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Hepatocyte Nuclear Factor 3 Is a Major Determinant of *CYP2C6* Promoter Activity in Hepatoma Cells

PETER M. SHAW, MARY C. WEISS, and MILTON ADESNIK

Department of Cell Biology, New York University School of Medicine and The Kaplan Cancer Center, New York, New York 10016 (P.M.S., M.A.), and Unite de Genetique de la Differentiation, URA 1149 du Centre National de la Reserche Scientifique, Institute Pasteur, 75015, Paris, France (P.M.S., M.C.W.)

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SUMMARY

Cytochrome P450 2C6 (CYP2C6) is a developmentally regulated, constitutively expressed form of rat liver microsomal cytochrome P450 that in the liver of adult male rats is induced to a limited extent by phenobarbital. The gene is not expressed at detectable levels in the lung, kidney, or brain. It is expressed and inducible by phenobarbital in differentiated Reuber hepatoma cells that express many hepatocyte-specific genes but not in dedifferentiated derivatives lacking the majority of hepatocyte-specific functions. A 505-base pair proximal segment of the *CYP2C6* promoter is highly efficient in driving transcription of a linked chloramphenicol acetyltransferase reporter gene in the differentiated rat hepatoma cell line FGC4, is much less effective in a related dedifferentiated variant H5, and has no measurable activity in nonhepatic C33 human cervical carcinoma cells. The activity of the *CYP2C6* promoter in the differentiated hepatoma cells is strongly dependent on hepatocyte nuclear factor (HNF)3, which acts at a complex site just upstream of the TATA motif. *Trans*-activation experiments show that the D-site-binding protein (DBP) may also contribute to *CYP2C6* promoter activity, via a site that is adjacent to the proximal HNF3 site. A substantial contribution to promoter activity by the base pair −505 to −316 segment is observed in FGC4 and H5 cells but not in HepG2 cells; deletion of this segment causes a marked diminution in promoter activity only in the former two cell lines. Although footprinting experiments have permitted the definition of three protein binding sites in this region (two HNF3 and one unidentified), mutation of these sites does not diminish promoter activity. The functionally important *cis* sequences in this region therefore remain to be defined. In HepG2 cells the distal region does not contribute to promoter activity. This most likely accounts for the low promoter activity in HepG2 and implies a deficiency in the relevant *trans*-acting factor(s).

The hepatic microsomal P450s are members of a superfamily of monooxygenases that function in the oxidative metabolism of a wide variety of endogenous and exogenous substrates and play a central role in the detoxification of drugs and the metabolic activation of carcinogens (1–3). More than 20 different forms of P450 with characteristic broad but overlapping substrate specificity have been detected in rat liver (4). Although initial studies on these P450s focused on enzymatic forms that are induced to high levels after treatment with various specific inducing compounds, recently there has been much interest in those forms that are constitutively expressed. Many of these constitutive P450s are interesting from a regulatory perspective because their expression may manifest developmental and hormonal regulation as well as tissue specificity of expression (1, 2, 3, 5).

CYP2C6 is a constitutively expressed form of rat liver P450. Phenobarbital treatment of adult rats, however, does lead to a 2–4-fold increase in CYP2C6 hepatic mRNA levels (6). In addition, for the organs examined in adult male rats, CYP2C6 mRNA is expressed in the liver but not in the brain, kidney, or lung, although it is expressed in the brains of adult female rats (7). Moreover, CYP2C6 mRNA is developmentally regulated in the liver, with significant levels being present only 3 weeks after birth (8). The liver-specific expression of the *CYP2C6* gene is also manifested in cell culture; its mRNA is present in cells of differentiated rat hepatoma clonal lines derived from the Reuber hepatoma but not in dedifferentiated variants of these cells (9).

The differential expression of the various P450s and other detoxifying enzymes within hepatocytes and the modulation of their levels by both endogenous (e.g., hormones) and exogenous (xenobiotic inducers) factors are, to a great extent, controlled

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ABBREVIATIONS: P450 or CYP, cytochrome P450; bp, base pair(s); CAT, chloramphenicol acetyltransferase; C/EBP, CCAAT/enhancer-binding protein; CMV, cytomegalovirus; DBP, D-site-binding protein; DEI, distal element I; EMSA, electrophoretic mobility shift assay; HNF, hepatocyte nuclear factor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SVe, simian virus early region; SV40, simian virus 40; TTR, transthyretin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PCR, polymerase chain reaction.

at the level of gene transcription (Refs. 8 and 10 and a recent review in Ref. 5). Although little information is currently available, the hepatocyte-specific transcription of the genes for these enzymes is likely to depend critically on hepatocyte-enriched transcription factors known to act in concert to sustain the transcription of genes encoding other specific products of the hepatocyte (for reviews, see Refs. 11 and 12).

In the work presented here, we have used a combination of transient expression experiments with hepatoma and nonhepatic cell lines, DNase I footprinting assays, and EMSAs to identify *cis* elements that determine the hepatocyte-specific expression of the *CYP2C6* gene and to identify the *trans*-acting factors that recognize them. Moreover, the availability of expression plasmids encoding various hepatocyte-enriched transcription factors has made it possible to demonstrate directly, in cotransfection experiments, the ability of individual transcription factors to contribute to the activity of the promoter.

Materials and Methods

Cell culture and transfection. FGC4 (13), H5 (14), and HepG2 (15) cells were maintained in modified Ham's F-12 medium supplemented with 5% fetal calf serum, as described previously (14, 16). Because it is well known that HepG2 cells that are grown in different laboratories have very different properties, it seems appropriate to specify that the HepG2 cells used here were received directly from Dr. B. Knowles (Jackson Laboratories, Bar Harbor, ME). They grow extremely slowly (generation time, 3–4 days) and form tightly confluent, strictly epithelial monolayers, with no evidence of rounded clusters of cells. After thawing, stocks were maintained for 2 months only (at which time rounded cells began to appear). Human C33 cervical carcinoma cells (17) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were transfected by the calcium phosphate precipitation procedure and harvested as described previously (18). Briefly, cells were plated 1 day before transfection, FGC4 cells at 1×10^6/10-cm dish, H5 cells at 0.5×10^6/10-cm dish, and HepG2 cells at 1×10^6/6-cm dish. As specified in the figure legends, between 5 and 20 μg of precipitated DNA, including 0.5–2 μg of pCMVβ (a plasmid that encodes *Escherichia coli* β-galactosidase to permit corrections for variations in transfection efficiency and cell protein recovery), were added to monolayer cultures overnight. The following morning, cells were washed three times with serum-free medium and renewed with medium-containing serum. Forty-eight hours later, the cells were harvested by trypsinization followed by centrifugation. Crude cytosolic extracts were prepared from the cell pellet by 'freeze-thaw lysis' and subsequent centrifugation at 14,000 rpm at 4° for 10 min in a benchtop centrifuge and were assayed for CAT and β-galactosidase activities as described previously (19).

