Cytochrome P450 Enzymes in Bile Acid Biosynthesis and Fatty Acid Metabolism

Studies on Members of the Porcine CYP4A and CYP8B Subfamilies

BY

KERSTIN LUNDELL
Dissertation for the Degree of Doctor of Philosophy (Faculty of Pharmacy) in Pharmaceutical Biochemistry presented at Uppsala University in 2003

ABSTRACT


The present investigation is devoted to studies on porcine members of the cytochrome P450 4A (CYP4A) and CYP8B1 subfamilies, which are involved in bile acid biosynthesis and fatty acid metabolism.

Hyocholic acid is considered to fulfil the requirements for trihydroxy bile acids in the domestic pig (Sus scrofa) in the absence of cholic acid. Hyocholic acid is a 6α-hydroxylated product of chenodeoxycholic acid and the enzyme catalyzing the 6α-hydroxylation was cloned and found to be an atypical member of the CYP4A subfamily. The primary structure of this porcine enzyme, designated CYP4A21, shows about 75% overall sequence identity to members of the CYP4A subfamily expressed in rabbit and man. Divergent amino acids in a “signature sequence” in the active site of all hitherto known CYP4A fatty acid hydroxylases, were found to be important determinants for the 6α-hydroxylase activity of CYP4A21.

Two homologous CYP4A fatty acid hydroxylases, designated CYP4A24 and CYP4A25, expressed in pig liver and kidney were cloned. These two cDNAs encode proteins of 504 amino acids similar to CYP4A21. The overall identity between CYP4A24 and CYP4A25 is 97% compared to 94% identity to CYP4A21. Whereas CYP4A21 clearly deviates regarding structural features and catalytic activity it is more difficult to establish whether CYP4A24 and CYP4A25 are distinct enzymes or allelic variants of a single enzyme.

Cloning of the CYP4A21 gene showed a conserved organization compared to CYP4A genes in other species. A segment of the CYP4A24 gene was also cloned and comparison with the CYP4A21 gene revealed an extensive sequence identity also within introns as well as within the proximal promoter regions. This indicates that CYP4A21 and CYP4A fatty acid hydroxylases have a common origin and evolved by gene duplication. The CYP4A21 and CYP4A fatty acid hydroxylases, however, show distinct patterns of expression.

The key enzyme in cholic acid biosynthesis, CYP8B1, was markedly expressed in fetal pig liver compared to livers from young pigs. The opposite was shown for the expression of CYP4A21. An apparently conserved pig CYP8B1 gene was cloned and was intronless, similar to CYP8B1 genes from other species. The pig gene encoded a protein of 501 amino acids with 81% identity to CYP8B1 expressed in rabbit and man. Unlike other CYP8B1 genes, the pig promoter lacked a TATA-box. This might offer one explanation for the unusual expression pattern, which appears to be restricted to pig fetal life.

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To those who made it possible
Kjell, Matti, and Ronnie

Thank you, it has been a pleasure
List of original papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. Lundell, K., Hansson, R., and Wikvall, K.
Cloning and expression of a pig liver taurochenodeoxycholic acid 6α-hydroxylase (CYP4A21)

II. Lundell, K.
Cloning and expression of two novel pig liver and kidney fatty acid hydroxylases [cytochrome P450 (CYP4A24 and CYP4A25)]

III. Lundell, K., and Wikvall, K.
Gene structure of pig sterol 12α-hydroxylase (CYP8B1) and expression in fetal liver: comparison with expression of taurochenodeoxycholic acid 6α-hydroxylase (CYP4A21)
Submitted

IV. Lundell, K.
Porcine taurochenodeoxycholic acid 6α-hydroxylase (CYP4A21): gene organisation and aspects on the evolution of the CYP4A subfamily
Manuscript

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<table>
<thead>
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<th>Trivial Names</th>
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<tbody>
<tr>
<td>AOX</td>
<td>Acyl-CoA oxidase</td>
</tr>
<tr>
<td>APR</td>
<td>Apolipoprotein regulatory protein</td>
</tr>
<tr>
<td>BSEP</td>
<td>Bile salt excretory pump</td>
</tr>
<tr>
<td>Chenodeoxycholic acid</td>
<td>$3\alpha,7\alpha$-Dihydroxy-$5\beta$-cholanoic acid</td>
</tr>
<tr>
<td>Cholic acid</td>
<td>$3\alpha,7\alpha,12\alpha$-Trihydroxy-$5\beta$-cholanoic acid</td>
</tr>
<tr>
<td>CMC</td>
<td>Critical micelle concentration</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>Deoxycholic acid</td>
<td>$3\alpha,12\alpha$-Dihydroxy-$5\beta$-cholanoic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetate</td>
</tr>
<tr>
<td>EET</td>
<td>Epoxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>HETE</td>
<td>Hydroxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>3-Hydroxy-3-methylglutaryl Coenzyme A</td>
</tr>
<tr>
<td>HNF4</td>
<td>Hepatic nuclear factor 4</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>Hyocholic acid</td>
<td>$3\alpha,6\alpha,7\alpha$-Trihydroxy-$5\beta$-cholanoic acid</td>
</tr>
<tr>
<td>Hyodeoxycholic acid</td>
<td>$3\alpha,6\alpha$-Dihydroxy-$5\beta$-cholanoic acid</td>
</tr>
<tr>
<td>I-BABP</td>
<td>Ileal bile acid binding protein</td>
</tr>
<tr>
<td>IBAT</td>
<td>Ileal bile acid transporter</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>Lithocholic acid</td>
<td>$3\alpha$-Hydroxy-$5\beta$-cholanoic acid</td>
</tr>
<tr>
<td>LXR</td>
<td>Liver X receptor</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
</tr>
<tr>
<td>NTCP</td>
<td>Sodium taurocholat co-transporter protein</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisomal proliferator-activated receptor</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoic X receptor</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SHP</td>
<td>Small heterodimer partner</td>
</tr>
<tr>
<td>SRE</td>
<td>Sterol regulatory element</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory element binding protein</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>Ursodeoxycholic acid</td>
<td>$3\alpha,7\beta$-Dihydroxy-$5\beta$-cholanoic acid</td>
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Introduction

Cytochrome P450

The cytochrome P450 (P450) enzymes constitute a large superfamily of heme-thiolate proteins with an ability to catalyze the monooxygenation of a wide variety of both endogenous and exogenous compounds. The P450 enzymes are classified into families and subfamilies based on their sequence similarities (1). Updated listing of P450 genes, sequences and activities can be found at http://drnelson.utmem.edu/CytochromeP450.html. All P450 isoforms have in common a heme cofactor (iron protoporphyrin IX) that is required for oxygen binding and formation of active oxidant species. A completely conserved cysteine residue in P450 proteins serves as the proximal ligand to the iron atom in the heme cofactor. This S-Fe bond is a defining characteristic of P450 proteins that give rise to their unique spectroscopic absorbance at 450 nm upon reduction and binding of carbon monoxide (2). The general reaction for P450-mediated monooxygenation of hydrocarbon substrates involves cleavage of molecular oxygen by the sequential input of two reducing equivalents, supplied by NADPH in eucaryotic cells. A single oxygen atom is thus inserted into the substrate to produce an oxygenated metabolite, with concomitant generation of water from the second oxygen atom as illustrated by the reaction:

\[
\text{RH} + \text{O}_2 + 2\text{e}^- + 2\text{H}^+ \xrightarrow{\text{P450}} \text{ROH} + \text{H}_2\text{O}
\]

The electron transfer pathway from NADPH to the heme differs among microcomal and mitochondrial P450 enzymes (3). For microsomal P450 enzymes this electron transfer reaction is catalyzed by the membrane-bound flavoprotein NADPH-cytochrome P450 reductase. For some cytochrome P450 enzymes, cytochrome b5 can serve as an effector or electron donor and enhance the catalytic activity in cooperation with NADPH-cytochrome P450 reductase (2).
Evolution of cytochrome P450

Cytochrome P450 enzymes are found in virtually all living organisms. All genes encoding P450 enzymes are believed to be derived from a single ancestral gene existing more than 3.5 billion years ago (4). The start of accumulation of free oxygen in the atmosphere concords with the time for the prokaryote-eucaryote divergence, approximately 2 billion years ago (5). A steady increase in atmospheric oxygen correlates with radiation of animal phyla and colonization of land about 400 million years ago. The rise in atmospheric oxygenation thus seems to conform closely to the evolution of eukaryotic species, and also with the evolution of cytochrome P450 families and subfamilies (6). Mammalian P450 families that are involved in critical endogenous pathways, for example P450 enzymes that participate in bile acid biosynthesis, are in general highly conserved between mammalian species. Numerous species-specific gene duplication and conversion events on the other hand account for a diversification of other families, i.e. the drug-metabolizing cytochrome P450 enzymes. The driving force for evolution of these enzymes has been ascribed to the animal-plant interaction or "warfare" (7,8).

Structural features of cytochrome P450 enzymes

Although the sequence identity between P450 enzymes with known three-dimensional structures is low (≤ 20%), all share similar structural and functional domain architecture. Eucaryotic P450 enzymes are membrane bound through a hydrophobic amino terminal sequence. For many years the hydrophobic natures of the eucaryotic enzymes hampered crystallization and hence structure determination. Recently, X-ray data from the modified eucaryotic CYP2C5 was made available (9). Comparison with previously described crystal structures of soluble prokaryotic P450 shows a similar conserved overall topology.

The P450 protein is shaped like a triangular prism; the overall structure might be roughly divided into a smaller domain, rich in β-sheets, and a larger domain, rich in α-helices. These domains, however, do not constitute independent folding units but are assembled from discontinuous secondary structure segments along the polypeptide chain. A diagrammatic representation of the secondary structure topology of the prokaryotic P450 bm3 (CYP102) and a ribbon drawing of the spatial arrangement of the structural elements in this P450 protein are shown in Fig. 1. A typical conserved P450 core structure is the four-helix bundle composed of helices denoted D, E, L and I. The middle part of the long I-Helix within this bundle is positioned in the active site over the heme prosthetic group, which is buried in the interior of the protein. The substrate access is limited by the dimension of the active site. The substrate specificity for each individual P450 enzyme is governed by interactions with variable regions found among P450 families and subfamilies (2,10). Six putative substrate recognition sites (SRRs) were originally defined for the CYP2 family (11).
**Fig. 1** Top panel: A ribbon drawing indicating the spatial arrangement of structural elements in the prokaryotic P450bm3 (CYP102). Bottom panel: A schematic drawing showing the position of secondary structure elements within CYP102. Helices are represented by black bars and the strands of β-sheets are shown as arrows. The position of the heme is shown by the square at the amino terminal end of L-Helix. (Reproduced with permission from: Cytochrome P450 (1995) P.R. Ortiz de Montellano (Ed), Plenum Press, New York)
The overall conserved topology among P450 proteins, around a core of conserved structural elements, enables comparison between P450 enzymes with known crystal structure and those without. Homology alignment and three-dimensional models have thus frequently been used to achieve information regarding the localization of secondary structures in P450 enzymes without crystal structure data (12,13).

The Cytochrome P450 4A (CYP4A) subfamily

Members of the CYP4 family are found in mammals as well as in insect species. The CYP4 family are microsomal enzymes that are classified into several subfamilies, which appear to have diverged relatively early in evolution, about 1.3 billion years ago (14). The CYP4A subfamily are abundant among mammalian species and are found in various tissues including the liver, kidney, lung, intestine, brain, prostate, uterus and placenta (15). The CYP4A proteins are fatty acid ω- and (ω-1) -hydroxylases with preference for medium chain fatty acids (C12-C20), including arachidonic acid and prostaglandins. Whereas other P450 enzymes, particularly isozymes within the CYP2 family, possess (ω-1)-hydroxylase activity towards fatty acids the ability to hydroxylate the thermodynamically disfavored terminal carbon (ω-hydroxylation) of fatty acids is characteristic for the CYP4A subfamily.

