Transcriptional Regulation of the Human Cholesterol 7α-Hydroxylase Gene†

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ABSTRACT: As an initial step toward understanding the transcriptional regulation of cholesterol 7α-hydroxylase (CYP7) in man, we isolated and functionally characterized the 5′-flanking region of the human CYP7 gene. The nucleotide sequences of the first exon and 1.6 kb preceding the exon were determined and found to contain a TATA box at position −30, a modified CAAT box at position −92, three potential hepatocyte nuclear factor 3 (HNF-3) recognition sites at nucleotides −316, −288, and −255, respectively, and a modified sterol response element at position −271. DNA sequences containing 1.3 kb of the 5′-flanking region and 29 nucleotides of the first exon were linked to the chloramphenicol acetyltransferase gene and transiently transfected into several cell lines. Promoter activity was very strong in the human hepatoma cell line HepG2 but absent in cells of nonhepatic origin. Mutational analysis of the promoter identified several regions that function in the transcriptional regulation of CYP7. Introduction of a fragment containing the region from −432 to −220 upstream of a heterologous promoter, in either orientation, resulted in a tremendous stimulation of activity in HepG2 cells. DNase I footprint analysis identified three regions within this fragment which were protected from digestion. The overexpression of HNF-3 in HepG2 cells resulted in a 4-fold stimulation of CYP7 transcriptional activity. We suggest that the region between −432 and −220 functions as a cell-specific enhancer whose activity is controlled, in part, by HNF-3.

The state of cholesterol equilibrium in the body is the result of a balance between cholesterol uptake, synthesis, and catabolism. All three of these pathways are tightly controlled and contribute to the homeostatic regulation of serum cholesterol (Turley & Dietschy, 1982). The molecular mechanisms responsible for controlling the rate of receptor-mediated uptake of cholesterol and the de novo synthesis of cholesterol have been studied in great detail in recent years (Sudhof et al., 1987; Osborne et al., 1988; Smith et al., 1988). These studies have defined an octanucleotide sequence, termed the sterol response element (SRE), in the promoter of critical genes in these pathways, which is essential for the end-product suppression of gene transcription (Goldstein & Brown, 1990). In the presence of sterols, this DNA sequence represses the positive transcriptional activity of a nearby positive DNA element. In this way, the body decreases cholesterol uptake and synthesis when cellular cholesterol is in excess. Unfortunately, we currently have a poor understanding of the regulatory mechanisms that are responsible for controlling the rate of cholesterol catabolism.

The cholesterol catabolic pathway, which is exclusive to the liver, is comprised of 10 or more enzymatic reactions which convert cholesterol into hydrophilic bile acids (Danielsson, 1973). The newly synthesized bile acids are then conjugated with either taurine or glycine and secreted from the liver and stored in the gall bladder as a component of bile. Upon ingestion of a meal, the bile is released into the duodenum where it facilitates the absorption of fatty nutrients. Bile acids are then actively reabsorbed from the terminal ileum into the portal circulation and transported back to the liver (Malet & Soloway, 1988). The rate of return of bile acids to the liver is a major influence on the rate of synthesis and secretion of bile acids. Bile acids in the portal blood stimulate the secretion of bile acids by the hepatocytes but inhibit the synthesis of bile acids (Myant & Mitropoulos, 1977). In the process of enterohepatic circulation, 500–800 mg of bile acids escapes absorption and is excreted in the feces daily. This pathway accounts for greater than 50% of the cholesterol which is excreted each day by man and thus plays a major role in the maintenance of cholesterol homeostasis (Turley & Dietschy, 1982).

