Characterization of Hepatic-specific Regulatory Elements in the Promoter Region of the Human Cholesterol 7α-Hydroxylase Gene*

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Cholesterol 7α-hydroxylase is the rate-limiting enzyme in the degradation of cholesterol to bile salts and plays a central role in regulating cholesterol homeostasis. The mechanisms involved in the transcriptional control of the human gene are largely unknown. HepG2 cells represent an appropriate model system for the study of the regulation of the gene. To identify liver-specific DNA sequences in the promoter of the human CYP7 gene, we first examined the DNase I hypersensitivity in the 5'-region of the gene. An area of hypersensitivity was observed in the region from −50 to −200 of the human gene in nuclei from transcriptionally active HepG2 cells, but was absent in transcriptionally inactive HeLa cell nuclei or in free DNA. Various 5'-promoter deletion constructs were made and transfected into HepG2 cells. About 300 base pairs of upstream sequence are required for high level promoter activity of the human CYP7 gene in HepG2 cells. DNase I footprinting of the hypersensitive region revealed nine protected sequences. Gel retardation experiments demonstrated binding of HNF-3 to the segment from −80 to −70 and of hepatocyte nuclear factor HNF-4 (and ARP-1) to the segment from −148 to −127 of the human CYP7 promoter. Deletion of either of these sites depressed promoter activity in HepG2 cells. A third region from −313 to −285 is bound by members of the HNF-3 family and acts as an enhancer. Additionally, the segment from −197 to −173 binds a negative regulatory protein that is present in Chinese hamster ovary cell extracts and in HepG2 cell extracts. These experiments define the key control elements responsible for basal transcription of the human CYP7 gene in HepG2 cells.

Cholesterol 7α-hydroxylase catalyzes the rate-limiting step in the pathway that leads to the catabolism of cholesterol to bile acids (for review, see Ref. 1). Cholesterol 7α-hydroxylase is a microsomal enzyme member of the cytochrome P-450 family. In human and rat, the major products of this metabolic pathway are cholic acid and chenodeoxycholic acid. Bile acids have a central role in regulating cholesterol homeostasis; the mechanisms involved in the transcriptional control of the human gene are largely unknown. HepG2 cells represent an appropriate model system for the study of the regulation of the gene. To identify liver-specific DNA sequences in the promoter of the human CYP7 gene, we first examined the DNase I hypersensitivity in the 5'-region of the gene. An area of hypersensitivity was observed in the region from −50 to −200 of the human gene in nuclei from transcriptionally active HepG2 cells, but was absent in transcriptionally inactive HeLa cell nuclei or in free DNA. Various 5'-promoter deletion constructs were made and transfected into HepG2 cells. About 300 base pairs of upstream sequence are required for high level promoter activity of the human CYP7 gene in HepG2 cells. DNase I footprinting of the hypersensitive region revealed nine protected sequences. Gel retardation experiments demonstrated binding of HNF-3 to the segment from −80 to −70 and of hepatocyte nuclear factor HNF-4 (and ARP-1) to the segment from −148 to −127 of the human CYP7 promoter. Deletion of either of these sites depressed promoter activity in HepG2 cells. A third region from −313 to −285 is bound by members of the HNF-3 family and acts as an enhancer. Additionally, the segment from −197 to −173 binds a negative regulatory protein that is present in Chinese hamster ovary cell extracts and in HepG2 cell extracts. These experiments define the key control elements responsible for basal transcription of the human CYP7 gene in HepG2 cells.

while their presence in the intestine facilitates the solubilization of dietary fats and is required for the absorption of cholesterol and fat-soluble vitamins. Because of the importance of these functions, bile acid synthesis in the liver is carefully regulated to maintain cholesterol homeostasis (1). To date, little is known about the molecular mechanisms that control cholesterol catabolism and bile acid synthesis.

The cDNAs and genes for cholesterol 7α-hydroxylase have been isolated from rat (2–4), human (5, 6), hamster (7), and mouse (8). CYP7 mRNA is found exclusively in the liver (9), making this gene a target for the study of the molecular mechanisms implicated in hepatic-specific gene expression. Work by several groups has demonstrated that CYP7 mRNA levels are modulated in cultured cells by a number of effectors. For example, in cultured rat hepatocytes (10) and human hepatoma (HepG2) cells, the addition of bile acids to the culture media suppresses CYP7 mRNA levels (11), and dietary cholesterol and dexamethasone increase CYP7 mRNA levels (12). In vivo, however, cholesterol feeding increases but dexamethasone reduces CYP7 mRNA levels in rats (12, 13). The elegant work by Lavery and Schibler (14) demonstrated that in rats, CYP7 gene expression follows a strict diurnal rhythm, with mRNA levels peaking in the evening; this phenomenon is controlled at the level of transcription by a specific transcription factor, DBP.

