Hormonal Regulation of the Human CYP27A1 and CYP7B1 Genes

WANJIN TANG
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Abstract

CYP27A1 and CYP7B1 are widely expressed in various human tissues and are key enzymes involved in the pathways for conversion of cholesterol to bile acids. Also, CYP27A1 is involved in bioactivation of vitamin D3 and CYP7B1 plays a role in 7alpha-hydroxylation of dehydroepiandrosterone and other steroids. Both enzymes have been reported to be relevant to prostate cell proliferation. The current study examines the hormonal regulation of CYP27A1 and CYP7B1.

CYP7B1 was shown to be regulated by estrogens and androgens in human embryonic kidney HEK293 and prostate cancer LNCaP cells. Quantitation of CYP7B1 mRNA in adult and fetal human tissues showed markedly higher CYP7B1 mRNA levels in fetal tissues compared with the corresponding adult ones, except in the liver. This indicates a tissue-specific, developmental regulation of CYP7B1 and suggests an important function for this enzyme in fetal life. CYP7B1 regulation by estrogens may be of importance in fetal development and in other processes where CYP7B1 is involved, including cholesterol homeostasis, cellular proliferation, and CNS function. The regulation of CYP7B1 by sex hormones also suggests an important role for CYP7B1 in balancing prostate hormone levels in human cells.

Results show that CYP27A1 can be regulated by dexamethasone, growth hormone, IGF-1, PMA, estrogens and androgens in liver-derived HepG2 cells. Dexamethasone, growth hormone and IGF-1 stimulated the promoter and endogenous activity of CYP27A1, whereas thyroid hormones and PMA inhibited CYP27A1. The regulatory effects of estrogens and androgens are different depending on the cell types. Thus, the results imply that human CYP27A1 gene is a target for estrogens and androgens, and the expression of CYP27A1 may be affected by endogenous sex hormones and pharmaco logical compounds with estrogenic or androgenic effects.

The mechanism for the dexamethasone-induced effect on the human CYP27A1 promoter was examined. A GRE was identified important for GR-mediated regulation of CYP27A1 transcriptional activity.

Keywords: CYP27A1, CYP7B1, cholesterol, estrogen, androgen, prostate, glucocorticoid receptor, fetal development

Wanjin Tang, Department of Pharmaceutical Biosciences, Box 591, Uppsala University, SE-75124 Uppsala, Sweden

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To my late grandparents
List of Papers

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

I  Araya, Z., Tang, W., and Wikvall, K
Hormonal regulation of the human sterol 27-hydroxylase gene (CYP27A1).

II  Tang, W., Eggertsen, G., Chiang, J.Y.L., and Norlin, M.
Estrogen-mediated regulation of CYP7B1: a possible role for controlling DHEA levels in human tissues.

III  Tang, W., and Norlin, M.
Regulation of steroid hydroxylase CYP7B1 by androgens and estrogens in prostate cancer LNCaP cells.

IV  Tang, W., Norlin, M., and Wikvall, K.
Regulation of human CYP27A1 by estrogens and androgens in HepG2 and prostate cells.

V  Tang, W., Norlin, M., and Wikvall, K.
Manuscript

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Contents

Introduction .................................................................................. 11
Cholesterol homeostasis and bile acid biosynthesis ............................. 11
Cytochrome P450 monooxygenase system ........................................ 11
Steroid hormones and their receptors ............................................... 13
Estrogens ....................................................................................... 15
Androgens ...................................................................................... 16
Glucocorticoids .............................................................................. 16
Prostate .......................................................................................... 17
Sterol 27-hydroxylase (CYP27A1) .................................................... 17
CYP27A1, sex hormones and prostate ............................................. 18
Oxysterol 7α-hydroxylase (CYP7B1) and DHEA ................................. 18
CYP7B1, sex hormones and prostate .............................................. 19
Previous studies on the regulation of CYP27A1 ............................... 20
Previous studies on the regulation of CYP7B1 ................................. 20

Aims of the Present Investigation .................................................. 22

Experimental Procedures .............................................................. 23
Materials ......................................................................................... 23
Cell culture ...................................................................................... 23
Transient transfection ................................................................. 24
Promoter-luciferase reporter assay ................................................. 24
Quantitation of CYP7B1 and CYP27A1 mRNA .............................. 24
Assay of CYP7B1 enzyme activity ............................................... 25
Assay of CYP27A1 enzyme activity ............................................. 25
Electrophoretic mobility shift assay ............................................. 26
Site-directed mutagenesis ............................................................ 26
Statistical analysis ......................................................................... 26
Other methods ................................................................................ 26

Results and Discussion .................................................................. 28
CYP7B1 ......................................................................................... 28
Human CYP7B1 expression is age-dependent and tissue-specific (Paper II) . 28
Estrogens regulate CYP7B1 (Paper II, III) ...................................... 29
E2 strongly stimulates CYP7B1 promoter in the presence of ERα in HEK293 cells ................................................................. 29
E2 increases CYP7B1 mRNA levels in the presence of ERα in HEK293 cells ................................................................. 29
E2 stimulates CYP7B1 activity in the presence of ERα in HEK293 cells ................................................................. 29
Implication of estrogen regulation on CYP7B1 in the presence of ERα ................................................................. 30
E2 affects CYP7B1 enzyme activity in the absence of ERα in HEK293 cells ................................................................. 31
Implication of estrogen effect on CYP7B1 in the absence of ERα ................................................................. 31
Overexpression of ERα increases CYP7B1 promoter activity in LNCaP cells (Paper III) ................................................................. 31
Adiol and E2 suppress CYP7B1 promoter activity in LNCaP cells (Paper III) .................................................................32

Androgens Regulate CYP7B1 (Paper III) .................................................. 32
DHT suppresses CYP7B1 promoter activity and CYP7B1-mediated catalytic activity in HEK293 cells .................................................................32
DHT suppresses CYP7B1 promoter activity in LNCaP cells .......................33
Possible implications of hormonal regulation of CYP7B1 for prostate proliferation .................................................................33

CYP27A1......................................................................................................... 34

PMA, dexamethasone, thyroid hormones, growth hormone and IGF-1 regulate CYP27A1 (Paper I)................................................................. 34
Growth hormone, IGF-1 and dexamethasone stimulate the human CYP27A1 promoter activity in HepG2 cells .................................................34
Thyroid hormones and PMA repress the human CYP27A1 promoter activity in HepG2 cells .................................................................35
Effects of PMA, dexamethasone, thyroid hormones, growth hormone and IGF-1 on the endogenous CYP27A1 activity in HepG2 cells ..........35
Experiments with progressive deletion constructs of the human CYP27A1 promoter .................................................................36

Estrogens and androgens regulate CYP27A1 (Paper IV) ......................... 36
Estrogens and androgens affect CYP27A1 promoter activity in HepG2 cells .............................................................................36
Estrogens and androgens affect endogenous CYP27A1 enzyme activity and mRNA levels in HepG2 cells .................................................36
Effects of estrogens and androgens on CYP27A1 promoter activity in human prostate cells .................................................................37
Experiments with deletion constructs of the CYP27A1 promoter ..........37
Sequence analysis of the human CYP27A1 5’-flanking sequence ..............38
Biological significance of regulation of CYP27A1 by sex hormones .........40

Dexamethasone-induced effect on the CYP27A1 promoter is mediated via GR (Paper V) ................................................................. 41
Mifepristone abolishes the dexamethasone-induced effect on CYP27A1 promoter activity .................................................................41
Deletion construct analysis of GR-mediated effects on the CYP27A1 promoter .................................................................42
Identification of GR-binding sites in the CYP27A1 promoter .................42
Site-directed mutagenesis of sequences in the CYP27A1 promoter ...........42
Mechanism and biological role of glucocorticoid-mediated induction of CYP27A1 transcription .........................................................42

Summary and Conclusions ....................................................................44

Acknowledgements ..............................................................................46

References ............................................................................................48
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>3βAdiol</td>
<td>5α-Androstan-3β,17β-diol</td>
</tr>
<tr>
<td>27-hydroxycholesterol</td>
<td>5α-Cholestene-3β,27-diol</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P 450</td>
</tr>
<tr>
<td>DHT</td>
<td>5α-dihydrotestosterone</td>
</tr>
<tr>
<td>DHEA</td>
<td>Dehydroepiandrosterone</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>DPN</td>
<td>2,3-bis(4-Hydroxyphenyl)-propionitrile</td>
</tr>
<tr>
<td>E2</td>
<td>17β-estradiol</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ERE</td>
<td>Estrogen response element</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>GRE</td>
<td>Glucocorticoid response element</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>HRE</td>
<td>Hormone response element</td>
</tr>
<tr>
<td>MR</td>
<td>Mineralocorticoid receptor</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PPT</td>
<td>4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>PRE</td>
<td>Progestrone response element</td>
</tr>
<tr>
<td>RAR, RXR</td>
<td>Retinoic acids receptor</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative light units</td>
</tr>
<tr>
<td>TR</td>
<td>Thyroid hormone receptor</td>
</tr>
<tr>
<td>TRE</td>
<td>Thyroid hormone response element</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
</tr>
</tbody>
</table>
Introduction

Cholesterol homeostasis and bile acid biosynthesis

Cholesterol is an indispensable component of all cellular and intracellular membrane structures. Approximately 50-75% of the total cholesterol is synthesized endogenously, most of which is formed in the liver. It can be metabolized to various products such as bile acids and steroid hormones. (Norlin and Wikvall, 2007). Cholesterol is mainly removed from the body by the conversion into bile acids and the biliary excretion. Every day about 500 mg of cholesterol is converted into bile acids in the adult human liver. This bile acid production is 90% of the cholesterol that is actively metabolized in the body, and steroid hormone biosynthesis accounts for the remaining 10%. Newly synthesized bile acids are secreted into the bile and delivered to the lumen of the small intestine where they are emulsifiers of dietary lipids, cholesterol, and fat-soluble vitamins. The solubilized nutrients bind to lipoproteins, which are delivered to the liver and metabolized. Bile acids are transported back from the intestine to the liver via the portal circulation and then resecreted into the bile. About 95% of bile acids are recovered in this way, and the lost 5% are replaced by new synthesis in the liver (Russell, 2003).

Cholesterol is metabolized into bile acids by pathways that involve about 17 different enzymes, many of which are preferentially expressed in the liver. There are two main pathways for the conversion of cholesterol into bile acids: the neutral/classic pathway and the acidic/alternative pathway (Fig. 1). There are also minor pathways such as via 24-hydroxylation of cholesterol or 25-hydroxylation of bile acid intermediates (Norlin and Wikvall, 2007). Bile acid synthesis is tightly regulated to maintain cholesterol homeostasis and to provide adequate emulsification in the intestine. When an organism is normal, excess bile acids suppress further synthesis, and conversely when bile acids are in short supply, synthesis is increased. Disturbance of cholesterol homeostasis and bile acid synthesis is related to serious diseases, for instance, atherosclerosis, Alzheimer's disease and various liver diseases. Thus, the regulation of the key enzymes in those two pathways is of great importance for understanding the mechanism of these diseases. These key enzymes may also be drug targets for curing these diseases.

