

FUNCTIONAL ANALYSIS OF 5'-FLANKING REGION OF CYTOCHROME P450
GENES THROUGH MOLECULAR CLONING AND TRANSFECTION *IN VITRO* AND
IN VIVO

by

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Cytochrome P450 (CYP) enzymes are an important class of heme-containing proteins that catalyze oxidation reactions leading toward the removal of a wide variety of endogenous and exogenous substrates including prescription drugs. The activities of CYP enzymes are regulated primarily at the transcription level involving the regulatory sequences at the 5'-flanking region of the CYP genes. The objective of this dissertation study was to characterize the function of the 5'-flanking sequences of selected CYP genes primarily responsible for drug metabolism.

Various sequences from the 5'-flanking regions of different CYP genes (CYP1A2, CYP2C9, CYP2C18, CYP2D6, CYP2E1, and CYP3A4) were cloned in expression vectors and tested for their activity in driving reporter gene expression in mouse livers and in transfected HepG2, 293, and BL-6 cells under optimized conditions. It was demonstrated that among the tested 5'-flanking regions of CYP genes, the CYP2D6 promoter showed the highest activity both *in vivo* and *in vitro*. The activities of various 5'-flanking regions of CYP genes in sustaining transgene expression were then tested in mouse liver and compared to those of other promoter sequences. As a result, the CYP2D6 promoter showed the highest activity and its activity was comparable to that of many established promoters. The mechanism

underlying CYP promoter activities *in vivo* and *in vitro* were then studied using the CYP2C9 promoter as a model. Activities of various 5'-flanking sequences of CYP2C9 were evaluated by using deletion mutations of plasmid constructs in combination with transfection in mouse livers and in HepG2 cells. Finally, the role of PXR and CAR nuclear receptors in regulating CYP2C9 activation was investigated. The results show that both CAR and PXR are essential for CYP2C9 activation and that the regulatory elements reside in the proximal 1-2 kb region upstream of the CYP2C9 gene.

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LIST OF ABBREVIATIONS

5'-FR	5'-flanking region
5'-FS	5'-flanking sequence
ACT	beta actin
ADR	adverse drug reaction
AhR	aryl-hydrocarbon receptor
APP	amyloid precursor protein
ARNT	AhR-nuclear translocator
BAC	bacterial artificial chromosome
BOP	TCPOBOP or 1,4-bis[2-(3,5-Dichloropyridyloxy)]benzene
BSA	bovine serum albumin
C/EBP	CCAAT/enhancer binding protein
CAR	constitutive androstane receptor
CAR-RE	CAR-responsive elements
CMV	cytomegalovirus
CYP	cytochrome P450
DBD	DNA binding domain
DDI	drug-drug interaction
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FXR	farnesol X receptor
GFP	green fluorescent protein
hAAT	human alpha 1-antitrypsin
HNF	hepatic nuclear factor
HS	heat shock
LBD	ligand binding domain
LXR	liver X receptor
mRNA	messenger ribonucleic acid
NFkB	nuclear factor kappa B
PAH	polycyclic aromatic hydrocarbon
PBS	phosphate buffered saline
PCN	pregnenolone-16 α -carbonitrile
PCR	polymerase chain reaction
PEI	polyethylenimine
PHB	phenobarbital
PPAR	peroxisome proliferators-activated receptor
PXR	pregnane X receptor
RIF	rifampicin
RLU	relative light units
RSV	Rous sarcoma virus
RXR	retinoid X receptor

SA	serum albumin
SRC-1	steroid receptor coactivator-1
TE	Tris-EDTA
VDR	vitamin D receptor
XRE	xenobiotic responsive element

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LITERATURE REVIEW

1.1 BACKGROUND

Before any drug can be approved for the clinical use, extensive studies have to be done to evaluate its efficacy and safety. The safety of drug treatment is a major concern since the adverse drug reactions (ADR's) have been identified as a significant factor in patient mortality. ADR can be defined as "an appreciably harmful or unpleasant reaction, resulting from an intervention related to the use of a medical product, which predicts hazard from future administration and warrants prevention or specific treatment, or alteration of the dosage regimen, or withdrawal of the product" (1). In the United States, ADR's occur in 1 out of 15 patients and represent the fourth to sixth leading cause of death to which 106,000 to 140,000 fatalities per year are attributed (2, 3). Between 1975 and 1999, ADR's led to the withdrawal of 10% of approved drugs from the market (4). The economic burden resulting from drug-related morbidity and mortality exceeded \$177.4 billion in year of 2000 (5). A better understanding of the mechanism of ADR's is necessary to prevent the significant outcomes of such a major health concern.

Drug-drug interactions (DDI's) are a major cause of ADR's, since 20-30% of all ADR's are caused by such interactions (6, 7). DDI's occur when administration of a drug results in undesirable modification of the pharmacological action of a second

concurrently administered drug (8). DDI's can be categorized as either pharmacodynamic or pharmacokinetic. Pharmacodynamic drug interactions occur as result of competition for the same drug receptor site, resulting in synergistic or antagonistic drug action (3, 9). Pharmacokinetic interactions are adverse drug events caused by altered absorption, distribution, metabolism, or excretion (3, 8, 10). The pharmacologic or toxicologic effect of a drug is related to the persisting level of the drug within the body where any modification in that level might alter body's biochemical, biological, and/or physiological homeostasis.

In general, DDI's take place in the liver where most drugs are metabolized and most of the metabolic enzymes are expressed (11, 12). Drug metabolism is carried out by a set of enzymes among which the cytochrome P450 (CYP) family considered to be the most involved. The expression of CYP is susceptible to modification by a variety of factors, including gender, age, genetic makeup, drugs, and dietary or environmental chemicals. Modification in drug metabolism may entail either enzyme induction or inhibition. A variety of ADR's due to induction of CYP have been reported (13). Metabolic reactions commonly yield inactive metabolites, however, metabolites with equal or greater pharmacological or toxicological activity can be generated. Accordingly, the induction in expression of metabolizing enzymes could result in reduced efficacy or induced toxicity (14).

