription factors has led to the conclusion that the co-operation of liver-enriched transcription factors with ubiquitous transactivating factors (such as Sp1 and NF-I) is necessary for the maintenance of liver-specific gene expression [26]. These two groups of transcription factors have also been shown to act in concert in the activation of the liver-specific gene transcription of many genes, including albumin [27].

The transcription factor family HNF-4 consists of three members, i.e. HNF-4 α , HNF-4 β and HNF-4 γ , and controls numerous important metabolic functions, including the metabolism of

glucose, cholesterol and fatty acids [25]. The three members of the HNF-4 family display overlapping tissue expression, but HNF-4 α plays the major role in liver. HNF-4 belongs to the large family of nuclear receptors and is considered to be an orphan receptor, but fatty acyl-CoA thioesters have been suggested as ligands for HNF-4 α [28]. HNF-4 α participates in the development and maintenance of the hepatic phenotype [29]. Interestingly, the transcription of some hepatic genes requires the constant presence of HNF-4 α , while others, such as PXR (pregnane X receptor), only need to be switched on during development [30,31]. Along with many other liver-expressed genes, several members of the cytochrome P450 superfamily are also under the direct regulation of HNF-4 α [32]. Especially, many members of the CYP 2 family have putative HNF-4 α binding sites in the proximal promoter. Some of these genes, such as the rabbit 2C1, CYP2C2 and CYP2C3, and the human CYP2C9 and CYP2D6, have actually been found to be regulated by HNF-4 α [32]. However, rat CYP2C7, CYP2C11, CYP2C12 and CYP2C13, for example, are not regulated to any major extent by HNF-4 α , despite the putative binding site in the promoter [32].

HNF-4 participates in the hepatic regulation of some CYP2A forms. Yokomori et al. [24] found that HNF-4 is able to bind to the *Cyp2a4* proximal promoter and activates the *Cyp2a4* gene. Furthermore, Jover et al. [33] found that adenoviral transfection of HNF-4 α antisense to human hepatocytes strongly down-regulated *CYP2A6* expression, suggesting that HNF-4 α plays an important role in the regulation of *CYP2A6*. In the present study, we showed that the *Cyp2a5* gene is also regulated by HNF-4 α . This is supported by several lines of evidence. The putative HNF-4-binding site in the *Cyp2a5* proximal promoter is able to bind HNF-4 α . Co-transfection of the HNF-4 α expression plasmid to COS-1 cells activates the transcription of the *Cyp2a5* promoter construct, but this activation is abolished by mutation of the HNF-4-binding site. Furthermore, in mouse primary hepatocytes, mutation of the *Cyp2a5* promoter HNF-4-binding site strongly decreases the transcriptional activation of the *Cyp2a5* promoter at both -271 bp and -3033 bp of the 5'-flanking region. The HNF-4-binding

site is located at position -63 to -47 . However, the promoter region -63 to $+10$ produced very little transcriptional activity. Apparently, the sequence immediately upstream from the binding site is necessary for proper functioning of HNF-4. This sequence may be required for binding, or alternatively, co-operation with factors that interact with a region further upstream may be necessary.

NF-I proteins mediate both the initiation of transcription and DNA replication, and they are encoded by four distinct genes (NF-IA, NF-IB, NF-IC and NF-IX), which are conserved in every vertebrate species examined, including mouse [34] and humans [35,36]. NF-I proteins have a conserved N-terminal region that mediates DNA binding and dimerization [37]. Each NF-I gene transcript can be alternatively spliced, generating various isoforms of NF-I proteins [38]. Different NF-I isoforms contain variable C-terminal transactivating domains [37]. Some isoforms can also repress transcription [39]. NF-I isoforms form stable homo- and hetero-dimers in all possible combinations [40], but homodimers have been shown to be transcriptionally more active than heterodimers [41].

