Satu Arpiainen

TRANSCRIPTIONAL REGULATION OF THE HEPATIC CYTOCHROME P450 2A5 GENE
SATU ARPIAINEN

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Academic dissertation to be presented, with the assent of the Faculty of Medicine of the University of Oulu, for public defence in the Auditorium of the Department of Pharmacology and Toxicology, on October 5th, 2007, at 12 noon

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Abstract
Cytochrome P450 (CYP) enzymes are the major metabolizers of xenobiotics, e.g. drugs, and
environmental toxins. Thus, changes in CYP expression have an important impact on drug
metabolism and susceptibility to chemical toxicity.

In the present study, the transcriptional mechanisms of both constitutive and inducible regulation
of the Cyp2a5 gene in mouse liver were investigated. Mouse primary hepatocyte cultures were used
as the main model system together with cell and molecular biology methods.

The key activation regions of the Cyp2a5 5' promoter were determined using reporter gene assays.
Two major transcription activation sites of the Cyp2a5 5' promoter, called the proximal and the distal,
were found. Transcription factors hepatocyte nuclear factor-4 (HNF-4) and nuclear factor I were
shown to bind to the proximal promoter. Aryl hydrocarbon receptor nuclear translocator (ARNT) and
upstream stimulatory factor bound to a common palindromic E-box element in the distal promoter
region. All three response elements were shown to be essential for constitutive expression of
CYP2A5 in murine hepatocytes. ARNT appeared to control Cyp2a5 transcription without a
heterodimerization partner suggesting active involvement of the ARNT homodimer in mammalian
gene regulation.

Aryl hydrocarbon receptor (AHR) ligands were shown to induce Cyp2a5 transcriptionally by an
AHR-dependent mechanism, and established Cyp2a5 as a novel AHR-regulated gene. The AHR
response element and the E-box, identified in these studies, were located near to each other and close
to a separately defined nuclear factor (erythroid-derived 2)-like 2 binding site in the distal region of
the Cyp2a5 promoter, suggesting cooperation between these elements.

Peroxisome proliferator-activated receptor gamma coactivator-1α was shown to up-regulate
Cyp2a5 transcription through coactivation of HNF-4α. This indicates that xenobiotic metabolism can
be regulated by modification of co-activation.

The present results show that CYP2A5 is regulated by several different cross-regulatory
pathways. The regulatory mechanisms involved in the transcription of the Cyp2a5 gene may also
control other CYP genes, especially the human ortholog CYP2A6, and may explain some of the
individual variations in the metabolism of xenobiotics.

Keywords: aryl hydrocarbon receptor, aryl hydrocarbon receptor nuclear translocator,
CYP2A5, cytochrome P450 enzyme system, hepatocyte nuclear factor 4, nuclear factor I,
peroxisome proliferator-activated receptor gamma coactivator-1alpha, upstream
stimulatory factors
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Abbreviations

3-MC  3-methylcholanthrene
AHR  aryl hydrocarbon receptor
AHRR  AHR repressor
ARE  antioxidant response element
ARNR  aryl hydrocarbon receptor nuclear translocator
bHLH  basic helix-loop-helix
bp  base pair(s)
C/EBP  CAAT/enhancer-binding protein
cAMP  cyclic adenosine monophosphate
CAR  constitutively active receptor
cDNA  complementary deoxyribonucleic acid
ChiP  chromatin immunoprecipitation
COH  coumarin 7-hydroxylase
CREB  cAMP response element binding protein
CTF  CAAT box transcription factor
CYP  cytochrome P450
DBD  DNA binding domains
DBP  D-site binding protein
E-box  enhancer box
EMSA  electromobility shift assay
HNF  hepatocyte nuclear factor
hnRNP  heterogeneous nuclear RiboNucleoProtein
HO  heme oxygenase
kb  kilo base pair(s)
LBD  ligand binding domain
LETF  liver-enriched transcription factor
Luc  luciferase
mRNA  messenger ribonucleic acid
NF  nuclear factor
NQO  NAD(P)H:quinone oxidoreductase
Nur2  nuclear factor (erythroid-derived 2)-like 2
OM  olfactory mucosa
P450  cytochrome P450
PAS  Per-ARNT-Sim homology
PCR  polymerase chain reaction
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>PGC</td>
<td>PPAR γ coactivator</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PXR</td>
<td>pregnane X receptor</td>
</tr>
<tr>
<td>Q-PCR</td>
<td>quantitative PCR</td>
</tr>
<tr>
<td>RXR</td>
<td>9-cis-retinoic acid receptor</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>SiRE</td>
<td>stress response element</td>
</tr>
<tr>
<td>TAD</td>
<td>transcription activation domain</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA box binding protein</td>
</tr>
<tr>
<td>TCDD</td>
<td>2,3,7,8-tetrachlorodibenzo-p-dioxin</td>
</tr>
<tr>
<td>TCPOBOP</td>
<td>1,4-bis[2-(3,5-dichloropyridyloxy)]benzene</td>
</tr>
<tr>
<td>TF</td>
<td>transcription factor</td>
</tr>
<tr>
<td>TSS</td>
<td>transcription start site</td>
</tr>
<tr>
<td>USF</td>
<td>upstream stimulatory factor</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>XRE</td>
<td>xenobiotic response element</td>
</tr>
</tbody>
</table>
List of original articles

This thesis is based on the following articles, which are referred to in the text by Roman numerals I to IV:


* equal contribution
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1 Introduction

The metabolism of xenobiotics is a critical liver function that allows the elimination of lipophilic, foreign compounds. Cytochrome P450 (abbreviated CYP or P450) enzymes are the major metabolizers of xenobiotics, including drugs, dietary constituents and environmental toxins. P450s are also elevated in amount and activity by xenobiotic compounds, and this phenomenon, called induction, is a common source of drug-drug interactions. (Pelkonen et al. 1998, Parkinson 2001.) Furthermore, alterations in CYP expression have a major impact on the toxicology and carcinogenicity of chemicals (Park et al. 1996). In addition to xenobiotics themselves, gender, tissue, and different physiological or pathological states can affect CYP expression and activity (Pelkonen et al. 2001). Besides elucidating the mechanisms behind the changes in CYP expression, it is important to determine the differences in CYP regulation between species so that results can be extrapolated from animals to man in drug testing and development.

Mouse cytochrome P450 2A5 (CYP2A5) and its human ortholog CYP2A6 metabolize several toxic substances, such as nicotine, nitrosamines and aflatoxins (Yamazaki et al. 1992, Camus et al. 1993, Kirby et al. 1994, Murphy et al. 2005). CYP2A5 also participates in the degradation of an endogenous substrate, bilirubin, that is a breakdown product of heme (Abu-Bakar et al. 2005). In addition to hepatocytes, CYP2A5 and CYP2A6 are expressed in some extrahepatic tissues, especially nasal mucosa (Kaipainen & Lang 1985, Honkakoski et al. 1993, Su et al. 1996, Koskela et al. 1999). The regulation of CYP2A5 is complex and in many cases different from that of the other major xenobiotic-metabolizing CYP enzymes, and both transcriptional and post-transcriptional mechanisms appear to be essential (Su & Ding 2004). CYP2A5 expression is strain-, gender-, and tissue-specific (Wood & Conney 1974, van Iersel et al. 1994, Su et al. 1998), and physiological conditions, such as energy homeostasis and circadian rhythm, affect the level of CYP2A5 (Lavery et al. 1999, Bauer et al. 2004). Coumarin 7-hydroxylase (COH) activity, catalyzed predominantly by CYP2A5 and CYP2A6 (Pelkonen et al. 1997), is inducible by a number of structurally diverse compounds, including nuclear receptor ligands, different hepatotoxins, and porphyrinogenic substances (Aida & Negishi 1991, Hahnemann et al. 1992, Salonpaa et al. 1995, Donato et al. 2000). In addition, the level of CYP2A5 increases in different types of liver damage and in tumours (Su & Ding 2004). Heterogeneous nuclear RiboNucleoProtein A1 (hnRNP A1) has been shown to mediate CYP2A5 induction by pyrazole post-transcriptionally.
(Raffalli-Mathieu et al. 2002) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is involved in the up-regulation of Cyp2a5 by cadmium (Abu-Bakar et al. 2007). Circadian expression of CYP2A5 in mouse liver is at least partly regulated by the transcription factor D-site binding protein (DBP) (Lavery et al. 1999). However, most of the molecular mechanisms behind constitutive, tissue specific expression of Cyp2a5 are not known and several pathways involved in the induction remain to be characterized.

The aim of this research was to elucidate the mechanisms behind both constitutive and inducible regulation of the hepatic CYP2A5 enzyme and thereby also characterize its role in the liver. CYP2A6 has been shown to respond to many of the regulators of CYP2A5, in a manner similar to it, and therefore these studies are assumed to give valuable information that may be applicable to humans as well.
2 Review of the literature

2.1 Cytochrome P450 (CYP) enzymes

Cytochrome P450 enzymes form a superfamily of heme-containing monooxygenases. They are involved in the biosynthesis of physiologically important compounds, such as steroids, fatty acids, eicosanoids, fat-soluble vitamins, and bile acids, and act as the major xenobiotic metabolizing enzymes. The P450 mediated metabolism of xenobiotics is an important part of the hepatic detoxification system and a major element in the modification and degradation of drugs (drug metabolism) that converts lipophilic chemical compounds into more readily excreted products. However, the metabolism of foreign chemicals can also produce toxic metabolites or reactive oxygen species, which may cause birth defects and other forms of toxicity, as well as tumour initiation and progression. (Park et al. 1995, Nebert & Russell 2002, Nebert & Dalton 2006.)

2.1.1 Nomenclature

The P450 enzymes were named after a unique optical absorption peak at wavelength 450 nm in the presence of a reducing agent and carbon monoxide (Klingenberg 1958). Omura and Sato (1962) reported that this pigment, P-450, from liver microsomes was a heme protein and hence called it a cytochrome. Since then, it has been discovered that P450s are expressed in almost every organism including animals, plants, fungi and bacteria (Nelson et al. 1996) and more than 6700 distinct cytochrome P450 sequences in over 700 families are known (Cytochrome P450 homepage, http://drnelson.utmem.edu/CytochromeP450.html, April 11, 2007). The diversity of CYP enzymes has led to the need to classify single CYP forms into families and subfamilies. The nomenclature is based on sequence similarity and evolutionary divergence: The amino acid sequences of the enzymes in the same family are at least 40% identical, and in the same subfamily over 55% identical. The abbreviation CYP is followed by an Arabic number indicating the family, a letter for the subfamily and an Arabic numeral representing the individual gene within the subfamily. Genes within a subfamily are numbered in order of discovery, regardless of species. Gene names are printed in italics. Human and most other species CYP gene names are written all in capital letters, whereas mouse and Drosophila Cyp gene
names are lower-case, except for the first letter. The cDNAs, RNAs and proteins in all species are printed in capital letters without italics. Pseudogenes are marked with ‘P’, or ‘ps’ in mouse and Drosophila, after the gene number. (Nelson et al. 1996.)

2.1.2 Structure and Function

Eukaryotes need CYP enzymes to produce sterols, which work as plasma membrane components and, in highly differentiated organisms, as hormonal mediators of development (Nebert 1991, Nebert & Russell 2002). Eukaryotic CYP enzymes are bound to membranes; most of them are attached to the endoplasmic reticulum (microsomes) but certain P450 forms are found in inner mitochondrial membranes. Small amounts of P450 enzymes have been detected in various sub-cellular compartments, such as the outer nuclear membrane and plasma membrane (Guengerich 2001). All mammalian P450 enzymes are formed from approximately 500 amino acids and a heme group (iron protoporphyrin IX). P450s catalyze mono-oxygenase reactions of lipophilic compounds by incorporating one atom of molecular oxygen into the substrate and one atom into water with the help of reducing equivalents from NADPH.

\[
\text{RH} + \text{O}_2 + \text{NADPH} + \text{H}^+ \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{NADP}^+
\]

In the endoplasmic reticulum, NADPH – cytochrome P450 reductase donates electrons to P450s. In mitochondria, ferredoxin and ferredoxin reductase transfer the electrons from NADPH to CYP enzymes. The attached hydroxyl group is used as a reactive group by other enzymes for further modifications. (Parkinson 2001.) Other reactions catalyzed by CYPs include epoxidations, peroxidations, deaminations, desulfurations, dehalogenations and reductive reactions (Guengerich 2001, Isin & Guengerich 2007).

Although the substrate specificities of several CYP enzymes have been studied extensively, the structural basis of substrate recognition was long unclear. Structure models were for a long time based on bacterial, water soluble P450 structures. Some very well conserved regions can be found in almost all CYPs, and the secondary structures in particular can be modelled with the help of bacterial CYPs. Substrate binding regions are usually specific for every CYP, but otherwise it is possible to recognize some functionally similar regions, such as the core region surrounding the heme group, the reductase recognizing region that takes part in redox reactions and the membrane binding region in mammalian
CYPs (Nelson & Strobel 1989). Williams et al. (2000) determined the first mammalian 3-D structure for a rabbit CYP2C5 in the year 2000 and thereafter several CYP structures have been published.

2.1.3 Endogenous substrate-metabolizing CYP enzymes

Cytochrome P450s participate in several important biosynthesis pathways and a number of their endogenous substrates are important signalling molecules that are involved in the regulation of homeostatic processes (Nebert & Russell 2002, Nebert & Dalton 2006). P450s involved in the metabolism of endogenous compounds usually have very selective substrate specificities (listed in Table 1) and they are highly conserved between species (Nelson 1999).

Table 1. Functions of CYP enzymes involved in the metabolism of endogenous compounds (modified from Lewis et al. 2004 and Nebert et al. 2006).

<table>
<thead>
<tr>
<th>CYP family</th>
<th>Substrates/function</th>
</tr>
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<tbody>
<tr>
<td>CYP4</td>
<td>Fatty acids</td>
</tr>
<tr>
<td>CYP5</td>
<td>Thromboxane A2 synthesis</td>
</tr>
<tr>
<td>CYP7</td>
<td>Cholesterol and bile acid synthesis</td>
</tr>
<tr>
<td>CYP8</td>
<td>Prostacyclin and bile acid synthesis</td>
</tr>
<tr>
<td>CYP11</td>
<td>Steroidogenesis</td>
</tr>
<tr>
<td>CYP17</td>
<td>Steroid 17-hydroxylase, 17/20-lyase</td>
</tr>
<tr>
<td>CYP19</td>
<td>Estrogen aromatization</td>
</tr>
<tr>
<td>CYP21</td>
<td>Steroid 21-hydroxylase</td>
</tr>
<tr>
<td>CYP24</td>
<td>Vitamin D3 24-hydroxylase</td>
</tr>
<tr>
<td>CYP26</td>
<td>Retinoic acid hydroxylation</td>
</tr>
<tr>
<td>CYP27</td>
<td>Bile acid biosynthesis, vitamin D3 hydroxylations</td>
</tr>
<tr>
<td>CYP39</td>
<td>24-hydroxycholesterol 7-hydroxylase</td>
</tr>
<tr>
<td>CYP46</td>
<td>Cholesterol 24-hydroxylase in the central nervous system</td>
</tr>
<tr>
<td>CYP51</td>
<td>Lanosterol 14-demethylase</td>
</tr>
</tbody>
</table>

Although members in families CYP1-3 are considered to be xenobiotic metabolizers, they also have endogenous substrates, such as steroids, bile acids, fatty acids, eicosanoids, and retinoids (Lewis 2004, Nebert & Dalton 2006).

2.1.4 Xenobiotic-metabolizing CYP enzymes

Xenobiotic substrates of CYP enzymes include both natural and manufactured chemicals, such as pharmaceuticals, industrial chemicals, pesticides, pollutants,
alkaloids, secondary plant metabolites, and toxins produced by moulds, plants, and animals. In the reactions of xenobiotic metabolism, lipophilic substances are biotransformed into more water-soluble metabolites that can be excreted from the body. These reactions are usually divided into two phases; I and II. (Parkinson 2001.) CYP enzymes comprise a major proportion of all known phase I xenobiotic metabolizing enzymes (Lewis 2004). Phase I reactions are chemical modifications that add or reveal a functional group, which can be used to attach a conjugate. Many P450s add a hydroxyl group in the Phase I step of xenobiotic metabolism. The hydroxyl group then serves as the site for further modifications in Phase II metabolism. Phase II biotransformation reactions include glucuronidation, sulfation, acetylation, methylation, and conjugation with glutathione and amino acids. Conjugation converts the compound being metabolised into a highly hydrophilic form that can be readily excreted in urine through the kidneys or with bile out of the body (Parkinson 2001).

The P450s that metabolize foreign chemicals are almost exclusively members of families 1-3 and to a lesser degree CYP4 (Waxman 1999). Xenobiotic metabolizing CYP enzymes are expressed predominantly in the liver, but they are also found in several extrahepatic tissues, such as the gastrointestinal tract, lung, kidney, olfactory epithelia, skin and central nervous system (Gonzalez & Lee 1996). A characteristic of xenobiotic metabolizing enzymes is their wide and partly over-lapping substrate specificity. CYP1s are typically active in the metabolism of aromatic hydrocarbons, whereas substrate specificities within members of subfamilies CYP2 and CYP3 vary noticeably from each other. CYP3A4, CYP2Cs, CYP2D6, and CYP1A2 appear to be the most important drug-metabolizing P450 enzymes in human liver and nearly 90% of the clinically used drugs metabolized by cytochrome P450s are substrates for these enzymes. (Lewis 2004.) The expression of P450 genes between species differs substantially, and the xenobiotic metabolizing enzymes in particular often have distinct functions in different species (Nelson 1999).

A feature that is essential for the function of xenobiotic metabolizing P450 enzymes is their adaptive increase, in other words induction, in response to foreign chemicals, which further elevates the elimination rate of xenobiotics from the body. Induction is also a major source of adverse drug interactions, since changes in CYP enzyme activity may affect the metabolism and clearance (the rate at which a substance is removed from the body) of various drugs. For drugs that are active in their parent form, induction may increase the elimination of the drug and decrease its pharmacological effect. For prodrugs, compounds that
require metabolic activation and whose effects are produced by the active metabolites, enhanced pharmacodynamic effects may be expected. (Pelkonen et al. 1998.) P450s may also convert xenobiotics to chemically active metabolites that can cause various forms of toxicity, including cellular necrosis, hypersensitivity, teratogenicity, and carcinogenicity (Park et al. 1995). In addition to xenobiotics, the nutritional and pathophysiological stages of the organism can also cause alterations in CYP expression (Walter-Sack & Klotz 1996, Morgan 1997).

Fig. 1. Scheme of the metabolism of xenobiotics (modified from Nebert & Dalton 2006).