The enzymes of CYP family play an important role in causing DDI's, therefore, investigating their gene expression and understanding the mechanisms of their regulation are critical for preventing ADR's. The biochemistry of drug metabolism

and the roles played by individual CYP enzymes in drug metabolism are important areas of molecular pharmacology and have been studied over the past decade. However, most of these studies were conducted using *in vitro* systems such as cell lines and hepatocytes in primary cultures. Unfortunately, liver specific gene expression is extinguished in these systems since crucial transcription factors are lost in culture. Moreover, cell culture cannot capture the full spectrum of hepatic responses to xenobiotic agents. Although transgenic mice have been useful in the investigation of gene regulation *in vivo*, considerable time, money, and the breeding of large numbers of animals over several generations are required. Therefore, development of an animal system modeling human drug metabolism and allowing identification of potential adverse effects of a drug prior to human use presents an urgent need in healthcare and the pharmaceutical industry. In the following sections a number of aspects of the CYP enzymes, and the systems used to study their regulation are reviewed.

1.2 CYTOCHROME P450 ENZYMES

Cytochrome P450 (CYP) enzymes are heme-containing, membrane-bound, and endoplasmic reticulum-located proteins (15, 16) that catalyze the initial step in the oxidative metabolism of a plethora of endogenous (steroids, bile acids, fatty acids, prostaglandins, leukotrienes, and biogenic amines) and exogenous (drugs, carcinogens, dietary supplements, pollutants, pesticides, and environmental chemicals) substances (17-22). They were first named in 1961, because the

cytochrome pigment (P) has a 450 nm ultraviolet spectral peak when reduced and bound to carbon monoxide (23-25). The general catalytic reaction cycle for CYP was first presented in 1971 and can be summarized as $(RH + O_2 + 2H^+ + 2e^- \rightarrow ROH + H_2O)$ where RH represents the drug molecule (26).

Based on similarities in their protein sequences, CYP enzymes have been divided into families and subfamilies (18, 27). Enzymes with $\leq 40\%$ sequence similarity are grouped into different families, designated by an Arabic number (e.g. CYP2). The enzymes with 40-55% similarity are grouped into different subfamilies, designated by a letter (e.g. CYP2C). Enzymes with $\geq 55\%$ similarity are classified as members of the same subfamily, designated by an Arabic number (e.g. CYP2C9).

Fifty seven functional CYP genes and eighteen families have been identified in humans, among them only the first three families are involved in drug metabolism (23, 28-31). Table 1 summarizes all the known human CYP families along with their functions. A recent study showed that among 403 tested drugs, 25% were eliminated unchanged and 55% were metabolized via CYP enzymes (32). The liver is considered the major site for drug metabolism mediated by CYP (15). The major human hepatic CYP450 enzymes are CYP2C and CYP3A and they account for 20% and 30% of the total CYP protein in the liver. The CYP enzymes CYP1A2, CYP2E1, CYP2A6, CYP2D6, and CYP2B6 account for 13%, 7%, 4%, 2%, and $< 1\%$, respectively (33). The enzymes of CYP3A (mostly CYP3A4) subfamily are responsible for metabolism of 51% of the commonly prescribed drugs, followed by CYP2D6, CYP2C (mostly CYP2C9), CYP1A2, and CYP2E1 which are responsible for metabolism of 24%,

19%, 5%, and 1%, respectively (34). Each of these CYP enzymes has its distinct tissue distribution and genetic expression (Table 2).

The human CYP1A subfamily consists of two members, CYP1A1 and CYP1A2, which are located in chromosome 15 (35). CYP1A2 is expressed almost exclusively in the liver, although low expression has also been detected in the lungs and intestines. CYP1A2 is responsible for bio-activation of nitrosamines, arylamines, polycyclic aromatic amines (PAHs), and aflatoxin B1 into intermediates that can bind DNA and induce mutation (36-39). It has been speculated that 90% of all known pro-carcinogens are activated by CYP1A1 and CYP1A2 (40). CYP1A2 is responsible for metabolism of some food supplements and drugs such as caffeine and theophylline (41, 42). CYP1A2 expression is highly inducible by cigarette smoking, charbroiled foods, and cruciferous vegetables (43, 44).

The human CYP2C subfamily consists of four genes, CYP2C8, CYP2C9, CYP2C18, and CYP2C19, which are located in chromosome 10 (45). CYP2C9 is the most abundant CYP2C protein expressed in the liver. Lower levels of CYP2C9 expression have been detected in the kidneys and intestines. CYP2C9 is responsible for metabolizing many drugs such as tolbutamide and S-warfarin (46, 47). Its expression is subjected to induction by many drugs including phenobarbital and rifampicin (13, 48).

Within the CYP2D subfamily in humans, CYP2D6 is the only active gene which is located in chromosome 22 (49). CYP2D6 is expressed in the liver and to lesser extent

in the intestines and brain. It is responsible for metabolizing a wide variety of prescribed drugs such as dextromethorphan and debrisoquine (50). The CYP2D6 gene is characterized by its high genetic polymorphism and its resistance to induction (51, 52).

The human CYP2E subfamily contains a single gene, CYP2E1, which is located in chromosome 10 (53). CYP2E1 is expressed mostly in the liver and to a lesser extent in the kidneys and lungs. It is responsible for metabolism of many compounds including ethanol and chlorzoxazone (54, 55). CYP2E1 expression is subject to induction by a variety of compounds such as ethanol and isoniazid (55-57).

The human CYP3A subfamily consists of four genes, CYP3A4, CYP3A5, CYP3A7, and CYP3A43, which are located in chromosome 7 (58). CYP3A4 is highly expressed in the liver and intestines and to a lesser extent in the lungs. CYP3A4 is responsible for metabolism of a wide variety of drugs including nifedipine and erythromycin (59, 60) and bio-activation of many carcinogens such as PAHs and aflatoxin B1 (36, 38). CYP3A4 is the most highly inducible CYP gene, and numerous pharmaceutical compounds, including rifampicin and dexamethasone, are able to enhance the expression of this gene (61, 62).