Cytochrome P450 monoxygenase system

Cytochrome P450 is a cellular chromophore. It was first named in 1961 because when it is reduced and bound to carbon monoxide, the pigment (P) has an absor-
Figure 1. Biosynthesis of primary bile acids from cholesterol.

bance peak at 450-nm. Sequence comparisons indicated extensive similarity between cytochromes P450 identified in man and bacteria, and suggested that the superfamily originated from a common ancestral gene some three billion years ago (Nebert and Gonzalez, 1987).

Cytochrome P450 proteins are divided further into families and subfamilies on the basis of percentage amino acid sequence identity (Nebert and Gonzalez, 1987) (Nebert et al., 1987). Enzymes that share ≥40% identity are classified into a particular family designated by an Arabic numeral, whereas those sharing ≥55% identity make
Hormonal Regulation of the Human CYP27A1 and CYP7B1 Genes

up a particular subfamily designated by a letter. For example, in the CYP7 family, enzymes share ≥40% sequence identity. CYP7A and CYP7B are subfamilies, because their protein sequences are <55% identical. (If an additional enzyme was found to have ≥55% identity with the CYP7A, then it would be named CYP7A2, etc.)

Up till now, it is known that human beings have 57 CYP genes and 58 pseudogenes arranged into 18 families and 43 subfamilies (Nelson et al., 2004). These 57 genes code for enzymes that can be related to: metabolism of drugs and other foreign chemicals, cholesterol homeostasis, bile acid biosynthesis, steroid synthesis and metabolism, metabolism of arachidonic acid and eicosanoids, and vitamin D₃ synthesis and metabolism; retinoic acid hydroxylation; and some have still unknown function. Mutations in many CYP genes will result in inborn errors of metabolism and cause many diseases (Nebert and Russell, 2002).

Every Cytochrome P450 enzyme contains a single protein and one haem group. The haem group binds oxygen through electron transfer reactions from NADPH and this reaction finally incorporates one atom of oxygen into the substrate. The typical mono-oxygenation reaction catalysed by cytochrome P450 can be described as follows:

\[
P₄₅⁰\quad RH+NADPH+H^++O_2 \rightarrow NADP^−+H_2O+R-OH
\]

R represents a substrate, for instance, a steroid, fatty acid or compound with an alkene, alkane, aromatic ring or heterocyclic ring substituent that serves as a site for oxygenation (Chang and Kam, 1999).

First the substrate binds to the oxidized cytochrome P450. The formed complex is then reduced by an electron transferred from NADPH. For microsomal cytochrome P450 enzymes, this electron transfer reaction is catalyzed by NADPH-cytochrome P450 reductase. For mitochondrial enzymes, this reaction involves ferredoxin and ferredoxin reductase. Then after a series of reactions, the oxygen-oxygen bond will be broken, and one atom of oxygen is inserted into the substrate, the other atom will form H₂O. Finally the hydroxylated substrate and H₂O leave cytochrome P450.

Cytochrome P450 information is updated on the internet: http://drnelson.utmem.edu/CytochromeP450.html

Steroid hormones and their receptors

Steroid hormones include glucocorticoids, mineralocorticoids, progesterone, estrogens and androgens. They are mainly produced in the adrenals, gonads, placenta and nervous system. Other tissues might also synthesize some though in much smaller quantities. The precursor for steroid hormones is cholesterol which is from three sources: de novo synthesis from acetate, pools of cholesterol esters in steroidogenic tissues and dietary sources. 80% of the cholesterol for hormone synthesis is from dietary sources. Most of the enzymes involved in steroid hormone synthesis are CYP450 enzymes (Gardner, 2007) (Fig. 4).

The receptors of steroid hormones, thyroid hormones and vitamin D belong to the nuclear receptor superfamily. They have variable N-terminal and C-terminal domains, which are activation function (AF) domains. There is a central DNA-binding domain (DBD), which targets the receptor to specific DNA sequence: hormone response ele-
Wanjin Tang

ment, in the target gene promoter. A hinge domain is in the middle. The C-terminal half of the receptor contains the ligand binding domain (LBD), which is for hormone recognition (Fig. 2). After being activated, these receptors can regulate target gene transcription by binding to their corresponding hormone response element in the target gene promoter region. (Mangelsdorf et al., 1995) (Fig. 3).

![NH3 AF-1 DBD Hinge LBD AF-2 COOH](image)

*Figure 2. Structural motifs of classical nuclear receptors.*

Some receptors can also be activated by interacting with other proteins which will then bind to the corresponding response elements in the promoter and modulate gene transcription. For example, ERs can interact with the fos/jun transcription factor complex on AP1 site to affect gene transcription. Besides AP1, ERs can also interact with the Sp1 and NF-kB proteins (Nilsson et al., 2001).

Non-genomic actions are a common property of steroid hormone receptors. These actions usually involve various protein-kinase cascades. It has been suggested that there are ERs existing in the plasma membrane which can affect gene transcription through non-genomic action. The possible convergence of genomic and nongenomic actions of estrogen can finely regulate gene expression (Björnström and Sjöberg, 2005).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Element</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR, MR, PR, AR, ER</td>
<td>HRE</td>
<td>AGAACANNTGTCT</td>
</tr>
<tr>
<td>ER</td>
<td>ERE</td>
<td>AGGTCANNTGACCT</td>
</tr>
</tbody>
</table>

*Figure 3. Response elements for main nuclear hormone receptors. The TRE is written as a direct repeat, but may also exist as a palindrome or an inverted palindrome. n = 3, 4, 5 are preferred for binding of the VDR, TR or RAR/RXR, respectively.*
Hormonal Regulation of the Human CYP27A1 and CYP7B1 Genes

Figure 4. Pathways of synthesis of steroid hormones and the active form of vitamin D3.

Estrogens

Estrogens are named for their importance in the estrous cycle, and functioning as the primary female sex hormone. Estrogens are used as part of some oral contraceptives and also in estrogen replacement therapy of postmenopausal women. Like other steroid hormones, estrogens readily diffuse across the cell membrane; inside the cell, they interact with ERs (Nussey, 2001).

Estrogens are produced mainly by developing follicles in the ovaries, the corpus luteum, and the placenta. Estrogens promote the development of female secondary sex characteristics and are also involved in the thickening of the endometrium and other aspects of regulating the menstrual cycle. In males estrogen regulates certain functions of the reproductive system important to the maturation of sperm (Hess et al., 1997). Estrogens also influence cell proliferation e.g. in neurogenesis (Galea et al., 2006).

Xenoestrogens are synthetic substances that also possess estrogenic activity (Fang et al., 2001). Phytoestrogens are natural plant products with estrogenic activity.

Selective estrogen receptor modulators (SERM) are a class of compounds that act on the ER. A characteristic that distinguishes these substances from receptor agonists and antagonists is that their action is different for various tissues, thereby granting the possibility to selectively inhibit or stimulate estrogen-like action in various tissues, such as tamoxifen and raloxifene. They act as agonists of ER in one tissue and antagonists in another. Tamoxifen and toremifen are used for treating breast cancer, and raloxifene is mainly for prevention and treatment of osteoporosis. Designing a compound that acts in only selected tissues is one of the goals of SERM development.
Androgens

Androgens are steroid hormones, that stimulate or control the development and maintenance of masculine characteristics in vertebrates by binding to ARs. This includes the activity of the accessory male sex organs and development of male secondary sex characteristics. Androgens, which were first discovered in 1936, are also called androgenic hormones or testoids. Androgens are also the original anabolic steroids. They are also the precursor of all estrogens, the female sex hormones. The primary and most well-known androgen is testosterone. Dihydrotestosterone (DHT), a metabolite of testosterone, is a more potent androgen in that it binds more strongly to ARs.

Recent results show that androgens inhibit the ability of some fat cells to store lipids by blocking a signal transduction pathway that normally supports adipocyte function (Singh et al., 2006). Androgens also promote the enlargement of skeletal muscle cells and probably act in a coordinated manner to enhance muscle function by acting on several cell types in skeletal muscle tissue (Sinha-Hikim et al., 2004).

Circulating levels of androgens can influence human behavior because some neurons are sensitive to steroid hormones. Androgen levels have been implicated in the regulation of human aggression (Giammanco et al., 2005) and libido.

Glucocorticoids

Glucocorticoids are a class of steroid hormones. They exert their effects via interaction with their cognate receptor. Glucocorticoids are distinguished from mineralocorticoids and sex steroids by the specific receptors, target cells, and effects. Technically, the term corticosteroid refers to both glucocorticoids and mineralocorticoids, but is often used as a synonym for glucocorticoid.

Cortisol (or hydrocortisone) is the most important human glucocorticoid. It is essential for life and regulates or supports a variety of important cardiovascular, metabolic, immunologic, and homeostatic functions. Glucocorticoid receptor (GR) is found in the cells of almost all vertebrate tissues.

Glucocorticoids have potent anti-inflammatory and immunosuppressive properties. This is particularly evident when they are administered at pharmacological doses, but also is important in normal immune responses. Thus, glucocorticoids are widely used as drugs to treat inflammatory conditions such as rheumatoid arthritis, inflammatory bowel disease, and asthma and dermatitis. They are also prescribed in combination therapy for treatment of certain malignancies primarily due to their role in immune cell apoptosis and for their ability to palliate the side effects resulting from other chemothapeutic agents (Smoak and Cidlowski, 2004).

Chronic glucocorticoid excess leads to Cushing’s syndrome. Clinical features are obesity, skin changes, hirsutism, hypertension, gonadal dysfunction, central nervous system and psychologic disturbances, muscle weakness and osteoporosis.

Dexamethasone is a potent synthetic member of the glucocorticoids. It is used as anti-inflammatory or immunosuppressive agent. As a glucocorticoid, dexamethasone is roughly 20—30 times more potent than hydrocortisone. It is one of the longest acting corticosteroids used in clinical medicine.
Prostate

Prostate is the organ most commonly afflicted with either benign or malignant neoplasms. AR is expressed in this organ, both at the epithelium and stroma. Androgens play a major role in normal prostate development, benign prostatic hyperplasia (BPH) and in established prostate cancer. Major circulating androgen is testosterone. DHT predominates in prostate and binds to AR with greater affinity and lower dissociation constant than does testosterone. Consequently, DHT is the most potent intraprostatic androgen (Parnes et al., 2005). ERα is localized in the prostatic stroma and ERβ is located in the epithelium in most species. It has been reported that ERβ expression is often altered in diseased prostate. 3β-Adiol is an important ligand for ERβ in the prostate (Weihua et al., 2002b).