Different levels of alternatively spliced NF-I transcripts are present in different cell types and tissues, suggesting a potential role for these transcription factors in tissue-specific gene expression [34,35]. In concert with liver-enriched transcription factors, NF-I proteins have been shown to regulate the liver-specific transcription of many genes, including albumin [27] and vitellogenin [42]. NF-I proteins have also been shown to participate in the regulation of CYP gene expression. NF-IC is essential for the transcription of the *CYP17* gene in human adrenal NCI-H295A cells [27,43]. Moreover, the PBREM (phenobarbital-responsive enhancer module) of the mouse *Cyp2b10* gene contains an NF-I-binding site flanked by two nuclear-receptor-binding sites. This NF-I site is important for both the induction response and the basal expression of the *Cyp2b10* gene [44]. The expression of the rat *CYP1A2* gene, expressed selectively in liver and olfactory mucosa, also seems to be regulated by NF-I [45].

The present paper provides evidence that the expression of the *Cyp2a5* gene in mouse primary hepatocytes is under the control of transcription factor NF-I. DNase I footprinting of the proximal promoter region from -271 to the transcriptional start site revealed that mouse primary hepatocyte nuclear proteins interacted with the promoter region from -132 to -107 corresponding to a putative binding site of the transcription factor NF-I. Using an EMSA, a protein complex, which was supershifted by an antibody specific against NF-I, was found to interact with the identified DNA sequence region. Site-directed mutagenesis of the NF-I-binding site in the *Cyp2a5* promoter constructs confirms the functional contribution of NF-I to the activation of *Cyp2a5* gene expression in hepatocytes.

In 1998, Zhang and Ding [46] identified a conserved NF-I-like-binding site in the promoter region of the rat *CYP2A3* gene, designated NPTA (nasal predominant transcriptional activating element), and proposed that this conserved binding site contributes to tissue-selective expression of the rat *CYP2A3*, mouse *Cyp2a5* and human *CYP2A6* genes in olfactory mucosa. In the present study, we showed that NF-I proteins are of importance for liver-enriched expression of *Cyp2a5*, and that an NF-I sequence motif in the *Cyp2a5* promoter similar to the *CYP2A3* NPTA element is involved. However, it seems probable that different NF-I isoforms are responsible for the transcriptional activation of *Cyp2a5* in olfactory mucosa and liver. The NPTA-binding site of the rat *CYP2A3* gene was reported to interact only with nuclear proteins from olfactory mucosa and not with liver nuclear extracts [46]. This is contradictory to our present observation showing that, in the EMSAs, the NPTA element of the *CYP2A3* gene was able

Figure 7 Alignment of the *Cyp2a5* and *CYP2A6* promoter sequences

The conserved TATA box, HNF-4 and NF-I elements are indicated by shading.

to compete with the *Cyp2a5* promoter NF-I site for binding of the mouse liver nuclear proteins. The *CYP2A3* gene is not expressed in liver to any appreciable extent, and the NF-I/NPTA element may therefore not be the sole determinant of liver-specific expression, but interaction with additional factors is possibly also required.

In the present study, we identified the two major factors activating the *Cyp2a5* promoter. Both of these factors, HNF-4 and NF-I, act on the proximal promoter. An additional activation region was located at a further upstream region -3033 to -2014 . Nevertheless, the function of HNF-4 appears to be indispensable also for the function of the distal activation region, as mutation of the proximal HNF-4 site decreased transcription to only 1.6% of the control activity in the context of the -3033 to $+10$ promoter. Mutation of the NF-I site decreased the transcriptional activity from the *Cyp2a5* -3033 -Luc construct significantly, but to a lesser extent than the HNF-4 site mutation. While separate mutations of the HNF-4- and NF-I-binding sites both strongly decreased transcription, the double mutation almost completely abolished transcription, indicating the vital role of these two transcription factors for constitutive regulation of *Cyp2a5* gene expression. The crucial role of HNF-4 is emphasized further by the fact that the low levels of this transcription factor detected in primary hepatocytes are necessary and sufficient for *Cyp2a5* transcription. In intact liver expressing higher levels of HNF-4, the contribution of HNF-4 to transcriptional activation of *Cyp2a5* is probably also quantitatively even more important. The promoters of mouse *Cyp2a5* and human *CYP2A6* display significant sequence similarity (Figure 7). The HNF-4- and NF-I-binding sites are conserved in both genes, suggesting that these binding sites may also be important for the transcriptional regulation of *CYP2A6* in human liver.