The first reaction of the catabolic pathway of cholesterol involves the hydroxylation of the steroid nucleus at the 7 position. This reaction is catalyzed by a member of the cytochrome P450 gene family, cholesterol 7α-hydroxylase (CYP7), and serves as the rate-limiting step and major site of regulation of bile acid biosynthesis (Myant & Mitropoulos, 1977). The mechanism by which bile salts repress CYP7 enzymatic activity is unknown at this time. However, recent studies employing cDNAs which encode CYP7 have provided insight into the regulatory mechanisms involved in the feedback suppression of bile acid biosynthesis. The data indicate that rat liver CYP7 is expressed exclusively in the liver and that CYP7 mRNA levels are suppressed by dietary bile acids and induced by bile acid sequestrants, cholesterol, and mevalonate (Jelinek et al., 1990; Noshiro et al., 1990; Sundseth & Waxman, 1990). This information appears to indicate that the pathways of cholesterol uptake, biosynthesis, and catabolism may share certain regulatory mechanisms.

As a first step toward understanding the molecular mechanisms responsible for the cell-specific expression of CYP7 and its regulation by sterols and bile acids, we isolated and functionally characterized the upstream sequence of the human CYP7 gene. We have defined the nucleotide sequence elements necessary for CYP7 transcriptional activity and provide evidence indicating that the nuclear factor HNF-3 plays a role

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in the hepatocyte-specific expression of CYP7.

Experimental Procedures

cDNA Cloning of Human Liver CYP7 mRNA. We synthesized two oligonucleotide primers, 5′-GATCGGAATTCATGATGACTATTTCTTTGATTT-3′ and 5′-GATCGGAATTCGTGTCCAAATGCCTTCGCAGA-3′, which correspond to nucleotides 36–57 and the inverse complement of nucleotides 348–368 of the published rat CYP7 cDNA sequence (Noshiro et al., 1990) with the addition of five stabilizing bases and an *Eco*RI site at the 5′-end. These primers were added to a standard PCR cocktail containing 1 μL of a human liver cDNA library as template (Clontech Laboratories). The major reaction product of 330 bp was isolated and cloned into the *Eco*RI site of pGEM3(−). The DNA sequence was determined by the chain termination technique. Sequence analyses were performed using the GCG software package.

Cloning of the 5′-Flanking Region of the CYP7 Gene. We screened 250000 clones of a human genomic library (Clontech Laboratories) with our ^{32}P-labeled HCYP7 cDNA and isolated 4 positive clones. Restriction analysis and Southern blot analysis revealed that one of the clones, CYP9211-2, contained a 4.4-kb *Eco*RI–*Sal*I fragment which strongly hybridized to our cDNA probe. This fragment was subcloned into pGEM3(−) for further analysis. A 2.3-kb *Pst*I–*Sph*I fragment which hybridized to our probe was isolated and sequenced.

Primer Extension Analysis. A ^{32}P-labeled oligonucleotide complementary to nucleotides +81–109 of Figure 2 (5′-GCATGCTGCTATAGCAATCCCCCAAATCA-3′) was annealed to the indicated amount of human liver poly(A$^+$) RNA (Clontech Laboratories) for 20 h at 30 °C. Fifty units of reverse transcriptase was added to the mixture to allow for cDNA synthesis. The reaction was terminated after 2 h by the addition of 1 μL of 0.5 M EDTA. Five nanograms of RNAase was added to each tube, and the reaction was allowed to proceed for 30 min at 37 °C. The products of the reaction were isolated by organic extraction and precipitation and resolved on a 6% denaturing polyacrylamide gel as described (Sambrook et al., 1989).

Plasmid Constructions. To construct pBLCYP7, we synthesized an oligonucleotide corresponding to nucleotides −1291 to −1270 of the CYP7 upstream sequence with a 5′-*Sph*I site and an oligonucleotide corresponding to the inverse complement of nucleotides +11 to +29 with a 5′-*Sal*I site. These oligonucleotides served as primers in a PCR utilizing our 2.3-kb *Pst*I–*Sph*I genomic fragment as template. The resultant 1.3-kb product was cloned into the *Sph*I–*Sal*I sites of pBLCAT3 (Luckow & Schutz, 1987). Plasmids pBLCYP7-24, -37, -38, -26, -39, -33, -47, and -34 were cloned in a similar manner with the exception that the 5′-primer started at the sequences designated in Figure 4. pBLCYP7-36 and -50 were created by synthesizing primers which started at the appropriate sequence, −432 and −220 for pBLCYP7-36 and −220 and −432 for pBLCYP7-50, and performing PCR as described above. The resultant PCR products were cloned into the *Sph*I–*Sal*I sites of pBLCAT2 (Luckow & Schutz, 1987). Plasmid DNA was purified by standard techniques, and the integrity of the DNA was verified by sequencing.