2.1.5 Polymorphisms

Mutations in several CYP genes involved in the biosynthesis of endogenous compounds are responsible for inborn errors in metabolism, which contribute to several clinically relevant hereditary diseases. Many of the xenobiotic-metabolizing CYP genes also are highly polymorphic, and variations can be relevant to drug or toxin metabolism and disease susceptibility. (Nebert & Russell 2002.) For example, over 50 alleles or haplotypes of the CYP2A6 gene and several additional single nucleotide polymorphisms have been found (CYP2A6 allele nomenclature, www.cypalleles.ki.se/cyp2a6.htm). Mutations have also been detected in the regulatory DNA. In humans, the allele nomenclature uses the * symbol for alleles (e.g. CYP2A6*2). A database for human CYP allelic variants is maintained by the Human Cytochrome P450 Allele Nomenclature Committee (www.cypalleles.ki.se).
2.2 CYP2A enzymes

CYP2As have received relatively little attention compared to other xenobiotic metabolizing P450 families, since human CYP2As have restricted substrate specificities and their percentage of the total CYP content in human liver is relatively small. However, interest in them has increased, especially after CYP2A6 and CYP2A13 were shown to be high-affinity metabolizers of nicotine and several carcinogenic nitrosamines.

2.2.1 Evolution and chromosomal localization

Xenobiotic metabolizing P450s probably evolved from the steroidogenic P450s and then began to oxidatively degrade dietary chemicals that could not be easily eliminated from animals unless they were converted to more hydrophilic derivatives (Nelson & Strobel 1987, Nebert et al. 1989, Gonzalez & Nebert 1990). The particular diversity of the CYP2 family has been shown to be associated with the diversification of plants (Nelson & Strobel 1987). Coumarin and related phenylpropanoid lactones are toxins found in many plants, suggesting that the CYP2A enzyme evolved in mammals to counter the toxicity of plant chemicals (Gonzalez & Nebert 1990).

According to the human and mouse genome projects there are 57 putatively functional CYP genes and 58 pseudogenes in human, and the respective figures for mouse are 102 functional genes and 88 pseudogenes. CYP2 is the largest P450 family in mammals and the six members of the mammalian CYP2ABFGST cluster are uniquely intermingled in the same chromosomal region. The close proximity of so many related genes has given rise to some gene recombinations, and alternative splicing of these genes adds another layer of complexity to the cluster. (Nelson et al. 2004.) The mouse CYP2 cluster includes 22 loci from the same six CYP2 subfamilies (Cyp2a, Cyp2b, Cyp2f, Cyp2g, Cyp2s and Cyp2t) that are found in the human cluster. Twelve of these loci are functional genes and 10 are pseudogenes. Some parts of the mouse and human gene clusters are similarly arranged, but different series of duplication events have produced the gene organizations in the two species. (Hoffman et al. 2001, Wang et al. 2003.) The mouse Cyp2a-t cluster is located on chromosome 7 and this region is syntenic to most of the q arm of human chromosome 19 (Kim et al. 2001a) (Fig. 2.). The entire region is in the opposite orientation relative to the centromere in the two
species (Wang *et al.* 2003). CYP2 genes typically have nine exons, and the intron-exon boundaries are preserved in location and phase (Nelson *et al.* 2004).

![Diagram of mouse Cyp2a-t gene cluster on chromosome 7](modified from Wang *et al.* 2003. A region of 300 kb in the middle of the cluster (dashed line) contains no Cyp genes.

### 2.2.2 Species, tissue distribution and substrate specificities

Phillips *et al.* (1985a) were first to publish the isolation and part of the sequence of CYP2A-cDNA. Since then, several members of the CYP2A family from different species have been characterized and shown to have significant differences in substrate specificity, tissue distribution, and regulation (Fernandez-Salguero & Gonzalez 1995, Chang & Waxman 1996, Su & Ding 2004). CYP2A enzymes can be distinguished by their preference for two types of model substrates; coumarin and 3-ketosteroids (Pearce *et al.* 1992), but their substrates also include several other compounds, particularly different toxic xenobiotics. Besides the liver, CYP2A enzymes are expressed in high levels in the olfactory tissues of several animal species, and may participate in the metabolism of odorants and the processing of olfactory signals (Su *et al.* 1996, Ding & Kaminsky 2003, Ling *et al.* 2004a). Lewis and co-workers led the way in the use of comparative models based on the crystal structures of bacterial or other mammalian CYP forms to study CYP2A1, -2A4, -2A5 and -2A6 protein structures (Lewis & Lake 1995, Lewis & Lake 2002, Lewis *et al.* 2003). Recently, the CYP2A6 structure was determined by X-ray crystallography (Yano *et al.* 2005).

#### Human CYP2As

Prior to the 1993 nomenclature update (Nelson et al. 1993), CYP2A6 and CYP2A7 were called CYP2A3 and CYP2A4, respectively, based on sequence homology with rat CYP2A1 and CYP2A2. CYP2A6 is expressed in the liver, constituting 1-13% of total human liver CYP content (Yun et al. 1991, Shimada et al. 1994, Imaoka et al. 1996). Lower levels of CYP2A6 expression have been observed in the oesophagus, skin, and tissues of the respiratory tract, including the nasal mucosa, trachea and lung (Koskela et al. 1999, Janmohamed et al. 2001, Saeki et al. 2002). The CYP2A7 gene produces mRNA in the liver, but no catalytic activity for the protein has been determined (Yamano et al. 1990, Ding et al. 1995). The highest levels of CYP2A13 are expressed in respiratory tissues, including the nasal mucosa, lung, and trachea (Koskela et al. 1999, Su et al. 2000). Prenatal expression of CYP2A enzymes is low or absent in human liver (Hakkola et al. 1994), but some CYP2As can be detected in fetal nasal tissues (Gu et al. 2000a, Chen et al. 2003). No gender differences have been shown in the hepatic expression of CYP2A6 (Parkinson et al. 2004), but nicotine metabolism, which is mediated mainly by CYP2A6, is faster in women than in men (Benowitz et al. 2006).

Human 2As do not possess the steroid 15α- and 7α-hydroxylase activities characteristic of rodent CYP2As. CYP2A6 is the major coumarin 7-hydroxylase (Miles et al. 1990, Waxman et al. 1991, Pelkonen et al. 1997) and nicotine C-oxidase in human liver (Cashman et al. 1992, Messina et al. 1997, Yamazaki et al. 1999). Nicotine is primarily metabolized to cotinine, and cotinine is further metabolized to trans-3’-hydroxycotinine, also by CYP2A6 (Nakajima et al. 1996). The other known substrates for CYP2A6 include several pharmaceuticals, such as halotane, losigamone, and SM-12502 (Nunoya et al. 1996, Spracklin et al. 1996, Torchin et al. 1996), and many toxins and carcinogens, including aflatoxin B1 and tobacco-related N-nitrosamines (Yamazaki et al. 1992, Camus et al. 1993, Tiano et al. 1994, Liu et al. 1996, Patten et al. 1996, Su et al. 2000). Dietary nitrosamines that are activated by CYP2A6 represent a potential factor for chemical carcinogenesis (Sweeney et al. 2002). There are also numerous xenobiotics, nutritional substances and different disease states that inhibit coumarin 7-hydroxylase in human liver (Draper et al. 1997, Pelkonen et al. 2000). CYP2A6 is highly polymorphic and some alleles have been linked to smoking behaviour and susceptibility to tobacco smoke induced cancers (Pianezza et al. 1998, Raunio et al. 2001, Tyndale & Sellers 2002). CYP2A13 shows overlapping substrate specificity with CYP2A6, also catalyzing the

Murine CYP2As

There are three functional members of the mouse CYP2A family: CYP2A4 (testosterone 15α-hydroxylase), CYP2A5 (coumarin 7-hydroxylase), and CYP2A12 (testosterone 7α-hydroxylase) (Burkhart et al. 1985, Juvonen et al. 1988, Iwasaki et al. 1993). Wang et al. (2003) discovered a new full-length Cyp2a locus, located between the Cyp2a5 and Cyp2a12 genes, named Cyp2a22. There are also partial Cyp2a pseudogenes – Cyp2a20-ps, Cyp2a23-ps and Cyp2a21-ps (Wang et al. 2003). Females in several inbred mouse strains have high hepatic CYP2A4 levels, whereas its expression is transcriptionally repressed by pulsatile growth hormone in males (Burkhart et al. 1985, Aida & Negishi 1993). CYP2A4 is also expressed in the kidney, and at low levels in the olfactory mucosa, where sex differences are not observed (Su et al. 1996). No expression of CYP2A4 and CYP2A5 proteins have been detected in the breast, bone marrow, testis, prostate, ovary, or uterus (Su et al. 1998). The prenatal expression of CYP2A4/5 also appears to be very low or absent in mouse (Choudhary et al. 2003). CYP2A5 expression and substrates are reviewed in detail in the following chapter, 2.3 CYP2A5. CYP2A12 is expressed in the liver (Su et al. 1996). The expression sites for CYP2A22 have not been determined yet.

Rat CYP2As

In rats, testosterone metabolizing CYP2A1 (steroid 7α-hydroxylase) and CYP2A2 (testosterone 15α-hydroxylase), are predominantly expressed in the liver, whereas CYP2A3, which is a testosterone 15α-hydroxylase and a relatively weak coumarin 7-hydroxylase, is expressed in the lung, olfactory mucosa and intestine, but not in the liver (Nagata et al. 1987, Matsunaga et al. 1988, Kimura et al. 1989, Su et al. 1996). CYP2A1 is female-predominant and CYP2A2 is male specific (Nagata et al. 1987, Matsunaga et al. 1988), and they are expressed at different developmental stages. CYP2A1 is detected within 1 week of birth in both male and female rats, but is suppressed at puberty in males, in association with activation of CYP2A2. (Gonzalez et al. 1989, Imaoka et al. 1991.) No
gender difference has been detected in the expression of CYP2A3 (Su et al. 1996).

**CYP2As in other species**

The CYP2A family has also been characterized in many other mammalian species, such as rabbit (CYP2A10 and CYP2A11), Syrian and Chinese hamster (CYP2A8, CYP2A9, CYP2A14, CYP2A15, CYP2A16 and CYP2A17), cow, pig, dog and non-human primates (Fernandez-Salguero & Gonzalez 1995, Su & Ding 2004) (Cytochrome P450 homepage, http://drnelson.utmem.edu/CytochromeP450.html).

### 2.3 CYP2A5

The mouse Cyp2a5 locus was first identified as a strain difference in coumarin hydroxylase activity between the liver microsomes of the DBA/2 and C57BL/6 strains (Wood & Conney 1974, Lush & Andrews 1978, Wood & Taylor 1979, Wood 1979). The purification (Kaipainen et al. 1984, Juvonen et al. 1988) and cDNA cloning (Negishi et al. 1989) of a mouse P450 established that the coumarin 7-hydroxylase is a member of the CYP2A family. Lindberg et al. (1992) characterized two forms of CYP2A5, CYP2A5-COh\text{high} and CYP2A5-COh\text{low} that are allelic variants with high and low coumarin hydroxylase activities, respectively. These allelic variants differ only in a single nucleotide associated with the codon alanine 117 in CYP2A5-COh\text{low} and valine 117 in CYP2A5-COh\text{high} (Lindberg et al. 1992). The Cyp2a5 and Cyp2a4 genes are extremely similar, and they show 98% and 96% homology in exon and intron sequences, respectively. These two enzymes have only 11 amino acid substitutions in their protein sequences, yet these cause their substrate preferences to be totally different. The substrate specificity of CYP2A5 is converted from coumarin 7- to testosterone 15\alpha-hydroxylase activity by the substitution of phenylalanine at position 209 with leucine (Lindberg & Negishi 1989). *Mus Musculus* has both Cyp2a5 and Cyp2a4 genes, whereas its close relative *Mus spretus* appears to have two nearly identical Cyp2a5-like loci. Therefore, Aida et al. (1994) have suggested that Cyp2a4 was formed by the alteration of a critical residue in a previously duplicated copy of the Cyp2a5 gene.
2.3.1 Expression

The highest levels of CYP2A5 are found in the liver, kidney, and olfactory mucosa (OM). CYP2A5 is also expressed to a lesser extent in the lung. (Kaijainen & Lang 1985, Honkakoski et al. 1993, Su et al. 1996.) However, it is problematic to estimate the percentage of the basal level of CYP2A5 compared to other CYP enzymes in murine liver, since CYP2A5 expression is dependent on the mouse strain, gender, and circadian rhythm, and due to difficulties in separately measuring CYP2A5 and CYP2A4 mRNAs or proteins (see 2.6.1 Methodological issues in the study of CYP2A5 regulation). In OM microsomes, CYP2A5 and CYP2G1 account for more than 30% of the total P450 content (Ling et al. 2004a).

In addition to the endoplasmic reticulum, CYP2A5 can also be found in high levels in mitochondrial membranes (Honkakoski et al. 1988). Mitochondrial CYP2A5 appears to have the same molecular weight (56.7 kDa) and immunochemical and catalytic properties as the corresponding microsomal enzyme, but very little is known about its function and regulation (Honkakoski et al. 1988).

2.3.2 Substrates

CYP2A5 is the major oxidase of the plant alkaloid coumarin (Pelkonen et al. 1997), and this activity is used as a specific marker to measure the functionality of CYP2A5 (Aitio 1978). CYP2A5 also metabolizes several other xenobiotic and potentially harmful substrates, such as nicotine, nitrosamines, aflatoxins and acetaminophen (Camus et al. 1993, Kirby et al. 1994, Genter et al. 1998, Murphy et al. 2005). According to recent results from Abu-Bakar et al. (2005), CYP2A5 participates in the degradation of an endogenous substrate, bilirubin, that is a breakdown product of heme. Several compounds also suppress CYP2A5 activity by inhibiting its substrate binding, such as pilocarpine and methoxalen (Kinonen et al. 1995, Damaj et al. 2007).

2.3.3 Orthologs

It has been proposed that mouse CYP2A5 is a true ortholog of human CYP2A6, as they have very similar amino acid sequences (84% identical), they are located at similar positions within the two chromosomal clusters and their enzyme
activities are similar (Raunio et al. 1988a, Wang et al. 2003). The regulation of both enzymes also seems to be alike and they respond in a comparable way to many inducers (Donato et al. 2000). Rat CYP2A3 is also considered orthologous to CYP2A5, although CYP2A3 is not expressed in rat liver or kidney (Kimura et al. 1989).

2.4 Mechanisms of CYP regulation

The expression of a gene can be controlled at many levels. Most regulatory studies on P450 enzymes have focused on transcriptional control, but several post-transcriptional mechanisms of CYP regulation have also been elucidated.

2.4.1 Transcriptional regulation

The expression of any gene is achieved principally through the interaction of transcription factor proteins (trans-acting factors) with certain nucleotide sequences (cis-acting elements) located in the control regions of the gene. Usually these control regions, or promoters, are located near to or upstream from the actual coding DNA region, but regulatory elements can be located even tens of thousands of bases away from the transcription start site (TSS). Eukaryotic promoters usually have core elements, such as the TATA box or other sequences that specify the TSS and regulatory elements, which can either enhance or repress transcription. (Alberts et al. 2002.) The core elements of the promoter form the site for assembly of the transcription preinitiation complex. This consists of RNA polymerase II and other components of the transcription machinery, including the general transcription factors TATA box binding protein (TBP), transcription factor IIA (TFIIB), TFIID, TFIIE, TFIIF and TFIH (Roeder 1996, Hahn 2004). Typically, gene-specific transcription factors bind near to the TSS and recruit different coregulators, the mediator complex and the preinitiation complex to the core promoter. Furthermore, RNA polymerase II elongation factors attach to the multiprotein complex to enable mRNA transcription. (Albets et al. 2002, Hahn 2004, Li et al. 2007). The binding of a specific combination of transcription factors to DNA activates or inhibits the preinitiation complex, by either direct or indirect interaction, and confers cell- and time-specific mRNA synthesis (Duncan et al. 1998, Wolberger 1999).

Transcription factors can be classified by their DNA binding domains (DBD), which include basic, zinc-coordinating, helix-turn-helix and beta-scaffold
domains. In addition, there are some other forms of DNA-binding proteins that are not included in these classes. (Pabo & Sauer 1992, Stegmaier et al. 2004.) Transcription factors are usually highly conserved among mammals, and they generally bind the same ligands and similar response elements in different species. However, some receptors have been shown to have different binding affinities in different species, and their tissue distribution may also be different (Jones et al. 2000).

Transcription factors are regulated by growth factors and cytokines, hypoxia, oxidative stress, lipids and polyunsaturated fatty acids, light, stress, mechanical forces such as stretching, or xenobiotics. These signals can control transcription factor activity in several different ways, including transcription factor synthesis, activation by ligand binding, protein modifications such as phosphorylation, stimulation of nuclear entry by removal of an inhibitory protein that would otherwise prevent the regulatory protein from entering the nucleus, and release of a transcription factor from a cytosolic membrane. In the nucleus, transcription factors recognize their response elements in DNA sequences and bind to them usually as homo- or heterodimers. (Alberts et al. 2002.)

Gene transcription is essentially dependent on the chromatin environment. Genomic DNA is organized into chromatin to pack it in a dense manner into the nucleus. Chromatin is composed of nucleosome core particles formed from 146 bp of DNA coiled around a histone octamer, which includes two copies of each of the four histones H2A, H2B, H3 and H4. Neighbouring nucleosomes are associated via the H1 linker histone, which facilitates further compaction. (Kornberg & Thomas 1974, Spencer & Davie 1999.) Due to packaging of the DNA, only a small percentage of it is available to transcription factors. Therefore, different chromatin modifications, including acetylation or ubiquitination of histone lysines and phosphorylation of histone serines and threonines, DNA methylation, and ATP-dependent chromatin remodelling play important roles in the regulation of transcription. (Bradbury 1992, Kornberg & Lorch 1999, Spencer & Davie 1999, Li et al. 2007.) Transcriptional coactivators and corepressors function by catalyzing the enzymatic modification of histones to modulate the repression by nucleosomes (Giordano & Avantaggiati 1999, Rosenfeld et al. 2006).
Cytosine residues in the sequence 5’CpG are often post-synthetically methylated in animal genomes. The methyl-CpG-binding proteins (MeCP1 and MeCP2) interact specifically with methylated DNA and mediate transcriptional repression. Genes are usually silenced by methylation in parental-specific imprinting and in X chromosome inactivation in female mammals, but methylation has also been shown to play a role in tissue-specific regulation and in certain disease states. (Robertson & Wolffe 2000.)

2.4.2 Post-transcriptional regulation

Post-transcriptional mechanisms include RNA precursor processing, mRNA stabilization or degradation, control of translational efficiency, and post-translational events, such as protein stabilization and modification (Porter & Coon 1991, Aguiar et al. 2005).

Expressed mRNA can be regulated by RNA-binding proteins, such as heterogeneous nuclear ribonucleoproteins (hnRNPs), which mediate processing, nucleocytoplasmic transport and stabilization of the mRNA (Day & Tuite 1998, Wilkinson & Shyu 2001). For instance, mouse CYP2A5 and human CYP2A6 are regulated by mRNA stabilization due to binding of the hnRNP A1 to the 3’
poly(A) tail of their mRNA (Glisovic et al. 2003a, Christian et al. 2004). Another example is that two nuclear proteins, regulated by 3-methylcholanthrene, bind to the poly(U)motif in the 3'-UTR of mouse CYP1A2 (Raffalli-Mathieu et al. 1997). Recent studies showed that insulin-mediated destabilization of rat CYP2E1 and CYP2B1 mRNAs is mediated by a 60-kDa cytosolic protein that binds to a 16-nucleotide sequence in the 5' region of the mRNA (Moncion et al. 2002, Truong et al. 2005).