Our long-term goal is to elucidate the molecular mechanisms that operate to regulate transcription of the human CYP7 gene in the liver as well as those that promote modulation by diet and hormones. As a first step toward our goal, we have pursued the identification of liver-specific elements that regulate basal transcription of the human CYP7 gene in hepatic cells. HepG2 cells have been used successfully as a model system to study CYP7 gene expression in a number of laboratories (11, 15, 16). Up-regulation of CYP7 mRNA by cholesterol as well as down-regulation by bile acids have been demonstrated (11).

Our rationale has been to use DNase I hypersensitivity as a tool to map liver-specific elements that are relevant in vivo, followed by a thorough analysis of the underlying hepatic control elements in HepG2 cells prior to testing them in vivo animal models. Using this approach, we established that liver-specific promoter elements of the CYP7 gene lie between −213 and +1. Within this region are functional binding sites for the liver-enriched transcription factors HNF-3 (−80 to −70), HNF-4, and ARP-1 (−144 to −127). Furthermore, a ubiquitous transcription factor that binds to the region from −197 to −173 reduces promoter activity of the human CYP7 gene, and an enhancer resides within an HNF-3-like binding site at −300 to −293.

EXPERIMENTAL PROCEDURES

Plasmid Construction—All constructs were derived from plasmid pD230 20.5-1 (kindly provided by the late Dr. Mike Komaromy). This plasmid contains the segment from −780 to +133 of the human cho-
lesterol 7α-hydroxylase gene and was constructed by PCR\(^1\) using oligonucleotide primers derived from the GeneBank\textsuperscript{TM} data base sequence. The genomic sequence is flanked by BamHI sites, allowing it to be excised by digestion with BamHI. Construct –764CAT was made by insertion of the –764/+46 BamHI fragment into a pOCAT vector (17) that had been digested with BamHI and treated with calf intestinal phosphatase. Plasmid –764CAT was used as a template for the remaining 5′-promoter deletion plasmids. The 5′-primers used for amplification were as follows: –341 to –317 for the –341CAT construct, –313 to –289 for the –313CAT construct, –285 to –261 for the –285CAT construct, –268 to –244 for the –268CAT construct, –227 to –203 for the –227CAT construct, –213 to –189 for the –213CAT construct, –91 to –67 for the –91CAT construct, and –65 to –41 for the –65CAT construct. In every case, the sequence of the primer started with CGC

\[ \text{primer 1, from} \]

Prepare plasmid by ligation to the pOCAT vector that had been digested with BamHI. To prepare plasmid –313CAT (–80/–70), the following PCR primers were used: primer 1, from –313 to –292 (with the BamHI site at the 5′-end); primer 2, from –55 to –95, but without the sequence from –70 to –80; primer 3, from –95 to –55 without –80 to –70, and primer 4, from –55 to –95 with the BamHI site at the 5′-end. The first PCR utilized primers 3 and 4 and generated an intermediate product spanning from –95 to +46 without –80 to –70. The two intermediate PCR products were mixed, denatured, renatured, and used as primers on a third PCR to generate a product spanning from –313 to +46 without –80 to –70. This BamHI fragment was then cloned into pOCAT. The orientation of the insert was determined by PCR. Constructs –227CAT (–197/–173), –213CAT (–144/–127), and –313CAT (–500/–293) were made in a similar manner as the construct described above. Construct –313mut1 was made by introducing a primer in which five point mutations (CGTAC instead of AAACCA at –298 to –294) were made to disrupt HNF-3 binding. The DNA sequence of every promoter deletion construct was verified by sequencing.

**Tissue Culture and Transient Transfection Assays—**Human hepatoma cells (HepG2), Chinese hamster ovary (CHO) cells, and HeLa cells were grown as described previously (18). Transient transfections with the various plasmid constructs were performed by the calcium phosphate coprecipitation method as described previously (19) with 7\(2\)\(5\)\(2\) nucleotide primers derived from the GeneBank\textsuperscript{TM} data base sequence.