Cell Culture and Transient Expression. Several cell lines were employed as recipient cells for transfection. Chinese hamster ovary CHO-K1, mouse fibroblast NIH3T3, human epithelial HeLa, and human hepatoma HepG2 were obtained from the American Type Tissue Collection (Parklawn, MD) and maintained according to their instructions. The cells were seeded in 60-mm plates and grown to approximately 65% confluency. The medium was changed, and the test plasmids (2.5 μg) and standard plasmid, pCH110 (Pharmacia) (1 μg), were cotransfected as a calcium phosphate precipitate. In the studies which involved the addition of the HNF-3 expression plasmid, a triple transfection was performed which included the test plasmid (2.5 μg), pNH3-α (2.5 μg), and pCMVβ (0.5 μg). After 6 h, the precipitate was removed, and the cells were fed with fresh media. After 48 h, the cells were harvested, and cell extracts were prepared. CAT activity was determined by the diffusion assay (Neumann et al., 1987). Briefly, 50 μg of protein was added to an aqueous mixture containing chloramphenicol and [^{3}H]acetyl-CoA (NEN) and overlaid with organic scintillation cocktail. The quantity of [^{3}H]-acetylchloramphenicol produced was determined by scintillation counting at 30-min intervals. The data are calculated as the slope of the line generated from four time points minus the activity generated by vector sequences. The data are then corrected for transfection efficiency by dividing the slope with the β-galactosidase generated by the standard plasmid.

DNase I Footprinting. DNase I footprinting was performed as described by Brenowitz et al. (1991). pBLCYP7-36 was digested with *Sal*I and end-labeled with [^{32}P]ATP by T4 polynucleotide kinase. The labeled plasmid was further digested with *Sph*I, and the resultant labeled 220 bp insert was isolated by gel purification. Sixty micrograms of rat liver or spleen nuclear extracts (Costa et al., 1989) was incubated with 2 ng of the ^{32}P-end-labeled probe (−432 to −220) and digested with the indicated concentration of DNase I for 2 min at 0 °C. The products were organically extracted, ethanol-precipitated, and resuspended in 50% formamide. They were then separated on a 6% polyacrylamide gel and visualized by autoradiography.

Results

Isolation and Characterization of CYP7 Genomic Clones. We isolated a 330 bp fragment of the human liver CYP7 cDNA through the use of the polymerase chain reaction (data not shown). The nucleotide sequence of this DNA fragment was found to share 100% identity with the recently published human liver CYP7 cDNA sequence (Noshiro & Okuda, 1990). We then screened a human genomic library with our 330 bp human CYP7 cDNA probe and isolated four positive clones. One of the positive clones, CYP9211-2, which contained a 13.6-kb insert, was further characterized by restriction enzyme mapping and Southern blot analysis. A 2.3-kb *Pst*I–*Sph*I fragment was identified which hybridized to the CYP7 cDNA. Sequence analysis revealed that this DNA fragment contained 1.6 kb of the 5′-flanking region, the first exon, and part of the first intron of the human CYP7 gene (Figure 1). The nucleotide sequence of the 141 bp first exon is 100% homologous to the cDNA in this region. This fact, coupled with our knowledge that CYP7 exists as a single gene in man (data not shown), leads us to believe that we have cloned sequences of the human CYP7 gene.

A comparison of the nucleotide sequence of the first 600 bases of the human CYP7 promoter with the recently published rat sequence (Jelinek & Russell, 1990) reveals a 59% identity. A consensus TATA box is located at −30 and a modified CAAT box at −93. Three regions which are highly homologous to a consensus recognition site for the transcription factor HNF-3 [TATTGA(C/T)TT(A/T)G] (Grayson et al., 1989) are located at −255 to −243, −288 to −276, and −316 to −304. A sequence at −271 shares 6/8 bases of sequence identity with the hamster LDL receptor SRE (Smith et al., 1990).