In transfection experiments that made use of expression plasmids encoding transcription factors, the total amount of DNA was kept constant in the controls by including an equal amount of the expression vector plasmid that did not contain the transcription factor cDNA insert. This ruled out the possibility that the results were biased by the expression vector competing with the reporter plasmid for common transcription factors.

DNase I footprint analysis and EMSAs. Radiolabeled fragments were prepared by filling in *CYP2C6* promoter fragments, obtained after restriction endonuclease cleavage, using the Klenow fragment of DNA polymerase in a reaction mixture containing the various deoxyribonucleotides and either [α-^{32}P]dCTP or [α-^{32}P]dATP. The DNA was then digested with a second endonuclease and the desired end-labeled fragment was purified by polyacrylamide gel electrophoresis. In a typical binding reaction for footprinting, 1 ng of end-labeled fragment was incubated for 120 min on ice in a final volume of 25 μl containing 15 mM HEPES, pH 7.6, 44 mM KCl, 0.1 mM EDTA, 0.35 mM dithiothreitol, 3.5% glycerol, 5 mM $MgCl_2$, 5 μg of poly(dI·dC), and 20–30 μg of rat nuclear extract prepared from animals sacrificed at 10 a.m. and therefore not containing DBP binding activity (20), as described previously (21). After treatment with DNase I, the reaction mixture was extracted with phenol/chloroform and the DNA was recovered by ethanol precipitation and analyzed on a 6% denaturing polyacrylamide sequencing gel. In competition experiments, double-stranded oligonucleotides were included in the binding reaction at a 100-fold molar excess over the end-labeled fragment.

For EMSAs, double-stranded oligonucleotides were end-labeled with [γ-^{32}P]dATP and T4 polynucleotide kinase. Labeled oligonucleotide (0.2 ng) was incubated for 10 min on ice with either 5 μg of nuclear extract prepared from FGC4 cells (22) or 5 μg of reticulocyte lysate extract, kindly provided by Dr. E. Lai (Memorial Sloan-Kettering Cancer Center, New York, NY), in a final volume of 14 μl containing 10 mM HEPES, pH 7.9, 10% glycerol, 9 mM magnesium acetate, 9 mM spermidine, 1 mM sodium phosphate, 0.2 mM EDTA, 0.05 mM EGTA, 1.5 μg of poly(dI·dC), and 0.5 μg of salmon sperm DNA. The protein-DNA complexes were resolved by electrophoresis in a 6% polyacrylamide gel using 22.5 mM Tris, 22.5 mM boric acid, 0.5 mM EDTA, as a buffer. The gel was then fixed in 10% methanol/10% acetic acid and dried before exposure to X-ray film. In competition experiments, nonradioactive double-stranded oligonucleotides were included in the incubation at a 100-fold molar excess over the labeled oligonucleotide.

Double-stranded oligonucleotides synthesized or obtained from others for these experiments included the following: oligo 1, $^{-68}$GATTAGTCAATTATGCAATATTGATTCAGCAGCC^{-35}; oligo 3, $^{-411}$GAGGGCTAATATCCAAAATCTATAAAGAACTCAA^{-378}; oligo 4, $^{-434}$TTTCACTAACTTGACCTCTGATAGAGGGC^{-408}; oligo 5, $^{-487}$CAAAGGCACATAGTCAATAGGGAATATC^{-460}; DEI, TTCTTTTTGGCAAGGATGGTATGATT; HNF3, GTTGACTAAGTCAATAATCAGAATCAG; HNF4, GCGCTGGGCAAAGGTCACCTGC. The HNF3 and HNF4 double-stranded oligonucleotides contained, in addition, a 5′ overhang of GATC and TCGA, respectively. The DEI sequence was from the proximal albumin promoter (23), the HNF3 sequence from the TTR promoter (24), and the HNF4 sequence from the human apolipoprotein CIII gene (25).

Construction of chimeric and mutant plasmids. Initially, a 5′ flanking segment of the *CYP2C6* gene that extended from bp −1246 to bp +14 was reconstructed by removing an *Eco*RI-*Hph*I fragment from a *CYP2C6* genomic clone (26), kindly provided by F. Gonzalez (National Institutes of Health, Bethesda, MD), and attaching *Bgl*II linkers after blunt ending. This fragment was cloned into the *Bgl*II site of pBLCAT3 (27). From this clone, a bp −1246 to bp −45 *Bgl*II-*Hinf*I fragment was removed and ligated to a synthetic double-stranded oligonucleotide that encompassed the natural genomic sequence extending from bp −44 to bp +14 and containing a cohesive *Hinf*I site at the 5′ end and a cohesive *Bgl*II site at the 3′ end. The ligated fragment was cloned into the *Bgl*II site of the reporter plasmid pBLCAT3. The *CYP2C6* 505-bp promoter construct was made by *Hind*III digestion of the 1246-bp *CYP2C6* promoter-CAT plasmid, to remove the 5′ portion of the promoter fragment that extended to the *Hind*III site at bp −505, and ligation of the remaining portion of the plasmid. Subsequent 5′ deletions to form 316-bp and 110-bp promoter constructs were made by digestion of the *Bgl*II 1246-bp insert with *Bgl*II-*Mae*III and *Bgl*II-*Mae*I, respectively, isolation of the required fragment, addition of *Bgl*II linkers, and subsequent ligation into the *Bgl*II site of pBLCAT3.

Base cluster mutations were made by designing two complementary oligonucleotides spanning the region to be mutated, which included at least six mismatches to the natural sequence and also contained a *Pst*I or *Not*I site. Two separate PCRs were set up, the first using a sense primer that spanned the *Hind*III restriction site at the 5′ end of the 505-bp *CYP2C6* promoter-CAT construct and as antisense primer the lower strand mutant oligonucleotide and the second containing an antisense primer matching a region in the CAT coding region and as sense primer the upper strand mutant oligonucleotide. These PCRs generated the 5′ and 3′ portions of the 505-bp *CYP2C6* promoter fragment that overlapped and contained either a *Pst*I or *Not*I site encompassing the mutation at their overlapping ends. The 5′ fragment

PCR mixture was digested with *Hind*III and either *Pst*I or *Not*I, whereas the 3′ fragment reaction mixture was digested with *Xho*I (to cleave in the polylinker of pBLCAT3) and either *Pst*I or *Not*I. The fragments were isolated by agarose electrophoresis, purified using Geneclean (Bio 101 Inc., La Jolla, CA) according to the manufacturer's instructions, mixed, and ligated with pBLCAT3 that had been digested with *Hind*III and *Xho*I. Sequence analysis of the PCR-constructed mutants revealed a substitution of a guanine for an adenine at base −410 in mutant 3. This base change did not appear to change other footprints at sites 4 and 5 (see DNase I footprint results).