A unique feature for the CYP4 family of proteins is the covalent attachment of the heme prosthetic group to the protein. A conserved glutamic acid (Glu) residue in the middle of the I-Helix has been shown to participate in formation of an ester bound between the protein and the heme group (16,17). The heme group is covalently bound by an autocatalytic mechanism and has been shown to increase with time under turnover condition (18). The Glu residue thus involved in the covalent binding to heme is part of a conserved sequence which has been regarded as a signature sequence for CYP4A and CYP4B enzymes (19). This Glu residue is also among the three residues (Tyr 120, Glu 321, Val 386) predicted to be the major determinants for the ω-hydroxylation of lauric acid in a three-dimensional model of human CYP4A11 (20).

Although members of the CYP4A subfamily are structurally conserved enzymes, specialized in fatty acid hydroxylation, a comparison between species and CYP4A isozymes indicates a diversified species-specific evolution. The number of CYP4A enzymes and the substrate specificity towards various fatty acids and prostaglandins differ among isozymes and species as do the regulation of the genes (21). An incomplete understanding of the biological significance of CYP4A-mediated fatty acid hydroxylation precludes the rational for this diversification of CYP4A genes among species. A role in fatty acid catabolism is suggested for CYP4A expressed in the liver. CYP4A expressed in kidney has been more extensively studied due
to its participation in generation of hydroxylated metabolites of arachidonic acid, especially the \( \omega \)-hydroxylated metabolite 20-HETE, which is a potent vasoconstrictor of clinical significance (22).

**CYP4A and fatty acid metabolism**

Fatty acids are ubiquitous molecules that are essential for a variety of cellular processes including energy storage, synthesis of cellular membranes and generation of lipid-containing messengers in signal transduction. Also, fatty acid oxidation plays a central role in energy homeostasis and disturbances in fatty acid metabolism and regulation can contribute to conditions such as hyperlipidemia, obesity, insulin resistance, and atherosclerosis.

Oxidation of fatty acids occurs by \( \beta \)-oxidation in mitochondria and peroxisomes (23). Mitochondrial \( \beta \)-oxidation is responsible for the oxidation of short, medium and long-chain fatty acids and the process contributes to energy generation via ATP. Long-chain fatty acids constitute the bulk of dietary fat. The peroxisomal \( \beta \)-oxidation is responsible for the oxidation of very-long-chain fatty acids (> C\(_{20}\)) as well as long-chain dicarboxylic acids, eicosanoids, and some bile acid precursors. Whereas mitochondrial \( \beta \)-oxidation produces energy, the peroxisomal \( \beta \)-oxidation generates \( \text{H}_2\text{O}_2 \) and does not go to completion as the chain-shortened acyl-CoAs are exported to the mitochondria for the completion of \( \beta \)-oxidation.

The CYP4A enzymes have been considered to be involved in the microsomal \( \omega \)-hydroxylation of fatty acids (23–25). This is a minor alternative pathway of fatty acid metabolism to prevent accumulation of fatty acids or the CoA derivatives in hepatocytes. The microsomal \( \omega \)-hydroxylation of fatty acids generates an initial \( \omega \)-hydroxy fatty acid, which is oxidized by dehydrogenases in the cytosol to a dicarboxylic acid. The dicarboxylic acid is further converted to its CoA derivative by acyl-CoA synthetase present in the endoplasmatic reticulum. The long-chain dicarboxyl-CoAs thus derived from initially \( \omega \)-hydroxylated fatty acids are the preferred substrates for the peroxisomal \( \beta \)-oxidation. Significant quantities of dicarboxylic acids can be formed from \( \omega \)-oxidation of long-chain fatty acids under conditions of fatty acid overload in the liver; for example in diabetes and in situations in which the mitochondrial \( \beta \)-oxidation is impaired (23).

The preferred substrates for the CYP4A subfamily are saturated medium-chain fatty acids (\( \leq C_{20} \)), and the enzyme activities tend to fall with an increase in fatty acid chain length from \( C_{12} \) to \( C_{20} \). This is in conflict with a role for CYP4A in \( \omega \)-hydroxylation of very long chain fatty acids (21). It has, however, been demonstrated that the amount of NADPH-cytochrome P450 reductase and also cytochrome b\(_5\) may affect the substrate specificity towards longer fatty acids. Conformational changes affecting the substrate access channel have been suggested to explain this effect (26,27).
CYP4A enzymes have, under certain conditions, been shown to participate in generation of reactive oxygen species (ROS). Oxidative stress, defined as an imbalance between pro-oxidant and antioxidant chemical species with an accompanying oxidative damaging to cellular macromolecules, is a contributing factor in development of steatohepatitis. The CYP2E1 subfamily is, however, responsible for generation of most ROS (28,29).

CYP4A and bioactivation of fatty acids in kidney

Oxidized species of arachidonic acid (eicosanoids), such as the ω-hydroxy product (20-HETE), are of potential clinical significance. These lipid-derived mediators are subjected to rapid turnover, which makes them efficient on/off molecular switches for intra- or intercellular signaling (30). 20-HETE is the major metabolite of arachidonic acid in the kidney. CYP4A enzymes along with members of the CYP4F subfamily are important mediators of 20-HETE generation in the kidney (Fig. 2) (31-35).

![Diagram of CYP4A-catalyzed reactions]

**Fig. 2** Products generated from arachidonic acid by CYP4A-catalyzed reactions. The major pathways are formation of 20-HETE and 19-HETE whereas epoxidation by CYP4A probably constitutes a minor pathway.
Renal blood flow is precisely controlled to maintain glomerular filtration and flow as well as electrolyte homeostasis. 20-HETE, along with other arachidonic acid metabolites, participates in this regulation by controlling renal vascular resistance and salt and water excretion (36). The rapid elevation of blood pressure in the spontaneously hypertensive rat (SHR) is associated with increased production of 20-HETE (37). 20-HETE has been characterized as a powerful vasoconstrictor in the renal and also cerebral microcirculation, and acts by blocking calcium-dependent K⁺ channels (Kᵥ channels) (38-40). Activation of the mitogen-activated protein kinase (MAPK) signal transduction pathway, and downstream stimulation of tyrosine kinase, is a proposed mechanism for the decreased activity of the Kᵥ channels (41). Also, 20-HETE has a major role in the regulation of salt and water excretion in kidney by modulation of Na⁺-K⁺-2Cl⁻ co-transporter and Na⁺/K⁺-ATPase activity in the medullary thick ascending limb and proximal tubule, respectively (38,40). Nitric oxide has been shown to act as a modulator of ω-hydroxylase activity by binding to the heme moiety of CYP4A enzymes and inhibit formation of 20-HETE (42,43).

The production of hydroxylyase- and epoxygenase- metabolites of arachidonic acid is regulated by hormonal and paracrine factors. 20-HETE has been characterized as a second messenger or mediator of the vasoconstrictor response to endothelin-1 and angiotensin II as well as a modulator of epithelium growth factor (EGF) and parathyroid hormone signaling (36,38-40,44). Similarly, generation of 20-HETE and activation of the RasMAPK signal transduction pathway are components in norepinephrine signaling (45).

The (ω-1)-hydroxy metabolite of arachidonic acid (19-HETE), also produced by CYP4A enzymes, has been shown to increase Na⁺ reabsorption and thus acts opposite to the major inhibitory effect of 20-HETE on Na⁺/K⁺ ATPase in the proximal tubule (46). An additional arachidonic acid metabolite identified as the epoxide 11,12-EET has been shown to be generated by rat CYP4A isoforms, which adds yet another vasoreactive eicosanoid to the list of CYP4A-generated products (Fig. 2) (26, 34).

Regulation of CYP4A enzymes by PPARα

For many years the regulation of CYP4A enzymes has been intimately associated with the peroxisome proliferator-activated receptor α (PPARα; NR1C1) (25). This nuclear receptor mediates the peroxisome proliferator-dependent transcriptional activation of genes involved in lipid metabolism, including those encoding peroxisomal enzymes and CYP4A, as well as peroxisome proliferation in rodents. The latter effect is responsible for naming of the receptor. The PPAR nuclear receptor family includes two additional isoforms, PPARβ(δ) and PPARγ, with distinct tissue distribution and functions (47). It is generally believed that these PPARs are constitutively localized in the nucleus, independent of ligand binding (48). PPARα is expressed in tissues with a high oxidative capacity such as liver, kidney, and heart. A broad spectrum of endogenous and exogenous compounds,
collectively referred to as peroxisome proliferators, activate PPARα. Synthetic peroxisome proliferators include certain hypolipidemic drugs such as clofibrate, and also phthalate ester plasticizers, herbicides, food flavors, and leukotriene D4 receptor antagonists. Natural ligands include fatty acids, fatty acid derivatives, such as fatty acid methyl esters and branched chain fatty acids like phytanic acid, and arachidonic acid metabolites including 8(S)-hydroxyeicosatetraenoic acid and leukotriene B4 (LTB4) (23,48). A CYP4A-derived ω-hydroxy metabolite of EET has been identified as a potent PPARα ligand, indicating a direct involvement of CYP4A isozymes in generation of PPARα ligands (49).

<Diagram>

**Fig. 3** A simplified scheme showing activation of PPARα and binding in a heterodimer complex with RXR to PPRE in the promoter of a target gene, exemplified by a CYP4A gene, upstream of the transcriptional start (TS) site. Besides the PPARα/RXR complex, transcriptional activation of a target gene involves a series of associated cofactors which are not depicted.

Ligand-bound PPARα forms a heterodimer complex with the nuclear retinoic X receptor (RXR) and binds responsive elements (PPRE) in the promoter of target genes. A PPRE is formed by a direct repeat of 6 nucleotides, separated by one spacer nucleotide (DR-1) and has the consensus sequence AGGTCAnAGGTCA. An illustration of PPARα- induced gene activation is shown in Fig. 3. The molecular mechanisms by which nuclear receptors trans-activate in a gene-, tissue- and species-specific fashion involve a series of cofactors (23). Recruitment of some cofactors is ligand dependent. The inhibitory effect of long-chain fatty acyl esters on PPARα activity has been proposed to be cofactor dependent (50). The DR-1 binding motif allows for cross-talk between PPARα and other nuclear receptors, such as hepatic nuclear factor 4α (HNF4α; NR2A1) (51) and apolipoprotein regulatory protein 1 (ARP-1; NR2F2) (52) through a common affinity for the DNA binding motif and also co-activators. Likewise, triiodothyronine (T3) acting through its nuclear
receptor, the thyroid hormone receptor (TR), is suggested to suppress peroxisome proliferative responses in rodents by competing with PPARα for heterodimerization with RXR and binding to PPRE in target genes, such as the CYP4A gene (53).

The trans-activating ability of PPARα is tightly controlled at multiple levels and via different mechanisms. The abundance of PPARα is regulated at the level of transcription by a variety of hormones whereas the trans-activating activity can be modulated by phosphorylation of the receptor. Glucocorticoids induce PPARα protein expression at the transcriptional level (54) whereas insulin treatment decreases PPARα levels (55). Insulin has on the other hand been reported to enhance the trans-activating ability of PPARα by phosphorylation of the receptor (56,57). These dual effects of insulin are proposed to reflect long-term and short-term effects, respectively, of exposure to the hormone (56).

Growth hormone (GH) has diverse effects on metabolism and growth, some of which are indirectly mediated by insulin-like growth factor-1 but many of which reflect the direct effect of GH on gene expression. GH, like many cytokines and growth factors, binds specific cell-surface receptors and induces activation of tyrosine kinases in the cell that subsequently activate cytoplasmic signal transducer and activator of transcription (STAT) proteins (58). Activated STAT proteins accumulate in the nucleus and act to regulate transcription. GH and prolactin induce activation of STAT5b. Both rodent and human STAT5b have been shown to inhibit the transcriotional activity of PPARα (59). This inhibitory effect may in part be mediated by inhibition of PPARα gene expression since the basal level of PPARα and its target genes was elevated in STAT5b−/− mice. These findings support the hypothesis that GH and potentially other endogenous activators of STAT5b help to maintain liver PPARα function at low basal level. A difference in the GH secretory pattern between male and female rodents could explain the observed sex differences seen in responsiveness to peroxisome proliferators, including activation of CYP4A, in mouse and rat liver (60). Acute inflammation and associated inflammatory cytokines are also known to modulate CYP4A activity. Mediators of inflammation such as arachidonic acid, prostaglandins, leukotrienes and HETEs are activators of PPARs (61).