Identification of the Transcriptional Initiation Site.

FIGURE 1: Nucleotide sequence of the 5'-flanking region, the first exon, and part of the first intron of the human CYP7 gene. The transcriptional initiation site, as determined by primer extension analysis, is indicated by the asterisk (+1). The TATA box is denoted by a single overline, a modified SRE is indicated by a single underline, and the putative HNF-3 binding sites are indicated by the double overlines. The beginning of the first intron is shown by lower case letters.

Table I: Transcriptional Activity of the CYP7 Promoter in Several Cell Types[a]

cell type	CAT activity (% pSV2CAT)
HepG2	23 ± 4
HeLa	ND
CHO	ND
NIH3T	ND

[a] Subconfluent cultures were transfected with pBLCYP7 (2.5 μg) or pSV2CAT (2.5 μg). Forty-eight hours later, the cells were harvested, and CAT activity was determined as described under Experimental Procedures. The data are presented as the mean of three experiments (±SD). ND, none detected.

Identification of the CYP7 transcriptional initiation site was achieved by primer extension analysis. A 29-mer synthetic oligonucleotide complementary to the sequence 45 bp downstream from the translational start site was used as the primer. One elongation product was detected corresponding to 109 nucleotides (Figure 2). This implies that the transcriptional start site is 64 nucleotides upstream of the ATG.

Analysis of Promoter Activity. To determine the DNA elements essential for the transcriptional activity of the CYP7 promoter, we cloned a 1.3-kb (−1291 to +29) DNA fragment into pBLCAT3. The construct, pBLCYP7, was transiently transfected into several cell lines to assess its promoter activity. As shown in Table I, the promoter was extremely active in the human hepatoma HepG2 but inactive in the HeLa, CHO, and NIH3T3 cells, cells of nonhepatic origin. Therefore, this DNA sequence appears to contain the regulatory information required for the liver-specific transcription of CYP7.

Characterization of the Enhancer Element. To further define the sequence elements which are responsible for the transcriptional activity of the CYP7 gene, we constructed a number of deletion mutants and tested their promoter activity in HepG2 cells. As shown in Figure 3, it is readily apparent that this promoter is quite complex. A deletion to −400 (pBLCYP7-37) has no effect on transcriptional activity. Further deletion from −400 to −301 (pBLCYP7-39), which removes the most distal HNF-3 recognition site, results in a 55% decrease in promoter activity. A deletion of an additional 25 nucleotides to −276 (pBLCYP7-33) removes a second potential HNF-3 binding site and virtually abolishes transcriptional activity. The graded nature of the initial decline in promoter activity and the precipitous drop in activity upon deletion from −301 to −276 suggest that there are multiple regulatory elements within this region.

To determine whether the DNA sequence between −432 and −220 could enhance the activity of a heterologous promoter, we inserted this DNA fragment, in either orientation, into the *Sal*I–*Sph*I site of pBLCAT2, a plasmid which contains the herpes simplex virus thymidine kinase promoter driving the CAT gene. Transient transfection of both constructs (pBLCYP7-36, pBLCYP7-50) into HepG2 cells resulted in a 14-fold and 3.5-fold increase in promoter activity, respectively, as compared to pBLCAT2 (Figure 3). Transfection of these constructs into HeLa or CHO cells did not result in the production of CAT activity. The enhancement of the transcriptional activity of a heterologous promoter in an orientation-independent fashion provides evidence for the existence of an enhancer within the region of −432 to −220 of the CYP7 promoter. As shown in Figure 3, this region contains all three of the sites which share significant homology with the HNF-3 consensus sequence.