Oligonucleotides used to generate the various mutants were as follows: mutant 1, $^{-59}$AATTATGCAACTGCAGTTCAGCAGCCAC^{-33} and $^{-33}$GGCTGCTGGAACTGCAGTTGCATAATTGA^{-61}; mutant 2, $^{-119}$ATTGTCCCTAGGATCTGCAGCATGTAAAAGAAAA^{-85} and $^{-85}$TTTTCTTTTACATGCTGCAGATCCTAGGGACAAT^{-119}; mutant 3, $^{-403}$ATATCCAAAACTGCAGAAGAACTCAAAA^{-376} and $^{-376}$TTGAGTTCTTCTGCAGTTTTGGATATTA^{-405}; mutant 4, $^{-439}$TCACTAACTTCTGCAGTGATAGAGGG^{-407} and $^{-406}$GCCCTCTATCACTGCAGAAGTTAGTGAA^{-433}; mutant 5, $^{-484}$AAGGACATAGCTGCAGGGAAATATCAGCA^{-456} and $^{-454}$CTGATATTTCCCTGCAGCTATGTCCTTTG^{-486}; mutant 6, $^{-499}$GCTTCTGTAAGGCCTGCAGCATAGTCAATAGGG^{-486} and $^{-486}$CCCTATTGACTATGCTGCAGGCCTTACAGAAGCTT^{-502}; mutant 7, $^{-403}$ATATCCAAGCGGCCGCAAGAACTCAAAA^{-376} and $^{-376}$TTGAGTTCTTGCGGCCGCTTGGATATTA^{-405}; upstream *CYP2C6* primer, $^{-552}$GAACTCTAATTGCTCATACTCTAAG^{-535}; pBLCAT3 primer, GAGCTAAGGAAGCTAAAATGGAG.

Plasmids and extracts kindly provided to us by other investigators included pLENHNF3α (an expression vector for HNF3α under control of a metallothionein promoter and the SV40 enhancer) (24) and pCMVβ (containing the *E. coli* β-galactosidase gene linked to a CMV promoter) from Dr. E. Lai and Dr. J. Darnell (Rockefeller University, New York, NY); a rabbit reticulocyte lysate containing *in vitro* synthesized HNF3α (24) was kindly provided by Dr. E. Lai (previously at the Rockefeller University), pSVeCAT and the 150-bp albumin promoter-CAT plasmid E1 described previously (28–30) were provided by Dr. M. Yaniv (Institut Pasteur, Paris, France), and pSCTODBP (an expression vector for DBP under control of the CMV promoter) and pSCTO (the corresponding vector lacking the cDNA insert) were obtained from Dr. U. Schibler (University of Geneva, Switzerland). The control plasmid for pLENHNF3 was prepared by removal of the HNF3α insert and recircularization of the plasmid. All plasmids used in the transfection experiments were transformed into DH5α and then isolated from large-scale preparations using the lysozyme/Triton method and two cycles of isopycnic banding in cesium chloride gradients (31).

Results

Differences in *CYP2C6* promoter activity in hepatocyte-derived and nonhepatocyte cell lines. We measured the activities of the *CYP2C6*, SV40, and albumin promoters in three different hepatoma cell lines and in one nonhepatic cell type, the C33 cervical carcinoma cell line. The hepatoma cell lines used included FGC4, a derivative of the Reuber hepatoma cell line FaO that expresses numerous liver-specific genes, including those for albumin, tyrosine aminotransferase, phenylalanine hydroxylase, phospho(enol)pyruvate carboxykinase, and CYP2B1 (13), and H5, a dedifferentiated variant of the same cell family that fails to express the ensemble of hepatocyte functions characteristic of the parent (14). Human HepG2 cells, which also express many characteristic hepatocyte products (32) and which have been successfully used to test the promoter activity of many liver-specific genes (33, 34), were also examined.

As shown in Fig. 1, when compared with pSVeCAT the 505-bp *CYP2C6* promoter was highly active only in FGC4 cells (and in the essentially equivalent FaO cells) (data not shown), showing a 10-fold lower activity in H5 cells and, surprisingly,

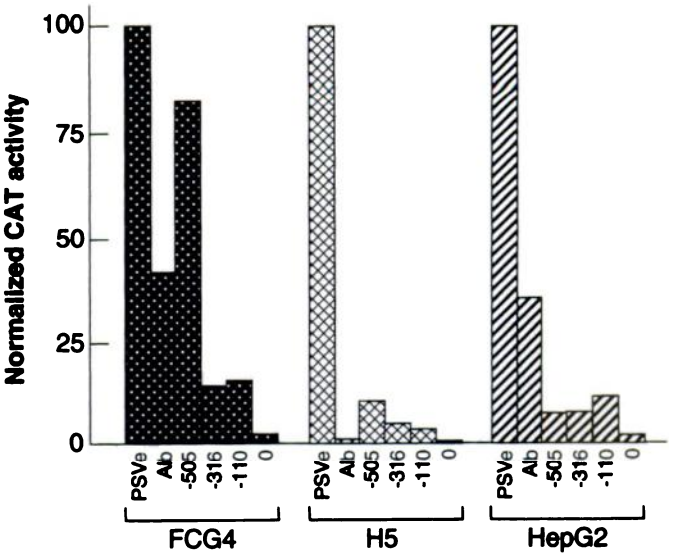


Fig. 1. Cell-specific expression of the *CYP2C6* promoter and effects of 5′ deletions on promoter activity in various hepatoma cell lines. FGC4 and H5 cells were transfected with 10 μg of the indicated chimeric CAT construct and 2 μg of pCMVβ, and HepG2 cells were transfected with 5 μg of the indicated chimeric CAT construct and 1 μg of pCMVβ. *Alb*, albumin; *0*, promoterless pBLCAT3 vector. Cells were harvested and extracts were prepared and analyzed as described in Materials and Methods. The normalized activities are expressed as a percentage of the pSVeCAT activity, in each cell line. The results are the average of at least duplicate determinations but in some instances represent the average of eight determinations.

in HepG2 cells as well. In all cases, transfection with the *CYP2C6* 505-bp promoter construct yielded a much higher CAT activity than obtained with the promoterless vector pBLCAT3. In contrast, in human C33 cervical carcinoma cells the *CYP2C6* construct did not show activity greater than that of pBLCAT3 (see below).

When compared with the 150-bp albumin promoter, the 505-bp *CYP2C6* promoter was more active in FGC4 cells, showed significant activity, compared with essentially background activity, in H5 cells, and was much less active in HepG2 cells. The proximal 150-bp albumin promoter contains several well characterized *cis*-acting elements, but only the most proximal one (the HNF1 binding site) and the TATA element are required for activity in cell lines such as FaO and FGC4 (35). The low activity of the albumin promoter in H5 cells correlates with, and has been attributed to, the absence of HNF1 activity (36). The lower activity of the *CYP2C6* promoter in H5 than in FGC4 cells presumably could reflect a requirement for HNF1. However, because activity of the *CYP2C6* promoter is clearly higher than that of the albumin promoter in dedifferentiated H5 cells, other factors present in H5 cells must make a substantial contribution to *CYP2C6* promoter activity. Taken together, these results indicate that the 505-bp proximal promoter sequence contains *cis* elements that are recognized by several liver-enriched transcription factors, the complement and concentration of which appear to vary considerably in the three different hepatoma cell lines tested (see below).