Most studies regarding regulation of CYP4A enzymes by PPARα have been performed with rat and mouse. However, a species difference in response to peroxisome proliferating agents has been shown. Humans and guinea pigs are among the “non-responding” species (62). The level of PPARα in human liver is low compared to rodents (63). Reduced expression level of functional human PPARα could allow PPREs to be occupied in vivo by other nuclear receptors, such as homodimers of RXR, ARP-1 or HNF4, that bind to similar sequences, and thus diminish responsiveness to peroxisome proliferators (63). Results from studies using HepG2 cells that stably overexpress PPARα suggest that humans retain a capacity for PPARα regulation of mitochondrial fatty acid oxidation and ketogenesis but is refractory to peroxisome proliferation and the induction of peroxisomal enzymes seen in rodents (64). Human CYP4A11 was shown to be inducible in HepG2 cells
overexpressing PPARα (65). A truncated splice variant of PPARα has been found in cytoplasm of human but not rat tissues. This truncated PPARα was found to act in a dominant negative fashion (66). Expression of two splice variants in humans allows for one more level of transcriptional control of PPARα responsive genes, and adds a new dimension to the complexity of species- tissue- and ligand-dependent effects of PPARα \textit{trans}-activation ability, including the regulation of CYP4A genes.

Structure of CYP4A genes

The CYP4A genes from rat, mouse, rabbit and human show a high degree of conservation regarding intron-exon organization and intron boundaries (67-71). The two human genes CYP4A11 and CYP4A22 share a considerable sequence identity between intron sequences (71). The rat CYP4A1 and CYP4A2 genes, on the other hand, exhibit little sequence identity within introns (67). The murine Cyp4A10, Cyp4a12, and Cyp4A14 genes have been shown to be physically linked on chromosome 4 (68). The human CYP4A genes are located on chromosome 1 (72). Both the murine and human CYP4A genes have further been shown to be linked to the CYP4B genes in the chromosome, unlike the CYP4F genes that are found on distinct chromosomes (68).

All CYP4A genes possess the same splicing sites within the coding region with an identical pattern of intron phases. The 5’ and 3’ intron junctions all display consensus donor (GT) and acceptor (AG) sites, respectively, which are essential elements for the splicing reaction (73). CYP4A genes are split into 12 exons with the exception for rat CYP4A1 and rabbit CYP4A6 where the 3’ untranslated region is split by a 12th intron, yielding a total of 13 exons (67,69).

A common feature among CYP4A genes is the lack of a canonical TATA-box upstream of the transcription start site. All CYP4A genes have a consensus polyadenylation signal in the 3’ untranslated region. Despite these similarities, the transcriptional regulation of these CYP4A genes differs. Steroids for example, appear to regulate transcription of the rabbit CYP4A4 gene, which is induced during pregnancy, and by treatment with progesterone or dexamethasone. The human CYP4A11 is also induced by dexamethasone (65). Peroxisome proliferators do not induce the rabbit CYP4A4 gene or the rabbit CYP4A5 gene. The rabbit CYP4A6, on the other hand, is readily inducible by peroxisome proliferators in a PPARα-dependent manner similar to most rat and mouse genes.
The biochemistry of bile acids

Bile acids are synthesized in the liver and secreted together with cholesterol, phospholipids, and electrolytes into bile. The bile is delivered via the bile duct and (in most mammals) gallbladder into the lumen of the small intestine and act to facilitate the uptake of dietary lipids i.e. triglycerides, cholesterol, and lipid-soluble vitamins. About 95% of the bile acids are retrieved by reabsorption from the intestine and returned to the liver via the portal blood. This recirculation, whereby the supply of bile acids is secured, is referred to as the enterohepatic circulation (74). The precursor for bile acids is cholesterol and conversion into bile acids and biliary secretion of free cholesterol are the two most important routes for removal of overabundant cholesterol from the body (74). The liver plays a critical role in maintaining cholesterol homeostasis by balancing de novo synthesis, dietary cholesterol uptake, catabolism and secretion.

**Fig. 4** The enterohepatic circulation of bile acids. Bile acid transporters in enterocytes and hepatocytes as well as the hepatic mdr2/MDR3 transporter for phospholipid secretion into the bile are shown.
Kerstin Lundell

The liver metabolizes many other endogenous and exogenous compounds that are predominantly hydrophobic molecules. Oxidative metabolism of these compounds followed by conjugation with e.g. glucuronic acid, sulphate or glutathione yield amphipatic substances which are secreted into the bile and solubilized by the mixed micelles for ultimate excretion from the body (74).

Bile acids are present as anion (often referred to as bile salts) at physiological pH and possess amphiphatic properties that promote micelle formation (or polymolecular aggregates) above a critical concentration (CMC). The transport of bile acids across membranes and within cells is facilitated by a number of transporter proteins as illustrated in Fig. 4. The bile salt excretory pump (BSEP) is the principal bile acid transporter across the canalicular membrane into the bile duct (75). Bile acids returning to the liver can enter by the hepatic basolateral sodium taurocholate co-transporter protein (NTCP) (76), by a sodium independent organic anion transporter (OATP) or by passive diffusion, depending on the properties of the bile acids (77). The passive uptake of unconjugated hydrophobic bile acids can occur along most of the entire length of the intestine (77). In the terminal part of the ileum, bile acids are taken up by the action of the ileal bile acid transporter (IBAT) located in the brush border membrane of enterocytes. Once taken up into the cell, bile acids are bound to proteins such as the ileal bile acid binding protein (I-BABP) for intracellular trafficking (78). Biliary secretion of phospholipids by the transporter multidrug resistance associated protein 2 (mdr2) in mouse is tightly coupled to secretion of free cholesterol and bile acids (79). MDR3 is the corresponding gene in humans (80). Biliary phospholipids, mostly phosphatidylycholine, form mixed micelles with bile acids and are believed to reduce the detergent action of bile acids in the bile ducts and thereby protect the epithelium of the small bile ducts.

Bile acid biosynthesis

Biosynthesis of primary bile acids proceeds by introduction of hydroxyl groups in the steroid nucleus and side chain of the cholesterol molecule. Side chain hydroxylation initiates the side chain shortening. Distinct enzymes located in different cellular compartments perform these reactions (81,82). The two major pathways in bile acid biosynthesis, the neutral (classic) and the acidic (alternative) pathways, are initiated by microsomal 7α-hydroxylase (CYP7A1) and mitochondrial sterol 27-hydroxylase (CYP27A1), respectively. The relative importance of these two pathways depends on the species and pathological conditions (83). Unlike CYP7A1, the expression of CYP27A1 is not restricted to the liver. The acidic pathway may be considered as a reverse cholesterol transport process for removing excess oxidized cholesterol from the peripheral tissue (84). Completion of bile acid biosynthesis by the alternative pathway is performed by hepatocytes. Hepatic CYP27A1 also participates in a downstream step in the neutral pathway, by a 27-hydroxylation that initiates the side chain shortening.
Hydroxylation of bile acid intermediates in the 12α-position by the microsomal sterol 12α-hydroxylase (CYP8B1) represents a branch point, which destines bile acid intermediates to be further converted into cholic acid. Cholic acid and chenodeoxycholic acids are the main primary bile acids in most mammals, including human. The primary bile acids are conjugated with taurine or glycine in the liver before being secreted. A fraction of the primary bile acids is converted to secondary bile acids in the intestine by the action of microbial enzymes. The 7α-dehydroxylation of cholic acid and chenodeoxycholic acid produces deoxycholic acid and lithocholic acid, respectively, which are the most common secondary bile acids. A simplified scheme for conversion of cholesterol into primary and secondary bile acids is shown in Fig. 5.

**Fig. 5** A simplified scheme for the conversion of cholesterol into primary and secondary bile acids. The positions for introduction of hydroxyl groups in the steroid nucleus and side chain of the cholesterol molecule are indicated by numbers.

Hyocholic acid, a primary bile acid in pig

A number of unique bile acids have been found in mammals. Many of these can be considered as hydroxylated products of chenodeoxycholic acid (85). This is the case for hyocholic acid in the domestic pig (Sus scrofa). Hyocholic acid is formed by a 6α-hydroxylation of chenodeoxycholic acid (Fig. 5) and is, besides chenodeoxycholic
acid, a main primary bile acid in pig (86). The bile of adult pig is devoid of cholic acid (87). The enzyme responsible for 6α-hydroxylation of chenodeoxycholic acid (or its conjugate) has been purified and characterized (88,89). The secondary bile acid hyodeoxycholic acid is formed from hyocholic acid by the 7α-dehydroxylase activity of the intestinal microflora (Fig. 5). Hyodeoxycholic acid has also been reported be present in germ-free pigs (90).

Cytochrome P450 8B1 (CYP8B1)

Sterol 12α-hydroxylase, the key enzyme in cholic acid biosynthesis, was initially cloned from a rabbit liver cDNA library (91). Based on a sequence similarity to prostaglandin synthetase (CYP8A) the rabbit sterol 12α-hydroxylase was designated CYP8B1, the first member of the CYP8B subfamily. The genes encoding sterol 12α-hydroxylase have been cloned from mouse, human, and rat (92,93). The sterol 12α-hydroxylase is highly conserved between species and hence all enzymes are denoted CYP8B1. A conservation of the CYP8B1 genes between species is reflected in the unusual gene structure which is devoid of introns. This is a unique feature among eucaryotic P450 genes, including CYP8A. Based on phylogenetic analysis the evolutionary ages of CYP8A and CYP8B, however, appear to be similar (92).

Due to long untranslated regions, the mRNA of human CYP8B1 is larger (4 kb) than the corresponding mRNAs from rat and mouse (< 3 kb). The CYP8B1 genes from rat, mouse, and human all have a canonical TATA-box upstream from the transcription start site but lack a consensus polyadenylation signal in the 3′ untranslated region. The sparse occurrence of conserved cis-acting elements between mouse and human CYP8B1 genes indicates a difference in transcriptional regulation between species (92). CYP8B1 is expressed exclusively in the liver, which is consistent with its role in bile acid biosynthesis. The CYP8B1 has been shown to 12α-hydroxylate a number of 7α-hydroxylated C_{27}-steroids but 7α-hydroxy-4-cholesten-3-one, the substrate for CYP8B1 in the classical pathway of bile acid biosynthesis, is most efficiently hydroxylated (94). The activity of CYP8B1 is an important determinant for the relative amount of cholic acid versus chenodeoxycholic acid formed. These two bile acids have distinct physiochemical properties and the ratio cholic acid to chenodeoxycholic acid will have an impact on several aspects of lipid metabolism, as well as gallstone formation. Suppression of the sterol 12α-hydroxylase activity has been suggested as a possible therapeutic strategy for dissolution of cholesterol gallstones (92).

Regulation of bile acid biosynthesis and transport

Bile acid biosynthesis is highly regulated by many factors including nutrients, hormones and bile acids themselves. The main target for regulation of bile acid biosynthesis is CYP7A1, the first and rate limiting step in the neutral pathway. The nuclear receptor, liver X receptor α (LXRα; NR1H3) has been shown to act as
key regulator of cholesterol and bile acid homeostasis in rodents (95). Mice lacking LXRα accumulate massive amounts of cholesterol in their livers when fed a high cholesterol-rich diet which establish LXRα as the cholesterol sensor responsible for feed-forward regulation of CYP7A1 expression (96). LXRα is activated by metabolites of cholesterol, so called oxysterols, and enhances clearance of cholesterol by increasing bile acid synthesis and excretion. Ligand-bound LXRα binds and induces transcription of the CYP7A1 gene in a heterodimer complex with RXR (97,98). Compared to rodents, LXRα has much less effect on the hamster and human CYP7A1 genes, which lack the LXRα binding site found in the mouse and rat genes (99). Hamster is also much more sensitive to the cholesterolemic effects of dietary cholesterol compared to rat (100).