Effect of Overexpressed HNF-3 on CYP7 Transcriptional Activity. The presence of multiple HNF-3 recognition sites

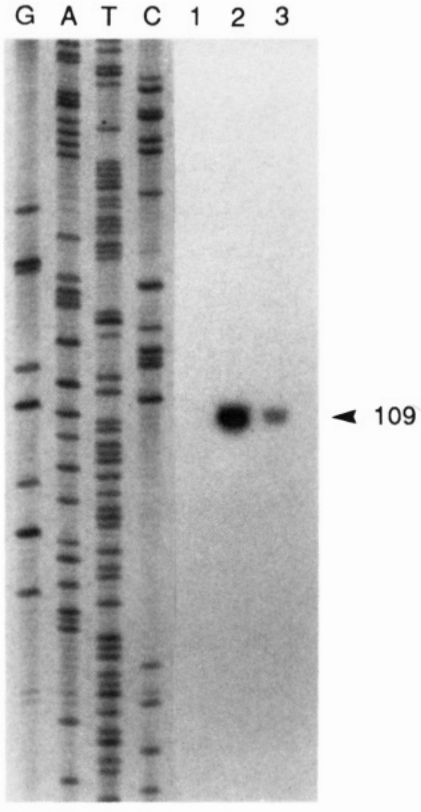


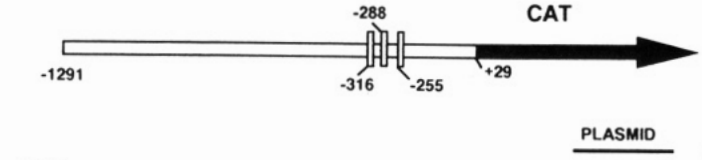
FIGURE 2: Determination of the transcriptional start site by primer extension analysis. Human liver poly(A^+) RNA (lane 1, no RNA; lane 2, 20 μg; lane 3, 5 μg) was hybridized to a ^{32}P-labeled oligonucleotide complementary to nucleotides +81–109 of Figure 1, extended with reverse transcriptase, and digested with RNase. The products were analyzed by polyacrylamide gel electrophoresis as described under Experimental Procedures and exposed to film for 4 days. A nonhomologous DNA sequencing ladder was used to correctly estimate the size of the products.

within the CYP7 enhancer element clearly suggested that HNF-3 played an important role in the hepatocyte-specific transcriptional activity of this gene. To test this hypothesis, we cotransfected a plasmid (pNH3-α) containing the rat liver HNF-3 α cDNA under the control of the SV40 promoter (Lai et al., 1991) with our original construct, pBLCYP7, pBLCYP-37, and pBLCYP-36. This expression plasmid has previously been shown to trans-activate the mouse transthyretin promoter in HepG2 cells (Lai et al., 1991). The overexpression of HNF-3 in HepG2 cells increased the transcriptional activity of each construct approximately 3–4-fold (Figure 3) but had no effect on pRSVCAT transcription (data not shown). These data suggest that at least one of the putative HNF-3 sites between −400 and −220 is functional. Interestingly, overexpression of HNF-3 in HeLa cells did not stimulate promoter activity, implying that other factors are also necessary for the hepatocyte-specific expression of CYP7 (data not shown).

DNase I Footprint Analysis of the CYP7 Enhancer. The location of the three putative HNF-3 recognition sites and the modified SRE within the DNA fragment which functioned as an enhancer, and responded to overexpressed HNF-3, suggested that the positive transcriptional activity of this sequence may be mediated by the binding of trans-acting factors to these sites. To test this hypothesis, we performed DNase I protection assays using nuclear extracts isolated from rat liver and spleen. Extracts from liver protected a region from −340 to −317 and from −315 to −306 and caused the formation of a new hypersensitive site at position −276. Extracts from spleen protected a region from −315 to −306 (Figure 4). The common footprint between liver and spleen represents the most distal HNF-3 site. These two tissues are known to contain HNF-3 (J. Darnell, personal communication). Deletion of this site results in a 30% drop in transcriptional activity (Figure 3). The liver-specific footprint from −340 to −317 is just 5′ of this site and also corresponds to an area of functional significance. In addition, the newly created DNase hypersensitive site at position −276 and subsequent footprint (−276 to −260) occur in the area of the modified SRE. A deletion in this region (pBLCYP7-33) results in a drastic drop in transcriptional activity.