Contribution of 5′ upstream sequences to the *CYP2C6* promoter activity in different hepatoma cell lines. Having established that the *CYP2C6* promoter functions in a cell-specific manner, the next step was to identify the *cis* elements contributing to its activity. Examination of the effects of a series of 5′ deletions revealed major differences in the extent

to which different regions contribute to the promoter activity in the various cell lines. As shown in Fig. 1, removal of the distal 200 bp led to a major loss in promoter activity in FGC4 cells, and further deletion to bp −110 had little additional effect. These 5′ deletions showed similar relative activities in the dedifferentiated variant H5. In contrast, the deletions to bp −316 and −110 had little effect in HepG2 cells. The positive *cis* elements located upstream of bp −316 are, therefore, important in maintaining expression in both FGC4 and H5 cells but not in HepG2 cells. In fact, the higher activity of the 505-bp promoter in FGC4 cells than in HepG2 cells can be attributed, at least in part, to the contribution of *cis* elements upstream of bp −316 that are inactive in HepG2 cells. These findings indicate that some transcription factors required for basal *CYP2C6* promoter activity are common to both the FGC4 and H5 rat hepatoma lines and that others present in differentiated cells but lacking in the dedifferentiated variants account for the overall higher activity of the promoter in FGC4 cells.

Identification of transcription factor binding sites in the *CYP2C6* promoter and activities of altered promoters containing site-specific mutations. Computer analysis of the promoter sequence was carried out to identify potential DNA binding sites for known liver-enriched transcription factors. Two possible recognition sites for HNF4 (25) were identified between bp −489 and −477 and between bp −108 and −96, whereas three sites resembling an HNF3 consensus (24) sequence were found between bp −479 and −469, between bp −398 and −388, and between bp −50 and −40. These sites are shown in Fig. 2 and are referred to as sites 1–6 (proximal to distal). The sequences designated as site 0 and site 4 correspond to regions of the promoter (bp −6 to 4 and bp −429 to −411, respectively) that are footprinted by liver nuclear extract (see below).

We evaluated the roles of these potential transcription factor binding sites on promoter activity by analyzing mutants in which each of the sites was individually destroyed by a base cluster replacement. The promoters containing mutations at sites 1–6 are referred to as mutants 1–6, respectively, whereas mutant 7 contains a double mutation at site 3 and site 5. The results of these experiments, shown in Fig. 3, demonstrate that the only mutation that had a significant deleterious effect was that of site 1, encompassing an HNF3 site; the other mutations had insignificant effects, causing a reduction of <40%. Finally, the properties of the double mutant reinforced the conclusion, because it too failed to have a deleterious effect.

The deletion analysis showed that elements making a major contribution to promoter activity reside between bp −505 and −316. In FGC4 cells, the severe effect of site 1 mutation on the *CYP2C6* promoter activity implies that transcription factors that bind to site 1 are essential for promoter function, even though they support only minimal promoter activity. However, the deletion analysis showed that full activity requires the distal 200 bp. Nevertheless, none of the base cluster mutations in this region had any significant effect.

DNase I footprint analysis of the *CYP2C6* promoter. DNase I footprint analysis using rat liver nuclear extracts was performed on the *CYP2C6* promoter to identify regions where nuclear factors actually bind. Two footprinted regions were observed on the lower strand of the *CYP2C6* proximal promoter region downstream of bp −94, as shown in Fig. 4A. Footprint 0 encompasses bp −6 to +4 and spans the transcription start site. The larger protected region, footprint 1, between bp −73 and −40 appears to include several components. It encompasses first the putative HNF3 binding site (bp −40 to −50) and second an adjacent sequence (bp −59 to −50) that matches the recently reported consensus binding site for DBP (37). It should be noted, however, that the nuclear extracts used in these experiments should not contain any DBP (see Materials and Methods). In addition, at its 5′ extremity this region appears to contain a third component. When a double-stranded oligonucleotide spanning residues −68 to −35 (oligo 1, including both the HNF3 and DBP sites) was present during the incubation, footprint 1 was nearly completely eliminated, with only some protection remaining in the extreme 5′ portion. More importantly, an oligonucleotide containing a segment of the TTR gene promoter that includes a well characterized HNF3 site (24) but does not contain a potential DBP binding site showed an identical competition pattern. Oligos 3 and 5, which also contain HNF3 binding sites (see Fig. 4B), affected footprint 1 in a manner identical to that of the HNF3 oligonucleotide (data not shown). On the other hand, DEI, an oligonucleotide that contains the C/EBPα binding site from the albumin

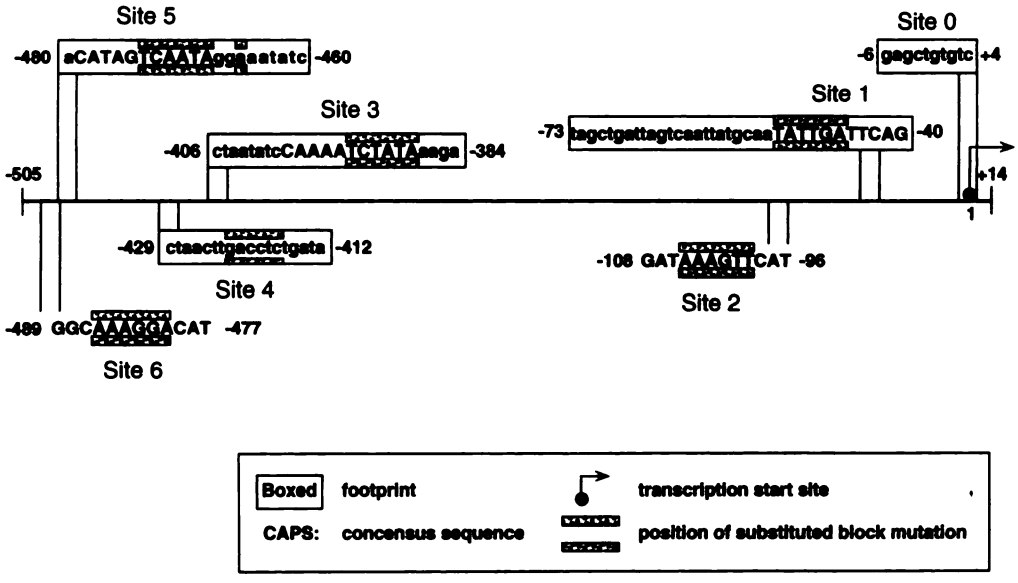
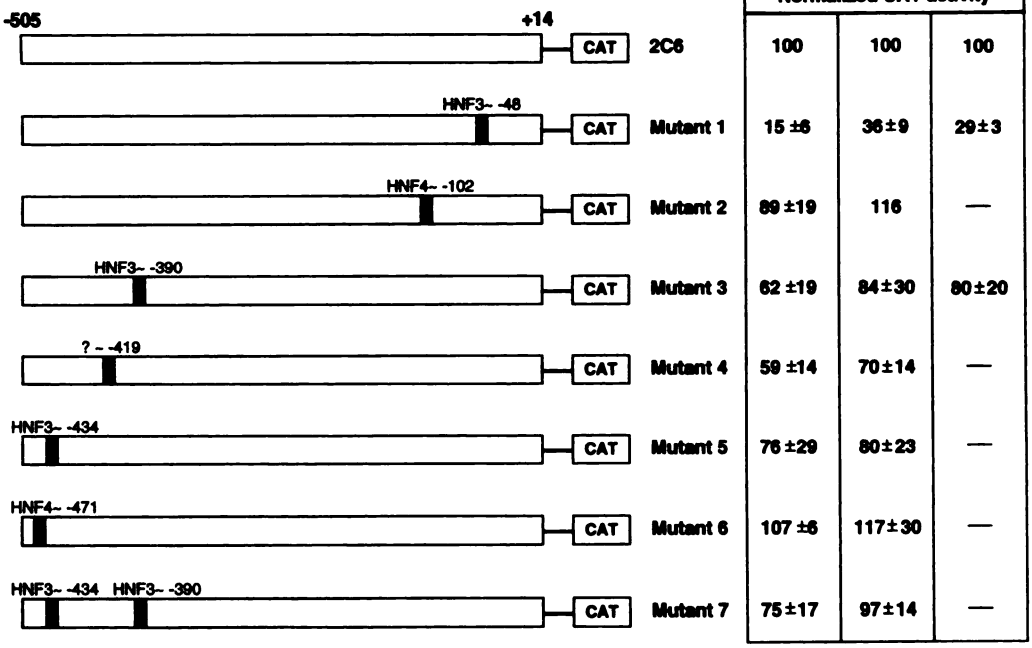