In addition to HNF-4 and NF-I, several additional factors may participate in the regulation of *Cyp2a5*. Indeed, it has been shown that the DBP factor regulates *Cyp2a5* transcription in a circadian fashion [17]. Furthermore, induction of *Cyp2a5* transcription by such factors as phenobarbital and cAMP [4,18] may require the action of additional transcription factors. Nevertheless, HNF-4 and NF-I appear to be the major factors controlling the constitutive expression of *Cyp2a5* and play essential roles in hepatic expression.

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REFERENCES

- Nelson, D. R., Koymans, L., Kamataki, T., Stegeman, J. J., Feyereisen, R., Waxman, D. J., Waterman, M. R., Gotoh, O., Coon, M. J., Estabrook, R. W. et al. (1996) P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* **6**, 1–42

- 2 Negishi, M., Lindberg, R., Burkhardt, B., Ichikawa, T., Honkakoski, P. and Lang, M. (1989) Mouse steroid 15 α -hydroxylase gene family: identification of type II P-450_{15 α} as coumarin 7-hydroxylase. *Biochemistry* **28**, 4169–4172
- 3 Lang, M. A., Juvonen, R., Jarvinen, P., Honkakoski, P. and Raunio, H. (1989) Mouse liver P450Coh: genetic regulation of the pyrazole-inducible enzyme and comparison with other P450 isoenzymes. *Arch. Biochem. Biophys.* **271**, 139–148
- 4 Wood, A. W. and Conney, A. H. (1974) Genetic variation in coumarin hydroxylase activity in the mouse (*Mus musculus*). *Science* **185**, 612–614
- 5 Pellinen, P., Stenback, F., Rautio, A., Pelkonen, O., Lang, M. and Pasanen, M. (1993) Response of mouse liver coumarin 7-hydroxylase activity to hepatotoxins: dependence on strain and agent and comparison to other monooxygenases. *Naunyn Schmiedeberg's Arch. Pharmacol.* **348**, 435–443
- 6 Kojo, A., Heiskanen, R., Rytönen, A. L., Honkakoski, P., Juvonen, R. and Lang, M. (1991) Inducibility of P450Coh by pyrazole and its derivatives. *Biochem. Pharmacol.* **42**, 1751–1759
- 7 Hahnemann, B., Salonpää, P., Pasanen, M., Maenpää, J., Honkakoski, P., Juvonen, R., Lang, M. A., Pelkonen, O. and Raunio, H. (1992) Effect of pyrazole, cobalt and phenobarbital on mouse liver cytochrome P-450 2a-4/5 (Cyp2a-4/5) expression. *Biochem. J.* **286**, 289–294
- 8 Salonpää, P., Iscan, M., Pasanen, M., Arvela, P., Pelkonen, O. and Raunio, H. (1992) Cerium-induced strain-dependent increase in Cyp2a-4/5 (cytochrome P4502a-4/5) expression in the liver and kidneys of inbred mice. *Biochem. Pharmacol.* **44**, 1269–1274
- 9 Viitala, P., Posti, K., Lindfors, A., Pelkonen, O. and Raunio, H. (2001) cAMP mediated upregulation of CYP2A5 in mouse hepatocytes. *Biochem. Biophys. Res. Commun.* **280**, 761–767
- 10 Wastl, U. M., Rossmann, W., Lang, M. A., Camus-Randon, A. M., Grasl-Kraupp, B., Bursch, W. and Schulte-Hermann, R. (1998) Expression of cytochrome P450 2A5 in preneoplastic and neoplastic mouse liver lesions. *Mol. Carcinog.* **22**, 229–234
- 11 Raunio, H., Juvonen, R., Pasanen, M., Pelkonen, O., Paakko, P. and Soini, Y. (1998) Cytochrome P4502A6 (CYP2A6) expression in human hepatocellular carcinoma. *Hepatology* **27**, 427–432
- 12 Kirby, G. M., Chemin, I., Montesano, R., Chisari, F. V., Lang, M. A. and Wild, C. P. (1994) Induction of specific cytochrome P450s involved in aflatoxin B1 metabolism in hepatitis B virus transgenic mice. *Mol. Carcinog.* **11**, 74–80
- 13 Niemela, O., Parkkila, S., Juvonen, R. O., Viitala, K., Gelboin, H. V. and Pasanen, M. (2000) Cytochromes P450 2A6, 2E1, and 3A and production of protein-aldehyde adducts in the liver of patients with alcoholic and non-alcoholic liver diseases. *J. Hepatol.* **33**, 893–901