DISCUSSION

The major purpose of the experiments described here is to gain insight into the transcriptional regulation of the human CYP7 gene. This information will allow us to address questions concerning the cell-specific expression of CYP7 and the



	PLASMID	PROMOTER ACTIVITY	PROMOTER ACTIVITY WITH HNF-3
-1291	pBLCYP7	100	420±53
-921	pBLCYP7-24	101 ± 8	
-400	pBLCYP7-37	105 ± 5	390±42
-360	pBLCYP7-38	85 ± 9	
-333	pBLCYP7-26	79 ± 9	
-301	pBLCYP7-39	49 ± 8	
-276	pBLCYP7-33	4 ± 2	
-243	pBLCYP7-47	2 ± 1	
-126	pBLCYP7-34	0	
-432 -220	pBLCYP7-36	352 ±47	1156±167
-220 -432	pBLCYP7-50	84 ±14	

FIGURE 3: Schematic representation of the pBLCYP7 plasmids and their corresponding CAT activity. CAT activity is presented as the percent of pBLCYP7 and represents the mean of 3–6 experiments ± the standard deviation.

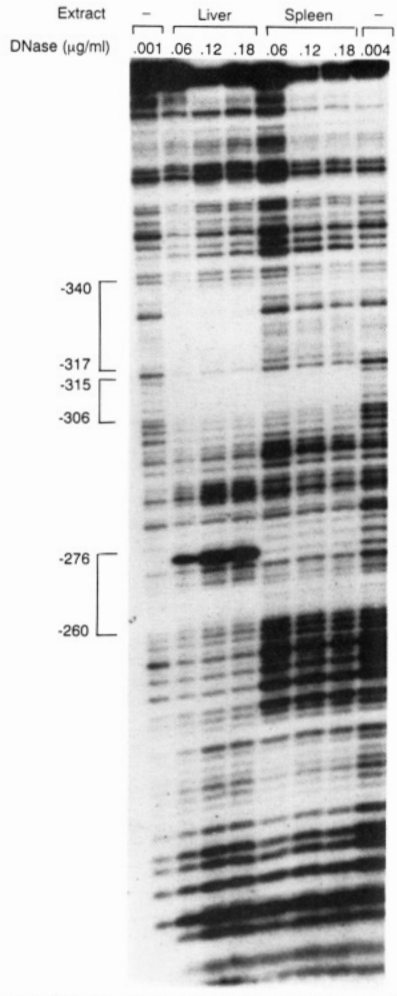


FIGURE 4: DNase I footprint analysis of the human CYP7 enhancer. A DNA fragment from pBLCYP7-36 (−432 to −220) was 5′-end-labeled and used as template in a footprint reaction as described under Experimental Procedures. The labeled fragment was incubated in the presence or absence of nuclear extract for 30 min at 0 °C and subjected to DNase I digestion at the indicated concentration. Regions of protection were determined by comparison to a DNA sequencing ladder and are designated by the bracketed regions.

regulation of cholesterol catabolism. Our approach was to clone a small 5′-portion of the human CYP7 cDNA and to use this probe to isolate the 5′-flanking region of the human CYP7 gene. We then structurally characterized the promoter and rigorously analyzed the function of this sequence of DNA. Our results have provided some preliminary answers to the question of the hepatocyte-specific expression of CYP7.

A measurement of the steady-state levels of CYP7 mRNA in the rat demonstrated that its expression is exclusive to the liver (Jelinek et al., 1990). To investigate the molecular mechanisms which regulate the hepatocyte-specific expression of CYP7, we analyzed the transcriptional activity of the human CYP7 promoter in several cell lines. As the in vivo data would predict, the promoter was inactive in cells of nonhepatic origin but quite active in the human hepatoma HepG2 (Table I). We further analyzed the transcriptional activity of this promoter by making a series of deletion mutants and testing their ability to stimulate transcription. Our results clearly indicate that this promoter is complex. The data imply that the functional elements of the promoter required for the cell-specific transcription of CYP7 reside within the first 400 nucleotides.