Post-translational modifications can modulate the enzyme activity, target the protein to specific cellular compartments, or tag it for proteosomal degradation. These covalent modifications include cleavage of signal peptides, formation of disulfide bonds, and numerous modifications of amino acid residues, such as phosphorylation, nitration, glycosylation, methylation, sulfation, acetylation, and prenylation. Ubiquitination leads to the degradation of a number of P450s by the 26S proteosomal pathway (Aguiar et al. 2005). Post-translational regulation of CYP2E1 has been studied extensively: CYP2E1 is induced by its substrates acetone, ethanol, and pyrazole, which cause protein stabilization by their presence (Gonzalez 2007).

### 2.5 Factors involved in transcriptional regulation of xenobiotic-metabolizing CYP enzymes in the liver

Regulation of P450 enzymes is usually specific for species, strain, and tissue. Many of the CYP genes are also regulated in a sex-dependent manner in the liver, and gender specificity is particularly striking in rodents (Wiwi et al. 2004). P450s that participate in different biosynthesis pathways are typically regulated by hormones and growth factors. Xenobiotic-metabolizing P450s are also regulated by different endogenous factors, but in particular, they show remarkable induction by foreign compounds.

The transcription factors involved in the regulation of xenobiotic-metabolizing P450s are usually divided into constitutive regulators of transcription and mediators of induction by xenobiotics. Typically, ubiquitously expressed and tissue-specific factors that are not activated by exogenous ligands belong to constitutive regulators, and xenobiotic ligand binding receptors are thought to mediate the induction. However, constitutive regulators are also controlled by ligand-activated cell-surface receptors, such as growth factor and cytokine receptors, which transmit gene activation signals to the nucleus via the
phosphorylation of a family of signal transducer and activator of transcription (STAT) proteins. (Darnell et al. 1994, Paukku & Silvennoinen 2004.)

The tissue selective expression of genes is usually regulated by the availability of the right combination of transcription factors and their coregulators, and the accessibility of the binding sites in the chromatin structure. Alternative promoters may also be used in different tissues (Mendelson et al. 2005). Eventually, complex regulatory networks are formed by ubiquitously expressed, tissue-specific and ligand-binding transcription factors.

Besides induction of P450s, the repression of the transcription of these enzymes also plays an important role in their regulation. However, the mechanisms involved in suppression of P450 expression are poorly understood. This is partly due to difficulties in measuring the down-regulation of enzymes whose basal level of expression may be nearly undetectable in dedifferentiated in vitro cell models or systems.

2.5.1 Liver-enriched transcription factors

The liver is the major site for P450 mediated metabolism. The transcription rate of genes encoding liver-specific proteins is distinctly higher in hepatocytes compared to other cell types (Powell et al. 1984). The regulatory sequences of these genes typically contain a combination of some or all of the so-called liver-specific shared motifs (Benvenisty & Reshef 1991, Aran et al. 1995) and it seems to be the combination rather than a single element that is required for liver-specific gene expression. Several liver-enriched transcription factors (LETFs) have been identified through these shared motifs (De Simone & Cortese 1991, Lai & Darnell 1991, Aran et al. 1995), including hepatocyte nuclear factor (HNF) -1, HNF-3, HNF-4, HNF-6, CAAT/enhancer-binding proteins (C/EBPs) and D-site binding protein (Cereghini 1996, Schrem et al. 2004). LETFs are not exclusively expressed in the liver, but also play critical roles elsewhere, such as in pancreatic β-cells (Vaisse et al. 1997). The coordinated activity of LETFs is typical for the developmental regulation of hepatic genes (Costa et al. 2003). LETF expression and activity is determined by a complex hierarchal series of transcriptional and post-transcriptional mechanisms. LETFs can regulate each other’s expression, forming reciprocal regulatory loops; for example HNF4α positively regulates HNF-1α, and HNF1α in turn negatively regulates HNF4α (Tian & Schibler 1991, Kuo et al. 1992, Bailly et al. 2001, Wiwi et al. 2004). Besides the combination of LETFs, cooperation between LETFs and more ubiquitously expressed
transcription factors is usually needed for liver-specific transcription (Gonzalez & Lee 1996).

**HNFs**

Hepatocyte nuclear factor (HNF) families 1, 3, 4 and 6 have different DNA-binding motifs and they all contain several submembers. HNF-1α is a homeodomain containing transcription factor involved in the regulation of several hepatic genes, including genes essential for hepatocyte differentiation (Courtois et al. 1987, Frain et al. 1989, Akiyama & Gonzalez 2003). Cheung et al. (2003) studied the expression CYP genes in the livers of mice lacking HNF-1α and found both reducing and inducing effects on CYP expression. The HNF-3 subfamily contains three proteins α, β and γ [also called forkhead box (Fox) A1, A2 and A3 proteins, respectively] that use a winged helix motif for DNA binding, whereas HNF-6 belongs to the one-cut class of homeodomain proteins. HNF3 and HNF6 are both relevant to liver function (Schrem et al. 2002), and it has been proposed that they act in a synergistic manner to positively regulate certain female-specific CYP genes (Delesque-Touchard et al. 2000).

HNF-4 appears to be one of the most crucial HNFs for liver development and function based on its high conservation among species and the lethality of HNF-4 knockout mice. HNF-4 is involved in critical liver functions, including the regulation of several hepatic genes involved in liver development, the metabolism of cholesterol, fatty acids and glucose, the synthesis of blood coagulation factors, hepatitis B infections and detoxification mechanisms. (Sladek 1993, Chen et al. 1994b, Stoffel & Duncan 1997, Hayhurst et al. 2001, Watt et al. 2003, Odom et al. 2004.)

HNF-4 subfamily members include HNF-4α, HNF-4β and HNF-4γ. HNF-4s are nuclear receptors, but they can activate transcription in the absence of exogenous ligands (Sladek 1993). Hertz et al. (1998) suggested that fatty acyl-CoA thioesters function as ligands of HNF-4α. It was later shown by crystallization studies that fatty acids are endogenous ligands for HNF-4α and γ (Dhe-Paganon et al. 2002, Wisely et al. 2002). HNF-4 forms a homodimer (Jiang et al. 1995), and binds to its response element, which contains direct repeats of the hexamer AGGTCA separated by one base. HNF-4 activity is regulated by several different mechanisms, including interactions with other transcription factors and several coregulators, and post-transcriptional modifications, such as phosphorylation and acetylation (Mietus-Snyder et al. 1992, Schrem et al. 2002,
Rhee et al. 2003). Seven distinct HNF-4α splicing variants have been identified in human and mouse, but their impact on regulation is still mostly unknown (Schrem et al. 2002).

The promoters of several CYP family members were found to include so-called HepG2 cell specific P450 factor I (HPF-I) elements that were further shown to be recognized by HNF-4 (Chen et al. 1994a). In addition to numerous members of the cytochrome P450 family (Chen et al. 1994a, Yokomori et al. 1997, Jover et al. 2001, Akiyama & Gonzalez 2003), HNF-4 controls the expression of other transcription factors and coregulators, which further regulate P450s, like pregnane X receptor (PXR) and peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) (Kamiya et al. 2003, Wiwi et al. 2004). It has also been shown to cooperate with other factors, as in the induction of CYP3A4 by constitutive androstane receptor (CAR) and PXR (Tirona et al. 2003). HNF-4α is also likely to regulate the sex-dependence of some P450 genes, since several male- and female-predominant CYPs are significantly down-regulated in HNF4α-deficient mouse liver (Wiwi et al. 2004).

**C/EBP**

CAAT/enhancer-binding proteins (C/EBPs) have a DNA binding basic domain and a leucine zipper dimerization domain. Members of this family include C/EBPα, -β, -γ, -δ, -ε, and -ζ. C/EBPα and β are the predominant forms in adult hepatocytes. C/EBP dimerization is a prerequisite for DNA binding and C/EBPs form both homo- and heterodimers. Dimers bind selectively to the CCAAT motif of target gene promoters. (Lekstrom-Himes & Xanthopoulos 1998.) C/EBPs are involved in the regulation of the cellular differentiation of hepatocytes and energy metabolism, and they control several P450 genes, such as CYP2A6, CYP2Bs, CYP2C9, CYP2D6 and CYP3A4 (Landschulz et al. 1988, Gonzalez & Lee 1996, Luc et al. 1996, Crosson et al. 1997, Ourlin et al. 1997, Jover et al. 1998, Arizmendi et al. 1999, Jover et al. 2002, Pitarque et al. 2005). The lung-specific isoform, C/EBPδ regulates CYP2A13 (Ling et al. 2007).

C/EBPβ can be translated into full-length and amino-terminally-truncated forms that are called liver activating protein (LAP) and liver inhibitory protein (LIP), respectively. A truncated C/EBPβ lacks the transactivation domain and acts as a transcriptional inhibitor. (Descombes & Schibler 1991.) Changes in the LAP:LIP ratio in hepatocytes are seen during liver development and in certain pathophysiological conditions (Descombes & Schibler 1991, An et al. 1996,
DBP

D-site binding protein (DBP) is a member of the proline and acidic amino acid rich basic leucine zipper (PAR bZip) transcription factors. It was originally identified as a transcription factor that regulates expression of the albumin gene by binding to a region in its promoter called the D-site (Mueller et al. 1990). DBP displays circadian accumulation in rodent liver and some other tissues, and is likely to control the circadian behaviour of the animals (Lopez-Molina et al. 1997). Among the P450 enzymes, Cyp2a5 and Cyp2a4 have been shown to be DBP target genes in the liver (Lavery et al. 1999).

2.5.2 Ubiquitously expressed transcription factors

Ubiquitously expressed transcription factors can be found in several different tissues and cell types. They are thought to be needed as general components of the transcriptional machinery and regulate numerous genes together with tissue-enriched and signal-responding factors (Gonzalez & Lee 1996). However, different quantities of distinct isoforms and splicing variants of ubiquitously expressed transcription factors are expressed in different tissues, and therefore these factors are also likely to play roles in tissue-specific expression. Several ubiquitously expressed factors have been reported to participate in P450 regulation, including specificity protein 1 (Sp-1), nuclear factor I (NF-I), and upstream stimulatory factor (USF) (Gonzalez & Lee 1996, Pickwell et al. 2003, Su & Ding 2004).

NF-I

Nuclear factor I (NF-I) proteins mediate both the initiation of transcription and DNA replication. Multiple isoforms are expressed from four distinct genes (NF-IA, NF-IB, NF-IC and NF-IX) through alternative splicing (Jones et al. 1987, Gronostajski 2000). Different NF-I isoforms have a conserved N-terminal region that mediates DNA binding and dimerization, but variable C-terminal transactivating domains (Mermod et al. 1989). NF-I isoforms form homo- and heterodimers in all possible combinations (Kruse & Sippel 1994), and are able to
both activate and repress their target genes (Gronostajski 2000). NF-I proteins regulate several CYP genes, such as rat CYP2A3 (Ling et al. 2004b) and CYP1A2 (Zhang et al. 2000), and down-regulation of CYP1A1 by oxidative stress is controlled by NF-I (Morel & Barouki 1998). In addition, the phenobarbital-responsive element of the mouse Cyp2b10 gene contains an NF-I binding site important for both the induction response and the basal expression of CYP2B10 (Honkakoski & Negishi 1998). Although NF-Is are ubiquitously expressed, distinct isoforms can activate promoters in a cell-specific manner, and it has been suggested that multiple NF-I splicing variants play roles in the tissue-specific regulation of P450s (Zhang & Ding 1998, Gronostajski 2000, Zhang et al. 2000).

**USF**

Upstream stimulatory factors (USFs) 1 and 2 are members of the basic-helix-loop-helix (bHLH) -leucine zipper transcription factor family (Sawadogo & Roeder 1985, Sawadogo et al. 1988, Sirito et al. 1992, Sirito et al. 1994). They are both ubiquitously expressed, but distinct cell specific ratios of homo- and heterodimers are found in different cell types (Sirito et al. 1994, Viollet et al. 1996). USF dimers bind to the symmetric enhancer box (E-box) sequence CACGTG and thereby regulate their numerous target genes, which are involved in stress- and the immune response, in cell cycle control and in glucose and lipid metabolism (Corre & Galibert 2005). USFs also regulate some xenobiotic metabolizing P450 enzymes, such as human CYP1A2 (Pickwell et al. 2003) and rabbit CYP1A1 (Takahashi et al. 1997).

**2.5.3 Transcription factors mediating CYP gene induction**

Classically, the term induction is used to indicate *de novo* synthesis of new protein molecules as a result of increased transcription of the respective gene after an appropriate stimulus. In the case of enzymes, induction usually means an increase in the amount and activity of an enzyme, and the post-transcriptional mechanisms are also considered to be induction pathways. Xenobiotic induction is usually tissue-specific, rapid, dose-dependent, and reversible upon removal of the inducer. P450 enzymes in subfamilies CYP1-4 are highly inducible by xenobiotics, which can be their own substrates or other compounds. In addition to the P450s, other xenobiotic-metabolizing enzymes, transporters and numerous genes in different physiological systems, including intracellular redox potential
regulation, steroid metabolism, cholesterol and bile acid metabolism, heme synthesis and breakdown, endoplasmic reticulum proliferation, and tumour promotion, can be induced by xenobiotics. (Pelkonen et al. 1998.)

Aromatic hydrocarbons represent one prototypical class of compounds that mainly induce CYP1As and -1Bs (Poland et al. 1974, Hankinson 1995). Induction of hepatic microsomal drug metabolism by barbiturates was reported in the 1960s (Remmer & Merker 1963), and later it was found that phenobarbital, other barbiturates, and several other compounds that exhibit a similar induction pattern (called phenobarbital-type inducers) activate the transcription of CYP2A, -2B, -2C and -3A genes. The same P450s were also activated by another group of inducers, namely glucocorticoid (dexamethasone/rifampicin) -type compounds. Peroxisome proliferators typically elevate CYP4A levels. (Denison & Whitlock 1995.)

Ligand-activated receptors were found to play critical roles in mediating the xenobiotic induction of P450s: arylhydrocarbon receptor (AHR) was identified as regulating CYP1s, and the nuclear receptors constitutive androstane receptor (CAR), pregnane X receptor (PXR) and peroxisome proliferators activated receptor (PPAR) CYP2s, CYP3s and CYP4s, respectively. More recently, extensive cross-regulation between these receptors has been reported (Honkakoski & Negishi 2000). The induction potency of several xenobiotics is drastically different in different species, suggesting that multiple mechanisms or receptors may operate to produce the response (Denison & Whitlock 1995). Indeed, many ubiquitous and tissue-specific factors together with coregulators are also needed for induction.

In addition to xenobiotics, other factors can induce P450 expression. For instance, oxidative stress up-regulates nuclear factor (erythroid-derived 2)-like 2 (Nrf2) that further induces xenobiotic metabolizing enzymes, particularly in Phase II metabolism, but also CYP2A5 (Rushmore & Kong 2002, Kwak et al. 2003, Abu-Bakar et al. 2007).

**AHR and ARNT**

AHR is a ligand-activated bHLH-Per-ARNT-Sim (PAS) transcription factor (Poland et al. 1974, Burbach et al. 1992, Ema et al. 1992). Polycyclic and halogenated aromatic hydrocarbons, such as 3-methylcholanthrene (3-MC), benzo[a]pyrene, and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), are environmental pollutants and AHR ligands, and mediate most of their toxic and
carcinogenic responses through AHR (Hankinson 1995, Fernandez-Salgueiro et al. 1996, Mimura & Fujii-Kuriyama 2003, Kawajiri & Fujii-Kuriyama 2007). AHR also appears to play a role in normal physiology and development, since Ahr knock out mice exhibit several defects, including abnormalities in the liver and immune system (Fernandez-Salgueiro et al. 1995b, Schmidt et al. 1996, Lahvis & Bradfield 1998), and AHR has many endogenous ligands, such as bilirubin and biliverdin (Phelan et al. 1998).

Unliganded AHR exists in a cytoplasmic complex associated with a dimer of the molecular chaperone heat shock protein 90 (hsp90), AHR interacting protein [AIP, also called immunophilin homolog ARA9 or hepatitis B virus (HBV)-X-associated protein 2, XAP2], and co-chaperone p23 (Kazlauskas et al. 2001). Ligand binding releases AHR from the complex and triggers its translocation from the cytosol into the nucleus, where AHR forms a heterodimer with aryl hydrocarbon receptor nuclear translocator (ARNT) (Hankinson 1995). Despite its name, ARNT does not appear to be directly involved in translocating AHR into the nucleus (Pollenz et al. 1994). Together, AHR and ARNT bind to the response elements in DNA called xenobiotic response elements (XREs) located in the 5’ flanking regions of the target genes, including xenobiotic metabolizing enzymes, particularly CYP1s, and genes involved in cell proliferation, cell cycle regulation, apoptosis and immune responses (Hankinson 1995, Swanson 2002, Mimura & Fujii-Kuriyama 2003, Kawajiri & Fujii-Kuriyama 2007).

AHR has also been shown to play a role in oxidative stress, since it induces the expression of several Phase II detoxifying enzymes directly and indirectly through up-regulation of Nrf2 (Nebert et al. 2000, Miao et al. 2005). CYP1A1 and CYP1A2 metabolism generates reactive oxygenated metabolites and therefore induction of CYP1s may trigger oxidative stress, which can be ameliorated by parallel up-regulation of the Phase II enzymes that control the cellular redox status (Nebert et al. 2000).

AHR and ARNT dimerize through their HLH and Per-ARNT-Sim (PAS) homology domains, whereas the basic domain binds to DNA. The carboxy-terminal segments of AHR and ARNT contain transactivation domains (TADs). (Reisz-Porszasz et al. 1994, Fukunaga et al. 1995.) Numerous coactivators for AHR and ARNT have been identified (Beischlag et al. 2002, Hankinson 2005). Several alternative mechanisms have also been described for AHR-mediated gene regulation such as protein-protein interactions between estrogen receptor, retinoblastoma protein and nuclear factor κB (NF-κB) (Tian et al. 1999, Puga et al. 2000, Frueh et al. 2001, Brunnberg et al. 2003, Ohtake et al. 2003). AHR
repressor (AHRR) can heterodimerize with ARNT and bind to XREs, but it does not activate the transcription and it has therefore been proposed that it down-regulates AHR target genes. AHRR expression is induced in an AHR-dependent manner, indicating that AHR and AHRR form a regulatory feedback loop (Mimura et al. 1999).