Prostate cancer is the most common tumor diagnosed in the western countries. It is one of the leading causes of cancer deaths in men. By early detection and improved local therapies, a large number of men will be cured, but unfortunately, a significant number of men will experience relapse of disease and require continued surveillance and ongoing therapy (Walczak and Carducci, 2007). Prostate cancer develops most frequently in men over fifty. Many factors, such as genetics and diets, have been implicated in the development of prostate cancer. Prostate cancer is most often discovered by physical examination or by screening blood tests, for instance, the PSA (prostate specific antigen) test. Prostate cancer can be treated with surgery, radiation therapy, hormone therapy, occasionally chemotherapy, or some combination of these. Hormonal therapy uses medications or surgery to block prostate cancer cells from getting DHT, a hormone produced in the prostate and required for the growth and spread of most prostate cancer cells.

Sterol 27-hydroxylase (CYP27A1)

CYP27A1 (sterol 27-hydroxylase, P450c27, mitochondrial vitamin D₃ 25-hydroxylase) is a multifunctional mitochondrial P450 enzyme. It is present in many human body tissues such as liver, kidney, lung, macrophages, brain, vascular endothelium, skin and prostate (Gascon-Barre et al., 2001; Norlin and Wikvall, 2007; Quinn et al., 2005). It catalyzes hydroxylations in both the neutral and acidic bile acid biosynthesis pathways. In the neutral pathway, CYP27A1 initiates degradation of the C₂₇-steroid side chain. In the acidic pathway, it catalyzes the initial 27-hydroxylation of cholesterol (Vlahcevic et al., 1997). CYP27A1 is also involved in the bioactivation of vitamin D₃ (Sawada et al., 2000). It catalyzes the 25-hydroxylation of vitamin D₃ (Hosseinpour and Wikvall, 2000; Sawada et al., 2000). CYP27A1 expressed in extrahepatic tissues appears to have multiple functions, such as in cholesterol metabolism and transport and in the formation of active vitamin D metabolites (Babiker et al., 1997; Gascon-Barre et al., 2001; Quinn et al., 2005; Theodoropoulos et al., 2001). CYP27A1 is reported to have anti-atherogenic effects and play an important role in the regulation of cholesterol homeostasis by formation of oxysterols and elimination of cholesterol from cells (Babiker et al., 1997; Quinn et al., 2005). The active forms of vitamin D₃, formed by CYP27A1, are found to have antiproliferative and pro-differentiating effects on a variety of malignant cells, such as human prostate cancer cell lines (Chen and Holick, 2003; Tokar and Webber, 2005b; Tuohimaa et al., 2005).
In addition, a major biologic role of CYP27A1 is believed to be the generation of ligands such as 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid, which activate nuclear receptors regulating the expression of genes that govern many aspects of cholesterol homeostasis in various cells (Björkhem et al., 2002) (Norlin and Wikvall, 2007; Russell, 2000). 27-Hydroxycholesterol formed by CYP27A1 suppresses, via SREBP, the rate-limiting enzyme in cholesterol biosynthesis, HMG-CoA reductase (Chiang, 2002; Schroepfer, 2000). There are several possible mechanisms in which CYP27A1 could play important roles for elimination of cholesterol from extrahepatic cells. 27-Hydroxycholesterol upregulates the genes for ABC-transport proteins in e.g. macrophages and thus stimulates the transport of cholesterol from the cell via reverse (HDL) cholesterol transport (Fu et al., 2001). Also, 27-hydroxycholesterol and other 27-oxygenated products formed by CYP27A1 are excreted from the cell more efficiently than cholesterol (Babiker et al., 1997) (Björkhem et al., 1994). 27-Oxygenated products formed in extrahepatic tissues can be transported to the liver and eliminated through conversion to bile acids.

Thus, CYP27A1 and its gene may be potential targets for drug development. The regulation of the human CYP27A1 gene, such as by hormones, is of interest not only in therapy but also in understanding development of diseases related to disturbances in cholesterol homeostasis and vitamin D metabolism. Hormones like pituitary growth hormone, thyroid hormones and the protein kinase C stimulator phorbol 12-myristate 13-acetate (PMA) are known to regulate some enzymes in cholesterol metabolism such as the cholesterol 7α-hydroxylase (CYP7A1). Thyroid hormones and PMA have been reported to repress the promoter activity of the human CYP7A1 gene (Drover et al., 2002; Wang et al., 1996). In previous studies, there is no information about the effects of these hormones or PMA on the activity of the human CYP27A1 gene promoter or the CYP27A1 enzyme activity.

CYP27A1, sex hormones and prostate

It has been suggested that vitamin D₃ inhibits growth and invasion by up-regulating CYP27A1 in human prostate cancer cells (Tokar and Webber, 2005a; Tokar and Webber, 2005b). Treatment of prostate cancer cells with vitamin D₃ increases cell differentiation and apoptosis and decreases proliferation (Chen and Holick, 2003) (Tuohimaa et al., 2005). These effects have been linked to CYP27A1 in prostate cells since this enzyme is involved in formation of the active vitamin D₃ metabolites 25-hydroxyvitamin D₃ and 1,25-dihydroxyvitamin D₃ (Tokar and Webber, 2005a; Tokar and Webber, 2005b).

Androgens and estrogens are known to influence cell proliferation in prostate development and growth (Galea et al., 2006; WeiHua et al., 2002a). There are no previous data reported on the possible regulation of the human CYP27A1 gene by estrogen or androgen at promoter level.

Oxysterol 7α-hydroxylase (CYP7B1) and DHEA

CYP7B1 is widely expressed in human tissues. It catalyses 7α-hydroxylation of sev-
eral steroid substrates, including cholesterol derivatives, neurosteroids and steroids involved in hormonal signalling (Norlin and Wikvall, 1998; Rose et al., 1997; Schwarz et al., 1997; Weihua et al., 2002a; Vlahcevic et al., 1999; Wu et al., 1999). The steroid hormone precursors dehydroepiandrosterone (DHEA) and pregnenolone, and the oxysterols 25-hydroxycholesterol and 27-hydroxycholesterol are well-known substrates for CYP7B1.

High CYP7B1 mRNA levels are reported in human brain, particularly in the hippocampus, the brain region considered most important for memory and learning (Rose et al., 2001; Yau et al., 2006; Yau et al., 2003). It is reported that CYP7B1 mRNA levels in brains from patients with Alzheimer’s disease were decreased (Yau et al., 2003). DHEA and pregnenolone, which are produced locally in the brain and have been termed neurosteroids, are believed to exert multiple effects in the CNS (Wolf and Kirschbaum, 1999). Several studies, most of which have been performed with rodents, suggest that these steroids may modulate neuronal transmission and synaptic plasticity and affect GABA and NMDA receptors (Wolf and Kirschbaum, 1999). Other suggested functions for DHEA and/or 7α-hydroxy-DHEA include immunostimulating properties (Dulos et al., 2005).

CYP7B1 displays high catalytic activity towards DHEA. Mainly produced in the adrenals, DHEA is an important precursor for androgens and estrogens (Pepe and Albrecht, 1995; Rainey et al., 2004). Large amounts of DHEA and DHEA-sulfate, its sulfated ester, are secreted from the adrenal gland into the circulation (Rainey et al., 2002). CYP7B1-mediated 7α-hydroxylation of DHEA results in a different metabolic fate for these steroids, which does not lead to formation of sex steroids.

CYP7B1, sex hormones and prostate

In recent years it has been proposed that CYP7B1-mediated metabolism may play an important role for growth of the prostate and other tissues via effects on ligands of ERβ, which modulates cellular proliferation and differentiation (Dupont et al., 2000; Jakobsson et al., 2004; Martin et al., 2004; Omoto et al., 2005; Pak et al., 2005; Weihua et al., 2002a).

Growth, differentiation and function of the prostate are strongly influenced by androgens which mediate their effects through AR (Brinkmann et al., 1999; Cude et al., 1999; Cunha et al., 2004). DHT is believed to play an essential role for prostate function and growth, both in normal and disease conditions. Interestingly, DHT is enzymatically converted in the prostate to 3βAdiol, an estrogenic compound (Weihua et al., 2002a). 3βAdiol is present in much higher levels than E2 in human prostate and is reported to be a major intraprostatic estrogen (Weihua et al., 2002a; Voigt and Bartsch, 1986). Thus the most potent prostate androgen is metabolized in the prostate to a compound with estrogenic effect.

In studies using strains of knockout mice, Weihua et al. (Weihua et al., 2002a) observed that the prostates of CYP7B1-/- mice are hypoproliferative and that treatment with 3βAdiol, a ligand of ERβ and a substrate for CYP7B1, decreases proliferation in wild-type but not in ERβ-/- mice. From these findings the authors proposed a pathway for control of prostate growth involving ERβ, 3βAdiol and CYP7B1. According to this pathway CYP7B1 regulates the function of ERβ by metabolizing its
ligand 3βAdiol and thereby counteracts anti-proliferative action of ERβ. In this fashion, a balance between hormonal signalling via ER and AR is maintained. CYP7B1 may play a key role in the maintenance of this balance.

Figure 5. CYP 7B1 is involved in an endocrine pathway that regulates growth of the rodent ventral prostate. - Modified from Weihua et al. [Proc. Natl. Acad. Sci. U S A 99(2002)13589-13594]

Previous studies on the regulation of CYP27A1

CYP27A1 activity and mRNA levels are decreased by bile acids in the rat, but unaffected in the rabbit and mouse (Vlahcevic et al., 1996). A coordinate regulation of sterol 27-hydroxylase (CYP27A1) and cholesterol 7α-hydroxylase (CYP7A1) by bile acids in rats has been reported (Vlahcevic et al., 1997). However, in humans, there appears to be little or no coordinate regulation of CYP7A1 and CYP27A1 at the transcriptional level, and human CYP27A1 is not subject to a negative feedback control by bile acids (Björkhem et al., 2002). Cholesterol stimulates sterol 27-hydroxylase levels in the rabbit (Norlin and Wikvall, 2007). It was reported that glucocorticoids, such as dexamethasone, probably increase post-transcriptionally sterol 27-hydroxylase mRNA in rat hepatocytes. It was suggested that dexamethasone also increases its protein mass and decreases its rate of protein degradation (Stravitz et al., 1996). Another report indicated that dexamethasone stimulates transcriptional activity of the human CYP27A1 gene (Segev et al., 2001).