It is interesting to note that the DNA sequence between −432 and −220, which appears to confer cell specificity on the transcription of human CYP7, shares only 51% homology with the rat CYP7 promoter sequence and that several of the relevant regulatory elements are absent in the rat. It may be that similar but not identical regulatory mechanisms are at work in the two species. It is clear, however, that CYP7 is regulated by the concentration of bile acids in the portal circulation in both rat and human liver (Jelinek et al., 1990, Reihner et al., 1989).

All three of the recognition sites for the liver-specific transcription factor HNF-3 and a sequence which is strikingly similar to the hamster LDL receptor SRE (Smith et al., 1990) are contained within the region from −432 to −220. HNF-3 is highly enriched in liver cell nuclear extracts, relative to those of other tissues, and has been demonstrated to be functionally important in the hepatocyte-specific expression of the mouse transthyretin and α_1-antitrypsin genes (Costa et al., 1988). HNF-3 binding sites have also been detected in the enhancers of the α-fetoprotein and albumin genes, two other hepatically expressed genes (Grayson et al., 1989). Our finding that the overexpression of HNF-3 in HepG2 cells greatly increases the transcriptional activity of the human CYP7 gene and that nuclear proteins from liver and spleen specifically protect the most distal HNF-3 site from DNase digestion clearly indicates that this protein contributes to the transcriptional activity of CYP7. However, the inability of HNF-3 to stimulate transcription in HeLa cells and the presence of two liver-specific footprints in the enhancer region provide evidence for the existence of other trans-acting factors which are required for the cell-specific expression of CYP7. Further mutational and biochemical analyses will more clearly define the role of these binding sites in CYP7 transcription.

Analyses of other genes involved in the maintenance of cholesterol homeostasis have uncovered what appears to be a common regulatory mechanism involving two tandem elements. It has been proposed that the suppression of the transcriptional activity of these genes by sterols is the result of a push–pull mechanism which is mediated by these tandem elements (Dawson et al., 1988). For example, in the LDL receptor gene, there is an SP1 site juxtaposed to the sterol response element (SRE). Although the protein which binds to the SRE has not been identified, it is presumed that in the presence of sterols, this factor disrupts the binding of SP1 to some degree and therefore represses the rate of transcription. It is possible that a similar mechanism is responsible for the end-product suppression of CYP7. Our finding that a modified SRE is positioned in between two putative HNF-3 binding sites is reminiscent of the human LDL receptor gene where the SRE is positioned in between two SP1 sites. A detailed mutational analysis of the human LDL receptor SRE has demonstrated that a C to A transversion, such as that which occurs in the CYP7 SRE, severely diminishes transcriptional activity within the context of the LDL receptor promoter (Smith et al., 1990). This brings into question the proposed activity of this sequence element in the human CYP7 promoter. However, it is reasonable to assume that the CYP7 SRE would not behave identically to the LDL receptor SRE given that CYP7 transcription increases upon cholesterol feeding and LDL receptor transcription decreases. Indeed, in contrast to the LDL receptor gene, we can detect a liver-specific footprint over the modified SRE whereas no binding activity has been detected with the LDL receptor SRE to date. We did not, however, detect binding of nuclear proteins to the two HNF-3 sites which flank the putative SRE. It is conceivable that under the conditions tested, the binding of HNF-3 is impaired. In vivo footprinting studies and transcription assays in transgenic mice will allow us to test the hypothesis that bile acids suppress

CYP7 transcription by interfering with HNF-3 binding to its recognition sites.

In conclusion, we have isolated and functionally characterized the 5′-flanking region of the human CYP7 gene. A detailed mutational analysis of this region indicates that the mechanisms underlying the transcriptional control of CYP7 are extremely complicated. Complete transcriptional activity requires the presence of a number of sequence elements which implies the involvement of multiple transcription factors. As a first step toward unraveling these complex regulatory mechanisms, we have demonstrated that the region between −432 and −220 functions as a cell-specific enhancer whose activity is controlled, in part, by HNF-3. Further analysis of the human CYP7 promoter will ultimately lead to a better understanding of the homeostatic mechanisms responsible for controlling the rate of cholesterol catabolism.

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