Table 1. Human CYP Families and Their Functions

Family	Subfamilies	Genes	Substrates and Functions
CYP1	2	3	Foreign chemicals, arachidonic acid, eicosanoids
CYP2	13	16	Foreign chemicals, arachidonic acid, eicosanoids
CYP3	1	4	Foreign chemicals, arachidonic acid, eicosanoids
CYP4	5	12	Fatty acids, arachidonic acid, eicosanoids
CYP5	1	1	Thromboxane A ₂ synthase
CYP7	2	2	Cholesterol, bile acid synthesis
CYP8	2	2	Prostacyclin synthase, bile-acid synthesis
CYP11	2	3	Steroidogenesis
CYP17	1	1	Steroid 17 α -hydroxylase, 17/20-lyase
CYP19	1	1	Aromatase to form estrogen
CYP20	1	1	Unknown
CYP21	1	1	Steroid 21-hydroxylase
CYP24	1	1	Vitamin D ₃ 24-hydroxylase
CYP26	3	3	Retinoic acid hydroxylation
CYP27	3	3	Bile-acid biosynthesis, vitamin D ₃ hydroxylations
CYP39	1	1	24-hydroxycholesterol 7 α -hydroxylase
CYP46	1	1	Cholesterol 24-hydroxylase
CYP51	1	1	Lanosterol 14 α -desmethylase

Adapted from reference (23)

Table 2. Expression Sites of CYP Enzymes

CYP	Site of Expression
1A2	Liver (63), brain (64), duodenum (65), umbilical vein (66), lung (67), esophagus (68)
2C9	Liver (69-71), intestine (72), kidney (73)
2D6	Liver (74), intestine (75, 76), brain (77), lung (78), bladder (79), kidney (80)
2E1	Liver (81), kidney (82), lung (83), lymphocytes (84), placenta (85)
3A4	Liver (86-89), small intestine (76, 89, 90), intestine (91), lung (92)

1.2.1 Regulation of CYP Expression

Induction in drug metabolism can be defined as “increase in the amount and/or activity of a drug metabolizing enzyme as result of an exposure to an inducing chemical whatever the underlying mechanism” (93). The induction of CYP was first demonstrated in 1956 following the administration of phenobarbital and 3-methylcholanthrene to animals (94). A few years later, it was found that the induction was attributed to elevation in the transcriptional activity of CYP genes (95, 96). Many CYP subfamilies including CYP1A, CYP2B, CYP2C, and CYP3A, are highly inducible by xenobiotics and their induction is usually tissue specific, rapid, dose-dependent, and reversible (29).

Induction of CYP enzymes can cause clinically significant DDI's. The outcome of enzyme induction depends on the pharmacological activity of the parent compounds and their metabolites. If the parent compound is the active therapeutic agent, then the net effect of enzyme induction will be loss of the pharmacological efficacy. for example, rifampicin increases the CYP3A4-dependent metabolism of cyclosporine resulting in rejection of the transplanted organ by the body (97). On the other hand, when the metabolite is more active than the parent compound, then the induction will increase the chance for toxicity. For example, ethanol increases the CYP2E1-dependent metabolism of acetaminophen resulting in formation of its hepatotoxic metabolite (N-acetyl-p-benzoquinoneimine) (98). The examples mentioned as well as many others all reveal the clinical consequences of CYP induction and suggest that

more studies are required in order to fully understand the mechanisms underlying their regulation.

In the last few years, extensive efforts have been made to understand the molecular mechanisms underlying the expression of CYP enzymes. It has been found that the induction of CYP enzymes is primarily regulated by a group of orphan nuclear receptors. They are called orphans because they were identified without knowing their endogenous or exogenous ligands (32). These receptors share two essential functional domains that include the N-terminal DNA-binding domain (DBD) and the C-terminal ligand-binding domain (LBD) (99). The conserved DBD acts to link the receptor to specific 5'-flanking region (5'-FR) element in its target gene called the xenobiotic responsive element (XRE) (32). The less conserved LBD has at least four functions: ligand binding, binding of co-activators or co-repressors, dimerization, and transactivation (100). Nuclear receptors were considered prime candidates for mediating hepatic drug induction for several reasons (29). First, their ligands are small and lipophilic similar to those of CYP enzymes. They bind to specific DNA elements similar to those found in the 5'-flanking sequences (5'-FSs) of CYP genes. Furthermore, they are expressed in specific tissues where most CYP enzymes are expressed. Finally, they play key roles in many physiological processes in which P450 enzymes are involved.

The most studied nuclear orphan receptor is the pregnane X receptor (PXR). PXR was isolated and identified as a key regulator in CYP3A expression in 1998 (101-103), although recent studies have disclosed its regulatory role for other CYP genes such as CYP2C and CYP2B (104, 105). PXR is expressed predominantly in the liver and

intestines and to a lesser extent in the kidneys and lungs (106). Many chemicals including prescription drugs, steroids, and environmental factors are able to bind and activate PXR (101, 107). For example, the antibiotic rifampicin and the antidepressant herbal product hyperforin are potent PXR activators (108, 109). The biochemical process of PXR activation has been illustrated (99). Upon ligand binding, a conformational change in the LBD creates a co-activator (e.g. steroid receptor coactivator-1, SRC-1) binding surface; and transcriptional activation occurs after recruitment of co-activator to the receptor (100). Subsequently, PXR regulates gene expression by forming a heterodimer with the retinoid X receptor (RXR α) and then regulation is achieved by binding of the PXR-RXR α heterodimer to XRE present in the 5'-FR of the target gene. The unique feature of PXR-mediated induction is its species specificity, primarily due to the differences in LBD (101). For example, rifampicin is a potent activator for human PXR but not the rodent isoform, whereas pregnenolone-16 α -carbonitrile (PCN), an anti-glucocorticoid, is a rodent-specific activator. PXR humanized mice have been generated (110) and in these transgenic mice the profile of PXR-based induction was similar to the human profile.

Another important nuclear receptor is the constitutive androstane receptor (CAR). CAR was first isolated in 1994, but its role in CYP2B induction was not appreciated until 1998 (111-113). CAR also can regulate other CYP genes such as CYP3A and CYP2C (114, 115). CAR is expressed predominantly in the liver and intestines and can be activated by many drugs such as phenobarbital and phenytoin. The mechanism of CAR activation is more complex than that of PXR. CAR is cytosolic protein and upon activation, it translocates into the nucleus and forms a heterodimer with RXR α .