A second point of regulation is CYP8B1, which will determine the ratio of cholic acid to chenodeoxycholic acid. In contrast to stimulation of rodent CYP7A1 gene expression, cholesterol feeding decreases CYP8B1 expression in rat (84). CYP8B1 has been shown to be activated by members of the sterol regulatory element-binding proteins (SREBPs). SREBPs is a family of membrane-bound transcription factors that act as sensors of membrane-embedded lipids. Activation of SREBPs in sterol-depleted cells occurs in a two step cascade to release the amino terminal of SREBP which binds to sterol response elements (SRE) in the promoter of target genes. Several genes involved in cholesterol biosynthesis and uptake is induced in this manner (101). The rat CYP8B1 promoter contains several SREs and is activated by the SREBP-1 isoform whereas a second isoform SREBP-2 has been reported to suppress the promoter activity in an indirect manner (102).

Bile acids down-regulate their own synthesis and modulate bile acid transport through the farnesoid X receptor (FXR; NR1H4) (103). Bile acid-bound FXR forms a heterodimer with RXR and regulates the hepatic expression of a small heterodimer partner 1 (SHP-1; NR0B2), an atypical member of the nuclear receptor family that lacks a DNA binding domain (95). SHP-1 represses target genes by forming a heterodimer complex with nuclear factors involved in activation of gene expression. SHP-1 has been shown to interact with members of the NR5A2 family of nuclear receptors. The NR5A2 includes mouse liver receptor homologue (LRH), human cholesterol 7α-hydroxylase promoter factor (CPF) (104) or human α-fetoprotein transcription factor (FTF) (110). FTF is the recommended name by Genomic Data Base Nomenclature Committee. This nuclear receptor is required for the hepatic expression of CYP7A1 and also for expression of SHP-1 itself, thus providing a route for feed-back regulation of the SHP-1 gene. An alternative route for induction of SHP-1 gene expression is through the c-Jun N-terminal kinase (JNK) pathway, a cell-stress activated pathway induced, for example, by bile acids. The SHP-1 gene is a direct target of activated c-Jun. Down-regulation of CYP7A1 transcription via activation of c-Jun and increased expression of SHP-1 has been reported (106).

Expression of CYP8B1 is also repressed by SHP-1 (107,108). SHP-mediated suppression of CYP8B1 has been shown to involve interaction with the hepatic nuclear factor 4α (HNF4α;NR2A1) (109) besides FTF (105). The rat and
human CYP8B1 genes have overlapping binding sites for HNF4α and FTF and these two receptors may differently regulate CYP8B1 gene expression (109,111). SHP-1 has been shown to suppress trans-activation by HNF4α and RXR through competition with co-activators and also via a direct transcriptional repressor function (112). Studies on FXR null mice have shown that this receptor is essential also for regulation of genes encoding BSEP in hepatocytes (75) and I-BABP in enterocytes (Fig. 4) (77,113,114). Unlike the mechanism for repression of CYP7A1 and CYP8B1, ligand-bound FXR directly binds and activates these genes. The hepatocyte transporter NTCP is, on the other hand, down-regulated by bile acids (84).

Additional routes for regulation of bile acid biosynthesis have been reported to occur through phosphorylation of nuclear receptors or competition between nuclear receptors for binding to response elements. Phosphorylation of HNF4α in a MAP-kinase dependent manner has been suggested as a mechanism for down-regulation of CYP7A1 by insulin (115). The PPARα/RXR complex has been reported to inhibit expression of CYP7A1 by interfering with binding of HNF4 to the gene (116). Treatment with the peroxisome proliferator WY14643, on the other hand, upregulates CYP8B1 in a PPARα-dependent manner in mice (117).

Taken together, bile acid biosynthesis and transport are regulated at multiple levels to provide adequate emulsification capacity in the bile and intestine and, at the same time, ensure that sufficient amounts of cholesterol are catabolized to maintain homeostasis.

Physiochemical properties of bile acids

Bile acids are curved molecules and possess two distinct hydrocarbon surfaces, the convex hydrophobic side, which is devoid of hydrophilic groups, and the concave hydrophilic side with the hydroxyl substituents as shown in Fig. 6. (118). The solubility of a bile acid in water depends on the number of hydroxyl groups, which in turn is a determinant for the critical micelle concentration (CMC). Trihydroxy bile acids are more soluble in water and thus have a higher CMC compared to dihydroxy bile acids that are more prone to aggregate. The hydrophilic side of bile acids is a determinant for the type of micelle formed as this side will be the external surface of the primary micelle and will govern micelle growth and secondary micelle formation (118).

The number, position, and orientation of hydroxyl groups are key determinants for the physiochemical properties of bile acids including their ability to solubilize cholesterol. The term hydrophobic-hydrophilic balance was introduced to describe the physiochemical properties of bile acids as a function of their relative retention time (RRT) in C18 reversed-phase HPLC. Hydrophobic bile acids with a higher affinity to the column have longer RRT. A correlation was found between the RRT of a bile acid and the ability to solubilize cholesterol, indicating that the hydrophobic bile acid more effectively solubilizes cholesterol (118). Cholic acid
is, however, a much more powerful promoter of intestinal cholesterol absorption than the more hydrophobic chenodeoxycholic acid and deoxycholic acid although the latter are more potent detergents (119-121). It has been suggested that the 6α-hydroxyl group, present in hyocholic acid, is intrinsically more hydrophilic than other hydroxyl substituents, with the exception of the 7β-hydroxyl group in ursodeoxycholic acid (118). These hydrophilic bile acids do not support cholesterol absorption.

![Schematic depiction of the amphiphilic structure of chenodeoxycholic acid, cholic acid, and hyocholic acid, showing the hydrophobic (convex) side and the hydrophilic (concave) side, with its hydroxyl groups.](image)

Fig. 6 Schematic depiction of the amphiphilic structure of chenodeoxycholic acid, cholic acid, and hyocholic acid, showing the hydrophobic (convex) side and the hydrophilic (concave) side, with its hydroxyl groups.

In humans, increased levels of deoxycholic acid, the 7α-dehydroxy metabolite of cholic acid, have shown to be an important factor in the pathogenesis of cholesterol gallstone formation (122). Likewise cholic acid is a prerequisite for gallstone formation in a murine model (121). The hydrophilic ursodeoxycholic acid has, on the other hand, frequently been used to dissolve cholesterol gallstones (123). The mechanism is related to decreased cholesterol crystallization at increasing bile salt hydrophilicity (124). Administration of hyocholic acid or its 7α-dehydroxy
metabolite, hydoxycholic acid, has also been shown to protect against gallstone formation during experimentally induced cholelithiasis in various animal species (125-127). In hamster, a major effect of treatment with these 6α-hydroxylated bile acids were decreased LDL-cholesterol concentration, stimulation of hepatic cholesterol biosynthesis, and an excessive loss of cholesterol in feces (128). Therapy with hyocholic acid or hyodeoxycholic acid in humans would theoretically be possible but these hydrophilic bile acids are not useful in practice since 6α-hydroxylated bile acids are preferably glucuronidated and lost from the hepatic circulation via urinary excretion (129).

Higher ratios of phospholipids to bile acids prevent cholesterol crystallisation. Bile acids have different effects on phospholipid secretion into the bile through the mdr2 transporter (Fig 5). It has been suggested that regulation of mdr2 proceeds via an indirect mechanism involving protein kinase C activation by bile acids. Hydrophobic bile acids increase phospholipid secretion to a greater extent than hydrophilic bile acids (79).

The hydrophobicity index (HI) of bile acids is, similarly to the hydrophobic-hydrophilic balance, based on bile acid retention in reverse-phase liquid chromatography (130). Correlations of the HI of bile acids assessed by RRT indicate that the same physiochemical properties, which determines their detergency, may also determine their potency as regulators of bile acid and cholesterol biosynthesis (120). Chenodeoxycholic acid has been shown to be the most potent activator of FXR followed by hydrophobic bile acids such as deoxycholic acid and lithocholic acid whereas cholic acid is less effective (131,132). Hydrophilic bile acids including several di- and trihydroxy bile acids with a hydroxyl group in the C-6 position were inactive (121). Bile acids also participate in regulation of cholesterol biosynthesis through an effect on HMG-CoA reductase, which is the rate-limiting step in cholesterol biosynthesis. The effect is suggested to be mediated through a post-translational regulation of the enzyme. More hydrophobic bile acids as chenodeoxycholic acid, cholic acid and deoxycholic acid decrease HMG-CoA reductase activity whereas hydrophilic bile acids as ursodeoxycholic acid, hyocholic acid and hyodeoxycholic acid have no effect (120,133).

Taken together the physiochemical properties of the circulating bile acid pool will influence several aspects of lipid metabolism including intestinal absorption of dietary lipids, biliary cholesterol and phospholipid secretion, cholesterol and bile acid biosynthesis, and also gallstone formation. The HI calculated for the pool of bile acids shows a marked variation among species. The rat, which can hydroxylate bile acids in the 6β-position has a bile salt pool consisting primarily of very hydrophilic α- and β-muricholic acid. The bile of dogs contains primarily cholic acid and has a more hydrophobic bile compared to rats. The bile of hamster, with a mixture of cholic acid and chenodeoxycholic acid, is still more hydrophobic. Human bile, containing cholic acid, chenodeoxycholic acid and also a substantial fraction of deoxycholic acid, is the most hydrophobic bile among the four species (130).
Developmental aspects on bile acid biosynthesis in human and pig

*Human* - In humans, the ability to form bile acids is well developed in the early gestation period (weeks 13 to 19) (134,135). The composition of bile acids in the fetal human bile is, however, different from that of the healthy adults and more similar to the bile acids found in adults with severe cholestatic liver diseases (136). A limited fetal synthesis of cholic acid has been suggested to reflect an immature hepatic 12α-hydroxylase activity early in life (134,135). The predominance of chenodeoxycholic acid appears to be a feature of fetal life. During early gestation a relatively high proportion of polyhydroxylated bile acids (30% of all bile acids) has been found (135). The presence of hyocholic acid and several 1β-hydroxycholanolic acid isomers indicates that C-6 and C-1 hydroxylations in the steroid nucleus are important pathways in bile acid biosynthesis during development (135-137). Also, C-4 hydroxylated species have been identified in human fetal bile (weeks 16 to 19) and amniotic fluid (135,136).

The bile acid to cholesterol ratio in fetal serum has been reported to be higher than in the adults. A low hepatic clearance of bile acids from the immature liver could account for an increase in the level of bile acids in serum (138). Sulfatation and glucuron-conjugation are poorly developed in fetal and newborn life (137-139). It has been speculated that the human fetus, which is maintained under conditions of high level of hepatic bile acids, attempts to excrete bile acids into urine (amniotic fluid) by increasing polarity (137). The polyhydroxylated bile acids found could thus help to protect the fetal liver against accumulation of more hydrophobic cytotoxic bile acids at a time when other detoxification pathways are poorly developed.

Total biliary bile acid concentration is low before week 17 of gestation but thereafter the concentration markedly increases (134). Major changes in bile acid biosynthesis occur between week 20 of the gestation and full term (136). Between weeks 20 and 37 of gestation chenodeoxycholic acid and cholic acid were the major components measured in fetal blood samples and umbilical cord (138) with chenodeoxycholic acid as the main bile acid. In the newborn, however, cholic acid predominates (136).

A shift from a predominance of chenodeoxycholic acid in fetal life to cholic acid in newborn indicates that the bile acid pool during development becomes progressively more hydrophilic. However, the presence of polyhydroxylated bile acids, such as hyocholic acid, reduces the hydrophobicity of fetal bile (138). Hyocholic acid appears as early as week 20 and is present throughout the gestational period. Also, 6α-hydroxylated bile acids are found in newborn infants (140).

