REVIEW

Control of Constitutively-expressed Developmentally-activated Rat Hepatic Cytochrome P450 Genes

Frank J Gonzalez

Laboratory of Molecular Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

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Abstract. Cytochromes P450 (P450) collectively refer to a superfamily of heme-containing enzymes that use O$_2$ and electrons from NADPH to insert a single atom of oxygen into any one of a large number of substrates. Two general classes of P450s exist; a relatively limited number of P450 forms, expressed in specialized tissues that are associated with pathways of steroidogenesis and a large number of forms responsible for metabolism of foreign compounds. Most of the latter P450s are expressed in liver, the primary site for metabolism of drugs, unusual dietary compounds and environmental pollutants. Numerous forms of P450 are expressed in liver of untreated animals and these are regulated quite differently. Both developmentally-programmed and sex-specific expressions have been observed. In this review, I will summarize recent findings on the mechanisms by which two P450 genes are regulated in livers of developing rats. The CYP2E1 gene is transcriptionally activated within a few hours after birth while the CYP2C6 gene is activated just prior to rats reaching puberty. These genes are under control of two transcription factors, HNF-1α and DBP, respectively, that themselves are developmentally controlled. (Keio J Med 41 (2):68-75, June 1992)

Key words: transcription factors, constitutive gene expression

Introduction

Cytochromes P450 (P450) are a superfamily of enzymes able to oxidize a large number of structurally diverse substrates.$^1$ P450s range in size from 40 to 65 kilodaltons and contain a single molecule of iron complexed as heme in protoporphyrin IX. This complex enables the enzyme to metabolize O$_2$. Upon chemical reduction and saturation of the protein with carbon monoxide, a typical Soret spectral band with a maximum absorption of about 450 nm is obtained. All P450s display this property.

P450s are named with the root symbole CYP followed by an Arabic numeral designating family number, a letter for the subfamily and another Arabic numeral denoting the specific P450 form. For example, CYP1A1 is P450 form 1, family 1, subfamily A. Families and subfamilies are defined solely based on percentages of amino acid sequence similarities of various P450 forms. Names of genes are the same except that they are italicized. The foreign compound-metabolizing P450s are found in families 1, 2 and 3. Family 4 enzymes can oxidize fatty acids and drugs containing long fatty acyl side chains.

The most common pathways of P450 metabolism involve C hydroxylation or sometimes epoxidation followed by spontaneous or enzymatic hydrolysis to the dihydrodiol, of hydrophobic chemical substrates. The hydroxyl groups serve as points of attack of various transferases which add sulfate, glutathione, and glucuronic acid to the molecule. The net result is conversion of a water-insoluble compound into a soluble derivative that can be easily excreted from the body. Numerous other reactions can be carried out by P450s that result from electronic rearrangement and migration at the enzyme's active site.$^1$ These include N oxidation, ketone formation, N, O and S dealkylations, deaminations, desaturations, dehalogenation, and even nitric oxide formation.

The precise number of foreign compound-metabolizing P450s expressed in an animal is unknown but estimates range from 30 to over 100, depending on the
species. A single P450 form is capable of metabolizing numerous structurally-diverse chemicals thus accounting for the ability of this group of enzymes to metabolize innumerable compounds.

In some cases, a single compound can be metabolized by several different P450s, albeit at differing rates. Certain compounds can be oxidized at different positions on the molecule by two or more P450s illustrating the unique nature of the P450 substrate-binding capacity.

In addition to the fascinating enzymology of P450, these enzymes are also regulated in diverse ways including transcriptional and posttranscriptional mechanisms.\(^{2-5}\) Transcriptional induction of the CYP1A1 gene by dioxins and polycyclic aromatic hydrocarbons has been the most well studied.\(^{3}\) This gene, which is well conserved in mammals, is regulated by the interaction of a segment of DNA, located about 1 kilobase pairs upstream of its transcription start site, with a receptor molecule that is complexed with its ligand. The CYP4A gene P450 subfamily is also regulated by a receptor that binds hypolipidemic drugs.\(^{6}\) Several P450 genes are transcriptionally activated by phenobarbital and related compounds but the mechanism of this regulation is largely unknown: No receptor or DNA element associated with a phenobarbital-inducible gene has been described.\(^{5}\)

Most P450 genes are constitutively expressed in adults. The most well studied are those expressed in livers of rats. Several distinct modes of expression have been described: 1 Activation of expression in both males and females within a day or two after birth. 2 Activation of expression in both sexes just after birth followed by a loss of expression in males when animals reach puberty (3 to 4 weeks of age). 3 Activation of expression in both males and females when rats reach puberty. 4 Activation of expression in males at puberty, and 5 Activation of expression in females at puberty. The rat hepatic P450 genes that are differentially activated during development are given in Table 1. The physiological stimuli responsible for these differing modes of expression is, in most cases, growth hormone secretory patterns which are determined by steroid hormones.\(^{25}\) Thyroid hormone also plays a role in maintenance of P450 expression.

