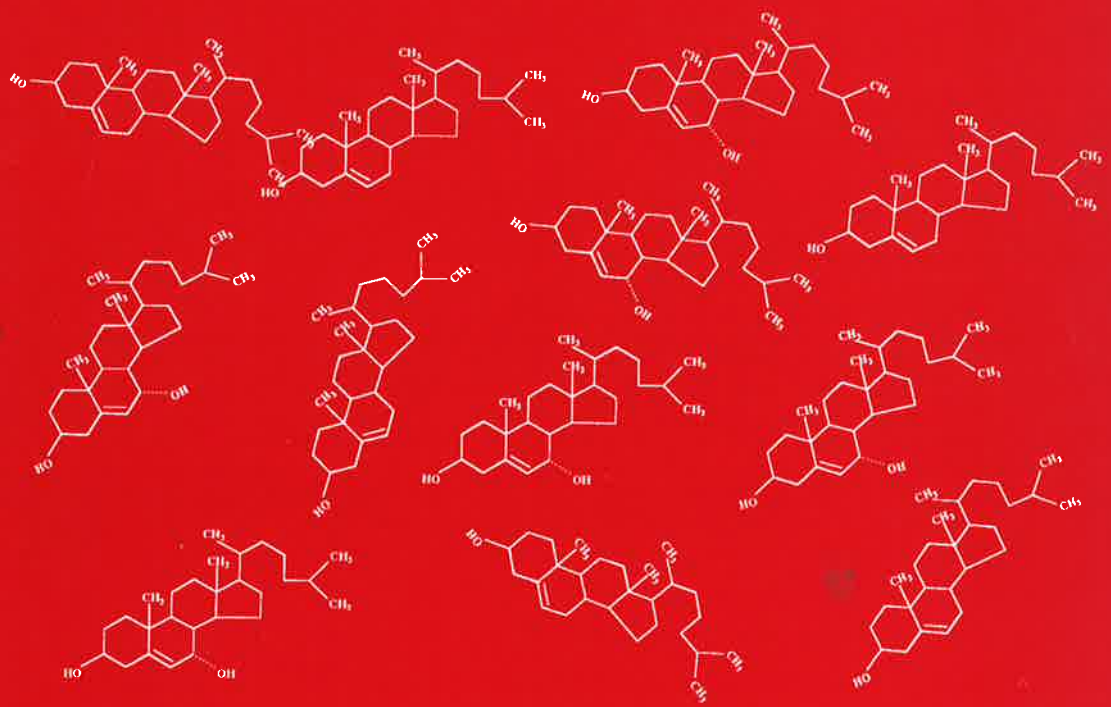
REGULATION OF CHOLESTEROL 7 α-HYDROXYLASE GENE EXPRESSION IN THE RAT



Marco Hoekman

REGULATION OF CHOLESTEROL 7α-HYDROXYLASE GENE EXPRESSION IN THE RAT

Cover : (7α-hydroxy) Cholesterol molecules playing the 'conversion' game

The studies presented in this thesis were performed at the Department of Biochemistry and Molecular Biology of the Vrije Universiteit, Amsterdam, and the Gaubius Laboratory, TNO-PG, Leiden. This work was supported by HGO-TNO, grant 900-523-138 to M.F.M. Hoekman and J.M.J. Rientjes and the Netherlands Heart Found., grant 89.079 to J. Twisk.

VRIJE UNIVERSITEIT

Regulation of cholesterol 7α-hydroxylase gene expression in the rat

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor aan
de Vrije Universiteit te Amsterdam,
op gezag van de rector magnificus
prof.dr E. Boeker,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der scheikunde
op donderdag 26 januari 1995 te 13.45 uur
in het hoofdgebouw van de universiteit, de Boelelaan 1105

door

Marcus Franciscus Maria Hoekman

geboren te Amsterdam

Promotor	:	prof.dr R.J. Planta
Copromotoren	:	dr W.H. Mager
		dr H.M.G. Princen
Referent	:	dr G. AB

aan mijn ouders

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.1 BILE ACID BIOSYNTHESIS

An elevated serum cholesterol concentration is considered to be a major risk factor in the development of atherosclerosis, main cause of death in Western society (1-4). For this reason many efforts have been made to gain more insight in cholesterol-metabolism. Cholesterol (Fig.1), precursor of steroid hormones, vitamins and bile acids, but also an important constituent of cell membranes, is transported through the mammalian body in the form of different classes of lipoproteins: very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) (5-9). LDL cholesterol is thought to be the most important contributor to the formation of atherosclerotic plaques, whereas HDL cholesterol is considered to have a reducing effect on the cholesterol concentration in the blood (10).

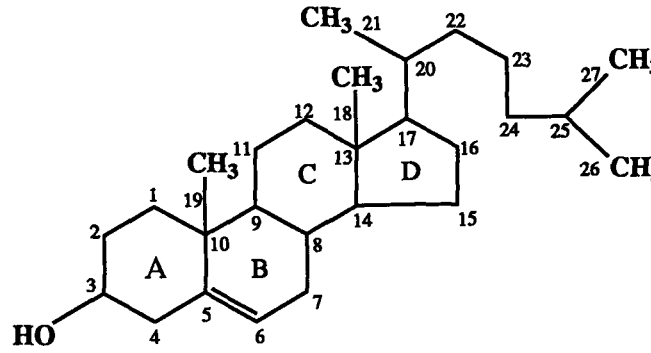


Fig. 1 Molecular structure of cholesterol. The numbering of the respective carbon atoms is indicated.

Cholesterol homeostasis is maintained through the coordinate regulation of uptake, synthesis and catabolism of cholesterol (11). Fig. 2 shows a schematic representation of cholesterol metabolism in rat liver. Two pathways supply cholesterol to cells: an endogenous biosynthetic route, in which acetate is converted into cholesterol, and an exogenous route, in which members of the low density lipoprotein receptor family bind and internalise cholesterol-carrying particles from the blood (12). Catabolism of cholesterol involves the conversion of cholesterol into bile acids (13). Formation of bile is considered to be the only way by which mammals can excrete cholesterol, either in the form of unchanged sterol, or after its conversion into bile acids (14,15).

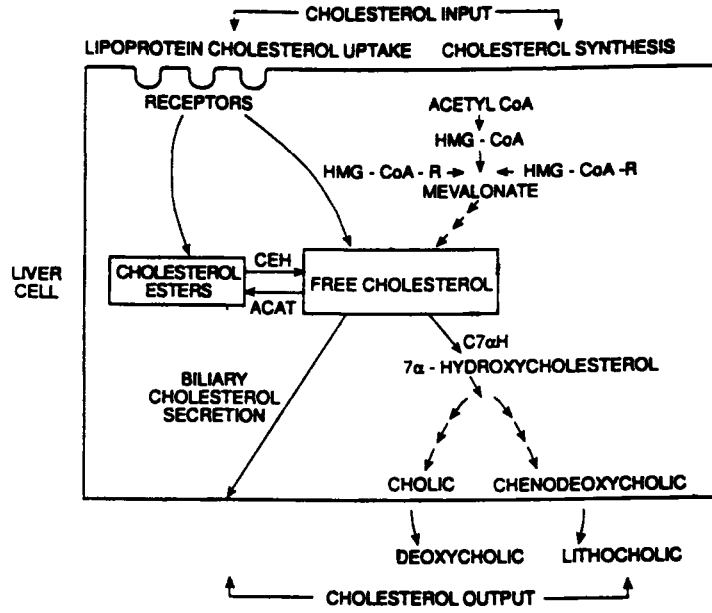


Fig. 2 Input and output pathways of cholesterol metabolism in the liver. Input pathways consist of uptake of lipoprotein cholesterol via specific receptors plus *de novo* synthesis of cholesterol. The output pathways consist of bile acid synthesis from cholesterol and biliary cholesterol secretion. Cholesterol in the liver exists in the form of metabolically active free cholesterol and cholesterol ester (a storage form). At least four enzymes participate in the maintenance of cholesterol homeostasis in the liver: HMG-CoA reductase (HMG-CoA-R), the rate-limiting enzyme in the cholesterol biosynthetic pathway; cholesterol 7α-hydroxylase (CYP7A), the rate-limiting enzyme in the bile acid biosynthetic pathway; acyl CoA-cholesterol acyltransferase (ACAT), which esterifies free cholesterol; and cholesteryl ester hydrolase (CEH), which hydrolyses cholesterol esters. The activity of these four enzymes plus LDL receptors regulate cholesterol homeostasis in the liver under physiological and pathophysiological conditions. Primary bile acids and bile acids returning from the intestine via the enterohepatic circulation (EHC) down-regulate CYP7A and HMG-CoA-R activities, whereas free cholesterol up-regulates CYP7A activity. The multiple steps in the cholesterol and bile acid biosynthetic pathways are depicted with multiple arrows, since a large number of intermediary reactions are involved.

The conversion of cholesterol into bile acids is a process comprising a sequence of metabolic reactions taking place exclusively in the liver. Fig. 3 illustrates the main cholesterol-catabolic pathways in the rat. The biosynthesis of bile acids from cholesterol requires at least 14 different enzymes located in the cytosol, microsomes, mitochondria and peroxisomes (16, 17). The main pathway is termed the neutral route, which is initiated by the introduction of a hydroxyl

group in the cholesterol molecule, at position 7 in the α-orientation, forming 7α-hydroxycholesterol. This reaction is catalysed by a microsomal P-450 isoenzyme, called cholesterol 7α-hydroxylase (CYP7A; 18). CYP7A is believed to be the rate-limiting and most highly regulated enzyme in the neutral pathway of bile acid biosynthesis (15). The second step in this pathway is catalysed by the microsomal 3β-hydroxy-Δ5-C-27 steroid oxidoreductase. The intermediate 7α-hydroxy-4-cholesten-3-one can either be 12α-hydroxylated or reduced to 7α-hydroxy-5β-cholestan-3-one by the enzyme Δ4-3-ketosteroid-5β-reductase (19, 20). This enzyme also catalyses the conversion of the product of 12α-hydroxylation, 7α,12α-dihydroxy-4-cholesten-3-one. All subsequent enzymatic reactions are shared by the two intermediates. A soluble 3α-hydroxysteroid dehydrogenase catalyses both oxidation and reduction of a number of different substrates (21). Final step in the neutral route involves the removal of the side chain, a process taking place mainly in the peroxisomes (22). Prior to their secretion from the hepatocyte, the bile acids cholic acid and chenodeoxycholic acid are conjugated at position C-24 to either glycine or taurine (23).

In the alternative acidic route, side-chain degradation precedes chemical modification of the steroid nucleus, forming acidic intermediates. Therefore, this pathway leading to the formation of chenodeoxycholic acid, has been termed acidic pathway (17). The first reaction in this bile acid-synthetic route is catalysed by the mitochondrial sterol 27-hydroxylase (24,25). 27-Hydroxycholesterol is subsequently metabolised to 3β-hydroxy-5-cholenoic acid (26), a reaction taking place in the peroxisomes of rat liver, eventually leading to the formation of chenodeoxycholic acid (27-30). As stated before, the neutral bile acid-biosynthetic pathway is considered to be the main route under 'normal' conditions (31,32). However, it has been shown that the acidic route is contributing considerably in the production of bile acids, and, under pathological conditions, may even become the major supplier of bile acids (17).

Together with cholesterol, phospholipids and electrolytes, bile acids are secreted in the form of bile. In man and pig, bile is stored in the galbladder, in contrast to the situation in the rat (11,33). Bile is postprandially discharged into the duodenum, where bile acids play an important role in the solubilisation of fats and cholesterol, enhancing their uptake by enterocytes (34). Secondary bile acids are formed from primary bile acids within the intestinal tract by the action of intestinal bacteria. Deoxycholic acid and lithocholic acid are synthesized from cholic acid and chenodeoxycholic acid respectively, by 7α-dehydroxylation. Approximately 95% of the bile acids is reabsorbed in the terminal ileum and transported back to the liver via the enterohepatic circulation (EHC), either by passive diffusion or by active transport over the intestinal membrane (33). As a consequence of the EHC, the bile acid

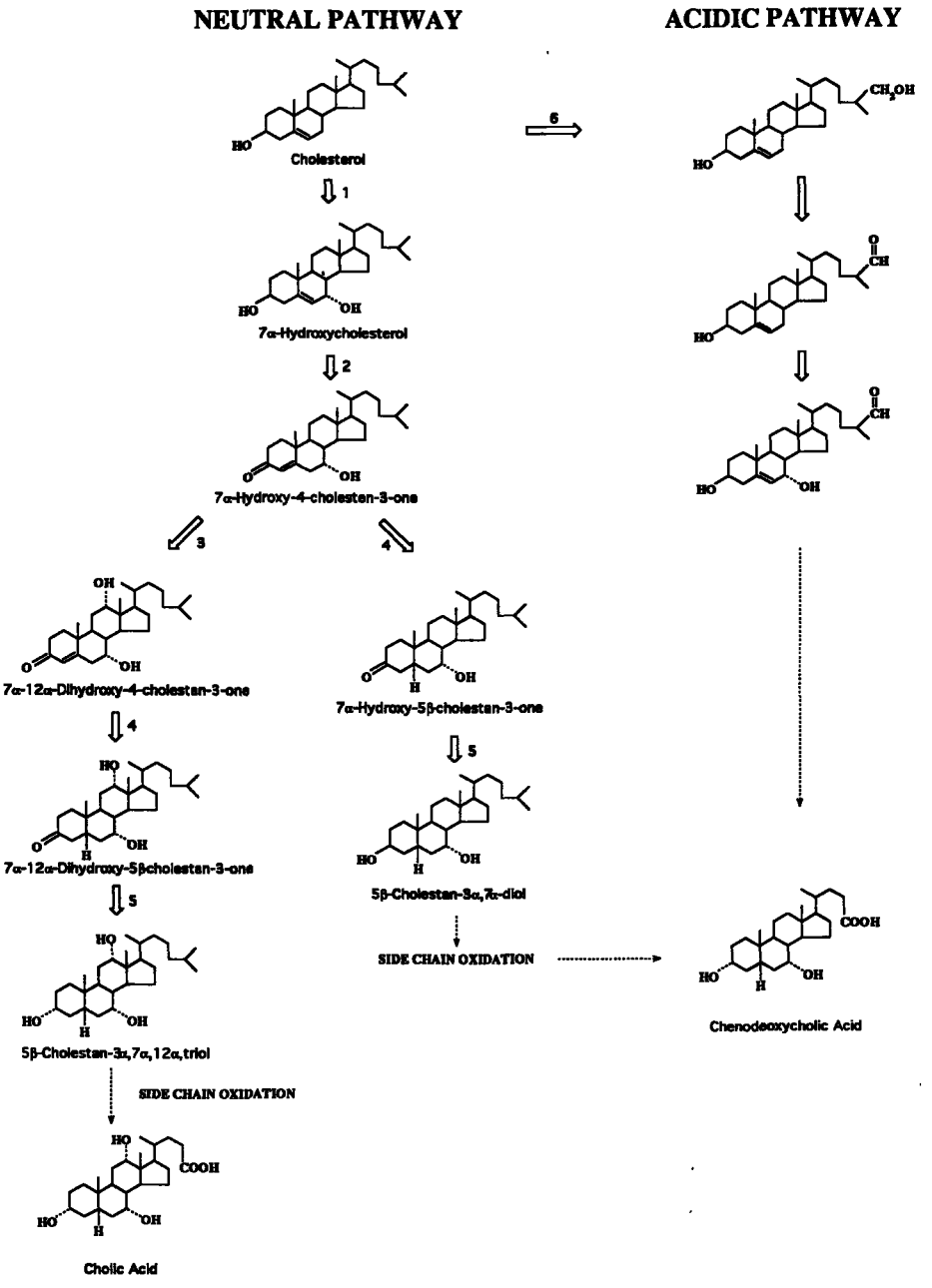


Fig. 3 Neutral and acidic pathways in the biosynthesis of bile acids in the rat. See text for details. 1, Cholesterol 7α-hydroxylase (CYP7A); 2, 3β-Hydroxy-Δ^5C_{27}-steroid oxidoreductase; 3, Sterol 12α-hydroxylase; 4, Δ^4-3-Ketosteroid-5β-reductase; 5, 3α-Hydroxysteroid-dehydrogenase; 6, Sterol 27-hydroxylase.

synthesis -and subsequently the breakdown of cholesterol- is suppressed. This knowledge has been successfully used in the treatment of hypercholesterolemic patients. Cholesterol-catabolism was enhanced by the interruption of the EHC, either by ileal bypass, or by administration of the bile acid-binding resins cholestyramine or cholestid (35-38). This therapy was successful in lowering serum cholesterol levels in a number of clinical trials and studies. However, the effect appeared to be limited (serum cholesterol dropped only 20-30%) and the drug had a poor patient acceptibility (39,40). Therefore, in the last decade many studies have been undertaken in order to develop more efficient regulators of the cholesterol-metabolism.

1.2 Liver morphology and bile acid synthesis

The liver is composed of liver lobules (41), with blood coming in at the corners through the portal veins and hepatic arteries , and flowing towards the central vein via the sinusoids (Fig. 4). The portal canals consist of small branches of the portal vein, the hepatic artery and the bile duct, whereas the central vein is a terminal branch of the hepatic vein. Within a lobule the parenchymal liver cells (*i.e.* hepatocytes) are arranged in plates situated radially towards the central vein. These plates of hepatocytes are interconnected, forming a three-dimensional network. Fenestrated endothelium cells form the wall of the sinusoids, while Kupfer cells are located in the sinusoids. The so-called Ito-cells, being fat-storing cells, are found in the spaces between the sinusoids and the hepatocytes. Bile acids are synthesized in the hepatocytes, and are then secreted into the bile canaliculi and transported to the hepatic duct. On the other hand, bile acids returning from the intestine to the liver are reabsorbed by the parenchymal liver cells where they actively suppress bile acid formation.

Apart from cholesterol metabolism, the liver plays an important role in the homeostatic regulation of carbohydrates, amino acids and lipids. Directly linked to this multifunctional role of the liver, a large degree of heterogeneity in uptake, storage, conversion and release of various compounds along the portocentral axis appears to exist (42-45). It has been postulated that the heterogeneous acinar distribution of liver-specific enzymes is a major determinant for efficient regulation of several liver-specific functions (46). The importance of heterogeneity within the liver in relationship to the regulation of bile acid biosynthesis, will be discussed in Chapter 6.

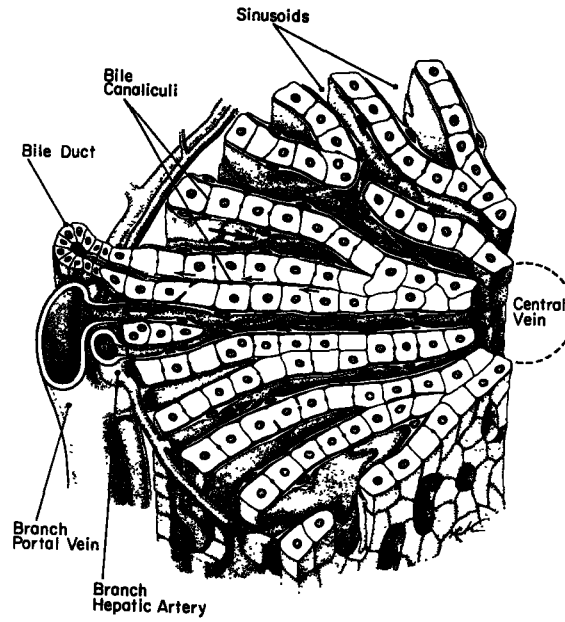


Fig. 4 Diagram representing the radial architecture of parenchymal cells, sinusoids and bile canaliculi, situated around the central vein. Large arrows indicate the portocentral flow of sinusoidal blood, originating from afferent portal veins (carrying nutrients from the intestine) and hepatic arteries (providing oxygen); small arrows represent centrifugal flow of bile towards the bile ducts. Reprinted from Bloom *et al.* (1975) A textbook of Histology.

1.3 Cholesterol 7α-hydroxylase

As mentioned above, the first and rate-limiting step in the conversion of cholesterol into bile acids, *viz.* the 7α-hydroxylation of cholesterol, is catalysed by the P-450 isoenzyme cholesterol 7α-hydroxylase (CYP7A). CYP7A is located in the endoplasmatic reticulum of the liver cell and a member of the P-450 superfamily (47). In general, these enzymes catalyse oxidative reactions in which one atom of an oxygen -molecule is introduced into the substrate, the other atom being reduced to water. CYP7A functions as a terminal oxidase in an electron-transport pathway, in which NADPH is the electron-donor, and electrons are subsequently transferred to the cytochrome P-450 by NADPH-cytochrome P-450 reductase (47). In 1985 Andersson *et al.* purified the protein to near homogeneity (48). Later, the enzyme was obtained in sufficient quantities, to allow sequencing the protein, producing antibodies against

it and cloning the corresponding gene (49-57). In the rat, the CYP7A protein consists of 503 amino acids and has a molecular weight of 57 KD. It consists of a highly hydrophobic aminoterminal membrane-anchoring region, a heme-binding domain (generally conserved in P-450 enzymes), and a sterol-binding site. These regions were shown to be completely identical in human, rat and hamster. Amino-acid comparison with other P-450 enzymes revealed a low degree of homology. Therefore, CYP7A could not be classified as a member of any of the known P-450 gene families as listed by Nebert *et al.*(58), and was considered to constitute a novel P-450 family (CYP7) (49,59).

In 1989 Noshiro *et al.* reported the isolation of the rat CYP7A cDNA from a rat liver cDNA library using specifc antibodies (50). Subsequently, the genes encoding rat, human, hamster and mouse CYP7A have been cloned and characterised (56, 60-62). The rat gene, present in a single copy in the genome, spans 11 kilobases (kb), comprising six exons. Northern-blotting experiments revealed the presence of multiple mRNAs, only detectable in the liver. Four different CYP7A mRNAs, with reported lengths of 1.8, 2.1, 3.6 and 4.0 kb, could be demonstrated *in vivo* and in primary cultures of rat hepatocytes (53,63). This variability in mRNA-length is a consequence of multiple poly adenylation sites present in the 3'-untranslated region (UTR), generating mRNAs of variable size. The mRNA half-life has been shown to be about four hours (63). This, for eukaryotic mRNAs, relatively short half-life is thought to be linked with the presence of several AU-rich sequences in the 3'-UTR, known to enhance mRNA-degradation (64-66).

2.1 CYP7A REGULATION

The major pathway responsible for elimination of cholesterol from the mammalian body, involves the degradation of cholesterol to bile acids. The enzyme catalysing the first and rate-limiting reaction in the 'neutral' bile acid biosynthetic pathway, CYP7A, has been reported to display a response to various physiological signals (67). The most important type of regulation is thought to be exerted by bile acids returning from the gut to the liver, where they actively suppress CYP7A-activity. This negative feedback control is a long term regulation, requiring several days to be established fully. Furthermore, CYP7A-activity is known to display a diurnal rhythm, with -for rat- high enzyme activity at night and low enzyme activity during the day. Finally, CYP7A is responsive to short term regulation by various hormones and dietary factors (for instance cholesterol-feeding), which can modulate CYP7A-activity in a few hours. These different types of CYP7A-regulation will be discussed in more detail below.

2.2 Regulation by bile acids

In 1957 the concept of bile acid-induced feedback suppression of the cholesterol breakdown was postulated by Eriksson *et al* (68). As stated before, bile acid-production in the rat was shown to be stimulated either by diversion of the biliary tract, or by administration of bile acid-binding resins, preventing bile acids from returning to the liver (69-72). Moreover, when bile acids were injected intraduodenally or intravenously in biliary diversed rats, the synthesis of bile acids was normalised (73). Interestingly, biliary drainage and cholestyramine treatment stimulated CYP7A-activity up to 8-fold, whereas the activities of the enzymes catalysing subsequent reactions in the formation of bile acids were either much less or not affected at all (69). Experiments performed by Shefer *et al.*, showing the inhibition of conversion of radiolabeled mevalonate and cholesterol, but not of 7α-hydroxycholesterol, after intraduodenal infusion of the bile acid taurocholate, confirmed the hypothesis of 7α-hydroxylation being the major site of feedback regulation (14). It is now believed that in man too, bile acid biosynthesis is regulated mainly via CYP7A.

However, it was not clear whether bile acids had to be regarded as the actual regulators of CYP7A-activity. The possible involvement of unknown intestinal factors entering the liver in an EHC-dependent manner, could not be excluded. This idea was supported by the initial failure to detect any effect of bile acids on the bile acid biosynthesis in rat hepatocytes in suspension, or in monolayer cultures of rat and rabbit (74-77). In 1988, Kwekkeboom *et al.* demonstrated an *in vivo*-like negative CYP7A feedback control by bile acids in primary

cultures of pig hepatocytes (78). Later, Twisk *et al.* showed that addition of physiological concentrations of bile acids to the culture medium of primary rat hepatocytes, resulted in a strong decrease of CYP7A-activity, steady-state mRNA levels and gene transcription (63).
Although it is now well established that bile acids directly suppress CYP7A-activity, the inhibiting capacity differs from one bile acid to the other (79). It has been postulated that the degree of hydrophobicity is determining the bile acid-suppressing potential (80,81). This view was substantiated by experiments using rats fed with different bile acids. Relatively hydrophobic bile acids, such as cholate, chenodeoxycholate and deoxycholate had a clear suppressing effect on CYP7A-activity in the order of increasing hydrophobicity, whereas relatively hydrophilic bile acids (ursocholate, ursodeoxycholate, hyocholate and hyodeoxycholate) did not affect CYP7A-activity. However, because only a limited number of bile acids were used, a statistically relevant correlation between the degree of hydrophobicity and CYP7A-inhibitory effect could not be established.

2.3 Diurnal variation

In the rat, bile acid biosynthesis and CYP7A activity have been shown to undergo strong diurnal changes, reaching a maximum at mid-dark and falling to a minimum during the day (15,51,64). In man, probably as a result of an opposite waking and feeding pattern, production of bile acids peaks at day-time (9 a.m.) (82). The peak in CYP7A activity parallels a maximum of CYP7A steady-state mRNA levels and protein mass.
These diurnal changes have been linked directly to a regulation by hormones. Since the observed variations follow the circadian pattern of the plasma corticosteron levels, the diurnal rhythm of bile acid synthesis has been proposed to be regulated by glucocorticoids (83). Interestingly, diurnal changes are abolished by adrenalectomy, indeed suggesting that glucocorticoids, directly or indirectly, play an important role in this phenomenon (84). In addition, when the secretion of adrenocorticotrophine (ACTH) was suppressed, diurnal variation of CYP7A activity was totally abolished (85). Recently, Lavery and Schibler reported that the circadian cycle of rat CYP7A gene-transcription probably involves the liver-enriched DBP (albumin element D binding protein) transcriptional factor (86). It was suggested that DBP-transcription itself is directly regulated by corticosteron levels. The daily fluctuations of CYP7A activity would then be the consequence of a DBP-mediated transcriptional regulation. However, the precise molecular mechanisms underlying CYP7A diurnal variations still have to be elucidated.

2.4 Hormonal Regulation

Several hormones have been implicated in the regulation of CYP7A activity using both *in vivo* and *in vitro* models (ref. 15 for a review). Glucocorticoids, as already suggested above, are thought to be involved in the specific up-regulation of CYP7A activity. It was shown that adrenalectomy or administration of corticosteroids causes a rise in bile acid synthesis and CYP7A activity (84). Princen *et al.* demonstrated that dexamethasone and other members of the glucocorticoid family stimulate CYP7A activity in primary cultures of rat hepatocytes (87). Interestingly, administration of both dexamethasone and the thyroid hormone T4 were shown to have a strong, synergistical up-regulating effect on CYP7A activity, indicating a hormonal cross-talk between glucocorticoids and thyroid hormones (88). Thyroid hormone has also been reported to increase CYP7A activity *in vivo* (89). Thyroidectomy decreases enzyme activity, whereas Ness *et al.* reported a rapid increase in CYP7A mRNA levels in hypophysectomised rats following injection of thyroid hormones (90). The pancreas-derived hormones insulin and glucagon also are known to have a clear effect on bile acid synthesis. Patients suffering from diabetes mellitus appeared to have an abnormal high level of bile acids (91). After treatment with insulin these levels are normalised, indicating a role for this hormone in the regulation of bile acid biosynthesis. Later, insulin was shown to actively suppress CYP7A steady state mRNA levels and mRNA synthesis (92). Moreover, glucagon was reported to down-regulate CYP7A activity in primary cultures of rat hepatocytes (93). It is not yet known whether these hormonal effects are direct or indirect, but the available data suggest that several hormones are capable of regulating CYP7A gene expression.

2.5 Regulation by cholesterol

Different lines of evidence indicate that cholesterol, being the substrate of the CYP7A enzyme, plays a role in the biosynthesis of bile acids. Several groups of investigators reported that the administration of cholesterol to the diet of rats, resulted in stimulation of CYP7A-activity (53,54,94,95). It remains unclear, however, whether cholesterol exerts this effect directly or indirectly, since it was shown that the actual delivery of cholesterol to the liver is not required for CYP7A up-regulation (96). Furthermore, it has been reported that specific cholesterol-rich lipoproteins are capable of up-regulating CYP7A-activity, mRNA levels and gene transcription *in vitro* (97). In contrast to βVLDL, the lipoproteins LDL and HDL were found not to have a CYP7A stimulating effect. Without any doubt exogenous cholesterol is involved in the regulation of bile acid biosynthesis via CYP7A. On the other hand, the effect of endogenous

(*de novo*) cholesterol is controversial. Data obtained with rats supplemented with the cholesterol-precursor mevalonate or HMG-CoA reductase inhibitors (e.g. lovastatin) appeared to be contradictory (98,99). Therefore, it is still not clear whether endogenous cholesterol exerts an effect on CYP7A regulation.

3.1 CYP7A TRANSCRIPTIONAL REGULATION

At the time these investigations were started, little was known about CYP7A transcriptional regulation. It was only at the end of 1989 that Noshiro *et al.* succeeded in isolating the rat CYP7A cDNA (50). This was the beginning of extensive investigations by several groups to elucidate the molecular mechanisms underlying CYP7A gene expression. By the end of 1990 it was clear that CYP7A activity is mainly regulated at the level of transcription. More specifically, all abovementioned types of regulation had been shown to be primarily transcription-mediated processes. In the next section general aspects of transcriptional regulation in eukaryotes will be discussed briefly.

3.2 Transcriptional regulation in eukaryotes

Essentially, transcriptional control of eukaryotic genes is the result of a complex network of interactions between regulatory DNA-elements (*cis*-acting elements) and site specific DNA-binding proteins (*trans*-acting factors). The upstream regions of genes transcribed by RNA polymerase II (class II genes), usually contain two different *cis*-elements: the TATA box and the enhancer elements.

The TATA box, present in most of the promoters of genes transcribed by RNA polymerase II, is usually located about 30 bp upstream from the transcriptional start site. Several studies indicate that the TATA box is an essential element both for the basal activation of transcription and for determining the exact point of transcription initiation (100,101). The fact that in some cases the TATA box is the only element required for a basal level of accurate transcription, indicates that this *cis*-element can be classified as a core promoter element, and that cellular factors exist with an intrinsic capacity to functionally interact with this element. Indeed, it was shown that initiation of mRNA synthesis in TATA box containing promoters requires the formation of a multiprotein preinitiation complex in the vicinity of the TATA box and the transcriptional start site of the gene (102,103). The complex consists of Polymerase II and several general initiation factors: transcription factor IIA (TFIIA), TFIIB, TFIID, TFIIE, TFIIF, TFIIH and TFIIJ. Assembly of the preinitiation complex starts with the binding of TFIID to the TATA element. Subsequently, the other general initiation factors and RNA polymerase II join the complex in an ordered fashion (104,105). Interestingly, TFIID appeared to be the only general factor binding directly to the TATA box. It is clear now that TFIID is a complex of multiple subunits, consisting of a so-called TATA box binding Protein (TBP) and a variety of tightly bound TBP-associated factors (TAFs) (106,107).

Unlike the TATA element, enhancers affect transcription in an orientation- and position-independent manner. Functional enhancer elements were found in positions far up- or downstream (several kb) from the transcription initiation site of several genes. The actual effect on transcription is exerted by transcriptional activator or repressor proteins binding to the enhancer elements. Whereas the preinitiation complex formed at the core promoter element determines accurate, basal transcription, proteins interacting with enhancer elements promote or repress transcription. To execute their regulatory function, these proteins interact, directly or indirectly, with one or more of the factors forming the preinitiation complex or with RNA polymerase itself (108-110). In this way enhancers can confer either cell-type specific transcription or act in response to exogenous physiological signals, *e.g.* steroid hormones and heavy metals.

An important part of transcriptional control in eukaryotes is considered to take place through interactions between regulatory proteins binding upstream (promoter) elements and general transcription factors forming the preinitiation complex (PIC) in the vicinity of the transcription initiation point (see Fig.5). These protein-protein contacts may affect PIC-formation (assembly) or PIC-function, as a consequence of which transcription of specific genes is controlled in a positive or negative way (111,112). Over the past several years it has become clear that activator-proteins contain both site-specific DNA binding domains, which anchor them to distinct upstream motifs, and transcription activation domains which directly or indirectly interact with the general transcriptional machinery (113). Three different types of activation domains have been identified sofar: acidic, glutamine-rich and proline-rich. Recent studies suggest that each type of activation domain has its own specific mode of interaction with different targets (114). Consistent with its central role in the assembly of the preinitiation complex, TFIID was implicated as a main target for activators. Indeed, several *in vitro* studies showed direct and indirect contacts between TFIID and different activator-proteins, such as the yeast GAL4 and the HSV-VP16 (115,116). However, the exact site of binding within TFIID has yet to be elucidated. More recent studies provided evidence for a role of the so-called TAF's (TBP associated factors), as a link between activators and the basal transcription complex (114,117,118). Besides TFIID, the general transcription factors TFIIB, as well as TFIIA and TFIIH have also been suggested as targets for specific activators, amongst others the abovementioned GAL4 and VP16 proteins, known to be involved in TFIID-mediated transcriptional control. The picture emerging now is a complex one, in which transcriptional activators, after binding to specific upstream sequences, contact -directly or indirectly- several general transcription factors.

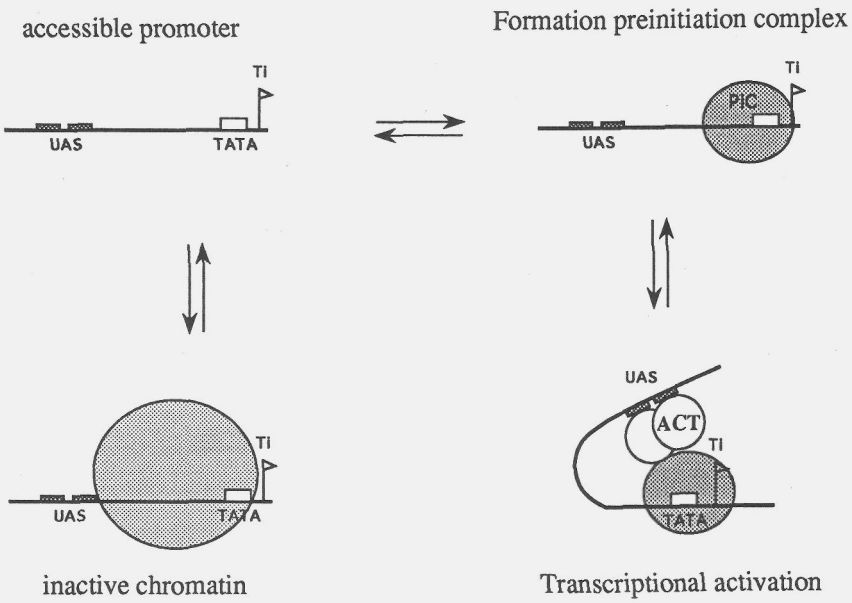


Fig. 5 Schematic representation of different stages before activation of eukaryotic gene transcription. In the so-called 'ground-state', the promoter region is incorporated into a nucleosome or a higher order chromatin structure. It is imagined that escape from the inactive chromatin state could be achieved by interactions of transcription factors with DNA elements either when present in nucleosomes or following transient structural chromatin modifications presenting a more accessible promoter. Two states of activation are indicated ; a basal state which results from the intrinsic ability of general initiation factors to form an active preinitiation complex (PIC), and an activated state, which results from the ability of activators (ACT) binding to upstream activating sequences (UAS), to increase the rate and extent of preinitiation complex formation. The extent of basal transcription and of activator-dependent transcription will depend on the effective concentrations and activities of the various positive and negative factors, which collectively determine the actual level of transcription.

As depicted in Fig. 5, an additional general mechanism to control eukaryotic gene-transcription involves the incorporation of DNA into nucleosomes and higher order chromatin structures (119,120). Eukaryotic DNA is packaged by multiple levels of chromatin condensation, the nucleosomal core particle being the building block of the eukaryotic chromosome. Each nucleosome contains approximately 160 bp of DNA wound in two turns around a histone

octamer (a tetramer of histones H3 and H4 and two histone H2A-H2B dimers), whereas a single histone H1 is involved in folding the linker DNA located between two subsequent nucleosomes. In turn, nucleosomes are packaged in higher order chromatin. As described above, transcription of eukaryotic genes is controlled by factors binding regulatory DNA elements. In general, the assembly of eukaryotic promoters into nucleosomes prevents the binding of these regulatory and basal transcription factors, in this way inhibiting gene transcription. However, it is now clear that chromatin is present in the cell in a more or less dynamic state, allowing transient access to transcription factors upon modification or positioning of specific nucleosomes. It has long been known that nucleosome depletion in yeast results in high levels of transcription from many genes (121,122). More recent studies revealed that nucleosomes are involved not only in repression of transcription, but also can facilitate transcription-initiation (123). The data obtained thusfar envision a central role for the so-called 'positioned nucleosome' (124). In a positioned nucleosome regulatory DNA-elements which are accessible for *trans*-acting factors are exposed to the surrounding solution or are located in the linker DNA, connecting subsequent nucleosomes. On the other hand, motifs orientated towards the nucleosomes are not available for protein-contacts. However, these protected DNA-elements can be 'freed' from the nucleosome by a relative simple post-translational modification or dissociation of a specific histone. Moreover, folding of DNA segments can bring widely separated regulatory elements in close proximity, as a consequence of which activation of genes is facilitated.

4.1 Primary cultures of rat hepatocytes as a model sytem

In our study we have used primary cultures of rat hepatocytes as an *in vitro* model system. Previous work has demonstrated that cultured pig and rat hepatocytes exhibit *in vivo* liver-specific characteristics, when cultured under well-specified conditions (63,78,87,125). In particular, bile acid-synthesis and CYP7A-activity were shown to be responsive to the addition of bile acids and hormones to the culture medium. Above all, the system of primary rat hepatocytes in culture has the advantage that the effects of regulatory agents on the parenchymal cells can be examined independent of effects exerted by other factors.

4.2 Aim and outline of the study

The aim of the study reported in this thesis is to gain further insight in the molecular mechanisms underlying CYP7A gene expression in the rat, through the identification and characterisation of *cis*-acting elements and *trans*-acting factors involved in the transcriptional regulation of this gene. In Chapter 2 and 3 we present the structural and functional analysis of the proximal part of the CYP7A promoter in the rat. Through transient expression experiments and *in vitro* protein-DNA analysis, we were able to localise a distinct *cis*-acting element, present in the proximal promoter and involved in the activation and regulation of the CYP7A transcription. Transcriptional regulation of CYP7A upon the addition of bile acids and insulin to the culture-medium, is described in Chapters 4 and 5. In Chapter 6 *in situ* hybridisations are shown, demonstrating a heterogeneous acinar distribution of the CYP7A gene expression in rat liver.

REFERENCES

1. Kannel, W.B., Castelli, W.D., and McNamara, P.M. : Serum cholesterol, lipoproteins, and the risk of coronary heart disease: The Framingham Study. Ann. Int. Med. 74 (1971) 1-12.
2. Martin, M.J., Hulley, S.B., Browner, W.S., Kuller, L.F., and Wentworth, D. : Serum cholesterol, blood pressure, and mortality: implications from a cohort of 361662 men. Lancet 2 (1986) 933-936.
3. Castelli, W.P. : The triglyceride issue, a view from Framingham. Am. Heart J. (1986) 432-437.
4. Austin, M.A. : Plasma triglyceride and coronary heart didease. Arteriosclerosis 11 (1991) 2-14.
5. Davis, R.A. : Lipoprotein structure and secretion. In: Biochemistry of lipids, lipoproteins and membranes. New York Comprehensive Biochemistry Vol.20, Neuberger, A. and van Deenen, L.L.M., eds., Elsevier Science Publishers B.V., Amsterdam (1991) 403-426.
6. Eisenberg, S. : High density lipoprotein metabolism. J. Lipid Res. 25 (1984) 1017-1058.
7. Havel, R.J., and Kane, J.P. : Introduction, structure and metabolism of plasma lipoproteins. In: The metabolic basis of inherited disease. 6th ed New York, NY, McGraw-Hill (1989) 1129-1138.
8. Gibbons, G.F. : Assembly and secretion of hepatic very low density lipoprotein. Biochem. J. 268 (1990) 1-13.
9. Johnson, W.J., Mahlberg, F.H., Rothblat, G.H. and Philips, M.C. : Cholesterol transport between cells and high density lipoproteins. Biochim. Biophys. Acta 1085 (1991) 273-298.
10. Gordon, T., Castelli, W.P., Hjortland, M.C., Kammel, W.B., and Dawber, T.R. : High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. Am. J. Med. 62 (1977) 707-714.
11. Turley, S.D., and Dietschy, J.M. : Cholesterol metabolism and excretion. In: The liver, biology and pathology. Arias, I.M., Jakoby, W.B., Popper, H., Schachter, D., and Shafritz, D.A., eds., New York, Raven Press (1988) pp 467-492.
12. Rilling, H.C., and Chayet, T. In : Sterols and bile acids. Danielsson, H. and Sjövall, J., eds., Elsevier, Amsterdam (1985) pp 1-39.
13. Danielsson, H., and Sjövall, J. : Bile acid metabolism. Ann. Rev. Biochem. 44 (1975) 233-253.
14. Shefer, S., Hauser, S, Bekersky, I., and Mosbach, E.H. : Biochemical site of regulation of bile acid synthesis in the rat. J. Lipid Res. 11 (1970) 404-411.
15. Myant, N.B., and Mitropoulos, K.A. : Cholesterol 7α-hydroxylase. J. Lipid Res. 18 (1977) 135-152.
16. Danielsson, H., and Einarsson, K. In: The biological basis of medicine. Bitten, E.E., and Bittar, N., eds., Acad. Press (1969) pp 279-314.
17. Axelson, M, and Sjövall, J. : Potential bile acid precursors in plasma, possible indicators of biosynthetic pathways to cholic acid and chenodeoxycholic acid in man. J. Steroid Biochem. 36 (1990)

631-640.

18. Gonzalez, F.J. : The molecular biology of cytochrome P450s. Pharmacol. Rev. 40 (1989) 243-288.

19. Furuebisu, M., Deguchi, S., and Okuda, K. : Identification of cortisone 5β-reductase. Biochim. Biophys. Acta 912 (1987) 110-114.

20. Onishi, Y., Noshiro, M., Shimasato, T., and Okuda, K. : Molecular cloning and sequence analysis of cDNA encoding Δ4-3-ketosteroid-5β-reductase of rat liver. FEBS Lett. 283 (1991) 215-218.

21. Penning, T.M., Abrams, W.R., and Pawlowski, J.E. : Affinity labeling of 3α-hydroxysteroid dehydrogenase with 3α-bromoacetoxyandrosterone and 11α-bromoacetoxyprogesterone. Isolation and sequence of active site peptides containing reactive cysteines; sequence confirmation using nucleotide sequences from a cDNA clone. J. Biol. Chem. 266 (1991) 8826-8834.

22. Wikvall, K. : Hydroxylations in biosynthesis of bile acids. Isolation of cytochrome P-450 from rabbit liver mitochondria catalysing 26-hydroxylation of C-27 steroids. J. Biol. Chem. 259 (1984) 3800-3804.

23. Pedersen, J.I., and Gustafsson, J. : Conversion of 3α,7α,12α-trihydroxy-5β-cholestanoic acid into cholic acid by rat liver peroxisomes. FEBS Lett. 121 (1980) 345-348.

24. Björkhem, I., Gustafsson, J., Johansson, G., and Persson, B. : Biosynthesis of bile acids in man: hydroxylation of the C-27 steroid side chain. J. Clin. Invest. 55 (1975) 478-486.

25. Mitropoulos, K.A., Avery, M.D., Myant, N.B., and Gibbons, G.F. : The formation of cholest-5-en-3β,26-diol as an intermediate in the conversion of cholesterol into bile acids by liver mitochondria. Biochem. J. 130 (1972) 363-371.

26. Krisans, S.K., Thompson, S.L., Pena, L.A., Kok, E, and Javitt, N.B. : Rat liver synthesis in rat liver peroxisomes: metabolism of 26-hydroxycholesterol to 3β-hydroxy-5-cholestenoic acid. J. Lipid. Res.26 (1985) 1324-1332.

27. Danielsson, H. : Formation and metabolism of 26-hydroxycholesterol. Ark. Kemi 17 (1961) 373-379.

28. Wachtel, N., Emerman, S., and Javitt, N.B. : Metabolism of cholest-5-ene-3β,26diol in the rat and the hamster. J. Biol. Chem. 243 (1968) 5207-5212.

29. Anderson, K.E., Kok, E., and Javitt, N.B. : Bile acis synthesis in man: metabolism of 7α-hydroxycholesterol-^{14}C and 26-hydroxycholesterol-^{3}H. J. Clinic. Invest. 51 (1972) 112-117.

30. Swell, L., Gustafsson, J, Schwartz, C.C., Halloran, L.G., Danielsson, H., and Vlahcevic, Z.R. : An *in vivo* evaluation of the quantitative significance of several potential pathways to cholic and chenodeoxycholic acids from cholesterol in man. J. Lipid. Res. 21 (1980) 455-466.