Previous studies on the regulation of CYP7B1

There were some reports about the regulation of CYP7B1 before I started work on my thesis. Sp1 was suggested to play a role in the basal transcription of CYP7B1 (Wu
Hormonal Regulation of the Human CYP27A1 and CYP7B1 Genes

and Chiang, 2001; Wu et al., 1999). CYP7B1 transcription was suppressed by sterol response element binding protein (SREBP), indicating a link between oxysterol-sensitive regulators and oxysterol metabolism (Norlin and Chiang, 2004). Rodent CYP7B1 expression has been shown to be affected by bile acids. CYP7B1 levels in rat hepatocytes are affected by cholesterol and squalestatin, a cholesterol synthesis inhibitor (Pandak et al., 2002). In the rat CYP7B1 and CYP7A1 were found to undergo diurnal variation, with peak and trough values for CYP7B1 lagging approximately 6 hours behind CYP7A1 (Ren et al., 2003). CYP7B1 levels were reported to be affected by thyroid hormones, glucocorticoids and cytokines (Norlin and Wikvall, 2007). An age-related expression pattern of CYP7B1 in the pig was reported in a study by Norlin (Norlin, 2002). This study showed marked age-dependent, tissue-specific variations in renal and hepatic CYP7B1 levels, suggesting that hormonal factors may be involved in the regulation of this enzyme (Norlin, 2002).
Aims of the Present Investigation

The aims of the present investigation were:

1. to study if hormones such as glucocorticoids, estrogens and androgens regulate human CYP27A1 and CYP7B1 genes in human tissues. If so, further aims are as follows

2. to study how different hormones regulate CYP27A1 and CYP7B1 in human tissues/cells, including liver for CYP27A1, embryonic kidney for CYP7B1 and prostate for both enzymes

3. to understand the implication of their regulation

4. to study the mechanisms of their regulation.
Hormonal Regulation of the Human CYP27A1 and CYP7B1 Genes

Experimental Procedures

Materials

27-Hydroxycholesterol, prepared from kryptogenin, was kindly provided by Dr. L. Tökes, Syntex, Palo Alto, CA, USA. Expression vectors containing murine ERβ (originally cloned by Dr. Jan-Åke Gustafsson, Sweden (Kuiper et al., 1996)) or ERα were gifts from Dr. Kenneth Korach, National Institute of Health, NC, USA. The expression vectors containing human ERα and ERβ (HEG0), respectively were generously provided by Dr. Pierre Chambon, Institut de génétique et de biologie moléculaire et cellulaire, Strasbourg, France. The pSV-AR expression plasmid containing human AR was a kind gift from Dr. A. Brinkmann, Erasmus Medical Centre, Rotterdam, the Netherlands. Empty pSV vector, used as control in experiments with AR, was prepared from the AR expression vector by digestion with SmaI, which removes most of the AR gene fragment, followed by religation of the vector. The expression plasmid containing human GR was kindly provided by Professor Ronald M. Evans, Howard Hughes Medical Institute, The Salk Institute, San Diego. CYP7B1 promoter-luciferase reporter gene constructs were generous gifts from Dr. John Chiang, Northeastern Ohio Univ. College of Medicine, Rootstown, USA and were generated as previously described (Wu and Chiang, 2001; Wu et al., 1999). The 4.2 kb DNA fragment of human CYP27A1 upstream of the translation-initiation codon was a generous gift from Professor Eran Leitersdorf (Hadassah University Hospital, Jerusalem, Israel). The CYP27A1-4.2kb-luciferase plasmid and three progressive deletion constructs of CYP27A1 promoter ligated in pGL2 vectors were prepared as described in Paper I. All remaining materials were purchased from commercial sources.

Cell culture

HEK293 cells and HepG2 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin and streptomycin. LNCaP cells were cultured in RPMI 1640 medium (Invitrogen no. 21875-034) supplemented with 10% (v/v) fetal bovine serum and antibiotics. RWPE-1 cells were grown in complete keratinocyte serum-free medium containing 50 µg/ml bovine pituitary extract and 5 ng/ml epidermal growth factor supplements and 1% antibiotics. Medium was changed every 72 h. When cells were 90% confluent, they were lifted by trypsin/EDTA and passaged.

Cells were treated with E2, DHT, 3βAdiol or other hormones dissolved in ethanol, dimethylsulphoxide (DMSO) or 45% (2-hydroxypropyl)-β-cyclodextrin. Some
Wanjin Tang

cells were starved of serum overnight or for several hours before hormone treatment. The same solvent volume was added to control cultures. Cells were cultured in 6-well tissue culture plates for assay of CYP7B1-mediated or CYP27A1-mediated catalytic activity and in 24-well plates for assay of reporter activity.

**Transient transfection**
In experiments to study the effects of ER, AR, GR on CYP7B1 or CYP27A1 activity, cells were transiently transfected with expression vectors containing ERα, ERβ, AR or GR using SuperFect transfection reagent (Qiagen), calcium coprecipitation or Lipofectamine 2000 reagent (Invitrogen). Control experiments were performed with empty vector to normalize the amounts of DNA transfected. The CYP7B1 reporter vector contained a DNA fragment spanning from -2771 to +189 of the human CYP7B1 promoter sequence (Wu and Chiang, 2001; Wu et al., 1999). Calcium coprecipitation was used for HEK293 and HepG2 cells, and Lipofectamine 2000 was for LNCaP and RWPE-1 cells.

**Promoter-luciferase reporter assay**
In experiments to study the effects of hormone treatment on the CYP27A1/CYP7B1 promoter activity, cells were transiently transfected with a human CYP27A1/CYP7B1 promoter-luciferase reporter gene and pCMV β-galactosidase plasmid (to control transfection efficiency). In control experiments, the empty vectors of the receptor plasmids were transfected to normalize the amounts of DNA transfected. Luciferase and β-galactosidase activities were assayed as previously described (Elfolk et al., 2006; Wu and Chiang, 2001). Luciferase reporter activity was expressed as relative light units (RLU) divided by β-galactosidase activity (expressed as absorbance at 420 nm). Luciferase activity was measured using TD-20/20 Luminometer, Turner Designs. The β-galactosidase reagent contains 100xMg-solution: ONPG (4mg/ml): 0.1M sodium phosphate buffer (ratio, 1:22:67).

**Quantitation of CYP7B1 and CYP27A1 mRNA**
Total RNA was isolated from HEK293 cells or HepG2 with RNeasy total RNA Mini isolation kit (Qiagen). Quantitation of CYP7B1 mRNA in HEK293 cells and in multiple human tissue cDNA panels (BD Biosciences Clontech), and CYP27A1 mRNA in HepG2 cells were performed by real time RT-PCR as described by Holmström et al. (Holmström et al., 2003). Real time PCR analysis (TaqMan assay) was performed with cDNA obtained by reverse transcription of total RNA isolated from the cells or from multiple human tissue cDNA panels, using an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems) or Bio-Rad iCycler, according to the manufacturers’ recommendations. The amount of cDNA in the tissue panels (BD Biosciences Clontech) had been normalized to mRNA expression levels of several housekeeping genes to standardize the amount of cDNA present within each panel. PCR amplification
Hormonal Regulation of the Human CYP27A1 and CYP7B1 Genes

was performed with the gene-specific TaqMan probes and primers for human CYP7B1 designed by Applied Biosystems (article no: Hs00191385_m1). CYP27A1 probes and primers are also designed by Applied Biosystems (article no: Hs00168003_m1). Eukaryotic 18S rRNA (Applied Biosystems, article no: Hs99999901_s1) and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems, article no: Hs99999905_m1) were used as endogenous control. Target and control were amplified in duplicates or triplicates as singleplex assays. All expression data were normalized to the endogenous control. The relative mRNA expression levels were calculated according to the comparative \( \Delta \Delta CT \) method (for CYP7B1 expression in HEK293 cells) or the standard curve method (for CYP7B1 expression in multiple tissue cDNA panels and for CYP27A1 expression in HepG2 cells) as described by the manufacturer.

**Assay of CYP7B1 enzyme activity**

Endogenous CYP7B1 activity in HEK293 cells was measured by incubation with 27-hydroxycholesterol (5 \( \mu \)M) or DHEA (9 \( \mu \)M, 1 \( \mu Ci \)), dissolved in DMSO or 45% (2-hydroxypropyl)-\( \beta \)-cyclodextrin. After incubation with substrate for 1-24 hours, cells were harvested and the medium was analysed for 7\( \alpha \)-hydroxylated products. With regard to the use of assay with 27-hydroxycholesterol as substrate, it should be noted that, although several hepatic oxysterol 7\( \alpha \)-hydroxylases have been reported (Norlin et al., 2000), CYP7B1 is the only sterol 7\( \alpha \)-hydroxylase present in the kidney.

In some experiments, CYP7B1 activity was assayed in microsomes prepared from HEK293 cells. Preparation of microsomes from untreated cells was conducted as described by Zissimopoulos and Lai (Zissimopoulos and Lai, 2005) and incubations with substrate were carried out at 37°C for 60 min. Microsomes (0.3 mg) were incubated with DHEA (9 \( \mu \)M), 1 U of NADPH-cytochrome P450 reductase and 1 \( \mu \)mol NADPH in a total volume of 0.5 ml of 50 mM Tris acetate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. In incubations with cell microsomes, E2 was added to the incubations in concentrations varying between 0-0.6 \( \mu \)M.

Incubations with medium from cell cultures or microsomes were extracted with ethyl acetate and 7\( \alpha \)-hydroxylated products formed from DHEA or 27-hydroxycholesterol were analysed by HPLC as previously described (Norlin et al., 2000).

**Assay of CYP27A1 enzyme activity**

HepG2 cells were transfected with the different receptor plasmids using calcium co-precipitation method. After 4 h the cells were treated with hormones which were dissolved in ethanol. The control cells were transfected with the corresponding empty vector and treated with ethanol. Cells were treated for 18 or 42 h. The CYP27A1 substrate 7\( \alpha \)-hydroxy-4-cholesten-3-one (5 \( \mu \)M) dissolved in 45% (2-hydroxypropyl)-\( \beta \)-cyclodextrin was added to the medium and incubated during the last 13 or 24 h of treatment. CYP27A1 converts 7\( \alpha \)-hydroxy-4-cholesten-3-one into 7\( \alpha \),27-dihydroxy-4-cholesten-3-one. The harvested medium was extracted with ethyl acetate and analysed directly for 27-hydroxylated product by HPLC as described (Norlin et
The mobile phase was hexane/isopropanol (92:8). The retention time was about 7 min for the formed 27-hydroxylated product.

In some experiments, radiolabelled 5β-cholestane-3α,7α-diol was added as CYP27A1 substrate to the cells. The medium and the cells were extracted with acidified diethyl ether or trichloroethane/methanol (2:1, v/v), and analysed for 27-hydroxylated product by TLC and radioactivity scanning (Wikvall 1984).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was carried out according to methods described (Norlin and Chiang, 2004) and in Paper V. Human GR protein was translated in vitro using the TNT® Transcription/Translation System (Promega).