Similar to PXR, it is the heterodimer that binds to target gene sequence and activates transcription (116-118). Phenobarbital activates CAR by facilitating its nuclear translocation through a phosphorylation-based mechanism (116, 118, 119). The only molecules shown to directly bind CAR were androstanol and clotrimazole which are inverse agonists that deactivate the response (120). Like PXR, CAR shows species differences in its induction profile, for example 1,4-bis[2-(3,5 dichloropyridyloxy)]benzene (TCPOBOP) was found to be specific mouse CAR activator (120, 121). The broad role of CAR and PXR in regulating many metabolizing enzymes and transporters and cross-regulation of gene expression has been reported (122).

Perhaps the most well studied nuclear receptor is the aryl-hydrocarbon receptor (AhR). For more than thirty years, AhR has been known to be a CYP1A regulator (123-125). AhR is a helix-loop-helix protein that belongs to the polycyclic aromatic hydrocarbon (PAH) family of transcription factors. Similar to CAR, AhR is a cytosolic protein and becomes activated once activated by its ligand. Consequently, the activated receptor translocates into the nucleus, form a heterodimer with its nuclear translocator protein (ARNT), binds to XRE sequences upstream of CYP1 genes, and activates gene transcription (126, 127). AhR-dependent induction is conserved among many cell types and across animal species. A significant number of substances were found to be ligands for AhR including omeprazole as well as several important environmental carcinogens found in auto exhaust and cigarette smoke (128, 129).

Other nuclear receptors are also involved in CYP regulation. For example, the peroxisome proliferator-activated receptor (PPAR α) regulates CYP4A (130-132) and the vitamin D receptor (VDR) regulates CYP3A, CYP2B, CYP2C, and CYP24 (133-135). The liver X receptor (LXR) and the farnesol X receptor (FXR) both regulate the expression of CYP7A (136-138).

Additionally, some transcriptional factors play crucial role in CYP regulation. For example, the hepatic nuclear factor (HNF1 α) regulates the expression of CYP2E1, CYP1A2, CYP7A1, and CYP27 (139) and the HNF4 α regulates CYP3A, CYP2C, CYP2D6, CYP2A6, and CYP2B (140-142). Other transcriptional factors such as the HNF3 γ regulates CYP2C (143) while the CAAT/enhancer binding protein (C/EBP) regulates the expression of CYP2B, CYP2D, and CYP2C (144).

1.2.2 Systems Used to Assess the Induction of CYP

The induction of CYP enzymes can be evaluated directly in the human body using different substrate-probes following repeated administration of a putative inducer drugs (Table 3). The metabolic ratio of the probe to its metabolite represents the metabolic activity of a single CYP enzyme. The increase in CYP metabolic activity stimulated by a given drug indicates that the drug is working as an inducer for that specific enzyme. For obvious ethical reasons, the use of human studies is limited only to compounds that are at a late stage of clinical development. Moreover, human trials are expensive and the subjects are difficult to recruit. Such limitations have prompted the use of animals, often rodents, as an alternative to test in humans.

Compared to clinical studies, studies of drug metabolism in animals have provided a plethora of information about animal CYP genes, their enzymatic action, substrate specificity, and gene regulation. Unfortunately, it is now well known that drug metabolism in animals often differs from humans and data obtained from animal studies cannot be directly extrapolated into humans. Differences in the action of important signaling molecules and pathways, the extreme sexual dimorphism of rodent CYP, the marked species differences in the activation profiles of key nuclear receptors, and the differences in the layout of nuclear receptor responsive elements within target genes make it difficult to use animals directly as *in vivo* models to predict the induction of CYP enzymes in humans (145). To minimize such differences, many lines of transgenic animals expressing human CYP genes (CYP3A4, CYP2D6, CYP1B1 and CYP2E1) or nuclear receptors (PXR, CAR, AhR, and PPAR α) have been generated (110, 141, 146-152)

Studies have also been conducted using *in vitro* systems, including purified CYP enzymes, subcellular fractions (microsomes, or cell extracts), hepatoma cell lines, and primary hepatocytes cultures. More complex systems like liver slices and isolated liver have also been employed (12). While cell free systems are convenient and have been widely employed for biochemical studies of CYP, non-cellular systems cannot be used for gene expression studies.

Before the discovery of PXR and CAR, cultured human hepatocytes were considered to be the suitable system to study CYP induction. In these systems, incubation of

hepatocytes for 24-72 hours with a compound is followed by assessment of CYP metabolic activities using specific substrates. The activity measured in the induced cells is then compared with that in untreated control cells (153). Despite the positive aspects, the use of hepatocytes has certain disadvantages including their availability, quality, involvement of phase II enzymes, and inter-individual variability toward xenobiotic responses as well as basal CYP expression (145). Furthermore, hepatocytes in primary culture tend to lose their ability to respond to CYP enzyme inducers rather quickly.

A relatively new and more sensitive system for studying CYP induction involves transfection with plasmids containing reporter genes under the control of regulatory sequence of human CYP genes into cells with characteristically low CYP gene and nuclear receptor expression, but exhibiting normal expression of cofactors such as RXR. In general, this approach involves insertion of human CYP regulatory sequences into the immediate 5' end of a reporter gene into a plasmid. The construct is then transfected into cells. Similarly, a plasmid containing the coding region of a nuclear receptor can also be introduced to the same cells through co-transfection. Drug specific induction is determined by the relative level of reporter gene expression over that of cells without drug treatment. The major advantage of the transfection-based approach is its convenience. Since plasmids containing nuclear receptors can be co-transfected with a reporter gene under control of regulatory elements of human CYP genes, various compounds or substances can be tested for their activity in inducing CYP gene expression.