Male and female rats differ in their growth hormone secretory patterns with males having a pulsatile secretion characterized by high peaks and low troughs while females have a constant and generally higher level of secretion than males. Administration of growth hormones to hypophysectomized rats, to mimic male and female secretory patterns, results in partial or complete restoration of expression of characteristic male forms in females and female forms in males. Differences can exist, however, in the mechanisms of expression of male-specific forms. For example, CYP2C11 and CYP2C13 are male-specific and controlled by growth hormone. However, CYP2C11 is activated by the pulsatile male pattern of growth hormone secretion whereas CYP2C13 is activated by the relatively low growth hormone levels in males (independent of the pulsatile secretion) and is repressed in females by their high levels growth hormone secretion.\(^{19}\) Other P450s such as CYP2A2 and CYP3A2 are repressed in adult females by high continuous growth hormone levels.\(^{11,26}\)

The CYP2C6 and CYP2C7 genes are activated just prior to puberty in both males and females.\(^{14,15}\) The latter gene is expressed at higher levels in females than males due to a stimulative effect of continuous growth hormone secretion.\(^{15}\) Expression of the CYP2C6, gene on the other hand, is totally independent of growth hormone and thus appears to be regulated quite differently from the other P450 genes.

The remaining portion of this review will focus on the molecular mechanisms of developmental activation of the CYP2E1 and CYP2C6 genes which are expressed in the liver of rats of both sexes just after birth and before puberty, respectively. These genes are both under control of liver-enriched transcription factors that themselves are expressed during certain stages of development.

### Hepatocyte-enriched Transcription Factors

Due to the relative homogeneity and easy accessibility of liver tissue, it has been the most actively studied mammalian organ, for tissue-specific gene regulation.
Among the first genes cloned was that encoding rat serum albumin which is expressed at high level in hepatocytes. The role of transcription factors in liver-specific transcription has been reviewed.27,28

Four families of transcription factors are known to be preferentially expressed in liver (Table 2). Three of these families exhibit DNA binding domains typical of other known transcription factors such as the zinc-finger motif of SP-1, the leucine zipper first identified in C/EBP and the POU-homeodomain proteins. HNF-4 possesses a region of low sequence similarity to the zinc finger domain and is a member of the steroid hormone receptor superfamily. Most transcription factors and their cDNAs were isolated by assaying the binding of their products to DNA upstream of the mouse and rat albumin promoters.28

HNF-1α is believed to control transcription of the promoters for the α- and β-fibrinogen, α1-antitrypsin and other genes expressed in liver. This factor is also capable of trans-activating the albumin promoter. HNF-1α forms homodimers and heterodimers with HNF-1β, a factor expressed at lower level than HNF-1α and expressed in certain hepa-toma cell lines in the absence of HNF-1α. The precise role of HNF-1β in liver gene expression is unknown but it is also able to trans-activate the albumin promoter. Another protein designated DCoH, has recently been identified that is required for dimerization of HNF-1 factors.30 As will be discussed below, HNF-1α is probably the primary factor responsible for expression of the CYP2E1 gene.

The factor C/EBP was originally isolated and named based on its ability to bind a sequence having the CCAAT box, found in many eukaryote promoter regions. This factor is present in adult liver and fat cells and is able to trans-activate the albumin gene. C/EBP contains a basic region leucine zipper which defines the bZIP transcription factor family. Other members of this family of transcription factors include Fos-Jun and the cAMP response element. CRP factor genes (Table 2) were identified by screening genomic libraries with the DNA binding domain-encoding region of the C/EBP cDNA.29 The C/EBP and CRP2 proteins are highly expressed in liver and to a lesser degree in lung. CRP1 protein expression has not been detected in liver or any other tissues examined. All of these bZIP factors are capable of forming homo- and heterodimers.29 Although C/EBP and CRP2 are capable of trans-activating the albumin gene, expression of this gene in adult animals appears to be due to the factor DBP.