**DNase I Footprinting of the 5′-End of the Human CYP7 Gene**—The DNase I hypersensitivity studies suggest that hepatic-specific transcription factors bind to the region from –50 to –200, thus causing its hypersensitivity. Binding of hepatic-specific nuclear proteins to the 5′-proximal region of the human CYP7 gene was examined by DNase I footprinting. Nine protected regions were observed in the DNA segment from –341 to +46 using nuclear extracts from HepG2 cells (Fig. 2A and B). Footprint 1 extends from –35 to –48, footprint 2 from –54 to –62, footprint 3 from –67 to –81, footprint 4 from –91 to –104, footprint 5 from –129 to –144, footprint 6 from –174 to –191, footprint 7 from –213 to –227, footprint 8 from –268 to –285, and footprint 9 from –313 to –341. The protected regions have been evolutionarily conserved, as evidenced by a

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\(^1\) The abbreviations used are: PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; CHO, Chinese hamster ovary; bp, base pair(s).
correspondence among the footprints in human, mouse, and rat liver cells as shown in Fig. 2 (C and D), suggesting that the nuclear proteins that bind to these sequences have an important functional role in CYP7 gene transcription.

Functional Assays to Test the Role of the Footprints in Promoter Activity—The combined results from the hypersensitivity studies and DNase I footprinting strongly suggest that key liver-specific promoter elements reside in the 300 bp immediately upstream of the transcriptional start site of the CYP7 gene. This hypothesis was tested as follows. Several constructs were made in which 5'-segments of the human CYP7 gene of varying lengths were cloned upstream of the reporter CAT gene, and their promoter activity was tested in transient transfection assays in HepG2 cells. A schematic illustration of the constructs and their promoter activities are shown in Fig. 3.

The construct with the largest 5'-extension was −764CAT, followed by −341CAT. To elucidate the potential functional role of the footprinted regions, subsequent constructs were designed to exclude either one footprint at a time or the region between two footprints. Transient transfections into HepG2 cells revealed a small but reproducible reduction of CAT activity (−20%) upon deletion of sequences between −764 and −341, suggesting that weak positive control elements reside in this region. On the other hand, a weak negative element may reside between −341 and −313 (footprint 9), as judged by the 30% increase in CAT activity of the −313CAT construct as compared with the −341CAT construct. Deletion of the segment from −313 to −285 caused a 40% decrease in CAT activity, suggesting that this segment may contain binding sites for a positive regulatory element. Further deletions from −285 to −227 and from −227 to −213 increased CAT activity by 30% each, respectively, suggesting that footprints 7 and 8 may harbor binding sites for negative regulatory elements. The −213CAT construct exhibited the highest promoter activity, suggesting that most of the important elements are localized within 213 bp upstream of the start site, in agreement with the DNase I data of Fig. 1. Deletion of the segment from −213 to −91 that includes footprints 4–6 caused a 40% reduction in promoter activity, suggesting the removal of a positive element. The activity of the −65CAT deletion was 2-fold lower than that of the −91CAT construct, suggesting that a positive element resides between −91 and −65. Therefore, from the data in Fig. 3, we conclude that the segment from −213 to +1 contains the minimal regulatory elements required for transcription from

**Fig. 2.** DNase I footprinting of the 5'-flanking region of the human CYP7 gene. In A, lane 1 shows the reaction in the absence of nuclear extract from HepG2 cells. Lanes 2–4 show the data with increasing amounts of nuclear extract from cells exposed to serum-depleted medium for 24 h. Lanes 5 and 6 show the footprints generated with nuclear proteins from HepG2 cells grown in complete medium. The positions of footprints (FP) 4–9 are indicated by the open boxes to the right of the panel, with the nucleotides at the start and end of each footprint also indicated. In B, the first two lanes show the Maxam and Gilbert sequencing reactions. Lane 1 shows the reaction in the absence of nuclear extract. Lanes 5–7 show the data with increasing amounts of HepG2 extract. Footprints 1–3 are shown to the right of the panel. In C, the layout is similar to that in B, except that different amounts of mouse liver extract were used in lanes 3 and 4. Lanes 1 and 2 show the reaction in the absence of nuclear extract. In D, lanes 1, 2, and 8 represent control reactions in the absence of nuclear extract. HepG2 nuclear proteins were used in lane 3, and rat liver extract in lanes 4–7. The footprints are shown to the right of the panel, using the same labeling system as for A.
the human CYP7 promoter in transient assays in HepG2 cells.