Table 3. Substrate Probes Used to Assess CYP Activities in Human

CYP	Substrate (Sampling Procedure)
CYP1A2	Caffeine (breath test, urine)
CYP2C9	Tolbutamide (urine), Diclofenac (blood), Phenytoin (blood)
CYP2D6	Sparteine (urine), Debrisoquine (urine), Dextromethorphan (urine)
CYP2E1	Chlorzoxazone (urine, blood)
CYP3A4	Erythromycin (breath test), Dapsone (urine), Midazolam (blood)

Adapted from reference (153)

1.3 RESEARCH OBJECTIVES

Many cases of clinically relevant ADR's have been attributed to the induction in CYP expression. As a result, these reactions led to significant mortality and an economic burden. Studies that enable us to understand the mechanism of CYP induction in order to prevent such reactions are critically needed. In addition, to reduce the time and cost of developing new drugs, there is a concerted effort in the pharmaceutical industry to identify potential drug interactions early in the drug discovery process. The difficult process of accurately predicting potential interactions by new drug candidates could be augmented by a procedure through which selected human CYP genes are introduced into a mouse and their responses to the candidate drug studied under the physiological conditions. The work of this dissertation project was designed to establish such an *in vivo* system wherein human CYP gene sequences can be introduced and studied in the hepatocytes of mice. The goal of this study was to establish optimal conditions for transfecting mouse hepatocytes; to validate the system for CYP related studies; and to determine the functional role of 5'-FRs of human CYP genes.

MATERIALS AND METHODS

2.1 MATERIALS

GeneChoice PCR kits that were used to amplify CYP 5'-FSs of p1A2-luc, p2C9-luc, p2C18-luc, p2D6-luc, p2E1-luc, and p3A4-luc were purchased from PGC Scientific Corporation (Frederick, MD) while PCR primers were designed and ordered from MWG-Biotech (High Point, NC). Failsafe PCR kits that were used to amplify CYP2C9 5'-FSs of p2C9-0.2K-luc, p2C9-1K-luc, p2C9-3K-luc, p2C9-5K-luc, and p2C9-10K-luc were purchased from Epicentre (Madison, WI) while PCR primers were designed and ordered from IDT (Coralville, IA). A BAC clone of RP11-208C17 was obtained from CHORI (Oakland, CA). Luciferase assay kits were purchased from Promega (Madison, WI). The protein assay reagent was from Bio-Rad (Hercules, CA). Cell culture media were purchased from Invitrogen (Grand Island, NY). Polyethylenimine (PEI) (Branched, 10 KD) was synthesized according to a previously published procedure (154). Rifampicin, phenobarbital, 1,4-bis[2-(3,5-Dichloropyridyloxy)]benzene (TCPOBOP), pregnenolone-16 α -carbonitrile (PCN), and DMSO were obtained from Sigma-Aldrich (St. Louis, MO). Bacto™-tryptone, Bacto™-yeast extract, and Bacto™-agar were purchased from Difco (Detroit, MI). Restriction enzymes and T4 DNA ligase were purchased from New England BioLabs (Beverly, MA). All other chemicals were of cell culture grade and obtained from

Sigma Chemical Co. (Saint Louis, MO). CD-1 mice (female, 18-20 g) were from Charles River (Wilmington, MA).

2.2 METHODS

2.2.1 Plasmid Construction

All 5'-FSs for construction of p1A2-luc, p2C9-luc (or p2C9-2K-luc), p2C18-luc, p2D6-luc, p2E1-luc, p3A4-luc, p2C9-0.2K-luc, p2C9-1K-luc, p2C9-3K-luc, p2C9-5K-luc, and p2C9-10K-luc (all named based on the CYP name or the length of the inserted CYP 5'-FSs) were cloned into pGL3-Basic vector (Promega, Madison, WI). The primers (forward (F) or reverse (R)) for PCR amplification, the cloned CYP 5'-FSs, and the enzymes used in primer sequences modifications are listed in Tables 4 and 5. Primer sequences of p1A2-luc, p2C9-luc, p2C18-luc, p2D6-luc, p2E1-luc, and p3A4-luc were selected based on previously published CYP gene sequences (74, 155-158). All CYP related primers were synthesized with the *KpnI* or *SacI* site attached to the 5' end of the forward primer and the *MluI*, *BglII*, or *XhoI* site to the 5' end of the reverse primer, with the exception of the reverse and the forward primers used to generate fragment 1 (F1) and 2 (F2) of p2C9-10-luc, respectively (Table 5). The genomic DNA from human liver was used as the PCR template for 5'-FSs of p1A2-luc, p2C9-luc, p2C18-luc, p2D6-luc, p2E1-luc, and p3A4-luc. The BAC clone of RP11-208C17 was used as the PCR template for 5'-FSs of p2C9-0.2K-luc, p2C9-1K-luc, p2C9-3K-luc, p2C9-5K-luc, and p2C9-10K-luc.

On arrival in our laboratory, the BAC clone was inoculated into Luria Broth (LB) culture medium (1% Bacto™-tryptone, 0.5% Bacto™-yeast extract, 1% NaCl) including 20 µg/ml chloramphenicol, the BAC was then extracted using a Qiagen Plasmid Maxi Kit (Valencia, CA). BAC DNA concentration was measured spectrophotometrically by absorbance at 260 nm and 280 nm. The Identity of the extracted BAC DNA was confirmed by restriction enzymes digestion (*Bam*HI or *Eco*rV) and by PCR using the extracted BAC DNA as the template and F: CCCACACTGTACGCACAATC and R: GGAGTTGAGAAAAACCAAGGG as primers.

Different strategies were used to clone each 5'-FSs into plasmid vector. To clone CYP 5'-FSs into the pGL3-Basic vector, PCR products of p2C9-0.2K-luc, p2C9-1K-luc, p2C9-3K-luc, p2C9-5K-luc, and p2C9-10K-luc were concentrated by ethanol precipitation, digested with the appropriate enzymes, separated on 1% agarose gel, and the correct fragment extracted with a Qiagen QIAquick gel extraction kit (Valencia, CA). PCR products of p2C9-10K-luc F1 and F2 were purified, digested with *Xba*I (they share the same *Xba*I digestion site), and then ligated together before digestion with *Kpn*I/*Xho*I. The modified PCR products were then ligated to a linear pGL3-Basic vector (linearized with the same pair of restriction enzymes by which the inserted PCR product was digested).

PCR products of p1A2-luc, p2C9-luc, p2C18-luc, p2D6-luc, p2E1-luc, and p3A4-luc were concentrated, purified, and then ligated into pGEM easy vector (Promega,

Madison, WI). Each CYP 5'-FSs in pGEM easy vector was then digested with the proper pair of restriction enzymes, and ligated to linear pGL3-Basic vector.