31. Princen, H.M.G., Meijer, P., Wolthers, B.G., Vonk, R.J., and Kuipers, F. : Cyclosporine A blocks bile acid synthesis in cultured hepatocytes by specific inhibition of chenodeoxycholic acid. Biochem. J. 275 (1991) 501-505.

32. Dahlbäck-Sjöberg, H., Björkhem, I., and Princen, H.M.G. : Selective inhibition of mitochondrial 27-hydroxylation of bile acid intermediates and 25-hydroxylation of vitamin D3 by cyclosporin A. Biochem. J. 293 (1993) 203-206.

33. Carey, M.C., and Cahalane, M.J. : Enterohepatic circulation. In: The Liver , Biology and Pathology. Arias, I.M., Jakoby, W.B., Popper, H., Schachter, D., Shafritz, D.A., eds., Raven Press, New York (1988) pp. 573-616.

34. Carey, M.C., Small, D.M., and Bliss, C.M. : Lipid digestion and absorption. Annu. Rev. Physiol. 45 (1983) 651-677.

35. Grundy, S.M. : Treatment of hypercholesterolemia by interference with bile acid metabolism. Arch. Intern. Med. 130 (1972) 638-648.

36. Sheperd, J., Packard, C.J., Bicker, S., Lawrie, T.D.V., and Morgan, H.G. : Cholestyramine promotes receptor-mediated low density lipoprotein catabolism. N. Eng. J. Med. 320 (1980) 1219-1222.

37. Kovanen, P.T., Bilheimer, D.W., Goldstein, J.L., Jaramillo, J.J., and Brown, M.D. : Regulatory role for hepatic low density lipoprotein receptors *in vivo* in the dog. Proc. Natl. Acad. Sci. U.S.A. 78 (1981) 1194-1198.

38. Lipid Research Clinics Program : The lipid research clinics coronary primary prevention trial results. J. Am. Med. Assoc. 251 (1984) 365-371.

39. Packard, C.J., and Sheperd, J. : The hepatobiliary axis and lipoprotein metabolism: effects of bile acid sequestrants and ileal bypass surgery. J. Lipid. Res. 23 (1982) 1081-1098.

40. Brown, M.S., and Goldstein, J.L. : In: The pharmalogical basis of therapeutics. Gilman, A.F., Goodman, L.S., Rall, T.W., and Murad, F., eds., MacMillan Publishing Company, New York (1985) 827-845.

41. Lamers, W., Hilberts, A., Furt, E., Smith, J., Jonges, G.N., van Noorden, C.J.F., Gaasbeek Janzen, J.W., Charles, R., and Moorman, A.F.M. : Hepatic enzymatic zonation : A reevaluation of the concept of the liver acinus. Hepatology 10 (1989) 72-76.

42. Jungermann, K., and Katz, N. : Functional hepatocellular heterogeneity. Hepatology 2 (1982) 385-395.

43. Gumucio, J.J., and Miller, D.L. : Liver cell heterogeneity. In: The liver, biology and pathology. Arias, I.M., Jakoby, W.B., Popper, H., Schachter, D., and Shafritz, D.A., eds., New York, Raven Press (1982) pp 647-661.

44. Jungermann, K., and Katz, N. : Functional specialization of different hepatocyte populations. Physiol. Rev. 69 (1989) 708-764.

45. Gebhardt, R. : Metabolic zonation of the liver: regulation and implications for liver function. Pharmac. Ther. 53 (1992) 275-354.

46. Jungermann, K., and Sasse, D. : Heterogeneity of liver parenchymal cells. Trends Biochem. Sci. 3

(1978) 198-202.

47. Hansson, R., and Wikvall, K. : Hydroxylations in biosynthesis and metabolism of bile acids: Catalic properties of different forms of cytochrome P-450. J. Biol. Chem. 255 (1980) 1643-1649.

48. Andersson, S., Boström, H, Danielsson, H., and Wikvall, K. : Purification of cholesterol 7α-hydroxylase from rat liver microsomes. Methods Enzymol. 111 (1985) 364-377.

49. Ogishima, T., Deguchi, S., and Okuda, K. : Purification and characterisation of cholesterol 7α-hydroxylase from rat liver microsomes. J. Biol. Chem. 262 (1987) 7646-7650.

50. Noshiro, M., Nishimoto, M., Morohashi, K., and Okuda, K. : Molecular cloning of cDNA for cholesterol 7α-hydroxylase from microsomes. FEBS Lett. 257 (1989) 97-100.

51. Chiang, J.Y.L., Miller, W.F., and Lin, G.M. : Regulation of cholesterol 7α-hydroxylase in the liver: Purification of cholesterol 7α-hydroxylase and the immunochemical evidence for the induction of cholesterol 7α-hydroxylase by cholestyramine and circadian rhythm. J. Biol. Chem. 265 (1990) 3889 -3897.

52. Nguyen, L.B., Shefer, S., Salen, G., Ness, G.C., Tanaka, R.D., Packin, V., Thomas, P., Shore, V., and Batta, A. : Purification of cholesterol 7α-hydroxylase from human and rat liver and production of inhibiting polyclonal antibodies. J. Biol. Chem. 265 (1990) 4541-4546.

53. Jelinek, D.F., Andersson, S., Slaughter, C.A., and Russell, D.W. : Cloning and regulation of cholesterol 7α-hydroxylase, the rate-limiting enzyme in bile acid biosynthesis. J. Biol. Chem. 265 (1990) 8190-8197.

54. Li, Y.C., Wang, D.P., and Chiang, J.Y.L. : Regulation of cholesterol 7α-hydroxylase in the liver: Cloning, sequencing and regulation of cholesterol 7α-hydroxylase mRNA. J. Biol. Chem. 265 (1990) 12012-12019.

55. Sundseth, S.S., and Waxman, D.J. : Hepatic P-450 cholesterol 7α-hydroxylase. Regulation *in vivo* at the protein and mRNA level in response to mevalonate, diurnal rhythm, and bile acid feedback. J. Biol. Chem. 265 (1990) 15090-15095.

56. Jelinek, D.F., and Russell, D.W. : Structure of the rat gene encoding cholesterol 7α-hydroxylase. Biochemistry 29 (1990b) 7781-7785.

57. Nishimoto, M., Gotoh, O., Okuda, K., and Noshiro, M. : Structural analysis of the gene encoding rat cholesterol 7α-hydroxylase, the key enzyme in bile acid biosynthesis. J. Biol. Chem. 266 (1991) 6467-6471.

58. Nebert, D.W., and Gonzalez, F.J. : P-450 genes: structure, evolution, and regulation. Ann. Rev. Biochem. 556 (1987) 945-993.

59. Waxman, D.J. : Rat hepatic cholesterol 7α-hydroxylase: Biochemical properties and comparison to constitutive and xenobiotic-inducible cytochrome P-450 enzymes. Arch. Biochem. Biophys. 247

(1986) 335-345.

60. Cohen, J.C., Cali, J.J., Jelinek, D.F., Mehrabian, M., Sparkes, R.S., Lusis, A.J., Russell, D.W., and Hobbs, H.H. : Cloning of the human cholesterol 7α-hydroxylase gene (CYP7) and localisation to chromosome 8q11-q12. Genomics 14 (1992) 153-161.

61. Crestani, M., Galli, G., and Chiang, J. Y. L. : Genomic cloning, sequencing, and analysis of the hamster cholesterol 7α-hydroxylase gene (CYP7). Arch. Biochem. Biophys. 306 (1993) 451-460.

62. Tzung, K., Ishimura-Oka, K., Kihara, S., Oka, K., and Chan, L. : Structure of the mouse cholesterol 7α-hydroxylase gene. Genomics 21 (1994) 244-247.

63. Twisk, J., Lehmann, E.M., and Princen H.M.G. : Differential feedback regulation of cholesterol 7α-hydroxylase mRNA and transcriptional activity by rat bile acids in primary monolayer cultures of rat hepatocytes. Biochem. J. 290 (1993) 685-691.

64. Noshiro, M., Nishimoto, M., Morohashi, K., and Okuda, K. : Rat liver cholesterol 7α-hydroxylase. Pretranslational regulation for circadian rhythm. J. Biol. Chem. 265 (1990) 10036-10041.

65. Shaw, G., and Kamen, R. : A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selctive mRNA degradation. Cell 46 (1986) 659-667.

66. Binder, R., Hwang, S.P.L., Ratnasabapathy R., and Williams, D.L. : Degradation of apolipoprotein II mRNA occurs via endonucleotic cleavage at 5'-AUU-3', 5'-UAA-3' elements in single-stranded loop domains of the 3'-noncoding region. J. Biol. Chem. 264 (1989) 16910-16918.

67. Vlahcevic, Z.R., Pandak, W.M., Heuman, D.M., and Hylemon, P.B. : Function and regulation of hydroxylases involved in the bile acid biosynthesis pathways. Seminars in liver disease. vol. 12, no 4 (1992) 403-419.

68. Eriksson, S. : Biliary excretion of bile acids and cholesterol in bile fistula rats. Proc. Soc. Exp. Biol. Med. 94 (1957) 578-582.

69. Danielsson, H., Einarsson, K., and Johansson, G. : Effects of biliary drainage on individual reactions in the conversion of cholesterol to taurocholic acid. Eur. J. Biochem. 2 (1967) 44-49.

70. Thompson, J.C., and Vars, H.M. : Biliary excretion of cholic acid and cholesterol in hyper-, hypo-, and euthyroid rats. Proc. Soc. Exp. Biol. Med. 83 (1953) 246-248.

71. Shefer, S., Hauser, S, Bekersky, I., and Mosbach, E.H. : Feedback regulation of bile acid biosynthesis in the rat. J. Lipid Res. 10 (1969) 646-655.

72. Mosbach, E.H., Rothschild, M.A., Bekersky, I., Oratz, M., and Mongelli, J. : Bile acid synthesis in the isolated, perfused rabbit liver. J. Clin. Invest. 50 (1971) 1720-1730.

73. Pries, J.M., Gustafsson, A., Wiegand, D., and Duane, W.C. : Taurocholate is more potent than cholate in suppression of bile acid synthesis in the rat. J. Lipid Res. 24 (1983) 141-146.

74. Botham, K.M., Lawson, M.E., Beckett, G.J., Percy-Robb, I.W., and Boyd, G.S. : The effect of portal

bile salt concentrations on bile acid synthesis in rat liver. Biochim. Biophys. Acta 666 (1981) 238-245.

75. Davis, R.A., Highsmith, S.E., Malone-McNeal, M., Archambault-Schexnayder, J., Hyde, P.M., and Kuan, J.C.W. : Bile acid synthesis by cultured rat hepatocytes: Inhibition by mevinolin but not by bile acids. J. Biol. Chem. 258 (1983) 4079-4082.

76. Kubaska, W.M., Gurley, E.C., Hylemon, P.B., Guzelian, P.S., and Vlahcevic, Z.R. : Absence of negative feedback control of bile acid synthesis in cultured pig hepatocytes. J. Biol. Chem. 260 (1985) 13459-13463.

77. Whiting, M.J., Wishart, R.A., Lewis, G., and Mackinnon, A.M. : Bile acid synthesis in cultured rabbit hepatocytes: stimulation by three lipoprotein fractions. Biochim. Biophys. Acta 1005 (1989) 137-142.

78. Kwekkeboom, J., Princen, H.M.G., Van Voorthuizen, E.M., and Kempen, H.J.M. : Bile acids exert negative feedback control on bile acid synthesis in cultured pig hepatocytes by suppression of cholesterol 7α-hydroxylase activity. Hepatology 12 (1990) 1209-1215.

79. Heuman, D.M., Hernandez, C.R., Hylemon, P.B., Kubaska, W.M., Hatman, C., and Vlahcevic, Z.R. : Regulation of bile acid synthesis, effects of conjugated ursodeoxycholate and cholate on bile acid synthesis in the chronic bile fistula rat. Hepatology 8 (1988) 358-365.

80. Heuman, D.M., Hylemon, P.B., and Vlahcevic, Z.R. : Regulation of bile acid synthesis, III. Correlation between biliary bile salt hydrophobicity index and the activities of enzymes regulating cholesterol and bile acid synthesis in the rat. J. Lipid Res. 30 (1989) 1161-1171.

81. Stravitz, R.T., Hylemon, P.B., Heuman, D.M., Hagey, L.R., Schteingart, C.D., Ton-Nu, H.T., Hofmann, A.F., and Vlahcevic, Z.R. : Transcriptional regulation of cholesterol 7α-hydroxylase mRNA by conjugated bile acids in primary cultures of rat hepatocytes. J. Biol. Chem. 268 (1993) 13987-13993.

82. Duane, W.C., Levitt, D.G., and Mueller, S.M. : Regulation of bile acid synthesis: Presence of a diurnal rhythm. J. Clinic. Invest. 72 (1983) 1930-1936.

83. Guillemin, R., Dear, W.E., and Liebelt, R.A. : Nychthemeral variations in plasma free corticosteroid levels of the rat. Proc. Soc. Exp. Biol. Med. 101 (1959) 394-395.

84. van Cantfort, J. : Controle par les glucocortico-steroides de l'activité circadienne de la cholesterol 7α-hydroxylase. Biochimie 55 (1973) 1171-1173.

85. Gielen, J., van Cantfort, J., Robaye, B., and Renson, J. : Rhythme circadien de la cholesterol 7α-hydroxylase chez la rat. C. R. Acad. Sci. Paris 269 (1969) 731-732.

86. Lavery, D.J., and Schibler, U. : Circadian transcription of the cholesterol 7α-hydroxylase gene may involve the liver-enriched bZIP protein DBP. Genes and Dev. 7 (1993) 1871-1884.

87. Princen, H.M.G., Meijer, P., and Hofstee, B. : Dexamethasone regulates bile acid synthesis in monolayer cultures of rat hepatocytes by induction of cholesterol 7α-hydroxylase. Biochem. J. 262 (1989) 341-348.

88. Hylemon, P.B., Gurley, E.C., Stravitz, R.T., Litz, J.S., Pandak, W.M., Chiang, J.Y.L., and Vlahcevic, Z.R. : Hormonal regulation of cholesterol 7α-hydroxylase mRNA levels and transcriptional activity in primary rat hepatocyte cultures. J. Biol. Chem. 267 (1992) 16866-16871.

89. Balasubramaniam, S., Mitropoulos, K.A., and Myant, N.B. : Hormonal control of the activities of cholesterol 7α-hydroxylase and hydroxymethyl-glutaryl-CoA reductase in rats. In: Advances in bile acid research, III. Bile Acid Meeting, Freiburg. Matern, S., Hackenschmidt, J., Back, P., and Gerok, W., eds., Schattauer Verlag, Stuttgart (1975) 61-67.

90. Ness, G.C., Pendleton, L.C., Li, Y.C., and Chiang, J.Y.L. : Effect of thyroid hormone on hepatic cholesterol 7α-hydroxylase, LDL receptor, HMG-CoA-reductase, farnesyl pyrophosphate synthetase and apolipoprotein A-I mRNA levels in hypophysesectomized rats. Biochim. Biophys. Res. Commun. 172 (1990) 1150-1156.

91. Bennion, L.J., and Grundy, S.M. : Effects of diabetes mellitus on cholesterol metabolism in man. N. Eng. J. Med. 296 (1977) 1365-1371.

92. Twisk, J., Hoekman, M.F.M., Lehmann, E., Meijer, P., Mager, W.H., and Princen, H.M.G. : Insulin suppresses bile acid synthesis in cultured rat hepatocytes by down-regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase gene transcription (in press, Hepatology 1995).

93. Botham, K.M., Suckling, K.E., and Boyd, G.S. : The effect of glucagon-induced adenosine 3',5'-monophosphate concentrations on bile acid synthesis in isolated rat liver cells. FEBS Lett. 168 (1984) 317-320.

94. Pandak, W.M., Li, Y.C., Chiang, J.Y.L., Studer, E.J., Gurley, E.C., Heuman, D.M., Vlahcevic, Z.R., and Hylemon, P.B. : Regulation of cholesterol 7α-hydroxylase mRNA and transcriptional activity by taurocholate and cholesterol in the chronic biliary diverted rats. J. Biol. Chem. 266 (1991) 3416-3421.

95. Björkhem, I., Eggertsen, G., and Andersson, U. : On the mechanism of stimulation of cholesterol 7α-hydroxylase by dietary cholesterol. Biochim. Biophys. Acta 1085 (1991) 329-335.

96. Björkhem, I., Blomstrand, R., Lewenhaupt, A., and Svensson, L. : Effect of lymphatic drainage on cholesterol 7α-hydroxylase of cholesterol in rat liver. Biochem. Biophys. Res. Commun. 85 (1978) 532-540.

97. Twisk, J., van der Fits, L.T.E., Hoekman, M.F.M., de Wit, E.M.C., Mager, W.H., and Princen, H.M.G. : Selective up-regulation of cholesterol 7α-hydroxylase by βVLDL, and not LDL or HDL in cultured rat hepatocytes (submitted).

98. Pandak, W.M., Vlahcevic, Z.R., Chiang, J.Y.L., Heuman, D.M., and Hylemon, P.B. : Bile acid synthesis. VI Regulation of cholesterol 7α-hydroxylase by taurocholate and mevalonate. J. Lipid Res. 33 (1992) 659-668.

99. Jones, M.P., Pandak, W.M., Heuman, D.M., Chiang, J.Y.L., and Vlahcevic, Z.R. : Cholesterol 7α-hydroxylase: evidence for transcriptional regulation by cholesterol or metabolic products of cholesterol in rat. J. Lipid Res. 34 (1993) 885-892.

100. Wobbe, C.R., and Struhl, K. : Yeast and human TATA binding proteins have nearly identical DNA sequence requirements for transcription *in vitro*. Mol. Cell. Biol. 10 (1990) 3859-3867.

101. McKnight, S.L., and Kingsbury, R. : Transcriptional control signals of a eukaryotic protein-coding gene. Science 217 (1982) 316-324.

102. Buratowski, S., Hahn, S., Guarente, L., and Sharp, P.A. : Five intermediate complexes in transcription initiation by RNA polymerase II. Cell 56 (1989) 549-561.

103. Conaway, R.C., and Conaway, J.W. : General Initiation-factors for RNA polymerase II. Annu. Rev. Biochem. 62 (1993) 161-190.

104. Zawel, L., and Reinberg, D. : Initiation of transcription by RNA polymerase II: A multi-step process. Prog. Nucl. Acid Res. 44 (1993) 67-108.

105. Buratowski, S. : The basics of basal transcription by RNA polymerase II. Cell 77 (1994) 1-3.

106. Pugh, B.F., and Tjian, R. : Diverse transcriptional functions of the multisubunit eukaryotic TFIID complex. J. Biol. Chem. 267 (1992) 679-682.

107. Struhl, K. : Duality of TBP, the universal transcription factor. Science 263 (1994) 1103-1104.

108. Tjian, R., and Maniatis, T. : Transcriptional activation: A complex puzzle with few easy pieces. Cell 77 (1994) 5-8.

109. Ptashne, M. : How eukaryotic transcriptional activators work. Nature 335 (1990) 683-689.

110. Mitchell, P.J., and Tjian, R. : Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science 245 (1989) 371-378.

111. Yankulov, K., Blau, J., Purton, T., Roberts, S., and Bentley, D.L. : Transcriptional elongation by RNA polymerase II is stimulated by transactivators. Cell 77 (1994) 749-759.

112. Proline-rich activator CTF1 targets the TFIIB assembly step during transcriptional activation. Proc. Natl. Acad. Sci. USA 91 (1994) 4170-4174.

113. Latchman, D.S. : Eukaryotic transcription factors. Biochem. J. 270 (1990) 281-289.

114. Gill, G., Pascal, E., Tseng, Z.H., and Tjian, R. : A glutamine-rich hydrophobic patch in transcription factor SpI contacts the TAFII110 component of the *Drosophila* TFIID complex and mediates transcriptional activation. Proc. Natl. Acad. Sci. USA 91 (1994) 192-196.

115. Stringer, K.F., Ingles, C.J., and Greenblatt, J. : Direct and selective binding of an acidic transcriptional

activation domain to the TATA-box factor TFIID. Nature 345 (1990) 783-786.

116. Horikoshi, M., Carey, M.F., Kakidani, H., and Roeder, R.G. : Mechanism of action of a yeast activator: Direct effect of GAL4 derivates on mammalian TFIID-promoter interactions. Cell 54 (1988) 665-669.

117. Hoey, T., Weinzierl, R.O.J., Gill, G., Chen, J.-L., Dynlacht, B.D., and Tjian, R. : Molecular cloning and functional analysis of Drosophila TAF110 reveal properties expected of coactivators. Cell 72 (1993) 247-260.

118. Goodrich, J.A., Hoey, J., Thut, C.J., Admon, A., and Tjian, R. : Drosophila TAFII40 interacts with both a VP16 activation domain and the basal transcription factor TFIID. Cell 75 (1994) 519-530.

119. Felsenfeld, G. : Chromatin as an essential part of the transcriptional mechanism. Nature 355 (1992) 219-224.

120. Wolffe, A.P. : Transcription: In tune with the histones. Cell 77 (1994) 13-16.

121. Durrin, L.K., Mann, R.K., and Grunstein, M. : Nucleosome loss activates CUP1 and HIS3 promoters to fully induced levels in the yeast *saccharomyces cerevisiae*. Mol. Cell. Biol. 12 (1992) 1621-1629.

122. Han, M., Kim, U.J., Kayne, P., and Grunstein, M. : Depletion of histone H4 and nucleosomes activates the PHO5 gene in *saccharomyces cerevisiae*. EMBO J. 7 (1988) 2221-2228.

123. Schild, C., Claret, F.-X., Wahli, W., and Wolffe, A.P. : A nucleosome-dependent static loop potentiates estrogen-regulated transcription from the *Xenopus* vitellogenin B1 promoter *in vitro*. EMBO J. 12 (1993) 423-433.

124. Wolffe, A.P. : Nucleosome positioning and modofocation: chromatin structures that potentiate transcription. TIBS 19 (1994) 240-244.

125. Princen, H.M.G., and Meijer, P. : Maintenance of bile acid synthesis and cholesterol 7α-hydroxylase activity in cultured rat hepatocytes. Biochem. J. 272 (1990) 273-275.

CHAPTER 2

Transcriptional regulation of the gene encoding cholesterol 7α-hydroxylase in the rat

Marco F.M. Hoekman[a], Jeanet M.J. Rientjes[a], Jaap Twisk[b], Rudi J. Planta[a], Hans M.G. Princen[b] and Willem H. Mager[a]

[a]Department of Biochemistry and Molecular Biology, IMBW, BioCentrum Amsterdam, Vrije Universiteit, 1081 HV Amsterdam, The Netherlands; and [b]Gaubius Laboratory TNO-PG, P.O. Box 430, 2300 AK Leiden, The Netherlands.

Gene 130 (1993) 217-223

ABSTRACT

The cytochrome P450 enzyme, cholesterol 7α-hydroxylase (CYP7A), catalyses the first and rate-limiting step in the conversion of cholesterol to bile acids. Expression of the CYP7A gene is under complex physiological control, encompassing amongst others a feedback down-regulation by bile acids. With the CYP7A cDNA of the rat as a probe, we isolated a rat genomic clone containing the 5' part of the gene, including approximately 3.6 kb of upstream sequences. Sequence analysis revealed the presence of several putative regulatory nucleotide (nt) elements. Transient expression analyses of transfected primary hepatocytes demonstrated that the major transcription-activating region is located in the proximal 145 nt. Upon addition of taurocholate to the culture, a significant reduction of the transcriptional activity was observed, suggesting the presence of a bile acid-responsive element in the proximal region of the *CYP7A* promoter. In addition, evidence was obtained for the presence of a thyroxine-responsive site further upstream.

After addition of taurocholate, steady-state CYP7A mRNA levels, as judged by Northern analysis of hepatocyte RNA, are eightfold reduced. On the other hand, the transcriptional activity of CYP7A , as shown both in CAT assays and run-on experiments, revealed only a threefold decrease. These experiments suggest that both transcriptional control and regulation of CYP7A mRNA stability play an important part in the feedback regulation of CYP7A activity in the rat.

INTRODUCTION

Cholesterol 7α-hydroxylase catalyses the first and rate-limiting step in the conversion of cholesterol to bile acids in vertebrate liver (1). In fact, 7α–hydroxylation of cholesterol represents the major activity to remove cholesterol from the body and to maintain cholesterol homeostasis (2,3). The enzyme in question belongs to the extended family of cytochrome P450 isozymes and the corresponding gene is referred to as CYP7A (4). The cholesterol 7α-hydroxylase activity depends on a variety of physiological signals. Glucocorticoids, thyroxine and insuline affect the enzyme activity (5-8) and hormonal control most likely underlies the observed diurnal variations (9,10). A second important regulatory effect is exerted by the endproducts of the pathway: bile acids. Bile acids suppress the CYP7A activity via the enterohepatic circulation (11,12). Accordingly, administration of the bile-acid-binding resin cholestyramine or biliary drainage results in stimulation of enzyme activity (13).

Thanks to the recent availability of gene-specific DNA probes, studies could be started in order

to elucidate at which level of CYP7A expression these regulatory events take place. Results obtained by *in vivo* analyses indicated a correlation between enzyme activity and mRNA levels, suggesting that regulation primarily occurs at the transcript level (14). Comparison of the results of Northern hybridizations (steady-state mRNA amounts) and those obtained by run-on assays (*de novo* mRNA synthesis) led to the hypothesis that transcription of CYP7A may indeed be the major target for regulation (13,15). Recently, we were able to demonstrate that primary (rat and pig) hepatocytes in culture exhibit similar regulatory responses as observed *in vivo* (16,17).
These findings prompted us to isolate and characterize the promoter of rat CYP7A and examine its transcription by transfection of primary hepatocytes.

RESULTS AND DISCUSSION

Cloning of a rat liver *CYP7A* cDNA probe

Based on the published cDNA sequence coding for rat CYP7A (18), primers were selected to prepare a CYP7A-specific probe using the PCR (polymerase chain reaction) technique. As a source of template we used total liver RNA isolated from cholestyramine-fed rats, which was previously shown to contain a high content of CYP7A mRNA (9,19). Two oligo primers:

5'-AGCCGCCAAGTGACATCATCCAGTGTTCGCTTCTTCC **and**
5'-ATGATGACTATTTCTTTGATTTGGGGAATTGCCGTG,

which correspond to the termini of the coding region of the CYP7A mRNA and its cDNA, respectively, were synthesized. Polymerase chain reaction was performed according to the manufacturer's protocol (Superscript, Bethesda Research Laboratories). The major reaction product of 1.6 kb was isolated and cloned in pUC18. The identity of the CYP7A cDNA probe was confirmed by nt sequence analysis (result not shown) according to the chain termination technique (20).

Cloning and sequencing of the 5'-flanking region of rat *CYP7A*

With this cDNA fragment as a probe, from a λ-library of total rat genomic DNA (λEMBL3, kindly provided by Dr. W. Lamers; Academic Medical Centre, Amsterdam) several positive clones were isolated. A second screening was performed with the 5'-located PCR-primer to

select clones that might contain the 5' part of CYP7A. One of the resulting positive clones, designated R7α21, was further characterised by subcloning in pUC18 and restriction-site mapping. In Fig. 1 the physical map of the insert of this subclone pSSRλ3 is shown. This insert contains about 3.6 kb of 5'-flanking region, as well as the first intron and a part of the second intron. Sequence-specific oligonucleotides were used as primers in the sequence analysis of the flanking region. The primary structure of the 3.6-kb 5' flanking region of rat *CYP7A* is presented in Fig. 2. The proximal 590 bp are identical to the sequence published previously (21), except that we did not find an extra thymidine residue between nt positions -584 and -585.

A computer-aided search revealed the presence of several putative *cis*-acting elements in the upstream region of the gene (indicated in Fig. 2). It has been suggested that transcription of CYP7A is under positive control by cholesterol (15,19). Therefore, these authors assumed that the extended promoter of the gene may contain sterol-responsive sites, as has been found for other genes (22). However, analysis of the rat sequence, contrary to that of the recently published human gene promoter (23,24), did not reveal such a motif. In addition, HNF3 (Hepatic Nuclear Factor 3) sites, which were proposed to determine the liver-specific transcription of human CYP7A, are not present in the flanking region of rat CYP7A (see Fig. 2). On the other hand, elements homologous with the so-called human glucocorticoid-responsive elements (HGRE's), as well as sites designated as BTE (basic transcriptional element) and cognate to LF-B1 (liver factor B1), are present in the CYP7A upstream region (see Fig. 2).

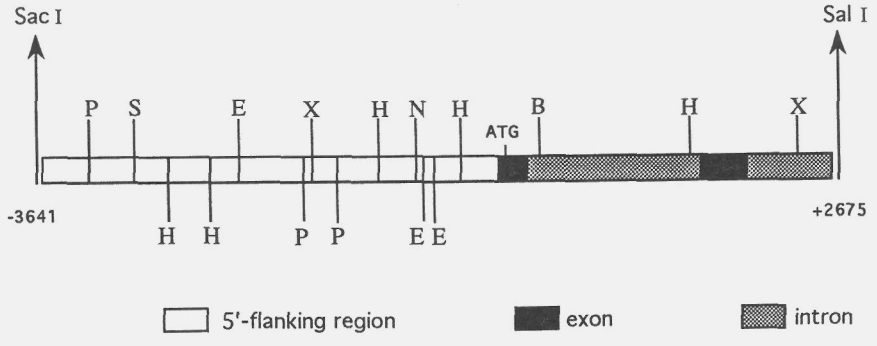


Fig. 1 Physical map of the 6.3 kb *Sac*I-*Sal*I insert of clone pSSRλ3. The organization of the gene is shown, drawn in scale. *Eco*RI (E), *Hin*dIII (H), *Pst*I (P), *Sma*I (S), *Xba*I (X), *Nco*I (N), *Bgl*II (B).

```
-3641 GAGCTCTACC CTTGCTCTGC TATTGTACTT TTTAATACAC AGTTCAATCA AATGTGCCAC CAGAATATGC ATGCTAACAG CTGTAGTGGT
      TGATTTTTCT TTCTACTCTT CTGTGTGTAA GACCCCATGT TTTATCAATT ATTTTTTAAT GATTTCTTTC TTCATGCATA TGTGTGGTTG
-3461 TCAGTGTGAG TCTGTGTGTA CAGCAGGTGC ACAGGTATCC ACAGAGGCCA GAGGTTCCCT GTAACTAGAA TTACAGGCAC TTGTGAACTT
      TCCTGTATGG GTGCTGGGAA GCAATCTGAG GTCTTCTGCA AGGGATCTTA ACCACTGACT TTCTAGCCTG CTTTGCCCAT TTCTATTTAT
-3281 GATGACTGGA AACTGGGCTT AGGCCTTATA TTCTCTGAGG CCAAAATCAA GTTCTTCCAA ACTGCAGGAT TTATGGTCTT CTATAGTATC
      CCACAGAAAT GGAAAAGAAA GTGACCCATT AGAGCAGTAT TAGAGTCGAA ATAAACTCAA CTTGGTATGC CAGGACTTTG GACAATAATA
-3101 ACCCTGTCTT TTCAGGGCAT CTATCTGTAC TGCTGCAATA GAAACTCCAC AGGTCAGGGT CACAGCTGTT GTGTTTTACA CAGTGTCCCC
      CGCATTAGTT CAGTGCCCAC CATGCAATAG GTGTCATGGT GTGTGTGTGT GTGTGTGTGC GTGTGTCGTG CTTGTGTGCA TGTGTGTGAG
-2921 ACACACACAC AGAGAGATAC AAAGACAGAA ACAGAAAATT AATAAAATTT TACCAACTAA AATAGGGAAT TAAAGAAAAG GAGGAGAAAA
      AGTTGGGCAT TCAACACCAT AAAGTCCCAG TACTATGCTA AGAACACCCA GCTGTCCTCA CACCCGGGCA TGAAACTTCA TGCACTGTTC
-2741 ATCAGAAAAT CGTTTACACA CATCCCCTTG CAGTCTACTT GTAGTTTTAA CAACTTCAGA GAGCACTAGC ATTTCCAGCC CCAGGTTAGA
      AGCTTTGGTA GATGCTGTTT GCGAGCACAG GATAGCAGCA AGAAGTGGAC TTGTTAGAAG GAAAGCCAAT GCCTATGTAA CAACGAAAAC
-2561 TAAGTATGAA TCTCGAATCT CCACTCTCGT GTGTCTGTGT CTCCATATAC GAGCTTGGGT GCCTGACATG GCAAGGTGTT ACAAGTAAGG
      GAGGAACAAG AAAAGGACAG GGTAGTGGAC ATCAGGATGA ATGCCAGCCA GGGCGACTGG AGAGAGTCTA CGCTGCTCTG AAGGTGGGTG
-2381 AAGAAGACCT CAGGAAGCTT TCTGAGGCTC CGAGAGTGCT TTTCCCTTCC CATGTTGAAA CATCCTTATT TGCAGAGAAT TCCAGGTTCA
      TGGGAATTTG TAAAGAGAAT ACTAAGAGGC CACCTGTGGC TTCTCCTATT TTTGTCTGCT GTCATTTATG GGACAGGGTT AGAGACCTGG
-2201 CTTGCTTGGC TATGAGGCTG TTGCTTCCTC GGTTACTCTG CTGTGGTTGG ATGCATTAGG GTTAGGCCCC TCAAGAGCCA TGTGTCATTT
      TATAAAAGCA ATATAAATAT ACTTAAGGTG CACAAAGCAT TAGGAGGTCT GAGATAATAG ATTCTGAGAA AATCTATCCT GCTGTGTAGC
-2021 AACTGATGTT TATGATTATA GTCCCAGACC ACACGATAAA GGATCTGTGG ACTCTGTTTA GGGAGGTCAA AAAACTATTG CAAATGGAGT
      CTATAGAGAA AACTAGACAG GACTCAATGC TCACCAATCG AGAATTAGTT GATGAGCTGG GGTAGTGACT TAGTGGATAA GAACACGGTC
-1841 CTTTCAGAGG TCCTGAGTTA AATCCCCAGC AAACACATGG TGGCTCATAA CCATCTATAT TGTGATTTGA TGCCCTCTTC TGGCATGCAG
      GTGTACATGC AGACTCGTAT ACATAAAATA AATAAATCTT GAAAAAATGA ATACGTTGAA TAAGTGTCCC CTCGGATAAC TTTCTGCAGA
-1661 ATTTTAAGCA CATGTCAATG GTAATAACAC ACACACACAC ACACACACAC ACACACACAC ACACACATAC ACACACCATA CAGATATGTA
      TCTAGAGACA TACACATGTA CATTTTATCT CTTTTATTTT CTTCTCCCCT CTTTGACATC AAGGAATAGA ATGCACTCAC TGTGGCCTAG
-1481 TGCCACACTC TACCTATTTC TTTGGCTTTA CTTTGTGCTA GGTGACCCGA AAGGTTTAAA TATCAAAAAT GCTAATGGCT CGATATTTAC
      ATCCCCAATT TCTCCTTTCT CCTTACCTCA GACTCTTACA TTCAGTTGAC AATTTGACAT CGTCTCCTGG ATTTTCAAAT GTTCAGCACA
-1301 CTGTACTGAT GTACTGCCTT CCAAGGCAAC CGGCACGATC CTCTCCCCAC TCCCAAGCAT CCCTCCATGA GCCACTGTTT GCTTATCTTC
      TTGACTCTTG TTTTAACCCA ACTCCTCCCC TATTCACTCT GCTCTAATTC ATTCATTCTA TATTTTCGCA CATCAGGCTC ATCCTTTGCT
-1121 CAGGAACTTC ACTTTTGCTT TCCGGTCTCC TGGAAATGTG TTTTCTTGGC TATTCCATCT CAAGACCATC TTTTCAGAAA AGCTTTTCCT
      ATCAACATAT TTAAAGCCCT CTTCATCCCC CAGTAGCTCT GGACACCTCA TTTTATGGAT ACACAACACA TATTTGCCAC CTGTCTCCCC
 -941 ATTAAAATAT AATCTTCAGT AGAGAAACTC CATATCTTGT TAATACCTGA AACAAGAATA TCTTCAAAGA GTTCCTGGGA CATAAAAACG
      CTCAATTAAT ATTTATGTTA AACAGGGATC TGGGGTATAT CACAGAGGTA GAGGGCTTAC CTAGGAGGAG TTGGGCCATG GGTTCAACTT
 -761 CCAGCACAGA ATGAAAGATT ATGTTAAATA AAGTTGGGAA GGATGTATGC CAGTCTATGA GTAGTATAGG AGGTAAATTA TGAATTCATA
      TTTACTTTTC GGACAAGAAG TGTTGTAGTC TTTATTTGAA ATAAAATACA TCTTAATTAC CAATAACAAT TGGTAAGGAG TGAATTCTCA
 -581 AGCTGTGGCT TCCTGGTAGA TGAGTCCTGG GAGGTTTTCT ATTTCGATGA TGGTAGATAG GTAACCTGTC ATATACCACA TGAAATACCT
      GTGGCTTTGT AAACACACCG AGCAGTCAAG CAGGAGAATA GTTCCATACA GTTCGCGTCC CTTAGGATTG GTTTCGGGAT ACTTCTGGAG
 -401 GTTCATTTAA ATAATTTTCC CCGAAGTACA TTATGGGCAG CCAGTGTTGT GATGGGAAGC TTCTGCCTGT TTTGCTTTGC GTCGTGCTCC
      ACACCTTTGA CAGATGTGCT CATCTGTTTA CTTCTTTTTC TACACACAGA GCACAGCATT AGCTGCTGTC CCGGCTTTGG ATGTTATGTC
 -221 AGCACATGAG GGACAGACCT TCAGCTTATC GAGTATTGCA GCTCTCTGTT TGTTCTGGAG CCTCTTCTGA GACTATGGAC TTAGTTCAAG
      GCCGGGTAAT GCTATTTTTT TCTTCTTTTT TCTAGTAGGA GGACAAATAG TGTTTGCTTT GGTCACTCAA GTTCAAGTTA TTGGATCATG
  -41 GTCCTGTGCA CATATAAAGT CTAGTCAGAC CCACTGTTTC GGgacagcct tgctttgcta ggcaaagagt ctccccttg gaaattttcc
      tgcttttgca aa
                  *
```

Fig. 2 Nucleotide sequence of the 5'-flanking region of rat CYP7A. The transcription start point, as determined previously (21), is indicated by the asterisk (+1). The TATA box is printed in boldface. The beginning of the leader region is shown by lower-case letters. Putative regulatory elements, which were identified on the basis of known *cis*-acting elements, are underlined, listed from right to left, upward: LFB1 (5'-GTTATT), liver factor B1 (31); BTE (5'-AGTAGGAGG), basic transription element (26); HGRE5 (5'-TGTTCT), human glucocorticoid responsive element 5 (32); HGRE7 (5'-AGTCCT), human glucocorticoid responsive element 7 (33); coreGRE (5'-AGGATGT), core sequence of the glucocorticoid responsive element (34). The nt sequence data for the CYP7A promoter sequence have been assigned the GenBank accession number Z18860.

Analysis of the CYP7A promoter activity

In order to identify the promoter elements that play a functional part in the regulation of transcription of CYP7A, different portions of the upstream DNA fragment were fused to the bacterial CAT-reporter gene (see Fig. 3). The resulting hybrid genes contain the transcription

initiation site of CYP7A as determined previously (21).To construct -348RCAT, we synthesized an oligo corresponding to nt -325 to -348 of the CYP7A upstream sequence containing a 5' *SalI* site and an oligo corresponding to the inverse complement of nt +23 to +2 containing a 5' *SphI* site. These oligos served as primers in a PCR experiment utilizing the 6.3 kb genomic fragment as a template. The resulting 371-bp product was cloned between the *SphI* and *SalI* sites of the SuperCAT vector, of which the *HindIII* site had been destroyed. SuperCAT is a derivative of pSV2CAT (25), containing the bacterial CAT gene and the simian virus 40 (SV40) splice and polyadenylation signals on a *HindIII-BamHI* fragment (nt 3370 to 5003 in pSV2CAT) cloned between the *Hin*dIII and *Nde*I sites of pUC12. By the use of the *SphI* site an 'upstream ATG' was introduced in the leader region of -348RCAT, which has been demonstrated to lower the translation efficiency of the respective mRNAs (26). To avoid this effect the *SphI* site was destroyed. Further 5' deletion of the CYP7A promoter (leading to constructs -145RCAT, -79RCAT and -49RCAT, respectively) was performed by digestion with BAL31 starting from nt -348. The deletion endpoints were determined by nt sequencing. For the construction of plasmids -1571RCAT, -2769RCAT and -3641RCAT an oligo was synthesized corresponding to nt -786 to -807. This oligo, together with the oligo corresponding to the inverse complement of nt +23 to +2 (containing the 5' *SphI* site) was used as a primer in a PCR-experiment in which clone pSSRλ3 served as a template. The product, a 830-bp DNA fragment, was cut with *Hin*dIII+*Nco*I and, in a triple ligation, fused to -348RCAT (cut with *Hin*dIII+*Sac*I) and the *Nco*I-*Sac*I fragment of pSSRλ3, giving rise to -3641RCAT. Subsequently, constructs -2769RCAT and -1571RCAT were made from -3641RCAT by digestion with *Sac*I+*Xba*I or *Sac*I+*Sma*I, respectively, isolation of the correct fragment, blunt-end formation with T4 DNA polymerase and religation with T4 DNA ligase. SV40CAT, used as a control in transfection studies, contains the SV40 early promoter on a *Pvu*II-*Hin*dIII fragment of pSV2CAT cloned into SuperCAT digested with *Sma*I+*Hin*dIII. The various deletion mutants were then tested as to their promoter activity in primary rat hepatocytes, cultured under conditions in which CYP7A and bile-acid-synthetic capacity were maintained (27). Fig. 3A shows the results of the CAT assays. From these data it is apparent that the proximal part of the CYP7A promoter region, up to -145, confers most of the transcription-activating capacity of the CYP7A promoter, which appeared to be about 10% of the activity observed with the SV40CAT used as a control (result not shown). Obviously, in this part of the promoter the major transcription-activating elements of CYP7A are located. We hypothesize that the BTE sequence present in this region (see Fig. 2) and previously shown to be involved in the transcription of other cytochrome P-450 genes (28),

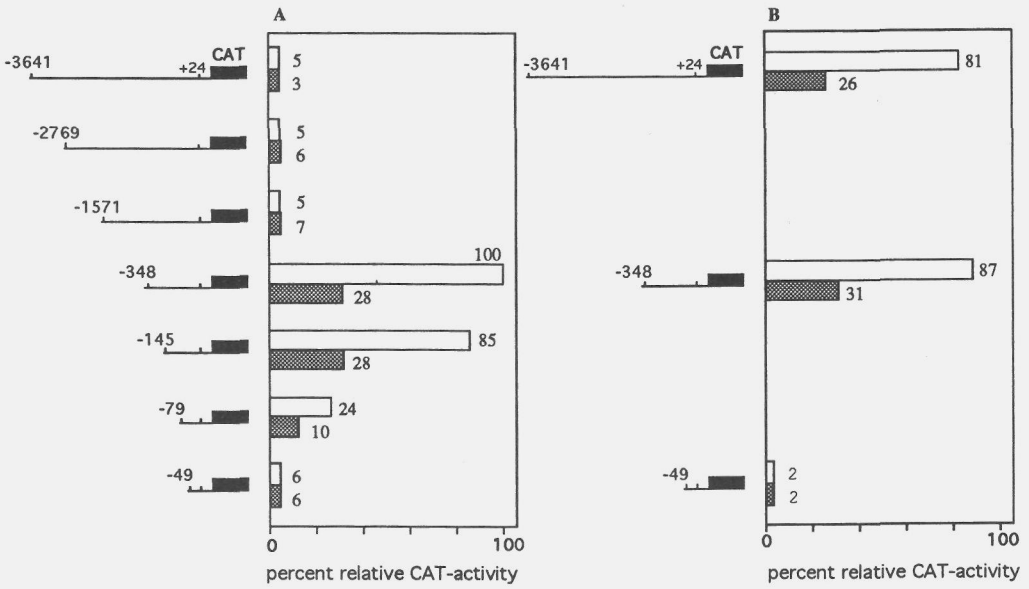


Fig. 3 Transient activity of the CYP7A promoter in primary rat hepatocytes. CAT activity was determined in primary cultures of rat liver cells transfected with the Ca.phosphate method. The used constructs contain various fragments of the 5'-flanking region of rat CYP7A fused to the CAT reporter gene (black bar) at +23 of the CYP7A sequence. The lengths of CYP7A fragments (in nt) are indicated relative to the cap-site (+1). The CYP7A promoter activity of each reporter construct is expressed relative to that of the -348RCAT construct (value set at 100%). Open bars represent CAT activity measured in hepatocytes without the addition of bile acids as opposed to hatched bars indicating CAT activity in hepatocytes cultured in the presence of 50 μM taurocholate. (**A**): Transfected cells were cultured in Williams E medium supplemented with 10% heat-inactivated FBS/ 2 mM L-glutamine/ 140 nM insuline/ 50 nM dexamethasone/ 100 IU penicillin and 100 μg streptomycin (per ml), in the presence or absence of 50 μM taurocholate. (**B**): Transfected cells were cultured in Williams E medium containing 1 μM thyroxine/ 100 nM dexamethason/ 1.4 μM insuline/ 2mM L-glutamine and 100 IU penicillin and 100 μg streptomycin (all per ml), in the presence or absence of 50 μM taurocholate. Data represent means of three independent experiments.