ARNT is a ubiquitously expressed nuclear bHLH-PAS transcription factor (Miller et al. 1983, Hoffman et al. 1991, Reyes et al. 1992, Carver et al. 1994, Eguchi et al. 1997). The ARNT protein, also called hypoxia inducible factor 1β, is a general heterodimerization partner for members of the bHLH-PAS transcription factor family, including AHR, hypoxia inducible factor 1α and developmental regulator single minded (SIM) (Gu et al. 2000b). ARNT is also able to homodimerize and bind the palindromic E-box sequence CACGTG (Sogawa et al. 1995, Swanson et al. 1995, Huffman et al. 2001, Card et al. 2005). The homodimer has been shown to activate transcription of reporter genes from the E-box located within the adenovirus major late promoter in mammalian cells in culture (Sogawa et al. 1995), but no mammalian target genes for the ARNT homodimer have been reported. ARNT also exists in an alternatively spliced form in both mouse and human, containing an additional 15 amino acids just N-terminal of the basic region encoded by exon 5 (Hoffman et al. 1991, Reisz-Porszasz et al. 1994).
Nuclear receptors

The nuclear receptor superfamily regulates diverse functions in eukaryotic development, differentiation, reproduction and metabolic homeostasis. Besides the classic steroid receptors, members of the nuclear receptor superfamily include receptors that can bind other ligands, such as vitamin D, thyroxin, retinoic acid, prostaglandins or fatty acids. (Mangelsdorf et al. 1995, Ribeiro et al. 1995, Giguere 1999, Nuclear Receptors Nomenclature Committee 1999, Robinson-Rechavi et al. 2003, Germain et al. 2006.) Novel nuclear receptors have been identified by searching DNA databases for sequences that share homology with highly conserved regions of known nuclear receptors. This type of analysis led to the cloning of CAR (Baes et al. 1994, Choi et al. 1997) and PXR (Bertilsson et al. 1998, Blumberg et al. 1998, Kliewer et al. 1998), which can bind several structurally different xenobiotics, and thereby yielded major breakthroughs in the study of xenobiotic-mediated transcriptional induction of CYP enzymes.

Nuclear receptors typically consist of a highly conserved DNA binding domain (DBD), which contains two zinc finger motifs, a carboxy-terminal ligand

Fig. 4. A schematic picture of the AHR signalling pathway (modified from Fujii-Kuriyama & Mimura 2005).
binding domain (LBD) and a ligand-dependent activation function 2 (AF-2) domain that is necessary for activation of transcription. The N-terminal region contains a cell- and promoter-specific transactivation function termed AF-1. (Lanz & Rusconi 1994, Bourguet et al. 2000.) In the simplified model of nuclear receptor-mediated regulation of gene expression, a ligand binds to the LBD, which results in a conformational change in the receptor such that it can interact with accessory proteins and bind to the DNA. The ligand-activated receptors then translocate to the nucleus and bind as dimers to their response sites in the DNA and activate transcription. (Moras & Gronemeyer 1998.) However, nuclear receptor signalling is actually far more complex and it can control transcription in numerous distinct ways, including both gene activation and repression. Some non-genomic actions have even been described for steroid receptors outside the nucleus. Nuclear receptor activities can also be ligand-independent and there are several so-called orphan receptors, for which regulatory ligands are still unknown or may not exist. Some nuclear receptors are constitutively localized in the cell nucleus regardless of the presence of a ligand (e.g. androgen receptor and mineralocorticoid receptor) and some (for instance, the retinoic acid receptors and the thyroid hormone receptors) exhibit a dual functionality, being able to act as transcriptional silencers in the absence of ligands, and activators in the presence of agonists. In addition to ligand-binding, the activity of nuclear receptors can be modulated by several mechanisms, including different post-translational modifications. Nuclear receptors can also be the targets of other signalling pathways and, reciprocally, can affect the activity of these pathways, for example by interfering with activator protein 1 (AP-1) and nuclear factor κB (NF-κB) activities. (Germain et al. 2006.)

Nuclear receptors bind to sequence-specific promoter elements on target genes as monomers, homodimers, or heterodimers with the general heterodimerization partner 9-cis-retinoic acid receptor (RXR). The nuclear receptor response elements are usually composed of two half-sites related to the hexamer AGGTCA. Steroid-hormone receptors bind as homodimers to palindromes with a 3 bp spacing, whereas other nuclear receptors typically form heterodimers with RXR and bind to the hexamers in a direct repeat (DR), inverted repeat (IR), or everted repeat (ER) orientation, with variable spacing. (Mangelsdorf & Evans 1995.) DNA binding is coupled to the recruitment of transcriptional coactivators or corepressors that determine the final transcriptional activity (Xu et al. 1999, Xu & Li 2003).
Nuclear receptor CAR is predominantly expressed in the liver (Baes et al. 1994, Choi et al. 1997). It was first found to activate an empirical set of retinoic acid response elements as a heterodimer with RXR in a constitutive, ligand-independent manner in vitro (Baes et al. 1994), but was reported to be quiescent in the cytoplasm of hepatocytes in vivo (Kawamoto et al. 1999). The steroids androstanol and androstenol bind to and inhibit the activity of CAR (Forman et al. 1998). CAR was shown to respond to phenobarbital and phenobarbital-like inducer 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), and to activate CYP2B genes through binding to DR-4 elements in phenobarbital responsive element modules in CYP2B promoters as a heterodimer with RXRα (Honkakoski et al. 1998, Sueyoshi et al. 1999a). TCPOBOP functions as an agonist ligand for CAR (Tzameli et al. 2000), but phenobarbital acts via a signalling pathway that induces translocation of CAR from the cytoplasm to the nucleus, probably involving phosphorylation events (Kawamoto et al. 1999). In addition to CYP2Bs, at least CYP2C and CYP3A genes are CAR targets (Handschin & Meyer 2003). CAR also appears to play an important role in regulating bilirubin clearance and bile acid detoxification (Sugatani et al. 2001, Huang et al. 2003, Xie et al. 2003, Zhang et al. 2004).

Several clinically relevant drug-drug interactions involving CYP3A induction are largely due to drug-mediated activation of PXR (Lehmann et al. 1998). PXR is also abundantly expressed in the same tissues as CYP3A4, including the liver and intestine (Kliewer et al. 1998). The large and flexible ligand-binding domain of PXR can accommodate various natural and synthetic pregnanes and other chemicals with different structures, including the prototypic activators pregnenolone 16α-carbonitrile (PCN) and rifampicin (Blumberg et al. 1998, Kliewer et al. 1998, Kliewer et al. 2002). Amino acid sequence differences
between the ligand binding domains of PXRs in rodents and human cause species specific induction by different inducers (Jones et al. 2000). Activation of PXR results in increased transcription of the phase I and phase II drug metabolizing enzymes, drug transporters, as well as heme and co-substrate synthesis (Kliewer et al. 2002).

PPARs mediate induction of the members of the CYP4A family by many drugs and other xenobiotics, including acidic chemicals classified as non-genotoxic carcinogens and peroxisome proliferators (Issemann & Green 1990, Muerhoff et al. 1992, Forman et al. 1997). In addition to CAR, PXR, and PPAR, some of the classical nuclear receptors previously mentioned, such as the vitamin D receptor (VDR) and glucocorticoid receptor, and some recently discovered receptors, including liver X receptor binding oxysterols and bile acid sensor farnesoid X receptor (FXR) are involved in the control of several P450 genes (Waxman 1999, Honkakoski & Negishi 2000, Drocourt et al. 2002, Handschin et al. 2002).

Nuclear receptors form interconnected regulatory networks where they are involved in extensive cross-talk (Handschin & Meyer 2003). Firstly, the response elements within a given gene can bind different nuclear receptors, for example, CAR can bind to PXR response elements and vice versa. Secondly, CAR and PXR also have common xenobiotic and steroid ligands. (Moore et al. 2000, Xie et al. 2000, Wei et al. 2002.) Furthermore, nuclear receptors can regulate each other’s expression. For instance, HNF-4α and the glucocorticoid receptor control the expression of PXR, CAR and FXR (Tirona & Kim 2005) and PXR appears to regulate the expression of CAR, AHR and itself (Maglich et al. 2002). In addition, nuclear receptors can affect the activity of other nuclear receptors by competition for coactivators (Makinen et al. 2002). The nuclear receptor short heterodimer partner (SHP) is an unusual nuclear receptor that lacks a DNA binding domain (Seol et al. 1996). It dimerizes with several transcription factors including CAR, HNF-4, AHR, and PXR and thereby inhibits the activation of their target genes, including P450s (Seol et al. 1996, Lee et al. 2000, Klinge et al. 2001, Ourlin et al. 2003, Bae et al. 2004). Very recent studies have evidenced that nuclear receptors, such as PPARα, FXR and VDR, also regulate the expression of particular fibroblast growth factors (FGFs) in response to different metabolic states. These factors, in turn, function as metabolic hormones and can further act through certain signalling pathways and control specific states of energy, bile acid, and phosphate and calcium homeostasis. (Moore 2007.)
Nrf2

Nrf2 is a member of the basic region-leucine zipper transcription factors. It regulates genes involved in cellular protection against oxidative stress, such as heme oxygenase 1 (HO-1), thioredoxin, glutathione S-transferase (GST) and NAD(P)H:quinone oxidoreductase 1 (NQO1) (Venugopal & Jaiswal 1996, Itoh et al. 1997, Alam et al. 1999, Kim et al. 2001b). Nrf2 is also reported to be involved in Cyp expression (Kwak et al. 2003, Abu-Bakar et al. 2007). Under normal conditions, Nrf2 associates with the Kelch-like ECH-related protein, Keap1, in the cytosol, but during oxidative or electrophilic stress, Nrf2 is released from Keap1 and translocated to the nucleus. In the nucleus, Nrf2 dimerizes with small Maf and binds to antioxidant response elements (AREs), also called stress response elements (StREs), in its target gene promoters (Rushmore et al. 1991, Itoh et al. 1997, Kang et al. 2005).
2.5.4 Coregulators

Activation or repression of transcription requires several coregulators that act through modifications of chromatin structure and regulate the recruitment of transcription factors and the preinitiation complex (Roeder 2005). Coactivator and corepressor proteins do not bind to DNA in a sequence-specific manner, but usually operate as components of multisubunit coregulator complexes through protein-protein interactions. Coregulators modify histone tails via several different enzymatic activities, including histone acetyltransferases (HATs), histone deacetylases (HDACs), methylases, demethylases, kinases, phosphatases and ubiquitinases. (Rosenfeld et al. 2006.) Some cofactors possess ATP-dependent remodelling activities, such as the members of the SWI/SNF (SWI=switch, SNF = sucrose nonfermenting) complex (Muchardt & Yaniv 1999).

Many coregulators have been found to play significant roles in mediating the actions of numerous different classes of transcription factors. On the other hand, specific transcription factors can use distinct combinations of coregulators, depending on the tissue, target gene, DNA-binding site, and the actions of various signalling pathways. (Hermanson et al. 2002.) Coregulator proteins participate in gene regulation, not only by being necessary parts of the transcriptional machinery, but by being the targets of developmental or physiological signals. The signal-dependent interactions of coactivators and corepressors with transcription factors can be controlled at several levels, including coregulator expression, post-translational modifications of coregulators and their targets, and in the case of nuclear receptors, ligand binding. (Spiegelman & Heinrich 2004, Rosenfeld et al. 2006.)

Although the roles of numerous transcription factors in the regulation of CYP genes have been studied extensively, relatively little is known about the coregulators of CYP expression. However, several AHR and nuclear receptor coregulators have been characterized and they are also likely to be involved in CYP transcription (Hermanson et al. 2002, Schrem et al. 2002, Hankinson 2005, Ding et al. 2006, Itoh et al. 2006). For example, members of the p160 coregulator family (molecular mass around 160 kDa) are known to interact ligand-dependently with a number of nuclear receptors (Cavailles et al. 1994, Glass & Rosenfeld 2000). p160s include nuclear coactivator 2 (NcoA-2), steroid receptor coactivator 1 (SRC-1) and p300/CBP cointegrator protein (p/CIP). They contain a highly conserved amino-terminal bHLH-PAS domain and several C-terminal interaction motifs with a consensus core LXXLL, where L is leucine and X is any
amino acid. (Leo & Chen 2000, Hermanson et al. 2002.) Through these domains the p160 coactivators trigger the assembly of multiple, distinct complexes, which contain diverse enzymatic activities. For instance, the C-terminal domains of p160 factors mediate interactions with the p300 protein and its homologue, the cAMP response element binding protein (CREB) -binding protein (CBP) (Kamei et al. 1996, Torchia et al. 1997, Rosenfeld et al. 2006). p300 and CBP further function by acting as molecular scaffolds, and by acetylating diverse substrates (Giordano & Avantaggiati 1999, Glass & Rosenfeld 2000, Rosenfeld et al. 2006). Peroxisome proliferator-activated receptor γ coactivator 1 (PGC-1), which is inducible by different signals from outside the cell, promotes transcription through the assembly of a complex that includes SRC-1 and CBP/p300 (Puigserver et al. 1999). Corepressors, such as nuclear receptor corepressor (NCoR) and its homolog silencing mediator of retinoid and thyroid hormone receptor (SMRT), interact with several unliganded nuclear receptors to repress their activity (Xu et al. 1999).

PGC-1α

Peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) is involved in numerous biological responses related to energy metabolism, thermal regulation, and glucose metabolism (Lin et al. 2005). Two related coactivators, PGC-1β and PGC-1-related coactivator, belong to the same protein family (Finck & Kelly 2006). PGC-1α is expressed in several tissues, especially those with very high oxidative metabolism, such as the heart, skeletal muscle, kidney, brown fat, brain, and liver (Spiegelman & Heinrich 2004). It is highly inducible, and key cellular signals that control energy and nutrient homeostasis, such as cAMP, strongly activate PGC-1α expression and function (Herzig et al. 2001, Koo et al. 2005). PGC-1α was originally identified through its interaction with peroxisome proliferator-activated receptor γ (Puigserver et al. 1998). It was later shown to coactivate several other nuclear receptors and transcription factors (Lin et al. 2005). Lack of PGC-1α has been shown to down-regulate the expression of several HNF-4α transactivated CYP genes (Martinez-Jimenez et al. 2006a) and CYP2A6 is induced by PXR only in the presence of the coactivator PGC-1α (Itoh et al. 2006).
2.6 Regulation of CYP2A5

The regulation of CYP2A5 is complex and involves several different mechanisms. CYP2A5 is also unique among the major CYP enzymes since it responds to certain signals in the opposite way to other P450s. CYP2A5 expression is specific for mouse strain, gender and tissue and is inducible by structurally diverse xenobiotic compounds. Pathological conditions, such as inflammation, microbial infection, and tumourigenesis elevate CYP2A5 levels. Both transcriptional and post-transcriptional mechanisms are involved in CYP2A5 regulation.

2.6.1 Methodological issues in the study of CYP2A5 regulation

The extremely homologous sequences of CYP2A4 and CYP2A5 have made the studies of each isoform difficult. The probes used in hybridization techniques, such as Northern blot, in situ hybridization and cDNA microarrays, as well as the antibodies against the proteins, do not distinguish CYP2A4 from CYP2A5 (Raunio et al. 1998, Wastl et al. 1998, Thai et al. 2003). Therefore, CYP2A4/5 is used to signify that both CYP2A4 and -2A5 mRNAs or proteins are probably detected in the assays done. However, measurement of coumarin hydroxylase activity specifically detects the CYP2A5 function (Wood & Taylor 1979, Negishi et al. 1989). The specific primers used in different PCR applications should also be able to differentiate between the two cDNAs.

Further problems in CYP2A4 and CYP2A5 research are due to circadian, and strain and sex-specific expression. Most of the in vivo studies have been carried out in DBA/2 mice that express the high activity form of CYP2A5 at a relatively high basal level in both genders (van Iersel et al. 1994). Usually male mice are used, because they express the female-predominant highly homologous CYP2A4 in only minor quantities. In some recent studies (Lavery et al. 1999, Maglich et al. 2002, Wei et al. 2002), CYP2A5 was measured in genetically modified animals that represented different mouse strains and therefore comparison with earlier results is complicated.

2.6.2 Strain and gender specific regulation

Strain specific differences in CYP2A5 activity have been shown both in constitutive level and in induction (Wood & Conney 1974, Lush & Andrews
1978, Wood & Taylor 1979, Wood 1979, Kaipainen & Lang 1985, Lang et al. 1989). Some of the variation in coumarin hydroxylase activity can be explained by allelic differences in the \( \text{Cyp2a5} \) locus (Lindberg et al. 1992), but the dissimilarities in the mRNA and protein expression are probably due to distinct regulation of CYP2A5 between the strains and sexes. Gender differences are more pronounced for CYP2A4 (Noshiro & Negishi 1986) than for CYP2A5, and they are thought to reflect the requirement for site-specific steroid hormone hydroxylation (Waxman 1988). van Iersel et al. (1994) determined hepatic microsomal coumarin 7-hydroxylase activity in male and female mice for the strains A/J, AKR, BALB/c, CBA/Ca, C3H/He, C57BL/6J, DBA/2 and 129. They demonstrated that in males, coumarin 7-hydroxylase activity was highest in livers from DBA/2 mice and lowest in BALB/c mice. In female mice, COH activity was highest in DBA/2 and 129 strains, intermediate in the CBA/Ca strain and relatively low in the other strains. In DBA/2, 129 and CBA/Ca strains, the CYP2A5 activity in females was significantly higher than in male mice (van Iersel et al. 1994). Some of the CYP2A5 inducers also have different effects in different mouse strains (Salonpaa et al. 1992, Salonpaa et al. 1995).

Pituitary growth hormone, whose excretion is regulated by gonadal hormones, has been shown to repress CYP2A4 expression in males (Burkhart et al. 1985, Aida & Negishi 1993), whereas oestrogen receptor \( \alpha \) is needed for the expression of CYP2A4 in females (Sueyoshi et al. 1999b). The transcription factor STAT5b responds to male pulsatile growth hormone expression and has been shown to mediate the effect of growth hormone on CYP2A4 expression (Udy et al. 1997, Teglund et al. 1998, Waxman 2000). Wiwi et al. (2004) reported that HNF-4\( \alpha \) also plays a role in regulating sexually dimorphic P450 genes in the liver. In their studies, HNF-4\( \alpha \) was found to be a negative regulator of \( \text{Cyp2a4} \) in male livers. In addition, methylation of the \( \text{Cyp2a4} \) promoter may play a role in the repression of its expression in males (Yokomori et al. 1995). However, the involvement of these mechanisms in \( \text{Cyp2a5} \) regulation remains to be studied.

2.6.3 Tissue specific regulation

The major sites of CYP2A5 expression are the liver and olfactory mucosa. Although the liver-enriched transcription factors and their role in CYP expression have been studied extensively, relatively little is known about tissue-specific expression of CYP2A5. In liver, HNF-4 binds the proximal promoter of several
CYP2A genes and activates hepatic expression of murine CYP2A4 and human CYP2A6 (Yokomori et al. 1997, Jover et al. 2001, Pitarque et al. 2005). In addition to HNF-4α, C/EBPα and octamer-binding transcription factor-1 bind to the proximal promoter and activate the transcription of CYP2A6, whereas C/EBPβ has been shown to decrease CYP2A6 promoter activity in the liver (Pitarque et al. 2005). According to results from Cheung et al. (2003) HNF-1 may repress CYP2A4/5 expression in mouse liver.