DBP is expressed in adult rat hepatocytes and is responsible for maintenance of transcription of the serum albumin gene.31,32 This factor bears similarity to the bZIP proteins across its DNA binding domain but lacks the characteristic leucine zipper motif required for dimerization of the C/EBP and CRP-relate factors. DBP protein is expressed primarily in liver of adult rats although its mRNA is found in numerous tissues. A second member of this class of transcription factor, designated TEF (Thyrotroph embryonic Factor), was discovered that is expressed at embryonic day 14 in the region of the anterior pituitary gland that gives rise to thyrotroph cells.33 This factor controls expression of the thyroid stimulating hormone gene and can form a heterodimer with DBP.

DBP expression is especially intriguing since it is under circadian control.32 Maximal expression of protein is seen only at night and its expression correlates with transcription of the serum albumin gene. It is likely that the DBP gene itself is controlled by the circadian circulation of serum cortisol levels. However, expression of DBP does not commence until just before rats reach puberty even though the albumin gene begins to be expressed soon after birth. Thus the albumin gene is probably controlled by other factors such as HNF-1α. As discussed below, the CYP2C6 gene, which is expressed in adult rats, is also apparently under control of DBP.

Regulation of the CYP2E1 Gene

CYP2E1 is expressed in liver and to a lesser degree in extrahepatic tissues such as kidney and lung.12,13 This P450 is thought to be involved in the propanediol pathway of gluconeogenesis in addition to its role in metabolizing foreign compounds.4 Transcription of the CYP2E1 gene is detected soon after birth and reaches maximal levels within two weeks of age.12 The putative role of this enzyme in glucose metabolism may be related to its early onset of expression at a time in which the newborn becomes independent of the mothers glucose. It is un-

Table 2  Hepatocyte-enriched Transcription Factors

<table>
<thead>
<tr>
<th>Family</th>
<th>Transcription Factor</th>
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<tbody>
<tr>
<td>POU-Homeodomain</td>
<td>HNF-1α (LF-B1, APF)</td>
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<tr>
<td></td>
<td>HNF-1β (lhHNF-1)</td>
</tr>
<tr>
<td>HNF-3</td>
<td>HNF-3α</td>
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<tr>
<td></td>
<td>HNF-3β</td>
</tr>
<tr>
<td></td>
<td>HNF-3γ</td>
</tr>
<tr>
<td>Zinc Finger</td>
<td>HNF-4</td>
</tr>
<tr>
<td>bZIP (leucine zipper)</td>
<td>C/EBP</td>
</tr>
<tr>
<td></td>
<td>CRP1</td>
</tr>
<tr>
<td></td>
<td>CRP2 (NF-IL6, LAP IL6-DBP)</td>
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<tr>
<td></td>
<td>CRP3</td>
</tr>
<tr>
<td></td>
<td>DBP</td>
</tr>
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</table>

Taken from Liu and Darnel28 and Williams et al.29 Abbreviations are HNF: Hepatocyte Nuclear Factor, C/EBP: CCAAT Enhancer Binding Protein, CRB: C/EBP Related Protein DBP; albumin D site Binding Protein

Gonzalez FJ: Regulation of Rat Liver P450 Gene Expression
Characterization of the cis-acting and trans-acting regulatory elements of the CYP2E1 gene

The human and rat CYP2E1 genes were cloned and completely sequenced. Comparisons of DNA upstream of both gene's RNA polymerase start sites revealed a high degree of nucleotide similarity between $-140$ bp upstream of start site) to $-10$ bp suggesting the presence of well conserved regulatory regions that might bind transcription factors.

To determine the mechanism by which the CYP2E1 gene is regulated during development and in adult animals, in vitro transcription experiments were carried out using protein extracts prepared from rat liver nuclei and a transcription template consisting of a portion of the 5' end of the rat CYP2E1 gene. A template containing $-4000$ bp of the CYP2E1 gene was actively transcribed in this system. Transcription was maintained at a high rate when DNA upstream of $-113$ bp was deleted. However, deletion to $-87$ bp markedly decreased the rates of transcription suggesting that DNA between $-113$ bp and $-87$ bp was largely responsible for controlling the CYP2E1 gene.