Fig. 2. Consensus sites for putative binding factors, actual DNase I protected regions, and positions of base cluster mutations. The sequences of putative transcription factor binding regions within the *CYP2C6* 505-bp promoter fragment are shown. *Upper case letters*, putative matching region for a consensus binding sequence of a known transcription factor. *Boxed regions*, regions protected from DNase I digestion by proteins in liver nuclear extracts. *Speckled thin rectangles above and below each sequence*, positions of the base cluster mutations analyzed in this work.



	FGC4	H5	HepG2
	Normalized CAT activity		
2C6	100	100	100
Mutant 1	15 ±6	36±9	29±3
Mutant 2	89 ±19	116	—
Mutant 3	62 ±19	84±30	80±20
Mutant 4	59 ±14	70±14	—
Mutant 5	76 ±29	80±23	—
Mutant 6	107 ±6	117±30	—
Mutant 7	75±17	97±14	—

Fig. 3. Activities of mutant *CYP2C6* promoter constructs. Site 1 has a major role in determining promoter activity. Six-base cluster mutations were made as described in Materials and Methods, and the mutant plasmids were transfected into the various hepatoma cells as described for Fig. 1. The activity of the wild-type 505-bp CAT construct, normalized to β-galactosidase activity, was set as 100% in each cell line. The results are the average of at least duplicate determinations but in some instances represent the average of six determinations.

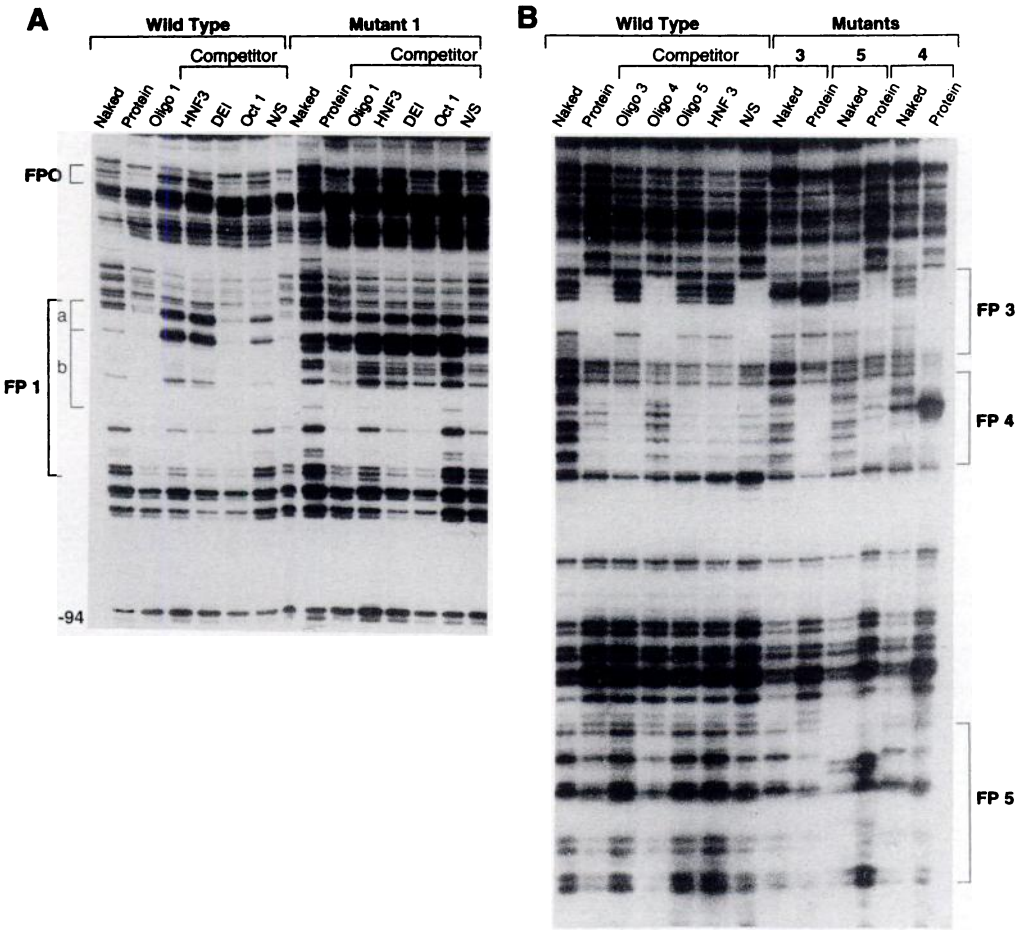


Fig. 4. DNase I footprint analysis of the *CYP2C6* promoter. HNF3 and DBP-C/EBP-like proteins bind to the *CYP2C6* promoter. Footprint analysis was performed as described in Materials and Methods, in the absence or presence of a 100-fold molar excess of the specific double-stranded competitor oligonucleotide specified above each lane. The oligonucleotide DEI contains the C/EBP-DBP binding site in the albumin proximal promoter. *N/S*, nonspecific oligonucleotide competitor; *Oct*, octamer. A, Comparison of the protected areas on the lower strand of the proximal region (bp −94 to +14) of the *CYP2C6* wild-type and mutant promoter. *Bracket FP 1*, protected region encompassing site 1; *brackets a* and *b*, regions within footprint 1 that match the consensus binding sites for HNF3 and DBP, respectively. B, Comparison of the footprint patterns over the region from bp −505 to bp −326 of the *CYP2C6* wild-type promoter and mutants 3, 4, and 5. *Bracket FP3*, protected region over site 3; *bracket FP4*, site 4; *bracket FP5*, site 5.

promoter and is known to bind DBP as well (38), failed to compete for either footprint, as expected. To our surprise, an oligonucleotide containing an octamer binding site, initially selected as a nonspecific competitor, competed very effectively for the extreme 5′ portion of the footprint as well as for the region spanning the DBP and HNF3 consensus sites. This was unexpected, because this region presents a match of only 6 of 10 bp with the octamer-binding consensus sequence. Another oligonucleotide that contained sequences from a different region of the *CYP2C6* promoter (site 4) was unable to compete

for any of the footprinted regions in the proximal promoter (Fig. 4A, *lane N/S*). The footprint encompassing the transcription start site, footprint 0, was also eliminated by oligonucleotides containing an HNF3 binding site, indicating that this region could bind HNF3 even though the sequence shows two mismatches from the HNF3 consensus sequence. The relevance of this binding to promoter activity has yet to be determined.