A conserved NF-I binding site has also been discovered in the proximal promoters of the Cyp2a5, CYP2A3, and CYP2A6 genes, and the site, designated nasal predominant transcriptional activating (NPTA) element, was shown to be essential for CYP2A3 promoter activity in the olfactory mucosa (Zhang & Ding 1998). Following more studies, it was suggested that an OM-specific form of NF-I, NF-I-A2, was involved in the tissue-selective transcription of CYP2A3 (Xie et al. 2001, Ling et al. 2004b). The NF-I element of CYP2A3 is inactive in the liver, probably due to methylation of the promoter (Ling et al. 2004b), but the NF-I element in the CYP1A2 promoter activates transcription, both in the liver and olfactory mucosa (Zhang et al. 2000). The NF-I element in the CYP2A6 promoter has four nucleotide changes, and in studies by Pitarque et al. (2005) it was shown to be inactive.

To study the species- and tissue-specific expression of CYP2A3, Su et al. (2002) generated a transgenic mouse, which expressed CYP2A3 under the control of its own 5’ flanking region (3.4 kb). The expression of CYP2A3 mimicked the tissue distribution of CYP2A5 suggesting that the differences in tissue specific expression between mouse and rat are not due to promoter sequences but are probably related to differences in the expression or function of transcription factors or to epigenetic mechanisms (Su et al. 2002).

Besides tissue-specific transcription factors, chromosome structure and distant regulatory modules may play roles in the tissue-specificity of CYP2A5 (Ling et al. 2004a, Zhuo et al. 2004). Methylation has been shown to inactivate at least CYP1s, CYP2A3 and CYP2A13 promoters, probably in a tissue-specific manner (Nakajima et al. 2003, Ling et al. 2004b, Ling et al. 2007).

### 2.6.4 Circadian rhythm

Lavery et al. (1997) reported that accumulation of CYP2A5 mRNA varied with high amplitude throughout the day, with peak accumulation detected in the evening. They later identified both Cyp2a5 and Cyp2a4 as having circadian
rhythm in the liver and showed that this rhythmic expression was regulated by DBP (Lavery et al. 1999). Three conserved DBP binding motifs were found on the proximal 5’ promoter (–300 to TSS) of both the Cyp2a5 and Cyp2a4 genes (Lavery et al. 1999). However, the circadian rhythm in CYP2A5 expression is not totally abolished in dbp−/− mice indicating that some other factor is also involved in this regulation (Lavery et al. 1999). Circadian variation of CYP2A5 is also found in the kidney, but not in the OM (Zhuo et al. 2004). The circadian expression of CYP2A4 is consistent with previous studies on daily fluctuations in the serum level of androgens, but no reason for daily variation in CYP2A5 levels has been described. It has been suggested that the circadian rhythm of Cyp2a5 might serve to protect the night-feeding mouse from toxic compounds, especially coumarins, in the food (Viitala et al. 2001).

2.6.5 Secondary messenger and protein phosphorylation pathways

In primary hepatocytes, stimulation of the cAMP signal transduction pathway by glucagon and isoproterenol produced several fold induction of CYP2A5 activity. Similarly, the protein kinase A stimulators dibutyryl-cAMP, forskolin and Sp-cAMP up-regulated CYP2A5 mRNA. Co-administration of phenobarbital and cAMP or PKA stimulators caused an additive inducing effect. (Salonpaa et al. 1994, Viitala et al. 2001.) In contrast, constitutive and phenobarbital induced COH activities were suppressed in cell culture by components in the serum (Salonpaa et al. 1994).

2-aminohipurine, a non-specific serine/threonine kinase inhibitor, up-regulated both basal and phenobarbital-induced CYP2A5 activity in hepatocytes. CYP2A5 increases were also seen with other protein kinase and phosphatase inhibitors, including roscovitine, K-252a and rapamycin. The serine/threonine phosphatase inhibitors tautomycin, calyculin A and okadaic acid all reduced both basal and phenobarbital induced CYP2A5 activity (Posti et al. 1999).

2.6.6 Xenobiotics

Hepatic CYP2A5 is induced by numerous, structurally different xenobiotics. Induction has been measured extensively both in vivo in mice and in cultured hepatocytes, but the mechanisms mediating the responses are still not well understood. Xenobiotics that induce CYP2A5 are listed in Table 2.
Table 2. Xenobiotics that induce CYP2A5 (Modified from Raunio et al. (2007))

<table>
<thead>
<tr>
<th>Class</th>
<th>Compound</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Nuclear receptor ligands and activators</td>
<td>Phenobarbital</td>
<td>Wood &amp; Conney 1974</td>
</tr>
<tr>
<td></td>
<td>TCPOBOP</td>
<td>Raunio et al. 1988</td>
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<tr>
<td></td>
<td>Rifampicin</td>
<td>Donato et al. 2000</td>
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<td></td>
<td>PCN</td>
<td>Cai et al. 2002</td>
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<td></td>
<td>Wy14,643</td>
<td>Cai et al. 2002</td>
</tr>
<tr>
<td>AHR ligands</td>
<td>TCDD</td>
<td>Gokhale et al. 1997</td>
</tr>
<tr>
<td>Hepatotoxins</td>
<td>Pyrazole</td>
<td>Juvonen et al. 1985</td>
</tr>
<tr>
<td></td>
<td>Cocaine</td>
<td>Pellinen et al. 1993</td>
</tr>
<tr>
<td></td>
<td>Carbon tetrachloride</td>
<td>Pellinen et al. 1993</td>
</tr>
<tr>
<td></td>
<td>Hexachlorobutadiene</td>
<td>Pellinen et al. 1993</td>
</tr>
<tr>
<td>Heavy metals</td>
<td>Cobalt</td>
<td>Legrum &amp; Netter 1980</td>
</tr>
<tr>
<td></td>
<td>Indium</td>
<td>Mangoura et al. 1989</td>
</tr>
<tr>
<td></td>
<td>Cerium</td>
<td>Arvela et al. 1991</td>
</tr>
<tr>
<td></td>
<td>Tin</td>
<td>Emde et al. 1996</td>
</tr>
<tr>
<td></td>
<td>Cadmium</td>
<td>Abu-Bakar et al. 2004</td>
</tr>
<tr>
<td>Porphyrinogenic compounds</td>
<td>Thioacetamide</td>
<td>Salonpää et al. 1995</td>
</tr>
<tr>
<td></td>
<td>Aminotriazole</td>
<td>Salonpää et al. 1995</td>
</tr>
<tr>
<td></td>
<td>Griseofulvin</td>
<td>Salonpää et al. 1995</td>
</tr>
</tbody>
</table>

**Nuclear receptor activators**

Phenobarbital elevates CYP2A5 at the mRNA, protein and enzyme activity level *in vivo* in different mouse strains (Wood & Conney 1974, Aida & Negishi 1991, Hahnemann et al. 1992). Also, in primary hepatocytes from DBA/2 mouse liver, CYP2A5 mRNA and coumarin hydroxylase activity are elevated after phenobarbital treatment, and an additional increase is seen when cyclic AMP is administered together with phenobarbital (Salonpaa et al. 1994, Viitala et al. 2001). It has been suggested that both transcriptional and post-transcriptional mechanisms could be behind CYP2A5 induction by phenobarbital (Aida & Negishi 1991, Salonpaa et al. 1994), but no mediator of the induction has been found so far. The specific CAR ligand TCPOBOP induces CYP2A5 (Raunio et al. 1988b) and CAR has been shown to mediate CYP2A4/5 mRNA induction by TCPOBOP in CAR knockout mice (Maglich et al. 2002, Wei et al. 2002) indicating that CAR might also be involved in CYP2A5 induction by phenobarbital. However, the phenobarbital induction of CYP2A5 appears to be differently regulated than that of other CYPs (Posti et al. 1999, Jover et al. 2000).
Rifampicin, which is a ligand for human PXR, has been shown to activate CYP2A5 activity in murine hepatocytes (Donato et al. 2000), and pregnenolone carbonitrile (PCN) induces CYP2A4/5 mRNA in vivo RXR dependently (Cai et al. 2002). In addition, the CYP2A4/5 mRNA level was observed to be elevated in a human PXR transgenic mouse model (Rosenfeld et al. 2003), and the human orthologue CYP2A6 is induced by PXR in the presence of the coactivator PGC-1α (Itoh et al. 2006). However, no direct evidence of the involvement of PXR in Cyp2a5 regulation has been shown.

Kojo et al. (Kojo et al. 1996) reported down-regulation of CYP2A5 in mRNA, protein and activity levels by peroxisome proliferator clofibrate. Later studies by Barclay et al. (1999) in PPARα knock out mice suggested that CYP2A5 inhibition by clofibrate is mediated via PPARα. In contrast, the PPARα ligand Wy14,643 has been reported to elevate CYP2A4/5 mRNA in vivo in an RXR dependent manner (Cai et al. 2002).

**AHR ligands**

It has been thought that among the cytochrome P450s, only members of the CYP1 family are regulated by the AHR pathway. However, CYP2A5-catalyzed COH activity is elevated by TCDD in mouse liver slices (Gokhale et al. 1997) and rat CYP2A3 is up-regulated by 3-methylcholanganthrene in the lung (Kimura et al. 1989), although no mechanisms behind these inductions have been shown.

**Post-transcriptional regulation by pyrazole and hnRNP A1**

Pyrazole and its derivatives, such as 4-iodo/bromo/methyl-pyrazole induce CYP2A5 activity both in mouse liver in vivo and in primary hepatocyte cultures (Juvonen et al. 1985, Juvonen et al. 1988, Kojo et al. 1991, Kojo et al. 1998). Induction by pyrazole has been shown to be post-transcriptional, and related to increases in mRNA stability (Aida & Negishi 1991, Hahnemann et al. 1992). Geneste et al. (1996) demonstrated that pyrazole treatment increases the level of a 44 kDa hepatic protein that can bind to the 3′-untranslated region (3′-UTR) of CYP2A5 mRNA, leading to protection of the poly(A) tail and increased stability of the transcript. Three protein duplexes (37/39, 45/48 and 70/72 kDa) that bind to the 3′-UTR of CYP2A5 mRNA have been discovered (Thulke-Gross et al. 1998). The 37/39 kDa protein was identified as heterogeneous nuclear RiboNucleoProtein A1 (hnRNP A1), which binds to a 71-nucleotide region of
CYP2A5 3′-UTR and protects the transcript from degradation (Tilloy-Ellul *et al.* 1999, Raffalli-Mathieu *et al.* 2002). hnRNP A1 was also shown to interact with the CYP2A6 3′ end (Gilmore *et al.* 2001, Christian *et al.* 2004).

Glisovic *et al.* (2003b) reported that inhibition of transcription in primary hepatocytes led to relocalization of hnRNP A1 from the nucleus to the cytoplasm, and to its increased binding to cytoplasmic CYP2A5 mRNA. In contrast, phenobarbital and cAMP stimulated transcription resulted in binding of nuclear hnRNP A1 to the proximal Cyp2a5 5′ promoter suggesting that hnRNP A1 also plays a role in transcriptional regulation.

**Heavy metals**

Heavy metals, including cobalt, indium, cerium, and tin have been shown to elevate CYP2A5 in mouse liver (Legrum & Netter 1980, Mangoura *et al.* 1989, Arvela *et al.* 1991, Kocer *et al.* 1991, Hahnemann *et al.* 1992, Emde *et al.* 1996). No mechanisms behind this induction have been suggested, but the gender and strain of mouse seem to play a role in induction by these compounds (Mangoura *et al.* 1989, Salonpaa *et al.* 1992). Cadmium induces CYP2A5 in an Nrf2-dependent manner (Abu-Bakar *et al.* 2004). Cadmium is known to alter the cellular redox state by reducing the intracellular ratio of glutathione to its oxidized form, and thereby activates Nrf2. This in turn regulates several genes in response to oxidative stress (Alam *et al.* 1999, Kim *et al.* 2001b, Ryter & Choi 2002, Kang *et al.* 2005). Nrf2 binds to a stress-response element (StRE) located in the Cyp2a5 5′ promoter within the region –2386 to –2377, and activates Cyp2a5 transcription (Abu-Bakar *et al.* 2007).

**Porphyrinogenic compounds**

Several porphyrinogenic compounds that disturb heme homeostasis up-regulate CYP2A5 in vivo. These compounds include thioacetamide, aminotriazole, griseofulvin, and cobalt chloride. (Kocer *et al.* 1991, Salonpaa *et al.* 1995.) In addition, the CYP2A5 inducer pyrazole and some of the heavy metals that cause CYP2A5 up-regulation have porphyrinogenic effects. An insufficient heme supply usually inhibits CYP mediated reactions, possibly through incomplete saturation of the P450 apoprotein (Halpert *et al.* 1994), but the involvement of heme in the transcriptional regulation of P450s has also been suggested (Dwarki *et al.* 1987, Rangarajan & Padmanaban 1989, Venkateswar & Padmanaban 1991).
In studies by Salonpää et al. (1995), CYP2A5 induction by aminotriazole and thioacetamide in mice could be abolished by addition of heme arginate, and the elevation by pyrazole was also decreased by heme addition, but this reversal did not occur after treatment with griseofulvin or phenobarbital. In contrast, heme appeared to actually stimulate the induction by griseofulvin (Salonpää et al. 1995), and a limited heme supply was shown to be associated with decreased phenobarbital induction of Cyp2a5 due to impaired gene transcription (Jover et al. 2000). Marked strain differences were also seen in response to porphyrinogenic compounds and heme between DBA/2 and C57BL/6 mice (Salonpää et al. 1995).

In primary hepatocytes, CYP2A5 was transcriptionally up-regulated by griseofulvin and the protoporphyrinogen oxidase inhibitor acifluorfen, whereas cobalt, aminotriazole, and thioacetamide either decreased or had no effect on CYP2A5 levels, indicating that a systemic effect might be needed for the induction of CYP2A5 by these compounds (Salonpää et al. 1997).

**Other hepatotoxic compounds**

In addition to pyrazole, heavy metals and porphyrinogenic compounds, some other hepatotoxic compounds, such as cocaine, carbon tetrachloride, and hexachlorobutadiene have been reported to induce CYP2A5 in vivo at mRNA, protein and activity levels (Pellinen et al. 1993, Pellinen et al. 1994). Camus-Randon et al. (1996) studied the effect of two well-known hepatotoxins, chloroform and thioacetamide, in the expression of hepatic CYP enzymes. In contrast to other CYPs (1A, 2B, 2C, 2E1, and 3A4), whose expression was either decreased or unaffected by these chemicals, the level and enzymatic activity of CYP2A5 was increased.

**2.6.7 Pathological conditions**

Unlike most other CYP enzymes (Morgan 1997, Morgan 2001, Renton 2004), CYP2A5 is up-regulated in certain disease states. Elevated CYP2A5 levels and activities have been detected in spontaneous, transplanted, or chemically induced mouse hepatomas and in liver injuries caused by chemical intoxication or microbial infection (Lange et al. 1990, Kobliakov et al. 1993, Jounaidi et al. 1994, Camus-Randon et al. 1996, Chomarat et al. 1997, Takagi et al. 1997, Wastl et al. 1998, Thai et al. 2003, Richardson et al. 2006). In addition, some parasitic
or viral infections that cause chronic inflammation in the liver, and, in some cases, specific types of cancers, are associated with high CYP2A5 expression in mouse liver (Kirby et al. 1994, Chemin et al. 1996, Chemin et al. 1999, Montero et al. 1999). In many studies, altered regional distribution of CYP2A5 in the liver is also seen in these pathological states, usually immediately adjacent to areas of inflammation (Chemin et al. 1996, Chomarat et al. 1997, Wastl et al. 1998). Similarly, human CYP2A6 is induced in alcoholic and non-alcoholic liver diseases, by parasitic infestation, and in hepatocytes that express the HBV surface antigen (Kirby et al. 1996, Satarug et al. 1996, Niemela et al. 2000). According to results from Raunio et al. (1998) CYP2A6 overexpression is associated with the presence of inflammatory reactions and cirrhosis, but does not seem to be an invariable phenotype in human hepatocellular carcinoma. However, the number of cases examined was rather small (n = 24).

In contrast, CYP2A5 was repressed in vivo similarly to other P450s during turpentine-induced tissue injury, which mimics aseptic inflammatory processes, and in inflammation mediated by the bacterial endotoxin lipopolysaccharide (LPS) (Siewert et al. 2000). Turpentine-induced inflammation down-regulated CYP2A5 interleukin 6 (IL-6) dependently, but IL-6 was not necessary in the LPS induced model of inflammation (Siewert et al. 2000, Gilmore et al. 2003). Richardson and Morgan (2005) studied the roles of PPARα and PXR in the regulation of CYP2A5 expression by LPS, but reported that these nuclear receptors were not involved in suppression.

### 2.6.8 Oxidative stress as a common inducer of CYP2A5

Given the structural diversity of CYP2A5 inducers and the variability of the pathological conditions that elevate CYP2A5 levels in the liver, it is currently thought that there may be a common mechanism behind many of the CYP2A5 inducing conditions. The fact that most inducing agents are hepatotoxins, which are often needed in high in vivo doses to obtain induction, also supports this view. It has been considered that hepatic inflammation in general could be a common precursor of CYP2A5/2A6 induction. However, CYP2A5 is down-regulated in liver inflammation induced by turpentine or LPS (Siewert et al. 2000, Gilmore et al. 2003), indicating that cellular damage rather than pro-inflammatory cytokines could regulate the induction.

Several chemical inducers of CYP2A5, such as carbon tetrachloride, cadmium, thiacetamide and cocaine have been shown to elicit cell death and
apoptosis in hepatocytes *in vivo* (Ledda-Columbano *et al.* 1991, Cascales *et al.* 1994, Habeebu *et al.* 1998, Shi *et al.* 1998). However, CYP2A5 induction occurs in the early stages of hepatocellular damage and appears not to be associated with terminal forms of injury, such as necrosis or apoptosis (Arvela *et al.* 1991, Tetri *et al.* 2002).

CYP2A5 induction in liver infected by *Helicobacter hepaticus* and in HBV transgenic mice is associated with oxidative damage (Chomarat *et al.* 1997, Sipowicz *et al.* 1997). Further, Konstandi *et al.* (1998) reported that restraint stress has an influence on CYP2A5 induction, but not on basal expression. Gilmore and co-workers (2003, 2004) proposed that induction of CYP2A5 during liver injury occurs via a mechanism involving alterations in the cellular redox status. The involvement of Nrf2 in Cyp2a5 induction by cadmium also suggests that oxidative stress in general may activate CYP2A5 (Abu-Bakar *et al.* 2007).