Methods (a) Isolation and culture of rat hepatocytes:

Male Wistar rats weighing 250-300 g were maintained on standard chow and water *ad libitum*. Two days before isolation of hepatocytes, rats were fed a diet supplemented with 2% cholestyramine (Questran, Bristol Myers B.V., Weesp, The Netherlands). Rat liver cells were isolated by perfusion with 0.05% collagenase/0.005% trypsin inhibitor, as described previously (35). Viability, as determined by trypan blue exclusion, was higher

than 90%. Cells were seeded on 60-mm diameter plastic tissue-culture dishes or 6-well cluster plates (Costar, Cambridge, MA, USA) at a density of $1.0x10^5$ cells/cm^2 in Williams E medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine/ 140 nM insuline/ 50 nM dexamethasone/ 100 IU penicillin/ 100 µg streptomycin (per ml), (unless stated otherwise), and maintained at 37^0C in a 5% CO_2/95% air atmosphere. After a 4-hr attachment period, medium was refreshed with culture medium supplemented with hormones as described above. Cells were left to recover for another 18 h before use for further experiments.

(b) Transfection experiments and CAT assays:

At 22 h after their isolation, cells were subjected to transfection. Recombinant plasmids to be used for transfections were purified by centrifugation to equilibrium in CsCl-ethidium-bromide density gradients (36). In transient-expression assays, 3 µg of test plasmid and 1 µg of standard LacZ plasmid (37) were used for cotransfection as a Ca.phosphate precipitate (38). After 4 h the precipitate was removed, cells were treated with Williams E medium containing 15% glycerol for 1 min, and supplied with fresh culture medium with and without taurocholate. At 42 h after transfection, cells were harvested and cell extracts were prepared. Preparation of cell extracts and CAT assays were performed essentially as described by Gorman *et al.* (25). Protein concentrations were determined with BCA protein-assay reagent (Pierce). The amounts of acetylated product as represented by signals on the autoradiograms were quantified with a Phosphor-imager 400B (Molecular Dynamics). Data were corrected for protein concentration and transfection efficiency.

may have an important functional role in the transcription activation of CYP7A in the rat. A further deletion to -79 led to a reduced CAT activity, while the -49RCAT construct showed only basic transcriptional activity, probably only due to the presence of the TATA box. Extending the promoter fragment up to -348 had only a slight effect on CAT activity, whereas the -1571, -2769 and -3641 constructs only showed a surprisingly low level of transcription activity, comparable to the basic transcriptional activity found for construct -49RCAT. These results suggest the presence of a negative regulatory element between nt -348 and -1571.

Recent results obtained by Hylemon *et al.* (29) indicated that CYP7A transcriptional activity is enhanced, when primary hepatocytes are cultured in a serum-free medium containing thyroxine. Therefore, constructs -49RCAT, -348RCAT and -3641RCAT were used for transfection of hepatocytes, cultured as described by Hylemon (Fig. 3B). As can be concluded from the presented data, constructs -49RCAT and -348RCAT displayed an activity similar to that in hepatocytes cultured without thyroxine. However, in contrast with the low level of CAT-activity in medium without thyroxine, the -3641RCAT construct showed a relatively strong transcriptional activity, comparable to that of the -348RCAT construct. These results

suggest the presence of a thyroxine-responsive element in the CYP7A promoter, between nt -348 and -3641. Obviously, this element can serve to compensate the repressing action of the putative negative control site between nt -348 and -1571.

Regulation of CYP7A expression

One of the most intriguing regulatory responses supposed to play a part in the expression of CYP7A is a feedback control exerted by bile acids. Previously obtained results indicated that taurocholate (as well as other conjugated and unconjugated bile acids) represses the CYP7A activity, both *in vivo* (30) and in primary hepatocytes in culture (16,17). In agreement with these findings, this inhibitory effect also appeared to be manifest at the cellular level of CYP7A mRNA concentrations (see Fig. 4A and 4C). Northern analysis of RNA isolated from primary hepatocytes, cultured in the absence or presence of 50 μM taurocholate, demonstrates that the addition of taurocholate led to an eightfold decrease in the steady-state level of CYP7A mRNA. These results indicate that the regulation of CYP7A expression upon addition of taurocholate mainly takes place at the transcript level. In order to further study this regulatory response, the CAT-constructs described above were used in transient-expression experiments in which primary hepatocytes were grown in a medium containing 50 μM taurocholate. The data presented in Fig. 3A show that the constructs -79 RCAT, -145R CAT and -348RCAT displayed a response upon the addition of bile acid to the medium. The CAT activity, reflecting the transcriptional activity of the proximal promoter fragments, appeared to be threefold decreased in taurocholate-exposed cells, whereas the control SV40CAT-signal was not affected (result not shown). Since the -49RCAT directed signal did not display a taurocholate response, the region between -79 and -49 of the CYP7A promoter apparently is essential for the bile acid response of CYP7A in the rat. Therefore this region is likely to contain a bile-acid-responsive element. The pertinent region of the CYP7A promoter contains some notable nucleotide motifs, for example, a direct repeat between nt -54 and -65 (*i.e.* TCAAGT). Experiments are in progress to identify the actual bile-acid-responsive site. The extent to which transcriptional control contributes to the negative regulation of CYP7A expression upon bile acid treatment can not entirely explain the eightfold reduced CYP7A mRNA levels as shown by the Northern analyses (see Fig. 4A and 4C). Consistently, run-on assays using isolated nuclei from primary hepatocytes cultured in the presence or absence of taurocholate (see Fig.4B and C) revealed a moderate (about twofold) feedback control.

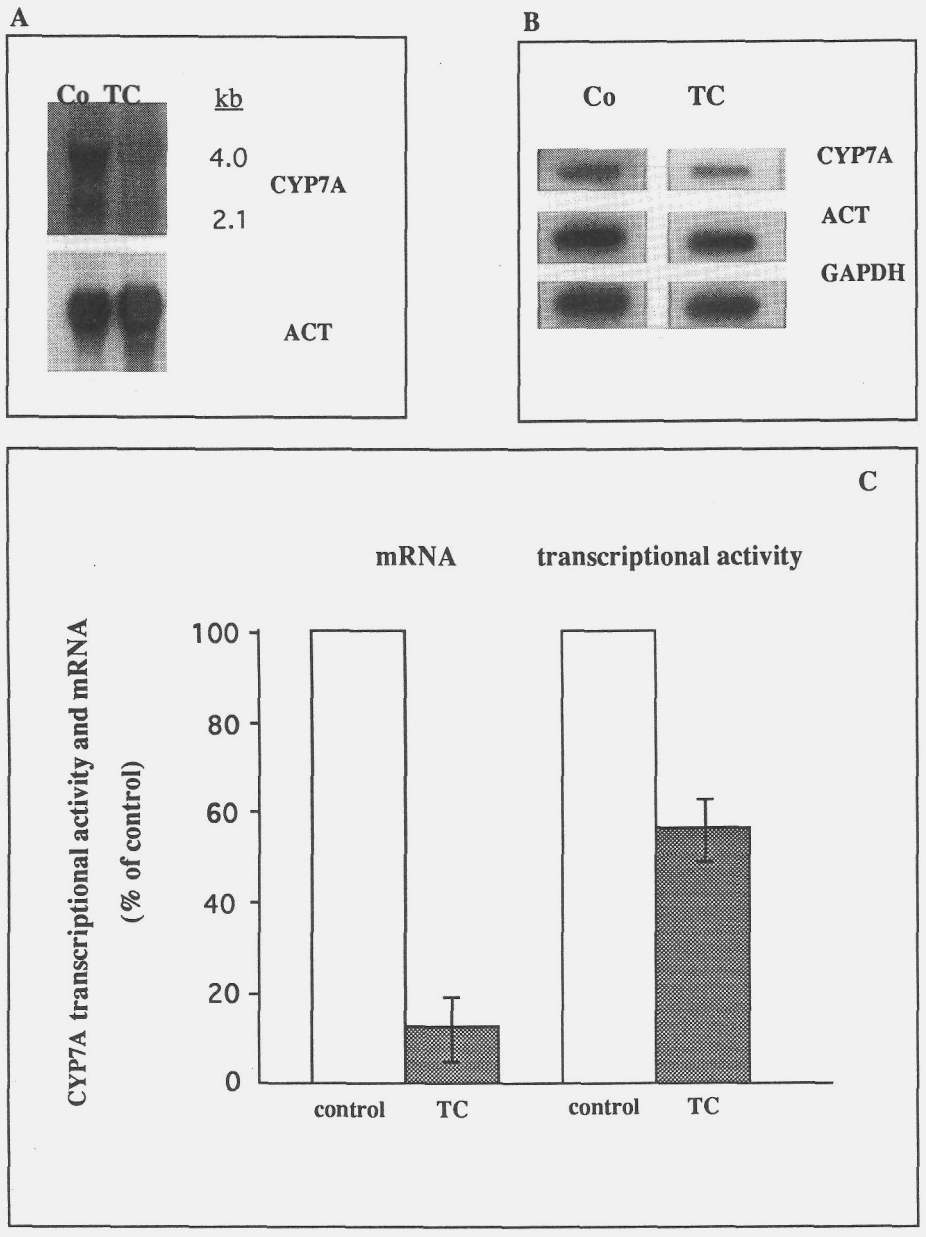


Fig. 4 CYP7A transcriptional activity and mRNA levels in hepatocytes cultured in the presence or absence of taurocholate. *Cells were exposed to 50 μM taurocholate* (TCh) for 24 h, between 18 and 42 h of culture, and were harvested simultaneously with untreated cells (control) for the isolation of nuclei and RNA. (**A**): Total RNA was isolated from cultured rat hepatocytes via the isolation procedure described by Chomczynski *et al.* (39). Northern blot analysis was performed with the 1.6 kb CYP7A cDNA and a 1.2 kb *Pst*I-fragment of hamster actin cDNA (ACT) as probes. ACT was used as a loading

control; 10 μg of total RNA was applied in both lanes. (B): Nuclear run-on experiments were performed as described previously by Twisk *et al.* (1993). As target DNA for slot-blot hybridizations plasmid DNA containing cDNA sequences of rat CYP7A, hamster actin (ACT) and rat GAPDH, respectively, were used. (C): Transcriptional activity of CYP7A is presented relative to that of actin (CYP7A transcriptional activity relative to GAPDH showed the same results). The amount of mRNAs or labelled transcripts were assessed by densitometric scanning of the respective autoradiographs. Data are expressed as means (+/- S.D.) of duplicate incubations, using hepatocytes from 12 (mRNA) or 3 (transcriptional activity) rats. S.D., standard deviation.

The data taken together, therefore, led us to propose that both transcriptional and post-transcriptional regulation play an important role in the expression of rat CYP7A. Post-transcriptional control, most likely, affects the stability of the CYP7A mRNA. Indeed, the occurrence of putative mRNA-destabilizing motifs in the respective trailer region has been indicated previously (18). It remains to be solved whether these elements actually are involved in the regulatory response to bile acids.

Conclusions

(a). A genomic clone containing approximately 3.6 kb of flanking region of rat CYP7A, was isolated and the primary structure of the flanking region was determined. A computer-aided search revealed the presence of several putative regulatory elements in this upstream region.
(b). Transient-expression experiments using cultured primary rat hepatocytes indicated that the major transcription-activating region is located within the proximal 145 nt of the CYP7A promoter. Furthermore, evidence was obtained for the presence of a bile-acid-responsive element between nt -49 and -79, and a possible thyroxine-responsive element between nt -348 and -3641.
(c). Combined data, obtained from Northern hybridizations, run-on transcription experiments and transient-expression studies in primary hepatocytes in culture, indicate that both transcriptional and posttranscriptional control play an important role in the feedback regulation of the CYP7A activity in the rat.

ACKNOWLEDGMENTS

We wish to thank Drs. Marcel Hoffmann for fruitful discussions and Miss Elly de Wit and Mrs Eline Lehmann for technical assistance during part of the experiments. This work was supported by HGO-TNO, grant 900-523-138 to M.F.M. Hoekman and J.M.J. Rientjes, and the Netherlands Heart Found., grant 89.079 to J. Twisk.

REFERENCES

1. Shefer, S., Hauser, S., Bekersky, I., and Mosbach, E.H. : Biochemical site of regulation of bile acid synthesis in the rat. J. Lipid Res. 11 (1970) 404-411.
2. Danielsson, H., and Sjöval, J. : Bile acid metabolism. Annu. Rev. Biochem. 44 (1975) 233-253.
3. Myant, N.B., and Mitropoulos, K.A. : Cholesterol 7α-hydroxylase. J. Lipid Res. 18 (1977) 135-152.
4. Gonzalez, F.J. : The molecular biology of cytochrome P450s. Pharmacol. Rev. 40 (1989) 243-288.
5. Princen, H.M.G., Meijer, P., and Hofstee, B. : Dexamethasone regulates bile acid synthesis in monolayer cultures of rat hepatocytes by induction of cholesterol 7α-hydroxylase. Biochem. J. 262 (1989) 341-348.
6. Ness, G.C., Pendleton, L.C., Li, Y.C., and Chiang, J.Y.L. : Effect of thyroid hormone on hepatic cholesterol 7α-hydroxylase, LDL receptor, HMG-CoA-reductase, farnesyl pyrophosphate synthetase and apolipoprotein A-I mRNA levels in hypophysesectomized rats. Biochim. Biophys. Res. Commun. 172 (1990) 1150-1156.
7. Subbiah, M.T.R., and Yanker, R.L. : Cholesterol 7α-hydroxylase of rat liver: an insulin sensitive enzyme. Biochem. Biophys. Res. Comm. 124 (1984) 896-902.
8. Vlahcevic, Z.R., Heuman, D.M., and Hylemon, P.B. : Regulation of bile acids synthesis. Hepatology 13 (1991) 590-600.
9. Chiang, J.Y.L., Miller, W.F., and Lin, G.M. : Regulation of cholesterol 7α-hydroxylase in the liver. Purification of cholesterol 7α-hydroxylase and the immunochemical evidence for the induction of cholesterol 7α-hydroxylase by cholestyramine and circadian rhythm. J. Biol.Chem. 265 (1990) 7646-7650.
10. Noshiro, M., Nishimoto, M., and Okuda, K. : Rat liver cholesterol 7α-hydroxylase: pretranslational regulation for circadian rhythm. J. Biol. Chem. 256 (1990) 10036-10041.
11. Björkhem, I. : Mechanism of bile acid biosynthesis in mammalian liver. In: Sterols and Bile Acids. Danielsson, H., and Sjöval, J., eds., Elsevier, New York, 1985, pp. 231-278.
12. Carey, M.C., and Cahalane, M.J. : Enterohepatic circulation. In: The Liver , Biology and Pathology. Arias, I.M., Jakoby, W.B., Popper, H., Schachter, D., Shafritz, D.A., eds., Raven Press, New York, 1988, pp. 573-616.
13. Pandak, W.M., Vlahcevic, Z.R., Chiang, J.Y.L., Heuman, D.M., and Hylemon, P.B. : Bile acid synthesis, VI. Regulation of cholesterol 7α-hydroxylase by taurocholate and mevalonate. J. Lipid Res. 33 (1992) 659- 668.
14. Sundseth, S., and Waxman, D.J. : Hepatic P-450 cholesterol 7α-hydroxylase. Regulation *in vivo* at the protein and mRNA level in response to mevalonate,diurnal rhythm and bile acid feedback. J. Biol.

Chem. 265 (1990) 15090-15095.

15. Li, Y.C., Wang, D.P., and Chiang, J.Y.L. : Regulation of cholesterol 7α-hydroxylase in the liver. Cloning, sequencing and regulation of cholesterol 7α-hydroxylase mRNA. J. Biol. Chem. 265 (1990) 12012-12019.

16. Twisk, J., Lehmann, E.M., and Princen H.M.G. : Differential feedback regulation of cholesterol 7α-hydroxylase mRNA and transcriptional activity by rat bile acids in primary monolayer cultures of rat hepatocytes. Biochem. J. 290 (1993) 685-691.

17. Kwekkeboom, J., Princen, H.M.G., Van Voorthuizen, E.M., and Kempen, H.J.M. : Bile acids exert negative feedback control on bile acid synthesis in cultured pig hepatocytes by suppression of cholesterol 7α-hydroxylase activity. Hepatology 12 (1990) 1209-1215.

18. Noshiro, M., Nishimoto, M., Morohashi, K., and Okuda, K. : Molecular cloning of cDNA for cholesterol 7α-hydroxylase from rat liver microsomes. FEBS Lett. 257 (1989) 97-100.

19. Jelinek, D.F., Andersson, S., Slaughter, C.A., and Russell, D.W. : Cloning and regulation of cholesterol 7α-hydroxylase, the rate limiting enzyme in bile acid biosynthesis. J. Biol. Chem. 265 (1990) 8190-8197.

20. Sanger, F., Nicklen, S., and Coulson, A.R. : DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. VS. 74 (1977) 5463-5469.

21. Jelinek, D.F., and Russell, D.W. : Structure of the rat gene encoding cholesterol 7α-hydroxylase. Biochemistry 29 (1990) 7781-7785.

22. Goldstein, J.L., and Brown, M.S. : Regulation of the mevalonate pathway. Nature 343 (1990) 425-430.

23. Molowa, D.T., Chen, W.S., Cimis, G.M., and Tan, C.P. : Transcriptional regulation of the human cholesterol 7α-hydroxylase gene. Biochemistry 31 (1992) 2539-2544.

24. Cohen, J.C., Cali, J.J., Jelinek, D.F., Mehrabian, M., Sparkes, R.S., Lusis, A.J., Russell, D.W., and Hobbs, H.H. : Cloning of the human cholesterol 7α-hydroxylase gene (CYP7) and localization to chromosome 8q11-q12. Genomics 14 (1992) 153-161.

25. Gorman, C. : High efficiency gene transfer into mammalian cells. In: DNA Cloning: A Practical Approach. Glover, D.M., ed., IRL Press, Oxford, England, 1985, vol.II, pp. 143-190.

26. Alam, J., Yu, N., Irias, S., Cook, J.L., and Vig, E. : Reduced chloramphenicol acetyl transferase activity observed with vectors containing an upstream *Sph*I recognition sequence. Biofeedback 10 (1991) 423-425.

27. Princen, H.M.G., and Meijer, P. : Maintenance of bile acid synthesis and cholesterol 7α-hydroxylase activity in cultured rat hepatocytes. Biochem. J. 272 (1990) 273-275.

28. Yanagida, A., Sogawa, K., Yasumoto, K.I., and Fujii-Kuriyama, Y. : A novel *cis*-acting DNA

element required for a high level of inducible expression of the rat P-450c gene. Mol. Cell. Biol. 10 (1990) 1470-1475.

29. Hylemon, P.B., Gurley, E.C., Stravitz, R.T., Litz, J.S., Pandak, W.M., Chiang, J.Y.L., and Vlahcevic, Z.R. : Hormonal regulation of cholesterol 7α-hyroxylase mRNA levels and transcriptional activity in primary rat hepatocyte cultures. J. Biol. Chem. 267 (1992) 16866-16871.

30. Heuman, D.M., Vlahcevic, Z.R., and Bailey, M.L. : Regulation of bile acid synthesis. II, Effect of bile acid feeding on enzymes regulating hepatic cholesterol and bile acid synthesis in the rat. Hepatology 8 (1988) 892-897.

31. Frain, M., Swart, G., Monaci, P., Nicosia, A., Stämpfli, S., Frank, R., and Cortese, R. : The liver-specific transcription factor LF-B1 contains a highly diverged homeobox DNA binding domain. Cell 59 (1989) 145-157.

32. von der Ahe, D., Janich, S., Scheidereit, C., Renkawitz, R., Schütz, G., and Beato, M. : Glucocorticoid and progesteron receptors bind to the same sites in two hormonally regulated promoters. Nature 313 (1985) 706-709.

33. Cato, A.C.B., Geisse, S., Wenz, M., Westphal, H.M., and Beato, M. : The nucleotide sequences recognized by the glucocorticoid receptor in the rabbit uteroglobin gene region are located far upstream from the initiation of transcription. EMBO J. 3 (1984) 2771-2778.

34. Langer, S.J., and Ostrowski, M.C. : Negative regulation of transcription *in vitro* by a glucocorticoid response element is mediated by a *trans*-acting factor. Mol. Cell. Biol. 8 (1988) 3872-3881.

35. Princen, H.M.G., Huijsmans, C.M.G., Kuipers, F., Vonk, R.J., and Kempen, H.J.M. : Ketoconazole blocks bile acid synthesis in hepatocyte monolayer cultures and *in vivo* in rat by inhibiting cholesterol 7α-hydroxylase. J. Clin. Invest. 78 (1986) 1064-1071.

36. Maniatis, T., Fritsch, E.F., and Sambrook, J. : Molecular Cloning , A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.

37. Hall, C V., Jacob, P.E., Ringold, G.M., and Lee, F.J. : Expression and regulation of *Escherichia coli lacZ* gene fusions in mammalian cells. Mol. Appl. Gen. 2 (1983) 101-109.

38. Pasco, D.S., and Fagan, J.B. : Efficient DNA-mediated gene transfer into primary cultures of adult rat hepatocytes. Lab. Meth. 8 (1989) 535-541.

39. Chomczynski, P., and Sacchi N. : Single-step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162 (1987) 156-159.

CHAPTER 3

Transcriptional control of cholesterol 7α-hydroxylase gene expression in the rat is mediated through a distinct proximal promoter element.

Marco F.M. Hoekman[a], Jaap Twisk[b], Jeanet M.J. Rientjes[a], Rudi J.Planta[a], Hans M.G. Princen[b] and Willem H. Mager[a].

[a]Department of Biochemistry and Molecular Biology, IMBW, BioCentrum Amsterdam, Vrije Universiteit, 1081 HV Amsterdam, The Netherlands; and [b]Gaubius Laboratory TNO-PG, P.O. Box 430, 2300 AK Leiden, The Netherlands.

Submitted for publication

ABSTRACT

Cholesterol 7α-hydroxylase (CYP7A) is the major rate-limiting enzyme in bile acid biosynthesis. It plays an important role in cholesterol homeostasis and is known to be regulated by several factors, including bile acids returning to the liver. We have previously reported the isolation, sequencing and initial functional characterization of 3.6 kb 5'-flanking region of the rat CYP7A gene (Hoekman *et al.* (1993) Gene 130, 217-223). In the present study, we extended these studies by constructing a more detailed deletion series of the CYP7A promoter and analyzing the respective promoter fragment CAT-reporter genes by transient expression assays in primary rat hepatocytes. The results obtained demonstrate that the major transcription activating element is located in the proximal part of the promoter, viz. between bp -47 to -79. Strikingly, most of the regulatory responses examined so far were also found to be mediated through the -47 to -79 element: down-regulation by cholic acid (3-fold), induction by retinoic acid (3-4x), suppression by insulin (3x) and induction by βVLDL-derived cholesterol (2x). The bile acid ursocholic acid did not cause a down-regulation of CYP7A gene transcription. In addition, dexamethasone appeared to have a general stimulatory effect on gene expression as was evident from promoter-reporter studies and measurement of CYP7A transcriptional activity and mRNA steady-state levels. Band shift analyses using rat liver nuclear extracts and the proximal 145 bp of the CYP7A promoter as a probe revealed the formation of at least two protein-DNA complexes. These complexes were found to be competed by a synthetic oligomer encompassing the -47 to -79 region. The data taken together indicate the presence of a composite element in the CYP7A promoter which plays a major role in transcriptional regulation by several physiological mediators.

INTRODUCTION

Uptake, synthesis and catabolism of cholesterol are tightly controlled processes, aimed at maintaining proper cholesterol homeostasis. The major pathway for the removal of cholesterol from the mammalian body is the formation of bile acids (1,2). The first and rate-limiting reaction in the major pathway in the conversion of cholesterol to bile acids, the 7α-hydroxylation of cholesterol, is catalysed by the P-450 isoenzyme cholesterol 7α-hydroxylase (CYP7A) (3,4). CYP7A has been reported to display a response to various physiological signals. In previous work we described the isolation and the initial functional characterization of 3.6 kb of the rat CYP7A 5'-flanking region (5). Comparison of the rat promoter region with known promoter sequences of human (6), hamster (7) and mouse (8) CYP7A genes, revealed

a high sequence homology in the proximal 250 bp, suggesting that this region fulfills an important role in the transcriptional regulation of the CYP7A gene. Interestingly, CAAT, LFB1, HRE, TGT3, and BTE motifs are clustered and partly overlapping each other in this region of the CYP7A promoter. Therefore, liver-enriched transcription factors such as HNF1 and HNF3 (binding the consensus motifs LFB1 and TGT3, respectively) are expected to play a role in the CYP7A gene expression. Molowa *et al.* (6) demonstrated that three cooperating TGT3 elements, all located within the first 400 bp of the human CYP7A promoter, can mediate liver-specific transcription. Recently, a rat liver-specific enhancer region, located 7 kb upstream of the transcription initiation site, and containing several binding sites for HNF3, was identified (9). In addition, studies by Lavery and coworkers (10) showed that the liver-enriched transcription factor DBP may be involved in the transcriptional regulation of the circadian CYP7A gene expression.

A negative feedback regulation through bile acids returning to the liver via the enterohepatic pathway, is thought to be an essential aspect of CYP7A regulation (11,12). As a consequence, biliary drainage or administration of the bile acid-binding resin cholestyramine causes increased CYP7A activity and mRNA levels (13,14). In previous work we have demonstrated the suppression of CYP7A by bile acids in cultured rat and pig hepatocytes (15), and regulation at the level of mRNA stability and transcription (16). Furthermore, we have shown that a bile acid-responsive region in the proximal promoter of the CYP7A gene may be involved in this transcriptional regulation (5).

In addition, several hormones have been implicated in control of CYP7A-activity. For instance, steroid and thyroid hormones have been reported to affect CYP7A gene expression. Thyroid hormone T3 was found to increase CYP7A enzyme activity and mRNA steady state levels *in vivo* as well as in HepG2 cells (17-19). Studies undertaken in our laboratory suggested the direct involvement of *cis*-elements located upstream of position -348 of the CYP7A promoter in the T3-mediated transcriptional response (5). The synthetic glucocorticoid dexamethasone is known to markedly increase CYP7A activity in *vivo* in adrenalectomised rats (20) and activity as well as mRNA steady-state levels in primary cultures of rat hepatocytes (21,22). Insulin has also been implicated to play an important role in cholesterol metabolism (23). However, the effects of this hormone on CYP7A regulation are less clear. It was notable that several putative hormone response elements (HREs) were found within the proximal part of the CYP7A promoter. This may indicate that the CYP7A hormone-mediated regulation takes place -at least in part- at the transcriptional level.

The role of cholesterol in the regulation of CYP7A gene expression is controversial. In *in*

vivo experiments, dietary cholesterol was found to increase cholesterol 7α-hydroxylase activity at the level of gene transcription (24). However, these latter findings were questioned by Björkhem *et al.* (25). They reported that a diet high in cholesterol causes bile acid malabsorption in rats, implying that the previously shown increase in CYP7A transcriptional activity may rather be a secondary effect, caused by lowering the amount of bile acids returning to the liver.

In this paper we report that the regulatory responses upon addition of bile acids, retinoic acid, insulin and exogenous cholesterol to the culture-medium of primary rat hepatocytes, are mediated through a distinct *cis*-acting region located in the proximal promoter of the CYP7A gene.

MATERIALS AND METHODS

Plasmid construction

In order to identify the promoter elements that play a functional part in the regulation of transcription of CYP7A, different portions of the upstream DNA fragment were fused to the bacterial CAT-reporter gene, as described previously (5; see also Fig.1) . The resulting hybrid genes contain the transcription initiation site of CYP7A as determined previously (26). Further 5' deletion of the CYP7A promoter (leading to constructs -166RCAT, -145RCAT, -121RCAT, -91RCAT, -76RCAT, -71RCAT, -68RCAT and -49RCAT respectively) was performed by digestion with BAL31 exonuclease, starting from position -348. The deletion endpoints were determined by DNA sequencing. A hybrid gene, harboring the proximal 348 bp of the CYP7A promoter but lacking the -79/-47 region fused to the CAT-reporter gene, was constructed using the pSelect system (Promega). For that purpose we synthesized an oligonucleotide corresponding to position -94 to -34 of the CYP7A rat promoter, but missing the -80 to -48 region. Construction of the resulting plasmid, Δ348RCAT, was verified by DNA sequencing.

Transfection experiments using cultured rat hepatocytes

The isolation and culturing of primary rat hepatocytes, as well as the transient expression experiments and CAT assays were performed as described previously (5,27).

Isolation of lipoprotein deficient serum and βVLDL

Lipoprotein deficient serum (LPDS) was isolated from Fetal Calf Serum (Boehringer Mannheim) by ultracentrifugation at 4^0C for 48 h after a density adjustment with solid KBr (28). The LPDS fraction (d>1.21 g/ml) was dialysed at 4^0C against 10 mM sodium phosphate, 0.15 M NaCl (pH 7.4) for 24 h, and subsequent dialysis against Williams E medium for an additional 24 h. Williams E medium was added up to the initial volume (100% LPDS). Before addition to the culture medium, the LPDS was filtered through a 0.22 μm membrane. For the isolation of βVLDL, blood was obtained from rats fed a diet supplemented with 0.5% (w/w) cholesterol for 7 days. βVLDL (d<1.006) was isolated from serum by ultracentrifugation according to Redgrave *et al.* (29) at 4^0C for 24 h.

Preparation of rat liver nuclear extracts

Liver nuclear extracts from 3-month-old male Wistar rats were prepared essentially as described by Gorski *et al.* (30) and Graves *et al.* (31). The animals were maintained on standard chow and water *ad libitum*. Separate groups of rats were fed a similar diet supplemented with 4% (w/w) cholestyramine or 0.5% (w/w) cholic acid, respectively, for seven days. All experiments were carried out at 0^0C to 4^0C. Usually 30 g of minced liver was suspended in homogenization buffer (10 mM HEPES.KOH (pH 7.6), 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2 M sucrose, 10 % glycerol) up to a final volume of 30 ml. It was then homogenised in the absence of air in a modified Waring blender. The homogenate was diluted 3 times in homogenization buffer, layered in 25 ml portions on 10 ml cushions of the homogenization buffer and centrifuged at 24,000 rpm for 45 min at 0^0C in a SW28 rotor (100,000 g) (32). This centrifugation step was repeated after resuspending the combined nuclear pellets in 50 ml homogenization buffer, layered on two 10 ml cushions of the homogenization buffer. The pellets were washed twice with nuclear lysis buffer (10 mM HEPES.KOH (pH 7.6), 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 10 % glycerol) and resuspended in 20 ml of nuclear lysis buffer, using an all-glass Dounce homogenizer (Wheaton pestle B). An aliquot was diluted 50 times in 0.5 % SDS and the A_{260nm} was measured. The nuclear suspension was diluted to approximately 10 A_{260nm} units per ml and extracted essentially as described by Parker and Topol (33). One-tenth volume of 4 M $(NH_4)_2SO_4$ (pH 7.9) was added directly and the extract was gently shaken and left on ice for 30 min. The viscous lysate was then centrifuged at 45,000 rpm for 90 min in a 50.2 Ti rotor at 0^0C to pellet the chromatin (200,000 x g). Subsequently solid $(NH_4)_2SO_4$ (0.3 g/ml) was added to the supernatant and allowed to dissolve by gentle agitation. After an additional 30

min on ice, precipitated proteins were collected at 26,000 rpm in a SW28 rotor at 0^0C. The protein pellet was stored overnight on ice, and subsequently suspended in dialysis buffer (25 mM HEPES.KOH (pH 7.6), 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, 10 % glycerol and 0.5 mM PMSF) at 1 ml/400 A_{260nm} units of nuclear lysate. The protein extract was dialysed against dialysis buffer. After dialysis the protein extract was centrifuged for 5 min in a microfuge to remove insoluble material. The protein concentration amounted usually to 4-8 mg/ml. Protein extracts were stored in aliquots under liquid nitrogen.

Electrophoretic mobility shift assays

Binding assays were performed essentially as described by Monaci *et al.* (34). The experiments were carried out either with a labelled double stranded oligonucleotide or with a suitable labelled DNA fragment. Nonspecific competitor (1µg pUC18 plasmid DNA) was first incubated with 0.5 to 10 µg of liver nuclear extract in a buffer containing 25 mM HEPES.KOH (pH 7.6), 7.5 % glycerol, 60 mM KCl, 50 mM $MgCl_2$, 4 mM spermidine, 0.75 mM DTT, 0.1 mM EDTA for 20 min at 0^0C. Then the probe was added and incubated for 15 min at room temperature. After this incubation 2 µl of 20 % Ficoll, 0.1 % xylene cyanol FF, 0.1 % bromofenol blue were added and the samples were loaded onto a 5 % polyacrylamide gel in 0.5 x TBE buffer and subjected to electrophoresis at 10 V/cm for 2-3 h.

RESULTS AND DISCUSSION

Analysis of the CYP7A promoter activity

In order to identify elements playing a role in the transcriptional activation of the rat cholesterol 7α-hydroxylase (CYP7A) gene, chimeric genes were constructed by fusing different parts of the CYP7A 5'-flanking region to the coding region of the CAT-reporter gene. The resulting hybrid genes, shown in Figure 1, were then tested for their ability to activate transcription after transfection of primary hepatocytes in culture. Cells were cultured under conditions at which CYP7A-activity and bile acid biosynthesis were maintained (27). As shown in Figure 1, the proximal 348 bp of the CYP7A-promoter (-348RCAT) appeared to confer the highest transcriptional activity, which is about 10% of the activity observed with the SV40CAT construct used as a reference (result not shown). On the other hand, the -49RCAT construct showed only a low, basal activity. The deletion-series progressing from position -348 to -49 in the CYP7A promoter, showed a more or less gradual decrease in CAT-activity. The first drop

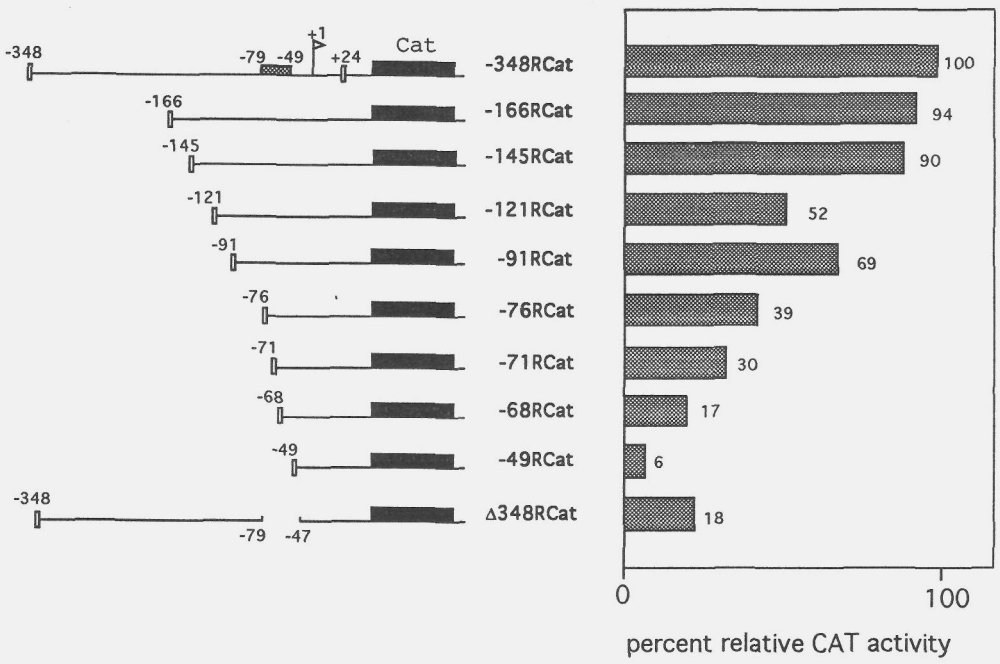


Fig. 1 Transient activity of the CYP7A promoter in primary rat hepatocytes. CAT activity was determined in primary cultures of rat liver cells transfected using the Ca-phosphate method. The hybrid plasmids contain various fragments of the 5'-flanking region of rat CYP7A fused to the CAT reporter gene (black bar) at +24 of the CYP7A sequence. The size of CYP7A fragments (in bp) is indicated relative to the cap-site (+1). The CYP7A promoter activity of each reporter construct is expressed relative to that of the -348RCAT construct (value set at 100%). The amounts of acetylated product as represented by signals on the autoradiograms were quantified with a Phosphor-imager 400B (Molecular Dynamics). Data were corrected for protein concentration and transfection efficiency. Data represent means of three independent experiments.

in transcription-activation was observed when 24 bp were deleted from the -145RCAT construct, creating -121RCAT. This may indicate the presence of (a) *cis*-element(s) located between bp -145 and -121 which positively regulates CYP7A-transcription. Indeed, a putative LFA1-site is positioned in this region of the CYP7A-promoter at position -132. The level of CAT-activity is decreased as a consequence of the further progressive deletion of 5'-flanking parts of the 7α-promoter, suggesting the presence of multiple, *cis*-acting elements in this region. It is clear from the presented data that most of the transcription activating capacity is located within the proximal 145 bp.

Previous results, obtained with 5'-deletion constructs, indicated that the region between position -79 and -49 of the rat CYP7A promoter may be sufficient to mediate the negative regulatory response by bile acids (5). To further investigate the role of this element in the transcriptional activation and regulation of the CYP7A gene, a hybrid gene was constructed, harboring the proximal 348 bp of the 7α-promoter but lacking the -79/-47 region. This internal deletion construct (Δ348RCAT) was used in transient expression experiments as described above. As can be concluded from Figure 1, the Δ348RCAT construct displayed an activity higher than the (basal) activity of -49RCAT, but considerably lower than the activity of the -348RCAT construct. We thus conclude that the -79/-47 region carries the major transcription activating element(s) in the proximal promoter of the CYP7A-gene.

As stated in the Introduction section, consensus sequences for CAAT, *LFB1*, *HRE* and TGT3 sites are present in the -79/-47 region in an overlapping arrangement. The abovementioned results suggest that CYP7A transcription is primarily activated by factors binding to motifs within this highly conserved region, such as C/EBP, HNF1 and HNF3. Furthermore, the results suggest that these transcriptional activators may act in concert with factors binding further upstream in the CYP7A promoter, for instance the BTE-binding protein or HNF4 (binding to the LFA1-motif).

Regulation of CYP7A expression

(a) Regulation by bile acids

In previous work (5) we showed that CYP7A transcription decreased 3-fold upon addition of 50 μM of the bile acid cholate to primary cultures of rat hepatocytes. Based on the results obtained with transient expression experiments, it was concluded that the element from position -79 to -49 within the CYP7A proximal promoter probably is involved in this transcriptional regulation by bile acids. To further investigate the role of the presumed bile acid-responsive region, we transfected primary hepatocytes in culture with the -348RCAT or the Δ348RCAT construct. The results, depicted in Figure 2A, confirm that upon addition of 50 μM cholic acid to the medium, the -348RCAT construct displayed a clear suppression (CAT-activity drops threefold), whereas the Δ348RCAT construct did not. Apparently, deletion of the -47/-79 region from the CYP7A proximal promoter, prevented occurrence of the cholate-mediated response. The data strongly suggest that element(s) within the -79/-47 region of the CYP7A proximal promoter play(s) an important role in conferring the response upon bile acids, such as cholate. As described above, the -79/-47 region contains several putative *cis*-

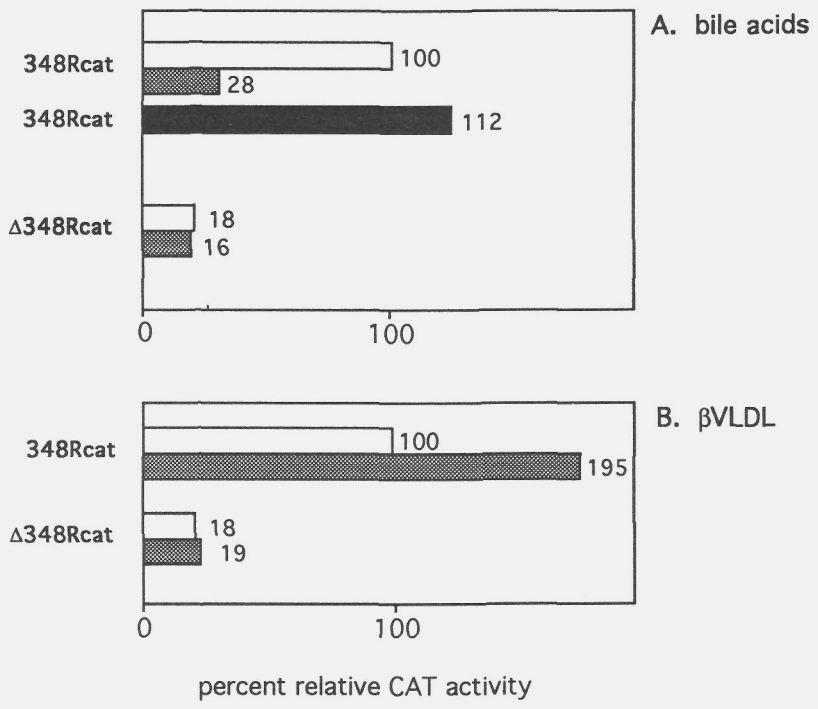


Fig. 2 Transient activity of the CYP7A promoter in primary rat hepatocytes. CAT activity was determined in primary cultures of rat liver cells transfected using the Ca-phosphate method. The CYP7A promoter activity of the reporter constructs is expressed relative to that of the -348RCAT construct (value set at 100%). Open bars represent CAT activity measured in hepatocytes without the addition of effectors. **(A)** Transfected cells were cultured in the presence of 50 μM cholic acid (hatched bars) or 50 μM ursocholic acid (black bar). **(B)** Transfected cells were cultured in the presence of 40 μg (protein)/ml rat βVLDL (hatched bars). The culturing medium was supplemented with 10% LPDS (lipoprotein deficient serum). The amounts of acetylated product as represented by signals on the autoradiograms were quantified with a Phosphor-imager 400B (Molecular Dynamics). Data were corrected for protein concentration and transfection efficiency. Data represent means of three independent experiments.

acting elements, amongst others an HRE-like sequence motif, which may represent a binding site for steroid(hormone) receptors. It has been postulated that the hypothetical bile acid receptor may be a member of the nuclear receptor super gene family. When this manuscript was in preparation, Chiang and Stroup (35) suggested that an until now unknown factor, designated BARP (bile acid response protein), may bind to a direct repeat (DR) at position -65 to -54 (CAAGNNCAAG). According to this hypothesis, BARP may serve as a positive

transcription factor, which is under negative control by bile acids. In addition to this proposed regulatory mechanism, we envision the possibility that the bile acid receptor may compete with another receptor in the formation of (hetero)dimers (see concluding remarks).

The potency of different bile acids to act as suppressors of CYP7A expression is found, amongst others, to vary with their relative hydrophobicity. Heuman *et al.* (36) and Kwekkeboom *et al.* (15) observed that relatively hydrophilic bile acids failed to suppress CYP7A, whereas relatively hydrophobic bile acids down-regulated the enzyme activity in the order of increasing hydrophobicity. Interestingly, primary hepatocytes transfected with the -348RCAT plasmid showed no response in CAT-activity after addition of 50 μM ursocholate, a relative hydrophilic bile acid. This is in agreement with results obtained by Twisk *et al.*(37), demonstrating that CYP7A mRNA-stability and transcription is not affected by addition of ursocholate to primary cultures of rat hepatocytes. It must be noted that addition of the bile acids cholic acid and ursocholic acid did not affect the control SV40CAT-signal (results not shown), indicating the specificity of the shown responses.

(b) Regulation by βVLDL

In order to investigate the role of exogenous cholesterol in the transcriptional regulation of the CYP7A gene, rat hepatocytes in culture were transfected with the -348RCAT or the Δ348RCAT plasmid in the presence or absence of the cholesterol-rich lipoprotein βVLDL. Figure 2B shows the results of the respective CAT assays. The proximal part of the CYP7A promoter, up to position -348, was found to confer a twofold increase in CAT-activity upon the addition of 40 μg/ml βVLDL. This result is in agreement with data obtained from run-on experiments performed with primary rat hepatocytes (Twisk *et al.*, unpublished results). In contrast, the Δ348RCAT construct did not display a response upon βVLDL administration. As a control, addition of βVLDL did not affect the SV40CAT-signal (result not shown).

Apparently, exogenous cholesterol, in the form of βVLDL, has a stimulatory effect on CYP7A-transcription. Furthermore, the data indicate the -79/-47 region to contain an element presumed to mediate the βVLDL -response in the hepatocyte, either directly or indirectly. However, no Sterol Response Elements (SREs), which have been implicated to mediate the regulation of genes involved in cholesterol biosynthesis, such as those encoding HMG-CoA synthase, HMG-CoA reductase and the LDL receptor (38), were found in this region nor in the rest of the first 3.6 kb of the rat CYP7A 5'-flanking region. This may indicate that (an)other, as yet unknown, *cis*-acting element(s) mediates the CYP7A-response upon addition of cholesterol to the hepatocyte.

(c) Regulation by hormones

- Insulin

Evidence from *in vivo* studies indicated that the bile acid pool and bile acid excretion is increased in man suffering from diabetes mellitus and in experimental diabetic animals, and that both parameters return to normal levels after administration of insulin (39-41). Twisk *et al.* (42) recently demonstrated that the addition of insulin to primary cultures of hepatocytes down-regulated bile acid biosynthesis by suppressing cholesterol 7α-hydroxylase and sterol 27-hydroxylase enzyme activity, their steady-state mRNA levels and transcriptional activity. In order to study the mechanism of the insulin-mediated regulation on CYP7A gene expression, primary hepatocytes in culture were transfected with the -348RCAT or the Δ348RCAT construct. As shown in Figure 3A, CAT-activity decreased 3-fold after addition of 140 nM insulin to -348RCAT transfected cells. However, cells transfected with the Δ348RCAT construct, displayed no decrease in CAT activity upon administration of insulin. Addition of insulin had no effect on the SV40CAT-control signal (result not shown). These results demonstrate the direct involvement of the proximal 348 bp of the CYP7A promoter in the insulin-mediated regulation. Furthermore, it is likely that the -79/-47 region contains a *cis*-acting element that is indispensable for this hormonal control.