Fig. 4A also shows the footprint pattern of the equivalent region of mutant 1 DNA, in which only the HNF3 consensus site was eliminated. The 3′ portion of footprint 1, encompassing the mutated HNF3 site, was abolished, whereas the 5′ portion, containing the DBP and putative octamer 1/2 binding sites, was hardly affected. The pattern of competition indicated that binding to the 5′ region was significantly weakened by the site 1 mutation, because DEI, a nonspecific oligonucleotide, oligo 1, and HNF3 could all compete for binding to the DBP site. The oligonucleotide containing an octamer 1/2 binding site, as before, was the most effective competitor; it completely eliminated the most 5′ portion of the footprint as well as the portion of footprint encompassing the putative DBP site.

These results indicate that an HNF3 family member binds to the putative HNF3 site contained within the 3′ segment of footprint 1 and that this binding can stabilize the binding of nuclear proteins to the upstream portion of the footprint that encompasses a DBP binding site. Thus, not only does an HNF3 oligonucleotide eliminate protein binding to the DBP site but also mutation of the HNF3 binding site apparently reduces but does not eliminate the affinity of that site for nuclear proteins.

The footprint pattern over the region of bp −505 to −326, which includes elements that contribute to *CYP2C6* promoter activity in the highly differentiated hepatoma cells, was also determined (Fig. 4B). Three fully or partially protected regions were observed; one (footprint 3) spans site 3 (bp −406 to −384), the second (footprint 4) protects bp −429 to −412 (site 4), which has no homology to any known transcription factor consensus sequences, and the third (footprint 5) spans site 5 (bp −480 to −460). The fully protected regions at site 3 and the partially protected region at site 5, which contained consensus binding sequences for HNF3, could be specifically competed for by an oligonucleotide containing either site 3 or site 5 and, more importantly, by the HNF3 oligonucleotide. The footprint at site 4 was specifically eliminated by an oligonucleotide spanning site 4, but not by the site 3, site 5, and HNF3 oligonucleotides. None of the footprints was affected by a nonspecific oligonucleotide from the *CYP2B1* promoter. These results establish that HNF3 family members bind to sites 3 and 5 and unidentified proteins bind to site 4. As expected, the base cluster mutations at sites 3, 4, and 5 abolished the footprint only in each corresponding region. Nevertheless, in spite of the fact that these sites can be bound by nuclear proteins, it is noteworthy that mutation of them, including a double mutation at sites 3 and 5, had an insignificant effect on promoter activity.

Characterization by EMSAs of transcription factor binding sites. We further studied the possible involvement of HNF3 in binding to site 1 of the *CYP2C6* promoter using EMSAs. Initially we used rat liver nuclear extracts for these studies but found that the banding pattern obtained was very complex (data not shown and see Ref. 34). Therefore, we repeated the experiments with nuclear extracts prepared from FGC4 cells.

Oligo 1, encompassing the site whose mutation essentially destroys promoter activity, formed one major and several minor shifted complexes (Fig. 5A). As expected, competition with the identical unlabeled oligonucleotide eliminated all of the shifted complexes. The HNF3 oligonucleotide competed efficiently for the formation of major shifted complex, and an oligonucleotide spanning the DEI element of the albumin promoter competed only partially for the formation of the major shifted complex. A nonspecific oligonucleotide containing an HNF4 site did not compete to a significant extent for any of the observed shifted complexes. When the HNF3 oligonucleotide was used as a probe, it formed one major complex with the FGC4 nuclear extract (Fig. 5B), consistent with what others have observed using similar hepatoma extracts (39). The formation of this complex was competed with fully by the unlabeled HNF3 oligonucleotide and partially by oligo 1 at a 100-fold molar excess but not by a nonspecific oligonucleotide.

Although these data provide evidence that HNF3 binds to the region that matches the HNF3 consensus sequence within the site 1 oligonucleotide, we definitively established that HNF3 binds to this site by using for the EMSAs an HNF3-enriched extract, obtained by programming a rabbit reticulocyte lysate with *in vitro* transcribed HNF3α mRNA. Two major complexes with oligo 1 (Fig. 5C, *arrows*) were formed by incubation with such a reticulocyte lysate, in addition to several minor complexes also formed in the control reticulocyte lysate. All of the complexes formed with oligo 1 were competed with effectively by unlabeled oligo 1, but only the two major bands present in the HNF3 mRNA-programmed reticulocyte lysate extract could be competed with by an HNF3 oligonucleotide. We presume that the higher mobility band formed in the HNF3 mRNA-programmed reticulocyte lysate contains a fragment of HNF3 that includes its DNA binding domain and is generated either by proteolytic cleavage or by translational initiation at

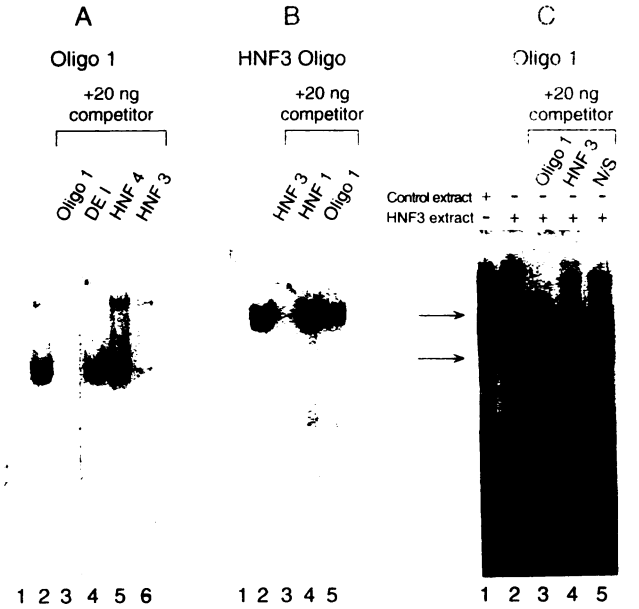


Fig. 5. Characterization by EMSA of nuclear proteins that bind to site 1. EMSA was performed as described in Materials and Methods, with FGC4 nuclear extracts (A and B) or reticulocyte lysate extracts (C) and the indicated labeled double-stranded oligonucleotide probe, in the absence or presence of the double-stranded competitor oligonucleotide specified above each lane. A, Site 1 oligonucleotide: lane 1, no protein, lane 2, no competitor; B, oligonucleotide encompassing the HNF3 binding site of the TTR promoter: lane 1, no protein, lane 2, no competitor; C, site 1 oligonucleotide, the arrows indicate the complexes formed with *in vitro* synthesized HNF3.