### 2.6.9 CYP2A5 induction in extrahepatic tissues

The induction of CYP2A5 has been studied extensively in the liver, but much less is known about its regulation in extrahepatic tissues. In addition to liver, pyrazole has been shown to activate CYP2A4/5 mRNA, protein, and COH-activity in the kidney, but not in the olfactory mucosa or lung (Verschoyle *et al.* 1997, Su *et al.* 1998). Several other known inducers of hepatic CYP2A5, including cobaltous chloride, stannous chloride, griseofulvin, thioacetamide, and aminotriazole also lack the ability to induce CYP2A5 in extrahepatic tissues (Su *et al.* 1998). In contrast, CYP2A5 is induced by sodium arsenite in the kidney but not in the liver (Seubert *et al.* 2002). Thus, the mechanisms involved in induction appear to be tissue-specific, at least for some inducers, but it is not known if they are related to tissue-selective toxicity or mechanistic differences in the regulation. Strain-specific differences in the induction of CYP2A5 in extrahepatic tissues have also been reported: cerium chloride induces CYP2A5 in the kidneys of DBA/2 but not C57BL/6 mice (Salonpaa *et al.* 1992).
3  Aims of the present study

The general aim of this study was to identify and characterize the mechanisms of cytochrome P450 induction and tissue specific expression. The study focused on characterization of transcriptional regulation of the murine Cyp2a5 gene in hepatocytes. The specific goals included:

1. To identify the specific 5′ promoter regions involved in transcriptional activation of the Cyp2a5 gene
2. To characterize transcription factors that bind to the identified promoter regions and activate Cyp2a5 expression in hepatocytes
3. To elucidate the impact of AHR and its ligands on Cyp2a5 induction
4. To investigate the mechanisms behind CYP2A5 induction caused by factors related to energy homeostasis, including fasting, glucagon, and cAMP
4 Materials and methods

4.1 Materials

The special reagents used in cell culture and their suppliers were as follows: collagenase solution (Worthington Biochemical Co., Lakewood, NJ, USA), William’s medium E, dexamethasone, ITS (insulin 5 mg/l, transferrin 5 mg/l, sodium selenate 5 µg/l), 3-methylcholanthrene (Sigma Chemical Co., St. Louis, MO, USA), gentamicin, fetal bovine serum, nucleoside-free α-minimal essential medium, Dulbecco’s Modified Eagle Medium with GlutaMAX™, PEST (100 U/ml penicillin, 100 µg/ml streptomycin) (Invitrogen, Paisley, Scotland), cycloheximide (Calbiochem, Merck KGaA, Darmstadt, Germany), and TCDD (National Cancer Institute Chemical Carcinogen Repository, Bethesda, MD, USA). Single-stranded oligonucleotides were purchased from Sigma Genosys and Oligomer (Helsinki, Finland). The antibodies used in these studies were: rabbit polyclonal anti-NF-I 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, USA.), goat polyclonal anti-HNF-4α antibody (C-19, sc-6556), rabbit polyclonal anti-HNF-4α antibody (H-171, sc-8987), rabbit polyclonal anti-USF-1 antibody (C-20, sc-229), rabbit polyclonal anti-ARNT antibody (H-172, sc-5580), rabbit polyclonal anti-PGC-1 antibody (H-300, sc-13067) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-ARNT antibody (ab14829, Abcam Ltd, Cambridge, UK), chicken polyclonal anti-CYP2A5 antibody (kindly provided by Dr. Risto Juvonen, University of Kuopio, Kuopio, Finland), mouse anti-actin antibody (A1978) Sigma Chemical Co., St. Louis, MO, USA), rabbit IgG and secondary HRP-rabbit anti-chicken/turkey IgG (Zymed Laboratories, South San Francisco, CA, USA).
4.2 Experimental set-up

Fig. 7. Schematic picture of experimental set-up.

4.3 Experimental animals

Hepatocytes were isolated from male DBA/2 (OlaHsd) (I-IV) and C57BL/6 (JOLAhsd) mice (III) (Center for Experimental Animals, University of Oulu, Finland). For in vivo studies, male DBA/2N and C57BL/6N mice and their F1 hybrids were obtained from IFFA-Credo (Lyon, France). Backcrosses of F1 and DBA/2N mice were generated at the International Agency for Research on Cancer (III).

The liver-specific Arnt-null mice were generated by breeding the Arnt-floxed (fl/fl) mouse (Tomita et al. 2000), with a transgenic mouse expressing the Cre transgene under control of the liver-specific albumin enhancer-promoter sequence (AlbCre) (Yakar et al. 1999), kindly provided by Dr. Derek Le Roith (NIDDK,
NIH, Bethesda MD, USA). The liver-specific Arnt-null mice and their Arnt(fl/fl) controls (without the AlbCre transgene) were on a mixed genetic background. The Arnt(fl/fl) mice were bred as littermates with the Arnt(fl/fl)-AlbCre mice in order to maintain the same genetic background (II).

For fasting experiments, male DBA2/J mice were caged in groups of five. The mice were kept at the Uppsala University animal house facilities with a 12 hour light-dark cycle. Control groups had access to standard animal chow and water, whereas the experimental groups had access only to water for 24, 48 and 72 hours (IV).

The animal experiments were approved by the local committees for laboratory animal welfare.

4.4 Cell models

4.4.1 Murine primary hepatocytes (I-IV)

CYP enzymes have rarely retained their expression in continuously growing cell lines and no CYP2A expressing cell line exists. Therefore the major model systems involved primary hepatocytes isolated from mouse liver. This culture system maintains CYP expression for a sufficiently long period of time to allow detailed regulatory studies and has the potential for CYP induction. Most of the studies were carried out with hepatocytes from DBA/2 mice that express the high activity form of CYP2A5 at a relatively high basal level (van Iersel et al. 1994). Male mice, aged 8 to 10 weeks, were used, because they express the female-predominant highly homologous CYP2A4 in only minor quantities.

Livers were perfused with collagenase solution (Worthington Biochemical Co., Lakewood, NJ, USA) as described previously (Salonpaa et al. 1994). After filtration and centrifugation, the isolated hepatocytes were dispersed in culture medium (William’s medium E containing dexamethasone, insulin, transferrin, sodium selenate, gentamicin and fetal bovine serum). The cultures were maintained at 37°C in a humidified incubator for 1 to 2 hours, after which non-attached cells were discarded by aspiration, and the medium was replaced with serum-free culture medium. The handling of the hepatocytes in the different assays is described comprehensively in original publications I-IV.
4.4.2 Continuous cell lines (I-III)

A subclone Hepa-1c1c7 of the mouse hepatoma cell line Hepa-1 and the Hepa1c1c7 mutant strains deficient in AHR (c12) and ARNT (c4) (Hankinson 1994) were kindly provided by Dr. Sirkku Saarikoski (Helsinki, Finland) (originally from Dr. Oliver Hankinson, UCLA, Los Angeles, CA, USA). The cell lines were cultured in nucleoside-free α-minimal essential medium supplemented with fetal bovine serum and PEST.

The human hepatoma cell line HepG2 and monkey kidney cell line COS-1 (American Type Culture Collection, Rockville, MD, USA) were cultured in Dulbecco’s Modified Eagle Medium, containing fetal bovine serum and PEST.

The cultivation and different treatments of the cell-lines are explained in detail in original publications I, II and III.

4.5 Promoter analysis (I-IV)

4.5.1 Reporter gene constructs

To identify and characterize the regulatory regions important for specific regulatory processes the Cyp2a5 5’–3033 to +10 (from the transcription start site) fragment of the Cyp2a5 5’ flanking region was amplified with PCR from DBA/2 mouse genomic DNA using Dynazyme EXT polymerase (Finnzymes, Helsinki, Finland). The fragment was then cloned into the pGL3-Basic vector (Promega, Madison, WI, USA) in front of the luciferase reporter gene. In addition, several shorter 5’ deletion constructs were prepared by PCR, using the Cyp2a5 5’–3033 to +10-Luc plasmid as a template and subcloning the PCR products. The Cyp2a5 5’-XRE-rAlbTATA-Luc plasmid was prepared by cloning the region Cyp2a5 5’–2513 GCTCACTCACGCACTCTGG –2495) in front of the rat albumin proximal promoter region –40 to +28 (Hakkola et al. 2003). The expected structures of the constructs were verified by sequencing. The reporter gene constructs used are listed in Table 3.

4.5.2 Site-directed mutagenesis

Site-directed mutagenesis of the putative transcription factor binding sites was performed using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer’s instructions. The mutations
were introduced into Cyp2a5 5'-Luc plasmids using mutated oligonucleotides described in original publications I-III. Correct assembly of the mutations was confirmed by sequencing. Mutated reporter gene constructs are listed in Table 3.

Table 3. Reporter gene constructs used in the studies.

<table>
<thead>
<tr>
<th>Reporter gene construct</th>
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<th>to</th>
<th>Element/Mutation</th>
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<tbody>
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<td>-3033</td>
<td>+10</td>
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<td>-3033</td>
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<td>+10</td>
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<tr>
<td>in pGL3-rAlbTATA</td>
<td>-2537</td>
<td>-2519</td>
<td>XRE</td>
</tr>
</tbody>
</table>

4.5.3 Expression vectors

The rat HNF-4α1 expression plasmid (Torres-Padilla & Weiss 2003) was a gift from Dr. Mary C. Weiss (Unité de Génétique de la Différenciation, Département de Biologie du Développement, Institut Pasteur, Paris Cedex 15, France). The expression plasmids coding pcDNA3-AHR (Fukunaga & Hankinson 1996) and pcDNA/Neo-ARNT (Reisz-Porszasz et al. 1994) expressing mouse AHR and ARNT, respectively, were provided by Dr. Oliver Hankinson (UCLA, Los Angeles, CA, USA). The full-length ARNT cDNA was restricted with BamHI and HindIII enzymes from the pcDNA/Neo construct and ligated to the pcDNA3.1(+) vector (Invitrogen, Paisley, Scotland). The ARNT deletion construct without the C-terminal transactivation domain (ARNTΔC) was prepared by polymerase chain reaction (PCR) using the pcDNA/Neo-ARNT as a template, as described in original publication II. The PCR product was subcloned into the pcDNA3.1(+) vector. The pCR3-hUSF-1 and pCR3-hUSF-2a expression vectors were provided by Dr. Benoit Viollet (Institut Cochin, Département de Génétique, Développement et Pathologie Moléculaire, Université René Descartes Paris 5, INSERM U567, Paris, France), and the pcDNA3-PGC-1α vector was from Dr.
JongsEEK Kim Kemper (Department of Molecular and Integrative Physiology, University of Illinois, Urbana-Champaign, Illinois, USA).

4.5.4 Transfection and luciferase assays

The reporter gene constructs were transfected into cells together with Renilla luciferase reporter vector (pRL3-TK) (Promega, Madison, WI, USA), which was used as an internal control. Tfx-20 reagent (Promega, Madison, WI, USA) was used according to the manufacturer’s protocol in Opti-MEM I medium (Invitrogen, Paisley, Scotland). In the co-transfection assays, expression vector DNA was transfected together with reporter gene vectors. 0.5 µg of reporter plasmid and 0.1 µg of pRL3-TK were transfected per 3 \times 10^5 hepatocytes, and 0.65 µg of reporter plasmid and 0.05 µg of pRL3-TK were transfected per one well (in a 24-well plate) of Hepa-1 or COS-1 cells. In the co-transfection assays, 0.1 µg and 0.05 µg of expression vector DNA was transfected into hepatocytes and Hepa-1 or COS-1 cells, respectively. The cell extracts were assayed for luciferase activity with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) and the experimental activities (firefly luciferase) were normalized against control activities (Renilla luciferase).

4.5.5 Computer based promoter analysis

Putative regulatory elements were identified using transcription factor search programs, such as the MatInspector professional program using Genomatix matrixes (http://www.genomatix.de) and by comparing the conserved promoter regions of closely related genes with alignment programs, e.g. BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and DiAlign TF (www.genomatix.de). MatInspector uses the core sequence similarity, the nucleotide distribution, the matrix similarity, the C_i-vector (which represents the conservation of the individual nucleotide positions in the matrix in numerical values), the optimized threshold and family information to scan sequences for matches to the consensus matrix description. The output of MatInspector consists of those matches that reach the user-defined minimum core and matrix similarity or the optimized matrix threshold for each matrix as cut-off criteria.
4.6 Virus infection (IV)

Since plasmid transfection is a relatively inefficient way of introducing DNA into primary hepatocytes, a recombinant adenovirus expressing mouse PGC-1α was prepared to study the effect of PGC-1α over-expression on the endogenous Cyp2a5 gene. The virus preparation is explained in original article IV. Mouse primary hepatocytes were infected with Ad-PGC-1α viruses or control adenovirus expressing a membrane-targeted GFP (Ad-GFP-GL-GPI) in Opti-MEM I medium (Invitrogen, Paisley, Scotland). The infected cultures were maintained at +37°C in a humidified incubator for 1 hour, after which the medium was replaced by serum-free William’s E medium. The cultures were maintained for an additional 48 hours before RNA and protein extraction or 24 hours before chromatin immunoprecipitation.

4.7 DNA-protein interaction studies

4.7.1 Preparation of nuclear extracts (I-III)

Nuclear extracts were prepared according to Schreiber et al. (1989) from cultured mouse primary hepatocytes, or HepG2, COS-1 and Hepa-1c1c7 cells as described in detail in original publications I-III. The protein content of the nuclear extracts was determined using the Bradford protein analysis method (Bradford 1976). Nuclear extracts from DBA/2 mouse liver were prepared similarly, except that the tissue was first homogenized in lysis buffer [20 mM Tris/Cl, 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).

4.7.2 In vitro–translation (II)

To produce ARNT protein for EMSA, ARNT cDNA was digested from the pcDNA/Neo-ARNT vector using Hind III and BamHI restriction enzymes and cloned into the pSP64 poly(A) vector (Promega, Madison, WI, USA). The TnT SP6 Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA) was used to generate ARNT protein from pSP64/ARNT. 1 µg of pSP64/ARNT, empty pSP64 or pSP64 Luciferase Control DNA was added to the rabbit reticulocyte lysate system and the reaction was performed according to the
manufacturer’s protocol. The translation results were analyzed by Western blot and by measuring the luciferase activity as a positive control.

4.7.3 Dnase I footprinting (I)

Dnase I footprinting is a method for determining the location and sequence of a protein binding site in DNA. The –271 to +10 fragment of the Cyp2a5 5’ flanking region was subcloned to the pCR 2.1-TOPO vector (Invitrogen, Paisley, Scotland), and the DNA probe for footprinting was prepared from the Cyp2a5 5’ – 271 to +10 fragment in pCR 2.1-TOPO by digestion with EcoRI. The fragment was treated with alkaline phosphatase and end labelled with T4 polynucleotide kinase using \([\gamma^{32}\text{P}]\). To produce a probe labelled at one end only, one of the labelled ends was cleaved by either with HindIII or with KpnI. The probe was purified using a QIAquick gel extraction kit (Qiagen, Venlo, The Netherlands).

DNase I footprinting analyses were performed with a Core Footprinting System (Promega, Madison, WI, USA) according to manufacturer’s instructions and as described in original publication I. 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 using a chemical A+G sequencing reaction (Maxam & Gilbert 1977).

4.7.4 Electromobility shift assays (I-III)

In an electromobility shift assay (EMSA), the DNA binding proteins are detected due to their ability to retard the mobility of a labelled DNA fragment through a non-denaturating gel. Double-stranded DNA probes were prepared by annealing the desired sense and antisense oligonucleotides. The annealed oligonucleotides were then 5’ end-labelled with \([\gamma^{32}\text{P}]\)ATP and T₄ polynucleotide kinase and purified using the QIAquick nucleotide removal kit (Qiagen, Venlo, The Netherlands). The labelled oligonucleotide probes were incubated together with nuclear extracts or in vitro-translated proteins under specific conditions, as described in original publications I-III. The samples were then separated by electrophoresis through 6% (w/v) polyacrylamide gel, and the retarded complexes were detected by autoradiography. For competition experiments, unlabelled competitor oligonucleotides (5 to 100-fold excess) were added to the mixtures. In supershift assays, the identity of binding proteins was studied by adding antibodies against a putative transcription factor to the nuclear extracts.
4.7.5 Chromatin immunoprecipitation (II and IV)

The chromatin immunoprecipitation (ChIP) method takes into account the role of chromatin and protein-protein interactions in the regulation of gene expression. In this method, DNA-protein complexes are fixed and precipitated with specific antibodies, and the DNA bound to these complexes is identified using the polymerase chain reaction (PCR).

Murine primary hepatocyte cultures (in 175 cm$^2$ flasks) were used for immunoprecipitation of protein-DNA complexes. Chromatin immunoprecipitations were done according to Väisänen et al. (2004) with certain exceptions. Transcription factors were cross-linked to DNA with 1% (v/v) formaldehyde for 10 minutes at 37°C. Cells were washed and collected, and pelleted cells were resuspended in 2 ml of SDS Lysis Buffer. Lysates were sonicated to reduce the DNA length to between 200 and 1000 base pairs. Precleared chromatin fractions were divided into 200-µl aliquots and diluted 10-fold in ChIP Dilution Buffer. 1 µg of a specific antibody or rabbit IgG as a negative control, was added to the chromatin solution and incubated overnight at 4°C. Immune complexes were collected with 60-100 µl of 50% (w/v) Protein A agarose slurry and the beads were washed with an immune complex wash buffer series. For the second immunoprecipitation (re-ChIP), the immuno-complexes collected and washed after first immunoprecipitation were eluted by adding 200 µl of 10 mM dithiothreitol and incubating at 22°C for 30 minutes with rotation. The beads were spun down and the supernatants were diluted 1:40 (v:v) in dilution buffer. 2 µg of antibody against the second protein of interest was added and incubated at 4°C overnight. The new immuno-complexes were collected by incubating with 120 µl of 50% (w/v) protein A agarose slurry at 4°C for two hours and washed in the same way as the first ChIP. DNA fragments from both ChIP- and re-ChIP samples were eluted and reverse crosslinked at +65°C overnight. The remaining proteins were digested with Proteinase K. The DNAs were purified by phenol:chloroform:isoamylalcohol (25:24:1, v:v:v) extraction and resuspended in 60 µl of H$_2$O. A dilution series was prepared from an input sample containing the total fragmented chromatin.

The AmpliQ Universal Real Time PCR Master Mix Kit (Ampliqon, Copenhagen, Denmark) was used for real-time PCR. The PCR mixtures contained 10 µl of the 2X master mix, 2 µl of Green DNA Dye diluted 1:2000 (v:v), 200 nM of each PCR primer (the sequences are listed in original articles II and IV) and 5 µl of precipitated DNA in a total volume of 20 µl. The samples were incubated at
95°C for 15 min, followed by 45 cycles of 95°C for 30 s, 55°C for 1 min and 72°C for 30 s in an Mx3000P QPCR system (Stratagene, La Jolla, CA, USA). The specificity of the PCR-products was confirmed with melting curve analysis and by size with agarose gel electrophoresis.