Site-directed mutagenesis

Mutations were introduced into reporter constructs by PCR-based site-directed mutagenesis using the QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Oligonucleotides with sequence changes corresponding to appropriate regions of the CYP27A1 promoter were designed as PCR-primers and used in mutagenesis with the wild-type construct designated 1094bp-CYP27A1-Luc (see Paper I) as template. This wild type construct contains a fragment spanning from −1094 to +128 of the CYP27A1 promoter sequence (Paper I). A reporter construct containing a mutation in a putative GRE-element between −824 to −819 was generated using the mutagenic primer

\[-849CTGGGTCCTTCATTCCTGGCTTGAAacgGcTCTTTGACCCTGTATT-\]

\[\text{TATTTTATAC}^{794}\]. Another construct contained a mutation in the putative GRE-element between −916 to −908 and was generated using the mutagenic primer

\[-941GCCTAATTGAACTTAATATGCCCTTTaaaCgCTGGCTGCCTGTAAAT\]

\[\text{CACCAATTTTTC}^{882}\].

Thermal cycling was performed with the designed primers and wild-type reporter construct as template and was followed by digestion of parental template DNA with DpnI. The PCRs were then transformed into XL-10 Gold ultracompetent cells (Stratagene). All mutations were verified by sequencing.

Statistical analysis

Analysis of statistical significance was performed using Student’s t-test. P values <0.05 were considered statistically significant.

Other methods

Assay of endogenous metabolism of E2 in HEK293 cells was performed by incubation of cell cultures with radiolabelled E2 (6 µM, 0.1 µCi) for 24 h and analysis by
silica gel thin layer chromatography with chloroform/ethyl acetate/acetone 60:20:10 (v/v/v) as the mobile phase (Williamson et al., 1985). Cell culture samples harvested immediately after addition of E2 were used as controls.

Protein concentrations in microsomes and cell homogenates were determined by the method of Lowry (Lowry et al., 1951).
Results and Discussion

**CYP7B1**

**Human CYP7B1 expression is age-dependent and tissue-specific (Paper II)**

CYP7B1 expression in different human tissues was compared. CYP7B1 mRNA levels were quantitated by real time PCR in human multiple tissue cDNA panels (Clontech), containing cDNA from a number of adult and fetal tissues. The results demonstrate a striking age-dependent variation in expression pattern among different human tissues. PCR data showed that the mRNA levels of CYP7B1 in almost all the fetal tissues were higher than the corresponding adult ones (by 2 to 20-fold, varying in different tissues), except in the liver where the level was higher in the adult than in the fetus. Adult liver contained about twice as much CYP7B1 mRNA as fetal liver. The highest fetal CYP7B1 levels were found in lung and kidney. In fetal tissues, the expression levels of CYP7B1 were in this order: lung >> kidney >> heart ≈ brain ≈ liver >> skeletal muscle; whereas the levels in adult tissues were: liver ≡ lung >> kidney >> brain > heart >> skeletal muscle. These results are in agreement with the previous data on the pig which showed an age-dependent increase in hepatic CYP7B1 levels whereas renal CYP7B1 decreased with age (Norlin, 2002). From the present data it may be concluded that human CYP7B1 expression is age-dependent and tissue-specific and that CYP7B1 levels are higher in fetal than in adult extrahepatic tissues.

The present data, showing age-dependent and tissue-specific differences in human CYP7B1 expression, indicate a developmental regulation of this enzyme. Also, the high levels found in fetal tissues suggest that CYP7B1 is important in fetal development. Tissue-specific variation in CYP7B1 expression has been reported in studies on CYP7B1 in other species (Bean et al., 2001; Norlin, 2002). Hepatic developmental expression of CYP7B1 seems to be different in mice as compared to humans and pigs, since hepatic CYP7B1 is reported to be undetectable in newborn mice (Bean et al., 2001). Bean et al. studied the ontogeny of CYP7B1 in mouse fetus and found widespread expression in fetal extrahepatic tissues which became restricted to fewer organs towards birth. Because the highest expression was observed in early gestation, when the fetus is most vulnerable to steroid excess, these authors suggested that the pattern of CYP7B1 expression could reflect a protective role for this enzyme in fetal development (Fig. 6).
Hormonal Regulation of the Human CYP27A1 and CYP7B1 Genes

**Figure 6.** In a series of experiments, CYP7B1 mRNA levels were quantitated by real-time PCR in human tissues of adult and fetal origin.

**Estrogens regulate CYP7B1 (Paper II, III)**

**E2 strongly stimulates CYP7B1 promoter in the presence of ERα in HEK 293 cells**

The effects of E2 and its receptors on a human CYP7B1 luciferase reporter gene were examined. Treatment with E2 in the absence of ER did not significantly affect CYP7B1 promoter activity. Cotransfection of ERα and treatment with E2 resulted in substantial stimulation (by two to four-fold) of CYP7B1 promoter activity. The effect of E2 under these conditions was dose-dependent. Similar experiments with ERβ showed much less effect on the CYP7B1 promoter.

**E2 increases CYP7B1 mRNA levels in the presence of ERα in HEK293 cells**

The mRNA levels of CYP7B1 were quantitated by real time RT-PCR in cells transfected with ER and treated with E2. Data showed a significant increase of CYP7B1 mRNA by E2 (about 50%) in the presence of ERα. Transfection of ERβ appeared to result in a slight increase of CYP7B1 mRNA, although not high enough to be statistically significant. Experiments were also performed to investigate if the mRNA level of CYP7B1 is affected by E2 in the absence of receptors. However no significant effects of E2 on CYP7B1 mRNA could be detected by real time RT-PCR under these conditions.

**E2 stimulates CYP7B1 activity in the presence of ERs in HEK293 cells**

HEK293 cells were transfected with expression vectors containing ERα or ERβ and cultured for various time periods prior to assay of CYP7B1 activity. Overexpression of ERα or β resulted in a significant E2-mediated increase of CYP7B1 activity. This is in contrast to the effect of E2 treatment alone, resulting in suppression of CYP7B1. Overexpression of ERα or β resulted in an E2-mediated increase of endogenous
CYP7B1 activity by about 50%. These findings indicate a role for ER in estrogen-mediated regulation of CYP7B1. It is possible that the different conditions used in our experiments may reflect situations in different cell types, suggesting that the effect of E2 is different depending on whether receptors are present or not.

Implication of estrogen regulation on CYP7B1 in the presence of ERs

CYP7B1 converts DHEA into a hydroxyderivative that does not serve as a precursor for estrogens. Therefore, up-regulation of CYP7B1 should lead to less DHEA available for estrogen synthesis, consequently resulting in less estrogen being formed. In this way CYP7B1 may divert excess DHEA from the sex hormone biosynthetic pathway into another metabolic route (Fig. 7). This route will probably lead to excretion of DHEA from the cell since the hydroxyderivative is comparatively polar. The widely expressed CYP7B1 may be important for controlling DHEA levels throughout the body, so as to maintain adequate cellular levels of estrogens and androgens. This function may be of particular importance during fetal development, which is one of the two periods in life when DHEA reaches its highest serum concentration (Rainey et al., 2002). Placental estrogen formation is dependent on C_{19} steroid precursors such as DHEA which is secreted in large amounts by the fetal adrenal cortex. However, excessive levels of estrogens or other sex steroids are deleterious to the development of fetus (McLachlan et al., 2001).

![Figure 7](image-url)

Figure 7. Suggested mechanism: up-regulation of CYP7B1 should lead to less DHEA available for estrogen synthesis, consequently resulting in less estrogen being formed.

Estrogen-mediated regulation of CYP7B1 also may have implications for our understanding of steroid action in other processes. E2, DHEA and pregnenolone are neuroactive steroids which are reported to have neuroprotective and neuroenhancing functions (Brinton, 2001; Rose et al., 1997). Rose et al., 1997 proposed that CYP7B1, which is highly expressed in the hippocampus, might be pivotal for the action of neurosteroids such as DHEA on cognition and behaviour. It is at present unclear whether there is a connection between the reported effects by estrogen and the proposed role(s) of DHEA and pregnenolone in the CNS. However, it may be concluded that regulation of CYP7B1 is a means by which estrogen could affect the levels of DHEA and pregnenolone in the brain, thereby modulating their action. Another
Hormonal Regulation of the Human CYP27A1 and CYP7B1 Genes

interesting potential connection between estrogens and CYP7B1 is related to cellular proliferation. Although the precise mechanisms remain to be elucidated, CYP7B1 action was recently associated with effects on cellular proliferation via ligands of ERβ (Martin et al., 2004; Weihsa et al., 2002a).

**E2 affects CYP7B1 enzyme activity in the absence of ERs in HEK 293 cells**

E2 (1 µM) was added to the culture medium of HEK293 cells. After treatment for 6 or 48 h and subsequent incubation with CYP7B1 substrate, the medium was extracted and the organic phase was analysed by HPLC. From earlier studies it is known that almost all of the 7α-hydroxylated product is secreted to the medium (Norlin et al., 2000). E2 significantly affected CYP7B1 levels, resulting in a marked inhibitory effect (by about 30-50%) of CYP7B1 activity.

The effects of E2 on CYP7B1 activity at different incubation time periods and concentrations were examined. Treatment times between 12-24 h resulted in the most pronounced effects. A significant decrease of CYP7B1 activity by about 40% was observed when cells were treated with E2 in concentrations between 0.04 nM and 1 µM.

**Implication of estrogen effect on CYP7B1 in the absence of ERs**

The mechanism for the potentially interesting ER-independent suppression by E2, observed in our experiments, is difficult to explain at this point. Since effects on the enzyme level seem to be excluded, it may be speculated that the suppression could be due to a post-transcriptional regulatory mechanism, perhaps through intracellular second messengers. Another possibility is that the observed ER-independent effects by E2 on CYP7B1 may reflect presently unknown cell-specific functions. It has been suggested that estrogen might affect DHEA production in the adrenal cortex through ER-independent mechanisms (Gell et al., 1998). Anyway, these results suggest that the effect of estrogen on CYP7B1 in cells that for some reason lose their expression of ER, such as some tumor cells, may be opposite to the effect in normal cells. This may have implications for the effect of estrogens in cancer treatment.