Screening of recombinant human cytochrome P450s recognized CYP3A4 as an enzyme capable to 6α-hydroxylate taurochenodeoxycholic acid and lithocholic acid (141). CYP3A4 has been shown to be involved in detoxification of the hepatotoxic lithocholic acid during cholestatic liver disease (142), and hyocholic acid is found in serum and urine during cholestasis (143). Whereas CYP3A4 predominates in the adult liver, the human fetal liver expresses mainly CYP3A7. These two members of the CYP3A subfamily have comparable catalytic properties (144). The reported
fetal expression of CYP3A7 (145,146) concords with the developmental-dependent formation of hyocholic acid, although the capacity of CYP3A7 to 6α-hydroxylate chenodeoxycholic acid has not yet been investigated.

**Pig** - The biliary bile acid composition in pig fetuses (weeks 4, 15 and 17 and after birth) has been investigated (87). The predominant bile acids in porcine fetal bile of the 4th week of gestation were cholic acid and chenodeoxycholic acid. The proportion of chenodeoxycholic acid did not change throughout the gestation period. The proportion of cholic acid was markedly decreased in the bile of late gestation period and was not found at all in the adult bile. The C-1 and C-4 hydroxylated bile acids present in human fetal liver, and also in other mammals such as rabbit and sheep, are not present in pig fetal bile (87). A major bile acid in the late gestation period was hyocholic acid, which was decreased after birth. This decrease could be explained by the increase in the proportion of hyodeoxycholic acid, the 7α-dehydroxy metabolite which is formed by the intestinal microflora.

Cultured hepatocytes from both weaned and unweaned piglets synthesized similar types of bile acids from cholesterol. Hyocholic acid was by far the major bile acid synthesized from radiolabeled cholesterol and constituted about 50% of all bile acids formed. Together, hyocholic acid and chenodeoxycholic acid accounted for more than 85% of total bile acid synthesis. In addition, hyodeoxycholic acid (7%) and muricholic acid (3%) and small amounts of lithocholic acid was identified (147).

Taken together, chenodeoxycholic acid is the main bile acid in the fetal life of both human and pig. In addition trihydroxy bile acids are also a common feature although bile acids and enzymes involved differ between species.
Aims of the present investigation

The initial aims were:

- to clone and acquire more knowledge of the specific porcine taurochenodeoxycholic acid 6α-hydroxylase that catalyzes formation of hyocholic acid from chenodeoxycholic acid. The enzyme had previously been purified and was believed to be a member of the CYP4A subfamily

- to study the 6α-hydroxylase from a developmental aspect as this enzyme appears to have evolved when the ancestor of the domestic pig lost the ability to synthesize cholic acid

Additional aims that were formulated during the course of the work:

- to clone porcine CYP4A fatty acid hydroxylases and compare their sequences and properties with the taurochenodeoxycholic acid 6α-hydroxylase (CYP4A21)

- to study if CYP8B1, the key enzyme in cholic acid biosynthesis, is expressed in pig. This study was initiated due to new data reported regarding the presence of cholic acid in bile of pig fetus

- to clone the taurochenodeoxycholic acid 6α-hydroxylase (CYP4A21) gene in order to get more information on the relationship between CYP4A21 and CYP4A fatty acid hydroxylases
Experimental procedures

Materials

RNase Midi Kits, QIAprep Spin Miniprep and Maxi kit were from Qiagen. Reverse Transcription System, and DNA 5’-End Labeling System were purchased from Promega. Oligonucleotides were obtained from Life Technologies and Thermo Hybaid (Interactive Division). TaqGold polymerase and oligo dNTP mix were from PE Applied Biosystems. A 1-kb DNA ladder, a Low mass DNA ladder, and 5’RACE system were from Life Technologies. Restriction enzymes were from AmershamPharmacia Biotech and New England BioLabs. SureClone Ligation Kit, ECL Western Blotting analysis system, Megaprime DNA labelling system, [γ-32P] dATP, [α-32P] dCTP, [1-14C]lauric acid (53 Ci/mol) and [1-14C]palmitic acid (56 Ci/mol) were from AmershamPharmacia Biotech. Tauro[24-3H]chenodeoxycholic acid (0.8 Ci/mol) was synthesized according to Norman (148). Porcine genomic library, Universal GenomeWalker kit, NucleoSpin Tissue kit, and Advantage genomic PCR kit, total RNA from human fetal liver (pooled from 38 spontaneously aborted male/female fetuses, ages 22-40 weeks) and adult human liver (27-year old male) were obtained from Clontech Laboratories.

Animals

Livers, kidneys and other tissues from castrated, otherwise untreated, six months old male pigs were obtained from the local slaughterhouse. Livers from pig fetus and pigs aged from a few days to three months were obtained from the Funbo-Lövsta Research Center, Department of Animal Breeding and Genetics, Swedish University of Animal Sciences, Ultuna. All tissues were frozen on dry ice and stored at -80°C until used.
Isolation of RNA, cDNA cloning by RT-PCR, and related methods

Isolation of RNA, RT-PCR, and purification of DNA fragments

Total RNA was isolated using Rneasy Midi kits (Qiagen). First strand DNA was obtained using a Reverse Transcription System (Promega) with an oligo(dT)$_{15}$ primer and AMV reverse transcriptase. Portions of the reverse transcription (RT) reaction was used directly as template for polymerase chain reaction (PCR). The PCR was performed in presence of 2.5 mM Mg$^{2+}$ and in some instances with 10% dimethyl sulfoxide (DMSO). PCR cycles were generally as follows: denaturation at 94 °C for 1 or 2 min, annealing temperature (50 °C to 60°C depending on primers) for 1 or 2 min, extension at 72°C for 2 min, for 30 cycles, and finally 72°C for 10 min. In most instances hot start AmpliTaq Gold was used for PCR amplification. The PCR products were analyzed by agarose gel electrophoresis using ethidium bromide to visualize DNA and a 1-kb ladder for size determination. Recovering of PCR fragments excised from agarose gels were mostly performed by electroelution into dialysis bags followed by phenol-chloroform extraction and ethanol precipitation. DNA fragments were resuspended in 10 mM Tris-Cl, pH 8.0 and the DNA concentration was determined by agarose gel electrophoresis and comparison with a Low DNA mass ladder.

Rapid amplification of 5’ and 3’ ends

The terminal ends of expressed mRNAs of interest were obtained using rapid amplification of cDNA ends (RACE) procedures. Extension in the 5’ direction was performed using 5’ RACE system (Life Technologies) according the manufacturer’s instruction with two reverse gene specific primers. Extension in the 3’ direction was performed by a terminal RACE procedure according to Frohman (149) using a primer directed against the natural poly(A) tail combined with forward gene specific primers. PCR fragments were analyzed and purified as previously described.

DNA sequence analysis

Sequencing service was provided by the Department of Animal Breeding and Genetics, Swedish University of Agricultural Sciences. Sequencing was performed using the Big Dye Terminator sequencing kit and an ABI 377 automatic sequencer (Perkin Elmer Applied Biosystem) or using a Dye Terminator cycle sequence kit and a Megabase 1000 sequenator (AmershamPharmacia Biotech). Plasmid DNA was sequenced with universal M13 reverse and forward primers and PCR fragments with gene specific primers.
Northern blot

Between 20 μg and 30 μg of total RNA were used for Northern blot analysis in different experiments. RNA was electrophoresed on 1.2% agarose gel containing formamide (150) and transferred to Hybond-N membranes (AmershamPharmacia Biotech). The cDNA probes were [³²P]-labeled using Megaprime labeling Kit (AmershamPharmacia Biotech). Oligoprobe were labeled using DNA 5'-End Labeling System (Promega). For details regarding hybridization conditions see Papers I and III. Sample loading was assessed by the intensity of the ribosomal 18S on the blotted filters. Attempts to use a labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe failed due to an age-dependent expression of this housekeeping gene. Developmental variations in the expression of both GAPDH and β-actin have been reported (151).

Southern blot

Separation of PCR fragments digested with restriction enzyme was performed by agarose gel electrophoresis and transferred to Hybond-N membranes (AmershamPharmacia Biotech). A [³²P]-labeled full-length cDNA probe of CYP4A21 was used for hybridization. For details regarding hybridization conditions see Paper II.

Expression of recombinant proteins

Expression of CYP4A21 in COS-1 cells

The cDNA of CYP4A21 was cloned into the expression vector pSVL (AmershamPharmacia Biotech). Care was taken to ensure the presence of a conserved eukaryotic initiation site (152). COS-1 cells were grown at 37 °C, 5% CO₂, in Dulbecco’s Modified Eagle Medium supplemented with fetal calf serum (10%), glutamine and antibiotics. Cells were transfected by the DEAE-dextran method (153) or by electroporation (settings 0.4 kV, 100 μFad). Transfected cells were grown for 48 h then washed and harvested. Microsomes from COS-cells were isolated by differential centrifugation according to Clark and Waterman (154), resuspended and homogenized in 100 mM potassium phosphate, pH 7.4, containing 0.1 mM EDTA. Protein concentration was determined using BCA reagent (Pierce) with bovine serum albumin as standard. COS-cell microsomes prepared from cells transfected by the pSVL vector without cDNA (mock-transfection) were used as control.
Expression of CYP4A24 and CYP4A25 in yeast

The cDNA of CYP4A24 and CYP4A25, respectively, was cloned into the expression vector pYeDP60 (a gift from Drs Denis Pompon and Philippe Urban, Centre de Génétique Moléculaire du CNRS, Gif-sur-Yvette, France) and transformed into S. Cerevisiae strain W(R) by the lithium acetate procedure (155). The S. Cerevisiae strain W(R) has been genetically modified to overexpress the yeast NADPH-cytochrome P450 reductase in presence of galactose (156). The transformed yeast cells were first grown to high density with glucose as the main energy source, thereafter galactose was added to induce expression of the pYeDP60 vector promoter and yeast NADPH-cytochrome P450 reductase. Yeast cells were grown in presence of galactose between 12 to 16 h at 28°C before harvest. Glass beads were used to disrupt the cells and microsomes were isolated by centrifugation (157). Protein concentration was determined by the method of Lowry et al (158) using bovine serum albumin as standard. Yeast cell microsomes prepared from cells transformed by the pYeDP60 vector without cDNA (mock-transformation) were used as controls.

Western blot analysis to detect recombinantly expressed proteins

SDS-PAGE was run on 7% gels according to Laemmli (159) with 15% acrylamide and 0.09% bisacrylamide slab gels containing 0.1% SDS. The gels were electrophoretically blotted onto nitrocellulose filters. Western blot analysis was performed using polyclonal antibodies raised against the purified 6α-hydroxylase (89) or rat CYP4A (AmershamPharmacia Biotech) as primary antibodies and horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (Bio-Rad). The immunoreacted protein bands were visualized with an ECL Western Blotting analysis system.

Enzyme activity assays

Recombinantly expressed proteins were subjected to analysis for 6α-hydroxylase activity towards taurocholodeoxycholic acid and ω-and (ω-1)-hydroxylase activities towards fatty acids.

[14C]-Labeled taurocholodeoxycholic was incubated with microsomes from transfected COS-cells and transformed yeast cells. Besides microsomes and substrate, the incubation mixtures contained 5 µM dithiothreitol and 2 µM NADPH in a total volume of 1 ml of 100 mM potassium phosphate, pH 7.4 containing 0.1 mM EDTA. Incubations with COS-cell microsomes were performed with an addition of 1 U NADPH-cytochrome P450 reductase prepared from pig liver microsomes (160). Incubations were performed at 37 °C for 20 or 30 min and terminated with 5 ml of ethanol. The incubation mixtures were prepared for thin layer chromatography as previously described (89). The chromatoplates
were analyzed by radioactivity scanning. The use of external standards and control preparations is described in Paper I. 

[1-14C]-labeled lauric acid and palmitic acid were incubated with microsomes from transformed yeast cells with 2 µM NADPH in a total volume of 0.2 ml of 0.1 M Tris-HCl, pH 7.5. Microsomes from COS-cells were incubated with lauric acid in 50 mM Tris/acetate, pH 7.4, containing 0.1 mM EDTA and 20% glycerol. Incubations were performed at 37°C for 20 min and terminated by the addition of 0.1 ml 6% acetic acid in ethanol (lauric acid) or 6% acetic acid in acetonitrile (palmitic acid). Samples were centrifuged and the supernatant analyzed by HPLC according to Okita et al (161) using a LichroCART 250 x 4 mm, RP-18 (5µM) column (Merck), and a Radiomatic detector (Packard Instrument).