4.8 RNA analysis

4.8.1 RNA extraction (II-IV)

Total RNA was isolated from cultured mouse hepatocytes (II-IV), Hepa-1 cells (III) or mouse liver and kidneys (II) by the guanidine thiocyanate-CsCl method (Chirgwin et al. 1979) or using the Tri-Reagent (Sigma Chemical Co., St. Louis, MO, USA) according to the manufacturer’s protocol for monolayer cells. The total liver RNA from mouse livers in original publication III was prepared using the RNAzol B reagent (TEL-TEST Inc. Friendswood, Texas, USA).

4.8.2 Northern blot (II-IV)

The total RNA was electrophoretically resolved and transferred on to a Hybond-N+ nylon membrane (Amersham Biosciences, Little Chalfont, UK) (II-IV) or on to a Gene screen Plus nylon membrane (Dupont Co. Boston MA, USA) (III). The RNA was fixed by UV-cross linking, and the membrane was hybridized with \([\alpha^{32}\text{P}]\) dCTP-labelled probes. The full-length CYP2A5 cDNA was kindly provided by Dr. Masahiko Negishi (NIEHS, Research Triangle Park, NC, USA), the CYP1A2 cDNA was a gift from Dr. Paavo Honkakoski (University of Kuopio, Finland), the CYP1A1 cDNA probe was prepared as described (Hakkola et al. 1996), the ARNT probe was prepared by digesting the pSP64/ARNT vector with SacI restriction enzyme yielding a 155 bp fragment, and the 18S probe was provided by Dr. Heikki Ruskoaho (University of Oulu, Finland).

4.8.3 Quantitative reverse transcriptase PCR (II and IV)

Two different chemistries were used for quantitative PCR (Q-PCR): SYBR green and TaqMan. RNA was first reverse transcribed to produce cDNA using either p(dN)6 random primers and M-MLV reverse transcriptase (Promega, Madison, WI, USA) or a First-Strand cDNA Synthesis Kit (Amersham Biosciences, Little
Chalfont, UK) according to the manufacturer’s instructions. Detailed explanations of Q-PCR reactions and the primer pairs used can be found in original articles II and IV. The RNA levels were normalized against the 18S control levels using the comparative $C_T$ ($\Delta\Delta C_T$) method, as described in the Methods and Application Guide, Introduction to Quantitative PCR (Stratagene, IN #70200-01/Revision #105002).

4.9 Protein analysis

4.9.1 Western blot (II-IV)

For western blots, 13 000 g supernatant proteins including microsome fractions of hepatocytes were subjected to SDS-polyacrylamide gel electrophoresis. The proteins were transferred on to a Hybond ECL nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK) and the membrane was incubated with specific primary antibodies and secondary HRP-linked antibodies. After washing, the immunoreactive bands were visualized with either an ECL+plus Western Blotting Detection System (Amersham Biosciences, Little Chalfont, UK) or using the Chemiluminescent Peroxidase Substrate 1 reaction (Sigma Chemical Co., St. Louis, MO, USA). The immunoreactive bands were quantitated using Quantity One software (Bio-Rad, Hercules, CA, USA).

4.9.2 Coumarin 7-hydroxylase assay (III and IV)

The catalytic activity of CYP2A5 was determined by measuring COH activity from the mouse livers according to Aitio et al. (1978). To determine the COH activity in murine primary hepatocytes, the cells were scraped, washed, suspended in phosphate-buffered saline, and sonicated. The sonicated suspensions were then centrifuged at 13000 g for 10 min. Pellets were discarded, and mouse liver COH activity was measured from the supernatants as described previously (Aitio 1978) using 100 µM coumarin (Sigma Chemical Co., St. Louis, MO, USA) as a substrate.
4.10 Cycloheximide chase (III)

The cycloheximide chase method was used to study if new protein synthesis was needed for up-regulation of CYP2A5. Murine primary hepatocytes were treated with 10 µg/ml cycloheximide (Calbiochem, Merck KGaA, Darmstadt, Germany) for 1 hour prior to the administration of the inducer to block protein synthesis. The cells were then treated with the inducer or vehicle only for a certain time, after which the mRNA levels were measured and compared to induced cells not treated with cycloheximide.

4.11 Statistical analysis

Student’s t test was used for comparisons between two groups. Comparisons of several groups were done with one-way ANOVA followed by the least significant difference post hoc test. Differences were considered significant when p<0.05.
5 Results

5.1 Characterization of the Cyp2a5 5' promoter

Preliminary characterization of the 5' sequence upstream from the Cyp2a5 transcription start site was done using computer-based promoter analysis programs. Putative transcription factor binding sites were searched from the Cyp2a5 5' region using the MatInspector professional program. Nearly 1000 putative binding sites were found in the −5.8 kb region upstream from the Cyp2a5 transcription start site when the all vertebrates Matrix Family Library and default matrix and core similarity values (0.75/optimized, respectively) were used. Because of the numerous false positive hits in these results, the search was limited to the conserved promoter regions by aligning the orthologous Cyp2a5 and CYP2A6 with the DiAlign TF program. However, the promoters of these genes are generally not very well conserved and primate promoters often include certain repeat elements not found in rodent DNA, which weaken the alignment of genomic sequences. Therefore, the alignment program was not able to compare the sequences properly, and no binding sites found by computer analysis in these studies were considered worth studying with in vitro methods.

No alternative promoters or transcription start sites were found for Cyp2a5 in literature or database searches [Mouse Genome Informatics (www.informatics.jax.org/) and UCSC Genome Browser on Mouse (http://genome.ucsc.edu/)]. Since mutations that affect gene expression levels have been found in the regulatory DNA of the CYP2A6 gene (Pitarque et al. 2004, von Richter et al. 2004), the sites surrounding these mutations were also located in the Cyp2a5 promoter and aligned with CYP2A6 sequences. No significant conservation between these regions in CYP2A6 and Cyp2a5 promoters was found and, therefore, it was then thought that these sites do not participate in Cyp2a5 regulation. However, in very recent studies, it has been shown that the lack of conservation does not guarantee that the sites are not functional (ENCODE Project Consortium 2007, Henikoff 2007, Odom et al. 2007). The TATA-box that binds the preinitiation complex can be found both in CYP2A6 and Cyp2a5 promoters (Lindberg & Negishi 1989, Pitarque et al. 2001).

To identify the active regions of the Cyp2a5 5' promoter in vitro, a series of Cyp2a5 sequences upstream from the transcription start site (3033, 2013, 1020, 271, and 63 bp) were cloned in front of the luciferase gene (Luc) in the pGL3
vector. The reporter vectors together with a control construct (pRL-TK) were transfected into mouse primary hepatocytes and the promoter activities were measured using the dual-luciferase system. Two major activation sites for Cyp2a5 were revealed: one at the proximal promoter region at −271 and the other at the distal enhancer between −3033 to −2014 from the transcription start site. Removal of the sequence from −2013 to −1021 did not noticeably affect luciferase activity compared with the Cyp2a5 −2013-Luc construct. Instead, the deletion of the sequence from −1020 to −272 increased the activity >3-fold relative to the Cyp2a5 −1020-Luc construct, indicating that repressing response elements might be present in the region between −1020 to −272. (Fig. 8).

![Diagram](image.png)

Fig. 8. Transcriptional activity of the Cyp2a5 5′-Luciferase (LUC) constructs transfected into murine primary hepatocytes (modified from original publication I). The two promoter constructs that had the strongest activity are indicated with arrows.

### 5.2 Activation of Cyp2a5 transcription by NF-I and HNF-4 (I)

In the first original publication, the role of the proximal promoter region in Cyp2a5 gene regulation was characterized. In DNAse I footprinting analysis, the nuclear proteins from mouse primary hepatocytes interacted with the promoter region from −132 to −107. The sequence of this region was homologous to a transcription factor NF-I binding site. Based on previous literature (Yokomori et al. 1997), it was hypothesized that HNF-4 might regulate the liver specific expression of Cyp2a5. Although the HNF-4 binding was not seen in DNAse I footprinting studies, probably due to the sensitive conditions needed for the DNA-protein interaction, a conserved HNF-4 response element was found at position −63 to −47 with a computational binding site search. Co-transfection of
the HNF-4α expression vector together with Cyp2a5 5′–271 to +10-Luc in COS-1 cells elevated the promoter activity significantly via the HNF-4 binding site. Both NF-I and HNF-4 bound to their respective binding sites in an electromobility shift assay.

According to deletion and site-directed mutations of the NF-I and HNF-4 binding sites, both elements were functional and needed for maximal activity of the Cyp2a5 promoter. HNF-4 also appeared to be essential for the function of the distal activation region, as mutation of the proximal HNF-4 site reduced transcription by the –3033 to +10 promoter to only 1.6% of the control activity. Mutation of the NF-I site decreased the transcriptional activity of the Cyp2a5 promoter significantly, but to a lesser extent than the HNF-4 site mutation. The double mutation of both HNF-4 and NF-I binding sites almost completely abolished the transcription. Although the HNF-4 binding site is also located in the shortest promoter construct, Cyp2a5 5′ –63 to +10-Luc, HNF-4 is likely to need the flanking regions of the motif for binding or cooperation with other transcription factors and thus does not activate the shortest promoter region.

### 5.3 ARNT and USF regulate Cyp2a5 via a common E-box site (II)

To characterize the distal activation region of the Cyp2a5 promoter, putative transcription factor binding sites were searched using computational analysis. Interestingly, a conserved, palindromic E-box site was found at position –2403 to –2398, between the xenobiotic response element, at –2514 to –2492 (characterized in original publication III), and Nrf2 response element, at -2386 to –2377 (Abu-Bakar et al. 2007) that were studied in our laboratory at the same time. The mutation of the E-box element in the Cyp2a5 5′ –3033 to +10-Luc construct caused a significant reduction in the promoter activity: only 20% of the activity was left when compared to the non-mutated promoter. Several factors can bind to the E-box. After an extensive literature search, it was considered that the ubiquitously expressed transcription factors ARNT and USF were the candidates most likely to regulate Cyp2a5 in the liver. In a chromatin immunoprecipitation assay, both ARNT and USF were able to bind to the E-box.

Previously, ARNT has been shown to bind to the palindromic E-box as a homodimer, but only in viral promoters (Sogawa et al. 1995, Swanson et al. 1995). To study if ARNT bound to the Cyp2a5 5′ E-box as a homodimer, in vitro-translated ARNT protein was used in gel shift assays. In these experiments, ARNT was shown to bind to the Cyp2a5 E-box without a heterodimerization
partner. Results from co-transfection assays in ARNT-deficient Hepa-1 cells also indicated that ARNT expression is needed for the maximal activation of the \textit{Cyp2a5} promoter. In the liver-specific \textit{Arnt-null} mice, the level of hepatic CYP2A5 mRNA was significantly decreased. The CYP2A5 protein level in these mice was also diminished even though the reduction was not significant due to the large variance in the data. The transcription activation domain (TAD) of ARNT was shown to be essential for \textit{Cyp2a5} transactivation, which further suggests that ARNT can act as a homodimer in \textit{Cyp2a5} regulation. Interestingly, the mRNA level of ARNT, and especially its splicing variant 1, was elevated together with CYP2A5 mRNA in hepatocyte cultures during the first 24 hours of cultivation. USF-1 and -2a also activated CYP2A5 transcription in transfection experiments done in COS-1 cells, but only in the presence of HNF-4\textalpha, whereas ARNT transactivation appeared to be independent of HNF-4\textalpha.

### 5.4 Regulation of \textit{Cyp2a5} involves an AHR dependent pathway (III)

It was previously assumed that among the xenobiotic-metabolizing CYP enzymes, only members of the CYP1 family were regulated by the AHR pathway. However, some CYP2 members have also been shown to be induced by AHR or its ligands (Kimura \textit{et al.} 1989, Kurose \textit{et al.} 1999, Rivera \textit{et al.} 2002), and CYP2A5-catalyzed COH activity has been reported to be elevated by the AHR ligand TCDD in mouse liver slices (Gokhale \textit{et al.} 1997). To further study this induction and the mechanisms behind it, murine primary hepatocytes were treated with TCDD. TCDD increased the CYP2A5 protein level 3-fold and a similar induction was seen in COH activity. To establish whether AHR regulates CYP2A5 in response to TCDD, the C57BL/6 and DBA/2 mouse strains with a genetically determined difference in AHR function (Chang \textit{et al.} 1993, Ema \textit{et al.} 1994) were used. CYP2A5 mRNA induction in the different mouse strains showed a response difference typical for AHR regulated genes. This difference in induction was seen both with TCDD in primary hepatocyte cultures and with 3-MC \textit{in vivo}. In addition, CYP2A5 was dose-dependently co-induced with CYP1A1 and CYP1A2, although the level of CYP2A5 induction was lower. The C57BL/6 and DBA/2 strains also differ in COH activity due to a mutation in the \textit{Cyp2a5} gene (Lindberg \textit{et al.} 1992), but, since it is assumed that the mutation does not affect the expression of \textit{Cyp2a5}, the mRNA levels should not be changed because of it. The experiments with the protein synthesis inhibitor cycloheximide indicate that synthesis of new proteins is not necessary for CYP2A5 induction by TCDD.
TCDD. This also suggests that a direct, putatively ligand-binding regulator is involved in CYP2A5 up-regulation by AHR ligands.

In reporter gene assays, the Cyp2a5 5’–3033 to +10 promoter mediated a 2-to 5-fold induction of luciferase activity by TCDD treatment in primary hepatocytes and in Hepa-1 hepatoma cells. The induction was also studied in Hepa-1 variant cell lines with deficiencies in the AHR/ARNT complex. The absence of ARNT in these cells abolished the TCDD induction of Cyp2a5, and a reduction in induction was seen in AHR-deficient cells. The remaining induction in AHR-deficient cells is probably due to the low, but existing levels of AHR in the cell line (Zhang et al. 1996). A putative AHR response element (XRE) was identified in the Cyp2a5 promoter at the position –2514 to –2492 and the element was found to interact with the AHR/ARNT heterodimer in a gel shift assay. The functionality of the XRE was investigated by mutating the element in a reporter gene construct. Transfection experiments with mutated and non-mutated constructs revealed that the XRE site partly mediates the TCDD induction of Cyp2a5. An additional AHR-dependent mechanism is likely to regulate the proximal promoter of the Cyp2a5 gene. Since no conserved AHR response elements were found in the proximal promoter, AHR may mediate the induction through another transcription factor.

5.5 Coactivator PGC-1α regulates Cyp2a5 through HNF-4α (IV)

The CYP2A5 enzyme has previously been shown to be induced by fasting (Bauer et al. 2004). In addition, glucagon, a major hormonal mediator of the fasting response, directs CYP2A5 induction through cAMP signalling (Salonpää et al. 1994, Viitala et al. 2001). PGC-1α, in turn, activates numerous hepatic fasting responses mediated by cAMP (Yoon et al. 2001, Bousted et al. 2003, Puigserver et al. 2003, Rhee et al. 2003). We hypothesized that PGC-1α was also involved in the nutritional regulation of drug metabolism and mediated the induction of CYP2A5 by cAMP. In murine primary hepatocytes, cAMP caused coordinated induction of PGC-1α and CYP2A5 mRNA. An increase of the PGC-1α expression level by adenovirus mediated gene transfer in primary hepatocytes induced CYP2A5 mRNA expression. In co-transfection studies, Cyp2a5 5’ promoter constructs with a PGC-1α expression plasmid demonstrated that PGC-1α was able to activate Cyp2a5 transcription. When the HNF-4 binding site in the Cyp2a5 promoter construct was mutated, the activation by PGC-1α was abolished, indicating that PGC-1α coregulates HNF-4α. Chromatin
immunoprecipitation assays showed that PGC-1α binds together with HNF-4α to the same region of the Cyp2a5 proximal promoter and that PGC-1α binding is enhanced when the PGC-1α expression level is elevated.
6 Discussion

The transcriptional mechanisms behind both constitutive and inducible regulation of CYP2A5 in murine hepatocytes have been identified and characterized in the original articles of this thesis. Transcription factors HNF-4 and NF-I are likely to be involved in the liver-specific expression of CYP2A5, whereas ARNT and USF may control CYP2A5 in different physiological circumstances. The AHR pathway, previously thought to regulate mainly CYP1 enzymes, was also shown to up-regulate CYP2A5. Finally, nutritional factors were shown to regulate CYP2A5 through transcriptional coactivator PGC-1α. The significance of these novel findings in relation to previously reported CYP2A5 regulatory mechanisms, as well as some methodological aspects of regulatory studies, will be discussed in the following chapters.

6.1 Methodological aspects

6.1.1 Cell models

Primary hepatocytes are considered the best model for hepatic CYP studies due to the well established loss of P450 activity in continuous cell lines. This loss is probably due to the down-regulation of liver specific transcription factors and their coactivators (Jover et al. 1998, Martinez-Jimenez et al. 2006a). Epigenetic factors, such as DNA methylation and histone modifications, may be involved in the suppression of these transcriptional activators or P450s directly in continuous cell lines, since a striking and wide-spread de novo methylation occurs in the immortalization of cells (Jones & Laird 1999). The CYP silencing in hepatocyte cell lines can be partially reversed by transfecting the missing transcription factors (Castell et al. 2006, Martinez-Jimenez et al. 2006b) or by demethylation and inhibition of histone deacetylases (Ling et al. 2007). Primary hepatocytes also undergo several rapid phenotypic changes and lose some of the characteristics of highly differentiated cells in culture (Waxman & Azaroff 1992). For instance, C/EBPα, HNF-1 and some CYP mRNA levels start to decrease during the cultivation of rat hepatocytes (Padgham et al. 1993).

When compared with hepatocytes in intact liver, the primary hepatocyte model has the apparent limitation that the normal growth restraints of hepatocytes in vivo are disrupted. In primary hepatocytes, the induction kinetics also appear to
be slower than in intact liver (Waxman & Azaroff 1992, Honkasoski et al. 1996, Salonpaa et al. 1997). With CYP2A5, some of the in vivo inducers do not elevate CYP2A5 in primary cells (Salonpaa et al. 1997, Kojo et al. 1998) indicating that systemic effects are involved in its regulation. Therefore, it is important to carefully plan the conditions and timing of the experiments carried out with primary hepatocytes and also to confirm the results obtained in vivo.

6.1.2 DNA constructs versus chromatin

An intrinsic limitation of transient transfection studies is that the plasmid DNA used does not contain the properties of native chromatin, where nucleosomal reorganization is known to be important for transcriptional regulation. The length of the sequences in artificial DNA constructs is also limited and promoters integrated with reporter genes may lack additional regulatory elements located in the coding DNA or intron regions. In these studies, the Cyp2a5 5’ promoter region spanning –3033 to +10 upstream of the transcription start site was used. It is usually assumed that the proximal promoter located in the first 500 bp upstream of the transcription start site confirms the constitutive activity of the promoter. However, tissue-specific regulatory regions responsible for the basal level activity of the CYP genes have been found much farther away (Martinez-Jimenez et al. 2005), and enhancer regions activated by specific inducers can be located even tens of thousands of base pairs away from the transcription start site.