**Gel Retardation Experiments Identify the Nuclear Factors Involved in CYP7 Promoter Activity**—The decline in transcriptional activity observed when sequences between −213 and −91 were deleted (Fig. 3) suggests that DNA sequences in this region play a key role in basal promoter activity. Three nuclear protein-binding sites, namely footprints 4–6, are located in this region.

To identify the protein factors responsible for footprints 4–6, gel retardation experiments were conducted. A double-stranded oligonucleotide corresponding to the segment from −2197 to −2173 (representing footprint 6) was incubated with nuclear extracts from HepG2 and CHO cells (Fig. 4). Two specific complexes were formed with HepG2 extracts (lane 1) that were competed for by an excess of unlabeled oligonucleotide (lane 2). The same specific complexes were seen when using CHO extracts (lanes 3 and 4), suggesting that the transcription factor binding to this region may be ubiquitous. The specific complex of lower molecular weight may represent a proteolytic fragment of the major binding protein since the intensity of this band varied from extract to extract. However, we cannot rule out the possibility that two different proteins bind to this sequence. An excess of unlabeled oligonucleotides representing in each case the binding site for a known transcription factor such as HNF-1, C/EBP, HNF-3, and HNF-4 failed to abolish complex formation (data not shown). Comparison of the DNA sequence from −2148 to −2127 to a data base of transcription factor-bindings sites revealed only some similarity to the binding site of the GATA factor that plays a role in erythroid expressed genes (25, 26).

The segment from −148 to −127 (representing footprint 5) exhibited sequence similarity to the binding sites for the liver-enriched transcription factors HNF-4 (27) and ARP-1 (28). These two proteins bind to the same DNA sequence with different affinities. When the labeled −148/−127 oligonucleotide was incubated with an extract from COS cells that had been transfected with an HNF-4 expression vector (Fig. 5, lane 2), we observed a complex that was not detected with control extract. This complex was competed for by an excess of unlabeled oligonucleotide (lane 3), confirming its identity as the HNF-4 complex. A weaker but specific HNF-4 DNA complex was also detected with HepG2 nuclear extracts (lanes 6 and 7). When the −148/−127 probe was incubated with an extract from COS cells that had been transfected with an ARP-1 expression plasmid, we observed formation of a retarded complex (lane 4), not evident with control extract, that was also specific (lane 5). However, the affinity of ARP-1 for the footprint 5 sequence appears to be severalfold lower than the affinity of HNF-4 for that same sequence.

Incubation of an oligonucleotide representing footprint 4 with nuclear extracts yielded one weak specific complex of high molecular weight (data not shown). Computer analysis of footprint 4 revealed no similarities to the binding sites of known transcription factors.

**Analysis of the Function of the Footprinted Sequences**—To ascertain the functional importance of the proteins binding to footprints 5 and 6, the effect of deleting these regions upon promoter activity was determined. The −144/−127 deletion (footprint 5) was made in the context of the −213CAT construct, and the −197/−173 deletion (footprint 6) was made in

**Fig. 3. Transcriptional activities of various promoter deletion constructs.** The left part of the figure shows the deletion constructs, with the 5′-end of each construct indicated on the left side. The transcriptional start site is shown as +1, with the arrow indicating the direction of transcription. The boxes represent the nine footprints described in the legend to Fig. 2. The fusion to the CAT gene begins at +46. The table on the right shows the absolute CAT activity of each construct expressed relative to the activity of the −764CAT construct, which was given the value of 1.0. The data are expressed as the mean ± S.D., with the number of independent transfections shown in parentheses.
the context of the −227CAT construct (Fig. 6). Deletion of the sequence from −197 to −173 (footprint 6) increased promoter activity by 2-fold, suggesting that it binds a negative regulatory element. On the other hand, deletion of the −144/−127 segment (footprint 5) decreased CAT activity by −2.5-fold, indicating that the protein(s) binding to footprint 5 also play a role in the transcriptional activation of the CYP7 gene. Having demonstrated that both HNF-4 and ARP-1 can bind to footprint 5 (Fig. 5), we examined the effect of an excess of each of these transcription factors upon the CAT activities of the wild-type and deletion constructs. To this end, the reporter constructs were cotransfected with expression plasmids for either HNF-4 or ARP-1. Cotransfection of the wild-type −213CAT construct with HNF-4 failed to alter promoter activity, suggesting that HNF-4 may not be limiting in our HepG2 cells. As expected, the additional HNF-4 protein did not affect the CAT activity of the −213CAT construct in which the HNF-4 site had been deleted (Fig. 6). An excess of ARP-1 protein reduced the transcriptional activity of the wild-type −213CAT construct by 2-fold, a result consistent with the fact that ARP-1, when binding to its cognate sequence, usually exerts a repressor effect (28). As expected, an excess of ARP-1 protein had no effect in the absence of the ARP-1-binding site.