**Genome Walking procedure**

Genomic DNA from pig kidney was purified using NucleoSpin kit (Clontech Laboratories). Construction and screening of GenomeWalker DNA libraries were performed according to the manual (Universal Genome Walker Kit, Clontech Laboratories). Briefly, separate aliquots of genomic DNA were digested with EcoRV, DraI, PvuII and SstI, respectively, to generate blunt ended fragments for subsequent ligation with an adopter sequence. Screening of the libraries was performed using nested primers that hybridize to the adopter sequence combined with gene specific primers.

**Screening of genomic libraries**

**Screening procedure**

A porcine genomic library constructed in the EMBL3 SP6/T7 vector was screened according to the manufacturer’s instruction (Clontech Laboratories). Briefly, dilutions of the library were incubated with the E. Coli host strain K802, spread on LB agar + MgSO4 plates and incubated at 37°C for about 7 h. Plaque lifts were performed using Nylon filters. The filters were denatured, neutralized, washed and dried prior to UV cross-linking. Hybridization was performed with either full-length [32P]-labeled CYP4A21 cDNA or a [32P]-labeled sequence of genomic CYP4A21, labeled using Megaprime DNA Labelling System. Filters were exposed to Fuji RX film and the developed film was aligned with the plaque plate. Plaques giving strong positive signals were picked and eluted from the agar plug in buffer. Successive rounds of plating of eluted plaques were performed to ensure single plaque purity.
Preparation of high-titer phage stocks and DNA purification

High-titer phage stocks were prepared by plating lytic phages from a single plaque at high density on LB agar + MgSO₄ plates. Plates were grown at 37°C to near confluence. Buffer was added to the plates, the plaque extract was collected and subjected to chloroform extraction and centrifugation. The supernatant was withdrawn and stored at +4°C.

Purification of phage DNA was performed using high-titer phage extract from several plates. The pooled extracts were subjected to treatment with DNase I and RNase A, extraction with chloroform and precipitation by polyethylene glycol. The precipitate was collected by centrifugation and resuspended in buffer. CsCl (0.5g/ml) was added followed by centrifugation (90 000xg for 2 h). The precipitate thus obtained was resuspended in buffer, treated with protein kinase K, and purified by sequential extractions with phenol:chloroform and chloroform, respectively, and finally precipitated by ethanol. The purified phage DNA was resuspended in water and the concentration of DNA was determined by UV absorbancy.

Analysis of high titer phage stocks and purified phage DNA

High-titer phage stocks were screened by PCR using gene specific primers to identify genomic clones corresponding to CYP4A21 and pig CYP4A fatty acid hydroxylases, respectively. For details regarding the primers see Paper IV.

Insert DNA was excised from purified recombinant phage using XhoI. Fragments from a recombinant phage, identified as the genomic sequence of CYP4A21, were obtained by successive digestion with SacI and PstI and ligated into pBluescript vector. The remaining sequence of this phage clone and two additional phages were obtained from PCR fragments generated with primers designed from putative exon sequences, based on the intron-exon structure of CYP4A11. For details regarding the procedure and primers used see Paper IV.
Results and discussion

Cloning of pig CYP4A21, CYP4A24, and CYP4A25. Papers I and II

Cloning of taurochenodeoxycholic acid 6α-hydroxylase (CYP4A21)

The porcine taurochenodeoxycholic acid 6α-hydroxylase has previously been purified and characterized (88,89). The N-terminal amino acid sequence and sequences of peptides obtained after partial trypsin digestion shown in Fig. 7A, suggested that this enzyme belongs to the cytochrome P450 4A (CYP4A) subfamily. Oligonucleotide primers were designed from the amino acid sequences contained in the N-terminal sequence (N2) and Fragment F (R:FEL) as shown in Fig. 7B. Based on an alignment with rabbit CYP4A7 these primers would generate a near full-length sequence of the 6α-hydroxylase.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-terminal</td>
<td>TVPALASVSGLLQVAALLGL</td>
</tr>
<tr>
<td>A</td>
<td>HSHAFMPFGGSR</td>
</tr>
<tr>
<td>B</td>
<td>EFQEESELQXNLK</td>
</tr>
<tr>
<td>C</td>
<td>WLWGTR</td>
</tr>
<tr>
<td>D</td>
<td>SALQNDIIYR</td>
</tr>
<tr>
<td>E</td>
<td>FAPGSR</td>
</tr>
<tr>
<td>F</td>
<td>FELAPDPISR</td>
</tr>
</tbody>
</table>

Fig. 7 A. The N-terminal amino acid sequence and sequences of peptides obtained after partial trypsin digestion of the purified 6α-hydroxylase (89). B. The primers designed from the amino acid sequences in the N-terminal sequence and Fragment F.
RT-PCR was performed with total RNA from pig liver. The 1430 bp PCR product obtained was gel-purified and cloned into pUC18 and transformed in E. Coli DH5α cells. A plasmid containing the insert was sequenced. The terminal 5’ and 3’ ends were obtained by RACE procedures. The compiled cDNA was 2,400 bp and contained a 37-nucleotide 5’ untranslated region, a 1,515-bp open reading frame, and an 848-bp 3’ untranslated region. No consensus polyadenylation signal sequence was found in the 3’ untranslated region. The cDNA encoded a protein of 504 amino acids. The primary structure of this porcine 6α-hydroxylase, designated CYP4A21, showed about 75% identity with known members of the CYP4A subfamily in rabbit and man. A peculiarity was that the conserved signature sequence previously found among members of the CYP4A and 4B enzymes (19) contained deviations as shown in Fig 8. For example, the otherwise conserved glutamic acid (E) residue in the middle of the I-Helix, which has been shown to participate in covalent binding to heme (16,17), is replaced by alanine (A). Investigations performed to verify these deviations found in the CYP4A21 sequence resulted in cloning of two additional porcine CYP4A enzymes as described below.

![Alignment of the deduced sequences between positions 308 and 320 of CYP4A21 and corresponding residues in the conserved sequence common to CYP4A and CYP4B subfamilies. Residues in CYP4A21 that deviate from the consensus sequence are underlined.](image)

Cloning of the porcine CYP4A fatty acid hydroxylases (CYP4A24 and CYP4A25)

The 1430 bp PCR product generated with the primer pair N2 and R:FEL, described above, was subjected to analysis by restriction enzymes and was found to contain more than one sequence. Similarly, sequences generated by PCR using primers that hybridize to internal sequences within the 1430 bp PCR product generated more than one population of sequences. It was thus concluded that the 1430 bp fragment contained a set of homologous sequences.
Using RT-PCR and the N2 and R:FEL primers, the 1430 bp fragment was generated from both pig liver and kidney RNA. Total RNA from tissues of four different individuals were used, two livers and two kidneys. Restriction enzyme analysis with PstI was used to distinguish between CYP4A21 and “conserved” CYP4A sequences due to the differences within the CYP4A signature motif. The use of EcoRI to differentiate between subpopulations of sequences in N2-R: FEL fragments was based on diverging sequences, regarding this restriction site, generated from the N2-R:FEL fragment using internal primers. An overview of expected restriction sites in subpopulations of sequences contained in the 1430 bp PCR product is shown in Fig. 9.

![Restriction Sites Diagram](image)

**Fig. 9** An outline of expected restriction sites and fragment sizes for two populations of sequences in the N2-R:FEL fragment using PstI (A) and EcoRI (B), respectively.

![Southern Blot](image)

**Fig. 10** Southern blot hybridization of PstI and EcoRI digests of the 1430 bp PCR product (N2-R:FEL fragment), using [32P]-labeled cDNA of CYP4A21 as probe. N2-R:FEL fragments were generated from total RNA obtained from livers from two individuals and from kidney likewise from two individuals. The digested fragments were separated by agarose gel electrophoresis and blotted.
The result of the *PstI* and *EcoRI* digest is shown in Fig. 10, which is a Southern hybridization of digested sequences separated by agarose gel electrophoresis. Restriction enzyme digest by *PstI* of the liver samples clearly shows the presence of two distinct populations of sequences. A strong band at 905 bp indicates that the CYP4A21 derived sequence predominates in both samples. The opposite is seen in the kidney samples. This is consistent with a high expression in the liver of enzymes that participate in bile acid biosynthesis. CYP4A fatty acid hydroxylases, on the other hand, are known to be highly expressed in kidney whereas the expression level in liver is comparatively low. The *EcoRI* digest shows an excess of uncleaved sequences in the liver, which indicate a high frequency of CYP4A21 derived sequences in the liver samples. The presence of two subpopulations of sequences, differently cleaved by *EcoRI*, is on the other hand, seen with the fragments generated from kidney. It is notable that the intensity of the two bands generated by *PstI* and *EcoRI*, respectively, differs in the kidney samples. This indicates the presence of a sequence, resistant to *EcoRI* digestion but distinct from CYP4A21 in the fragments generated from kidney. The pattern of restriction enzyme digest was identical between the two liver samples and the two kidney samples, respectively.

A new set of primers (5N1 and 3CB), designed from sequences in the 5’ and 3’ untranslated regions of CYP4A21, generated a fragment which was cleaved by *PstI* and *EcoRI* similar to the N2-R:RE1 fragment. Two CYP4A-fatty acid hydroxylases, designated CYP4A24 and CYP4A25, expressed in both liver and kidney were cloned using restriction enzyme analysis to distinguish between sequences. A full-length sequence of CYP4A21 was also obtained in the process. CYP4A24 and CYP4A25 share extensive sequence homology (~99% overall nucleotide identity). The two cDNAs encode proteins of 504 amino acids, similar to that of CYP4A21. The overall amino acid identity between these two sequences is 97% compared to a 94% overall identity to CYP4A21. An alignment between the three porcine CYP4A amino acid sequences is shown in Fig 11. The position of secondary structures indicated in the figure are based on secondary structure prediction using the PHD program (162) and using a secondary structure nomenclature adopted from Chang and Loew (20) with the addition of the membrane helix. In comparison with the topographical map of secondary structure elements, shown in Fig. 1, the β-sheet is missing, which is consistent with the variable presence of this β-sheet among P450s (10).
<table>
<thead>
<tr>
<th>Membrane helix</th>
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<tbody>
<tr>
<td><strong>CYP4A24</strong></td>
</tr>
<tr>
<td><strong>CYP4A25</strong></td>
</tr>
<tr>
<td><strong>CYP4A21</strong></td>
</tr>
<tr>
<td><strong>CVP4</strong></td>
</tr>
</tbody>
</table>