- Retinoic acid

The vitamin A-derived hormone *all-trans* retinoic acid (RA) and its natural and synthetic analogs (retinoids) affect a large number of biological processes (43). To investigate the possible role of RA in CYP7A gene expression, we performed transfection experiments using the -348RCAT or the Δ348RCAT plasmid in cultured primary rat hepatocytes. As shown in Figure 3B, the administration of 10 μM *all-trans* RA increased the -348RCAT activity 3- to 4-fold. The additional administration of 10 μM *9-cis* RA led to a significant decrease in CAT-activity. No effect was observed in the SV40CAT-control signal after addition of *all-trans* or *9-cis* retinoic acid (results not shown). It is known that *all-trans* RA acts via a retinoic acid receptor (RAR), which binds to a so-called RARE (retinoic acid response element) motif (44). Recently it became apparent that RARs form heterodimers with retinoic X receptors (RXRs) (45). These heterodimers, rather than RARs alone, bind RARE-motifs, thus regulating transcription. Evidence is emerging that *9-cis* RA has the capacity to stimulate RXR-homodimer formation, resulting in the inability for RARs to bind RXRs and modulate transcription (46). Our data are consistent with such interplay of *all-trans* and *9-cis* RA. It is tempting to speculate that the HRE-motif located within the -79/-47 region might represent the

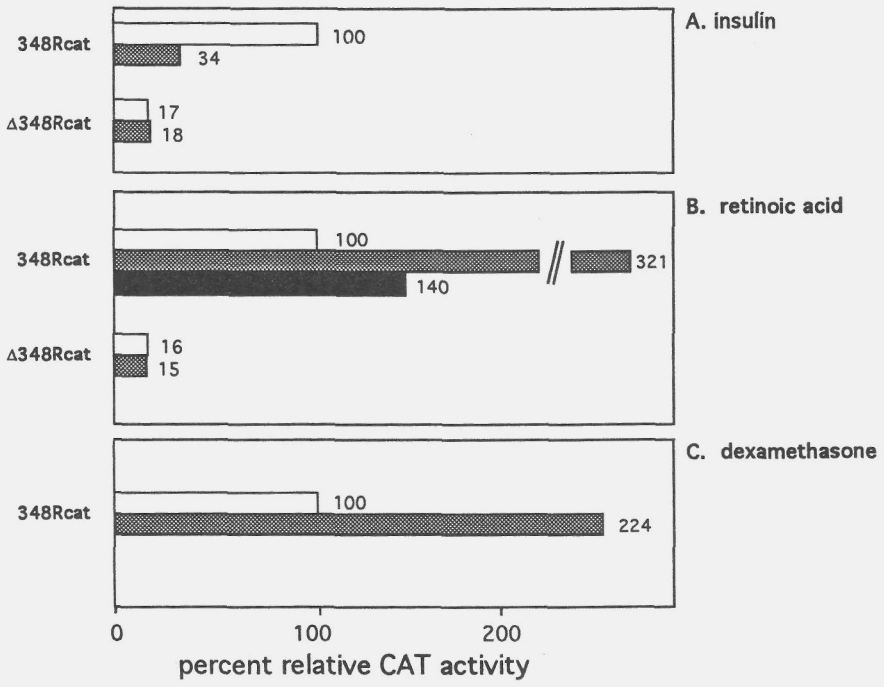


Fig. 3 Transient activity of the CYP7A promoter in primary rat hepatocytes.

(A) Transfected cells were cultured in the presence of 140 nM insulin (hatched bars). The culturing medium did not contain insulin. **(B)** Transfected cells were cultured in the presence of 10 μM *all-trans* retinoic acid (hatched bars) or in the presence of 10 μM *all-trans* and *9-cis* retinoic acid (black bar). (C) Transfected cells were cultured in the presence of 1 μM dexamethasone (hatched bars). The amounts of acetylated product as represented by signals on the autoradiograms were quantified with a Phosphor-imager 400B (Molecular Dynamics). Data were corrected for protein concentration and transfection efficiency. Data represent means of three independent experiments.

corresponding *cis*-element. Interestingly, no effect of *all-trans* RA was detected when the Δ348RCAT-plasmid was used, suggesting that the -79/-47 region is essential in conferring the *all-trans* RA-response (see Fig. 3B).

- Dexamethasone

Previous studies have shown that dexamethasone stimulated CYP7A enzyme-activity (21) and its steady-state mRNA levels (22) in cultures of rat hepatocytes. To further examine the effect of the glucocorticoid dexamethasone on CYP7A gene expression, primary cultures of rat hepatocytes were transfected, using the -348RCAT construct in the presence or absence of

this hormone analog. Upon addition of 1 μM dexamethasone CAT-activity markedly increased (Figure 3C). However, a parallel induction of CAT-activity was also seen in SV40CAT-transfected cells (results not shown), indicating that dexamethasone has a general stimulatory effect on gene expression. These results were confirmed by Twisk *et al.* (unpublished data), showing that the addition of dexamethasone to primary cultures of rat hepatocytes had a clear inducing effect on steady-state mRNA levels and transcriptional activity of CYP7A, sterol 27-hydroxylase, actin and albumin. The data suggest that dexamethasone affects the mRNA-stability in general, but the precise working mechanism has yet to be elucidated.

DNA-protein interactions

In an attempt to identify proteins showing affinity for the transcription activating region of the CYP7A gene, we performed electrophoretic mobility shift assays, using crude rat liver nuclear extract and the proximal 145 bp of the 5'-flanking region (-145 to +1) of the CYP7A gene. The band shift analysis depicted in Figure 4A, demonstrates the formation of at least two major protein-DNA complexes, C1 and C2 (lane 2). Increasing the amount of added protein did not lead to further retardation of the complexes (lane 3). Autocompetition provided evidence that the formed complexes are the result of specific binding (lane 4).

Since the transient expression experiments discussed above, demonstrated that the -79/-47 region within the proximal CYP7A promoter plays an important role in the activation and regulation of the CYP7A gene transcription, a synthetic oligomer encompassing this element was synthesized and used in band shift analyses. When the 145 bp fragment was used as a probe, the -79/-47 element was found to compete all complex-formation (lane 6), suggesting that this oligomer most likely encompasses the entire or major protein-binding element located in the proximal 145 bp of the CYP7A promoter. The band shift assay using the -79/ -47 oligo as a probe revealed only one distinct protein-DNA complex (Fig. 4B, lane 2). This complex was not further retarded when the amount of protein was increased (lanes 3 to 6). We hypothesise that binding of (a) protein(s) to the -79/-47 region is required for additional protein binding to the proximal promoter. Both the -145 bp fragment and the -79/-47 oligo were used as probes in band shift analyses with nuclear liver extracts from rats fed on different diets, *i.e.* control, cholestyramine and cholate fed rats. In none of the band shift experiments performed so far we were able to detect qualitative differences between different extracts (results not shown). When this manuscript was in preparation, similar results were published by Chiang and Stroup (35).

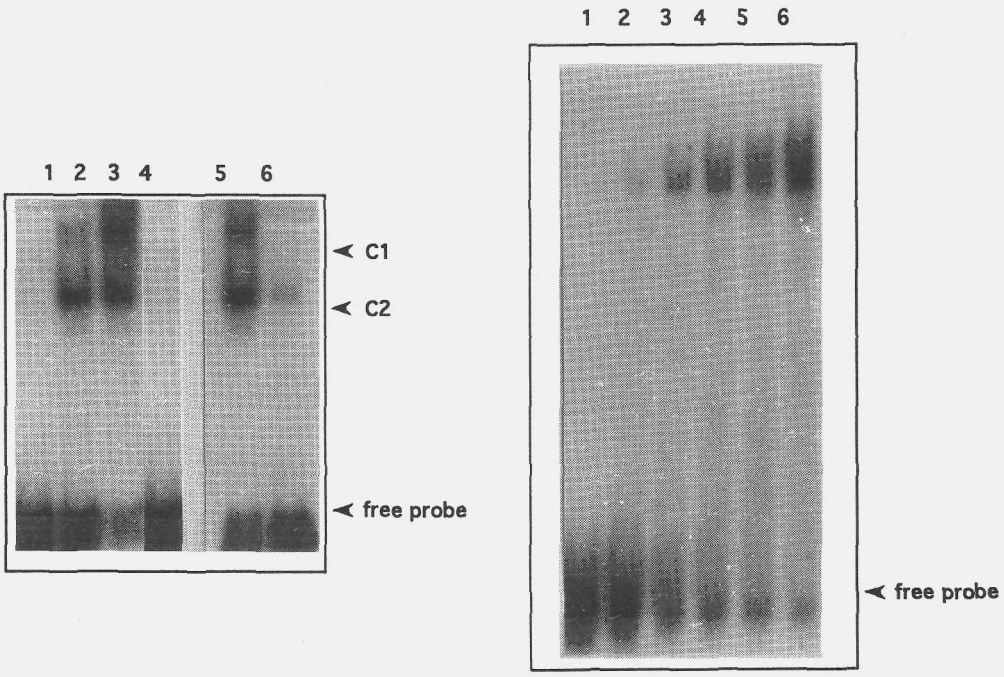
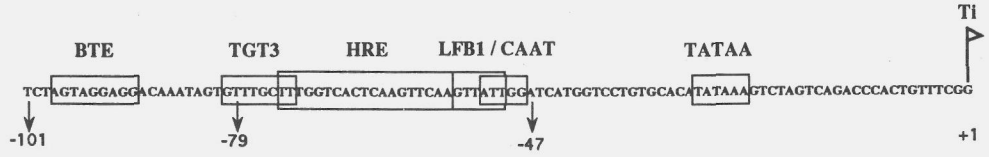


Fig. 4 Electrophoretic mobility shift assays of the proximal region of the CYP7A promoter. **(A)** The labelled proximal 145 bp of the CYP7A promoter was incubated in the presence of rat liver nuclear extract. Lane 1: free probe; lane 2,3: 5 and 10 μg of nuclear extract; lane 4: as lane 2, but in the presence of 50-fold excess unlabelled 145 bp. fragment; lane 5: 5 μg of nuclear extract; lane 6: as lane 5, but in he presence of 50-fold excess unlabelled -79/-47 oligomer. **(B)** The labelled -79/-47 region from the CYP7A promoter was incubated in the presence of rat liver nuclear extract. Lane 1: free probe; lane 2-6: 2, 4, 6, 8 and 10 μg of rat liver nuclear extract.

Concluding remarks

In conclusion, the data presented in this paper strongly suggest that a putatively composite element in the -79/-47 region of the proximal promoter of the rat CYP7A gene plays a major role in the transcriptional activation of this gene and, moreover, mediates several regulating responses investigated sofar. Figure 5B illustrates our model proposed for transcriptional regulation of the rat CYP7A gene. We suggest that general and liver-enriched factors (C/EBP, HNF1 and HNF3) bind cooperatively to the corresponding *cis*-acting elements within the -79/-47 region, forming an 'upstream' complex. In this model, transcription is activated through interactions between this 'upstream' complex and the preinitiation complex. Additional protein factors binding in the vicinity of the -79/-47 element, such as the BTE or LFA1 binding proteins, may contribute to activation of CYP7A transcription in concert with the composite

A.



B.

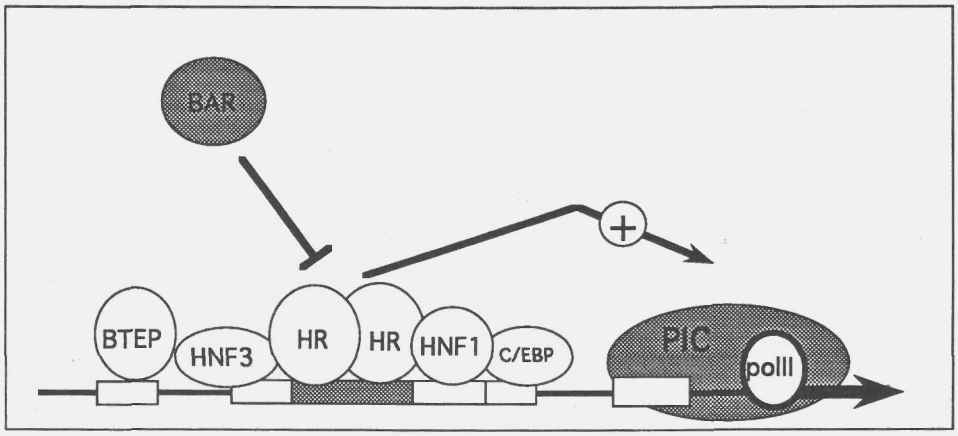


Fig. 5 Model of the transcriptional regulation of CYP7A gene expression. (A) Nucleotide sequence of the proximal 101 bp of the rat CYP7A promoter. The transcription initiation point as determined previously (23), is indicated (Ti; +1). Conserved regulatory elements are boxed: CAAT, reversed CAAT box; LFB1, Liver Factor B1; HRE, Hormone Responsive Element; TGT3, binding site for the Hepatic Nuclear Factor 3 (HNF3); BTE, Basic Transcription Element. (B) Representation of transcription factors binding to responsive motifs and regulating CYP7A transcription. See text for details. Proximal *cis*-acting elements, corresponding with the motifs indicated in Fig. 5A, are shown as rectangles. Proteins binding to these elements are represented symbolically and include the preinitiation complex (PIC), consisting of Polymerase II (pol II) and basic transcription factors (not shown). Putative DNA-binding proteins that may function through specific sequence elements are indicated: C/EBP, CAAT-element binding protein; HNF1 and HNF3, hepatic nuclear factors 1 and 3; HR, hormone receptor; BTEP, BTE binding protein. The putative Bile Acid Receptor (BAR) interacts either with the Hormone Receptors or binds to motifs within the HRE region (indicated by arrow). CYP7A transcription is subsequently down-regulated through interactions between the modulated basal complex and the preinitiation complex (indicated by arrow/minus).

upstream complex. In addition, we suggest the binding of hormone-responsive factors to motifs within the HRE region, mediating different types of CYP7A-transcriptional control. As suggested in Fig. 5B, after ligand-binding, two Hormone Receptors (HR) may form a dimer complex, interacting with the HRE. In line with this view, we envision the putative bile acid receptor (BAR) to be a member of the steroid hormone receptor family, which is activated by incoming bile acids and interacts with the HR-complex. BAR may eiher bind directly to the dimer, or may compete with the HR-complex for a binding motif within the HRE region. CYP7A transcription may thus be down-regulated through reduced or less efficient interaction between the upstream complex and the preinitiation complex (Fig. 5B). We hypothesise that the RA-mediated regulation of CYP7A gene expression has a similar working-mechanism. It has been shown that *all-trans* retinoic acid promotes dimerization of the RAR and RXR factors, forming an RAR-RXR heterodimer (45). We suggest this dimer to interact with the HR-complex, and exert its inducing effect on CYP7A gene transcription either by directly binding to the HR-dimer or by competing for a motif within the HRE region.

The picture emerging from our data is in agreement with the present knowledge of transcriptional regulation of other liver-specific genes: several *cis*-acting elements and *trans*-acting factors interacting in a complex and dynamic network, and ultimately resulting in an overall transcriptional activity of the gene. Identification and characterization of *cis*-elements involved in this process are the first steps in the elucidation of the transcriptional regulation of this gene, important in maintaining cholesterol homeostasis.

ACKNOWLEDGMENTS

We thank Miss Emelie Martijn for performing part of the band shift assays and Miss Elly de Wit for technical assistance during part of the experiments. This work was supported by HGO-TNO, grant 900-523-138 to M.F.M. Hoekman and J.M.J. Rientjes, and the Netherlands Heart Found., grant 89.079 to J. Twisk.

REFERENCES

1. Danielsson, H., and Sjöval, J. : Bile acid metabolism. Annu. Rev. Biochem. 44 (1975) 233-253.
2. Myant, N.B., and Mitropoulos, K.A. : Cholesterol 7α-hydroxylase. J. Lipid Res. 18 (1977) 135-152.
3. Shefer, S., Hauser, S., Bekersky, I., and Mosbach, E.H. : Biochemical site of regulation of bile acid synthesis in the rat. J. Lipid Res. 11 (1970) 404-411.
4. Gonzalez, F.J. : The molecular biology of cytochrome P450s. Pharmacol. Rev. 40 (1989) 243-288.
5. Hoekman, M.F.M., Rientjes, J.M.J., Twisk, J., Planta, R.J., Princen, H.M.G., and Mager,W.H. : Transcriptional regulation of the gene encoding cholesterol 7α-hyroxylase in the rat. Gene 130 (1993) 217-223.
6. Molowa, D.T., Chen, W.S., Cimis, G.M., and Tan, C.P. : Transcriptional regulation of the human cholesterol 7α-hydroxylase gene. Biochemistry 31 (1992) 2539-2544.
7. Crestani, M., Galli, G., and Chiang, J.Y.L. : Genomic cloning, sequencing, and analysis of the hamster cholesterol 7α-hydroxylase gene (CYP7). Arch. Biochem. Biophys. 306 (1993) 451-460.
8. Tzung, K., Ishimura-Oka, K., Kihara, S., Oka, K., and Chan, L. : Structure of the mouse cholesterol 7α-hydroxylase gene. Genomics 21 (1994) 244-247.
9. Ramirez, M.I., Karaoglu, D., Haro, D., Barillas, C., Bashirzadeh, R., and Gil, G. : Cholesterol and bile acids regulate cholesterol 7α-hydroxylase expression at the transcriptional level in culture and in transgenic mice. Mol. Cell. Biol. 14 (1994) 2809-2821.
10. Lavery, D.J., and Schibler, U. : Circadian transcription of the cholesterol 7α-hydroxylase gene may involve the liver-enriched bZIP protein DBP. Genes and Dev. 7 (1993) 1871-1884.
11. Björkhem, I. : Mechanism of bile acid biosynthesis in mammalian liver. In: Sterols and Bile Acids. Danielsson, H., and Sjöval, J., eds., Elsevier, New York (1985) pp. 231-278.
12. Carey, M.C., and Cahalane, M.J. : Enterohepatic circulation. In: The Liver, Biology and Pathology, Arias, I.M., Jakoby, W.B., Popper, H., Schachter, D., Shafritz, D. A., eds., Raven Press, New York (1988) pp. 573-616.
13. Danielsson, H., Einarsson, K., and Johansson, G. : Effects of biliary drainage on individual reactions in the conversion of cholesterol to taurocholic acid. Eur. J. Biochem. 2 (1967) 44-49.
14. Chiang, J.Y.L., Miller, W.F., and Lin, G.M. : Regulation of cholesterol 7α-hydroxylase in the liver: Purification of cholesterol 7α-hydroxylase and the immunochemical evidence for the induction of cholesterol 7α-hydroxylase by cholestyramine and circadian rhythm. J. Biol. Chem. 265 (1990) 3889-3897.
15. Kwekkeboom, J., Princen, H.M.G., Van Voorthuizen, E.M., and Kempen, H.J.M. : Bile acids exert negative feedback control on bile acid synthesis in cultured pig hepatocytes by suppression of

cholesterol 7α-hydroxylase activity. Hepatology 12 (1990) 1209-1215.

16. Twisk, J., Lehmann, E.M., and Princen H.M.G. : Differential feedback regulation of cholesterol 7α-hydroxylase mRNA and transcriptional activity by rat bile acids in primary monolayer cultures of rat hepatocytes. Biochem. J. 290 (1993) 685-691.

17. Ness, G.C., Pendleton, L.C., Li, Y.C., and Chiang, J.Y.L. : Effect of thyroid hormone on hepatic cholesterol 7α-hydroxylase, LDL receptor, HMG-CoA-reductase, farnesyl pyrophosphate synthetase and apolipoprotein A-I mRNA levels in hypophysectomized rats. Biochim. Biophys. Res. Commun. 172 (1990) 1150-1156.

18. Crestani, M., Karam, W.G., and Chiang, J.Y.L. : Effects of bile acids and steroid/thyroid hormones on the expression of cholesterol 7α-hydroxylase mRNA and the CYP7 gene in HepG2 cells. Biochem. Biophys. Res. Comm. 198 (1994) 546-553.

19. Balasubramaniam, S., Mitropoulos, K.A., and Myant, N.B. : Hormonal control of the activities of cholesterol 7α-hydroxylase and hydroxymethyl-glutaryl-CoA reductase in rats. In: Advances in bile acid research, III. Bile Acid Meeting, Freiburg. Matern, S., Hackenschmidt, J., Back, P., and Gerok, W., eds., Schattauer Verlag, Stuttgart (1975) 61-67.

20. van Cantfort, J. : Controle par les glucocortico-steroides de l'activité circadienne de la cholesterol 7α-hydroxylase. Biochimie 55 (1973) 1171-1173.

21. Princen, H.M.G., Meijer, P., and Hofstee, B. : Dexamethasone regulates bile acid synthesis in monolayer cultures of rat hepatocytes by induction of cholesterol 7α-hydroxylase. Biochem. J. 262 (1989) 341-348.

22. Hylemon, P.B., Gurley, E.C., Stravitz, R.T., Litz, J.S., Pandak, W.M., Chiang, J.Y.L., and Vlahcevic, Z.R. : Hormonal regulation of cholesterol 7α-hydroxylase mRNA levels and transcriptional activity in primary rat hepatocyte cultures. J. Biol. Chem. 267 (1992) 16866-16871.

23. Grundy, S.M. : Treatment of hypercholesterolemia by interference with bile acid metabolism. Arch. Intern. Med. 130 (1972) 638-648.

24. Pandak, W.M., Li, Y.C., Chiang, J.Y.L., Studer, E.J., Gurley, E.C., Heuman, D.M., Vlahcevic, Z.R., and Hylemon, P.B. : Regulation of cholesterol 7α-hydroxylase mRNA and transcriptional activity by taurocholic acid and cholesterol in the chronic biliary diverted rats. J. Biol. Chem. 266 (1991) 3416-3421.

25. Björkhem, I., Eggertsen, G., and Andersson, U. : On the mechanism of stimulation of cholesterol 7α-hydroxylase by dietary cholesterol. Biochim. Biophys. Acta 1085 (1991) 329-335.

26. Jelinek, D.F., and Russell, D.W. : Structure of the rat gene encoding cholesterol 7α-hydroxylase. Biochemistry 29 (1990) 7781-7785.

27. Princen, H.M.G., Meijer, P., Kwekkeboom, J. and Kempen, H.J.M. : Assay of cholesterol 7α-

hydroxylase activity in rat hepatocytes in primary monolayer culture. Anal. Biochem. 171 (1986) 158-168.

28. Havekes, L.M., Schouten, D., De Wit, E.C.M., Cohen, L.H., Griffioen, M., Van Hinsbergh, V.W.M., and Princen, H.M.G. : Stimulation of the LDL-receptor activity in the human hepatoma cell line HepG2 by high density serum fractions. Biochim. Biophys Acta 875 (1986) 236-246.

29. Redgrave, T.G., Roberts, D.C.K., and West, C.E. : Separation of plasma lipoproteins by density-gradient ultracentrifugation. Anal. Biochem. 65 (1975) 42-49.

30. Gorski, K., Carneiro, M., and Schibler, U. : Tissue-specific *in vitro* transcription from the mouse albumin promoter. Cell 47 (1986) 767-776.

31. Graves, B.J., Johnson, P.E., and McKnight, S.L. : Homologous recognition of a promoter domain common to the MSV LTR and the HSV tk gene. Cell 44 (1986) 565-576.

32. Ueno, T., and Gonzalez, F.J. : Transcriptional control of the rat hepatic CYP2E1 gene. Mol. Cell. Biol. 10 (1990) 4495-4505.

33. Parker, C.S., and Topol, J. : A Drosophila RNA polymerase II transcription factor contains a promoter-region-specific DNA-binding activity. Cell 36 (1984) 357-369.

34. Monaci, P., Nicosia, A., and Cortese, R. : Two different liver-specific factors stimulate *in vitro* transcription from the human α1-antitrypsin promoter. EMBO J. 7 (1988) 2075-2087.

35. Chiang, J.Y.L., and Stroup, D. : Identification and characterization of a putative bile acid-responsive element in cholesterol 7α-hydroxylase gene promoter. J. Biol. Chem. 269 (1994) 17502-17507.

36. Heuman, D.M., Hernandez, C.R., Hylemon, P.B., Kubaske, W.M., Hatman, C., and Vlahcevic, Z.R. : Regulation of bile acid synthesis, effects of conjugated ursodeoxycholate and cholate on bile acid synthesis in the chronic bile fistula rat. Hepatology 8 (1988) 358-365.

37. Twisk, J., Hoekman, M.F.M., Muller, L.M., Iida, T., Tamaru, T, IJzerman, A., Mager, W.H. ,and Princen, H.M.G. : Structural aspects of bile acids involved in regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase. Eur. J. Biochem. (1995), in press.

38. Goldstein, J.L., and Brown, M.S. : Regulation of the mevalonate pathway. Nature 1343 (1990) 425-430.

39. Villanueva, G.R., Herreros, M., Perez-Barriocanal, F., Bolanos, J.P., Bravo, P., and Marin, J.J.G. : Enhancement of bile acid-induced biliary lipid secretion by streptozotocin in rats; role of insulin deficiency. J. Lab. Clin. Med. 115 (1990) 441-448.

40. Bennion, L.J., and Grundy, S.M. : Effects of diabetes mellitus on cholesterol metabolism in man. N. Eng. J. Med. 296 (1977) 1365-1371.

41. Nervi, F.O., Severin, C.H., and Valdivieso, V.D. : Bile acid pool changes and regulation of cholate synthesis in experimental diabetes. Biochim. Biophys. Acta 529 (1978) 212-223.

42. Twisk, J., Hoekman, M.F.M., Lehmann, E., Meijer, P., Mager, W.H., and Princen, H.M.G. : Insulin suppresses bile acid synthesis in cultured rat hepatocytes by down-regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase gene transcription. Hepatology (1995), in press.

43. Roberts, A.B., and Sporn, M.B. : Cellular biology and biochemistry of the retinoids. In: The retinoids. (1984) 209-286. Academic press, Inc., Orlando, Fla.

44. Umesono, K., Murakami, K.K., Thompson, C.C., and Evans, R.M. : Direct repeats as selective response elements for the thyroid hormone, retinoic acid and vitamin D3 receptors. Cell 65 (1991) 1255-1266.

45. Zhang, X.K., Hoffmann, B., Tran, P., Graupner, G., and Phahl, M. : Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. Nature (London) 355 (1992) 441-446.

46. Zhang, X.K., Lehmann, J.M., Hoffmann, B., Dawson, M.I., Cameron, J., Graupner, G., Tran, P., and Phahl, M. : Homodimer formation of retinoid X receptor induced by *9-cis* retinoic acid. Nature (London) 358 (1992) 587-591.

CHAPTER 4

Structural aspects of bile acids involved in regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase

Jaap Twisk*, Marco F.M. Hoekman†, Linda M. Muller*, Takashi Iida‡, Tamaaki Tamaru‡, Ad IJzerman§, Willem H. Mager† and Hans M.G. Princen*

Gaubius Laboratory* TNO-PG, Leiden, Vrije Universiteit†, Department of Biochemistry and Molecular Biology, Amsterdam, and LACDR§, Division of Medicinal Chemistry, Leiden University, The Netherlands; and Nihon University‡, College of Engineering, Department of Industrial Chemistry, Koriyama, Fukushima-ken, Japan.

in press, Eur. J. Biochem (1995)

ABSTRACT

We have recently reported that coordinate down-regulation of cholesterol 7α-hydroxylase (CYP7A) and sterol 27-hydroxylase by bile acids, commonly found in rat bile, results in suppression of bile acid synthesis in cultured rat hepatocytes (Twisk *et al.* (1994) Biochem J : in press). In the current study, we have assessed the effects of a large group of different bile acids, both naturally occurring and synthetic, on these two key-enzymes, to elucidate structural features which render bile acids potent as a regulator of bile acid synthesis in cultured rat hepatocytes. Addition of 50 μM deoxycholate or cholate, two relatively hydrophobic bile acids, to the culture medium of hepatocytes resulted in strong suppression of CYP7A (-75 % and -88%) and sterol 27-hydroxylase activity (-76% and -72%). On the other hand, two hydrophilic bile acids, β-muricholate and ursocholate, yielded no effect. These differential effects were also reflected in mRNA levels and transcriptional activity for the two enzymes, as assessed by nuclear run-on assays, showing a parallel suppression of both parameters in response to cholate (-78% and -43%, respectively for CYP7A mRNA and transcription, and -76% and -42%, respectively for sterol 27-hydroxylase mRNA and transcription), and yielding no effect when ursocholate was added. Transient expression analysis, using a promoter-reporter construct containing the proximal part of the CYP7A promoter, in cultured rat hepatocytes, demonstrated a reduction of transcriptional activity by cholate (-72%), but not by ursocholate. In contrast, transcription of the gene encoding the key-enzyme in cholesterol synthesis, HMG-CoA reductase, was not affected by bile acids, while mRNA levels for this enzyme were slightly up-regulated by cholate (+47%), but not by ursocholate. Assessment of the effects of 27 different bile acids, varying in number, position and orientation (α/β) of hydroxyl groups on the steroid nucleus of the molecule, on CYP7A mRNA showed only a weak correlation with the hydrophobicity index of the bile acid involved ($r = 0.61$). Analysis of the three-dimensional structure of a number of these bile acids suggests that hydroxyl groups situated in close proximity of each other within the molecule, creating a hydrophilic environment as in the case of cholate, may be a prerequisite for strong inhibitory potency. Deviation from this situation leads to a markedly lesser effect on suppression of CYP7A and sterol 27-hydroxylase.

INTRODUCTION

Hepatic conversion of cholesterol into bile acids is a major route for elimination of cholesterol from the mammalian body (1,2). Based upon information obtained *in vivo* in rat (3-8) and

man (9,10), and *in vitro* using cultured pig (11,12) and rat (13-15) hepatocytes, it was concluded that cholesterol 7α-hydroxylase (CYP7A), the major rate-limiting enzyme in the bile acid biosynthetic pathway, is repressed by the flux of bile acids returning to the liver in portal blood. In a recent paper, however, we have shown that not only CYP7A, but also sterol 27-hydroxylase is down-regulated by bile acids (16). The latter enzyme is involved in the alternative routing of cholesterol to bile acids, a route which has been shown to contribute considerably to bile acid synthesis both *in vivo* in man (17), and *in vitro* in cultured human and rat hepatocytes (18,19). Both enzymes are coordinately down-regulated by taurocholate, one of the major bile acids in rat bile, at the level of mRNA and transcription, leading ultimately to suppression of bile acid synthesis (16).

With regard to the functional structure of a bile acid in terms of its potency to inhibit bile acid synthesis, it has been postulated that repressional activity is directly correlated to the hydrophobicity of a given bile acid (20,21). However, studies *in vivo* involving feeding of bile acids are difficult to interpret due to the many types of conversion taking place in the mammalian body. In addition, distinct views exist, originating from *in vivo* studies in rat and rabbit, reporting taurocholate not active, whereas taurodeoxycholate and taurolithocholate were strong inhibitors of bile acid synthesis. It was suggested that primary bile acids first have to be converted to secondary ones to become regulatory (22-24). Alternatively, specifically monohydroxy bile acids derived from either intestinal or hepatic sources, were reported to effectively down-regulate bile acid synthesis in the rabbit, indicating that the hydroxylation status *per se* is important for inhibitory potency (24). Shefer and co-workers (25) reported strong suppression of bile acid synthesis by taurocholate, but not by tauroursocholate, at the level of CYP7A *in vivo* in rat. Epimerisation of the 7-hydroxyl group, yielding such drastic differences in effect on this enzyme, suggests that specific three-dimensional structure of a bile acid might also be important, rather than hydrophobicity alone, in determining the ability to regulate. The latter is in agreement with previous results obtained with cultured pig and rat hepatocytes, in which cholate, hyodeoxycholate, chenodeoxycholate, and deoxycholate showed equal suppressive effects on bile acid synthesis and CYP7A, while differing significantly in hydrophobicity index (11-13).

In this study, we have assessed the effects of a large number of bile acids, both naturally-occurring and synthetic, and differing highly in number, position, and orientation of hydroxyl groups in the basic steroid structure, on CYP7A, to elucidate whether there exists a basic structural requirement for a bile acid to be able to exert suppressive effects on the expression of this enzyme. For reasons of comparison, a subset of these bile acids was also tested for effects

on sterol 27-hydroxylase. In these experiments, measures were taken to prevent or reduce interconversions of bile acids by the hepatocyte, facilitating interpretation of results.
Results presented indicate that, in general, both CYP7A and sterol 27-hydroxylase activities are coordinately down-regulated by similar bile acids at the level of mRNA and gene transcription. The correlation of the potency of bile acids to inhibit CYP7A, versus their hydrophobicity index (HI_X), however, is a weak one (r = 0.61), suggestive of additional important structural aspects involved in down-regulation.

MATERIALS AND METHODS

Materials and bile acids

Materials used for isolation and culturing of rat hepatocytes, and assaying CYP7A and sterol 27-hydroxylase activities, were obtained from sources described previously (26-28). Taurocholate, glycocholate, cholic acid and deoxycholic acid were obtained from Sigma Chemicals (St. Louis, MO, USA), chenodeoxycholic acid was from Serva (Heidelberg, Germany) and β-muricholic acid was obtained from Steraloids (Wilton, NH, USA). All other bile acids used in this study were obtained from and synthesized by Prof.Dr. Takashi Iida and Dr. Tamaaki Tamaru, from the Department of Industrial Chemistry, College of Engineering, Nihon University, Japan, as described previously (29-32). Ketoconazole was obtained from Janssen Life Sciences Products (Beerse, Belgium). Radiochemicals ([α-^{32}P]dCTP (3000 Ci/mmol), [α-^{32}P]UTP (400 Ci/mmol) and [^{14}C]-cholesterol (60 mCi/mol)) were obtained from The Radiochemical Centre, Amersham, Buckinghamshire, UK. Male Wistar rats weighing 250-350 g were used throughout and were maintained on standard chow and water *ad libitum*. Two days before isolation of hepatocytes, rats were fed a diet supplemented with 2% cholestyramine (Questran, Bristol Myers B.V. Weesp, The Netherlands) (13). For preparation of hepatocytes, animals were killed between 9 and 10 a.m. Institutional guidelines for animal care were observed in all experiments.

Rat Hepatocyte Isolation and Culture

Rat liver cells were isolated by perfusion with 0.05% collagenase and 0.005% trypsin inhibitor as described previously (26-28). Viability, as determined by trypan blue exclusion, was higher than 90%. The cells were seeded on 60-mm diameter plastic tissue culture dishes or 6-well cluster plates (Costar, Cambridge, MA, USA) at a density of 1.5 x 10^5 cells/cm^2 in Williams E

medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 140 nM insulin, 50 nM dexamethasone, 100 IU/ml penicillin, and 100 µg/ml streptomycin, and were maintained at 37°C in a 5% CO^2/95% air atmosphere (12,13,27). After a 4-hour attachment period, medium was refreshed with 1.0 ml (6-well plates) or 2.5 ml (dishes) of culture medium as described in the above. Bile acids, dissolved in 10% (v/v) DMSO, were added to the hepatocytes for 16 hours, during two consecutive 8-hour periods, between 26-42 hours of culture age. The final concentration of DMSO in the medium was 0.1% (v/v). Cells were harvested at the same time for measurement of CYP7A and sterol 27-hydroxylase activities, mRNA levels and transcriptional activities. To prevent the effect of conversion of added bile acids by the hepatocytes, and to prolong the period of time that the bile acids could be effective, medium containing bile acids was refreshed after the first 8-hour period and incubated for another 8-hour period. Alternatively, hydroxylation of bile acids by the hepatocytes was inhibited by simultaneous incubation of bile acids with 10 µM ketoconazole over a similar period as described above, in a separate set of experiments. The latter substance has been shown to effectively inhibit hydroxylation reactions catalysed by NADPH-cytochrome P-450 dependent monooxygenases (33), among them CYP7A (up to 94% at 10 µM) (26). Culturing of cells in the presence of ketoconazole thus made assessment of effects of bile acids on CYP7A activity impossible, but had no effect on expression of CYP7A mRNA levels (data not shown). Conversion of bile acids by the hepatocytes, either in the presence or absence of ketoconazole, was determined by gas liquid chromatography after incubation of cells with a given bile acid for an 8-hour period. Hepatocytes incubated with bile acids and ketoconazole were used for determination of mRNA levels.

Cell viability, after culturing with the various bile acids, or ketoconazole, was assessed by ATP measurements (34) and MTT-assays (35). The latter is dependent on the cellular reduction of MTT (Sigma Chemical Co., St. Louis, MO, USA) by the mitochondrial dehydrogenase of viable cells, to a blue formazan product which can be measured spectrophotometrically. The assay was performed essentially as described by De Vries *et al.* (35). In short, parallel with the various incubations, cells on 12-well plates (5 x 10^5 cells/well) in 0.5 ml medium were incubated with bile acids. At the end of the incubation period, 55 µl of MTT solution (1 mg MTT/ml Williams E medium) was added to each well and incubated for 2 hours at 37^0C. The medium was aspirated, and 1 ml 100% DMSO was added to solubilise the formazan crystals. Absorbance at 545 nm was measured immediately.

Assay of CYP7A and Sterol 27-Hydroxylase

CYP7A and sterol 27-hydroxylase activities in homogenates of cultured hepatocytes were measured as mentioned in references 27 and 36, respectively. [^{14}C]-labelled products were separated by thin layer chromatography, and the amount of [^{14}C]-7α-hydroxycholesterol and [^{14}C]-27-hydroxycholesterol was quantitated by scraping off and counting of the spots containing these products, using the [^{14}C]-cholesterol input as a recovery standard. Blank values, determined by running parallel incubations without a NADPH-generating system, were substracted before calculating enzyme activity. Protein and cholesterol were assayed as previously described (27).

RNA Isolation, Blotting and Hybridisation Procedures

Isolation of total RNA, and subsequent electrophoresis, blotting and hybridisation techniques were performed as described previously (13). Quantitation of mRNA levels was performed using slot-blotting techniques as described in ref. 37. The following DNA fragments were used as probes in hybridisation experiments: the 1.6 kb PCR-synthesized fragment of rat CYP7A cDNA, spanning the entire coding region (13), a 1.6 kb *Hin*dIII/*Xba*I fragment of rat sterol 27-hydroxylase cDNA, kindly provided by Dr. J. Strauss (38), and a 773 bp *Hin*dIII fragment of hamster HMG-CoA reductase cDNA (39). The sterol 27-hydroxylase cDNA was isolated from a rat liver cDNA library (38) using the rabbit sterol 27-hydroxylase cDNA, previously isolated by the group of Russell, as a probe (40). As controls a 1.2 kb *Pst*I fragment of hamster actin cDNA (41), and a 1.2 kb *Pst*I fragment of GAPDH-cDNA (42) were used. For all probes used in slot-blotting experiments, a linear relationship between areas under the curves and mRNA concentration was shown on an autoradiograph, using concentrations between 2 and 8 μg of total RNA. The hamster actin cDNA was used as an internal standard to correct for differences in the amount of total RNA applied onto the gel or filter.

Nuclear run-on Studies

Nuclear run-on studies were conducted essentially as described by Groudine *et al.* (43), with minor modifications (13). Hybridisation-target DNA, being 5 μg of plasmid material containing cDNA sequences of rat CYP7A, rat sterol 27-hydroxylase, hamster HMG-CoA reductase, hamster actin, rat GAPDH (see the above) and the empty vector pUC18, were slot-blotted onto strips of Hybond-N+ filter (Amersham), and crosslinked with 0.4 N NaOH for 30 min. The filters were preincubated for 30 min at 65°C in a sodium phosphate buffer as

described (13), and hybridised with the labelled RNA for 36 hours in the same buffer. [^{32}P]-UTP had been incorporated into nascent RNA, using isolated nuclei from cells which had been cultured with or without bile acids for 16 hours, between 26 and 42 hours of culture time. After hybridisation, the various filters were washed once for 5 min and twice for 30 min in 2 x SSC/1% SDS at 65°C, and exposed to Hyperfilm MP (Amersham) for 2-5 days. Quantitation of the relative amounts of mRNA synthesized was conducted as described (13).

Transfection experiments and CAT assays

At 22 hours after their isolation, cells were subjected to transfection (14). Recombinant plasmids to be used for transfection were purified by centrifugation to equilibrium in CsCl-Ethidium-bromide-density gradients (44). In transient-expression assays, 3 μg of test plasmid and 1 μg of standard LacZ plasmid (45), used as internal control for transfection efficiency, were cotransfected as a CaPi-precipitate (46). After 4 hours, the precipitate was removed, and cells were treated with Williams E medium containing 15% glycerol for 1 min, and supplied with fresh culture medium with or without bile acids (50 μM). At 42 hours after transfection, cells were harvested and cell extracts were prepared. Preparation of cell extracts and CAT assays were performed essentially as described by Gorman *et al* (47). Protein concentrations were determined with BCA protein-assay reagent (Pierce). The amounts of acetylated product were quantitated, after thin layer chromatography and autoradiography, with a Phosphor-imager 400B (Molecular Dynamics). Data were corrected for protein-content and transfection efficiency.

Determination of the hydrophobicity index (HI_X) of bile acids

HI_X of all bile acids, used in this study, was determined by methods described by Heuman *et al.* (21), using a Hitachi L-6000 High Performance Liquid Chromatograph. The HI_X of an individual bile acid is based on its capacity factor (k_X) in C18 reversed phase HPLC, expressed relative to the capacity factors of taurocholate and taurolithocholate; column used being a Capcell Pak C18 AG 120 (25 cm x 4.6 mm I.D.; 5 μm; Shiseido, Tokyo, Japan). The eluent consisted of a gradient of methanol-0.025M potassium phosphate buffer (pH=7.0), ranging from 4:1 to 7:3 (v/v) at 1 ml/min, with U.V.-detection set at 210 nm.

Molecular modelling of bile acids

The software package QUANTA/CHARm, implemented on a Silicon Graphics 4000 XZ workstation, was used to model the structures of bile acids. As a starting point, the crystal

structure of methylcholate (48) was retrieved from the Cambridge Crystallographic Database (Cambridge Crystallographic Data Center, Lensfield Road, Cambridge, U.K.). Polar hydrogen atoms were added, and the resulting structure was energy minimised using default values as provided by the program. The structure of allo-methylcholate was built by inverting the hydrogen atom at position 5, and subsequent energy minimisation. Likewise, other bile acids were analysed.

Statistical Analysis

Data were analysed statistically using Student's paired t-test with the level of significance selected to be $P < 0.05$. Values are expressed as means ± SD. In Figure 2, the line was fitted to the points by the method of least squares. Pearson correlation coefficients were calculated to determine the correlation between hydrophobicity index and suppression of CYP7A mRNA levels.

RESULTS

Effects of different bile acids on CYP7A and sterol 27-hydroxylase activity, mRNA and transcriptional activity.

Since hydrophobicity has been suggested to be a major determinant for the potency of a given bile acid to inhibit bile acid synthesis (12,13,15,20,21), the effects of the hydrophobic bile acids deoxycholate (HI_X=+0.60) and cholate (HI_X=+0.01) were compared with those of β-muricholate (HI_X=-0.76) and ursocholate (HI_X=-1.04), two hydrophilic bile acids.

Table 1 shows that 50 μM of deoxycholate added to medium of cultured hepatocytes for a period of 16 hours resulted in -75 ± 15% suppression of the CYP7A activity level. Previous data from our group (13,16) has shown that this dosage and culture period for bile acids yields maximal effects. Addition of cholate resulted in comparable suppression of enzyme activity (-88 ± 6%), while there was no effect of the 7β-epimer of cholate, ursocholate, nor of β-muricholate. Assessment of sterol 27-hydroxylase activity levels revealed similar effects of these bile acids on this enzyme, showing strong suppression by deoxycholate (-76 ± 12%) and cholate (-72 ± 20%), and no significant effects of ursocholate or β-muricholate. Cell viability was not affected by the concentration of the bile acids used (data not shown). Addition of the different bile acids had no effect on intracellular cholesterol levels (49 ± 10 nmol per mg cell protein for control cells), neither could the effects observed be explained by differences in

Table 1. Effect of hydrophobic and hydrophilic bile acids on cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity.

Bile acid added		HI_x	Enzyme activity level (% of control)	
			cholesterol 7α-hydroxylase	sterol 27-hydroxylase
control (0.1% DMSO)			100	100
deoxycholate	3α12α	+0.60	25 ± 15*	24 ± 12*
cholate	3α7α12α	+0.01	12 ± 6*	28 ± 20*
β-muricholate	3α6β7β	-0.76	96 ± 27	115 ± 32
ursocholate	3α7β12α	-1.04	121 ± 27	135 ± 33

Rat hepatocytes were incubated with different bile acids (50 μM) from 26 to 42 hours of culture, over two 8-hour periods. Medium and bile acids were refreshed in the second 8-hour period. Cells were harvested at 42 hours, and the cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity levels were determined as described in "Experimental Procedures". Data are expressed as a percentage of control (0.1% DMSO) and are a means ± SD of independent experiments using hepatocytes from 4-12 rats. The absolute values for both enzyme activities were 343 ± 130 pmol/h per mg of cell protein and 73 ± 29 pmol/h per mg cell protein, respectively. * Indicates a significant difference ($p<0.05$) compared with control values.

uptake by the hepatocyte in terms of accumulation as determined by gas liquid chromatography, which were similar for all bile acids concerned (data not shown). It should also be noted that interconversion by the hepatocyte does not play a role in the effects described in the above, as the bile acids cholate, ursocholate, and β-muricholate are not or only marginally metabolised. Only deoxycholate is partly converted into cholate (49), but these two bile acids have equal suppressive effects. The differential effects of the bile acids were also reflected by the steady-state levels of mRNA for both CYP7A and sterol 27-hydroxylase. Northern-blotting and hybridisation with various cDNA-probes of total RNA isolated from hepatocytes incubated with 50 μM of cholate or ursocholate, as compared with control cells, showed a parallel down-regulation of all three mRNAs for CYP7A (2.1, 3.6, and 4.0 kb) in size (13,50,51), and of the single mRNA for sterol 27-hydroxylase (2.4 kb in rat liver) (16,38) by cholate, and not by ursocholate (Fig.1). Actin and GAPDH, used as internal standards, showed no response to the bile acids added.