CYP constructs that contain the human alpha antitrypsin (AAT) reporter gene (p1A2-hAAT, p2C9-hAAT, p2D6-hAAT, and p3A4-hAAT) were cloned by ligating the digested CYP PCR products into linear pGL3-Basic vector from which the luciferase gene was removed using *HindIII* / *XbaI* and replaced by the hAAT gene. The hAAT gene was amplified by PCR using primers that were designed to include *HindIII* (attached to 5' end of the forward primer) and *XbaI* (attached to 3' end of the reverse primer) cutting sites (F: GCAAGCTTACAATGCCGTCTTCTGTCTCG and R: GCTCTAGACTTTAATGTCATCCAGGGAGGG).

Plasmids of pGL3-Basic vector to which CYP 5'-FSs were ligated were then transformed into *E. coli* DH-5 α by means of a Bio-Rad MicroPulser[®] in a E.coli Pulser Cuvette (Bio-Rad, Hercules, CA). The transformed cells were then spread on LB-1.5% agar plates containing 100 μ g/ml ampicillin, and the surviving cells were tested for their inclusion of the right plasmid. Plasmid DNA was extracted from cells either by the DNA boiling procedure or by a Qiagen Plasmid Mini Purification Kit (Valencia, CA). In the DNA boiling procedure, cells were boiled for 1 min in STET buffer (8% sucrose, 0.5% triton X-100, 50 mM EDTA pH 8, 10 mM Tris pH8) containing 50 μ g/ml lysozyme, centrifuged (12000 rpm, 1 min, 4°C), and DNA size examined on 1% agarose gel. Plasmid DNA was then confirmed by restriction enzyme digestion, PCR amplification, and sequencing.

Upon plasmid DNA confirmation, CYP plasmids were prepared and extracted in large scale using the lysozyme lysis method and cesium chloride (CsCl) gradient centrifugation (159). Briefly, the transformed E. coli cells were grown in Terrific Broth (TB) culture medium (1.2% Bacto™-tryptone, 2.4% Bacto™-yeast extract, 0.4% glycerol, 17 mM KH₂PO₄, 72 mM K₂HPO₄) containing 100 µg/ml ampicillin and collected by centrifugation (5000 rpm, 5 min, 4°C). Cells were then lysed with solution I (1% glucose, 25 mM Tris pH 8, 20 mM EDTA pH 8) including 100 µg/ml lysozyme, their DNA content denatured by solution II (0.2 M NaOH, 1% SDS), and collected by centrifugation (8000 rpm, 20 min, 4°C). Plasmid DNA was separated from chromosomal DNA and cell debris by solution III (3 M KC₂H₃O₂, 2 M C₂O₂ H₄) and centrifugation (7000 rpm, 20 min, 4°C). Plasmid DNA was then collected and further purified by isopropanol (0.6 X volume) and 5 M LiCl (1X volume) precipitation. The plasmid DNA was then re-suspended into Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), treated with 50% CsCl and ethidium bromide (0.25 mg/ml), and isolated by gradient centrifugation (64000 rpm, 18 hr, 25°C). Plasmid DNA was later isolated and freed of ethidium bromide by saturated butanol extraction and from CsCl by water dialysis using a Spectrum molecularporous membrane (Rancho Dominguez, CA). Plasmid DNA was then precipitated by ethanol and kept in 0.9% saline solution. The purity of the plasmid DNA was confirmed spectrophotometrically by the ratio of ultraviolet absorbance at 260 and 280 nm as well as by 1% agarose-gel electrophoresis.

The plasmid of pSA-luc was provided by Dr. Weidong Xiao (University of Pennsylvania, School of Medicine). Plasmids of pCMV-luc, pACT-luc, and pCMV-

GFP were provided by Dr. Leaf Huang (University of Pittsburgh, School of Pharmacy). Plasmid of pCMV-EBV-luc was provided by Dr. O Mazda (Kyoto Prefectural University of Medicine, Department of Microbiology, Kamikyo Kyoto, Japan). Plasmid of pAAT-luc was provided by Dr. Xiao Xiao (University of Pittsburgh, School of Medicine). Plasmid of pAPP-luc was provided by Dr. Debomoy Lahiri (Indiana University, School of Medicine). Plasmids of p2b10-luc, pCMX-CAR, pCMX-SXR, pCMX-VPCAR, and pCMX-VPSXR were provided by Dr. Wen Xie (University of Pittsburgh, School of Pharmacy). Plasmids of pRSV-luc, pNFkB-luc, and pHS-luc were constructed or provided by members of our laboratory.

Table 4. Cloned Regions, Primers, and Digestion Enzymes Used in CYP Plasmid Construction

Construct	Cloned Region	Primer Sequences	Enzymes
p1A2-luc	-1872/+38	F: GGTACCAAAGCCCACTCCAGTCTAAATC R: AGGCGTTGAGATTGGCAGGGTTGTAATG	<i>KpnI/MluI</i>
p2C9-luc	-2145/+2	F: GGTACCGATCTCAGATATCCCTTCTATC R: ACGCGTATTGTTGCCTTCTCTTCTTGAC	<i>KpnI/MluI</i>
p2C18-luc	-1224/-12	F: GGTACCTAGTGTTCAGTCTTGCAGATC R: ACGCGTCTTCTTAGTAAGACAACCTGGGG	<i>KpnI/MluI</i>
p2D6-luc	-1516/+ 11	F: ATGGTACCAGCCTGGACAACCTTGGGAAG R: ATACGCGTACACTCTCAGCACACCGAG	<i>KpnI/MluI</i>
p2E1-luc	-2670/+ 4	F: GAGCTCCCCCTACAGTATAAAGTATCCC R: AGATCTGGACAATCCTGTGGAAAGGAAG	<i>SacI/BglII</i>
p3A4-luc	-1088/+85	F: GGTACCCCATCATTGCTGGTCTTTG R: CTCGAGTCTCTCTCCTCTGAGTCTTC	<i>KpnI/XhoI</i>

Table 5. Cloned Regions, Primers, and Digestion Enzymes Used in CYP2C9 Plasmid Construction