These data suggest that both HNF-4 and ARP-1 can bind to the −144/−127 region. When HNF-4 binds, it promotes activation of transcription; when ARP-1 binds, transcription is decreased. This is analogous to the situation in the proximal promoter of the human apoB gene, where an HNF-4 (ARP-1) site resides (20).

**Role of HNF-3 in CYP7 Promoter Activity**—Computer analysis of the sequence of the proximal promoter region of the human CYP7 gene revealed a perfect match to the HNF-3-binding site (TGTTTGCT) (20) in the segment from −80 to −70, a sequence that is 100% conserved among the human, rat, and mouse genes. Binding of nuclear proteins from HepG2 cells and mouse liver to an oligonucleotide encompassing this sequence (−88 to −65) was examined. In Fig. 7, we observe that the labeled −88/−65 oligonucleotide forms two major retarded complexes with HepG2 extracts, designated as A and B (lane 3). The mobility of these complexes is identical to that of complexes A and B formed between a labeled oligonucleotide representing a consensus HNF-3-binding site and HepG2 nuclear proteins (lane 1). Specificity of binding is established in lane 2. Furthermore, the −88/−65 probe also formed two specific complexes with mouse liver nuclear extracts (lane 4) that were competed for by the homologous oligonucleotide (lane 5) as well as by the HNF-3 consensus oligonucleotide (lane 6). The HNF-3 consensus oligonucleotide formed retarded complexes with mouse liver proteins that were identical in mobility to those formed by the −88/−65 oligonucleotide (lane 7) and were specific (lane 8) and also competed for by the −88/−65 oligonucleotide (lane 9). These observations confirm that HNF-3 does bind to the −88/−65 segment of the CYP7 promoter.

The functional significance of this binding was studied by making a promoter construct in which the HNF-3 site at −80 to −70 was deleted in the context of the −313CAT construct (Fig. 8). The −313CAT construct has an activity of 1.10 as compared with the −764CAT construct. Deletion of the −80/−70 segment reduced promoter activity by 3-fold, demonstrating that binding of HNF-3 to this sequence is important for promoter activity in HepG2 cells. When an HNF-3α expression vector was cotransfected with our constructs, the activity of the wild-type −313CAT construct was 5-fold higher, implying that HNF-3α is limiting in HepG2 cells. When the region from −80 to −70 was deleted from the construct, transcriptional activity in the presence of an excess of HNF-3α was again reduced, thus underscoring the importance of this region in hepatic transcription of the CYP7 promoter.

Based on computer analysis, there are three other potential
HNF-3-binding sites within the region from −320 to −240 of the human CYP7 promoter, namely −316 to −306, −288 to −278, and −235 to −243 (29). The footprinting data of Fig. 2 revealed a protected region from −285 to −268. However, deletion of this segment did not affect promoter activity (data not shown), suggesting that the putative HNF-3 site at −285 to −268 is not functional in our assays. Similarly, the segment from −255 to −245 was not protected by nuclear proteins from liver or HepG2 cells (Fig. 2), suggesting that HNF-3 may not bind to that sequence, and deletion of the region from −268 to −227 did not significantly affect the activity of the human CYP7 promoter. To evaluate the possible role of HNF-3 in these regions, we cotransfected our promoter deletion constructs together with an HNF-3α expression vector (Fig. 9). The transcriptional activity of the −764CAT, −341CAT, and −313CAT constructs was 5–7-fold higher in the presence of excess HNF-3α. On the other hand, the activity of the −285CAT construct dropped 2-fold as compared with that of the −313CAT construct in the presence of cotransfected HNF-3α, suggesting that a functional HNF-3-binding site may reside in the segment from −313 to −285 and that the putative site at −243 to −235 may not be functional. Examination of the DNA sequence from −313 to −285 revealed two (5 out of 8 bp) matches to the HNF-3 recognition sequence (−297 to −293 and −290 to −286). The sequence of this segment of the human CYP7 gene is shown in Fig. 10A, with the two 5-bp matches to the HNF-3 recognition sequences indicated by brackets.