In this particular analysis, the mRNA level for HMG-CoA reductase was also determined, for reasons of comparison. In view of reports stating that, apart from enzymes involved in degradational pathways of cholesterol, mRNA (52) and activity (20,21,52) levels of this key-

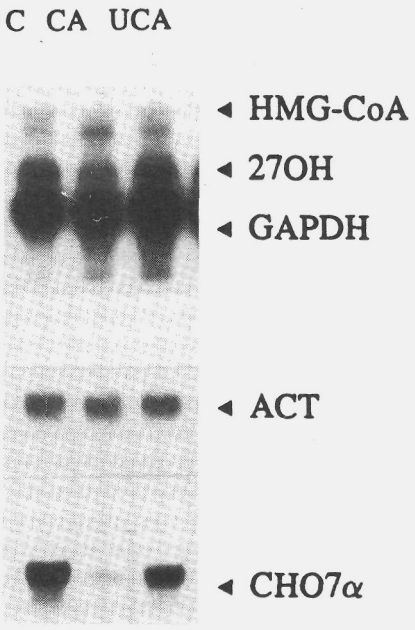


Figure 1. Effect of hydrophobic and hydrophilic bile acids on mRNA levels for cholesterol 7α-hydroxylase, sterol 27-hydroxylase and HMG-CoA reductase. Cells were exposed to different bile acids (50 μM) for 16 hours, between 26-42 hours of culture, for two 8-hour periods. Cells were harvested after 42 hours for isolation of total RNA; 10 μgr of total RNA was electrophoresed in a 0.8% agarose/1M formaldehyde gel, transferred to Hybond-N^+, and subsequently hybridized with probes for cholesterol 7α-hydroxylase (CHO7α), sterol 27-hydroxylase (27OH), HMG-CoA reductase (HMG-CoA), and GAPDH and actin (ACT). The latter two served as internal standards, as described under "Experimental Procedures". C: control (0.1% DMSO); CA: cholate; UCA: ursocholate.

Table 2. Effect of hydrophobic and hydrophilic bile acids on mRNA levels for cholesterol 7α-hydroxylase, sterol 27-hydroxylase and HMG-CoA reductase.

Bile acid added			mRNA level (% of control)		
		HI_x	cholesterol 7α-hydroxylase	sterol 27-hydroxylase	HMG-CoA reductase
control (0.1% DMSO)			100	100	100
deoxycholate	3α12α	+0.60	25 ± 16*	25 ± 16*	ND
cholate	3α7α12α	+0.01	22 ± 10*	24 ± 12*	147 ± 21*
β-murichloate	3α6β7β	-0.76	86 ± 14	86 ± 24	ND
ursocholate	3α7β12α	-1.04	106 ± 19	97 ± 22	95 ± 16

Rat hepatocytes were incubated with different bile acids (50 μM) from 26 to 42 hours of culture, for two 8-hour periods as described in the legends to table 1. Cells were harvested at 42 hours, and the cholesterol 7α-hydroxylase, sterol 27-hydroxylase and HMG-CoA reductase mRNA levels were assessed by slot-blotting and densitometric scanning of resulting autoradiographs, using the actin mRNA as an internal standard to correct for differences in the amount of RNA applied, as described in "Experimental Procedures". Data are expressed as a percentage of control (0.1% DMSO) and are a means ± SD of independent experiments using hepatocytes from 3-12 rats. * Indicates a significant difference ($p<0.05$) compared with control values. ND, not determined.

enzyme in cholesterol synthesis are also regulated by bile acids *in vivo*, evaluation of effects of different bile acids on HMG-CoA reductase mRNA could serve as a control. It is still not clear, however, whether effects of bile acids on this enzyme reflect a direct inhibition, or whether feeding of experimental animals with bile acids causes increased intestinal uptake of dietary cholesterol (53,54). The latter is a known suppressor of HMG-CoA reductase (55), and thus the effects of bile acids on the enzyme might in fact be exerted indirectly. The use of isolated hepatocytes as a model provides the possibility to discriminate between the two options. Hybridisation with pRED227 visualised two bands of 4.2 and 4.6 kb, as has been reported previously by Clarke *et al.* in rat liver *in vivo* (56). Interestingly, culturing of cells with cholate showed significant up-regulation of the smaller of the two bands, while yielding no effect when ursocholate was added to the medium (Fig. 1). Table 2 summarises the effects of the different bile acids tested on the levels of mRNA for the enzymes mentioned. Deoxycholate and cholate inhibited CYP7A (-75 ± 16% and -78 ± 10%, respectively) and sterol 27-hydroxylase (-75 ±16% and -76 ± 12%, respectively) mRNA levels considerably, while there was no effect by either β-muricholate or ursocholate. The level of both HMG-CoA reductase mRNAs together was increased (+47 ± 21%) by cholate, but remained unaltered upon addition of ursocholate.

Table 3. Effect of cholate and ursocholate on transcriptional activity of the cholesterol 7α-hydroxylase, sterol 27-hydroxylase and HMG-CoA reductase genes.

Gene	relative transcriptional activity (% of control)	
Bile acid added:	cholate	ursocholate
cholesterol 7α-hydroxylase	57 ± 8*	92 ± 10
sterol 27-hydroxylase	58 ± 9*	110 ± 18
HMG-CoA reductase	90 ± 23	94 ± 12
GAPDH	89 ± 24	100 ± 16

Cells were exposed to bile acids (50 μM) for 16 hours, from 26-42 hours of culture, in two 8-hour periods, as opposed to untreated cells, as described in the legends to table 1. Cells were harvested and transcriptional activity for cholesterol 7α-hydroxylase, sterol 27-hydroxylase, HMG-CoA reductase and GAPDH was assessed relative to that of actin, used as an internal standard. [^{32}P]-labeled total RNA was hybridized to immobilized corresponding cDNAs as indicated in the "Experimental Procedures" section, and the resulting blots were exposed to Hyperfilm for 48-120 hours. Non-specific hybridization was checked using the empty vector pUC18. Results shown are expressed as transcriptional activity relative to that of actin, and as a percentage of control (0.1% DMSO). Data are a means ± SD of independent experiments using hepatocytes from 3-4 rats. * Indicates a difference ($p < 0.05$) compared with control values.

Analysis of transcriptional activity (Table 3) in cells incubated for 16 hours with 50 μM cholate or ursocholate, showed suppression of transcription of both CYP7A and sterol 27-hydroxylase genes (-43 ± 8 and -42 ± 9%, respectively) relative to that of β-actin, used as an internal standard. There was no effect on HMG-CoA reductase gene transcription, suggesting that the observed up-regulation at the level of mRNA for this particular enzyme represents post-transcriptional changes. There was also no effect on the expression of the house-keeping gene GAPDH. Ursocholate showed no effect on transcription of any of the genes analysed.

Effect of cholate and ursocholate on CAT activity in cells transfected with a CYP7A promoter-CAT reporter construct.

Recent results obtained by Hoekman *et al* (14), using transient expression assays, indicated that the region between nucleotides -79 and -49 of the rat CYP7A promoter is essential for a bile acid induced response. We wanted to assess whether, using this system, a differentiation is made between bile acids. The -348RCAT construct, consisting of the proximal 348 nucleotides of the CYP7A promoter, fused to the CAT-reporter gene (14), was used in transient expression experiments. Table 4 shows that, while cholate had a strong inhibitory effect on promoter activity of the -348RCAT construct (-72 ± 8%), ursocholate did not. The SV40CAT-signal, showing a strong basal expression originating from the SV40-promoter, and used as an external standard in parallel incubations, showed no response to either bile acid (data not shown). These results confirm the conclusions drawn from nuclear run-on assays, showing that, at least in part, down-regulation of CYP7A mRNA levels is a result of decreased transcription. Furthermore, suppression of gene expression via a distinct DNA-element within the CYP7A promoter appears to be specific in terms of bile acid structure.

Table 4. CAT-activity of the cholesterol 7α-hydroxylase promoter-reporter construct -348*cat* in response to different bile acids in transfected cultured rat hepatocytes.

Bile acid added		Relative CAT-activity (% of control)
control (0.1% DMSO)		100
cholate	(3α7α12α)	28 ± 7*
ursocholate	(3α7β12α)	104 ± 10

Primary rat hepatocytes were transfected with the CaP_i-method, as described in the "Experimental Procedures" section. Transfected cells were subsequently incubated with 50 μM cholate or ursocholate for 42 hours, and CAT activity was assessed afterwards. The cholesterol 7α-hydroxylase promoter activity is expressed relative to that of control (0.1% DMSO), and as a mean of 4 independent experiments. * Indicates a significant difference ($P < 0.05$) compared with control values.

Structure-function relationships.

Previous studies have indicated that CYP7A mRNA levels decline rapidly in parallel with enzyme activity levels, in response to bile acids (T = 4h for both parameters), and the mRNA is therefore considered a good parameter in this respect (13). Twenty-seven different bile acids were therefore tested on their ability to down-regulate levels of CYP7A mRNA. Metabolism of bile acids by hepatocytes was reduced or prevented by short-term incubations and by renewing of medium and bile acids. Hydroxylation of bile acids by the hepatocytes was lowered by simultaneous incubation of bile acids in the presence of 10 μM ketoconazole, as demonstrated by the decrease in metabolism of chenodeoxycholate, a bile acid specifically subject to conversion (49). Without additional measures, 89% of chenodeoxycholate was converted to β-muricholate, as determined by gas liquid chromatography, over an 8-hour period by cultured rat hepatocytes. Simultaneous incubation with 10 μM ketoconazole lowered conversion to 25% over this period of time. Nonetheless, culturing cells for two 8-hour periods with 50 μM chenodeoxycholate in the absence of ketoconazole yielded a 45 ± 8% suppression of CYP7A mRNA, despite conversion of this bile acid to a non-active metabolite under these circumstances. The latter finding indicates a rapid effect of bile acids on mRNA levels for this enzyme, prior to appreciable conversion, in agreement with short-term down-regulation by bile acids as reported before (13). Simultaneous addition of both chenodeoxycholate and ketoconazole led to a further reduction (-85 ± 8%) of CYP7A mRNA (Table 5).

The bile acids used for analysis, differed in hydrophobicity index, and in number, position and orientation (α-β) of hydroxyl groups present on the ringstructure of the bile acid. Additional changes concerned the presence of a keto-group at the 7-, 12- or both positions instead of the normal hydroxyl-entity. In a few cases there was also variation in the orientation of the hydrogen atom located at the 5-position in the ring-structure (5α or 'allo' as opposed to the common 5β-epimers). Table 5 summarises the characteristics of each bile acid tested, and their inhibitory potency in terms of effect on CYP7A mRNA level. Both synthetic and naturally-occurring monohydroxy bile acids were powerful suppressors of both CYP7A and sterol 27-hydroxylase mRNA levels. These bile acids, however, had a very negative impact on cell-viability, which was decreased by approximately 50%, as judged by measurement of mitochondrial dehydrogenase activity (MTT-assay). These compounds were hence excluded from further analysis.

In general, hydrophobic bile acids were potent inhibitors, as demonstrated by the strong suppression of CYP7A mRNA by cholate (3α7α12α), be it conjugated or not (-91 ± 8%, -86 ± 5%, and -78 ± 10% for tauro-, glyco- and unconjugated cholate, respectively),

chenodeoxycholate (3α7α; -85 ± 8%), and deoxycholate (3α12α; -79 ± 14%). Nonetheless, Figure 2, in which CYP7A mRNA levels are plotted versus the hydrophobicity index of the bile acid in question, demonstrates that the effects of a marked number of bile acids can not be explained by hydrophobicity alone, resulting in a moderate correlation coefficient (r-value= 0.61). Evident exceptions were lagodeoxycholate (3α12β; -7 ± 21%) and allo-cholate (allo-3α7α12α; -2 ± 20%), in comparison with cholate (-78 ± 10%), which differ only very little in

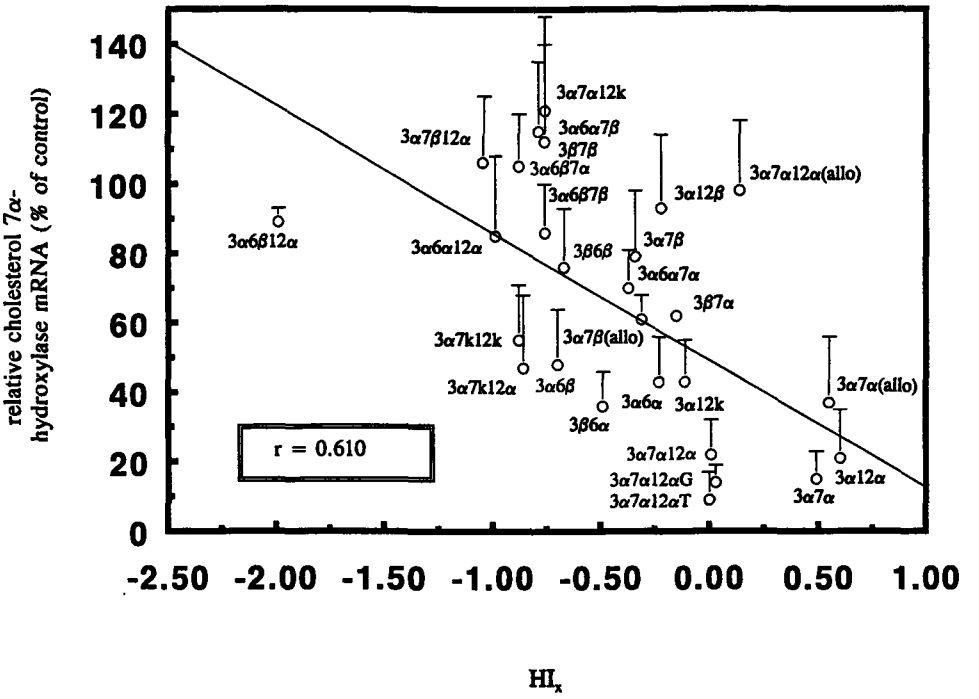


Figure 2. Effect of different bile acids on cholesterol 7α-hydroxylase mRNA levels versus their hydrophobicity index. Rat hepatocytes were incubated with different bile acids (50 μM) from 26 to 42 hours of culture, for two 8-hour periods, in the presence of 10 μM ketoconazole. Medium of cells was refreshed in the second 8-hour period, and fresh bile acids and ketoconazole were added. Cells were harvested at 42 hours, and the cholesterol 7α-hydroxylase mRNA levels were assessed as described in the legends to table 5. Hydrophobicity indices (HI_x) were determined by HPLC and converted to scale by graphic interpolation as proposed by Heuman et al. (21). Cholesterol 7α-hydroxylase is plotted versus HI_x. Structural characteristics depicted refer to bile acids as presented in Table 5.

Table 5. Effects of different bile acids (50 μM) on cholesterol 7α-hydroxylase mRNA in primary cultures of rat hepatocytes.

Bile acid	hydroxyl substituents	HI_x	Cholesterol 7α-hydroxylase mRNA (% of control)	n
I. conjugated bile acids				
1. glycocholate	3α7α12α	0.03	14 ± 5*	3
2. taurocholate		0.00	9 ± 8*	12
II. unconjugated bile acids				
3. hyodeoxycholate	3α6α	-0.23	43 ± 13*	4
4. murocholate	3α6β	-0.70	48 ± 16*	4
5. isohyodeoxycholate	3β6α	-0.49	36 ± 10*	3
6. isomurocholate	3β6β	-0.67	76 ± 17*	4
7. chenodeoxycholate	3α7α	0.49	15 ± 8*	3
8. allo-chenodeoxycholate	3α7α(allo)	0.55	37 ± 19*	4
9. ursodeoxycholate	3α7β	-0.34	79 ± 19*	5
10. allo-ursodeoxycholate	3α7β(allo)	-0.31	61 ± 7*	4
11. isochenodeoxycholate	3β7α	-0.15	62 ± 2*	3
12. isoursodeoxycholate	3β7β	-0.76	112 ± 28	4
13. deoxycholate	3α12α	0.60	25 ± 16*	4
14. lagodeoxycholate	3α12β	-0.22	93 ± 21	3
15. hyocholate	3α6α7α	-0.37	70 ± 11*	5
16. ω-muricholate	3α6α7β	-0.79	115 ± 20	3
17. α-muricholate	3α6β7α	-0.88	105 ± 15	2
18. β-muricholate	3α6β7β	-0.76	86 ± 14	3
19. -	3α6α12α	-0.99	85 ± 23	3
20. -	3α6β12α	-1.99	89 ± 4*	3
21. cholate	3α7α12α	0.01	22 ± 10*	3
22. allo-cholate	3α7α12α(allo)	0.14	98 ± 20	3
23. ursocholate	3α7β12α	-1.04	106 ± 19	7
III. bile acids with keto-substituents				
24.	3α12k	-0.11	43 ± 12*	3
25.	3α7k12α	-0.86	47 ± 21*	5
26.	3α7α12k	-0.76	121 ± 27	4
27.	3α7k12k	-0.88	55 ± 16*	4

Rat hepatocytes were incubated with different bile acids (50 μM) from 26 to 42 hours of culture, for two 8-hour periods in the presence of 10 μM ketoconazole. Medium was refreshed in the second 8-hour period, and fresh bile acids and ketoconazole were added. Cells were harvested at 42 hours, and the cholesterol 7α-hydroxylase mRNA levels were assessed by slot-blotting and densitometric scanning of resulting autoradiographs, using the actin mRNA as an internal standard to correct for differences in the amount of RNA applied, as described in "Experimental Procedures". Data are expressed as a percentage of control (0.1% DMSO) and are a means ± SD of independent experiments using hepatocytes from n rats. HI_x for each bile acid used was determined by HPLC and converted to scale by graphic interpolation, as proposed by Heuman et al. (21), and further described in "Experimental Procedures". * Indicates a significant difference ($P < 0.05$) compared with control values.

hydrophobicity. On the other hand, as stated in the above, cholate (3α7α12α), chenodeoxycholate (3α7α), and deoxycholate (3α12α) were equally active in suppressing CYP7A mRNA levels, allthough they differ significantly in their respective HI_X. Among the more hydrophilic bile acids there were also clear exceptions, as demonstrated by the strong suppressive capacity of isohyodeoxycholate (3β,6α; -64 ± 10%), and murocholate (3α,6β; -52 ± 16%). Other examples within this group were the synthetic bile acids 3α7κ12α (-43 ± 16%), and 3α7κ12κ (-45 ± 16%), both of which are hydrophilic compounds, nevertheless active as a suppressor of CYP7A. Taken together, these data indicate that apart from hydrophobicity as a general characteristic of a bile acid, other aspects of its structure may be important in determining inhibitory potency.

DISCUSSION

The present study shows hydrophobic bile acids to be powerful inhibitors of bile acid synthesis at the level of CYP7A and sterol 27-hydroxylase. However, apart from hydrophobicity, other structural features are important for determining inhibitory potency.

The results presented show down-regulation by the bile acids deoxycholate (3α12α) and cholate (3α7α12α) of both CYP7A and sterol 27-hydroxylase activity, as a result of an effect of these compounds on mRNA expression and transcriptional activity of the corresponding genes. In contrast, there was no effect of β-muricholate (3α6β7β) or of ursocholate (3α7β12α), two hydrophilic bile acids, on either enzyme. A simple 7β-epimerisation, as is the case for ursocholate versus cholate, renders the bile acid ineffective, in agreement with previous *in vivo* studies in rats showing a decrease in bile acid synthesis and similar decline of CYP7A activity after infusion of taurocholate, while yielding no effects after infusion of tauroursocholate (25). The current paper shows that the key-enzymes, involved in both major and alternative routing of cholesterol to bile acids, are sensitive to similar bile acids.

Analysis of the rat CYP7A promoter via the transient expression system described, revealed a preference for cholate over ursocholate in terms of inhibition of CAT activity of the CYP7A promoter construct -348RCAT. In a previous paper, Hoekman *et al.* (14) described the localisation of a specific DNA sequence, responsive to bile acids, between -79 and -49 nucleotides preceding the transcriptional initiation site. Apparently, this regulatory element differentiates, directly or indirectly, between bile acids in accordance with their differential effects *in vivo* and *in vitro*. Promoter regions of both CYP7A (nucleotides -49 to -79 in rat) and sterol 27-hydroxylase (nucleotides -254 to -280 in human) show similarities limited to a

region subject to interaction with several known transcriptional factors (14,57). For instance, a putative binding-site for the liver-specific transcription factor HNF1 is present within these regions. In accordance with the observed coordinate regulation of both enzymes by similar bile acids (16, and this study), this suggests that specific bile acids may interact in some way with a DNA sequence (bile acid response element, or BARE) common to the promoter region of both enzymes.

Several views have been expressed with respect to the nature of bile acids involved in suppression of CYP7A. It has been suggested that specifically monohydroxylated bile acids are active as inhibitor of the enzyme (24). Use of this particular subgroup of bile acids, however, yielded toxic effects, in accordance with reported hepatotoxicity of these compounds *in vitro* (58). Absence of an effect of infusion of cholate *in vivo* in rabbit, as opposed to an effect of either deoxycholate or lithocholate, has led to the suggestion that primary bile acids have to be converted intestinally in order to become active (22-24). Equal effects of both primary (e.g. cholate) and secondary (deoxycholate) bile acids (Table 5) rules out this possibility.

The generally accepted idea is that hydrophobicity determines inhibitory potency of bile acids (15,20,21). Nevertheless, in a previous paper we clearly show equal inhibitory effects of cholate, chenodeoxycholate, and deoxycholate, despite substantial differences in their respective hydrophobicity indices (12,13). We therefore determined the effects of a much larger group of bile acids on CYP7A, differing highly in number, position and orientation of the OH-groups present on the basic molecule. Other changes made concerned bile acids with a keto-group instead of a hydroxyl group, and isomerisation of the 5β-hydrogen atom, resulting in 5α- or allo-forms. Figure 2 shows that the correlation between inhibition of CYP7A mRNA and the hydrophobicity index of the bile acid involved is a weak one ($r = 0.61$). The low correlation coefficient cannot be attributed to conversion of bile acids by the hepatocyte. Only a few of the bile acids that do not follow the 'hydrophobicity rule' are subject to conversion, and even so are converted into bile acids that cannot explain observed effects. Chenodeoxycholate (3α7α) is converted into β-muricholate (3α6β7α), a non-active bile acid (Tables 1,2 and 5) for 89% within 8 hours. Nevertheless it is a potent suppressor of CYP7A mRNA levels (down to 55 ± 8%). Efforts to prevent conversion by refreshing medium and adding new bile acids within the 16-hour period, in combination with suppression of conversion using ketoconazole, lowered this value to 15 ± 8%. Taken together, it is apparent that the effects of the various bile acids tested take place very rapidly, preceding appreciable conversion by the hepatocyte. Addition of taurocholate to rat hepatocytes leads to a 50% reduction in CYP7A mRNA levels

within 4 hours (13), in agreement with this assumption. The short half-life of the messenger RNA is consistent with the reported half-life for the CYP7A enzyme *in vivo* in rat (59,60). In this respect, discrepancies between our results and those obtained by Stravitz and coworkers (15), can be explained by differences in time-curves in response to taurocholate. The latter work, in which primary rat hepatocytes were cultured with various bile acids in serum-free medium supplemented with thyroxine and dexamethasone, showed a 50% reduction of CYP7A mRNA only after 24 hours of incubation. Under the latter conditions, conversion of bile acids by the hepatocyte may play an important role in the effects observed.

Strong examples of effects of bile acids that can not be explained by hydrophobicity are cholate (3α7α12α) versus allo-cholate ((5α)3α7α12α) and lagodeoxycholate (3α12β), and murocholate (3α6β) versus β-muricholate (3α6β7β). In both cases, the bile acids differ only slightly in hydrophobicity index, but show marked differences in inhibitory potency. Two bile acids were therefore analysed with molecular modelling techniques, using the crystal structure of methylcholate as a starting point (48). Based on the molecular structure described, it was evident that the three hydroxyl groups present on methylcholate are in close proximity of each other, and that they are grouped together within the molecule to form a hydrophilic component within an otherwise relatively hydrophobic molecule (Figure 3a). Several bile acids were analysed by changing the basic cholate structure, and subsequent remodelling into an energetically minimised conformation. This analysis elucidated that allo-cholate, having a proton in the 5α-position, as opposed to cholate (5β), did not have the hydroxyl groups in proximity within the molecule. Calculation of the approximate distances between the three hydroxyl groups shows large differences, particularly between the 3α- and 12α-OH (8.4Å in allo-cholate as opposed to 6.4Å in cholate), and between 3α- and 7α-OH (6.4Å and 5.0Å, respectively). This particular epimerisation causes drastic changes in the conformation of the steroid backbone (Figure 3b), as a result of which the three hydroxyl groups are directed away from each other. Allo-cholate is a very poor regulator of CYP7A mRNA. In other cases, small or large changes in the relative position of the hydroxyl groups disrupting the hydrophilic microenvironment within the molecule, could be correlated to the effect of the particular bile acid on CYP7A. Epimerisation of the hydroxyl group in the 7-position, as is the case for cholate (3α7α12α) versus ursocholate (3α7β12α), chenodeoxycholate (3α7α) versus ursodeoxycholate (3α7β), and in the 12-position deoxycholate (3α12α) versus lagodeoxycholate (3α12β), weakened hydrophobic- hydrophilic compartimentalisation within the molecule. In each case, this resulted in a decreased ability to down-regulate CYP7A

mRNA.

The intramolecular organisation of cholate could be suggestive of some factor binding to the hydrophilic component of the molecule, acting perhaps as an anchor for interaction. It is feasible that binding of a protein factor regulating CYP7A gene transcription to a presumed BARE sequence (11,61), may be dependent on the relative orientation of the hydroxyl groups present on the bile acid molecule. As in the case of allo-cholate, the hydroxyl groups protrude from the steroid base, and may interfere sterically with binding to the factor. Presence of two hydroxyl groups or more requires them to be in close proximity three-dimensionally (3α7α12α, 3α7α, 3α12α), and any deviation therefrom leads to intermediate (3α7κ12κ, 3α7κ12α, 3α6β, 3β6α, 3α6α ορ 3α12κ) or no capacity at all (allo-3α7α12α, 3α7β12α, 3α12β, 3α7β) to down-regulate CYP7A.

Hydrophobicity, as determined by assessing relative distribution of a given bile acid within a

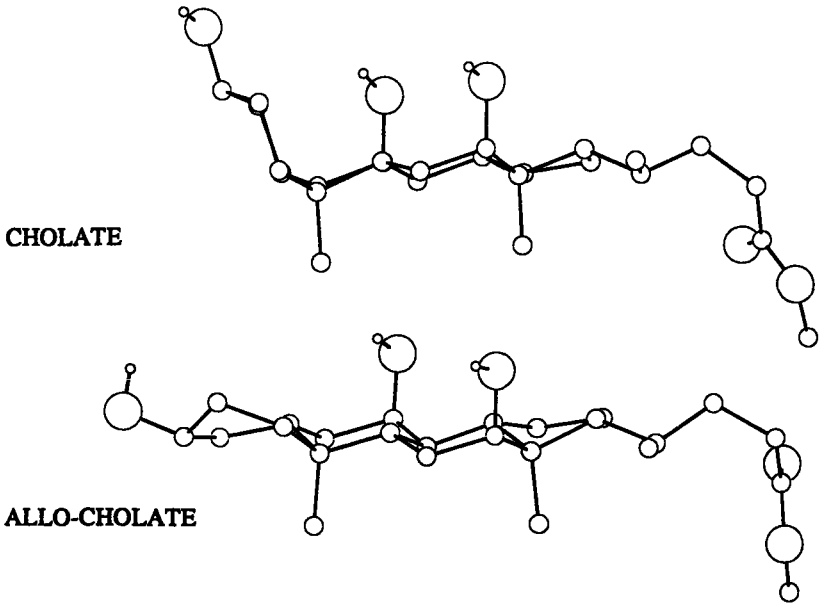


Figure 3. Three-dimensional structure of cholate versus allo-cholate. Three-dimensional depiction of cholate, based on the crystallographical studies of methylcholate by Miki et al. (48). The structure of allo-cholate was derived from the previous one by computer-modelling according to methods described in "Experimental Procedures". Small circles: hydrogen atoms (shown only when connected to oxygen atoms); intermediate circles: carbon atoms; large circles: oxygen atoms.

two-phase system as described, is dependent on the number, position and orientation of hydroxyl groups within the molecule. It is therefore difficult to segregate hydrophobicity per se from other structural aspects of a bile acid, with respect to its potency to affect CYP7A and sterol 27-hydroxylase. Nevertheless, from studying effects of a large group of bile acids, differing significantly in hydroxylation-status and hydrophobicity index, it can be concluded that the effects observed can not be explained by differences in hydrophobicity alone. We postulate that coordinate down-regulation of both CYP7A and sterol 27-hydroxylase at the level of gene transcription by bile acids requires specific structuralisation of the bile acid molecule. Compartimentalisation, in which the hydroxyl groups present on the bile acid are in close proximity and hence form a clear hydrophilic environment, may be a prerequisite for binding to a putative factor involved in interaction with regulatory sequences within the CYP7A and sterol 27-hydroxylase promoter.

ACKNOWLEDGEMENTS

The authors would like to thank Miss Elly C.M. de Wit for excellent technical assistance, and Miss Marisa Horsting for typing the manuscript. This work was supported by a grant from the Netherlands Heart Foundation (Grant 89.079 to Jaap Twisk), and from HGO-TNO (grant 900-523-138 to Marco F.M. Hoekman).

REFERENCES

1. Björkhem, I. : Mechanisms of bile acid biosynthesis in mammalian liver. In: Sterols and bile acids. Danielsson, H., and Sjövall, J., eds., (1985), pp. 231-278, Elsevier, Amsterdam.
2. Carey, M.C., and Cahalane, M.J. : Enterohepatic circulation. In: The Liver: Biology and Pathology. Arias, I.M., Jakoby, W.B., Popper, H., Schachter, D., and Shafritz, D.A., eds., (1988), pp. 573-616, Raven Press, NY.
3. Thompson, J.C., and Vars, H.M. : Biliary excretion of cholic acid and cholesterol in hyper-, hypo-, and euthyroid rats. Proc. Soc. Exp. Biol. Med. 83 (1953), 246-248.
4. Erikson, S. : Biliary excretion of bile acids and cholesterol in bile fistula rats. Proc. Soc. Exp. Biol. Med. 94 (1957), 578-582.
5. Danielsson, H., Einarsson, K., and Johansson, G. : Effects of biliary drainage on individual reactions in the conversion of cholesterol to taurocholic acid. Eur. J. Biochem. 2 (1967), 44-49.
6. Shefer, S., Hauser, S., Bekersky, I., and Mosbach, E.H. : Biochemical site of regulation of bile acid synthesis in the rat. J. Lipid Res. 11 (1970), 404-411.
7. Johansson, G. : Effect of cholestyramine and diet on hydroxylations in the biosynthesis and metabolism of bile acids. Eur. J. Biochem. 17 (1970), 292-295.
8. Spady, D.K., and Dietschy, J.M. : Hepatic bile acid synthesis: studies on regulation utilizing rats whose total body pool of cholesterol is uniformly labelled with ^{3}H. In: Bile acids in the liver. Paumgartner, G., Stiehl, A., and Gerok, W., eds., (1987), pp. 15-24, MTP Press, Lancaster.
9. Einarsson, K., Hellström, L., and Kallner, M. : Feedback regulation of bile acid formation in man. Metabolism 22 (1973), 1477-1483.
10. Reihnér, E., Björkhem, I., Angelin, B., Ewerth, S., and Einarsson, K. : Bile acid synthesis in humans: regulation of hepatic microsomal cholesterol 7α-hydroxylase activity. Gastroenterology 9 (1989), 1498-1505.
11. Kwekkeboom, J., Van Voorthuizen, E.M., Princen, H.M.G., and Kempen, H.J.M. : Feedback inhibition of bile acid synthesis in cultured pig hepatocytes. Biochem. Biophys. Res. Commun. 155 (1988), 850-856.
12. Kwekkeboom, J., Princen, H.M.G., Van Voorthuizen, E.M., and Kempen, H.J.M. : Bile acids exert negative feedback control on bile acid synthesis in cultured pig hepatocytes by suppression of cholesterol 7α-hydroxylase activity. Hepatology 12 (1990), 1209-1215.
13. Twisk, J., Lehmann, E.M., and Princen, H.M.G. : Differential feedback regulation of cholesterol 7α-hydroxylase mRNA and transcriptional activity by rat bile acids in primary cultures of rat hepatocytes. Biochem. J. 290 (1993), 685-691.

14. Hoekman, M.F.M., Rientjes, J.M.J., Twisk, J., Planta, R.J., Princen, H.M.G., and Mager, W.H. : Transcriptional regulation of thecholesterol 7α-hydroxylase gene encoding cholesterol 7α-hydroxylase in the rat. Gene 130 (1993), 217-223.

15. Stravitz, R.T., Hylemon, P.B., Heuman, D.M., Hagey, L.R., Schteingart, C.D., Ton-Nu, H.-T., Hofmann, A.F., and Vlahcevic, Z.R. : Transcriptional regulation of cholesterol 7α-hydroxylase mRNA by conjugated bile acids in primary cultures of rat hepatocytes. J. Biol. Chem. 268 (1993), 13987-13993.

16. Twisk, J., De Wit, E., and Princen, H.M.G. : Suppresion of sterol 27-hydroxylase mRNA and transcriptional activity by bile acids in cultured rat hepatocytes. Biochem. J. (1995), in press.

17. Axelsson, M., and Sjövall, J. : Potential bile acid precursors in plasma-possible indicators of biosynthetic pathways to cholic and chenodeoxycholic acids in man. J. steroid Biochem. 276 (1990), 631-640.

18. Princen, H.M.G., Meijer, P., Wolthers, B.G., Vonk, R.J., and Kuipers, F. : Cyclosporine A blocks bile acid synthesis in cultured hepatocytes by specific inhibition of chenodeoxycholic acid synthesis Biochem. J. 275 (1991), 501-505.

19. Dahlbäck-Sjöberg, H., Björkhem, I., and Princen, H.M.G. : Selective inhibition of mitochondrial 27-hydroxylation of bile acid intermediates and 25-hydroxylation of vitamin D3 by cyclosporin A. Biochem. J. 293 (1993), 203-206.

20. Heuman, D.M., Vlahcevic, Z.R., Bailey, M.L., and Hylemon, P.B. : Regulation of bile acid synthesis: II. Effects of bile acid feeding on enzymes regulating hepatic cholesterol and bile acid synthesis in the rat. Hepatology 4 (1988), 892-897.

21. Heuman, D.M., Hylemon, P.B., and Vlahcevic, Z.R. : Regulation of bile acid synthesis. III. Correlation between biliary bile salt hydrophobicity index and the activities of enzymes regulating cholesterol and bile acid synthesis in the rat. J. Lipid Res. 30 (1989), 1161-1171.

22. Stange, E.F., Scheibner, J., Lutz, C., and Ditschuneit, H. : Feedback regulation of bile acid synthesis in the rat by dietary vs. intravenous cholate or taurocholate. Hepatology 8 (1988), 879-886.

23. Stange, E.F., Scheibner, J., and Ditschuneit, H. : Role of primary and secondary bile acids as feedback inhibitors of bile acid synthesis in the rat *in vivo*. J. Clin. Invest. 84 (1989), 173-180.

24. Hall, R., Kok, E., and Javitt, N.B. : Bile acid synthesis: down-regulation by monohydroxy bile acids. FASEB J. 2 (1988), 152-156.

25. Shefer, S., Nguyen, L., Salen, G., Batta, A.K., Brooker, D., Zaki, F.G., Rani, I., and Tint, G.S. : Feedback regulation of bile-acid synthesis in the rat. J. Clin. Invest. 85 (1990), 1191-1198.

26. Princen, H.M.G., Huijsmans, C.M.G., Kuipers, F., Vonk, R., and Kempen, H.J.M. : Ketoconazole blocks bile acid synthesis in hepatocyte monolayer cultures and *in vivo* in rat by inhibiting

cholesterol 7α-hydroxylase. J. Clin. Invest. 78 (1986), 1064-1071.

27. Princen, H.M.G., Meijer, P., Kwekkeboom, J., and Kempen, H.J.M. : Assay of cholesterol 7α-hydroxylase activity in rat hepatocytes in primary monolayer culture. Anal. Biochem. 171 (1988), 158-165.

28. Princen, H.M.G., Meijer, P., and Hofstee, B. : Dexamethasone regulates bile acid synthesis in monolayer cultures of rat hepatocytes by induction of cholesterol 7α-hydroxylase. Biochem. J. 262 (1989), 341-348.

29. Iida, T., and Chang, F.C. : Potential bile acid metabolites. 6. Stereoisomeric 3,7-dihydroxy-5β-cholanoic acids. J. Org. Chem. 47 (1982), 2966-2972.

30. Iida, T., Momose, T., Tamura, T., Matsumoto, T., Chang, F.C., Goto, J., and Nambara, T. : Potential bile acid metabolites. 13. Improved routes to 3β,6β- and 3β,6α-dihydroxy-5b-cholanoic acids, J. Lipid Res. 29 (1988), 165-171.

31. Iida, T., Momose, T., Tamura, T., Matsumoto, T., Chang, F.C., Goto, J., and Nambara, T. : Potential bile acid metabolites. 14. Hyocholic and muricholic acid stereoisomers, J. Lipid Res. 30 (1989), 1267-1279.

32. Iida, T., Tamaru, T., Chang, F.C., Goto, J., and Nambara, T. : Potential bile acid metabolites. XVIII. Synthesis of stereoisomeric 3,6,12α-trihydroxy-5β-cholenoic acids. J. Lipid Res. 32 (1991), 649-658.

33. Higashi, Y., Omura, M., Suzuki, K., Inano, H., and Oshima, H. : Ketoconazole as a possible universal inhibitor of cytochrome P-450 dependent enzymes: its mode of inhibition, Endocrinol. Japon. 31 (1987), 105-115.

34. Lamprecht, W., and Trautschold, I. : Adenosine- 5'- triphosphat. Bestimmung mit Hexokinase und Glucose-6-phosphat Dehydrogenase, in Methoden der enzymatischen Analyse (Bergmeijer, H.U., ed.), pp. 2024-2033 (1970), Verlag Chemie, Berlin.

35. De Vries, E.G.E., Meijer, C., Timmer-Bosscha, H., Berendsen, H.H., De Leij, L., Scheper, R.J., and Mulder, N.H.: Resistance mechanisms in three human small cell lung cancer cell lines established from one patient during clinical follow-up. Cancer Res. 49 (1989), 4175-4178.

36. Björkhem, I., and Gustaffson, J. : Mitochondrial-hydroxylation of cholesterol side chain. J. Biol. Chem. 249 (1974), 2528-2535.

37. Krawczyk, Z., and Wu, C. : Isolation of RNA for dot hybridization by heparin- DNAseI treatment of whole cell lysate. Anal. Biochem. 165 (1987), 20-27.

38. Su, P., Rennert, H., Shayiq, R.M., Yamamoto, R., Zheng, Y.-M., Addya, S., Strauss III, J.F., and Avadhani, N.G. : A cDNA encoding rat mitochondrial cytochrome P450 catalyzing both the 26-hydroxylation of cholesterol and 25-hydroxylation of vitamin D3: gonadotropic regulation of the cognate mRNA in ovaries. DNA and Cell Biol 9 (1990), 657-665.

39. Chin, D.W., Gil, G., Russell, D.W., Liscum, L., Luskey, K.L., Basu, S.K., Okayama, H., Berg, P., Goldstein, J.L., and Brown, M.S. : Nucleotide sequence of the 3-hydroxy-3-methyl-glutaryl coenzyme A reductase, a glycoprotein of endoplasmatic reticulum. Nature 308 (1984), 613-617.

40. Andersson, S., Davis, D.L., Dahlbäck, H., Jörnvall, H., and Russell, D.W. : Cloning, structure and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. J. Biol. Chem. 264 (1989), 8222-8229.

41. Dodemont, H.J., Soriano, P., Quax, W.J., Ramaekers, F., Lenstra, J.A., Groenen, M.A.M., Bernardi, G., and Bloemendal, H. : The genes coding for the cytoskeletal proteins actin and vimentin in warm-blooded vertabrates. EMBO J. 1 (1982), 167-171.

42. Fort, Ph., Marty, L., Piechaczyk, M., Sabrouty, S.E., Dani, Ch., Jeanteur, Ph., and Blanchard, J.M. : Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. NAR 13 (1985), 1431-1442.

43. Groudine, M., Peretz, M., and Weintraub, H. : Transcriptional regulation of hemoglobin switching on chicken embryos. Mol. Cell. Biol. 1 (1981), 281-288.

44. Maniatis, T., Fritsch, E.F., and Sambrook, J. : Molecular cloning: a laboratory manual, 2nd edn (1982), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

45. Hall, C.V., Jacob, P.E., Ringold, G.M., and Lee, F.J. : Expression and regulation of Escherichia coli LacZ gene fusions in mammalian cells. Mol. Appl. Gen. 2 (1983), 101-109.

46. Pasco, D.S., and Fagan, J.B. : Efficient DNA-mediated gene transfer into primary cultures of rat hepatocytes. Lab. Methods 8 (1989), 535-541.

47. Gorman, C. : High efficiency gene transfer into mammalian cells, in DNA cloning: a practical approach (Glover, D.M., ed), pp. 143-190 (1985), IRL Press, Oxford, England.

48. Miki, K., Masui, A., Kasai, N., Goonewardena, W., Shibakami, M., Takemoto, K., and Miyata, M. : Structures of 1:1 addition compounds of methyl cholate with methanol and with 2-propanol. Acta. Cryst. (1992), 503-507.

49. Princen, H.M.G., and Meijer, P. : Hydroxylation, conjugation and sulfation of bile acids in primary monolayer cultures of rat hepatocytes. Biochem. Biophys. Res. Commun. 154 (1988), 1114-1121.

50. Jelinek, D.F., Andersson, S., Slaughter, C.A., and Russell, D.W. : Cloning and regulation of cholesterol 7α-hydroxylase, the rate-limiting enzyme in bile acid biosynthesis. J. Biol. Chem. 265 (1990), 8190-9197.

51. Li, Y.C., Wang, D.P., and Chiang, J.Y.L. : Regulation of cholesterol 7α-hydroxylase in the liver. Cloning, sequencing, and regulation of cholesterol 7α-hydroxylase mRNA. J. Biol. Chem. 265 (1990), 12012-12019.

52. Duckworth, P.F., Vlahcevic, Z.R., Studer, E.J., Gurley, E.C., Heuman, D.M., Beg, Z.H., and

Hylemon, P.B. : Effect of hydrophobic bile acids on 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity and mRNA levels in the rat. J. Biol. Chem. 266 (1991), 9413-9418.

53. Spady, D.K., Stange, E.F., Bilhartz, L.E., and Dietschy, J.M. : Bile acids regulate hepatic low density lipoprotein receptor activity in the hamster by altering cholesterol flux across the liver Proc. Natl. Acad. Sci. USA 83 (1986), 1916-1920 .

54. Russell, D.W., and Setchell, D.R. : Bile acid biosynthesis. Biochemistry 31 (1992), 4737-4749.

55. Goldstein, J.L., and Brown, M.S. : Regulation of the mevalonate pathway. Nature 343 (1990), 425-430.

56. Clarke, C.F., Fogelman, A.M., and Edwards, P.A. : Diurnal rhythm of rat liver mRNAs encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase J. Biol. Chem. 259 (1994), 10439-10447.

57. Leitersdorf, E., Reshef, A., Meiner, V., Levitzki, R., Schwartz, S.P., Dann, E.J., Berkman, N., Cali, J.J., Klapholz, L., and Berginer, V.M. : Frameshift and splice-junction mutations in the sterol 27-hydroxylase gene cause cerebrotendinous xanthomatosis in jews of moroccan origin. J. Clin. Invest. 91 (1993), 2488-2496.

58. Anwer, M.S., Engelking, L.R., Nolan, K., Sullivan, D., Zimniak, P., and Lester, R. : Hepatotoxic bile acids increase cytosolic Ca^{2+} activity of isolated rat hepatocytes. Hepatology 8 (1988), 887-891.

59. Einarsson, K., and Johansson, G.: Effect of Actinomycin D and puromycin on the conversion of cholesterol into bile acids in bile fistula rats. FEBS Lett. 1 (1968), 219-222.

60. Brown, M.J.G., and Boyd, G.S. : The specificity of rat-liver cholesterol 7α-hydroxylase. Eur. J. Biochem. 44 (1974), 37-47.

61. Chiang, J.Y.L., and Stroup, D. : Identification and characterization of a putative bile acid-responsive element in cholesterol 7α-hydroxylase gene promoter. J. Biol. Chem. 269 (1994), 17502-17507.