Construct	Cloned Region	Primer Sequences	Enzymes
p2C9-0.2K-luc	-211/+25	F: ATTGAGGTACCAGTGGACAATGGAACGAAGG R: AATTACTCGAGGCACAAGGACCACAAGAGAATC	<i>KpnI/XhoI</i>
p2C9-1K-luc	-1011/+25	F: ATTGAGGTACCCACTGAGCGTTTCACTTCTGC R: AATTACTCGAGGCACAAGGACCACAAGAGAATC	<i>KpnI/XhoI</i>
p2C9-3K-luc	-3024/+25	F: ATTGAGGTACCAAGGAAGGGAGAGAGAACACG R: AATTACTCGAGGCACAAGGACCACAAGAGAATC	<i>KpnI/XhoI</i>
p2C9-5K-luc	-5470/+25	F: ATTGAGGTACCCAGCGAACTAAGAATAGAGGAGG R: AATTACTCGAGGCACAAGGACCACAAGAGAATC	<i>KpnI/XhoI</i>
p2C9-10K-luc (F1)	-9900/-5485	F: ATTGAGGTACCTTGTGGAGGAAGTGAGTCCC R: GAATGTGTGCTGGATTTAGGC	<i>KpnI/XbaI</i>
p2C9-10K-luc (F2)	-6437/+25	F: TAGTGAAAGCAGTGGTTAGAGGG R: AATTACTCGAGGCACAAGGACCACAAGAGAATC	<i>XbaI/XhoI</i>

2.2.2 Cell Culture and Transfection

Three cell lines derived from different tissues were used, HepG2 (human hepatoma), 293 (human embryonic kidney fibroblasts) and BL-6 (murine melanoma). BL-6 cells were maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS). HepG2 and 293 cells were cultured in DMEM medium with 10% FBS. For a standard transfection, 5×10^4 cells per well were seeded into a 48-well plate and allowed to grow for 24 hours. For each well, 100 μ l of serum free medium containing the desired amount of plasmid DNA was mixed with 125 μ l of serum free medium containing an appropriate amount of PEI. The mixture was incubated for 15 minutes at room temperature followed by mixing with 25 μ l of FBS. The mixture (250 μ l total) was added to each well and the plates incubated for 24 hours. The transfection medium was then replaced with a fresh growth medium containing 10% FBS. Cells were cultured for an additional 24-72 hours to allow for gene expression. Prior to gene expression analysis, cells were washed three times with phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 5.4 mM Na₂HPO₄, 1.7 mM NaH₂PO₄, pH 7.4) and lysed with 100 μ l lysis buffer (0.1 M Tris-HCl, 0.1% Triton X-100, 2 mM EDTA, pH 7.8) at room temperature for 15 minutes. The cell lysates were collected and subjected to centrifugation (12,000 rpm, 10 min, 4°C) and the supernatant was used for measurement of luciferase activity and protein concentration.

2.2.3 Hydrodynamic Transfection of Animals

CD-1 mice (female, 18-20 g) were injected via the tail vein within 3-5 seconds with 1.8 ml of saline solution containing the desired amount of plasmid DNA according to our previously published procedure (160). For the CYP2C9 induction studies, two hours post transfection, animals were injected intraperitoneally with 100 μ l DMSO containing rifampicin (200 mg/kg), TCPOBOP (10 mg/kg), phenobarbital (200 mg/kg), or PCN (200 mg/kg). At the indicated time, animals were sacrificed and the desired tissue samples of approximately 200 mg were obtained, homogenized in 1 ml of lysis buffer, and centrifuged (12,000 rpm, 10 min, 4°C). Protein concentration and luciferase activity in the supernatant were then determined.

2.2.4 Luciferase Assay

Ten μ l of cell lysate from either *in vitro* transfected cells or supernatant of tissue homogenate from transfected animals were added to 100 μ l of substrate solution in the luciferase assay kit. Luciferase activity was measured in a luminometer (Autolumat LB953, EG & G, Berthold, Germany) with the time for the measurement set for 10 seconds. Luciferase activity in each sample was normalized to relative light units (RLU) per mg of extracted protein.

2.2.5 Analysis of Serum hAAT by ELISA

Serum samples were collected from the injected mice at appropriate times and diluted serially with 1% bovine serum albumin (BSA) in PBS-Tween buffer before a standard

ELISA was performed. A rabbit anti-hAAT polyclonal antibody (12.6 $\mu\text{g/ml}$ in carbonate buffer, pH 8.8) was used for coating of the ELISA plate (room temperature, overnight). The coated wells were blocked with 4% BSA in PBS-Tween buffer. After incubation with the diluted serum sample, biotinylated goat anti-hAAT polyclonal antibody (1:1000 dilution in 1% BSA in PBS-Tween buffer) was added and followed by incubation for 1 hour at room temperature. After washing, streptavidin–horseradish peroxidase conjugate (1:50000 dilution 1% BSA in PBS-Tween buffer) was then added to each well and incubated for 1 hour. With the exception of blocking buffer (200 μl per well) and the washing buffer (400 μl per well), the sample volume used was 100 μl per well. After addition of peroxidase substrate (3,3',5,5'-tetramethylbenzidine), the absorbency at 450 nm for each well was obtained using an ELISA reader. The hAAT concentration was calculated based on the standard curve established using pure hAAT. To minimize the plate-to-plate variation, a standard curve was established for each of the 96-well plates used. The concentration range for the standard curve was 0-32 ng/ml. The hAAT standard was prepared by dissolving lyophilized hAAT powder in normal mouse serum at a concentration of 500 $\mu\text{g/ml}$.

2.2.6 Analysis of Green Fluorescent Protein Gene Expression

Green fluorescent protein (GFP) expression in transfected cells in culture or in tissue samples was used to determine the transfection efficiency of the various methods employed. After transfection with a GFP-containing plasmid (pCMV-GFP) and at the indicated time, GFP positive cells were directly identified by fluorescence microscopy using 488 nm for excitation and 510-530 nm for emission. The transmitted light

image was recorded simultaneously. The transfection efficiency was estimated by the total number of GFP positive cells divided by the total number of cells in the same field detected under regular light (phase contrast). Approximately 500 cells in randomly selected fields were counted and used for calculation. For GFP expression in animals, liver samples were snap frozen on dry ice after removal from the animal. Tissue sections (10 μm) were made using a Cryostat and immediately observed under a fluorescence microscope.