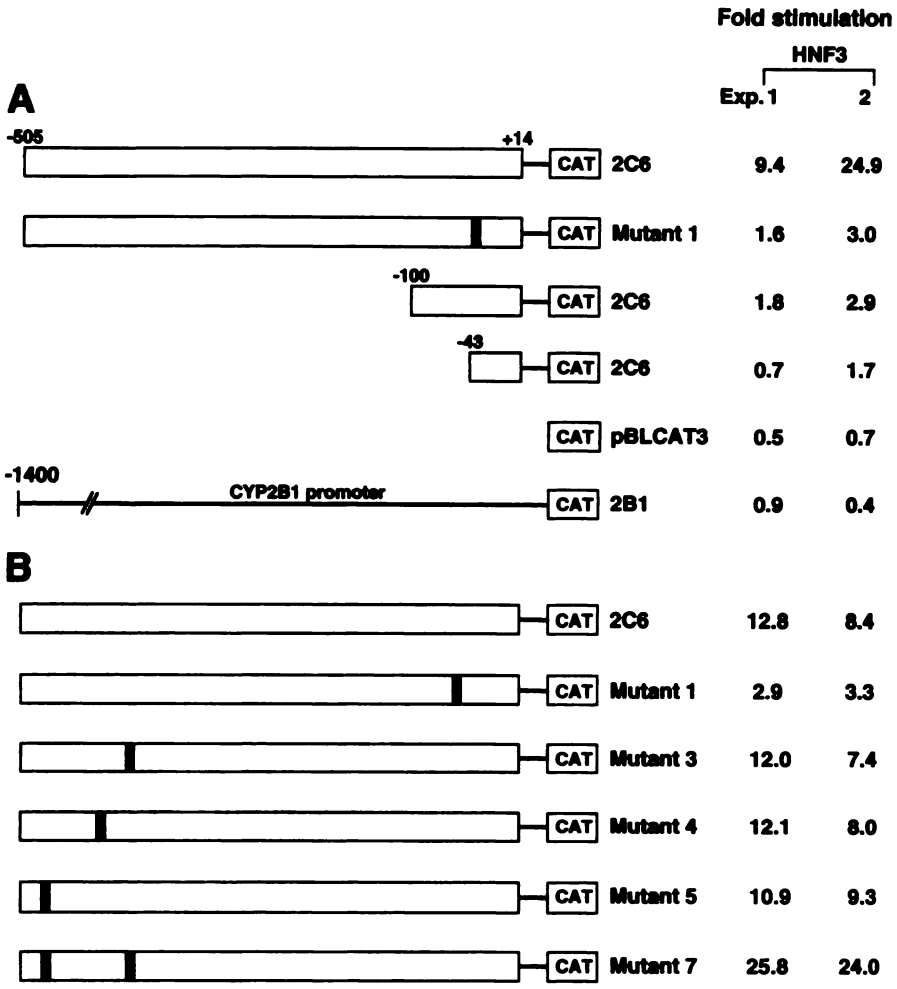


Fig. 6. HNF3 *trans*-activation of the *CYP2C6* promoter in C33 cells. Cells were transfected with a mixture of 2 μg of the CAT reporter plasmid, 3 μg of the HNF3 expression plasmid, and 0.5 μg of pCMVβ. All transfections employed equivalent amounts of DNA; the control contained an equivalent amount of the expression plasmid lacking the cDNA encoding HNF3. Transfection and assay procedures were as described in Materials and Methods. The fold activation by HNF3 is given. This was calculated by dividing the normalized CAT activity obtained when an expression plasmid for a transcription factor was included by the normalized activity of the control. The control activities corresponded to conversion of approximately 1% of the [^{14}C]chloramphenicol substrate to acetylated products. A, Analysis of 5 deletions and site 1 mutation; B, analysis of mutants within the bp −505 to bp −316 region.

an internal methionine codon in the cell-free system. These results definitively establish the presence of an HNF3 site in the promoter proximal region of the *CYP2C6* gene between bp −38 and −63.

***Trans*-activation of the *CYP2C6* promoter by exogenous HNF3α.** We attempted to demonstrate the presence of specific functional *cis* elements in the promoter by evaluating the capacity of transcription factors to stimulate the activity of the *CYP2C6* promoter constructs, using cotransfection assays. The nonhepatic C33 cell line was chosen for the initial experiments because it exhibits a low activity of the 505-bp *CYP2C6* promoter and, therefore, would presumably show a strong activation in the presence of functional liver-enriched transcription factors.

HNF3α was indeed able to *trans*-activate the 505-bp *CYP2C6* promoter, as demonstrated by the 10–25-fold increase in CAT activity resulting from cotransfection (Fig. 6A). The specificity of this *trans*-activation was demonstrated by the fact that the promoterless plasmid, pBLCAT3, and a 1.4-kilobase *CYP2B1* promoter construct were not significantly stimulated by HNF3α. The latter promoter could be stimulated 10–40-fold by C/EBPα.[1] Mutation at site 1 essentially destroyed the ability of the *CYP2C6* promoter to be *trans*-activated by HNF3α, indicating that it is a functional HNF3 site. Strikingly, the 100-bp promoter construct was only slightly *trans*-activated by HNF3α, confirming our conclusion that the binding of HNF3 to site 1 is not sufficient for full promoter activity. Indeed, all of these data concur to indicate that *trans*-activation of the *CYP2C6* promoter by HNF3 requires cooperation between HNF3 molecules that bind to site 1 and factors that bind to upstream sites.

In an attempt to define the sites within the distal fragment that contribute to promoter activity, the base cluster mutations were used in parallel *trans*-activation tests. Surprisingly, mutation of either of the sites to which HNF3 binds did not reduce *trans*-activation, nor did mutation of both of them (Fig. 6B).

DBP failed to *trans*-activate the *CYP2C6* promoter in C33 cells (data not shown). This was not due to a lack of DBP expression, because we could measure DBP binding activity in C33 cells transfected with the DBP expression vector (data not shown), but confirmed an observation made by others (34), that DBP cannot *trans*-activate this promoter in nonhepatic cells. However, in HepG2 cells both HNF3 and DBP were able to stimulate *CYP2C6* promoter activity, as demonstrated by the 9–12-fold and 12–33-fold increases in CAT activity obtained in cotransfection experiments with expression vectors for DBP and HNF3, respectively (Fig. 7). In addition, it is very clear that the two transcription factors failed to activate the promoter in a synergistic fashion.

[1] P. Van T. Luc, P. M. Shaw, and M. Adesnik. Regulation of the *CYP2B2* promoter activity *in vitro*: evidence for the involvement of C/EBP-related proteins in both the activation and repression of transcription. Manuscript in preparation.