CHAPTER 5

Insulin suppresses bile acid synthesis in cultured rat hepatocytes by down-regulation of cholesterol 7α-hydroxylase and sterol 27 hydroxylase gene transcription

Jaap Twisk, Marco F.M. Hoekman*, Eline M. Lehmann, Piet Meijer, Willem H.Mager*, and Hans M.G. Princen

Gaubius Laboratory IVVO-TNO, Leiden, and *Dept. of Biochemistry and Molecular Biology, Vrije Universiteit, Amsterdam, The Netherlands

in press, Hepatology (1995)

ABSTRACT

Evidence from *in vivo* studies indicates that the bile acid pool and bile acid excretion is increased during diabetes mellitus in man and experimental diabetic animals, and that both parameters return to normal levels after administration of insulin. To investigate the biochemical background of these changes, the effects of insulin on bile acid synthesis and CYP7A (CYP7A) and sterol 27-hydroxylase, two key-enzymes in routing of cholesterol towards bile acids, were studied in cultured rat hepatocytes. Mass production of bile acids was dose-dependently diminished, showing significant reduction (33 to 53%) at physiological concentrations of the hormone (1.4 to 14 nM) and a maximal decrease at 140 nM (65%). The decrease of bile acid synthesis correlated well with the suppression of CYP7A and sterol 27-hydroxylase activity. The enzyme activity for CYP7A, examined in more detail, was dose-dependently diminished upon incubation of hepatocytes with various concentrations of insulin, reaching maximal reduction at 14 nM insulin. Maximal decrease of the enzyme activity was seen after 8 h of incubation (70%). Insulin strongly reduced the rise in CYP7A activity induced by incubation with dexamethasone. Sterol 27-hydroxylase activity was inhibited up to -58% after 24 hours of incubation with 140 nM insulin. To study the mechanism of suppression of CYP7A and sterol 27-hydroxylase activity, the effects of insulin on their respective levels of mRNA and gene transcription were assessed. The decrease in enzyme activities could be explained by a concomitant reduction in the CYP7A (-76%) and sterol 27-hydroxylase (-62%) mRNA level. Transcriptional activity, as assessed by nuclear run-on assays, was decreased to the same extent, *i.e.* 60% for CYP7A and -75% for sterol 27-hydroxylase. Transient-expression experiments using a construct containing the proximal 348 basepairs of the CYP7A promoter fused to the CAT-gene (-348RCAT) showed a significant reduction of transcriptional activity (-64%) with insulin, indicating that a sequence important for an insulin induced transcriptional response is located within the first 348 basepairs, preceding the transcription start of the CYP7A promoter.

We conclude that physiological concentrations of insulin suppress bile acid synthesis by down-regulation of CYP7A and sterol 27-hydroxylase gene transcription, and that this effect is mediated through a direct action of the hormone on the hepatocyte. These results may provide an explanation for the increased bile acid pool and excretion as found during untreated diabetes mellitus in humans and with insulin deficiency in experimental animals.

INTRODUCTION

The liver plays an important role in the synthesis and catabolism of cholesterol (1). Conversion of cholesterol into bile acids takes place exclusively in the liver and excretion of cholesterol and bile acids via the bile represents the major pathway for elimination of cholesterol from the body (1,2). According to current concepts, the primary pathway of bile acid biosynthesis in rats and humans is initiated with 7α-hydroxylation of cholesterol. This reaction is catalysed by the microsomal cholesterol 7α-hydroxylase (CYP7A), a cytochrome P-450-dependent enzyme (3-5). Recently the mRNAs for rat and human 7α-hydroxylase have been isolated and cloned (6-9), presenting a valuable tool for regulation studies. CYP7A displays a response to various physiological signals, the most important of which is considered to be exerted through the enterohepatic circulation of bile acids. CYP7A activity (3,4,10) and mRNA (8,9,11) in animals and enzyme activity in humans (12,13) is subject to end-product suppression by bile acids. Regulation takes place through a direct effect of bile acids on the hepatocyte, at concentrations commonly observed in portal blood (14,15), both at a transcriptional and posttranscriptional level (11,15,16).

Additionally, several hormones have been implicated to play a physiological role in the regulation of CYP7A (5). Several observations suggest the involvement of glucocorticoids in modulation of the enzyme. Enzyme activity, protein and mRNA, in parallel with plasma corticosterone levels in rat, show a diurnal variation (17-19). Adrenalectomy subsequently abolishes the diurnal rhythm of CYP7A activity (20,21). Using primary monolayer cultures of rat hepatocytes our group has shown that glucocorticoids and no other steroid hormones induce bile acid synthesis by stimulation of CYP7A activity (22,23). Other investigators recently showed that regulation by dexamethasone takes place at the level of mRNA (24,25) and transcription (25) in a rat hepatoma cell line and cultured rat hepatocytes. Furthermore, thyroid hormone was found to increase CYP7A activity (26) and mRNA (27) *in vivo*. It has recently been reported that thyroxine stimulates CYP7A gene transcription in cultured rat hepatocytes (16,25).

Insulin has also been implicated to play an important role in cholesterol metabolism. However, the effects of this hormone on bile acid synthesis and CYP7A are less clear. Experimental diabetes in the rat (28-31) and uncontrolled diabetes mellitus in humans (32) have been shown to lead to a marked increase in bile acid pool and biliary lipid and bile acid excretion. These parameters returned to normal levels upon insulin administration (28,31,32). The higher bile acid excretion in diabetes was ascribed to an increased bile acid synthesis (28,32), however other investigators found no change in bile acid production (29). Furthermore, divergent

results have been reported with regard to the effect of experimental diabetes on CYP7A activity in rats. Both an increased (33), unchanged (29,34) and decreased (35) activity of CYP7A were found. In none of the latter studies the site of regulatory control was investigated.

There is accumulating evidence that in normal human subjects an alternative pathway towards bile acids exists, involving initial 27-hydroxylation of cholesterol (36). It has also been shown that the alternative route contributes substantially to bile acid synthesis *in vitro*, accounting for approximately 50% of total synthesis in cultured human and rat hepatocytes (37). Sterol 27-hydroxylase, responsible for catalysis of the initial step in alternative routing of cholesterol, is a member of the cytochrome P-450 superfamily, and located in the inner mitochondrial membrane. The enzymes from rabbit (38,39) as well as rat (40) and pig (41) liver have been characterised, as well as corresponding cDNA sequences for this enzyme from rabbit (42), rat (43,44) and human (45). Little is known about possible regulatory processes affecting sterol 27-hydroxylase. It has been suggested that the enzyme is of minor importance for the regulation of bile acid synthesis and composition of bile acids formed *in vivo* in rat (46). However, it is conceivable that in view of the reported significant contribution of the 27-hydroxylase pathway to total bile acid synthesis, any mediators of the latter may regulate the enzyme, as in the case of well-documented regulatory processes affecting CYP7A.

In this study we have assessed the effects of insulin on bile acid synthesis in primary monolayer cultures of rat hepatocytes and we have investigated the mechanism of regulation. Our data indicate that physiological concentrations of insulin inhibit bile acid synthesis by decreasing CYP7A and sterol 27-hydroxylase activity. Regulation takes place by down-regulation of gene transcription for both enzymes.

MATERIALS AND METHODS

Materials used for isolation and culturing of rat hepatocytes, determination of bile acid synthesis from radiolabelled cholesterol and of mass production of bile acids, and assaying CYP7A and sterol 27-hydroxylase activity were obtained from sources described previously (22,47,48). Insulin (Actrapid, 100 IE/ml) was from Novo Industri (Copenhagen, Denmark). [α-^{32}P]dCTP (3000 Ci/mmol), [α-^{32}P]UTP (400 Ci/mmol) and [4-^{14}C]-cholesterol (60 mCi/mol) were obtained from The Radiochemical Centre, Amersham, Buckinghamshire, UK. Male Wistar rats weighing 250-350 g were used throughout and were maintained on standard chow and water *ad libitum*. For preparation of hepatocytes, animals were sacrificed between 9 and 10 a.m. Institutional guidelines for animal care were observed in all experiments.

Rat hepatocyte isolation and culture

Rat liver cells were isolated by perfusion with 0.05% collagenase and 0.005% trypsin inhibitor as described previously (22,47,48). Viability, as determined by trypan blue exclusion, was higher than 90%. The cells were seeded on 60-mm diameter plastic tissue culture dishes or 6-well cluster plates (Costar, Cambridge, MA, USA) at a density of $1.5*10^5$ cells/cm^2 in Williams E medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin, and maintained at 37°C in a 5% CO^2/95% air atmosphere (22,47,48). After a 4-hour attachment period, and 20 h thereafter, medium was refreshed with 1.0 ml (6-well plates) or 2.5 ml (dishes) of culture medium with the appropriate insulin concentrations, as indicated. Hepatocytes were cultured for 2 days under these conditions. After a 48-hour culture period, cells were harvested for determination of bile acid synthesis, CYP7A and sterol 27-hydroxylase activity and assessment of mRNA and transcriptional activity levels. In time course experiments insulin was added to the hepatocytes at various times between 24 and 48 hours of culture age as indicated in the results section.

Quantification of bile acid synthesis

Synthesis of bile acids by rat hepatocytes was measured by determination of mass production of bile acids with gas liquid chromatography during the second 24-hour culture period from 24 to 48 hours, as described before (22). Bile acid synthesis was also determined by measuring conversion of pre-existing radiolabelled cholesterol (0.15 µCi of [4-^{14}C]-cholesterol per 10 cm^2 of cells) into bile acids during the same period, *i.e.* between 24 to 48 hours, as reported previously (22,47).

Assay of CYP7A and sterol 27-hydroxylase

CYP7A and sterol 27-hydroxylase activity were measured in homogenates of hepatocytes, or in microsomes and mitochondria, respectively, as reported previously (47-49). [^{14}C]-labelled products were analysed by thin layer chromatography, and the amount of [^{14}C]-7α-hydroxycholesterol and [^{14}C]-27-hydroxycholesterol was quantitated by scraping off and counting of the spots containing these products, using the [^{14}C]-cholesterol input as a recovery standard. Blank values, determined by running parallel incubations without a NADPH-generating system, were subtracted before calculating enzyme activity. Protein and cholesterol were assayed as previously described (48).

RNA isolation, blotting and hybridisation procedures

Total RNA was isolated from cultured rat hepatocytes as previously described (15). Equal amounts of total RNA from different incubations were fractionated by electrophoresis on a 0.8% agarose gel containing 1M formaldehyde, and transferred to Hybond-N^+ filter (Amersham). For slotblotting of total RNA, samples were diluted to appropriate concentrations and applied onto a filter using the Minifold II slotblotting apparatus (Schleicher and Schuell). After both procedures, filters were crosslinked with UV-light for 5 min and then hybridised with different probes as described previously (15). Each blot was hybridised with 25 ng of probe, labelled by the random-primer method (Mega-prime, Amersham) to approximately 6x 108 cpm/μg DNA. After hybridisation and washing, the filters were exposed to Hyperfilm MP (Amersham) together with an intensifying screen (Eastman-Kodak Co.) for 48-120 h at -80°C. For quantitation of the relative amounts of mRNA, the autoradiographs were scanned using a Shimadzu CS 910 chromatograph scanner, and areas under the curves were integrated using a data processor (Shimadzu Corp. Kyoto, Japan). The mRNA levels were quantitated by using three different amounts of total RNA, between 2 and 8 μg, giving a linear relation between the specific mRNA signal and the amount of RNA applied.

The following DNA fragments were used as probes in hybridisation experiments: a 1.6 kb PCR-synthesized fragment of rat CYP7A cDNA, spanning the entire coding region as described in detail in ref. 15; a 1.6 kb *Hin*dIII/*Xba*I fragment of rat sterol 27-hydroxylase cDNA, kindly provided by Dr. Jerome Strauss (44), and isolated from a rat liver cDNA library using the rabbit sterol 27-hydroxylase cDNA, previously isolated by Russell and coworkers (42), as a probe; a 773 bp *Hin*dIII fragment of hamster HMG-CoA reductase cDNA (50); a 1.2 kb *Pst*I fragment of hamster actin cDNA; and a 1.1 kb *Pst*I fragment of rat GAPDH. The latter two served as an internal standard to correct for differences in the amount of total RNA applied onto the gel or filter.

Nuclear run-on studies

Nuclear run-on studies were conducted essentially as described by Groudine *et al.* (51), with minor modifications (15).

Isolation of nuclei : Cells were washed, scraped using a rubber policeman, and collected by centrifugation at 500 x g at 4°C for 5 min. They were resuspended in NP40-lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP40, 1 mM PMSF, 1 mM DTT), and after being left on ice for 5 min, homogenised in a Potter Elvehjem tube with pestle B for 15 strokes at 4°C. Resulting nuclei were again centrifuged at 500 x g and resuspended in

NP40-lysis buffer. This procedure was repeated until the nuclei were free of cellular debris. They were then taken up in glycerol storage buffer (50 mM Tris-HCl pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM PMSF, 5 mM DTT), counted, and aliquoted at approximately 2 x 107/500 µl before being frozen at -80°C.

RNA labelling and isolation : An aliquot of frozen nuclei was spinned down and resuspended in 200 µl of transcription buffer (10 mM Tris-HCl pH 7.9, 140 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 1 mM of dGTP, dATP, dCTP, 0.1 mM s-adenosyl-1-methionine, 14 mM β-mercaptoethanol, 1 mg/ml heparin sulfate, 1.7 mM spermidine, 10 mM creatine phosphate, 40 µg/ml creatine kinase, 25% glycerol and 100 µCi of [α^{32}P]UTP), and incubated while shaking at 30°C for 30 min. 600 µl of a buffer containing 0.5 M NaCl, 50 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Tris-HCl pH 7.4 and 200 U/ml DNAseI (Bethesda Research Laboratories) was added, and the mixture was incubated for an additional 5 min. at 30°C. 200 µl of SDS/Tris (5% w/v SDS, 0.5 M Tris-HCl pH 7.4, 0.125 M EDTA) with 200 µg/ml Proteinase K (Boehringer Mannheim) was added, and the mixture was incubated for 30 min at 42°C. RNA was extracted with 1 volume of phenol/chloroform/isoamylalcohol (50:49:1), precipitated with 2.5 volumes of ethanol and 10 µg/ml tRNA, washed, and taken up in 50 µl of Tris/EDTA (10 mM Tris-HCl pH 7.4, 1 mM EDTA). Labelled RNA was separated from free nucleotides by passage over a Sephadex G50 (fine)-column (Boehringer Mannheim). The RNA was mildly degraded by a 10 min incubation on ice in 0.25 M NaOH, and the mixture was neutralised by addition of a half volume of 1 M HEPES and precipitated with 2 volumes of ethanol and 0.1 volume of 3 M NaAc. Incorporation of label was measured by liquid scintillation counting, and equal amounts of labelled RNA were added to the filters.

Hybridisation : Target DNA, being 5 µg of plasmid material containing cDNA sequences of rat CYP7A, rat sterol 27-hydroxylase, hamster HMG-CoA reductase, hamster actin and rat GAPDH were slotblotted onto strips of Hybond-N^+ filter (Amersham) and crosslinked. The filters were hybridised with the labelled RNA for 36 hours, washed and exposed to Hyperfilm MP (Amersham) for 2-5 days. Quantitation of relative amounts of mRNA synthesized was conducted as described above.

Transfection experiments and CAT-assays

At 22 hours after isolation cells were subjected to transfection using recombinant plasmid -348RCAT, and CAT-assays were performed, as described previously (16). -348RCAT contains the proximal 348 nucleotides of the CYP7A promoter fused to the bacterial chloramphenicol acetyltransferase gene, used as a reporter. The amounts of acetylated product

as represented by autoradiography were quantitated using a Phosphor-imager 400B (Molecular Dynamics). Data were corrected for protein and transfection efficiency.

Statistical analysis

Data were analysed statistically using Student's paired t-test. Values are expressed as means ± SD.

RESULTS

Effect of insulin on bile acid synthesis in cultured rat hepatocytes

To determine the influence of insulin on bile acid synthesis, hepatocytes were incubated with [4-^{14}C]-cholesterol as a substrate. Addition of 140 nM insulin to the culture medium resulted in a significant ($P < 0.005$) 58 ± 19% decrease in total bile acid synthesis, from 8760 ± 3370 dpm/24 h per mg of cell protein to 3680 ± 1320 dpm/24 h per mg of cell protein (means ± S.D., n = 4) in the period from 24-48 h. In these experiments, bile acid synthesis was

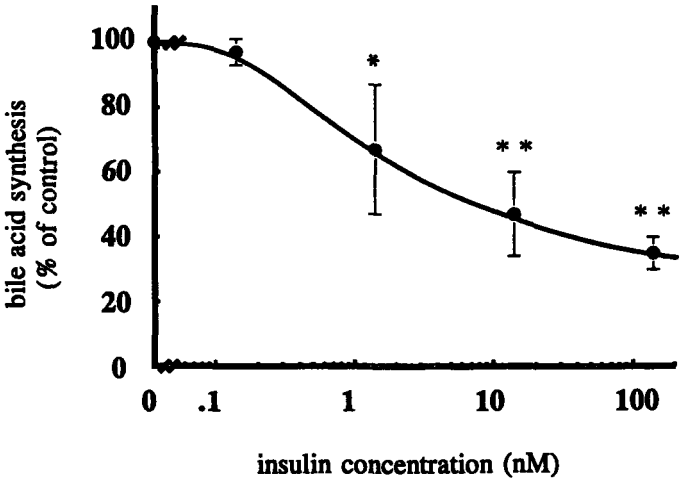


Figure 1. Effect of insulin concentration on mass production of bile acids in cultured rat hepatocytes. Hepatocytes were cultured as described in the Materials and methods section. After a 4-h attachment period and 20 h thereafter, cells were refreshed with medium without (control) or with the indicated insulin concentrations. Bile acid synthesis was measured in the period from 24-48 h in cells and media. Values shown are expressed as percentage of bile acid synthesis in control incubations and are means (± S.D.) of duplicate incubations of hepatocytes from six rats. Absolute synthesis rate in the absence of insulin was 1.29 ± 0.80 μg/24 h per mg cell protein. Cholic acid was 23 ± 7% of total bile acid synthesis, β-muricholic and cheno-deoxycholic acids 77 ± 7%. A significant difference between control and insulin-treated cells is indicated by an asterix (*, $P < 0.05$; **, $P < 0.005$).

determined by measuring conversion of pre-existent radiolabelled cholesterol into bile acids. To exclude the possibility that insulin decreased bile acid synthesis from exogenous cholesterol by changing the amount of total cholesterol available for bile acid formation, consequently leading to changes in the specific activity of the precursor pool of cholesterol, mass production of bile acids was determined. As can be seen in Fig. 1, addition of insulin to the cells caused a dose-dependent decrease in total bile acid synthesis. Significant inhibition (33 to 53%) was achieved at physiological concentrations of insulin (1.4 to 14 nM), whereas maximal inhibition was reached at 140 nM (65 ± 5%). The latter value compared well with the data obtained from the [^{14}C]-cholesterol conversion measurement. No change was observed in the proportion of individual bile acids synthesized in the presence or absence of insulin. The contribution of cholic acid to total bile acid synthesis was 23 ± 7% and of β-muricholic acid and chenodeoxycholic acid 77 ± 7%.

Effect of insulin on CYP7A and sterol 27-hydroxylase activity in cultured rat hepatocytes

The effect of incubation of hepatocytes with 140 nM insulin on CYP7A and sterol 27-hydroxylase activities is shown in Fig. 2. The suppression of the activities of both enzymes

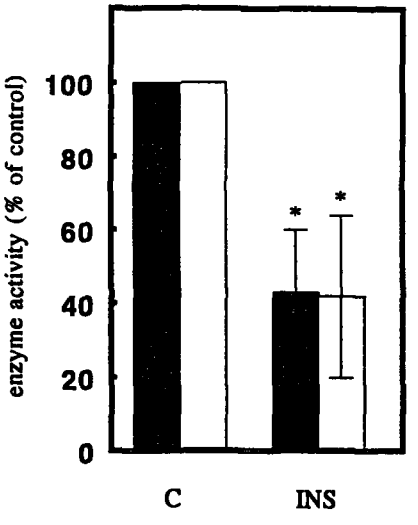


Figure 2. Effect of insulin on cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity in cultured rat hepatocytes. Experiments were performed as described in the legend to Fig. 1. Hepatocytes were harvested at 48 hours of culture, after a 24-hour incubation with insulin, or solute (controls), and enzyme activities were determined. Values shown are expressed as a percentage of enzyme activity in control incubations and are a means (± S.D.) of duplicate incubations, using hepatocytes from 5-6 rats. Cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity in control hepatocytes amounted to 203 ± 154 and 70 ± 24 pmol/h per mg of cell protein, respectively. A significant difference between control and insulin-treated cells is indicated by an asterix ($P < 0.005$).

(-57% for CYP7A and -58% for sterol 27-hydroxylase) paralleled the decrease in bile acid synthesis (-65%). The dose-dependency and time course of the suppression of CYP7A activity by insulin were studied in more detail.

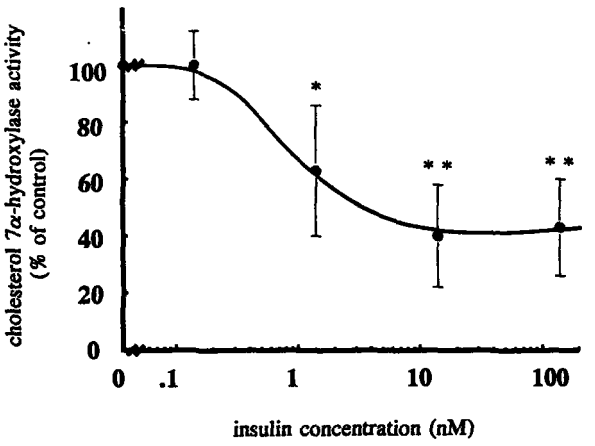


Figure 3. Effect of insulin concentration on cholesterol 7α-hydroxylase activity in cultured rat hepatocytes. Experiments were performed as described in the legend to Fig. 1. Hepatocytes were harvested at 48 h of culture and enzyme activity was determined. Values shown are expressed as percentage of enzyme activity in control incubations and are means (± S.D.) of duplicate incubations, using hepatocytes from 3-9 rats. Absolute values for cholesterol 7α-hydroxylase activity were 203 ± 154 pmol/h per mg of cell protein. A significant difference between control and insulin-treated cells is indicated by an asterix (*, $P < 0.05$; **, $P < 0.005$).

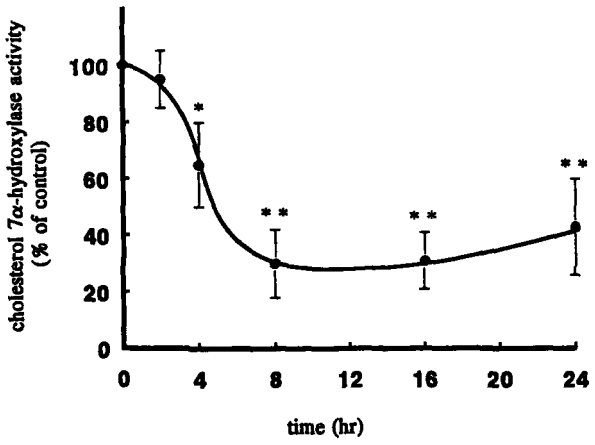


Figure 4. Time course of inhibition of cholesterol 7α-hydroxylase activity in cultured rat hepatocytes by insulin. At the indicated times before harvesting 140 nM insulin was added to the culture medium. Hepatocytes of all incubations were harvested simultaneously, at 48 h of culture time. Values shown are expressed as percentage of enzyme activity in control incubations and are means ± S.D. of duplicate incubations, using hepatocytes from 3-9 rats. Cholesterol 7α-hydroxylase activity in control hepatocytes amounted to 203 ± 154 pmol/h per mg of cell protein. A significant difference between control and insulin-treated cells is indicated by an asterix (*, $P < 0.05$; **, $P < 0.005$).

Addition of various concentrations of insulin to the culture medium resulted in a dose-dependent suppression of CYP7A activity between 24-48 h of culture (Fig. 3). Maximal reduction (60% ± 20%) was reached at 14 nM insulin whereas 1.4 nM was already sufficient to obtain significant inhibition. To assess the time course of inhibition, cells were exposed to 140 nM insulin for different lengths of time between 24 and 48 h of culture and hepatocytes were harvested simultaneously after 48 h of culture time. Figure 4 shows that maximal inhibition was achieved after an incubation period of 8 hours. The rat hepatocytes were refractory to induction of CYP7A activity during the first 24 h of culture. This finding is in line with previous reports on poor hormonal induction of enzymes, including CYP7A (22) and other mono-oxygenases (52) shortly after hepatocyte isolation (53,54).

We have previously shown that glucocorticoids stimulate bile acid synthesis in cultured rat hepatocytes by inducing CYP7A (22,23). To investigate whether insulin had an effect on the stimulation of the enzyme activity by dexamethasone, hepatocytes were incubated for 24 h without hormones, with 50 nM dexamethasone or in the presence of 50 nM dexamethasone and 140 nM insulin. The induction of 7α-hydroxylase activity by 50 nM dexamethasone (5.7-fold (S.D. = 2.5, n = 6) with respect to control cultures without hormones) was strongly blocked by simultaneous addition of 140 nM insulin. Stimulation was found to be only 2.0-fold (S.D. = 1.4, n = 6) in the presence of both hormones. The suppression of activity of either enzyme cannot be attributed to a decrease in substrate availability, since the cellular free cholesterol content was increased significantly ($P < 0.05$) from 16.4 ± 1.5 μg/mg of cell protein (n = 5) in control cells to 20.5 ± 1.9 μg/mg of cell protein (n = 5) in hepatocytes maintained in the presence of 140 nM insulin. Furthermore, free cholesterol from cells comprises only 33% of total free cholesterol in the enzyme assay (48), and the slight dilution of specific radioactivity of substrate as a result of the increase of cholesterol content of cells was corrected for in the enzyme assay. To determine whether insulin exerted its effect by direct inhibition of CYP7A, as suggested previously (33), or of sterol 27-hydroxylase, the hormone was added directly to the assay mixture. Both in incubations with isolated liver microsomes or mitochondria, and homogenates of hepatocytes, 140 nM insulin had no effect on enzyme activity (data not shown).

Effect of insulin on CYP7A and sterol 27-hydroxylase mRNA and transcriptional activity

To assess the level of regulation of both CYP7A and sterol 27-hydroxylase activity by insulin, steady-state mRNA levels and transcriptional activity of both genes were determined in hepatocytes which had been incubated with 140 nM insulin.

Northern-blot analysis of total RNA isolated from cultured hepatocytes shows a strong down-regulation of CYP7A as well as sterol 27-hydroxylase mRNA in response to 140 nM insulin, as opposed to the actin and GAPDH mRNA, used as internal standards (Fig. 5a). The three distinct mRNAs for CYP7A, as reported before (15), all show equal down-regulation by insulin. For reasons of comparison, mRNA levels for HMG-CoA reductase were also assessed. Figure 5a clearly shows that this particular messenger is strongly upregulated in response to an 8-hour incubation with insulin (6.5-fold), indicative for the fact that not all mRNAs for enzymes involved in maintainance of cholesterol homeostasis behave similarly in response to insulin. Stimulation was transient, and declined to normal levels after prolonged

incubation with the compound (results not shown). Results described herein are in good agreement with *in vitro* studies using cultured rat hepatocytes (55) and recent *in vivo* studies with streptozotocin-treated diabetic rats (56), which show rapid stimulation of HMG-CoA reductase activity and mRNA upon administration of insulin. Figure 5b shows a rapid decline of mRNA-levels for both CYP7A and sterol 27-hydroxylase in response to insulin. After 8 hours of incubation with the compound, the messenger level had decreased up to 76 ± 15% and -62 ± 19% for CYP7A and sterol 27-hydroxylase, respectively.

Nuclear run-on assays were conducted to assess whether insulin had any effect on transcriptional activity of the genes for the two key-enzymes. Nuclei were isolated from hepatocytes incubated in the presence or absence of 140 nM insulin for 8 hours. [α^{32}P]-labelled total RNA was hybridised to rat CYP7A cDNA, rat sterol 27-hydroxylase cDNA, hamster HMG-CoA reductase cDNA, rat GAPDH and hamster actin cDNA. The latter two served as transcriptional activity controls between the different samples and specific transcriptional activity is hence expressed relative to that of actin.

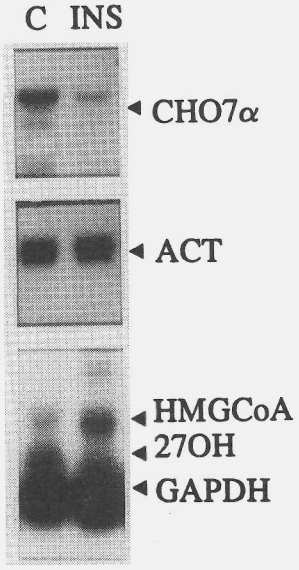
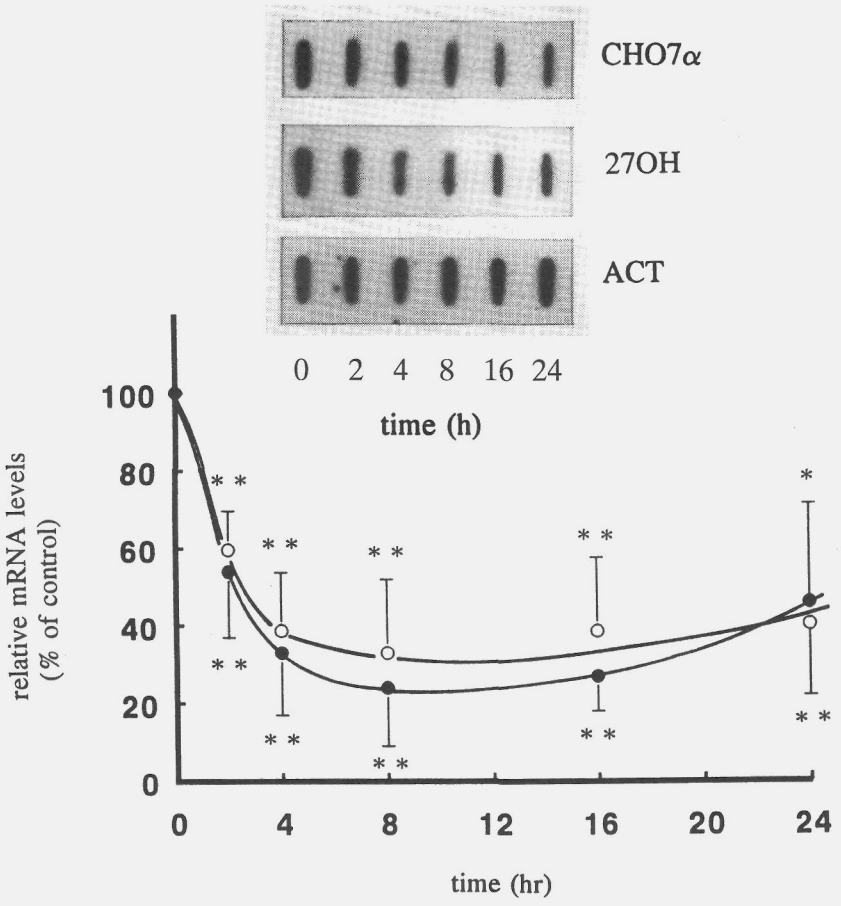


Figure 5. Time course of inhibition of cholesterol 7α-hydroxylase and sterol 27-hydroxylase mRNA by insulin in cultured rat hepatocytes.
A. Northern-blot hybridization of total RNA, isolated from hepatocytes incubated in the presence (INS) or absence (C) of 140 nM insulin for 8 h. 10 μg of total RNA was separated on a 1% agarose gel containing formaldehyde, transferred to a Hybond-N$^+$ filter, and hybridized with [^{32}P]-labelled cholesterol 7α-hydroxylase (CHO7α) cDNA, sterol 27-hydroxylase (27OH), HMG-CoA reductase (HMG-CoA) cDNA, GAPDH cDNA, or actin (ACT) cDNA as described under "Materials and Methods".



B. At the indicated times before harvesting, 140 nM insulin was added to the culture medium. Hepatocytes of all incubations were harvested simultaneously at 48 h of culture time, and mRNA was isolated as described under "Materials and Methods". The amount of cholesterol 7α-hydroxylase (CHO7α, closed symbols) and sterol 27-hydroxylase (27OH, open symbols) mRNA was assessed by slot-blotting and densitometric scanning of resulting autoradiographs (inset), using the actin (ACT) mRNA as an internal standard to correct for differences in the amount of total RNA applied to the filter. Values shown are expressed as percentages of mRNA levels in control incubations and are a means ± S.D. of duplicate incubations, using hepatocytes from 3-6 rats. A significant difference between control and insulin-treated cells is indicated by an asterix (*, $P < 0.05$; **, $P < 0.005$).

Figure 6 shows that addition of 140 nM insulin lowers the transcriptional rate of CYP7A by 60 ± 1%, and in the case of sterol 27-hydroxylase: by 75 ± 4%. There was no effect on transcriptional activity of the GAPDH gene. In contrast, insulin displays a strong stimulatory effect on HMG-CoA reductase gene expression, which is induced 4.0 ± 1.1-fold.

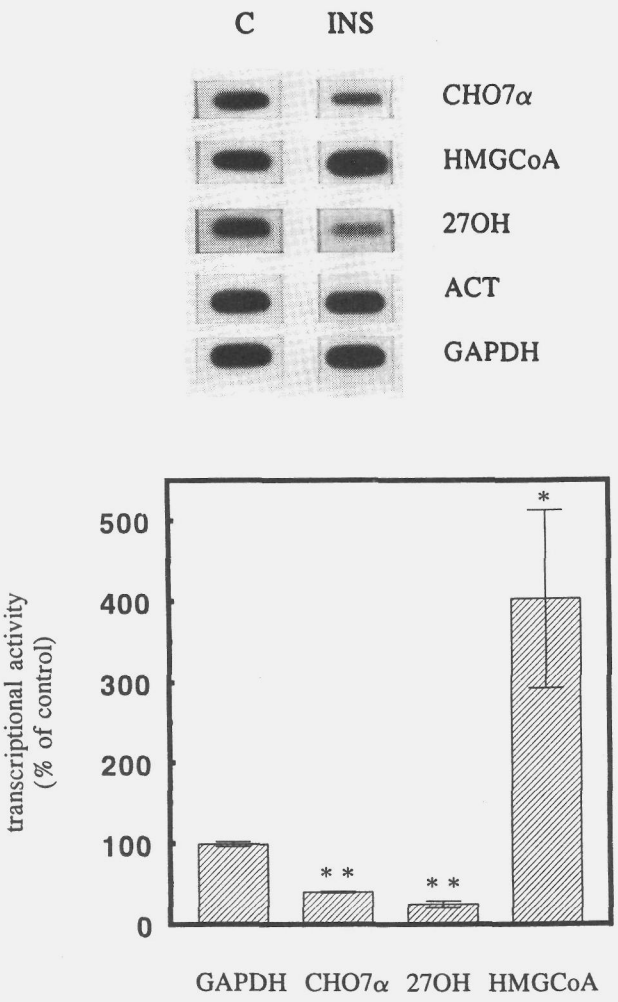


Figure 6. Effect of insulin on transcriptional activity of cholesterol 7α-hydroxylase and sterol 27-hydroxylase.

A. Transcriptional activity of several genes in nuclei isolated from hepatocytes incubated with 140 nM insulin. Cells were exposed to the hormone (INS) for 8 h, between 40 and 48 h of culture, and were harvested simultaneously with untreated cells (C) after this period. [^{32}P]-labelled total RNA was synthesized and isolated from nuclei, and hybridized to 5 μg of cholesterol 7α-hydroxylase (CHO7α) cDNA, sterol 27-hydroxylase (27OH) cDNA, HMG-CoA reductase (HMG-CoA) cDNA, actin (ACT) cDNA and GAPDH cDNA, as described under "Materials and Methods".

B. The amount of specific mRNA was assessed by densitometric scanning of resulting autoradiographs, using the actin mRNA signal as a transcriptional control. Data are expressed as transcriptional activity relative to that of actin, and as a percentage of control cells (no insulin added). Each value represents a mean ± SD of three independent experiments. A significant difference between control and insulin-treated cells is indicated by an asterix (*, $P < 0.05$; **, $P < 0.005$).

Effect of insulin on CAT-activity in cells transfected with a CYP7A promoter-CAT-reporter construct

Recently, Hoekman *et al* (16), by performing transient-expression experiments in primary cultures of rat hepatocytes, obtained evidence that a major transcription activating element of the CYP7A gene is located in the proximal region up to -348 nucleotides. We wanted to assess whether an insulin-responsive sequence might be localised within this proximal part of the CYP7A promoter. A -348RCAT construct, consisting of the first 348 basepairs of the CYP7A

promoter fused to the CAT-reporter gene (16), was used in transient-expression experiments. In accordance with down-regulation of transcriptional activity of the CYP7A gene as determined by nuclear run-on assays, insulin displayed a strong inhibitory effect (-64 ± 7 %) on the promoter activity of the -348RCAT construct (Fig.7)

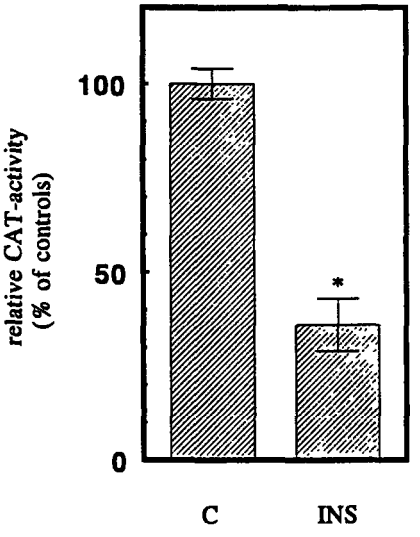


Figure 7. Effect of insulin on CAT-activity of cholesterol 7α-hydroxylase promoter-reporter construct -348R*cat* in transfected cultured rat hepatocytes. Transient-expression experiments were performed, using the -348R*cat* promoter-reporter construct described in ref. 16. After transfection, cells were cultured in standard medium in the presence (INS) or absence (C) of 140 nM insulin, and CAT-activity was assessed. Values expressed are a means ± SD of three independent experiments. *Indicates a significant difference ($P < 0.05$) between controls and insulin-treated cells.

DISCUSSION

The present study shows that bile acid synthesis in cultured rat hepatocytes is subject to down-regulation by physiological concentrations of insulin through a direct effect on the hepatocyte and that regulation takes place by suppression of transcription of the CYP7A and sterol 27-hydroxylase gene. Insulin caused a maximal inhibition (58%) of the conversion of [^{14}C]-labelled cholesterol, which is in good agreement with the 65% reduction of mass production of bile acids, *i.e.* cholic acid andβ-muricholic acid. The latter observation excludes the possibility that insulin might affect the routing of cholesterol from an intracellular precursor pool towards bile acids, thereby causing a shift from exogenous to endogenous cholesterol as a substrate. This would ultimately result in a decrease in utilisation of exogenous [^{14}C]-cholesterol. Addition of physiologically relevant concentrations of insulin (1.4 - 14 nM) already leads to a marked decrease in bile acid synthesis (33% to 53%, respectively), with a maximum (65%) at 140 nM. The magnitude of suppression agrees well with results obtained

in vivo in rat by other investigators, who showed a two-fold increase in bile acid pool size and biliary bile acid secretion in diabetic, insulin-deficient rats, which were restored to normal values after daily injection with insulin (28,31). Treatment of insulin resistance with insulin in patients having maturity-onset diabetes mellitus also reduced bile acid pool size and fecal bile acid excretion (32).

With regard to the mechanism of the increased bile acid synthesis in diabetic animals contradictory reports have appeared, showing unchanged (29,34), increased (33) and decreased (35) activity of CYP7A. Our results show that addition of insulin to the culture medium of hepatocytes results in a time- and dose-dependent decline of CYP7A activity. In addition, insulin displayed a concomitant suppressive effect on sterol 27-hydroxylase, involved in alternative routing of cholesterol to bile acids. The decrease of these enzyme activities correlated well with the suppression of bile acid synthesis.

It has been postulated that CYP7A activity, as that of other major enzymes involved in cholesterol homeostasis, are coordinately regulated by phosphorylation/dephosphorylation processes (57). Insulin does affect a number of enzyme activities through modulation of their phosphorylation state (58). However, we and others have not found indications for such a type of regulation (48,59). Down-regulation of CYP7A activity by insulin did not differ upon addition or absence of 50 mM fluoride (data not shown). Additionally, our results indicate that regulation by insulin takes place at the level of CYP7A mRNA, resulting in a 76% decrease of the steady-state mRNA levels after 8 h, similar to the suppression of enzyme activity.

In addition to normal routing of cholesterol towards bile acids via CYP7A as first and rate-limiting step, a substantial contribution to bile acids is made via an 'alternative' or '27-hydroxylase' pathway (36,37). Synthesis via initial 27-hydroxylation has been estimated to amount up to 50% in cultured human and rat hepatocytes (37). Down-regulation of CYP7A alone may thus not be sufficient to explain inhibitory effects of insulin on bile acid synthesis, since this would leave a major portion of synthesis unaffected. The results clearly show that, in addition to CYP7A, sterol 27-hydroxylase is affected to a similar extent. Insulin caused suppression of sterol 27-hydroxylase activity (-58%) and mRNA (-62%), comparable to the effects on CYP7A. Measurement of transcriptional activity for both genes via nuclear run-on assays and via transient expression assays for CYP7A shows that the inhibitory effect of insulin thereon can fully explain the decline in activity and mRNA for these key-enzymes. Insulin increased the intracellular amount of cholesterol by 25% (from 16.4 to 20.5 μg/mg cell protein). This is, however, not a mechanism by way of which the hormone can exert its suppressive effect on the key-enzymes described. As has been shown by other investigators

(8,60-63) dietary cholesterol has stimulatory effects on CYP7A activity, mRNA and gene expression, possibly by a reduced feedback inhibition due to cholesterol-induced malabsorption of bile acids (60). Sterol 27-hydroxylase is not affected by this mediator (J. Twisk, L. v.d. Fits, H.M.G. Princen, unpublished observation). In this light, the negative effect of insulin on CYP7A might well be underestimated due to simultaneous positive effects through enhanced intracellular cholesterol levels. The increase of cellular cholesterol may have multiple causes, since insulin has multiple effects on hepatic lipid and lipoprotein metabolism. Insulin has a stimulatory effect on the key enzyme in cholesterol synthesis, HMG-CoA reductase, both *in vivo* and in cultured rat hepatocytes (55,56,64-66). Streptozotocin-treated rats infused with insulin showed a rapid recovery of activity and mRNA levels for this enzyme (56). Similarly, treatment of primary monolayer cultures of rat hepatocytes with insulin resulted in an increase of both HMG-CoA reductase activity and mRNA (55). These effects can be explained by induction of gene expression (this study). Additionally, the hormone has been shown to increase the receptor-mediated uptake of lipoproteins (67,68), to decrease the synthesis and secretion of apoB-containing lipoproteins (69-71) and to inhibit bile acid synthesis (this study).

We conclude that physiological concentrations of insulin suppress bile acid synthesis in cultured rat hepatocytes by down-regulation of CYP7A and sterol 27-hydroxylase gene transcription. These findings may provide an explanation for the increased bile acid pool size and excretion as found in association with insulin resistance in untreated non-insulin-dependent diabetes mellitus in humans or insulin deficiency in experimental diabetic animals, and normalisation thereof upon insulin administration.

ACKNOWLEDGEMENTS

We thank Miss Marisa Horsting for typing the manuscript.

REFERENCES

1. Björkhem, I. : Mechanisms of bile acid biosynthesis in mammalian liver. In: Sterols and bile acids. Danielsson, H., and Sjövall, J., eds. Elsevier, Amsterdam (1985), 231-278.
2. Carey, M.C., and Cahalane, M.J. : Enterohepatic circulation. In: The Liver: Biology and Pathology. Arias, I.M., Jakoby, W.B., Popper, H., Schachter, D., and Shafritz, D.A., eds. Raven Press, New York (1988), 573-616.
3. Danielsson, H., Einarsson, K., and Johansson, G. : Effects of biliary drainage on individual reactions in the conversion of cholesterol to taurocholic acid. Eur. J. Biochem. 2 (1967), 44-49.
4. Shefer, S., Hauser, S., Bekersky, I., and Mosbach, E.H. : Biochemical site of regulation of bile acid synthesis in the rat. J. Lipid. Res. 11 (1970), 404-411.
5. Myant, N.B., and Mitropoulos, K.A. : Cholesterol 7α-hydroxylase. J. Lipid. Res. 18 (1977), 135-153.
6. Noshiro, M., Nishimoto, M., Morohashi, K., and Okuda, K. : Molecular cloning of cDNA for cholesterol 7α-hydroxylase from rat liver microsomes. FEBS Lett. 257 (1989) 97-100.
7. Noshiro, M., and Okuda, K. : Molecular cloning and sequence analysis of cDNA encoding human cholesterol 7α-hydroxylase. FEBS Lett 1990; 268: 137-140.
8. Jelinek, D.F., Andersson, S., Slaughter. C.A., and Russell D.W. : Cloning and regulation of cholesterol 7α-hydroxylase, the rate limiting enzyme in bile acid biosynthesis. J. Biol. Chem. 265 (1990), 8190-8197.
9. Li, Y.C., Wang, D.P., and Chiang, J.Y.L. : Regulation of cholesterol 7α-hydroxylase in the liver. Cloning, sequencing and regulation of cholesterol 7α-hydroxylase mRNA. J. Biol. Chem. 265 (1990), 12012-12019.
10. Heuman, D.M., Vlahcevic, Z.R., Bailey, M.L., and Hylemon, P.B. : Regulation of bile acid synthesis. II. Effect of bile acid feeding on enzymes regulating hepatic cholesterol and bile acid synthesis in the rat. Hepatology 8 (1988), 892-897.
11. Pandak, W.M., Vlahcevic, Z.R., Chiang, J.Y.L., Heuman, D.M., and Hylemon, P.B. : Bile acid synthesis. VI. Regulation of cholesterol 7α-hydroxylase by taurocholate and mevalonate. J. Lipid Res. 33 (1992), 659-668.
12. Coyne, M.J., Bonorris, G.G., Goldstein, L.I., and Schoenfield, L.J. : Effect of chenodeoxycholic acid and phenobarbital on the rate-limiting enzymes of hepatic cholesterol and bile acid synthesis in patients with gallstones. J. Lab. Clin. Med. 87 (1976), 281-291.
13. Reihnér, E., Björkhem, I., Angelin, B., Ewerth, S., and Einarsson, K. : Bile acid synthesis in humans: Regulation of hepatic microsomal cholesterol 7α-hydroxylase activity. Gastroenterology 97 (1989),

1498-1505.

14. Kwekkeboom, J., Princen, H.M.G., Van Voorthuizen, E.M., and Kempen, H.J.M. : Bile acids exert negative feedback control on bile acid synthesis in cultured pig hepatocytes by suppression of cholesterol 7α-hydroxylase activity. Hepatology 12 (1990), 1209-1215.