- 14 Camus-Randon, A. M., Raffalli, F., Berezat, J. C., McGregor, D., Konstandi, M. and Lang, M. A. (1996) Liver injury and expression of cytochromes P450: evidence that regulation of CYP2A5 is different from that of other major xenobiotic metabolizing CYP enzymes. *Toxicol. Appl. Pharmacol.* **138**, 140–148
- 15 Koskela, S., Hakola, J., Hukkanen, J., Pelkonen, O., Sorri, M., Saranen, A., Anttila, S., Fernandez-Salguero, P., Gonzalez, F. and Raunio, H. (1999) Expression of CYP2A genes in human liver and extrahepatic tissues. *Biochem. Pharmacol.* **57**, 1407–1413
- 16 Su, T., Sheng, J. J., Lipinskas, T. W. and Ding, X. (1996) Expression of CYP2A genes in rodent and human nasal mucosa. *Drug Metab. Dispos.* **24**, 884–890
- 17 Lavery, D. J., Lopez-Molina, L., Margueron, R., Fleury-Olela, F., Conquet, F., Schibler, U. and Bonfils, C. (1999) Circadian expression of the steroid 15 α -hydroxylase (Cyp2a4) and coumarin 7-hydroxylase (Cyp2a5) genes in mouse liver is regulated by the PAR leucine zipper transcription factor DBP. *Mol. Cell. Biol.* **19**, 6488–6499
- 18 Salonpää, P., Pelkonen, O., Kojo, A., Pasanen, M., Negishi, M. and Raunio, H. (1994) Cytochrome P4502A5 expression and inducibility by phenobarbital is modulated by cAMP in mouse primary hepatocytes. *Biochem. Biophys. Res. Commun.* **205**, 631–637
- 19 Lindberg, R., Burkhardt, B., Ichikawa, T. and Negishi, M. (1989) The structure and characterization of type I P-450_{15 α} gene as major steroid 15 α -hydroxylase and its comparison with type II P-450_{15 α} gene. *J. Biol. Chem.* **264**, 6465–6471
- 20 Torres-Padilla, M. E. and Weiss, M. C. (2003) Effects of interactions of hepatocyte nuclear factor 4 α isoforms with coactivators and corepressors are promoter-specific. *FEBS Lett.* **539**, 19–23
- 21 Schreiber, E., Matthias, P., Muller, 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
- 22 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
- 23 Maxam, A. M. and Gilbert, W. (1977) A new method for sequencing DNA. *Proc. Natl. Acad. Sci. U.S.A.* **74**, 560–564
- 24 Yokomori, N., Nishio, K., Aida, K. and Negishi, M. (1997) Transcriptional regulation by HNF-4 of the steroid 15 α -hydroxylase P450 (Cyp2a-4) gene in mouse liver. *J. Steroid Biochem. Mol. Biol.* **62**, 307–314
- 25 Schrem, H., Klempnauer, J. and Borlak, J. (2002) Liver-enriched transcription factors in liver function and development. Part I: the hepatocyte nuclear factor network and liver-specific gene expression. *Pharmacol. Rev.* **54**, 129–158
- 26 Hayashi, Y., Wang, W., Ninomiya, T., Nagano, H., Ohta, K. and Itoh, H. (1999) Liver enriched transcription factors and differentiation of hepatocellular carcinoma. *Mol. Pathol.* **52**, 19–24
- 27 Jackson, D. A., Rowader, K. E., Stevens, K., Jiang, C., Milos, P. and Zaret, K. S. (1993) Modulation of liver-specific transcription by interactions between hepatocyte nuclear factor 3 and nuclear factor 1 binding DNA in close apposition. *Mol. Cell Biol.* **13**, 2401–2410
- 28 Hertz, R., Magenheimer, J., Berman, I. and Bar-Tana, J. (1998) Fatty acyl-CoA thioesters are ligands of hepatic nuclear factor-4 α . *Nature (London)* **392**, 512–516
- 29 Watt, A. J., Garrison, W. D. and Duncan, S. A. (2003) HNF4: a central regulator of hepatocyte differentiation and function. *Hepatology* **37**, 1249–1253
- 30 Hayhurst, G. P., Lee, Y. H., Lambert, G., Ward, J. M. and Gonzalez, F. J. (2001) Hepatocyte nuclear factor 4 α (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. *Mol. Cell Biol.* **21**, 1393–1403