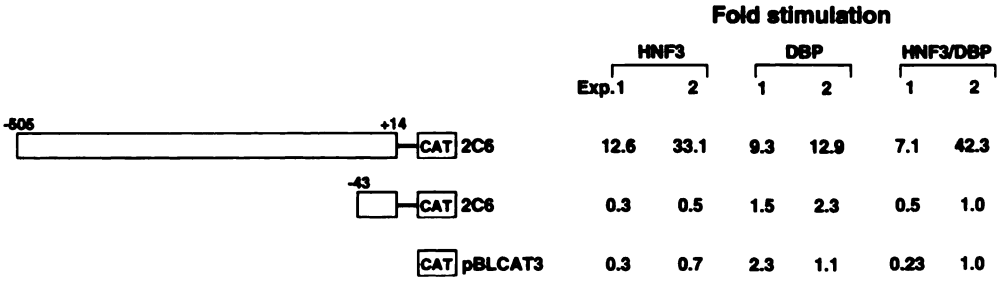


Fig. 7. HNF3 and DBP *trans*-activation of the *CYP2C6* promoter in HepG2 cells. This experiment was executed and the results were calculated as described in the legend to Fig. 6.

Discussion

The experiments presented here have identified one complex proximal *cis* element and provide evidence for the involvement of distal elements in determining the high constitutive activity of the *CYP2C6* promoter in well differentiated FGC4 rat hepatoma cells. FGC4 cells express the endogenous *CYP2C6* gene, and a 505-bp promoter construct is highly active in these cells. In contrast, the same construct is inactive in nonhepatic C33 cells. Moreover, the promoter construct is much less active in dedifferentiated hepatoma cells, such as H5 (see below). Because the relative activities of the albumin and *CYP2C6* promoters varied widely when the promoters were transfected in the various hepatoma cell lines, it appears that the activities of these two promoters are dependent upon different arrays of transcription factors.

Deletion and base cluster replacement mutagenesis experiments have permitted us to identify regions within the promoter that are required for high activity in FGC4 cells. Three potential HNF3 binding sites are present in the 505-bp promoter segment used in our studies. In DNase I footprinting experiments, each of these sites is protected by proteins present in rat liver nuclear extracts and all of the footprints are eliminated by the presence in the incubation of an oligonucleotide that contains the high affinity TTR promoter HNF3 binding sequence (24). For site 1, EMSA with *in vitro* synthesized HNF3α and FGC4 nuclear extracts, using the TTR oligonucleotide either as probe or as competitor, confirmed its identity as an HNF3 binding element. Site 1 is, by itself, most critical for promoter activity, because its mutation leads to an 80% reduction in reporter gene expression. The fact that this site, indeed, acts via interaction with an HNF3 family member is demonstrated by the finding that its mutation eliminates the capacity of the promoter to be *trans*-activated by exogenous HNF3α. Mutational inactivation of either or both of the other two putative HNF3 sites neither significantly influences promoter activity in FGC4 cells nor reduces *trans*-activation by exogenously added HNF3 in C33 cells. Nevertheless, deletion of the promoter segment extending from bp −505 to −316, which includes sites 3 and 5, does cause an 80% reduction in promoter activity in transfected FGC4 cells.

The fact that mutation of the proximal HNF3 site or elimination of the upstream region markedly reduced promoter activity demonstrates that widely spaced elements within the promoter act cooperatively to regulate *CYP2C6* expression. The contribution of the distal region to promoter activity could be due to a *cis* element that was not identified by the footprint analysis, because mutation of each of the protein binding sites is without effect. It should be noted, however, that sites 3–6, which are inactive in hepatoma cells in culture, nevertheless could play a role in transcription of the gene in adult liver.

We have confirmed the finding of Yano *et al.* (34) that the *CYP2C6* promoter can be activated by DBP in HepG2 cells. Those workers demonstrated that *trans*-activation by DBP is dependent on sequences between bp −103 and −38; deletion of these sequences from a 103-bp promoter construct did not affect basal promoter activity in HepG2 cells, but it did eliminate the 2–3-fold *trans*-activation resulting from cotransfection with a DBP expression plasmid.

Yano *et al.* (34) also demonstrated that purified DBP can protect from DNase I digestion the sequence of bp −64 to −43 in the proximal *CYP2C6* promoter. The true DBP binding site in this region is probably the 10-bp segment $^{-59}$A/GTTATGTAAC/T^{-50} that matches the consensus sequence for DBP (37). Our footprint analysis of this region of the promoter provides no new information concerning DBP binding. Indeed, Yano *et al.* (34) observed no difference in the footprint patterns over this region when the nuclear extract did or did not contain DBP. We, however, clearly demonstrated the presence of a functional HNF3 site at bp −50 to −40, which overlaps by 1 bp with the presumptive DBP binding site within site 1. Our DNase I footprint experiments indicate that both sites can be occupied simultaneously. Moreover, the capacity of the HNF3α-binding oligonucleotides to compete for the 5′ portion of the footprint that contains the DBP site suggest that HNF3α stabilizes protein binding to the DBP site.

The report of Yano *et al.* (34) on the possible role of DBP in regulating *CYP2C6* gene expression also showed that the time course of the developmental increase in rat liver *CYP2C6* mRNA levels paralleled the time course of appearance of DBP. This parallel suggested a mechanistic basis for the developmental activation of the *CYP2C6* gene, with DBP playing a dominant role in initiating and maintaining the transcriptional activity of the *CYP2C6* gene in adult rats. It is clear, however, that DBP does not participate in transcription from the *CYP2C6* gene and its promoter in FGC4 cells or its relatives, because no DBP protein can be detected in any cell line of the Reuber hepatoma.[2] Thus, although DBP may contribute to the very high basal activity of the *CYP2C6* gene in adult liver, its presence is not a condition required for expression of the gene.

Striking evidence that DBP plays a dominant role in regulating the transcription of the albumin and cholesterol 7α-hydroxylase (*CYP7*) genes *in vivo* comes from the observations that the transcription rates of these genes follow the same circadian rhythm as does the level of DBP itself, with both genes being transcribed at much higher rates at 8 p.m. than at 8 a.m. (20, 37). A similar demonstration would provide conclusive evidence that DBP plays a dominant role in regulating the *CYP2C6* gene in adult rats.

The behavior of the *CYP2C6* promoter is different in HepG2 and FGC4 cells. First, in HepG2 cells the 505-bp promoter has

[2] E. Schmidt and U. Schibler, personal communication.

does not diminish *CYP2C6* promoter activity in HepG2 cells. It would therefore appear that the factors that mediate the contribution of this upstream segment to *CYP2C6* transcriptional activity are substantially diminished or absent in HepG2 cells. These observations provide a provocative guide for future experiments aimed at defining the factor or factors required for transcriptional enhancement by the distal 200-bp segment of the *CYP2C6* promoter.

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Send reprint requests to: Milton Adesnik, Department of Cell Biology, New York University School of Medicine and The Kaplan Cancer Center, 550 First Ave., New York, NY 10016.