15. Twisk, J., Lehmann, E.M., and Princen, H.M.G. : Differential feedback regulation of cholesterol 7α-hydroxylase mRNA and transcriptional activity by rat bile acids in primary monolayer cultures of rat hepatocytes. Biochem. J. 290 (1993), 685-691.

16. Hoekman, M.F.M., Rientjes, J.M.J., Twisk, J., Planta, R.J., Princen, H.M.G., and Mager, W.H. : Transcriptional regulation of the gene encoding cholesterol 7α-hydroxylase in the rat. Gene 130 (1993), 217-223.

17. Chiang, J.Y.L., Miller, W.F., and Lin, G.-M. : Regulation of cholesterol 7α-hydroxylase in the liver. Purification of cholesterol 7α-hydroxylase and the immunochemical evidence for the induction of cholesterol 7α-hydroxylase by cholestyramine and circadian rhythm. J. Biol. Chem. 265 (1990), 3889-3897.

18. Noshiro, M., Nishimoto, M., and Okuda, K. : Rat liver cholesterol 7α-hydroxylase. Pretranslational regulation for circadian rhythm. J. Biol. Chem. 265 (1990), 10036-10041.

19. Sundseth, S.S., and Waxman, D.J. : Hepatic P-450 cholesterol 7α-hydroxylase. Regulation in vivo at the protein and mRNA level in response to mevalonate, diurnal rhythm, and bile acid feedback. J. Biol. Chem. 265 (1990), 15090-15095.

20. Van Cantfort, J. : Controle par les glucocorticoides de l'activité circadienne de la cholesterol 7α hydroxylase. Biochimie 55 (1973), 1171-1173.

21. Mitropoulos, K.A., and Balasubramaniam, S. : The role of glucocorticoids in the regulation of the diurnal rhythm of hepatic β-hydroxy-β-methylglutaryl-coenzyme A reductase and cholesterol 7α hydroxylase. Biochem. J. 160 (1976), 49-55.

22. Princen, H.M.G., Meijer, P., and Hofstee, B. : Dexamethasone regulates bile acid synthesis in monolayer cultures of rat hepatocytes by induction of cholesterol 7α-hydroxylase. Biochem. J. 262 (1989), 341-348.

23. Princen, H.M.G., and Meijer, P. : Maintenance of bile acid synthesis and cholesterol 7α-hydroxylase activity in cultured rat hepatocytes. Biochem. J. 272 (1990), 273-275.

24. Leighton, J.K., Dueland, S., Straka, M.S., Trawick, J., and Davis, R.A. : Activation of the silent endogenous cholesterol 7α-hydroxylase gene in rat hepatoma cells: a new complementation group having resistance to 25-hydroxycholesterol. Mol. Cell. Biol. 11 (1991), 2049-2056.

25. Hylemon, P.B., Gurley, E.C., Stravitz, R.T., Litz, J.S., Pandak, W.M., Chiang, J.Y.L., and Vlahcevic, Z.R. : Hormonal regulation of cholesterol 7α-hydroxylase mRNA levels and transcriptional

activity in primary rat hepatocyte cultures. J. Biol. Chem. 267 (1992), 16866- 16871.

26. Pauletzki, J., Stelaard, F., and Paumgartner, G. : Bile acid metabolism in human hyperthyroidism. Hepatology 9 (1989), 852-855.

27. Ness, G.C., Pendleton, L.C., Li, Y.C., and Chiang, J.Y.L. : Effect of thyroid hormone on hepatic cholesterol 7α-hydroxylase, LDL-receptor, HMG-CoA reductase, farnesyl pyrophosphate synthetase and apolipoprotein A-1 mRNA levels in hypophysectoctomized rats. Biochem. Biophys. Res. Commun. 172 (1990), 1150-1156.

28. Nervi, F.O., Severin, C.H., and Valdivieso, V.D. : Bile acid pool changes and regulation of cholate synthesis in experimental diabetes. Biochim. Biophys. Acta 529 (1978), 212-223.

29. Uchida, K., Takase, H., Kadowaki, M., Nomura, Y., Matsubara, T., Takeuchi, N. : Altered bile acid metabolism in alloxan diabetic rats. Japan. J. Pharmacol. 29 (1979), 553-562.

30. Hassan, A.S., Subbiah, M.T.R., and Thiebert, P. : Specific changes of bile acid metabolism in spontaneously diabetic wistar rats. Proc. Soc. Exp. Biol. Med. 164 (1980), 449-452.

31. Villanueva, G.R., Herreros, M., Perez-Barriocanal, F., Bolaños, J.P., Bravo, P., and Marin, J.J.G. : Enhancement of bile acid-induced biliary lipid secretion by streptozotocin in rats: role of insulin deficiency. J. Lab. Clin. Med. 115 (1990), 441-448.

32. Bennion, L.J., and Grundy, S,M. : Effects of diabetes mellitus on cholesterol metabolism in man. N. Engl. J. Med. 296 (1977), 1365-1371.

33. Subbiah, M.T.R., and Yunker, R.L. : Cholesterol 7α-hydroxylase of rat liver: an insulin sensitive enzyme. Biochem. Biophys. Res. Commun. 124 (1984), 896-902.

34. Mayer, D. : Hormones and 7α-hydroxylation of cholesterol. In: Advances in Bile Acid Research. Matern, S., Hackenschmidt, J., Back, P., and Gerok, W, eds., F.K. Schattauer Verlag, Stuttgart-New York (1957), 53-59.

35. Hansson, R. : Effect of diabetes, starvation, ethanol and isoniazid on rat liver microsomal 12α hydroxylase activity involved in bile acid biosynthesis. Biochem. Pharmacol. 38 (1989), 3386-3389.

36. Axelson, M., and Sjövall, J. Potential bile acid precursors in plasma - possible indicators of biosynthetic pathways to cholic and chenodeoxycholic acids in man. J. Steroid Biochem. 36 (1990), 631-640.

37. Princen, H.M.G., Meijer, P., Wolthers, B.G., Vonk, R.J., and Kuipers, F. : Cyclosporine A blocks bile acid synthesis in cultured hepatocytes by specific inhibition of chenodeoxycholic acid synthesis. Biochem. J, 275 (1991), 501-505.

38. Wikvall, K. : Hydroxylations in biosynthesis of bile acids. Isolation of a cytochrome P-450 from rabbit liver mitochondria catalyzing 26-hydroxylation of C27-steroids. J. Biol. Chem. 259 (1984), 3800-3804.

39. Dahlbäck, K., and Wikvall, K. : 25-Hydroxylation of vitamin D3 by a cytochrome P-450 from rabbit liver mitochondria. Biochem. J. 252 (1988), 207-213.

40. Okuda, K., Masumoto, O., and Ohyama, Y. : Purification and characterization of 5β-cholestane 3α,7α,12α-triol 27-hydroxylase from female rat liver mitochondria. J. Biol. Chem. 263 (1988), 18138-18142.

41. Bergman, T., and Postlind, H. : Characterization of mitochondrial cytochrome P-450 from pig kidney and liver catalysing 26-hydroxylation of 25-hydroxyvitamin D3 and C27-steroids. Biochem.J. 276 (1991), 427-432.

42. Andersson, S., Davis, D.L., Dahlbäck, H., Jörnvall, H., Russell, D.W. : Cloning, structure and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. J. Biol. Chem. 264 (1989), 8222-8229.

43. Usui, E., Noshiro, M., and Okuda, K. : Molecular cloning of cDNA for vitamin D3 25-hydroxylase from rat liver mitochondria. FEBS Lett. 262 (1990), 135-138.

44. Su, P., Rennert, H., Shayiq, R.M., Yamamoto, R., Zheng, Y.-M., Addya, S., Strauss III, J.F., and Avadhani, N.G. : A cDNA encoding rat mitochondrial cytochrome P-450 catalyzing both the 26-hydroxylation of cholesterol and 25-hydroxylation of vitamin D3: gonadotropic regulation of the cognate mRNA in ovaries. DNA and Cell Biology 9 (1990), 657-665.

45. Cali, J.J., Russell, D.W. : Characterization of human sterol 27-hydroxylase. A mitochondrial cytochrome P-450 that catalyzes multiple oxidations in bile acid biosynthesis. J. Biol. Chem. 266 (1991), 7774-7778.

46. Björkhem, I. : Mechanism of degradation of the steroid side chain in the formation of bile acids. J. Lipid Res. 33 (1992), 455-470.

47. Princen, H.M.G., Huijsmans, C.M.G., Kuipers, F., Vonk, R.J., and Kempen, H.J.M. : Ketoconazole blocks bile acid synthesis in hepatocyte monolayer cultures and *in vivo* in rat by inhibiting cholesterol 7α-hydroxylase. J. Clin. Invest. 78 (1986), (1986), 1064-1071.

48. Princen, H.M.G., Meijer, P., Kwekkeboom, J., and Kempen, H.J.M. : Assay of cholesterol 7α hydroxylase activity in rat hepatocytes in primary monolayer culture. Anal. Biochem. 171 (1988), 158-165.

49. Björkhem, I., Gustaffson, J. : Mitochondrial hydroxylation of cholesterol side chain. J. Biol. Chem. 249 (1974), 2528-2535.

50. Chin, D.J., Gil, G., Russell, D.W., Liscum, L., Luskey, K.L., Basu, S.K., Okayama, H., Berg, P., Goldstein, J.L., and Brown, M.S. : Nucleotide sequence of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase, a glycoprotein of endoplasmatic reticulum. Nature 308 (1984), 613-617.

51. Groudine, M., Peretz, M., and Weintraub, H. : Transcriptional regulation of hemoglobin switching on

chicken embryos. Mol. Cell. Biol. 1 (1981), 281-288.

52. McManus, M.E., Edwards, A.M., Stupans, I., Burgess, W., Lucas, C., and Birkett, D.J. : Effect of dexamethasone on cytochrome P-450 mediated metabolism of 2-acetylaminofluorene in cultured rat hepatocytes. Biochem. Pharmacol. 36 (1987), 237-243.

53. Ichihara, A., Nakamura, T., and Tanaka, K. : Use of hepatocytes in primary culture for biochemical studies on liver functions. Mol. Cell. Biochem. 43 (1982), 145-160.

54. Bissell, D.M., and Guzelian, P.S. : Phenotypic stability of adult rat hepatocytes in primary monolayer culture. Ann. N. Y. Acad. Sci. 349 (1980), 85-98.

55. Spence, J.T., Koudelka, A.P., and Tseng-Crank, J.C.L. : Role of protein synthesis in the carbohydrate-induced changes in the activities of actetyl-CoA carboxylase and hydroxymethylglutaryl-CoA reductase in cultured rat hepatocytes. Biochem. J. 227 (1985), 939-947.

56. Ness, G.C., Wiggins, L., and Zhao, Z. : Insulin increases hepatic 3-hydroxy-methylglutaryl coenzyme A reductase mRNA and immunoreactive protein levels in diabetic rats. Arch. Biochem. Biophys. 1 (1994), 193-194.

57. Scallen, T.J., and Sanghvi, A. : Regulation of three key enzymes in cholesterol metabolism by phosphorylation-dephosphorylation. Proc. Natl. Acad. Sci. USA 80 (1983), 2477-2480.

58. Denton, R.M., Brownsey, R.W., and Belsham, G.J. : A partial view of the mechanism of insulin action. Diabetologia 21 (1981), 347-362.

59. Berglund, L., Björkhem, I., Angelin, B., and Einarsson, K. : Evidence against *in vitro* modulation of rat liver cholesterol 7α-hydroxylase activity by phosphorylation-dephosphorylation: comparison with hydroxymethylglutaryl CoA reductase. Acta Chem. Scand. B. 40 (1986), 457-461.

60. Björkhem, I., Eggertsen, G., and Andersson, U. : On the mechanism of stimulation of cholesterol 7α-hydroxylase by dietary cholesterol. Biochim. Biophys. Acta 1085 (1991), 329-335.

61. Pandak, W.M., Li, Y.C., Chiang, J.Y.L., Studer, E.J., Gurley, E.C., Heuman, D.M., Vlahcevic, Z.R., and Hylemon, P.B. : Regulation of cholesterol 7α-hydroxylase mRNA and transcriptional activity by taurocholate and cholesterol in the chronic biliary diverted rat. J. Biol. Chem. 266 (1991), 3416-3421.

62. Shefer, S., Nguyen, L.B., Salen, G., Ness, G.C., Chowdhary, I.R., Lerner, S., Batta, A.K., and Tint, GS. : Differing effects of cholesterol and taurocholate on steady state hepatic HMG-CoA reductase and cholesterol 7α-hydroxylase activities and mRNA levels in the rat. J. Lipid Res. 33 (1992), 1193-1200.

63. Jones, M.P., Pandak, W.M., Heuman, D.M., Chiang, J.Y.L., Hylemon, P.B., and Vlahcevic, Z.R. : Cholesterol 7α-hydroxylase: evidence for transcriptional regulation by cholesterol or metabolic products of cholesterol in the rat. J. Lipid Res. 34 (1993), 885-892.

64. Gibbons, G.F., Björnsson, O.G., and Pullinger, C.R. : Evidence that changes in hepatic 3-hydroxy-3-

methylglutaryl coenzyme A reductase activity are required partly to maintain a constant rate of sterol synthesis. J. Biol. Chem. 259 (1984), 14399-14405.

65. Sample, C.E., and Ness, G.C. : Regulation of the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase by insulin. Biochem. Biophys. Res. Commun. 137 (1986), 201-207.

66. Hwa, J.J., Zollman, S., Warden, C.H., Taylor, B.A., Edwards, P.A., Fogelman, A.M., and Lusis, A.J. : Genetic and dietary interactions in the regulation of HMG-CoA reductase gene expression. J. Lipid Res. 33 (1992), 711-725.

67. Salter, A.M., Fischer, S.C., and Brindley, D.N. : Interactions of triiodothyronine, insulin and dexamethasone on the binding of human LDL to rat hepatocytes in monolayer culture. Atherosclerosis 71 (1988), 77-80.

68. Jensen, E., Florén, C.-H., and Nilsson, A. : Insulin stimulates the uptake of chylomicron remnants in cultured rat hepatocytes. Eur. J. Clin. Invest. 18 (1988), 226-232.

69. Durrington, P.N., Newton, R.S., Weinstein, D.B., and Steinberg, D. : Effects of insulin and glucose on very low density lipoprotein triglyceride secretion by cultured rat hepatocytes. J. Clin. Invest. 70 (1982), 63-73.

70. Patsch, W., Franz, S., and Schonfeld, G. : Role of insulin in lipoprotein secretion by cultured rat hepatocytes. J. Clin. Invest. 71 (1983), 1161-1174.

71. Sparks, J.D., Sparks, C.E., Bolognino, M., Roncone, A.M., Jackson, T.K., Amatruda, J.M. : Effects of nonketotic streptozotocin diabetes on apolipoprotein B synthesis and secretion by primary cultures of rat hepatocytes. J. Clin. Invest. 82 (1988), 37-43.

CHAPTER 6

Heterogenous expression of cholesterol 7α-hydroxylase and sterol 27-hydroxylase genes in the rat liver lobulus

Jaap Twisk[a], Marco F.M. Hoekman[b], Willem H. Mager[b], Antoon F.M. Moorman[c], Piet A.J. de Boer[c], Ludger Sheja[d], Hans M.G. Princen[a], and Rolf Gebhardt[d].

[a]Gaubius Laboratory, TNO-PG, P.O. Box 430, 2300 AK Leiden, [b]Vrije Universiteit and [c]Univ. of Amsterdam, Amsterdam, The Netherlands, and [d]Univ. of Tübingen, Tübingen, Germany

in press, J. Clinic. Invest. (1995)

ABSTRACT

We have investigated the lobular localisation and molecular level of expression of CYP7A (CYP7A) and sterol 27-hydroxylase, two key-enzymes in bile acid synthesis, in isolated periportal and pericentral hepatocytes, and by *in situ* hybridisation of rat liver. Enzyme activity, mRNA levels and transcription of CYP7A were predominant in pericentral hepatocytes of control rats, being 7.9-fold, 9.9-fold, and 4.4-fold higher than in periportal hepatocytes, respectively. A similar situation was found for sterol 27-hydroxylase; 2.9-fold, 2.5-fold, and 1.7-fold higher enzyme activity, mRNA levels, and gene transcription, respectively, in pericentral hepatocytes. Interruption of the enterohepatic circulation with colestid showed up-regulation of these parameters for both enzymes, as a result of stimulated gene expression mainly in the periportal zone. In contrast, mRNA levels and gene transcription of HMG-CoA reductase, showed opposite lobular distribution. Selective periportal expression for the latter was enhanced, but remained local, after colestid-treatment. *In situ* hydridisation showed unambiguously that CYP7A mRNA is located exclusively in the pericentral zone and that sterol 27-hydroxylase mRNA is expressed preferentially in the pericentral region, if less pronounced. Administration of colestid led to expression of both genes within a larger area of the liver lobulus. In conclusion, we suggest that CYP7A and sterol 27-hydroxylase are coordinately regulated by the bile acid gradient over the lobulus, resulting in predominant expression in the pericentral zone. Opposite lobular localisation of cholesterol and bile acid synthesis provides an alternative view on the interregulation of these metabolic pathways.

INTRODUCTION

The liver plays an important role in the homeostatic maintenance of a large number of nutrients in the blood, such as carbohydrates, amino acids and lipids, and is the main site of intermediary metabolism of these intermediates. It has become increasingly clear that not all hepatocytes contribute equally in this task. In contrast, contribution of hepatocytes to uptake, storage, interconversion and release of various compounds shows a large degree of heterogeneity along the portocentral axis, even up to a point that only a few cells are involved in a given function (1-4). The concept of "metabolic zonation" dictates that the heterogeneous expression of enzymes in the liver lobulus (or acinus) is a major determinant for the proper execution and regulation of various liver functions (5,6). Opposite metabolic pathways like gluconeogenesis and glycolysis are carried out simultaneously by hepatocytes in periportal and pericentral zones, respectively, and are separately localised within the liver (1,3). Importantly,

the distribution may change under different physiological and pathological conditions, in the sense that the liver may adapt to certain requirements by changes in distribution patterns. Liver-cell heterogeneity thus provides the basis for effective regulation and adaptation to different metabolic states.

It has previously been shown that cholesterol synthesis is predominantly localised in the periportal hepatocytes, as judged from the positive immunohistochemical staining for HMG-CoA synthase and HMG-CoA reductase protein in only 20% of the periportal cell fraction (7,8). Excretion of cholesterol into bile, either as free cholesterol or following its conversion into bile acids, is the predominant pathway for elimination of cholesterol from circulation in mammals (9,10). Rate of bile acid formation is therefore considered an important determinant for cholesterol homeostasis. On the other hand, bile acid synthesis and the major key-enzyme in routing of cholesterol to bile acids, CYP7A, are mainly localised pericentrally (11,12). Opposite lobular localisation of cholesterol synthetic and metabolic pathways poses the interesting question how the two are interregulated to achieve homeostasis.

Another aspect of bile acid synthesis concerns the different pathways of bile acid formation. According to current views, the initial and rate-determining step in routing of cholesterol to bile acids is catalysed by CYP7A (9). However, accumulating evidence has led to the suggestion that an alternative pathway exists, involving initial 27-hydroxylation of cholesterol via sterol 27-hydroxylase (13). Based both on *in vivo* studies in humans (13), and studies using cultured human and rat hepatocytes (14) it was concluded that this alternative pathway may contribute considerably to total bile acid synthesis (13,14). Hitherto, lobular expression of sterol 27-hydroxylase has not been assessed.

One of the major regulatory processes affecting bile acid biosynthesis is bile acid-induced feedback inhibition, which is exerted at the level of CYP7A by the flux of bile acids returning to the liver via portal blood (9). Bile acids are taken up efficiently by the periportal hepatocytes (15-19), thereby creating decreasing concentration gradients along the sinusoids (20). Consequently, periportal hepatocytes are exposed to a 6-fold higher concentration of bile acids, as compared with those in the pericentral area (15,16). In line with this, a concentration-dependent and direct down-regulation of bile acid synthesis, at the level of CYP7A, was found *in vitro* when cultured pig (21) and rat (22,23) hepatocytes were incubated with bile acids. In a recent paper, our group demonstrated that sterol 27-hydroxylase is regulated in parallel with CYP7A in cultured rat hepatocytes, resulting in coordinate down-regulation of both enzymes at the mRNA and transcriptional level by similar bile acids. Thus it was postulated that efficient down-regulation of bile acid synthesis by bile acids is accomplished by coordinate regulation

of both key-enzymes (24).

In the present study, we have assessed the distribution patterns for both CYP7A and sterol 27-hydroxylase within the liver lobulus. In addition, we have established the molecular level at which these expression patterns are imposed, by measuring mRNA levels in freshly isolated periportal and pericentral hepatocytes, and transcriptional activity levels for these enzymes using nuclear run-on assays. Dynamic aspects of lobular distribution of these cholesterol-metabolising enzymes, and relationship of their expression with lobular bile acid concentrations, were determined by treatment of rats with colestid. The bile-acid-sequestrant, like cholestyramine, has been shown to lead to up-regulation of bile acid synthesis (25), as a result of lowering the bile acid concentration in portal blood (26). Lobular distribution in control and stimulated rats was also assessed by *in situ* hybridisation of sections of rat liver.

The current study shows heterogeneous distribution of both CYP7A and sterol 27-hydroxylase. Heterogeneity resulted from coordinate differential transcriptional activity of both genes, and even more so of steady-state mRNA levels for the enzymes, residing primarily in the pericentral zone of the liver lobulus. The opposite situation was found for the HMG-CoA reductase gene. Treatment of rats with colestid led to a more overall recruitment of hepatocytes within the lobulus for bile acid synthetic capacity, resulting from increased expression of the CYP7A and sterol 27-hydroxylase gene within a large part of the liver lobule.

MATERIALS AND METHODS

Materials used for isolation of rat hepatocytes, determination of CYP7A and sterol 27-hydroxylase activity, and determination of mRNA and transcriptional activity levels, have been described previously (22,27-30).

Animals

Male Sprague-Dawley rats (200-280 gm) were used for isolation of hepatocytes. Animals were kept in a strictly controlled 12-hr light and dark cycle (lights on from 06:00-18:00 h) on standard chow (Alma H 1003, Botzenhardt, Kempten, F.R.G.) and tap water *ad libitum*. A separate group of animals was fed a similar diet supplemented with 5% (w/w) Colestid (Upjohn, Belgium), for a 7-day period prior to isolation (31). Time of isolation was between 08:00-9:00 h.

Isolation of hepatocytes

Total liver parenchymal cells were isolated by the two-step collagenase perfusion technique, modified as described (28). Periportal and pericentral subfractions of hepatocytes were isolated by the digitonin/collagenase perfusion technique, as described by Quistorff (32) and Lindros and Penttilä (33), with modifications described elsewhere (27). Viability, assessed by trypan blue exclusion, was higher than 90% and 80% for normal and isolated periportal and pericentral hepatocytes, respectively. Cell suspensions from digitonin/collagenase perfusions with a viability index of less than 70% were discarded.

The efficiency of enrichment of periportal and pericentral hepatocytes was monitored by measurements of glutamine synthetase, alanine aminotransferase and pyruvate kinase activity (Table 1).

Table 1. Activity of marker-enzymes in pericentral and periportal hepatocytes.

Enzyme	activity (nmoles/min per mg cell protein)		PC:PP ratio
	pericentral	*periportal*	
glutamine synthetase	632 ± 167 (6)	13 ± 6 (6)	48.6
alanine aminotransferase	189 ± 94 (6)	293 ± 105 (6)	0.65
pyruvate kinase	168 ± 72 (5)	137 ± 41 (4)	1.22

Pericentral and periportal hepatocytes were isolated as described in "materials and methods". Purity of hepatocyte preparations was assessed by determination of activities for various marker enzymes. Data are expressed as absolute values ± SD of enzyme activities, using hepatocytes from (n) rats, or as a ratio of PC:PP activity.

Enzyme assays

The activity of glutamine synthetase was determined by the glutamyltransferase assay with modifications reported previously (34). Alanine aminotransferase and pyruvate kinase were determined according to Bergmeyer (35). CYP7A and sterol 27-hydroxylase were assessed using homogenates as described in detail (14,29,30). Protein and cholesterol were assayed according to methods described (30).

RNA isolation, blotting and hybridisation procedures

Total RNA was isolated from whole livers or freshly isolated periportal and pericentral hepatocytes, and quantitation thereof was performed as previously described (22). Probes used in hybridisation experiments were labelled by the random-primer method (Mega-prime,

Amersham) to approximately 6 x 10^8 cpm/μg DNA. After hybridisation and washing, the filters were exposed to Hyperfilm MP (Amersham) together with an intensifying screen (Eastman-Kodak Co.) for 48-120 h at -80°C. For quantitation of the relative amounts of mRNA, the autoradiographs were scanned using a Shimadzu CS 910 chromatograph scanner, and areas under the curves were integrated using a data processor (Shimadzu Corp. Kyoto, Japan). The following DNA fragments were used as probes in hybridisation experiments: a 1.6 kb PCR-synthesized fragment of rat CYP7A cDNA, spanning the entire coding region as described in detail in ref. 22; a 1.6 kb *Hin*dIII/*Xba*I fragment of rat sterol 27-hydroxylase cDNA, kindly provided by Dr. Jerome Strauss (36), and isolated from a rat liver cDNA library using the rabbit sterol 27-hydroxylase cDNA, previously isolated by Russell and coworkers (37), as a probe; a 700 bp *Eco*RI fragment of hamster lithocholic acid 6β-hydroxylase cDNA (38); a 773 bp *Hin*dIII fragment of hamster HMG-CoA reductase cDNA (39); a 1.5 kb *Pst*I fragment of rat glutamine synthetase cDNA (40,41); a 1.2 kb *Pst*I fragment of hamster actin cDNA; and a 1.1 kb *Pst*I fragment of rat GAPDH. The latter two served as an internal standard to correct for differences in the amount of total RNA applied onto the gel or filter.

Nuclear run-on studies

Nuclear run-on studies were conducted essentially as described in ref. 22. Whole livers, or freshly isolated periportal and pericentral hepatocytes, from control and colestid-treated rats served as material for the isolation of nuclei. For whole liver preparations, livers were perfused with saline-solution (0.9% NaCl), cut into small fragments, washed and homogenised mechanically (10 strokes, 180 rpm) in NP40-lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP40, 1 mM PMSF, 1 mM DTT), at 4°C. The resulting suspension was filtered (100 μm) prior to an additional homogenizing step. Alternatively, directly after isolation, periportal and pericentral hepatocytes were washed and resuspended in NP40-lysis buffer. Both preparations were further homogenized, after being left on ice for 5 min, in a Potter Elvehjem tube with pestle B for 15 strokes at 4°C. Resulting nuclei were centrifuged at 500 x g and resuspended in NP40-lysis buffer. Washing procedures in NP40-lysis buffer were repeated until the nuclei were free of cellular debris. They were then taken up in glycerol storage buffer (50 mM Tris-HCl pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM PMSF, 5 mM DTT), counted, and aliquoted at approximately 2x 107/500 μl before being frozen at -80°C. RNA labelling and isolation was performed as described (22). Target DNA, being 5 μg of plasmid material containing cDNA sequences of rat CYP7A, rat sterol 27-hydroxylase, hamster HMG-CoA reductase, hamster lithocholic acid

6β-hydroxylase, hamster actin and rat GAPDH, or the empty vector pUC19, were slotblotted onto strips of Hybond-N^+ filter (Amersham) and crosslinked. The filters were hybridised with the [^{32}P]-labelled RNA for 36 hours, washed and exposed to Hyperfilm MP (Amersham) for 2-5 days. Quantitation of relative amounts of mRNA synthesized was conducted using a Phosphor-imager 400B (Molecular Dynamics).

In situ hybridisation experiments

Liver-tissue was fixed in 4% formaldehyde-solution and quickly frozen in liquid Freon 22, for preparation of sections (41). The *in situ* hybridisation experiments were performed on closely adjacent sections to allow easy comparison of the patterns of hybridisation. [^{35}S]-Labelled probes for *in situ* hybridisation were prepared using the multiprime DNA labeling method, to a specific activity of 5-10 x 10^8 cpm/μg of DNA. Prehybridisation treatments, hybridisation and autoradiography were carried out precisely as described previously (41,42). Negative controls included RNase-treated sections, and hybridisations with an empty vector (pBR322).

RESULTS

Heterogeneous distribution of CYP7A and sterol 27-hydroxylase

Pericentral and periportal hepatocytes were isolated by digitonin/collagenase perfusion as described (43), and purity of the different preparations was determined in terms of enrichment of specific marker enzymes known to be differentially expressed. Table 1 shows strong predominant expression of glutamine synthetase in the pericentral fraction (pericentral (PC): periportal (PP) ratio being 48.6), in agreement with expression of this enzyme within the most distal pericentral hepatocytes surrounding the central venules of rat liver (43,44). Other marker enzymes, alanine aminotransferase and pyruvate kinase, showed PC:PP ratio's of 0.65 and 1.22, respectively, in agreement with data reported by others (33). Taken together, the perfusion technique allows for a good separation of hepatocytes into a pericentral and periportal fraction, keeping differential expression of several known marker enzymes intact.
Northern-blotting of total RNA isolated from periportal and pericentral hepatocytes (Fig.1) shows the typical expression pattern for CYP7A in both cell preparations (mRNAs 2.1, 3.6, and 4.0 kb in size, as reported before (22,45,46)). Clearly, expression of cholesterol 7α-hydroxylase mRNA is particularly strong in the pericentral area, as is that of glutamine

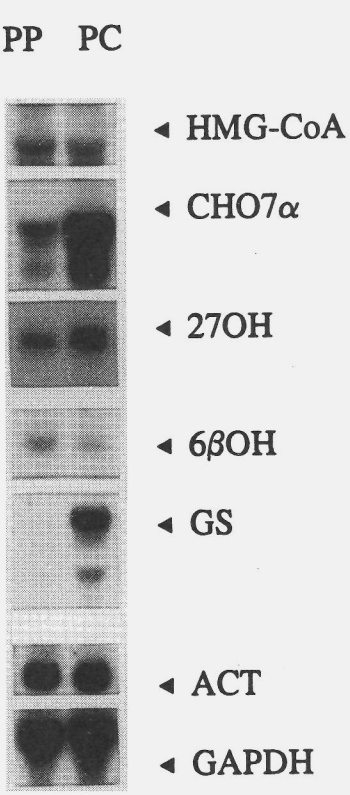
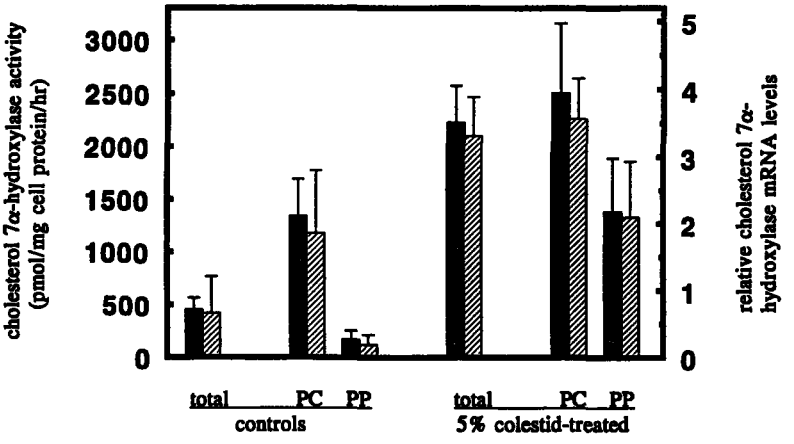


Figure 1. Distribution patterns for cholesterol 7α-hydroxylase and sterol 27-hydroxylase mRNA in periportal and pericentral hepatocytes. Periportal (PP) and pericentral (PC) hepatocytes were isolated by digitonin/collagenase perfusion, as described in "material and methods". Immediately after isolation of hepatocytes, total RNA was isolated for mRNA analysis. 10 μg of total RNA was electrophoresed in a 0.8% agarose/1 M formaldehyde gel, transferred to Hybond-N^+, and subsequently hybridized with [^{32}P]-labelled cDNA probes for HMG-CoA reductase (HMG-CoA), cholesterol 7α-hydroxylase (CHO7α), sterol 27-hydroxylase (27OH), lithocholic acid 6β-hydroxylase (6βOH), glutamine synthetase (GS), β-actin (ACT), and GAPDH. The latter two served as an internal standard. Resulting filters were subjected to autoradiography for 2-5 days, and specifically for detection of HMG-CoA reductase mRNA: 2-3 weeks.

synthetase (two mRNAs of 1.6 and 2.8 kb, as described (47)), assessed for reasons of comparison. The latter is in agreement with exclusive expression of both mRNA and protein (41,47) in a very limited fraction of pericentral cells. Sterol 27-hydroxylase (2.4 kb in rat liver) (24,36) is expressed less abundantly in rat liver, as compared with the messengers describedin the above. Nevertheless, this mRNA was also localised predominantly pericentrally. Figure 2

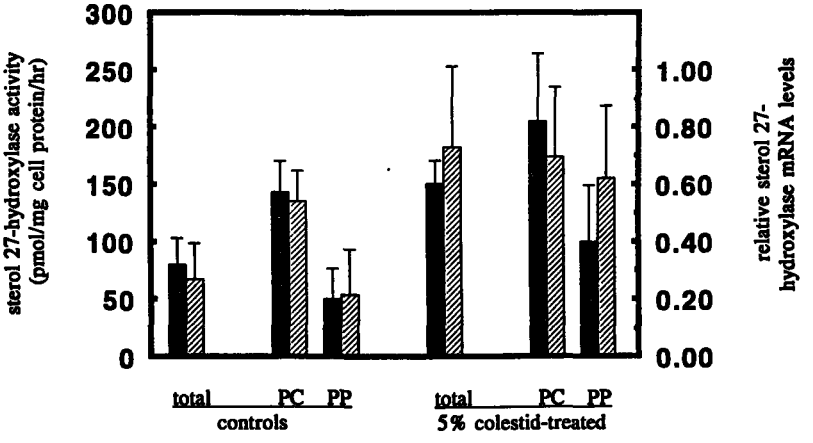
Figure 2. Distribution patterns for cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity and mRNA levels in isolated pericentral and periportal hepatocytes from control- and colestid- treated rats. Enzyme activities were assessed as described in Materials and Methods. Northern experiments were performed as in the legends to Fig.1. Rats used for hepatocyte preparations were either fed normal chow, or a diet supplemented with 5% colestid (w/w). Values are expressed in terms of absolute enzyme activity (solid bars), or in arbitrary units of mRNA relative to expression of β-actin (hatched bars), and are means (±SD) using hepatocytes from 4-7 rats. PC:PP ratio's are indicated at the bottom of the figure, as is the extent of stimulation in total hepatocyte preparations by the colestid-treatment. **(A)** cholesterol 7α-hydroxylase (CYP7A); **(B)** sterol 27-hydroxylase.

A.



	PC:PP-ratio		stimulation by 5% colestid
	controls	5% colestid	
activity	7.9	1.8	4.9x
mRNA-levels	9.9	1.7	5.0x

B.



	PC:PP-ratio		stimulation by 5% colestid
	controls	5% colestid	
activity	2.9	1.9	1.9x
mRNA-levels	2.5	1.1	2.7x

summarizes the distribution of CYP7A and sterol 27-hydroxylase activity and mRNA levels, the latter relative to β-actin mRNA. β-Actin mRNA was used as an internal standard, and did not vary between different cell-preparations, neither from control (Fig.1) -nor from colestid-treated rats (data not shown). Both CYP7A activity and mRNA levels are predominant in hepatocytes from the pericentral area, showing respective PC:PP-ratio's of 7.9 and 9.9 (Fig. 2a). For sterol 27-hydroxylase, heterogeneity of expression is less extreme, showing a 2.9 and 2.5-fold higher activity and mRNA-level, respectively, within the pericentral hepatocytes (Fig. 2b).

In view of reports on the periportal localisation of HMG-CoA reductase, a key-enzyme in the cholesterol biosynthetic route (7,8), it was of interest to assess the relative mRNA levels for this enzyme within the liver lobulus as well, thus acting as an additional internal control for the identity of periportal hepatocytes. HMG-CoA reductase mRNA levels were very low, both in periportal and pericentral hepatocytes, as indicated by the longer exposure time required (legend to Fig. 1). Low levels of HMG-CoA reductase mRNA in livers of control rats were reported by others (48,49) as well. Nevertheless, a mean PC:PP-ratio of 0.5 (n=3) was detected, in agreement with positive immunohistochemical staining for the HMG-CoA reductase protein of hepatocytes located in the periportal zone (7,8).

Expression of lithocholic acid 6β-hydroxylase, primarily involved in metabolism of secondary bile acids returning to the liver via portal blood (*i.e.* lithocholic acid; ref. 38), was found preferentially in the periportal zone (Fig. 1; PC:PP-ratio of 0.4, n=3). The latter indicates that not all mRNAs of enzymes involved in bile acid biosynthesis are similarly localised.

Effect of colestid-treatment of rats on the heterogeneity of mRNA patterns for different enzymes

Rats were treated with 5% colestid for 7 days prior to isolation of hepatocytes. The bile-acid-sequestering property of this agent, like that of cholestyramine, leads to up-regulation of bile acid synthesis in man (25) and rat (11,12), as a result of diminished bile acid concentrations in portal blood (26). Assessment of activity for marker enzymes, within different hepatocyte preparations from these rats, revealed PC:PP ratio's similar to those found in control rats (data not shown). As shown in Fig. 2a, feeding rats a colestid-supplemented diet resulted in overall stimulation of both enzyme activity and mRNA levels for CYP7A. The strong increase in CYP7A activity (4.9-fold) and mRNA levels (5.0-fold) in whole liver preparations of stimulated rats is mainly due to stimulation of both parameters in the periportal hepatocytes. Levels for CYP7A activity within this zone rose from 170 ± 81 to 1386 ± 504 pmol/hr per mg

of cell protein (8.2-fold), as did mRNA levels for this enzyme (11.1-fold), while both parameters were only stimulated 1.9-fold in the pericentral hepatocytes. Consequently, PC:PP-ratio's for CYP7A activity and mRNA were lowered to 1.8 and 1.7, respectively, in livers of stimulated rats.

Sterol 27-hydroxylase activity and mRNA (Fig. 2b) were also stimulated in colestid-treated rats (1.9-fold and 2.7-fold, respectively, as compared with control rats), be it less pronounced. Sterol 27-hydroxylase mRNA was specifically up-regulated in the portal zone (2.9-fold), thereby lowering the PC:PP-ratio from 2.5 in control rats to 1.1 in colestid-treated animals. Sterol 27-hydroxylase activity was mildly stimulated in hepatocytes from both zones, particularly in the portal fraction (2-fold).

HMG-CoA reductase mRNA levels were also increased in colestid-treated rats (4.5-fold, data not shown), in agreement with up-regulation of mRNA (48,49) and activity levels (49,50) for this enzyme by bile acid sequestrants. In contrast to CYP7A, however, this particular increase is not a result of overall expression of the messenger in livers from stimulated rats, but rather of selective up-regulation in the portal zone. HMG-CoA reductase mRNA was stimulated 7.8-fold in the portal region, and only 2.7-fold in the pericentral area. The PC:PP-ratio for mRNA of this enzyme was hence lowered even further, from 0.5 in control livers to 0.1 in livers from colestid-treated rats.

Transcriptional activity of the CYP7A and sterol 27-hydroxylase genes in different zones of the liver lobulus

Nuclei from freshly isolated pericentral and periportal hepatocytes were used in nuclear run-on assays. Fig. 3 shows a typical autoradiograph of a hybridisation experiment, in which [^{32}P]-labelled RNA from pericentral and periportal nuclei was hybridised to cDNAs specific for CYP7A, HMG-CoA reductase, sterol 27-hydroxylase, and lithocholic acid 6β-hydroxylase. As internal standards, transcriptional activities of β-actin and GAPDH genes were also assessed in these nuclear preparations. While no difference was found in expression patterns of the latter two, expression of the CYP7A gene is clearly pericentral (PC:PP-ratio amounting to 4.4, Fig. 4a). Similarly, transcriptional activity of the sterol 27-hydroxylase gene was found to be highest in the pericentral region (PC:PP-ratio of 1.7). In contrast, HMG-CoA reductase gene expression was preferentially localised in periportal cells (PC:PP-ratio of 0.7), in agreement with opposite lobular localisation of mRNA for this enzyme. The overall transcriptional activity is high for HMG-CoA reductase, reaching levels somewhat lower than

expression of β-actin, and GAPDH, indicating that posttranscriptional processes may be responsible for the low mRNA levels observed for this enzyme. Lithocholic acid 6β-hydroxylase gene expression was also localised predominantly periportally (PC:PP-ratio being 0.4), in agreement with expression of mRNA levels for this enzyme (Fig. 1).

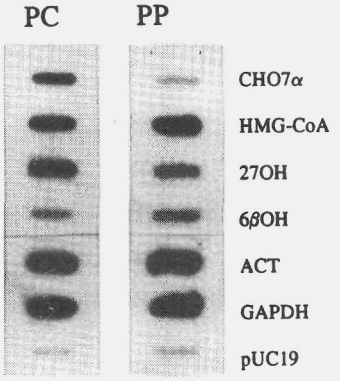
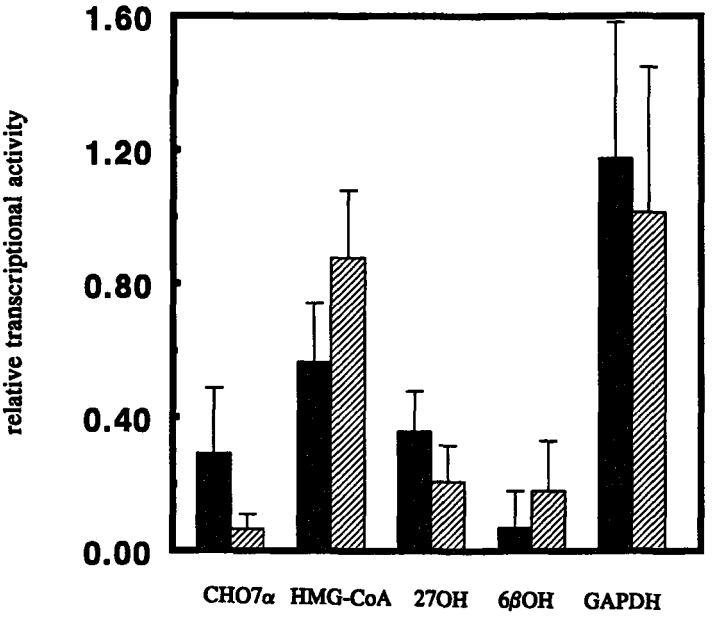


Figure 3. Transcriptional activity of the cholesterol 7α-hydroxylase and sterol 27-hydroxylase genes in isolated pericentral and periportal hepatocytes. Nuclei were prepared from freshly isolated pericentral (PC) and periportal (PP) hepatocytes, as described in "materials and methods". [^{32}P]-labelled total RNA was synthesized *in vitro* using these nuclear preparations, and hybridized to different cDNA-probes. Resulting filters were subjected to autoradiography. Probes used were cDNAs for cholesterol 7α-hydroxylase (CHO7α), HMG-CoA reductase (HMG-CoA), sterol 27-hydroxylase (27OH), lithocholic acid 6β-hydroxylase (6βOH), β-actin (ACT), and GAPDH. The latter two served as internal standards. Non-specific hybridization was checked using an empty vector (pUC19).

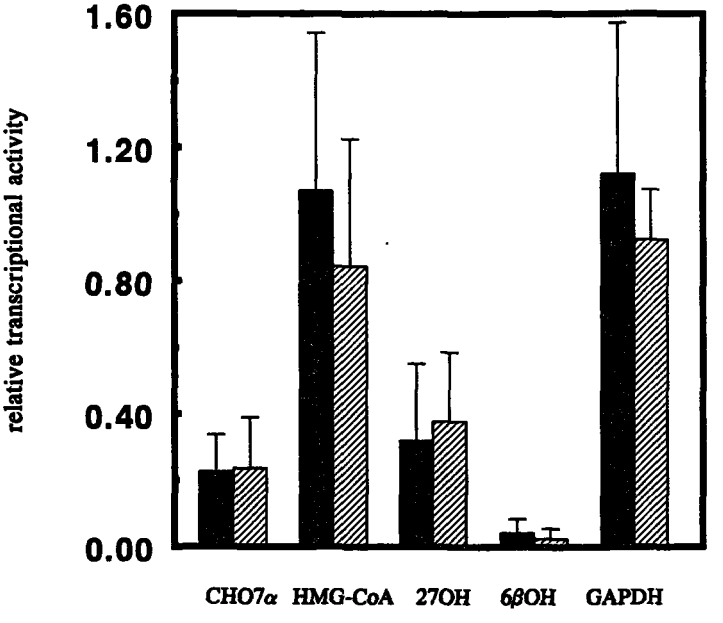
Treatment of rats with 5% colestid resulted in enhanced transcriptional activity in whole liver preparations of the CYP7A (3.6-fold), sterol 27-hydroxylase (2.2-fold) and HMG-CoA reductase (5.5-fold) genes. In contrast, lithocholic acid 6β-hydroxylase gene transcription was down-regulated (3.6-fold), while house-keeping genes β-actin and GAPDH were not affected by colestid-treatment. Analysis of gene expression in isolated pericentral and periportal cells from stimulated rats (Fig. 4b) showed marked expression of CYP7A and sterol 27-hydroxylase genes over the entire portocentral axis. Up-regulation of transcriptional activity in the pericentral zone only was found for the HMG-CoA reductase gene, while gene expression for lithocholic acid 6β-hydroxylase was specifically down-regulated in the portal zone.