Binding of the −313/−285 sequence to nuclear proteins from HepG2 cells was examined. As shown in Fig. 11A, the −313/−285 oligonucleotide probe formed five specific retarded complexes with HepG2 nuclear proteins, namely complexes A, B, C, and E and a lower molecular weight complex (lanes 1 and 2). The HNF-3 consensus oligonucleotide from the transthyretin promoter competes for formation of complexes A, B, and C, but not complex E or the smaller complex (lane 3). On the other hand, the HNF-3 consensus probe forms two major specific retarded complexes with HepG2 proteins, namely complexes C and D, and a lower molecular weight complex migrating between the nonspecific complex and complex E (lanes 4 and 5) that are partially competed for by the −313/−285 oligonucleotide (lane 6).

These data suggest that the −313/−285 segment does bind members of the HNF-3 family. The question arises as to whether these DNA/protein interactions are functionally significant. To elucidate the functional role of these sequences, we first mutagenized the putative HNF-3-binding site centered at −297 to −293 by altering those 5 bp as shown for the −313mut-1 construct in Fig. 10A. The CAT activity of the −313mut-1 construct was 3-fold lower than that of the wild-type construct, thus validating the importance of those 5 bp in the transcriptional activity of the human CYP7 promoter (Fig. 10B). The involvement of HNF-3α in binding to this segment was demonstrated by cotransfection of the wild-type and mutant −313CAT constructs with an HNF-3α expression plasmid. The transcriptional activity of the −313mut-1 construct in the presence of an excess of HNF-3α was reduced by >2-fold as compared with the wild-type construct (Fig. 10B). In Fig. 11B, the DNA binding properties of the −313mut-1 and wild-type sequences are compared. As shown above in Fig. 11A, the wild-type oligonucleotide forms four major specific retarded complexes with HepG2 nuclear proteins, namely complexes A, B, C, and E, with complex B being the strongest (lanes 1 and 2). The mutant oligonucleotide as well as the HNF-3 consensus oligonucleotide compete mainly for the formation of complex C.
The -313mut-1 oligonucleotide forms two major specific complexes, complex C and a lower molecular weight complex (lanes 5 and 6). Interestingly, both the HNF-3\(\alpha\) oligonucleotide and the wild-type -313/-285 sequence compete for formation of complex C (lanes 7 and 8). The HNF-3\(\alpha\) oligonucleotide forms complexes C and D (lanes 9 and 10).

Thus, a 5-bp mutation in the segment from -297 to -293 disrupts binding of the proteins responsible for the formation of complexes A and B, but not complex C, suggesting that the second HNF-3 site at -290 to -286 may be responsible for complex C formation and for the residual CAT activity of the -313mut-1 construct (Fig. 10). In parallel experiments, we deleted the segment from -300 to -293 in the context of the -313CAT construct. Gel retardation experiments with the -313CAT\((-300/-293)\) oligonucleotide revealed no retarded complexes (data not shown), suggesting that the 8-bp deletion can disrupt binding of nuclear proteins to the adjacent -290/-286 sequence. Transfections of the wild-type and mutant -313CAT\((-300/-293)\) constructs into HepG2 cells revealed a 5.5-fold decrease in CAT activity of the deletion construct as compared with the wild-type construct, thus confirming that the HNF-3 site at -300 to -293 is important for CYP7 promoter activity (Fig. 10). Cotransfection of the HNF-3\(\alpha\) expression plasmid with the wild-type and -313CAT deletion constructs yielded similar results, i.e. a 4-fold reduction in promoter activity of the deletion construct.
In summary, mutagenesis or deletion of the segment from -300 to -293 in the context of a -313CAT promoter construct severely impairs promoter activity, demonstrating that protein binding to this segment is functionally significant. Results of the gel retardation experiments show that members of the HNF-3 family of transcription factors are involved in binding to the -313/-285 region and thus play an important functional role in CYP7 gene regulation in HepG2 cells. Our overall data can be reconciled by postulating that, in transient assays, -213 bp of upstream sequence are required for basal promoter activity of the human CYP7 gene. However, in the context of the entire gene, the sequence from -313 to -285 binds HNF-3 and related proteins, and it functions as a transcriptional enhancer because deletion or mutagenesis of the HNF-3 sequence at -300 to -293 severely reduces transcription.