<table>
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<th>β1-2</th>
</tr>
</thead>
<tbody>
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<td>100</td>
<td></td>
</tr>
<tr>
<td>HIlVGHYSEFQEEESELPALLKRAVEXKPYSCDDALITTEHTRLHVDTPYIK</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>HIlVGHYSEFQEEESELPALLKRAVEXKPYSCDDALITTEHTRLHVDTPYIK</td>
<td>100</td>
<td></td>
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<td>150</td>
</tr>
<tr>
<td>VILRSEPQKVVRLLTVHICQGOLLNLNLNGQITFFRRRAHLTAFHVVDLH</td>
<td>150</td>
</tr>
<tr>
<td>VILRSEPQKVVRLLTVHICQGOLLNLNLNGQITFFRRRAHLTAFHVVDLH</td>
<td>150</td>
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<table>
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</tr>
</thead>
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<td>PVGELHAKSVQVWLKQJOVLREHDRDELIEVGPVLHLEHTDITIKC@FSSHOG</td>
<td>200</td>
</tr>
<tr>
<td>PVGELHAKSVQVWLKQJOVLREHDRDELIEVGPVLHLEHTDITIKC@FSSHOG</td>
<td>200</td>
</tr>
<tr>
<td>PVGELHAKSVQVWLKQJOVLREHDRDELIEVGPVLHLEHTDITIKC@FSSHOG</td>
<td>200</td>
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<tr>
<th>E-Helix</th>
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<tr>
<td>SAQGDSOSVQIORHLDKMNLSRTKSFQONDICVIALPSVRKHHARQ</td>
</tr>
<tr>
<td>SAQGDSOSVQIORHLDKMNLSRTKSFQONDICVIALPSVRKHHARQ</td>
</tr>
<tr>
<td>SAQGDSOSVQIORHLDKMNLSRTKSFQONDICVIALPSVRKHHARQ</td>
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<th>G-Helix</th>
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<tbody>
<tr>
<td>RRHAKYHTDVQVLOQKROGENYKKAHLDFDLTLLLLALLAEEKGNISL</td>
</tr>
<tr>
<td>RRHAKYHTDVQVLOQKROGENYKKAHLDFDLTLLLLALLAEEKGNISL</td>
</tr>
<tr>
<td>RRHAKYHTDVQVLOQKROGENYKKAHLDFDLTLLLLALLAEEKGNISL</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>J-Helix</th>
<th>I-Helix</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSTDVRVEVTDTFNQFAGHDETRSGISSLILVLAFLSHPEHQQRCREEIQGLLG</td>
<td>350</td>
</tr>
<tr>
<td>SSTDVRVEVTDTFNQFAGHDETRSGISSLILVLAFLSHPEHQQRCREEIQGLLG</td>
<td>350</td>
</tr>
<tr>
<td>SSTDVRVEVTDTFNQFAGHDETRSGISSLILVLAFLSHPEHQQRCREEIQGLLG</td>
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<table>
<thead>
<tr>
<th>K-Helix</th>
<th>β1-4</th>
<th>β2-1</th>
<th>β2-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>DGTST1TLCHDLLQNYTTP1ICKEALRLYVPGPVQVQVRELSKPIITFDDORSNLQ</td>
<td>400</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DGTST1TLCHDLLQNYTTP1ICKEALRLYVPGPVQVQVRELSKPIITFDDORSNLQ</td>
<td>400</td>
<td></td>
<td></td>
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<tr>
<td>DGTST1TLCHDLLQNYTTP1ICKEALRLYVPGPVQVQVRELSKPIITFDDORSNLQ</td>
<td>400</td>
<td></td>
<td></td>
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</tbody>
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<table>
<thead>
<tr>
<th>K’-Helix</th>
<th>Meander</th>
<th>β-bulge</th>
</tr>
</thead>
<tbody>
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<td>A@ITLSSLSTVOLVHPNQVNPENEFDSRAMLPSERGSRHRSHAFHPSOGSQRNGP</td>
<td>450</td>
<td></td>
</tr>
<tr>
<td>A@ITLSSLSTVOLVHPNQVNPENEFDSRAMLPSERGSRHRSHAFHPSOGSQRNGP</td>
<td>450</td>
<td></td>
</tr>
<tr>
<td>A@ITLSSLSTVOLVHPNQVNPENEFDSRAMLPSERGSRHRSHAFHPSOGSQRNGP</td>
<td>450</td>
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<table>
<thead>
<tr>
<th>L-Helix</th>
<th>β3-3</th>
<th>β4-1</th>
<th>β4-2</th>
<th>β3-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1GKOFANNENHKKVAVATLLLTFELAPDPFSRKIPATFPEVVELNHSKHGUHLKL</td>
<td>500</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1GKOFANNENHKKVAVATLLLTFELAPDPFSRKIPATFPEVVELNHSKHGUHLKL</td>
<td>500</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1GKOFANNENHKKVAVATLLLTFELAPDPFSRKIPATFPEVVELNHSKHGUHLKL</td>
<td>500</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
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Fig. 11 An alignment between the deduced amino acid sequences of CYP4A24, CYP4A25 and CYP4A21. Lines above the sequences indicate the position of secondary structures, identified as helices and β-sheets by the secondary structure prediction using the PHD program (162). The overall nomenclature, used for CYP4A11, is adopted from Chang and Loew (20) with addition of the membrane helix.
Recombinantly expressed CYP4A21, CYP4A24 and CYP4A25. Papers I and II

Expression of CYP4A21 protein in microsomal preparations from transfected COS-1 cells was confirmed by Western blot analysis. The 6α-hydroxylase activity towards taurochenodeoxycholic acid in COS-cell microsomes was assayed using different concentrations of radiolabeled substrate. For comparison, microsomal preparations from pig liver were equally analyzed. As shown in Table 1, microsomes from transfected COS-1 cells and from pig liver showed a similar range of activity, depending on the substrate concentration. A higher rate of 6α-hydroxylation with higher concentration of substrate is consistent with the high Km-value (62.9 μM) calculated for the purified enzyme (89), and also with a high concentration of bile acids in portal blood after a meal (50 μM to 170 μM) (74). Neither ω- nor (ω-1)-hydroxylase activity towards lauric acid was detected with recombinant CYP4A21.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Hydroxylation of Taurochenodeoxycholic acid (6α)</th>
<th>Hydroxylation of Lauric acid (ω- and (ω-1))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/mg microsomal protein/min</td>
<td></td>
</tr>
<tr>
<td>Microsomes from CYP4A21-transfected COS cells\a</td>
<td>5 (25 μM)</td>
<td>ND\b</td>
</tr>
<tr>
<td></td>
<td>28 (145 μM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>49 (200 μM)</td>
<td></td>
</tr>
<tr>
<td>Pig liver microsomes</td>
<td>12 (25 μM)</td>
<td>575 and 2950</td>
</tr>
<tr>
<td></td>
<td>39 (145 μM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>56 (200 μM)</td>
<td></td>
</tr>
</tbody>
</table>

\a Microsomes from mock-transfected COS cells (pSVL vector without insert) did not show any detectable hydroxylase activity towards the two substrates.

\b ND= Not Detected

Mutation of the CYP4A21 sequence was performed to insert the nucleotides encoding the conserved CYP4A signature sequence (Fig 8). Recombinant expression of this mutant in COS-1 cells showed a loss of 6α-hydroxylase activity towards taurochenodeoxycholic acid. With the present knowledge about the importance of the glutamic acid (E) residue for covalent attachment of the heme group (16,17), it is tempting to speculate that insertion of this residue will have significant impact on the active site conformation. A more sterically restricted active site due to covalently bound heme could contribute to the loss of 6α-hydroxylase activity with this mutant. Introduction of the conserved signature sequence did, however, not render
the enzyme ω- or (ω-1)-hydroxylase activities towards lauric acid, indicating the importance of other regions governing the substrate specificity. Microsomes from mock-transfected COS-cells were devoid of 6α-hydroxylase activity towards taurochenodeoxycholic acid.

CYP4A24 and CYP4A25 were expressed in yeast cells. The presence of recombinant proteins was shown by Western blot analysis. The ω- and (ω-1)-hydroxylase activities towards lauric acid and palmitic acid, as well as 6α-hydroxylase activity towards taurochenodeoxycholic acid were assayed using microsomes from transformed yeast cells. No hydroxylase activity towards taurochenodeoxycholic acid could be detected. The ω- and (ω-1)-hydroxylase activities towards lauric acid and palmitic acid are shown in Table 2. Both sequences clearly encode proteins that exhibit ω- and (ω-1)-hydroxylase activity towards fatty acids. Increasing the fatty acid length decreased the hydroxylation rates for both enzymes, which is consistent with reports on CYP4A from other species. An activity towards arachidonic acid was also detected (unpublished results) and was found to follow the same trend, a lower activity with an increase in fatty acid length. The ω/(ω-1) ratios for lauric acid are comparable with the previously reported activity of a purified pig kidney ω- and (ω-1)-hydroxylase (163). The only observed difference so far is that the expressed fatty acid hydroxylase activity of CYP4A24-transformed yeast cell microsomes is higher throughout all experiments compared to that of CYP4A25-transformed yeast cell microsomes. In the rat CYP4A2 and CYP4A3, the residues involved in an observed difference in the ω- and (ω-1)-hydroxylase regiospecificity towards lauric acid have been mapped to an insert (Ser14-Gly15-Ile16) present in CYP4A3 but not in CYP4A2 (164). The corresponding amino acids in CYP4A24 and CYP4A25 are those encoded by the nucleotide differentially cleaved by EcoRI. Microsomes from mock-transformed yeast cells were devoid of or had very low ω- and (ω-1)-hydroxylase activity towards lauric acid and palmitic acid, respectively.

Table 2: The ω- and (ω-1)-hydroxylation of lauric and palmitic acid in microsomal preparations of transformed yeast cells. Data presented are means ± S.D. for three separate determinations (pmol/mg of microsomal protein per min).

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Hydroxylation of Lauric acid</th>
<th>Palmitic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ω (ω-1)</td>
<td>ω (ω-1)</td>
</tr>
<tr>
<td>CYP4A24</td>
<td>372 ± 7</td>
<td>174 ± 10</td>
</tr>
<tr>
<td>CYP4A25</td>
<td>200 ± 22</td>
<td>77 ± 16</td>
</tr>
</tbody>
</table>

Taken together, these results indicate that at least three CYP4A enzymes with extensive overall sequence similarity are present in pig liver and kidney. CYP4A21 clearly deviates regarding structural features and catalytic activity. It is, however, more difficult to establish whether CYP4A24 and CYP4A25 are the products of
distinct genes or merely allelic variants of a single gene. The position of the variable regions between CYP4A24 and CYP4A25, which are confined to \( \beta \)-sheets 1 and 4 (Fig 11), indicates a possible difference in substrate specificity or regiospecificity. The \( \beta \)-sheet 1 is located at the entrance of the substrate access channel and \( \beta \)-sheet 4 is among the structural elements, proposed to be involved in substrate specificity of P450s (10).

Sterol 12\( \alpha \)-hydroxylase (CYP8B1) in pig. Paper III

Cholic acid is not detectable in bile of adult pig suggesting that the pig lacks the key enzyme in cholic acid formation, the sterol 12\( \alpha \)-hydroxylase (CYP8B1). From the presence of high amounts of the trihydroxy bile acid hyocholic acid in pig bile it has been assumed that the ability to form this bile acid has evolved as a consequence of a deficient cholic acid production in pig (85). Surprisingly, Kuramoto et al (87) recently found that the bile of pig fetus contains cholic acid whereas the adult pig, in consistence with previous observations, was devoid of this bile acid.

Using fetal pig liver, we were able to show that an mRNA encoding CYP8B1 is indeed expressed in fetal pig liver. Cloning of the pig CYP8B1 gene revealed that the gene is devoid of introns similar to CYP8B1 genes in other species (82,82). An apparent conserved gene encoding CYP8B1 is thus present in the pig genome. Among available CYP8B1 sequences, the pig gene is most similar to the human CYP8B1 both regarding the coding sequence (81% amino acid identity) and within the proximal 460 bp of the putative promoter region (70% nucleotide identity). So far, all known CYP8B1 genes have a canonical TATA-box upstream of the transcription start site. The pig CYP8B1 is, however, an exception. Whether this deviation accounts for the unusual expression pattern, which appears to be restricted to the fetal pig liver, remains to be seen. Preliminary results using transient transfection of HepG2-cells with the pig CYP8B1 promoter in a luciferase reporter vector indicate the presence of a functional promoter, despite the lack of a proper TATA-box. Among \textit{cis}-acting elements in the promoter of other CYP8B1 genes, binding sites for FTF and HNF4\( \alpha \) have been identified. These transcription factors have been shown to be important for the expression of CYP8B1 in rat, mouse and human and are also a target for regulation of gene expression by SHP-1 (84,111,112). A HNF4\( \alpha \) binding element was identified in the pig CYP8B1 promoter sequence between - 46 to - 34 nucleotides upstream of the transcriptional start site. HNF4\( \alpha \) is expressed very early in embryotic development and is essential for liver development and function (165). This receptor has been observed to have different regulatory functions in the context of fetal compared to adult hepatocytes (166). HNF4\( \alpha \) has also been shown to recruit TFIIB and facilitate assembly of a preinitiation complex (167). The involvement of HNF4\( \alpha \) and other factors in the developmental-dependent expression of CYP8B1 remains, however, to be determined.
Kerstin Lundell

Tissue-specific and developmental expression of CYP4A21 and CYP8B1 in pig. Papers I, III and IV

Total RNA from different tissues of pig (heart, muscle, intestine, spleen, thymus, lung, adrenal gland, kidney, liver) was subjected to RT-PCR to assess the tissue distribution of CYP4A21. The specific CYP4A21 primer (RNON-CONS) combined with an upstream primer (Xho-N) was used for PCR amplification. The primer sequences are given in Paper I. As shown in Fig. 12, an intense band of the expected size (956 bp) was seen using RNA from liver. Also, a faint band of the same size could be distinguished with the kidney sample, whereas total RNA from other tissues did not generate bands of similar size. As described above a low expression of CYP4A21 in kidney was indicated using BstI digest of the kidney N2-R:FEL fragments where a faint band at 905 bp, typical for a CYP4A21-derived sequence, was obtained (Fig. 10). The expression of CYP4A21 in kidney raises questions regarding the possibility of additional catalytic activities, besides the 6α-hydroxylase activity towards taurochenodeoxycholic acid.