- 31 Kamiya, A., Inoue, Y. and Gonzalez, F. J. (2003) Role of the hepatocyte nuclear factor 4 α in control of the pregnane X receptor during fetal liver development. *Hepatology* **37**, 1375–1384
- 32 Akiyama, T. E. and Gonzalez, F. J. (2003) Regulation of P450 genes by liver-enriched transcription factors and nuclear receptors. *Biochim. Biophys. Acta* **1619**, 223–234
- 33 Jover, R., Bort, R., Gomez-Lechon, M. J. and Castell, J. V. (2001) Cytochrome P450 regulation by hepatocyte nuclear factor 4 in human hepatocytes: a study using adenovirus-mediated antisense targeting. *Hepatology* **33**, 668–675
- 34 Chaudhry, A. Z., Lyons, G. E. and Gronostajski, R. M. (1997) Expression patterns of the four nuclear factor I genes during mouse embryogenesis indicate a potential role in development. *Dev. Dyn.* **208**, 313–325
- 35 Apt, D., Liu, Y. and Bernard, H. U. (1994) Cloning and functional analysis of spliced isoforms of human nuclear factor I-X: interference with transcriptional activation by NF1/CTF in a cell-type specific manner. *Nucleic Acids Res.* **22**, 3825–3833
- 36 Kulkarni, S. and Gronostajski, R. M. (1996) Altered expression of the developmentally regulated NF1 gene family during phorbol ester-induced differentiation of human leukemic cells. *Cell Growth Differ.* **7**, 501–510
- 37 Mermod, N., O'Neill, E. A., Kelly, T. J. and Tjian, R. (1989) The proline-rich transcriptional activator of CTF/NF-I is distinct from the replication and DNA binding domain. *Cell* **58**, 741–753
- 38 Kruse, U. and Sippel, A. E. (1994) The genes for transcription factor nuclear factor I give rise to corresponding splice variants between vertebrate species. *J. Mol. Biol.* **238**, 860–865
- 39 Liu, Y., Bernard, H. U. and Apt, D. (1997) NF1-B3, a novel transcriptional repressor of the nuclear factor I family, is generated by alternative RNA processing. *J. Biol. Chem.* **272**, 10739–10745
- 40 Kruse, U. and Sippel, A. E. (1994) Transcription factor nuclear factor I proteins form stable homo- and heterodimers. *FEBS Lett.* **348**, 46–50
- 41 Chaudhry, A. Z., Vitullo, A. D. and Gronostajski, R. M. (1998) Nuclear factor I (NF1) isoforms differentially activate simple versus complex NF1-responsive promoters. *J. Biol. Chem.* **273**, 18538–18546
- 42 Cardinaux, J. R., Chapel, S. and Wahli, W. (1994) Complex organization of CTF/NF-I, C/EBP, and HNF3 binding sites within the promoter of the liver-specific vitellogenin gene. *J. Biol. Chem.* **269**, 32947–32956
- 43 Lin, C. J., Martens, J. W. and Miller, W. L. (2001) NF-1C, Sp1, and Sp3 are essential for transcription of the human gene for P450c17 (steroid 17 α -hydroxylase/17,20 lyase) in human adrenal NCI-H295A cells. *Mol. Endocrinol.* **15**, 1277–1293
- 44 Honkakoski, P. and Negishi, M. (1998) Regulatory DNA elements of phenobarbital-responsive cytochrome P450 CYP2B genes. *J. Biochem. Mol. Toxicol.* **12**, 3–9
- 45 Zhang, J., Zhang, Q. Y., Guo, J., Zhou, Y. and Ding, X. (2000) Identification and functional characterization of a conserved, nuclear factor 1-like element in the proximal promoter region of CYP1A2 gene specifically expressed in the liver and olfactory mucosa. *J. Biol. Chem.* **275**, 8895–8902
- 46 Zhang, J. and Ding, X. (1998) Identification and characterization of a novel tissue-specific transcriptional activating element in the 5'-flanking region of the CYP2A3 gene predominantly expressed in rat olfactory mucosa. *J. Biol. Chem.* **273**, 23454–23462