DISCUSSION

The mechanisms by which expression of the CYP7 gene is regulated are worthy of study because of the important role that cholesterol 7α-hydroxylase plays in regulating overall cholesterol homeostasis. However, information available to date is fragmentary. Using transgenic mice, Ramirez et al. (30), using large constructs encompassing -1633 bp of the rat CYP7 gene 5'-upstream region, ligated to a mouse albumin enhancer and linked to the reporter LacZ gene, demonstrated that the regulation of the reporter gene by bile salts that is observed in vivo can be reproduced. However, in the absence of the mouse albumin enhancer, expression of the reporter gene in the liver was not detectable. Using a line of transformed cultured mouse hepatocytes and stable transfections, a liver-specific enhancer was localized some 7 kilobase pairs upstream of the transcriptional start site of the rat CYP7 gene and was required for high level transcription from the CYP7 promoter. But, this enhancer may not function in vivo in transgenic mice. Using HepG2 cells and transient transfection assays, Molowa et al. (31) implicated the transcription factor HNF-3 as playing a role in transcription of the human CYP7 gene by binding to a site located between -432 and -220. Chiang and Stroup (15), studying the proximal promoter region of the rat CYP7 gene, described two regions protected from DNase I digestion that they designated footprints A and B. Rat footprint A (-81 to -35) corresponds to human footprints 1–3 of Fig. 2, and footprint B is equivalent to our footprint 5. These investigators have suggested (based on computer analysis of the DNA sequence) that HNF-3, C/EBP, HNF-4, retinoic acid receptor, COUP, and glucocorticoid receptor may bind to the rat promoter region and influence transcription. They have also proposed that recognition sites for binding proteins that respond to corticosteroids as well as insulin and bile acids may also reside in this region. Lavery and Schibler (14) have shown that the liver-enriched basic leucine zipper protein DBP binds to an element centered at -225 of the rat CYP7 gene and plays an important role in the circadian transcription of the gene. A similar sequence is present in footprint 7 (-227 to -213) of the human gene (Fig. 2). Thus, the apparent complexity and multiplicity of interactions among various transcription factors that may regulate this gene warrant a comprehensive and systematic approach.

Our long-term goal with the human CYP7 gene is to identify all of the control elements that regulate this gene and that modulate its in vivo expression. Encouraged by our earlier results with the apoB gene (30) and by the suitability of HepG2 cells as a model system for hepatic-specific expression of the CYP7 gene (11, 32, 33), we began our studies by identifying a key DNase I-hypersensitive region in the segment from -50 to -200 of the human CYP7 gene. This DNase I-hypersensitive region is absent in cells in which the CYP7 gene is transcriptionally inactive, such as HeLa cells, and in free DNA, suggesting that it is the chromatin structural features of the CYP7 gene that make this region open and available for interaction with transcription factors.

Transfections of 5'-CAT deletion constructs illustrated in Fig. 3 yielded results that were consistent with those from the DNase I hypersensitivity studies. Together, both approaches
point to the location of key hepatic-specific elements between −213 and +1. Within this region, we found functional binding sites for HNF-3 (−80 to −70), HNF-4 (and/or ARP-1) (−144 to −127), and a ubiquitous transcription factor (−197 to −173) present also at high concentrations in CHO cell extracts. Deletion of either the HNF-3 or HNF-4 sites reduced promoter activity, while deletion of the −197/−173 sequence increased promoter activity. The HNF-3 and HNF-4 sites are also present in the rat gene, but their functional role has not been determined. HNF-4 and HNF-3 may act synergistically, as has been shown to occur in the promoter of another liver-specific gene, the apoA-I gene (34). The negative influence of the sequence from −197 to −173 upon transcription is of interest. It is not uncommon to find that a ubiquitous factor, working in conjunction with other cell-specific factors, plays a key role in transcriptional activation or repression. For example, in the human apoC-III gene, an interaction between SP1, bound to distal regulatory sites, and HNF-4, respectively. We also thank Rick Cuevas for assistance in the preparation of this manuscript and to Drs. Alan R. Brooks and Brian J. McCarthy for comments on the manuscript.

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