![Fig. 12 RT-PCR of total RNA to assess the distribution of CYP4A21 expression in different tissues. A CYP4A21 specific reverse primer was used. The PCR products were analyzed by agarose gel electrophoresis using a 1 kb DNA ladder for size determination. Samples were loaded as follows: lane 1, heart; lane 2, muscle; lane 3, intestine; lane 4, spleen; lane 5, thymus; lane 6, lung; lane 7, adrenal gland; lane 8, kidney; lane 9, liver.](image)

Northern blot analysis to compare expression of CYP4A21 and CYP4A fatty acid hydroxylases was carried out using oligo-probes. A description of these probes and the control hybridizations made is given in Papers III and IV. Total RNA from livers of unweaned piglets (2 to 6 weeks old) and weaned pigs (3 to 6 months old) were used. As shown in Fig. 13, the signal generated with the CYP4A21 probe is stronger compared to that of the CYP4A-fatty acid hydroxylase probe, despite the fact that the CYP4B subfamily and some isoforms of the CYP4F subfamily also hybridize to the latter probe. The estimated size of the hybridization positive band is 2.5 kb, which is consistent with the size of CYP4A21 mRNA. A low amount of CYP4A fatty acid hydroxylase-derived sequences was also present in the N2-R:FEL
fragment from liver relative to kidney, as shown by the PstI digest described above (Fig 10).

![Northern blot analysis of total RNA isolated from livers of eight pigs of varying ages. A [32P]-labeled CYP4A21-specific oligo-probe and a [32P]-labeled oligo-probe specific for CYP4 fatty acid hydroxylases, respectively, were used. Sample loading was assessed by the intensity of the ribosomal 18S on the blotted filter as shown in Paper IV.](image)

A comparison of the expression of CYP8B1 and CYP4A21 in fetal pig liver and livers from young pigs was made to investigate whether the expression of mRNA for these enzymes correlated with reported developmental-dependent formation of cholic acid and hyocholic acid, respectively. A cDNA probe of pig CYP8B1 and a CYP4A21-specific oligo probe were used. The CYP8B1 probe hybridized solely to the fetal samples whereas the CYP4A21 specific probe showed an opposite pattern, as can be seen in Fig. 14. These results are in agreement with the presence of cholic acid in fetal pig bile whereas hyocholic acid predominates in the neonatal period and thereafter. The estimated size of the bands detected by the CYP4A21-specific oligo is 2.5 kb which agrees with the size of CYP4A21 mRNA. The CYP8B1 probe hybridized to a position of approximately 4.3 kb, which is similar to the reported size of human CYP8B1 mRNA (91).

![Northern blot analysis of total RNA isolated from livers of fetal pigs and young pigs. A [32P]-labeled CYP8B1 cDNA probe and a [32P]-labeled CYP4A21-specific oligo probe, respectively, were used. Sample loading was assessed by the intensity of the ribosomal 18S on the blotted filter as shown in Paper III.](image)
RT-PCR with total RNA from fetal and human livers using CYP8B1 specific primers showed a low expression of CYP8B1 in fetal liver and a pronounced expression in adult liver, as shown in Paper III. Thus an opposite age-dependent pattern of CYP8B1 expression is seen in human liver compared to pig liver.

The CYP4A21 gene and comparison with other CYP4A genes. Paper IV

To get more information on the relationship between CYP4A21 and CYP4A fatty acid hydroxylases, the gene encoding CYP4A21 was isolated and sequenced. This was accomplished by screening a porcine genomic library and also by genome walking. Due to the high sequence identity between CYP4A21 and pig CYP4A fatty acid hydroxylases, a clone encoding CYP4A24 was also found among the hybridization positive recombinant phages during screening of the porcine genomic library. The identification of CYP4A21 and CYP4A fatty acid hydroxylases among positive clones was accomplished by PCR using the specific primers as described in paper IV. Gene regulation described in terms of cis-acting promoter elements and trans-acting transcription factors is presently a hot topic in the search to understand the molecular events governing gene expression. In an attempt to obtain the promoter region of CYP4A21, a genome walking procedure was undertaken in parallel with screening of the genomic library.

By compilation of the sequences contained in the two recombinant CYP4A21 clones found by library screening and the upstream sequence obtained by genome walking, a near full-length sequence of CYP4A21 was obtained. About 3000 bp in the first intron and adjacent nucleotides in the first and second exons are missing. The organization of the CYP4A21 gene was found to be extraordinarily conserved when compared with CYP4A genes in other species, in spite of the differences in catalytic activity. The CYP4A21 gene is about 12 kb and split into 12 exons as shown in Fig. 15. This is similar to CYP4A genes in other species with the exception for rat CYP4A1 and rabbit CYP4A6 where the 3' untranslated region is split by a 12th intron, yielding a total of 13 exons (67,69). A sequence spanning from the second exon to the ninth exon was obtained from the CYP4A24 clone (Fig 15). The degree of sequence identity shown by the two pig CYP4A genome sequences is exceptionally high and revealed an homology that is not confined to the exon sequences but extends into the introns. Recently, the genomic sequences of two human CYP4A genes, CYP4A11 and CYP4A22, were shown to have similar high overall identity (71). The high sequence identity found between CYP4A21 and CYP4A24 within introns is also seen in the proximal promoter region obtained by Genome Walking, as indicated in Fig 15.

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Similar to what has been reported for other CYP4A genes there is no consensus TATA-box upstream of the transcription start site. Using the Transcription Element Search Software (TESS) (http://www.cbil.upenn.edu/) multiple binding sites for the ubiquitous mammalian transcription factor, specificity protein 1 (Sp1) was identified within a 100 bp region upstream of the transcription start site. Promoters of TATA-less genes that exhibit this feature are most commonly housekeeping genes (168), i.e. genes whose products are required in all cells at low levels. Sp1 sites in the promoter of the housekeeping gene dihydroporpholate reductase (Dhfr) has been proposed to be a platform for assembling of the basal transcription machinery (169). Multiple Sp1-binding sites are present also in CYP4A genes from other species. Could it be that the CYP4A enzymes present today are descendants from an ancestral housekeeping gene? If so, the CYP4A subfamily might have maintained properties of regulation resembling that of a housekeeping gene, with specific transcription factors for conferring the tissue-, and isozyme-specific regulation. The high sequence identity (close to 100%) between the 5’ flanking sequences of CYP4A21 and CYP4A24, hampered the identification of cis-acting elements involved in gene-specific regulation. A difference in expression shown by Northern blot indicates that sequences further upstream are involved in their different pattern of expression.

The conclusion drawn from the conserved genome organization and overall sequence similarity is that CYP4A21 and the CYP4A fatty acid hydroxylases have a common origin and evolved by gene duplication. The classical model for origin of new gene functions by gene duplication proposes that one copy of a particular gene maintains the original function while the other copy can accumulate mutations for further evolution of new functions (170). It appears feasible that the evolution
CYP4A21 applies to this model, especially since selection has been demonstrated to play a significant role in the process. Domestication of the pig is believed to have occurred some 5000 years ago (171). It is conceivable that CYP4A21 and the ability to form hyocholic acid in pig have provided a selective advantage as this gene is fixed in the genome and not silenced as a null allele, which is a more common fate of duplicated genes (172).
Summary and conclusions

The porcine taurochenodeoxycholic 6α-hydroxylase was cloned and found to be an atypical member of the CYP4A subfamily. The primary structure of this porcine enzyme, designated CYP4A21, shows about 75% overall sequence identity with members of the CYP4A subfamily expressed in rabbit and man. The substrate specificity is, however, clearly different from that of other CYP4A enzymes. A signature sequence, common to all CYP4A enzymes in the middle of the long I-Helix, was found to contain deviations in CYP4A21. Studies using a mutant of CYP4A21, containing nucleotides encoding the signature sequence, indicate that residues in this region are of importance for the 6α-hydroxylase activity.

Investigations performed to verify the deviation in the otherwise conserved signature motif resulted in cloning of two homologous porcine CYP4A fatty acid hydroxylases, designated CYP4A24 and CYP4A25, showing ω- and (ω-1)-hydroxylase activities towards lauric acid and palmitic acid. Whereas CYP4A21 clearly deviates regarding structural features and catalytic activity from the two porcine CYP4A fatty acid hydroxylases, it is more difficult to establish whether CYP4A24 and CYP4A25 are products of distinct genes or merely allelic variants of one gene. The conserved genome organization of CYP4A21 and an extensive sequence identity to CYP4A24, also within introns, indicate that CYP4A21 and the CYP4A fatty acid hydroxylases have a common origin and evolved by gene duplication.

The key enzyme in cholic acid biosynthesis, the sterol 12α-hydroxylase (CYP8B1) was found to be expressed in fetal pig liver. This is consistent with a reported presence of cholic acid in pig fetal bile. An apparently conserved porcine gene encoding CYP8B1 was cloned. Similar to CYP8B1 genes from other species the porcine gene was devoid of introns. Among available CYP8B1 gene sequences, the pig gene is most similar to human CYP8B1 both regarding the encoded sequence (82% amino acid identity) and within the proximal 460 bp of the putative promoter region (70% nucleotide identity). Unlike other CYP8B1 genes the pig gene lacks a canonical TATA-box in the promoter region which could offer one explanation for the unusual expression pattern, which appear to be restricted to fetal pig life.

In adult pigs, hyocholic acid generated by CYP4A21 can fulfill the requirement for trihydroxy bile acids in the absence of cholic acid. Even though cholic acid and hyocholic acid both are trihydroxy bile acids, the position of the third hydroxyl group (12α and 6α position, respectively) has a profound effect on the hydrophobic-hydrophilic property of these bile acids. The presence of hyocholic acid in pig bile, instead of cholic acid, will not merely fulfill a basic requirement for trihydroxy bile acids but will probably also have an impact on cholesterol and lipid metabolism.
Dietary habits have been proposed as a factor governing evolution of P450 enzymes. There is also an association between mammalian bile salt type and the diet. Even though enzymes involved in bile acid biosynthesis are highly conserved among species, the composition of circulating bile acids varies, indicating a species-specific adaptation on the level of gene regulation. The lack of a consensus TATA-box in the pig CYP8B1 promoter is a distinct feature among CYP8B1 genes, which could contribute to the unusual expression pattern of the gene in this species. The lack of a consensus TATA-box is, on the other hand, common to all CYP4A genes hitherto cloned. Despite the overall homology between CYP4A21 and CYP4A24 genes, including the proximal promoter region, a distinct pattern of expression of these genes was shown. CYP4A21 appears to be more or less constitutively expressed in pig liver with a concomitant low basal expression of CYP4A fatty acid hydroxylases. These genes could aid in future studies to elucidate the principles for expression of the TATA-less CYP4A genes, and also contribute to our understanding of features governing the substrate specificity of CYP4A enzymes. Together, porcine CYP4A21 and the CYP8B1 genes could serve as tools for studies of factors contributing to developmental-dependent gene expression.
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