Figure 4. Transcriptional activity of cholesterol 7α-hydroxylase and sterol 27-hydroxylase genes in isolated pericentral and periportal hepatocytes from control- and colestid- treated rats. Nuclear extracts were prepared from control rats and animals fed chow supplemented with 5% colestid (w/w), and run-on assays were performed as desribed in 'Materials and Methods'. The level of transcriptional activity was calculated relative to expression of β-actin, used as an internal standard, and presented as a means (±SD) using hepatocytes from 4-7 rats. Freshly isolated pericentral (solid bars) and periportal (hatched bars) hepatocytes served as material for the isolation of nuclei. **(A)** control rats; **(B)** colestid-treated rats. PC:PP ratio's are indicated at the bottom of the figure.

A.



B.



	PC:PP-ratio				
controls	4.4	0.7	1.7	0.4	1.2
5% colestid	1.0	1.3	0.9	1.8	1.2

In situ hybridisation of livers from control and colestid-treated rats

Fig. 5 shows the heterogeneous expression of CYP7A and sterol 27-hydroxylase mRNA, as detected by *in situ* hybridisation of liver sections from control and colestid-treated rats. CYP7A (Fig. 5a) is allmost exclusively and abundantly expressed in a limited fraction of hepatocytes surrounding the terminal venules. Particular pericentral expression was also detected for glutamine synthetase (Fig. 5c), as reported previously (41,47), and assessed for reasons of comparison and positive zonal identification. *In situ* hybridisation of control livers with the rat sterol 27-hydroxylase cDNA-probe also showed positive staining of pericentral hepatocytes only, be it far less abundantly, and less discretely, in agreement with mRNA analysis shown previously for this enzyme (Fig. 2b). Rather, a slight gradient of weak positive staining, declining towards the periportal zone, was found (Fig. 5b).

Treatment with colestid caused a more abundant expression of CYP7A mRNA throughout a large section of the lobulus (Fig. 5d), concomitant with observed expression patterns of CYP7A activity and mRNA. Sterol 27-hydroxylase mRNA showed a similar, but less marked up-regulation by colestid-treatment, resulting in positive staining of a large fraction of hepatocytes within each lobular unit (Fig. 5e). Glutamine synthetase showed no colestid-induced effect, and remained pericentrally localised (Fig. 5f). The latter agrees well with reported rigid heterogeneity of this enzyme in rat liver (51,52).

DISCUSSION

The current study shows that key-enzymes involved in bile acid biosynthesis, CYP7A and sterol 27-hydroxylase, predominate in the pericentral area of the rat liver lobulus. Under normal feeding conditions, the distribution of both enzymes is accomplished by parallel expression of mRNA levels and transcriptional activity of the corresponding genes. The localisation is dynamic, and responds to reduced portal bile acid concentrations after colestid-treatment by changing distribution patterns for transcriptional activities, and particularly

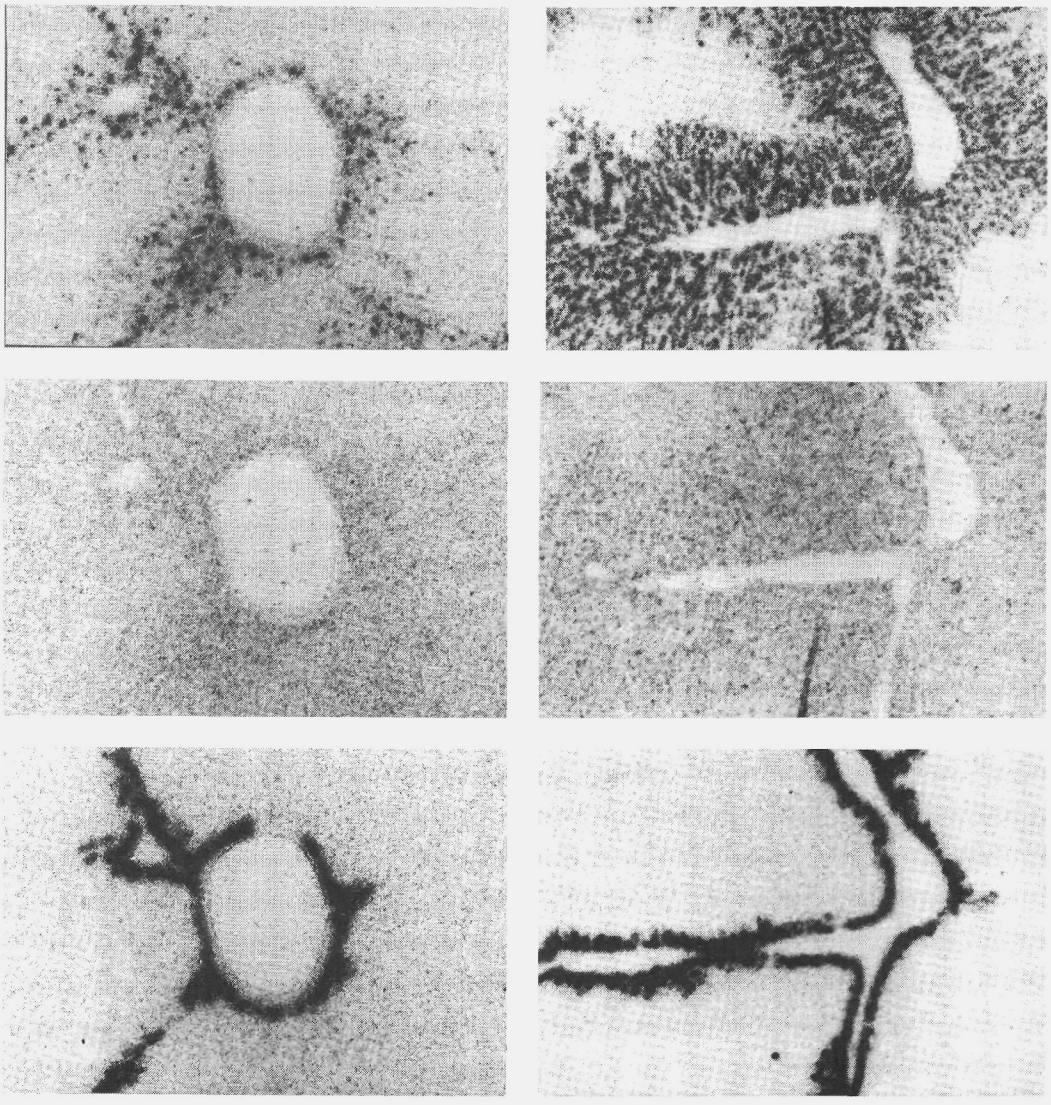
Figure 5. Localisation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase mRNA on serial liver sections by *in situ* hybridisation. Liver sections were made from control (a-c) and colestid-treated rats (d-f), as described in 'Materials and Methods'. The hybridisation experiments were performed using (^{35}S)-labelled probes for cholesterol 7α-hydroxylase (a and d), sterol 27-hydroxylase (b and e) and glutamine synthetase (c and f). The latter was assessed as a positive identification of the pericentral zone. Sections depicted are of a central vein with surrounding hepatocytes.

CONTROL

COLESTID

a-c

d-f



mRNA levels of these enzymes, to a more overall expression, resulting in recruitment of a larger portion of hepatocytes within the liver lobulus for bile acid synthetic purposes.

Parallel lobular distribution of CYP7A and sterol 27-hydroxylase.

A strong preferential expression of CYP7A activity, mRNA and gene transcription was detected within the pericentral zone (PC:PP-ratio's of 7.9, 9.9 and 4.4, respectively). These results provide the molecular basis for the reported predominantly pericentral expression of bile acid synthesis and CYP7A activity (11,12). In addition, the present data show that enzyme activity and mRNA level for sterol 27-hydroxylase are also higher in the pericentral zone (PC:PP-ratio's of 2.9 and 2.5, respectively), as a result of a higher transcriptional activity of the corresponding gene (1.7-fold) in this area. The latter enzyme is involved in alternative routing to bile acids via initial 27-hydroxylation of cholesterol, a pathway which has been shown to contribute considerably to bile acid synthesis, both *in vivo* in man (13) and in cultured human and rat hepatocytes (14).

Coordinate bile acid-induced feedback of bile acid synthetic enzymes.

Allmost nothing is known about regulation of sterol 27-hydroxylase. It has been suggested that the enzyme is of minor importance for regulation of bile acid synthesis and composition of bile acids formed in rat (53). However, recent *in vitro* studies showed that sterol 27-hydroxylase may be regulated by factors known to also affect CYP7A. Both enzymes were inhibited to a similar extent by bile acids (24) and insulin (54). Thus it is conceivable that the alternative pathway is also subject to regulation *in vivo*, and in maintenance of cholesterol homeostasis. The co-localised expression of both CYP7A and sterol 27-hydroxylase activities and mRNA levels, further confirmed with *in situ* hybridisation experiments, strongly suggests the involvement of both enzymes in determining expression of total bile acid synthesis within the lobulus.

The data presented support the concept that bile acid synthesis *in vivo* is regulated by the flux of bile acids returning to the liver through enterohepatic circulation. Allthough in principle all hepatocytes have equal uptake capacity for bile acids (19,55), the microanatomy of the liver lobule results in a lobular concentration gradient during blood-flow through the liver (15,16,55). We have suggested that high bile acid concentrations are responsible for major down-regulation of bile acid synthesis specifically in the portal area, whereas the route is hardly affected in the pericentral zone (11,12,56). A relationship between portal bile acid concentrations and bile acid biosynthetic capacity of hepatocytes has been assumed previously

(57,58). Further evidence for direct regulation of both CYP7A and sterol 27-hydroxylase by bile acids was obtained recently with *in vitro* studies, showing a concentration-dependent down-regulation of enzyme activities and mRNA by bile acids in cultured rat hepatocytes, manifest at the transcriptional level (24).

Treatment of rats with bile acid sequestrants has been shown to result in lowering of the bile acid gradient over the liver lobulus, as a consequence of diminished bile acid concentrations in portal blood (26). Subsequent up-regulation of bile acid synthesis as a result of colestid-treatment may be caused by a reduced down-regulation within the lobulus (11,12). In addition, as a result of diversion of bile acids in this way, loss of zonal heterogeneity of bile acid excretion and cell polarity, in terms of cellular translocation of bile acids has been demonstrated (67). The current study shows that stimulation of bile acid synthesis by colestid-treatment may result from de-repression of transcriptional activity of CYP7A and sterol 27-hydroxylase genes, and particularly mRNA levels of these enzymes, in the portal zone. The latter results provide further evidence that bile acids are directly involved in inducing distribution patterns for CYP7A and sterol 27-hydroxylase within the liver lobulus. Furthermore, the colestid-treatment shows that heterogeneity for these enzymes is not rigid, but responds to changing metabolic requirements.

The molecular level of regulation by bile acids.

The heterogeneous expression of mRNA levels for both enzymes is induced at the level of gene transcription. Nevertheless, allthough relative levels for sterol 27-hydroxylase mRNA and transciptional activity within the lobulus are closely linked, the stronger PC:PP-ratio for CYP7A mRNA, as compared with transcriptional activity of the gene (PC:PP-ratio's of 9.9 and 4.4, respectively), suggests that the synthesis and the amount of mRNA for this enzyme may be regulated at different levels. The distinct expression of CYP7A mRNA in only a few pericentral cells (Fig. 5a), whereas transcriptional activity for this gene is low, though not absent, in the periportal region, substantiates this view. This becomes even more apparent after colestid-treatment, resulting in up-regulated levels of CYP7A mRNA in both zones of the lobulus (11-fold and 2-fold, repectively, for PP and PC-zones), while the transcriptional activity of the gene is affected only in the portal area, and to a modest degree (2-fold). In line with this thought, it has been suggested that stability of the CYP7A messenger is an important determinant of steady-state mRNA levels for this enzyme with respect to regulation by bile acids (22,60). AU-rich sequences present in the 3'-noncoding region of CYP7A mRNA may be involved in such a regulatory scheme (61).

mRNA levels and transcriptional activity analysis for lithocholic acid 6β-hydroxylase and HMG-CoA reductase showed that not all enzymes involved in maintenance of cholesterol homeostasis are co-localised. Portal expression of lithocholic acid 6β-hydroxylase is conceivable in view of involvement of this enzyme in metabolism of secondary bile acids. Periportal hepatocytes, subject to the highest bile acid concentrations, are expected to be most active in conversion of lithocholic acid to murideoxycholic acid. Additionally, it has been demonstrated that the enzyme is up-regulated by feeding bile acids to hamsters (38). In agreement with this finding, we have shown that expression of the enzyme can be down-regulated at the level of transcription, by diversion of bile acids from the circulation (3.6-fold in whole liver preparations, and Fig. 4a and b). Consequently, high expression of lithocholic acid 6β-hydroxylase in the portal area, where blood rich in bile acids enters the liver, may reflect a protective mechanism of the liver to minimise hepatotoxic effects.

The link between cholesterol synthesis and bile acid formation.

Opposite lobular localisation of mRNA and transcriptional activity for HMG-CoA reductase is interesting, since it poses questions regarding interregulation of cholesterol synthetic- and bile acid synthetic routes, and how each may contribute to liver cholesterol homeostasis. Specifically with regard to functional pools of cholesterol contributing to bile acid formation, controversy exists. It has been postulated that newly synthesized cholesterol is the preferred substrate for CYP7A (62,63). Based on the results presented in this and other reports (7,8,11,12), enzymes involved in *de novo* cholesterol synthesis and bile acid synthesis are strictly separated, and therefore newly-synthesized cholesterol can not be the preferred substrate under normal physiological circumstances. In line with this assumption, Robins and coworkers have shown that liver-synthesized cholesterol is preferentially secreted into bile, without being metabolised (64). Furthermore, Scheibner and coworkers recently demonstrated that the bulk of bile acids synthesized in the first short period following bile duct ligation originates from preformed cholesterol (65).

Stimulation of *de novo* cholesterol synthesis in control rats by treatment with mevalonate did not result in an effect on CYP7A, while HMG-CoA reductase activity was profoundly inhibited (66). Similarly, administration of HMG-CoA reductase inhibitor to hyper-cholesterolemic patients had no impact on synthesis of acidic sterols in bile (67), nor did administration to gallstone patients have any effect on CYP7A activity (68). These data demonstrate that under normal circumstances, manipulation of the *de novo* cholesterol pool does not result in alteration of bile acid synthesis levels.

In this and previous studies (11,12), we have shown that treatment of rats with colestid leads to expansion of bile acid synthetic capacity within the lobulus. These results indicate a direct linkage between cholesterol synthesis and bile acid synthesis, as a result of diversion of bile acids, and may explain the increased use of *de novo* cholesterol under these circumstances (63,65,69,70). Interestingly, HMG-CoA reductase mRNA, though up-regulated by colestid, remained predominantly periportally localised. In agreement with these findings, it has been shown that activity of HMG-CoA reductase remains periportal after treatment of rats with cholestyramine, but that expression of the enzyme up to the pericentral area is accomplished when this treatment is combined with mevinolin-administration to rats (7,8).

In conclusion, the present study demonstrates that heterogeneous localisation of bile acid synthesis is accomplished by preferential transcriptional activity and mRNA stability for key-enzymes for this route, CYP7A and sterol 27-hydroxylase, in the pericentral zone of the liver lobulus. Co-localisation of the two enzymes provides insights on how feedback regulation of bile acid synthesis by bile acids is achieved, and how the total cholesterol pool within the liver is regulated to meet different metabolic demands. The concept of metabolic zonation provides the basis for this understanding, and may shed further light on interregulation of pathways involved in maintainance of cholesterol homeostasis in the liver.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Netherlands Heart Foundation (grant 89.079 to Jaap Twisk), and from HGO-TNO (grant 900-523-138 to Marco F.M. Hoekman). The authors wish to thank Miss Marisa Horsting for secretarial assistance.

REFERENCES

1. Jungermann, K., and Katz, N. : Functional hepatocellular heterogeneity. Hepatology 2 (1982), 385-395.
2. Gumucio, J.J., and Miller, D.L. : Liver cell heterogeneity. In: The Liver: Biology and Pathology. Arias, I., Popper, H., Schachter, D., and Shafritz, D.A., eds,. Raven Press, New York (1982), 747-661.
3. Jungermann, K., and Katz, N. : Functional specialization of different hepatocyte populations. Physiol. Rev. 69 (1989), 708-764.
4. Gebhardt, R. : Metabolic zonation of the liver: regulation and implications for liver function. Pharmac. Ther. 53 (1992), 275-354.
5. Jungermann, K., and Sasse, D. : Heterogeneity of liver parenchymal cells. Trends Biochem. Sci. 3 (1978), 198-202.
6. Lamers, W., Hilberts, A., Furt, E., Smith, J., Jonges, G.N., Van Noorden, C.J.F., Gaasbeek, L., Janzen, J.W., Charles, R., and Moorman, A.F.M. : Hepatic enzymatic zonation: A reevaluation of the concept of the liver acinus. Hepatology 10 (1989), 72-76 .
7. Singer, I.I., Kawka, D.W., Kazazis, D.M., Alberts, A.W., Chen, J.S., Huff, J.W., and Ness, G.C. : Hydroxymethylglutaryl-CoA reductase-containing hepatocytes are distributed periportally in normal and mevinolone treated rat livers. Proc. Natl. Acad. Sci. USA 81 (1984), 5556-5560.
8. Li, A.C., Tanaka, R.D., Callaway, K., Fogelman, A.M., and Edwards, P.A. : Localization of 3 hydroxy-3-methylglutaryl CoA reductase and 3-hydroxy-3-methylglutaryl CoA synthase in the rat liver and intestine is affected by cholestyramine and mevinoline. J. Lipid Res. 29 (1988), 781-796.
9. Björkhem, I. : Mechanisms of bile acid biosynthesis in mammalian liver. In: Sterols and bile acids. Danielsson, H., and Sjövall, J., eds., Elsevier, Amsterdam (1985), 231-278.
10. Carey, M.C., and Cahalane, M.J. : Enterohepatic circulation. In: The Liver: Biology and Pathology. Arias, I.M., Jakoby, W.B., Popper, H., Schachter, D., and Shafritz, D.A., eds., Raven Press, New York (1988), 573-616.
11. Ugele, B., Kempen, H.J.M., Gebhardt, R., Meijer, P., Burger, H.-J., and Princen, H.M.G. : Heterogeneous distribution of cholesterol 7α-hydroxylase among periportal and perivenous hepatocytes. In: Trends in Bile Acid Research. Paumgartner, G., Stiehl, A., and Gerok, W., eds., Academic Publishers, Dordrecht, Boston, London (1988), 53-61.
12. Ugele, B., Kempen, H.J.M., Gebhardt, R., Meijer, P., Burger, H.-J., and Princen, H.M.G. : Heterogeneity of rat liver parenchyma in cholesterol 7α-hydroxylase and bile acid synthesis. Biochem. J. 276 (1991), 73-77.

13. Axelsson, M., and Sjövall, J. : Potential bile acid precursors in plasma-possible indicators of biosynthetic pathways to cholic and chenodeoxycholic acids in man. J. steroid Biochem. 36 (1990), 631-640.

14. Princen, H.M.G., Meijer, P., Wolthers, B.G., Vonk, R.J., and Kuipers, F. : Cyclosporine A blocks bile acid synthesis in cultured hepatocytes by specific inhibition of chenodeoxycholic acid synthesis. Biochem. J. 275 (1991), 501-505.

15. Jones, A.L., Hradek, G.T., Renston, R.H., Wong, K.Y., Karlaganis, G., and Paumgartner, G. : Autoradiographic evidence for hepatic acinar concentration gradient of bile acid derivatives. Am. J. Physiol. 238 (1980), 233-237.

16. Groothuis, G., Hardonk, M.J., Keulemans, K.P.T., Miervenhuis, P., and Meijer, D.K.F.: Autoradiographic and kinetic demonstration of acinar heterogeneity of taurocholate transport. Am. J. Physiol. 243 (1982),455-462.

17. Buscher, H.P., Gerok, W., Kurz, G., and Schneider, S. : Visualization of bile acid transport with fluorescent derivative. In: Enterohepatic Circulation of Bile Acids and Sterol Metabolism. Paumgartner, G., Stiehl, A., and Gerok, W., eds., MTP Press, Lancaster, UK (1985), 243-247.

18. Sherman, I.A., and Fischer, M.M. : Hepatic transport of fluorescent molecules: *in vivo* studies using intravital TV microscopy. Hepatology 6 (1986), 444-449.

19. Ugele, B., M. Locher, H.-J. Burger, and R. Gebhardt (1986) Is there a heterogeneity of liver parenchyma in taurocholate uptake ? In: Bile Acids and the Liver. G. Paumgartner, A. Stiehl, and W. Gerok, eds., MTP Press Limited, Lancaster, Boston; The Hague, Dordrecht. 153-160.

20. Goresky, C.A. : Uptake in the liver: the nature of the process. In: Liver and Biliary Tract Physiology I. Javitt, N.B., ed., University Park, Baltimore (1980). 65-101.

21. Kwekkeboom, J., H.M.G. Princen, E.M. van Voorthuizen, and H.J.M. Kempen (1990) Bile acids exert negative feedback control on bile acid synthesis in cultured pig hepatocytes by suppression of cholesterol 7α-hydroxylase activity. Hepatology 12: 1209-1215.

22. Twisk, J., E.M. Lehmann, and H.M.G. Princen (1993) Differential feedback regulation of cholesterol 7α-hydroxylase mRNA and transcriptional activity by rat bile acids in primary monolayer cultures of rat hepatocytes. Biochem. J. 290: 685-691.

23. Stravitz, R.T., P.B. Hylemon, D.M. Heuman, L.R. Hagey, C.D. Schteingart, H.-T. Ton-Nu, A.F. Hofmann, and Z.R. Vlahcevic (1993) Transcriptional regulation of cholesterol 7α-hydroxylase mRNA by conjugated bile acids in primary cultures of rat hepatocytes. J. Biol. Chem. 268: 13987-13993.

24. Twisk, J., E. de Wit, and H.M.G. Princen (1994) Suppression of sterol 27-hydroxylase mRNA and transcriptional activity by bile acids in cultured rat hepatocytes. Biochem. J. in press.

25. Fears, R. (1985) Pharmacological control of cholesterol 7α-hydroxylase activity. In: cholesterol 7α-

hydroxylase (7α-monooxygenase). R. Fears, and J.R. Sabine, editors. CRC Press, Boca Raton, Florida. 115-132.

26. Botham, K.M., M.E. Lawson, G.J. Beckett, I.W. Percy-Robb, and G.S. Boyd (1981) Portal blood concentrations of conjugated cholic and chenodeoxycholic acids relationship to bile salt synthesis in liver cells. Biochim. Biophys. Acta. 665: 81-87.

27. Burger, H.J., R. Gebhardt, C. Mayer, and D. Mecke (1989) Different capacities for amino acid transport in periportal and perivenous hepatocytes isolated by digitonin/collagenase perfusion. Hepatology 9: 22-28.

28. Gebhardt, R., and W. Jung (1982) Biliary secretion of sodium fluorescein in primary cultures of adult rat hepatocytes and its stimulation by nicotinamide. J. Cell. Sci. 56: 233-244.

29. Princen, H.M.G., C.M.G. Huijsmans, F. Kuipers, R.J. Vonk, and H.J.M. Kempen (1986) Ketoconazole blocks bile acid synthesis in hepatocyte monolayer cultures and *in vivo* in rat by inhibiting cholesterol 7α-hydroxylase. J. Clin. Invest. 78: 1064-1071

30. Princen, H.M.G., P. Meijer, J. Kwekkeboom, and H.J.M. Kempen (1988) Assay of cholesterol 7α-hydroxylase activity in rat hepatocytes in primary monolayer culture. Anal. Biochem. 171: 158-165.

31. Kempen, H.J.M., Vos-van Holstein, M.P.M., and de Lange, J. : Bile acids and lipids in isolated rat hepatocytes: content, synthesis, and release, as affected by cholestyramine treatment of the donor rats. J. Lipid Res. 23 (1982), 823-830.

32. Quistorff, B. : Gluconeogenesis in periportal and pericentral hepatocytes of rat liver, isolated by a new high-yield digitonin-collagenase perfusion technique. Biochem. J. 229 (1985), 221-226.

33. Lindros, K.O., and Penttilä, K.E. : Digitonin-collagenase perfusion for efficient separation of periportal or perivenous hepatocytes. Biochem. J. 228 (1985), 757-760.

34. Gebhardt, R., and Williams, G.M. : Amino acid transport in established adult rat liver epithelial cell lines. Cell. Biol. Toxicol. 2 (1986), 9-20.

35. Bergmeyer, H.U., and Bernt, E. : Methoden der enzymatischen Analyse. Verlag Chemie , Weinheim (1974).

36. Su, P., Rennert, H., Shayiq, R.M., Yamamoto, R., Zheng, Y.-M., Addya, S., Strauss III, J.F., and Avadhani, N.G. : A cDNA encoding rat mitochondrial cytochrome P-450 catalyzing both the 26-hydroxylation of cholesterol and 25-hydroxylation of vitamin D3: gonadotropic regulation of the cognate mRNA in ovaries. DNA and Cell. Biol. 9 (1990), 657-665.

37. Andersson, S., Davis, D.L., Dahlbäck, H., Jörnvall, H., and Russell, D.W. : Cloning, structure and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. J. Biol. Chem. 264 (1989), 8222-8229.

38. Texeira, J., and Gil, G.: Cloning, expression, and regulation of lithocholic acid 6β-hydroxylase. J.

Biol. Chem. 266 (1991), 21030-21036.

39. Chin, D.W., Gil, G., Russell, D.W., Liscum, L., Luskey, K.L., Basu, S.K., Okayama, H., Berg, P., Goldstein, J.L., and Brown, M.S. : Nucleotide sequence of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase, a glycoprotein of endoplasmtic reticulum. Nature 308 (1984), 613-617.

40. De Groot, C.J., Ten Voorde, G.H.J., Van Andel, R.E., Kortschot, A., Gaasbeek Janzen, J.W., Wilson, R.H., Moorman, A.F.M., Charles, R., and Lamers, W.H. : Reciprocal regulation of glutamine synthetase and carbamoyl phosphate synthetase levels in rat liver. Biochim. Biophys. Acta. 908 (1987), 231-240.

41. Moorman, A.F.M., de Boer, P.A.J., Geerts, W.J., van den Zande, L., Lamers, W.H., and Charles, R. : Complementary distribution of carbamoylphosphate synthetase (ammonia) and glutamine synthetase in rat liver acinus is regulated at a pretranslational level. J. Histochem.Cytochem. 36 (1988), 751-755.

42. Moorman, A.F.M., de Boer, P.A.J., Vermeulen, J.L.M., and Lamers, W.H. : Practical aspects of radio-isotopic *in situ* hybridization on RNA. Histochem. J. 25 (1993), 251-266.

43. Gebhardt, R., and Mecke, D. : Heterogeneous distribution of glutamine synthetase among rat liver parenchymal cells *in situ* and in primary culture. EMBO J. 2 (1983), 567-570.

44. Lindros, K.O., Pentilla, K.E., Gaasbeek Janzen, J,W., Moorman, A.F.M., Speisky, H., and Israel, Y. : The γ-glutamyltransferase/glutamine synthetase activity ratio. A powerful marker for the acinar origin of hepatocytes. J. Hepatol. 8 (1989), 338-343.

45. Jelinek, D.F., Andersson, S., Slaughter, C.A., and Russell, D.W. : Cloning and regulation of cholesterol 7α-hydroxylase, the rate limiting enzyme in bile acid biosynthesis. J. Biol. Chem. 265 (1990), 8190-8197.

46. Li, Y.C., D.P. Wang, and J.Y.L. Chiang (1990) Regulation of cholesterol 7α-hydroxylase in the liver. Cloning, sequencing and regulation of cholesterol 7α-hydroxylase mRNA. J. Biol. Chem. 265: 12012-12019.

47. Gebhardt, R., Ebert, A., and Bauer, G. : Heterogeneous expression of glutamine synthetase mRNA in rat liver parenchyma revealed by *in situ* hybridization and Northern blot analysis of RNA from periportal and perivenous hepatocytes. FEBS Lett. 241 (1988), 89-93.

48. Clarke, C.F., Edwards, P.A., Lan, S.-F., Tanaka, R.D., and Fogelman, A.M. : Regulation of 3 hydroxy-3-methylglutaryl-coenzyme A reductase mRNA levels in rat liver. Proc. Natl. Acad. Sci. USA 80 (1983), 3305-3308.

49. Liscum, L., Luskey, K.L., Chin, D.J., Ho, Y.K., Goldstein, J.L., and Brown, M.S. : Regulation of 3-hydroxy-3-methylglutaryl Coenzyme A reductase and its mRNA in rat liver as studied with a monoclonal antibody and a cDNA probe. J. Biol. Chem. 258 (1983), 8450-8455.

50. Tanaka, R.D., Edwards, P.A., Lan, S.-F., Knöppel, E.M., and Fogelman, A.M. : The effect of

cholestyramine and mevinolin on the diurnal cycle of rat hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase. J. Lipid Res. 23 (1982), 1026-1031.

51. Gebhardt, R., and Mecke, D. : Cellular distribution and regulation of glutamine synthetase in liver. In: Glutamate Metabolism in Mammalian Tissues. Häussinger, D., Sies, H., eds., Springer Verlag, Heidelberg (1984). 98-121.

52. Gebhardt, R. : Heterogeneous intrahepatic distribution of glutamine synthetase. Acta Histochem. Suppl. XL. (1990), 23-28.

53. Björkhem, I. : Mechanism of degradation of the steroid side chain in the formation of bile acids. J. Lipid Res. 33 (1992), 455-471.

54. Twisk, J., Hoekman, M.F.M., Lehmann, E., Meijer, P., Mager, W.H., and Princen, H.M.G. : Insulin suppresses bile acid synthesis in cultured rat hepatocytes by down-regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase gene transcription. Hepatology (1995), in press.

55. Buscher, H.-P., Fricker, G., Gerok, W., Kurz, G., Müller, M., Schneider, Schramm, S., and Schreyer, S. : Hepatic transport systems for bile salts: localization and specificity. In: Bile acids and the Liver. Paumgartner, G., Stiehl, A., and Gerok, W., eds., MTP Press, Lancaster (1987), 95-110.

56. Twisk, J., Lehmann, E.M., and Princen, H.M.G. : Feedback regulation of bile acid synthesis in primary monolayer cultures of rat hepatocytes by suppression of cholesterol 7α-hydroxylase mRNA and transcriptional activity. In: Bile Acids and the Hepatobiliary System. Paumgartner, G., Stiehl, A., and Gerok, W., eds., MTP Press, Lancaster (1993), 58-72.

57. Hoffmann, A.F. : Bile acid transport by the hepatocyte. In: Communication in liver cells. Popper, H., Bianchi, L., Gudat, F., and Reutter, W., eds., MTP Press, Lancaster (1980), 43-49.

58. Cronholm, T., Crustedt, T., and Sjövall, J. : Formations of bile acids and glycerophosphatides in liver. In: Metabolic compartimentation. Sies, H., ed., Academic Press, New York (1982), 331-359.

59. Baumgartner, U., Schölmerich, J.,Karsch, J., Gerok, W., and Farthmann, E.H. : Loss of zonal heterogeneity and cell polarity in rat liver with respect to bile acid secretion after bile drainage. Gastroenterology 100 (1991), 1054-1061.

60. Hoekman, M.F.M., Rientjes, J.M.J., Twisk, J., Planta, R.J., Princen, H.M.G., and Mager,W.H. : Transcriptional regulation of the gene encoding cholesterol 7α-hyroxylase in the rat. Gene 130 (1993), 217-223.

61. Noshiro, M., Nishimoto, M., and Okuda, K. : Rat liver cholesterol 7α-hyroxylase: pretranslational regulation for circadian rhythm. J. Biol. Chem. 265 (1990), 10036-10041.

62. Balasubramanian, S., Mitropoulos, K.A., and Myant, W.B. : Evidence for compartmentalization of cholesterol in rat liver microsomes. Eur. J. Biochem. 34 (1979), 77-83.

63. Björkhem, I., and Lewenhaupt, A. : Preferential utilization of newly synthesized cholesterol as

substrate for bile acid synthesis. J. Biol. Chem. 254 (1979), 5252-5257.

64. Robins, S.J., Fasulo, J.M., Collins, M.A., and Patton, G.M. : Evidence for separate pathways of transport of newly synthesized and preformed cholesterol into bile. J. Biol. Chem. 260 (1985), 6511-6513.

65. Scheibner, J., Fuchs, M., Schiemann, M., Tauber, G., Hörmann, E., and Stange, E.F. : Bile acid synthesis from newly synthesized vs. preformed cholesterol precursor pools in the rat. Hepatology 17 (1985), 1095-1102.

66. Vlahcevic, Z.R., Pandak, W.M., Hylemon, P.B., and Heuman, D.M. : Role of newly synthesized cholesterol or its metabolites on the regulation of bile acid biosynthesis after short-term biliary diversion in the rat. Hepatology 18 (1993), 660-668.

67. Grundy, S.M., and Bilheimer, P.W. : Inhibition of 3-hydroxy-3-methylglutaryl- CoA reductase . by mevinolin in hypercholesterolemia heterozygotes: effects on cholesterol balance. Proc. Natl. Acad. Sci. USA 81 (1986), 2538-2542.

68. Reihnér, E., Rudling, M., Stahlberg, D., Berglund, L., Ewerth, S., Björkhem, I., Einarsson, K., and Angelin, B. : Influence of pravastatin, a specific inhibitor of HMG-CoA reductase on hepatic metabolism of cholesterol. N. Engl. J. Med. 323 (1986), 224-228.

69. Long, T.T., Jakoi, L., Steven, R., and Quarfordt, S.: The sources of rat biliary cholesterol and bile acid. J. Lipid Res. 19 (1978), 872-878.

70. Kempen, H.J.M., Vos-Van Holstein, M.P.M., and de Lange, J. : Bile acids and lipids in isolated rat hepatocytes. II. Source of cholesterol used for bile acid formation, estimated by incorporation of tritium from tritiated water, and by the effect of ML-236B. J. Lipid Res. 24 (1983), 316-323.

Samenvatting

Regulering van de cholesterol 7α-hydroxylase gen expressie in de rat

Cholesterol is een essentieel bestanddeel in het lichaam van alle zoogdieren, inclusief de mens. Het maakt deel uit van de membraan die een cel omgeeft, maar is tegelijkertijd ook de basisstof voor de aanmaak van galzuren en een grote groep van hormonen (steroïde hormonen). Zoals bekend, vormt een te hoge cholesterolconcentratie in het bloed een verhoogde risicofactor voor het ontstaan van galstenen en hart- en vaatziekten. Deze zgn. coronaire aandoeningen zijn in de laatste 30-40 jaar uitgegroeid tot doodsoorzaak nr. 1 in de westerse wereld. Dat is reden waarom tegenwoordig veel onderzoek wordt verricht naar het ontstaan van atherosclerose.
Na het bovenstaande is duidelijk dat het voor het lichaam van groot belang is de hoeveelheid cholesterol nauwkeurig te controleren. Behalve inname via de voeding, is er ook de mogelijkheid cholesterol aan te maken en af te breken, processen die uitsluitend plaatsvinden in de lever. In de afbraakroute van cholesterol, uiteindelijk leidend tot de vorming van galzuren, wordt de eerste reactie - de hydroxylering van cholesterol op de 7-positie in de α-oriëntatie - gekatalyseerd door het enzym cholesterol 7α-hydroxylase, afgekort CYP7A. Uit voorafgaand onderzoek is gebleken dat de mate waarin dit enzym aanwezig is in de levercel, uiteindelijk bepalend is voor de snelheid waarmee cholesterol in galzuren wordt omgezet. De informatie nodig voor de leverspecifieke productie van het CYP7A eiwit ligt besloten in het gen coderend voor CYP7A, gelegen ergens in de genetische bibliotheek, het DNA. Dit specifieke gen komt alleen tot productie (expressie) van CYP7A in de lever, waarbij de mate van expressie afhankelijk is van tal van externe condities. Zo kan onder bepaalde omstandigheden de expressie worden gestimuleerd of geremd, met als gevolg een resp. verhoogde of verlaagde cholesterolafbraak. De regulering van de voor eiwitten coderende genen blijkt over het algemeen plaats te vinden op het niveau van de transcriptie (het overschrijven van het DNA naar RNA, in dit geval door RNA polymerase II -Pol II). Bij dit proces binden verschillende zgn. transcriptiefactoren specifiek aan regulerende DNA-elementen, meestal gelegen in de flankerende gebieden van de betreffende genen. Gevolg hiervan is een stimulatie dan wel remming van de gentranscriptie door Pol II.
Het in dit proefschrift beschreven onderzoek, waarbij het gen voor cholesterol 7α-hydroxylase centraal staat, werd verricht in het kader van een samenwerkingsproject tussen de vakgroep Biochemie en Moleculaire Biologie van de Vrije Universiteit te Amsterdam en het Gaubius

Laboratorium, TNO-PG, te Leiden. Het betreft moleculair-biologisch en fysiologisch onderzoek, enerzijds gericht op het opsporen en karakteriseren van DNA-elementen en eiwitfactoren betrokken bij de regulering van de CYP7A genexpressie, anderzijds met als doel een breder inzicht te verkrijgen in de fysiologische mechanismen die ten grondslag liggen aan de werking van cholesterol 7α-hydroxylase in de rattelever. De experimenten zijn uitgevoerd met de rat als ideaal modelsysteem voor de bestudering van CYP7A genexpressie en fysiologie, met name door gebruik te maken van gekweekte rat hepatocyten.

Teneinde promotorstudies te kunnen uitvoeren, werd eerst het 5'-flankerend gebied van het ratte CYP7A gen gekloneerd, waarna de nucleotide-volgorde in het DNA werd bepaald (Hoofdstuk 2). Na vergelijking met de humane en hamster CYP7A-promotor, bleek vooral het meest proximale gedeelte sterk geconserveerd (in de evolutie behouden) te zijn. Dit is een sterke aanwijzing dat zich in dit gebied belangrijke regulerende elementen (zgn. in *cis* werkende elementen) zouden kunnen bevinden. Een computer-search van de eerste 3600 nucleotiden (nt) van de betreffende promotor, waarbij werd gezocht naar bekende regulerende elementen, leverde inderdaad enkele potentiële in *cis* werkende elementen op.

De functionele analyse van de ratte CYP7A promotor wordt beschreven in de Hoofdstukken 2 en 3. Daartoe werd gebruik gemaakt van zgn. transiënte expressie experimenten (metingen van de transcriptie-activiteit), aangevuld en onderbouwd door mRNA steady-state bepalingen (Northern analyses), run-on metingen (ook een bepaling van de transcriptionele activiteit) en band-shift analyses (DNA-eiwit interacties). De transiënte expressie studies werden uitgevoerd in in kweek gebrachte levercellen (hepatocyten) uit de rat. Deze cellen werden getransfecteerd met constructen, waarin CYP7A promotor-fragmenten zijn gefuseerd aan het bacteriële chlooramfenicolacetyltransferase (CAT) gen, dat dienst doet als reporter. De gemeten CAT-activiteit is een maat voor de activiteit van het betreffende promotor-fragment. Op deze wijze werden verschillende CYP7A promotor-regio's geïdentificeerd, betrokken bij de regulering van de CYP7A transcriptie. Zo kon worden aangetoond dat tussen nt -348 en -3650 een of meer thyroxine (schildklierhormoon)-gevoelige elementen gelocaliseerd zijn. Uit verschillende daarop volgende deletie-analyses bleek dat een element (nt -47/-79), gelegen in de proximale promotor, betrokken is bij zowel de activering van de CYP7A transcriptie als de regulering ervan. Er werd vastgesteld dat galzuren en insuline in staat zijn de transcriptie te verlagen via dit [-47/-79] promotorgebied, terwijl hetzelfde element essentieel is voor de verhoging van de CYP7A transcriptie door cholesterol (in de vorm van βVLDL) en retinoïde zuur. Verder werd duidelijk dat het element in staat is één of meer eiwitten te binden uit een ruw rattelever kernextract. Dit laatste duidt op een directe interactie tussen de regulerende sequentie en

regulerende eiwit-factoren (in *trans* werkende factoren). Northern- en run-on experimenten bevesigden dat de regulering van de CYP7A genexpressie onder bovengenoemde omstandigheden vooral plaatsvindt op het niveau van transcriptie. Aan de hand van de in de Hoofdstukken 2 en 3 verkregen resultaten werd een algemeen model geponeerd voor de regulering van de CYP7A-transcriptie, waarbij een centrale rol wordt ingenomen door een aantal in *trans* werkende factoren die direct of indirect binden aan het [-47/-79] element.

Zoals gezegd, remmen galzuren hun eigen aanmaak via een verlaging van de CYP7A-transcriptie. In Hoofdstuk 4 worden experimenten beschreven waaruit blijkt dat niet alle galzuren in dezelfde mate aktief zijn in de door galzuur gemedieerde terugkoppeling. Er werden 27 verschillende galzuren getest op verlaging van de CYP7A mRNA steady-state niveau's, variërend in hydrofobiciteit en in aantal, positie en oriëntatie van de OH-groepen op de sterole kern. Er bleek slechts een geringe correlatie te bestaan tussen de mate van remming en de hydrofobociteit. Nadere analyse d.m.v. computer-modelling van de 3-dimensionale structuur van een potente remmer, het galzuur cholaat, toonde aan dat enkele OH-groepen op het molecuul, door zich in elkaars nabijheid te bevinden, een hydrofiele groep creëren. Inderdaad blijkt in minder sterk remmende galzuren deze hydrofiele groep, die mogelijk betrokken is bij de binding van het galzuur aan een hypothetische receptor, niet in dezelfde vorm aanwezig te zijn. Transiënte expressie experimenten bevestigden de verschillen tussen galzuren onderling in hun remmende werking op de CYP7A-transcriptie.

Het is reeds lang bekend dat patiënten leidend aan suikerziekte een verhoogde galzuur-productie vertonen. Door deze personen te behandelen met insuline wordt de ophoping van galzuur teruggebracht tot normale proporties. In Hoofdstuk 5 wordt d.m.v. experimenten met gekweekte rat hepatocyten blootgesteld aan insuline, aangetoond dat het insuline-hormoon inderdaad een remmend effekt heeft op de enzymactiviteit, mRNA steady-state niveau's en transcriptionele activiteit van zowel cholesterol 7α-hydroxylase als sterol 27-hydroxylase, het snelheidsbepalende enzym in de alternatieve afbraakroute van cholesterol, met als direct gevolg een verlaagde galzuursynthese.

In Hoofdstuk 6 tenslotte, ligt de nadruk op de heterogene localizatie van CYP7A en sterol 27-hydroxylase binnen de leverlobulus. Enzymactiviteit, mRNA niveau's en transcriptie van de betreffende genen blijken vooral in de pericentrale regio gesitueerd te zijn, dit in tegenstelling tot HMG-CoA reductase (het sleutelenzym in de synthese route van cholesterol), dat voornamelijk periportaal tot expressie komt. Dit gegeven doet vermoeden dat onder 'normale' condities vooral exogeen cholesterol gebruikt wordt voor de galzuursynthese, en dat in verband met een efficiënte regulering van de cholesterolspiegels, de synthese- en afbraakroutes van

cholesterol binnen de leverlobulus strict zijn gescheiden. Galzuren spelen dus een belangrijke rol in de regulering van hun eigen synthese. Ong. 95% van de galzuren die een functie vervullen bij de vertering van vetten, wordt gereabsorbeerd in de dikke darm en keert via het portale bloed terug in de lever. Daar worden ze opnieuw opgenomen door de hepatocyten, waardoor een galzuur-gradient ontstaat over de leverlobulus: relatief hoge concentraties periportaal, relatief lage concentraties pericentraal (waar het bloed de lever weer verlaat). Direct gevolg is een omgekeerde gradient voor de CYP7A en sterol 27-hydroxylase enzymactiviteit, mRNA niveau's en transcriptie: pericentraal relatief hoog, periportaal relatief laag. Deze resultaten werden bevestigt door *in situ* hybridizaties, terwijl ook werd aangetoond dat de toediening aan ratten van een galzuurbindend hars (colestid) resulteert in een verhoogde CYP7A en sterol 27-hydroxylase transcriptie als gevolg van een verminderde galzuur-flow door de lever-lobulus.

Dankwoord

Tot slot wil ik graag van de mogelijkheid gebruik maken iedereen te bedanken die direct of indirect, in wetenschappelijke of niet-wetenschappelijke zin, bewust of onbewust, heeft meegewerkt aan de totstandkoming van dit proefschrift. Enkele personen wil ik hier met name noemen :

Mijn promotor, prof Planta, dank ik voor de interesse in het onderzoek en voor de mogelijkheid dit project op de vakgroep uit te voeren, en zo aan zelfvertrouwen te winnen.
Pim Mager, mijn directe begeleider (en co-promotor) bedank ik voor alles en nog meer, zowel binnen als buiten het onderzoek. Beste Pim, een betere 'levens'-begeleiding kon ik mij gedurende de laatste 4 jaar niet wensen. Hou je taai, en rook d'r een sigaartje op !
Heel veel dank geldt Jeanet Rientjes, die mij de eerste 3 jaar heeft bijgestaan in de soms moeizame weg naar dit boekje. Veel geluk in Heidelberg, und auf wiedersehn !!
Niet te vergeten de collega's in Leiden. Co-promotor Hans Princen en mede AIO Jaap Twisk. Heren, uw impact-factor op dit werk was groot. Dank daarvoor !
Emelie Martijn dank ik voor de prachtige retardaties en de pittige discussie's.

Verder alle mensen in Amsterdam, Leiden en Groningen die ik gedurende mijn promotie-periode tegen het lijf ben gelopen en die iets voor mij betekend hebben. Ik hoop dat ik ooit iets voor jullie kan terugdoen. Dank, dank en bedankt !

Zoals velen weten was ik vaak (de laatste tijd zeker !) te bereiken bij mijn ouders. Officieel omdat de computer daar stond, officieus vanwege de koffie en de pasta (ik zal er maar niet langer omheen draaien) Pa en Ma, dit boekje is voor jullie !!

Ciaooo, Marco