Zhuo et al. (2004) reported that knocking out the Cyp2g1 gene, about 20 kb upstream from the Cyp2a5 coding DNA, suppressed Cyp2a5 expression in the liver. The mechanism of the suppression is not clear, but the authors suggested that neighbouring effects from the active transcription of the neo cassette in the third exon in the Cyp2g1 gene, in tissues that normally have an inactive Cyp2g1 allele, such as liver, disturb the chromosomal structure of the Cyp2a5 promoter. However, the circadian variation in the level of hepatic CYP2A5 mRNA was not altered, indicating that the proximal promoter, including the DBP response elements, worked normally. Thus, some tissue specific regulatory elements can be located far upstream from the transcription start site of the Cyp2a5 gene, and the promoter region used in the present studies may be too short and may lack additional regulatory elements needed for maximal Cyp2a5 transcription.
6.2 Constitutive and tissue-specific expression of Cyp2a5

Several different transcription factors appear to participate in the constitutive regulation of the Cyp2a5 gene. HNF-4α and NF-I are most likely involved in tissue-specific expression of CYP2A5 together with DBP, which controls the circadian rhythm of the CYP2A5.

It has been reported that NF-I isoforms are associated with tissue-specific regulation of P450s in the olfactory mucosa (Zhang et al. 2000, Xie et al. 2001, Ling et al. 2004b), and a liver-specific form/forms may also exist that is involved in hepatic regulation of Cyp2a5. NF-I has been shown to mediate CYP1A1 down-regulation by oxidative stress (Morel & Barouki 1998), but since Cyp2a5 appears to be up- rather than down-regulated by oxidative stress, different signalling pathways or NF-I forms are likely to be involved in CYP2A5 regulation.

HNF-4 is expressed in high levels in the liver and it has a critical role in liver development and in the expression of a large number of hepatic genes (Sladek 1993, Chen et al. 1994b, Odom et al. 2004). According to the results presented in original publication I, HNF-4α is the key factor in activating Cyp2a5 transcription and it is probably also needed in the regulation by other transcription factors and inducers of the gene. Somewhat contradictory to these results, Wiwi et al. (2004) reported that HNF-4α represses the transcription of the female-predominant Cyp2a4 gene in male mice indicating that HNF-4α has the opposite effect in controlling these two genes in male mice. Gender differences are more pronounced for CYP2A4 (Noshiro & Negishi 1986) than for CYP2A5 and it may be that HNF-4α regulates Cyp2a5 and Cyp2a4 differently. However, systemic effects are usually needed for gender specific gene regulation and thus it will be important to determine the role of HNF-4α in CYP2A5 regulation in vivo in mice.

ARNT and USF are also involved in the constitutive expression of the Cyp2a5 gene in hepatocytes (II). Since they both mediate their transactivation effects through a common E-box element, they are likely to compete on binding to this site. It is not known exactly how the target gene’s preference for different factors binding to the same E-box sequence is determined. Binding is likely to be dependent on the concentrations or modifications of the transcription factors and their coregulators in the cell. Distinct physiological or pathophysiological conditions may alter the expression or activity of ARNT and USF in hepatocytes and thereby determine their contribution in Cyp2a5 regulation. However, the exact role of these E-box binding factors has still to be clarified.
The location of the E-box motif between the AHR response element and the stress response element activated by Nrf2 in a short ~140 bp region suggests that the E-box might cooperate in regulation by AHR and Nrf2. Indeed, USF-1 and -2 have previously been shown to be involved in the transcription of genes related to stress response, and to participate in the induction of heme oxygenase-1 and metallothionein I genes by cadmium (Sato et al. 1989, Sato et al. 1990, Li et al. 1998).

Both USF and ARNT have also been associated with the regulation of glucose and lipid metabolism, although the dimerization partner for ARNT was not specified in these studies (Vaulont et al. 2000, Carriere et al. 2005, Gunton et al. 2005). E-box sequences are involved in the glucose response of several genes and USFs mediate this response in many cases (Vaulont et al. 2000). The cooperative binding of USF and HNF-4, which was also seen in the regulation of Cyp2a5, has been reported to drive the transcription of various genes involved in energy metabolism (Aida & Negishi 1991, Ribeiro et al. 1999, Pastier et al. 2002).

### 6.3 The role of AHR and Nrf2 in Cyp2a5 regulation

In original publication III, the AHR mediated pathway was shown to activate CYP2A5 expression. The induction of CYP1 enzymes by AHR is well established, and these enzymes also metabolize AHR ligands. However, no xenobiotic AHR ligands have been shown to be CYP2A5 substrates and the significance of CYP2A5 induction by AHR is not apparent. The extent of Cyp2a5 induction by AHR is also modest compared to Cyp1 family induction and especially that of Cyp1a1. Cyp1a1 contains several copies of XREs in its 5' regulatory region, while only one site was identified in the Cyp2a5 promoter. Interestingly, the single XRE is located very near to the Nrf2 binding site StRE in the distal Cyp2a5 5' region. Interaction between the AHR and Nrf2 pathways has previously been shown: AHR is able to activate some Phase II genes through XRE sequence motifs, as well as indirectly through StRE sites via Nrf2 (Nebert et al. 2000). Furthermore, AHR has been reported to be able to activate Nrf2 gene transcription by binding to the XREs of the Nrf2 promoter (Miao et al. 2005). Proteins that are regulated by Nrf2, such as HO-1 and NQO1, control cellular redox status and defend cells against oxidative damage (Ryter & Choi 2002, Cederbaum 2006, Gong & Cederbaum 2006b). Similarly to Cyp2a5, the XRE and ARE sites are located near to each other in the murine nqo1 promoter (Nioi & Hayes
Therefore, the role of AHR in the regulation of \textit{Cyp2a5} might be linked to oxidative stress.

HO-1 catalyzes the degradation of heme to biliverdin, which is subsequently converted to bilirubin (Tenhunen \textit{et al.} 1969). Both biliverdin and bilirubin are potent antioxidants (Choi & Alam 1996), but excess concentrations of bilirubin are toxic and need to be controlled (Kapitulnik 2004). Bilirubin is normally conjugated with glucuronic acid and excreted in bile. When glucuronidation is impaired, oxidative bilirubin metabolism offers an alternative pathway for bilirubin degradation (Schmid & Hammaker 1963). It has been reported that CYP2A5 plays a role in microsomal bilirubin oxidation in conditions where the bilirubin level is elevated due to cadmium treatment and HO-1 induction (Abu-Bakar \textit{et al.} 2005). Furthermore, bilirubin itself has been shown to activate AHR-signalling in hepatoma cells (Phelan \textit{et al.} 1998). Thus, oxidative stress may activate \textit{Cyp2a5} both directly through Nrf2 and indirectly through AHR.

Besides binding to the XRE in the distal \textit{Cyp2a5} promoter, AHR dependent induction of the proximal promoter region was also detected. However, there are no conserved AHR response elements in the proximal promoter, and therefore it is likely that AHR mediates its response through an indirect pathway. The induction through the proximal promoter appears to be hepatocyte-specific, as it was detected in primary hepatocytes and in the hepatoma cell line Hepa-1, but not in COS-1 cells. Thus, hepatocyte-enriched factors are likely to be involved, and TCDD has been reported to induce, for instance, C/EBP\textbeta via AHR in mouse liver (Liu \textit{et al.} 1998).

### 6.4 Nutritional and circadian regulation of \textit{Cyp2a5} by PGC-1\textalpha

According to the results presented in original article IV, PGC-1\textalpha mediates the induced expression of CYP2A5 by cAMP in mouse hepatocytes through coactivation of HNF-4\textalpha. This suggests that PGC-1\textalpha may also mediate the fasting response of CYP2A5. However, the reason for the elevation of CYP2A5 in fasting is not known. Fasting is reported to induce bilirubin concentration, possibly due to increased activity of the hepatic HO-1 enzyme (Bakken \textit{et al.} 1972), and the physiological purpose of CYP2A5 induction during fasting could thus again be to prevent a dangerous increase of bilirubin levels.

Very recent results from Liu \textit{et al.} (Liu \textit{et al.} 2007) show that PGC-1\textalpha is rhythmically expressed in mouse liver and that PGC-1\textalpha itself up-regulates the expression of proteins involved in the control of circadian rhythm, such as ARNT.
isoform brain and muscle ARNT-like 1 (BMAL-1) and Clock. Circadian rhythms in physiology and behaviour are believed to enable organisms to adapt to rhythmic changes in their environment. It has been suggested that the CYP2A5 level increases in response to regulators of circadian rhythm to prime the night-feeding mouse and enable it to breakdown dietary compounds, especially toxic coumarins (Viitala et al. 2001). The transcription factor DBP is known to contribute to the circadian regulation of CYP2A5, but the circadian rhythm in CYP2A5 expression is not totally abolished in $dbp^{-/-}$ mice (Lavery et al. 1999). This indicates that some other factor, possibly PGC-1α, is also involved in circadian regulation of Cyp2a5.

6.5 Anatomy of the Cyp2a5 5’ promoter

A schematic picture of the regulatory mechanisms of the Cyp2a5 gene elucidated in the studies of this thesis work and in the previous literature is shown in Figure 9A. Two separate regions of the Cyp2a5 5’ promoter (proximal −271 and distal −3033 to −2014 from the TSS) appeared to activate the transcription of the gene. The proximal promoter region is suggested to play a role in constitutive, tissue-specific regulation of Cyp2a5, whereas the distal region may respond to different signals, such as oxidative stress. These two regions are likely to interact, possibly through the looping of DNA indicated by the cooperation of HNF-4α and USF (Fig. 9B).
Fig. 9. A schematic picture of the regulatory mechanisms of the Cyp2a5 gene.
6.6 Similarities in the regulation of Cyp2a5 and other genes

As described previously, CYP2A5 regulation appears to be different from, and in some cases even opposite to, other major CYP forms (Juvonen et al. 1985, Arvela et al. 1991, Hahnemann et al. 1992, Camus-Randon et al. 1996). However, some genes regulated in a similar manner to Cyp2a5 can be found. Cyp2a5 shares extensive homology with Cyp2a4, and besides similarities in the coding sequences, the 5’ flanking sequences of Cyp2a5 and Cyp2a4 are also over 90% homologous up to –3000 bp. Many of the transcription factor binding sites in the Cyp2a5 promoter, including response elements for HNF-4, NF-1, and DBP, E-box, XRE and StRE are conserved in the Cyp2a4 5’ region. HNF-4a and DBP have been shown to participate in Cyp2a4 regulation (Yokomori et al. 1997, Lavery et al. 1999). Both enzymes also have a circadian rhythm (Lavery et al. 1999). Thus, it is likely that common regulatory mechanisms for both enzymes exist.

The human orthologue of Cyp2a5, CYP2A6, has also been shown to be regulated by HNF-4a (Pitarque et al. 2005). However, the NF-1 binding site in its promoter appears not to be functional (Pitarque et al. 2005). PGC-1α has been shown to regulate CYP2A6 through PXR (Itoh et al. 2006), but it may also affect CYP2A6 expression through HNF-4a. The role of CYP2A6 in the metabolism of bilirubin and the effect of TCDD on CYP2A6 expression, as well as the functionality of a putative palindromic E-box site at –4096 bp from the CYP2A6 transcription start site remain to be elucidated.

Cyp2a5 and Cyp1a2 have several common characteristics in their regulation. Just as with CYP2A5, CYP1A2 is expressed preferentially in the liver and the olfactory mucosa (Genter et al. 1998). It also shares substrates with CYP2A5, such as acetaminophen and bilirubin (Genter et al. 1998, Zaccaro et al. 2001). NF-1 and USFs have been shown to participate in the hepatic expression of human CYP1A2 (Zhang et al. 2000, Pickwell et al. 2003). CYP1A2 is induced through an AHR mediated pathway, and similarly to Cyp2a5, AHR induces proximal and distal promoter regions of the CYP1A2 gene (Quattrochi et al. 1994). The distal enhancer region includes a single weak binding site for AHR/ARNT, E-boxes and activator protein 1 (AP-1) binding sites (Pickwell et al. 2003). A DNA element responsive to 3-MC but which does not bind AHR/ARNT was discovered in the proximal promoter of the rat CYP1A2 gene (Sogawa et al. 2004). An analogous response element sequence [CNRG-N_{5,c}-CNR(G/C)] was also identified within the promoter of the hamster CYP2A8 gene and the factor
binding to it was identified as nuclear factor 2d9 (NF-2d9) (Kurose et al. 2005). Interestingly, a similar element was also found in the proximal promoter of Cyp2a5 at the site –22 to –9.

Like CYP2A5, HO-1 is highly inducible by numerous stress stimuli (Ryter & Choi 2002). It catalyzes the rate-limiting step in the degradation of heme to form biliverdin, which is subsequently converted to bilirubin by biliverdin reductase (Tenhunen et al. 1969). Abu-Bakar et al. (Abu-Bakar et al. 2005) have shown that CYP2A5 participates in the degradation of bilirubin and they suggested that HO-1 and CYP2A5 may have coordinated regulation and a role in cellular defence. Indeed, HO-1 and CYP2A5 are both induced by oxidative stress caused by heavy metals, such as cadmium, and by different tissue injuries (Choi & Alam 1996, Abu-Bakar et al. 2004, Su & Ding 2004), and Nrf2 mediates this response for both enzymes (Gong & Cederbaum 2006a, Abu-Bakar et al. 2007). HO-1 and Cyp2a5 are also up-regulated in fasting (Bakken et al. 1972, Bauer et al. 2004). In addition, HNF-4 and USF have been shown to play a role in the regulation of both enzymes (Sato et al. 1990, Takahashi et al. 2002).

6.7 The significance of the unique and complex regulation of Cyp2a5

The regulation of CYP enzymes, the main metabolizers of drugs, has been studied extensively, since changes in the rate of drug metabolism determine the duration and intensity of the pharmacological action of drugs. Xenobiotic metabolizing-CYP enzymes may also convert foreign compounds to toxic or carcinogenic forms, and therefore their induction increases susceptibility to chemical toxicity. During the last ten years, numerous signalling pathways and molecules have been found to control P450s in response to xenobiotic inducers. However, relatively little is still known of the mechanisms that control CYP expression in different tissues or in different physiological or pathophysiological states of the liver. Yet, the changes in energy supply or certain pathological conditions, for example, may have striking consequences for the metabolism of different classes of xenobiotics. In addition, the pathways that regulate CYPs’ basal level and tissue-specific expression, and response to different signals outside the cell, are likely to form a complex cross-regulatory network, and therefore, it is important to investigate the different mechanisms in-depth. The results of this thesis describe novel transcriptional mechanisms that control both constitutive and inducible expression of CYP2A5. These mechanisms are likely to be, at least partly,
applicable to other xenobiotic metabolizing enzymes as well, particularly to the human ortholog of CYP2A5, CYP2A6. CYP2A6 has an important role in the metabolism of several drugs, nicotine and tobacco-related toxic compounds, and it is of interest to see if, for instance, the supply of nutrition or substances causing oxidative stress also affect its activity and thereby smoking behaviour and susceptibility to tobacco smoke induced cancers. However, CYP2A6 regulation needs to be studied separately in detail, since several differences in the regulation and function of CYP enzymes between mouse and man are known to exist. And, for example, circadian regulation is not likely to be as pronounced for humans as it is for nocturnal mice.

The results of these studies also provide a novel mechanistic link between nutrition, disease and the metabolism of xenobiotics through the regulation of coactivation. PGC-1α and proteins that control its expression and activity are potential target molecules for new drugs for the treatment of several diseases, such as diabetes and obesity (Handschin & Spiegelman 2006). However, drugs that have an effect on PGC-1α expression or function may also directly influence the PGC-1α mediated regulation of xenobiotic-metabolizing enzymes, a factor which must be considered in the development of such drugs.

CYP2A5 has been shown to have a unique role among the P450s since its expression is regulated by several structurally different xenobiotics, and in many pathophysiological states of the liver it acts in an opposite way to other P450s. However, the significance of the exceptional and complex regulation of CYP2A5 is not clear and, in fact, it is not known if induction of CYP2A5 is beneficial or harmful to the liver. One of the goals of these studies was also to clarify the role of CYP2A5 in the liver. The two main functions of CYP2A5 are hypothesized based on previous literature and the results presented in the original publications in this thesis. Firstly, CYP2A5 appears to be induced by several compounds and conditions that cause oxidative stress in the cell, and CYP2A5 may be needed for the metabolism of cytotoxic amounts of bilirubin or other endogenous, potentially harmful compounds. Thus, CYP2A5 could be part of a protective network that maintains control of bilirubin levels and redox homeostasis. Secondly, CYP2A5 is likely to be needed in the metabolism of plant alkaloids in the diet, and its expression appears to be associated with the supply of nutrition. Furthermore, some dietary compounds or pathophysiological conditions linked to energy metabolism, such as fasting or diabetes, may cause oxidative stress and therefore induce CYP2A5.
The present studies have characterized several mechanisms involved in the transcriptional regulation of the \textit{Cyp2a5} gene in the liver. However, a number of important questions remain to be answered. For instance, what is the role of the promoter area between the two activation regions? Does it include inhibitory sequence motifs or does it provide appropriate spacing for the identified response elements? How is the preference for ARNT and USF binding to the common E-box determined, and is the ARNT homodimer able to bind to the E-boxes of the other USF target genes? Does PGC-1\(\alpha\) also induce other xenobiotic-metabolizing enzymes in response to changes in the supply of nutrition? How important is regulation through other coactivators? Do similar regulatory mechanisms also control \textit{Cyp2a5} in extrahepatic tissues as in the liver? And, what are the molecular mechanisms that link the various regulatory pathways of \textit{Cyp2a5}?
7 Conclusions

1. Two major regions of the Cyp2a5 5’ promoter, called the proximal and the distal, are involved in the constitutive regulation of Cyp2a5 transcription in murine hepatocytes.

2. HNF-4 and NF-1 transcription factors are the key activators of the proximal promoter of the Cyp2a5 gene, and they are needed for Cyp2a5 expression in hepatocytes.

3. The distal region of the Cyp2a5 promoter contains a symmetric E-box motif that can bind ARNT and USF. Both ARNT and USF are likely to be essential in the regulation of Cyp2a5 but the significance of these factors may vary under different physiological conditions in the liver.

4. ARNT appeared to control Cyp2a5 transcription without a heterodimerization partner suggesting active involvement of the ARNT homodimer in mammalian gene regulation.

5. AHR ligands up-regulate Cyp2a5 transcriptionally by an AHR/ARNT-dependent mechanism and established Cyp2a5 as a novel AHR-regulated gene. The AHR response element is located near to the E-box and the previously defined Nrf2 binding site in the distal region of the Cyp2a5 promoter, suggesting cooperation between the proteins binding to these elements.

6. PGC-1α regulates Cyp2a5 through coactivation of transcription factor HNF-4α in murine hepatocytes. PGC-1α is likely to be the major factor mediating the induction of CYP2A5 caused by fasting and cAMP.
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Original articles


Original articles are not included in the electronic version of the dissertation.


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