To determine if the $-113$ bp to $-87$ bp region bound to any known transcription factors, a double stranded oligonucleotide containing this sequence information was synthesized and used in gel mobility shift assays. In this assay, the oligonucleotide is labeled with $^{32}$P and mixed with rat liver nuclear extracts. The mixture is then subjected to polyacrylamide gel electrophoresis. Oligonucleotide not bound to protein will migrate rapidly in the gel whereas protein-bound oligonucleotide will migrate slowly. Addition of unlabeled oligonucleotide during the incubation will result in competition with labeled oligonucleotide if the interaction with protein is sequence-specific. Likewise, a related oligonucleotide will also compete if it binds the same protein. By use of this assay, the CYP2E1 region between $-113$ bp and $-87$ bp was found to bind the same protein as a double-stranded oligonucleotide that interacts with HNF-1 $\alpha$. This region was also found to be protected from DNase I digestion using the DNase I footprinting assay. Thus HNF-1 $\alpha$, or a protein displaying similar DNA binding characteristics, probably controls CYP2E1 transcription.

To confirm this possibility trans-activation experiments were performed as shown in Figure 1. Cloned transcription factor cDNAs are inserted into plasmids containing powerful viral promoters and used to cotransfect cells with the P450 promoter connected to the bacterial chloramphenicol acetyltransferase gene (CAT). A third plasmid containing the firefly luciferase gene is included in the transfection mixture to control for variation in transfection efficiency. Transfected cells will take up all three plasmids and if the transcription factor cDNA is capable of activating the P450 promoter, CAT activity will be obtained. Co-transfection of plasmids containing the cDNAs encoding C/EBP, DBP and HNF-1 $\alpha$ revealed that only the latter was able to trans-activate the rat CYP2E1 promoter linked to the CAT gene (S-Y Liu and FJ Gonzalez, unpublished results).

Further evidence for involvement of HNF-1 $\alpha$ in control of the CYP2E1 gene was provided by analysis of radiation deletion mice. These mice contain 1.2 centiMorgan deletion of DNA on chromosome 7 which, in the homozygous state results in premature death of the animal within a few days after birth. Since the deleted region of chromosome 7 contains the tyrosinase gene, encoding an enzyme involved in pigmentation, the normal homozygotes (ch/ch), heterozygotes (ch/14CoS) and mutant homozygotes (14CoS/14CoS) can be pheno-
typed by visualization of eye color (Fig 2). Analysis of mRNA encoding albumin, CYP2E1 and HNF-1α revealed that the latter two mRNAs were not expressed in 14CoS/14CoS mice. These data demonstrate a correlation between the lack of CYP2E1 and HNF-1α gene expression. Interestingly the HNF-1α gene is not present on the deleted region of chromosome 7 in these mice, suggesting the presence on this chromosome of a gene that controls expression of the HNF-1α gene.

Developmental Activation of CYP2E1 Gene Expression

To explore the mechanism by which the CYP2E1 gene is transcriptionally activated during development, nuclear extracts were prepared from livers of rats just prior to birth (prenatal) and within 6h after birth at which times the gene is inactive, and three days after birth, when the gene is active. In vitro transcription of the cloned CYP2E1 template by these extracts correlated with expression of the gene in intact animals. However, direct DNA binding experiments revealed that proteins exist in the rat liver that bound to the HNF-1α recognition site of the CYP2E1 gene when the transcriptional activity was nil. Slightly different mobilities of shifted bands were found in inactive extracts of prenatal and immediate postnatal rats as compared to adult liver extracts. The nuclear protein from these CYP2E1 gene transcriptionally inactive rat livers had an apparent molecular weight similar to that protein found in adult liver nuclei as revealed by oligonucleotide crosslinking studies. Thus it would appear that HNF-1α or a protein with similar binding properties to NHF-1α exists in liver even before the CYP2E1 gene is expressed.

Recent experiments revealed that indeed HNF-1α mRNA and protein are found in livers of rats just before birth but at a level that is one-fifth to one-tenth that of adults. The transcription factor is fully expressed by three days of age which is coincident with activation
of the CYP2E1 gene (Liu and Gonzalez, unpublished results). These data suggest that HNF-1α expression is the primary determinant of activation of CYP2E1 gene.