**Overexpression of ERs increases CYP7B1 promoter activity in LNCaP cells (Paper III)**

In the prostate ERβ should be the ER subtype of greater importance for CYP7B1 function. ERβ is reported to co-localize with CYP7B1 in human prostate epithelial cells, whereas ERα is expressed in the prostate stroma (Härkonen and Mäkelä, 2004) (Martin et al., 2004). Levels of ERβ are reported to decrease in prostate cancer (Härkonen and Mäkelä, 2004). Prostate cancer LNCaP cells were transfected with an ERβ expression vector and treated with DPN, an agonist of ERβ, or with ICI 182,780, an ER antagonist. Overexpression of ERβ and treatment with DPN resulted in two to three-fold stimulation of the CYP7B1 promoter-luciferase gene. Treatment with ICI 182,780, an ER antagonist, abolished the effect of ERβ on CYP7B1 promoter activity. Overexpression of ERα and treatment with the ERα agonist PPT in prostate cancer LNCaP cells showed a similar effect as ERβ + agonist on the CYP7B1 reporter gene, resulting in stimulation by about two-fold.
3βAdiol and E2 suppress CYP7B1 promoter activity in LNCaP cells (Paper III)

It has been reported that CYP7B1-mediated metabolism of 3βAdiol may play a role for ERβ-mediated effects on prostate proliferation. 3βAdiol is a well-known ERβ ligand which also binds ERα albeit with a lower affinity than for ERβ (Kuiper et al., 1997). Experiments were performed to examine the effects of treatment with this estrogen on the CYP7B1 promoter-luciferase gene in prostate LNCaP cells. Surprisingly, treatment with 3βAdiol suppressed CYP7B1 promoter activity. Significant suppression by this compound was observed both with and without overexpression of ERβ. The suppression of CYP7B1 promoter activity was about 30-50% for concentrations between 1-100 nM of 3β-Adiol. The effect by this compound on CYP7B1 may be mediated via ER even though the results of our experiments can not provide conclusive evidence that this is the case. Furthermore, the suppressive effect of 3βAdiol observed in our experiments was not counteracted by overexpression of ERβ. The endogenous expression of ERβ in LNCaP cells may be sufficient for mediating suppression of CYP7B1 by 3βAdiol.

Treatment of LNCaP cells with E2 resulted in suppression of CYP7B1 promoter activity in a similar fashion as was observed for 3βAdiol, by about 50% at a concentration of 100 nM.

From the present and previous data (Paper II), it seems that CYP7B1 transcription is affected by ERs. Surprisingly, even though overexpression of ER and treatment with ER agonists stimulated promoter activity, treatment with just the ER ligands 3βAdiol and E2 suppressed the CYP7B1 promoter in prostate LNCaP cells. These data indicate that in this cell type CYP7B1 expression is down-regulated also by estrogens.

The role of estrogenic action in carcinogenesis remains unclear due to conflicting data from many reports using different approaches and methodologies (Härkonen and Mäkelä, 2004). Possibly the physiological effects of estrogens may differ among individual estrogenic compounds and effects may vary depending on dosage.

Androgens Regulate CYP7B1 (Paper III)

DHT suppresses CYP7B1 promoter activity and CYP7B1-mediated catalytic activity in HEK293 cells

Treatment with DHT and cotransfection with AR significantly suppressed the CYP7B1 promoter-luciferase reporter gene in kidney HEK293 cells. In the kidney cells, suppression by DHT was dependent on cotransfection with AR. This is probably due to low endogenous AR expression in HEK293 cells. The suppressive effect by DHT on CYP7B1 promoter activity in the presence of AR was about 40% in human embryonic kidney 293 cells at a concentration of 1 μM DHT. Consistently, treatment with DHT and cotransfection with AR also resulted in significant suppression of endogenous catalytic CYP7B1 activity. Treatment with DHT without cotransfection of AR did not significantly alter endogenous catalytic activity or promoter activity of CYP7B1 in kidney 293 cells. These findings indicate that the effect of DHT on CYP7B1 is indeed mediated via AR.
DHT suppresses CYP7B1 promoter activity in LNCaP cells
The effects of treatment with DHT on a human CYP7B1 promoter-luciferase gene in prostate cancer LNCaP cells were examined. LNCaP cells are known to respond strongly to androgen treatment leading to induced proliferation (Cude et al., 1999). Treatment with DHT dramatically reduced CYP7B1 promoter activity, in a dose-dependent fashion. Suppression by DHT was about 40-80% for concentrations between 1-100 nM.

The effects of DHT on promoter activity were studied with different deletion constructs of the CYP7B1 promoter-luciferase gene. DHT inhibited the reporter activity of all constructs including the one containing the shortest promoter fragment. This indicates that sequence(s) involved in regulation by DHT are present in the proximal promoter region. The DHT-responsive element(s) in the CYP7B1 promoter remain to be identified.

Possible implications of hormonal regulation of CYP7B1 for prostate proliferation
The current study indicates that DHT is not only a precursor of 3βAdiol, but may also control 3βAdiol metabolism via effects on the CYP7B1 gene. These findings suggest that androgens might be able to control the intraprostatic levels of estrogen, a previously unknown connection between androgens and estrogens in the prostate. This may reflect a mechanism to balance the levels of androgen vs estrogen in this tissue. Since 3βAdiol is metabolized by CYP7B1, DHT-mediated suppression of CYP7B1 would lead to higher levels of 3βAdiol. Rising DHT levels in the prostate should therefore lead to rising levels also of 3βAdiol. This increase of estrogen levels may be a means to counteract effects of high androgen levels. It is possible that hormonal regulation of the CYP7B1 gene, may help to maintain a balance between androgen and estrogen levels adequate for prostate function and growth (Fig. 8).

In LNCaP cells

Figure 8. Suggested mechanism for regulation of sex hormone levels by CYP7B1 in LNCaP human prostate cancer cells: androgens (DHT) suppress CYP7B1 transcription, which will lead to decreased metabolism of 3βAdiol, thereby increasing the levels of 3βAdiol. As a result, rising levels of DHT would lead to rising levels of estrogen (3βAdiol). This may be a means to help maintain a balance between androgen and estrogen in the prostate.
A mechanism for control of estrogen and androgen levels involving CYP7B1 may not be unique for prostate and kidney. Gustafsson and coworkers (Omoto et al., 2005; Weihua et al., 2002a) proposed that since CYP7B1 is abundantly expressed in tissues throughout the body, this enzyme may play a widespread role in regulating AR and ER balance in the body. The current study supports this proposed role for CYP7B1 and expands it to involve human cells. It should be noted that the data obtained by Weihua et al. (Weihua et al., 2002a) in connection with hormone function related to proliferation were obtained from experiments with mouse and rat. The present study suggests that CYP7B1 action may play an important role in this context also in humans. It can not be excluded that the control of androgen/estrogen levels and the mechanism of CYP7B1 action might be different in different cell types. For instance we currently do not know whether the mechanisms for regulation of CYP7B1 are different in tumour cells as compared with normal cells.

The current results, indicating that human CYP7B1 expression is involved in the careful control of prostatic androgen and estrogen levels, suggest an important function for this enzyme in connection with hormonal action in human prostate.

CYP27A1

PMA, dexamethasone, thyroid hormones, growth hormone and IGF-1 regulate CYP27A1 (Paper I)

Growth hormone, IGF-1 and dexamethasone stimulate the human CYP27A1 promoter activity in HepG2 cells

Dexamethasone and growth hormone increased CYP27A1 promoter activity. The induction by dexamethasone was dose and time dependent. Maximal induction was found when the cells were treated with 1 µM of dexamethasone and harvested after 6 h of treatment. Treatment with 1 µM dexamethasone for 6 h increased the activity about three-fold compared with that in the untreated control cells. The induction by growth hormone was time dependent and the promoter activity increased up to at least 48 h. Treatment with 0.01-0.03 IU/ml of human growth hormone (2 IU/mg) for 48 h resulted in about twofold higher activity than in the controls.

Many biological effects of growth hormone are mediated by IGF-1. In contrast to the two-fold induction observed with growth hormone, the luciferase activity of the largest CYP27A1-promoter fragment was not affected by IGF-1. However, when a shorter promoter sequence (1094bp) was used, a significant stimulation was found.
Hormonal Regulation of the Human CYP27A1 and CYP7B1 Genes

Figure 9. The CYP27A1 promoter luciferase constructs, containing promoter fragments of different lengths, were used in Paper I, IV, V.

Thyroid hormones and PMA repress the human CYP27A1 promoter activity in HepG2 cells

Thyroxine (T₄), triiodothyronine (T₃) and PMA decreased the activity of the CYP27A1 promoter. The most marked repression was found when the cells, transfected with 4.2 kb-CYP27A1-Luc, were treated for 48 h with thyroid hormones and for 6 h with PMA. In the presence of 1 µM of thyroxine or triiodothyronine, the promoter activity was 30-40% of that in untreated, transfected control cells. PMA treatment resulted in an even more marked repression.

It has been reported that the combined addition of thyroid hormone and dexamethasone stimulates bile acid formation in primary rat hepatocytes but tends to decrease bile acid formation in primary human hepatocytes (Ellis et al., 1998). In the present study, a separate set of experiments was performed with the combined addition of dexamethasone and thyroxine or triiodothyronine (1 µM and 24 h). Dexamethasone+thyroxine and dexamethasone+triiodothyronine, respectively, suppressed the promoter activity to 75 and 79 % of that with control cells.

Effects of PMA, dexamethasone, thyroid hormones, growth hormone and IGF-1 on the endogenous CYP27A1 activity in HepG2 cells

In HepG2 cells 5β-cholestan-3α,7α-diol is 27-hydroxylated by CYP27A1. To study the effect of PMA, dexamethasone and thyroxine on the endogenous CYP27A1 activity, 5β-cholestan-3α,7α-diol was added as substrate to the transfected cells directly after addition of these compounds to HepG2 cells. The cells were treated for 6 h or 24 h. The medium was extracted and analysed for amount of 27-hydroxylated product formed during the period of treatment. PMA and thyroxine decreased the rate of formation of 27-hydroxylated product to, respectively, 15 and 55% of that in the control. Dexamethasone increased the endogenous CYP27A1 activity almost
Treatment of non-transfected cells with growth hormone or IGF-1 increased the CYP27A1 activity about twofold. These results are consistent with the effects observed on the promoter activity.

Experiments with progressive deletion constructs of the human CYP27A1 promoter
To identify regions of the human CYP27A1 promoter responsive to potential regulators, progressive deletion constructs were made for transient transfection assays in HepG2 cells (Fig. 9).

The luciferase vectors harboring the fragments of 1094, 792 and 451 bp upstream of the CYP27A1 translation start were tested for responsiveness to IGF-1, dexamethasone, thyroxine, PMA and growth hormone following transfection of HepG2 cells. IGF-1 increased the promoter activities of all constructs. IGF-1 increased the activity of 451 bp-CYP27A1-Luc two-fold. Dexamethasone increased the activity of the 1094-CYP27A1-Luc but not the constructs harboring shorter fragments. Thyroxine and PMA repressed the activity of all constructs and repressed the activity of the 451-bp-CYP27A1-Luc, harboring the shortest fragment, by 55 and 68%, respectively. Growth hormone had no significant effect on the activity of the constructs containing the shorter promoter fragments.

The results indicate that putative response elements for IGF-1, thyroxine and PMA are located in the nt -451/+42 fragment and suggest that putative response elements for dexamethasone are located in the nt -1095/-792 fragment. As mentioned above, growth hormone increased the activity of the 4.2 kb-CYP27A1-Luc but not that of the constructs with shorter promoter fragments. These data suggest that the response elements for growth hormone are located in a region upstream to nt -1094.

Estrogens and androgens regulate CYP27A1 (Paper IV)

Estrogens and androgens affect CYP27A1 promoter activity in HepG2 cells
In Paper IV, experiments were performed with liver-derived HepG2 cells to study the effects of estrogen and androgen on CYP27A1. In this study, ERα, ERβ or AR was overexpressed in the cells to examine receptor-mediated hormonal effects.

Overexpression of ERα or ERβ and treatment of the cells with estrogen inhibited CYP27A1 promoter activity by 60-70% in HepG2 cells. No suppression by estrogen was observed without overexpression of receptors. These data indicate that the effects of estrogens on CYP27A1 are mediated by ERs. Overexpression of AR and treatment of the cells with androgen resulted in three- to fourfold enhancement of CYP27A1 promoter activity. No enhancement by androgen was observed without overexpression of receptor indicating that the effects of androgen are mediated by AR.

Estrogens and androgens affect endogenous CYP27A1 enzyme activity and mRNA levels in HepG2 cells
Experiments were performed to examine whether the endogenous CYP27A1 gene in HepG2 cells was affected in the same way by sex hormones as the transiently
Hormonal Regulation of the Human CYP27A1 and CYP7B1 Genes

Transfected CYP27A1 promoter-luciferase reporter gene. The endogenous CYP27A1 enzyme activity was measured as the rate of 27-hydroxylation of 7α-hydroxy-4-cholestene-3-one. The endogenous CYP27A1 enzyme activity (27-hydroxylase activity) in HepG2 cells was significantly suppressed by estrogen in presence of ERs, and significantly increased by androgen in presence of AR. These results are in agreement with the effects observed on the promoter activity.

Experiments were carried out to quantitate CYP27A1 mRNA in HepG2 cells using real-time PCR. These experiments showed statistically significant effects by ERβ and AR on CYP27A1 mRNA levels. Transfection with AR and treatment with androgen resulted in about 30% increase of CYP27A1 mRNA levels. Transfection of cells with ERα or β and treatment with estrogen resulted in about 10-20% decrease of CYP27A1 messenger RNA levels. These effects on mRNA levels are consistent with the effects on endogenous enzyme activity by the respective treatment. Taken together, the data on the regulation of CYP27A1 promoter activity together with the effects on endogenous CYP27A1 activity and mRNA levels strongly support a receptor-mediated transcriptional regulation of the endogenous CYP27A1 gene by estrogen and androgen in HepG2 cells.

Effects of estrogens and androgens on CYP27A1 promoter activity in human prostate cells

Overexpression of ERα and treatment with estrogen resulted in a suppression of CYP27A1 promoter activity by about 70% in non-cancerous human prostate cells, RWPE-1 cells. Overexpression of ERβ and treatment of the cells with estrogen resulted in a suppression by about 50%. Treatment with androgen significantly suppressed CYP27A1 promoter activity in RWPE-1 cells.

In prostate cancer LNCaP cells (Elo et al., 1995), an ER-mediated enhancement of CYP27A1 promoter activity was found. Overexpression of ERα and treatment of human prostate cancer cells LNCaP cells with estrogen increased CYP27A1 promoter activity about three-fold. Overexpression of ERβ and treatment of the cells with estrogen resulted in about two-fold enhancement of the promoter activity. Thus, in contrast to the receptor-mediated suppression by estrogen in HepG2 and RWPE-1 cells, an ER-mediated enhancement of CYP27A1 promoter activity was found in prostate cancer LNCaP cells.

DHT had no significant effects on the promoter activity in LNCaP cells in contrast to a suppression found in RWPE-1 cells. These results indicate that androgens as well as estrogens have different effects on CYP27A1 promoter activity in different prostate cell lines.

Experiments with deletion constructs of the CYP27A1 promoter

Experiments were conducted with progressive deletion constructs of the human CYP27A1 promoter in order to identify regions of the human CYP27A1 promoter responsive to ERs and ARs (Fig. 9 and 10). In HepG2 cells overexpressed with ERα, estrogen inhibited the reporter activity only of the construct containing the largest fragment. The results indicate that sequences involved in ERα-mediated suppression by estrogen are present in a region upstream of –1094. Intriguingly, the reporter activity increased markedly with the construct containing the 792 bp fragment, indicating putative ERα-mediated enhancing sequences in the region –792 to –451.
In cells overexpressed with ERβ, estrogen inhibited the reporter activity of all constructs including the one containing the shortest promoter fragment. This indicates that sequence(s) involved in ERβ-mediated suppression by estrogen is present in the proximal promoter region. Interestingly, the results from the experiments with progressive deletion constructs indicate that estrogen signalling for ERα and ERβ may be different in HepG2 cells.

The effects of androgens on promoter activity with different deletion constructs of the CYP27A1 promoter-luciferase gene were also investigated. In HepG2 cells overexpressed with AR, androgen increased the reporter activity of the constructs containing the two largest fragments (4.2 kb and the 1094 bp fragments) two to threefold. Only small increase of reporter activity was obtained with the shorter fragments. The results indicate that sequence(s) most important for AR-mediated enhancement by androgen is present in a region upstream of –792. A putative androgen response sequence (ARE) can be found between nt –2453 and -2446 of the CYP27A1 promoter (Fig. 9 and 10).

Experiments were also performed in LNCaP cells with progressive deletion constructs of the human CYP27A1 promoter in order to identify regions of the human CYP27A1 promoter responsive to estrogens.

Experiments with promoter-deletion constructs in LNCaP cells revealed that, in this cell type, estrogen and ERα and ERβ stimulate the reporter activity of all constructs including the one containing the shortest promoter fragment. This indicates that sequence(s) involved in both ERα and ERβ-mediated upregulation by estrogen in LNCaP cells are present in the proximal promoter region. Thus, it seems that the ERβ-mediated effects in HepG2 and LNCaP cells are mediated by the same regions whereas ERα-mediated effects on CYP27A1 promoter are more complex.

Sequence analysis of the human CYP27A1 5’-flanking sequence

The CYP27A1 promoter sequence from -4158 bp to +128bp region was computer analyzed for putative response elements considered of relevance for the observed hormonal effects. The analysis was carried out using Transcription Element Search Software (TESS) [http://www.cbil.upenn.edu/] and the associated Transfac DATABase (Computational Biology and Informatics Laboratory, University of Pennsylvania) (Fig. 10). In this promoter region there are several putative ERE-halfsites and putative binding sites for AP-1 and Sp1. AP-1 and Sp1 are factors known to be involved in ER-mediated regulation of some other genes. A putative androgen response element (ARE) can be found between -2453 and -2446 of the CYP27A1 promoter. Five putative GRE sites could be found at –1183/-1177, -935/-929, -916/-908, -824/-819, and –707/-700 (Paper V) (Fig. 9 and 10).

Biological significance of regulation of CYP27A1 by sex hormones

The information on the regulation of CYP27A1, particularly in humans has been limited (Quinn et al., 2005; Segev et al., 2001).

The present study (Paper IV) is the first reporting effects of estrogens and androgens on the regulation of CYP27A1 at promoter level in any species. Because estrogens are used in oral contraceptives, in hormone therapy of postmenopausal women and in cancer treatment, the question arises how CYP27A1 is influenced by estrogens. The present studies clearly show certain effects of estrogens and androgens on CYP27A1
Several putative regulatory elements and transcription factor binding sites in the CYP27A1 promoter are underlined and labeled.

Figure 10.
gene expression in liver-derived HepG2 cells. The effects may be different in different tissues. The possibility of a tissue-specific regulation by sex hormones is supported by our results with LNCaP prostate cancer cells and non-cancerous prostate RWPE-1 cells. Whether this difference in regulatory effects is due to different properties of different prostate cell lines or to altered properties of the CYP27A1 regulation in prostate cancer remain to be established.

Hormonal action on the CYP27A1 promoter appears to be complex. In addition to cell-dependent effects, we also observed differences between receptor subtypes and different promoter deletion constructs. For instance, whereas ERα suppressed the full-length promoter in HepG2 cells, deletion of a 3.4 kb long part of the promoter resulted in the opposite response. On the contrary, the response of different promoter constructs to ERβ was similar. From the current data it seems that the CYP27A1 promoter should contain sequences able to mediate both stimulation and suppression by ER. ER-mediated regulation of transcription is often associated with binding of ER homodimers to estrogen response elements (ERE) in target promoters. However, regulation by ER can also involve interaction with sequences containing Sp1 and activator protein (AP-1) sites (Safe, 2001; Schultz et al., 2005). For instance, ERα-mediated transactivation may involve interaction between ERα and Sp1, affecting Sp1 sites and EREs or ERE halfsites (Safe, 2001). Interestingly, it has been reported that ER-mediated regulation involving AP-1 sites may lead to opposite effects depending on ER subtype. (Paech et al., 1997) reported that estrogen signalling via an AP-1 element resulted in stimulation by ERα but inhibition by ERβ. As mentioned above, the CYP27A1 promoter contains several putative binding sites for ER, AP-1 and Sp1. Further studies are required to evaluate the physiological role(s) of these sequences. It seems possible that cell-specific interactions with coactivators may be the reason for the different effects we observed with different cell types and receptor subtypes.

These results are consistent with reports on an increased risk for cardiovascular disease in postmenopausal women treated with estrogen plus progestin (Rossouw et al., 2002). Also, increased testosterone levels in men have been associated with a favorable lipid profile (Alexandersen and Christiansen, 2004; Barrett-Connor, 1995; Khaw and Barrett-Connor, 1991; Tchernof et al., 1997; Zmuda et al., 1997). The difference in prevalence of atherosclerotic coronary disease between men and women can obviously not be explained by effects of estrogens and androgens solely on CYP27A1 expression. However, the effects described here by sex hormones on the expression of CYP27A1 may have impact on several processes in cholesterol homeostasis (Steinberg, 2006; Stoll and Bendszus, 2006).

Activated vitamin D metabolites, that can be formed by CYP27A1, are known to have beneficial effects on cell growth in extrahepatic tissues, such as in prostate cells and prostate cancer cells (Chen and Holick, 2003; Gascon-Barre et al., 2001; Sawada et al., 2000; Tokar and Webber, 2005a; Tokar and Webber, 2005b). The current data indicate that estrogens and androgens might regulate the intracellular levels of active hydroxyvitamin D₃ metabolites in prostate cells via regulation of CYP27A1 gene expression.