A transcriptional hierarchy is involved in CYP2E1 gene expression. HNF-4 was found to control expression of the HNF-1α gene which in turn controls CYP2E1. It is believed that an extinguisher locus exists that represses the HNF-4 gene. This locus may reside on the deleted region of chromosome 7 in the 14CoS/14CoS mice.

Regulation of the CYP2C6 Gene

The CYP2C6 gene is expressed in livers of adult male and female rats with onset of transcription occurring at about 3 weeks of age. This gene is not under control of growth hormone and is not known to be expressed in any extrahepatic tissues. Analysis of the literature on developmental expression of liver-enriched transcription factors revealed that DBP expression commences around the onset of puberty. Thus, the role of DBP in transcription of CYP2C6 was investigated by the trans-activation protocol as described in Figure 1. Co-transfection of an expression plasmid containing the DBP cDNA with a CAT construct having −1225 bp of the CYP2C6 gene resulted in marked activation of CAT activity as compared to transfection of the CYP2C6 reporter gene alone. Co-transfection of vectors containing the HNF-1α and C/EBP cDNAs were ineffective in activation of the CYP2C6 promoter. This finding is especially intriguing since both DBP and C/EBP can activate the albumin promoter through binding to the same segment of DNA.

Analysis of CYP2C6-CAT constructs containing various amounts of upstream DNA revealed that a region of DNA between −103 bp and −38 bp was responsible for the trans-activation by DBP. DNase I footprinting experiments, using adult rat liver nuclear extracts, identified a protein-binding region between −63 bp and −43 bp. Gel mobility shift assays, using a double-stranded oligonucleotide containing this sequence information, also confirmed that this DNA could specifically interact with rat liver nuclear protein (s) and DBP made by recombinant DNA expression in bacteria. The CYP2C6 DBP recognition site contained two almost perfect half sites but displayed little similarity with the albumin gene DBP-binding region. Two half sites suggest that a dimer binds to the CYP2C6 sequence. Indeed, DBP is capable of forming heterodimers with another related bZIP factor TEF33 and can probably form homodimers with itself.

The lack of sequence similarity between the CYP2C6 and albumin gene DBP-binding region is especially intriguing. Several other functional differences exist between these two DNA segments. Despite the fact that both DNAs bind DBP and C/EBP, only the former trans-activates the CYP2C6 promoter. Maximal activation of the CYP2C6 promoter occurs with considerably less DBP as compared to the albumin gene promoter. This difference in trans-activation potential might be due to the marked 17-fold higher affinity of the CYP2C6 DBP-binding oligonucleotide for recombinant DBP. It is still unclear, what role this difference in trans-activation and affinity for DBP between CYP2C6 and the albumin gene plays in mediating the activation and expression of these genes in rat liver.

Expression of hepatic DBP protein during rat development was highly correlated with activation of the CYP2C6 gene. Both DBP protein and CYP2C6 mRNA increased at about 3 weeks of age, unlike albumin that begins to be expressed soon after birth at which time no DBP is expressed. It is likely that other transcription factors such as C/EBP are responsible for early expression of the albumin gene while DBP plays a role in maintenance of maximal expression in adults.

Footprinting and gel mobility shift assays revealed that proteins exist in livers of immature rats that do not contain DBP, that bind to the CYP2C6 DBP-binding sequence. This sequence also binds to recombinant DNA-expressed C/EBP. These data suggest that either DBP displaces another protein from the DBP-binding region or that the in vitro binding studies do not reflect binding in the intact liver cell chromatin.

In conclusion, these studies reveal that different constitutively-expressed P450s are under control of distinct liver-enriched transcription factors. Developmental activation of the genes encoding HNF-1α and DBP determines when a particular P450 gene will be expressed. It remains to be determined what physiological/hormonal or developmentally programmed signals control the genes encoding these transcription factors.

It should be interesting to determine the mechanisms of control of other P450 genes whose expressions are

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**CYP2C6 DBP-binding site**

5'-GTCAATTATGCAATATTGATT-3'
CAGTTAATACGTTATAACTAA

**Albumin gene DBP-binding site**

5'-TGGTATGATTTGTATAGGGG-3'
ACCATACTAAAACATTTACCCCC

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**Fig 3** Sequence of the CYP2C6 and albumin gene DBP binding region. Bold type shows the half sites in CYP2C6 sequence.
activated in liver during development, especially those that are under sex-specific control.

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