In summary, the human CYP27A1 gene has been identified as a target for estrogens and androgens. The results indicate that the effects might be different in different tissues and different cell types. The results imply that regulation of CYP27A1 may be affected by endogenous sex hormones and pharmacological compounds with estrogenic or androgenic effects.
Hormonal Regulation of the Human CYP27A1 and CYP7B1 Genes

In HepG2 cells

- Glucocorticoids
- Growth hormone
- IGF-1

CYP27A1

Androgens

+ + +

PMA

Thyroid hormones

- - -

Estrogens

Figure 11. A summary of hormonal regulation of CYP27A1 in HepG2 cells. Glucocorticoids, growth hormone, IGF-1 and androgens upregulate CYP27A1, whereas PMA, thyroid hormones and estrogens downregulate CYP27A1.

Dexamethasone-induced effect on the CYP27A1 promoter is mediated via GR (Paper V)

Mifepristone abolishes the dexamethasone-induced effect on CYP27A1 promoter activity

Report experiments were performed with HepG2 cells overexpressed with GR. Treatment of the cells with the GR agonist dexamethasone increased the CYP27A1 promoter activity more than four-fold. Treatment of GR-overexpressed cells with the GR-antagonist mifepristone did not result in induction of the promoter activity. Treatment of the cells with dexamethasone in presence of mifepristone almost completely abolished the dexamethasone-induced effect on the promoter activity. The results indicate that dexamethasone induction of the CYP27A1 transcriptional activity is mediated by GR.

Deletion construct analysis of GR-mediated effects on the CYP27A1 promoter

The effects of dexamethasone and GR on promoter activity were studied with different deletion constructs of the CYP27A1 promoter-luciferase gene. In HepG2 cells overexpressed with GR dexamethasone increased the reporter activity substantially with the constructs containing the two largest DNA fragments (the 4.2 kb and 1094 bp fragments) (Fig. 9). The results indicate that sequences involved in GR-mediated induction by dexamethasone are present in a region upstream of −792. The promoter sequence was analyzed for putative GRE in this region. Five putative GRE sites could be found at −1183/−1177, −935/−929, −916/−908, −824/−819, and −707/−700 (Fig. 10).
Identification of GR-binding sites in the CYP27A1 promoter

Analysis of the 5′-flanking region of the human CYP27A1 gene revealed a number of putative GRE sequences. Deletion construct analysis of the CYP27A1 promoter-luciferase reporter gene indicated that the region mediating the response to GR-mediated induction may be located upstream of –792 of the CYP27A1 promoter sequence. In order to investigate whether GR might bind to sequences in this part of the promoter, EMSA experiments were carried out with in vitro-translated GR protein and several probes designed based on putative GRE sites. Four DNA probes were used in the EMSA experiments, corresponding to –1192 to –1170, –942 to 901, –835 to –812 and –713 to –692 respectively, of the CYP27A1 promoter sequence. The sequence corresponding to nucleotides –942 to -901 contains two putative GRE halfsites. As a positive control for GR-binding, a probe was included based on a consensus GRE sequence from the rat tyrosine aminotransferase gene (CTAGGCTG-TACAGGATGCTGCCTAG) which is known to bind GR (Tseng et al., 2001). As expected, a shifted band indicating binding by GR protein was observed with the known GR-binding sequence used as positive control. EMSA analysis of CYP27A1 promoter sequences revealed strong binding by in vitro-translated GR protein also to the DNA probe corresponding to nucleotides –835 to –812 of the CYP27A1 promoter. Binding was also observed with the DNA probe corresponding to nucleotides –942 to –901. Binding by GR was not observed to the other two CYP27A1 promoter sequences (–1192 to –1170 and –713 to –692) analyzed in EMSA.

Site-directed mutagenesis of sequences in the CYP27A1 promoter

Experiments were carried out to compare the effects of GR and dexamethasone on wild-type reporter and two reporter constructs with mutations in putative GRE sequences. Mutagenesis in one of the GR-binding putative GRE sequences –824/–819 resulted in substantial decrease of the response to GR-mediated dexamethasone induction. A construct with this mutation and an additional mutated GRE –916/–908 did not reduce the response further. These findings suggest that the putative GRE at –824/–819 is important for GR-mediated regulation of CYP27A1 transcriptional activity (Fig. 10).

Mechanism and biological role of glucocorticoid-mediated induction of CYP27A1 transcription

Glucocorticoid-mediated effects may occur at both transcriptional and post-transcriptional levels. GR may bind dexamethasone and related ligands. The activated GR can interact with the regulatory regions of responsive genes to alter the level of gene expression. Variant forms of GREs with different affinities for GR binding and hence various activation potentials have been reported (Beato and Sanchez-Pacheco, 1996; Pearce et al., 1998; Tseng et al., 2001).

There have been different explanations for the mechanism by which dexamethasone increases CYP27A1 mRNA levels in the rat (Mullick et al., 2001; Stravitz et al., 1996). From studies with rat hepatocytes, Stravitz (Stravitz et al., 1996) suggested that the glucocorticoids appears to increase CYP27A1 mRNA posttranscriptionally
by e.g. stabilization. Mullick et al (Mullick et al., 2001) explained the dexamethasone-mediated transcription activation of the rat CYP27A1 promoter by a physical interaction and functional synergy between GR and Ets2 factor.

The current results may indicate that dexamethasone affects the human CYP27A1 gene by binding to GR which subsequently binds directly to a GRE at –824/–819 in the promoter resulting in the transcription activation of the human CYP27A1 gene.

The biological role of glucocorticoid-mediated induction of CYP27A1 transcription may be related to one or more of the several important functions of CYP27A1 in cholesterol homeostasis, bile acid biosynthesis, vitamin D metabolism and calcium homeostasis (Babiker et al., 1997; Gascon-Barre et al., 2001; Norlin, 2007; Sakaki et al., 2005). Glucocorticoids are among the most widely used agents for the treatment of inflammatory and autoimmune diseases. Glucocorticoids, such as the synthetic corticosteroid dexamethasone, were found to increase bile acid metabolism in rat hepatocytes (Princen et al., 1989). CYP27A1 also affects cholesterol transport from extrahepatic cells in different ways, such as reverse cholesterol transport (Babiker et al., 1997; Fu et al., 2001). It has been reported that glucocorticoids play an important role in the regulation of genes central in reverse cholesterol transport to get rid of the excess cholesterol (Sporstol et al., 2007).
In this thesis, the regulation of the human CYP27A1 and CYP7B1 genes is studied. Paper I reports hormonal regulation of the human CYP27A1 gene. HepG2 cells were transiently transfected with luciferase reporter gene constructs containing DNA fragments of the 5’ flanking region of the human CYP27A1 gene. Growth hormone, IGF-1, and dexamethasone increased the promoter activity two to three-fold whereas thyroxine and PMA repressed the activity as measured with luciferase activity expressed in the cells. The endogenous CYP27A1 enzyme activity in the cells was stimulated by growth hormone, IGF-1 and dexamethasone whereas thyroxine and PMA inhibited the activity. The results of this study imply that CYP27A1 is regulated in human cells by hormones and signal transduction pathways.

In paper II, the regulation of CYP7B1, a DHEA 7α-hydroxylase, by sex hormones is examined. Transfection with ERα and treatment with E2 in human embryonic kidney 293 cells significantly increased CYP7B1 catalytic activity and mRNA, and stimulated a human CYP7B1 reporter gene. In the absence of receptors, E2 suppressed CYP7B1 activity, suggesting that estrogenic effects may be different in cells not expressing receptors. Quantitation of CYP7B1 mRNA in adult and fetal human tissues showed markedly higher CYP7B1 mRNA levels in fetal tissues compared with the corresponding adult ones, except in the liver. This indicates a tissue-specific, developmental regulation of CYP7B1 and suggests an important function for this enzyme in fetal life. DHEA secreted by fetal adrenals is an essential precursor for placental estrogen formation. Since CYP7B1 diverts DHEA from the sex hormone biosynthetic pathway, ER-mediated up-regulation of CYP7B1 should lead to less DHEA available for sex hormone synthesis and may help to maintain normal levels of estrogens and androgens in human tissues, especially during fetal development.

Paper III reports effects of androgens and estrogens on human CYP7B1 transcription in prostate cancer LNCaP cells. Studies with rodents have suggested a role for the CYP7B1 enzyme in balancing cellular hormone levels important for prostate growth. This study showed strong suppression of a human CYP7B1 luciferase reporter gene by DHT in prostate cancer LNCaP cells. Also, DHT and overexpression of AR suppressed CYP7B1 promoter activity and CYP7B1-mediated catalysis in kidney-derived HEK293 cells. Effects on CYP7B1 transcription were observed also by ER. The effects appeared different for different estrogens. CYP7B1 was stimulated by synthetic ER agonists but suppressed by E2 and 3βAdiol in LNCaP cells. The data indicate an important role for CYP7B1 in balancing prostate hormone levels in human cells. In particular, the data suggest that androgens may control intraprostatic levels of estrogen via regulation of CYP7B1-mediated metabolism.

In paper IV, the regulation of the human CYP27A1 gene by estrogens and androgens was studied in human liver-derived HepG2 and prostate cells. The results show that the transcriptional promoter activity and enzymatic activity of CYP27A1 in HepG2 cells are downregulated by estrogen in presence of ERα or ERβ. Similar
Effects by estrogen were found in RWPE-1 prostate cells. In contrast, estrogen markedly upregulated the transcriptional activity of CYP27A1 in LNCaP prostate cancer cells. DHT and AR upregulated the transcriptional activity of CYP27A1 in HepG2 cells. Progressive deletion experiments were conducted to map the regions responsible for the effects of estrogen and androgen in the CYP27A1 promoter. The results imply that regulation of CYP27A1 may be affected by endogenous sex hormones and pharmacological compounds with estrogenic or androgenic effects.

In paper V the mechanism of the dexametasone-induced effect on the human CYP27A1 promoter is examined. Treatment of HepG2 cells overexpressed with GR with dexamethasone increased the CYP27A1 promoter activity more than four-fold. Treatment of the cells with dexamethasone in presence of the GR antagonist mifepristone almost completely abolished the dexamethasone-induced effect on the promoter activity. Progressive deletion analysis of the CYP27A1 promoter indicated that sequences involved in GR-mediated induction by dexamethasone are present in a region upstream of −792. EMSA experiments revealed that some of the putative GRE in this region could bind to GR. Site-directed mutagenesis identified a putative GRE important for GR-mediated regulation of CYP27A1 transcriptional activity.

The results of this study imply that CYP27A1 and CYP7B1 are regulated in human cells by hormones.
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Hormonal Regulation of the Human CYP27A1 and CYP7B1 Genes


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