2.2.7 Analysis of DNA by Southern Blot

Total DNA was extracted from the homogenized liver cells using DNAZOL[®] reagent following the manufacture's procedure (Invitrogen, Carlsbad, CA). DNA concentration was determined by spectrophotometry at 260 nm. Twenty five micrograms of DNA was digested with *HindIII* and separated on 1% agarose gel using a running buffer of 40 mM boric acid, 0.4 mM EDTA and 6% formaldehyde. The gel was then soaked with a 0.4 M NaOH, 0.6 M NaCl buffer for 30 minutes and incubated at room temperature overnight. DNA bands were transferred to GeneScreen Plus hybridization transfer membrane (MEN[™] Life Science Products, Boston, MA) using the soaking buffer. The membrane then was soaked with Tris buffer (0.5 M Tris pH 7.0, 1 M NaCl) for 15 minutes. After UV crosslinking, the membrane was pre-hybridized in hybridization buffer (0.25 M NaHPO₄, 0.25 M NaCl, 1 mM EDTA, 0.25 M SDS, 50% deionized formamide, 0.1 mg/ml salmon sperm ssDNA) at 42°C for 3 hours. The hybridization probe of ³²P-labelled luciferase cDNA made with a Random Primer DNA Labeling Kit (Invitrogen, Carlsbad, CA) was then added to the

hybridization buffer in a ratio of 10^6 cpm/ml, and the hybridization reaction was continued for 18 hours. The membrane then was washed with buffer I (0.3 M NaCl, 0.1 M $C_2H_3NaO_2$, 3.5 mM SDS), buffer II (25 mM $NaHPO_4$, 1 mM EDTA, 1.4 mM SDS), and buffer III (25 mM $NaHPO_4$, 1 mM EDTA, 14 mM SDS). The membrane was then dried and subjected to autoradiography.

2.2.8 Northern Blot Analysis

Total RNA was extracted from the homogenized liver cells using Trizol[®] reagent following the manufacture's procedure (Invitrogen, Carlsbad, CA). RNA concentration was determined by spectrophotometry at 260 nm. Twenty microgram of total RNA was separated on 1% agarose-3% formaldehyde gel using a running buffer of 40 mM boric acid, 0.4 mM EDTA and 6% formaldehyde. RNA bands were transferred to GeneScreen Plus hybridization transfer membrane (MEN[™] Life Science Products, Boston, MA) using a 25 mM $NaHPO_4$ transfer buffer. After UV crosslinking, the membrane was pre-hybridized in hybridization buffer (0.25 M $NaHPO_4$, 0.25 M NaCl, 1 mM EDTA, 0.25 M SDS, 50% deionized formamide, 0.1 mg/ml salmon sperm ssDNA) at 42°C for 3 hours. A hybridization probe of ³²P-labelled luciferase cDNA made with a Random Primer DNA Labeling Kit (Invitrogen, Carlsbad, CA) was then added to the hybridization buffer at a ratio of 10^6 cpm/ml, and the hybridization reaction continued for 18 hours. The membrane was then washed with buffer I (0.3 M NaCl, 0.1 M $C_2H_3NaO_2$, 3.5 mM SDS), buffer II (25 mM $NaHPO_4$, 1 mM EDTA, 1.4 mM SDS), buffer III (25 mM $NaHPO_4$, 1 mM EDTA, 14 mM SDS) and subjected to autoradiography.

EVALUATION OF RELATIVE PROMOTER STRENGTH OF HUMAN CYTOCHROME P450 GENES USING OPTIMIZED TRANSFECTION *IN VITRO* AND *IN VIVO*

3.1 INTRODUCTION

The expression of CYP genes is subject to diverse regulatory controls, which display tissue-specific, sex-specific and developmental patterns. CYP enzymes are primarily expressed in liver hepatocytes, and to a lesser extent, in cells of the intestines, lungs, kidneys, and brain (161). The CYP enzymes are important clinically in maintaining homeostasis by metabolizing invading xenochemicals. Striking differences in the amount and activity of the various CYP enzymes exist among individuals. At least some of these differences are responsible for the inter-individual variability in drug response that results in instances of treatment failure. Genetic mutations in CYP genes have been identified and linked to abnormal drug responses (162). However, less is known about how gene expression differs among the CYP genes.

Certain cytochrome P450s are constitutively expressed and the expression of others is known to be induced by various xenobiotics including prescription drugs. Most CYP regulation is at the transcription level, although post-transcriptional regulation is also seen. Studies on the relative strength of CYP gene promoters may yield information leading toward a better understanding of the polymorphic nature of drug metabolism and aid in maximizing the benefits of drug therapy. Two general approaches have

been previously used to estimate the relative strength of CYP gene promoters. The first of these involves measurement of the mRNA or protein level of individual CYP gene products using Northern, Western blot analysis, or substrate-based enzyme activity assays. The second approach involves an *in vitro* transfection of cells with a plasmid containing a reporter gene under the control of a CYP promoter (163, 164). Significant information has been obtained using these approaches. However, since these early studies were conducted by many investigators employing various cell lines and different reporter systems, it is difficult to directly compare the activity of the different CYP promoter sequences that have been studied to date. Furthermore, results from cell lines, primary cell culture and tissue samples obtained from patients suggest that transcription activity varies significantly depending on experimental conditions employed. Thus, progress in developing a cohesive picture of the relative strength of CYP gene promoters has been slow.

Recently, reliable techniques have been developed for *in vitro* transfer of DNA into cells using positively charged liposomes or cationic polymers (165-170). In addition, the method of hydrodynamic delivery for efficient DNA transfer into hepatocytes in mice has also been developed (160). These newer developments, plus the already available molecular biology techniques, provide a convenient means for cloning of CYP gene promoters into reporter containing plasmid constructs and the assessment of their relative promoter strength both *in vitro* and *in vivo*. Application of this methodology readily makes possible the side by side comparison of relative promoter strength under the same experimental conditions. In this chapter, we describe optimized conditions for such a comparison in different types of cells *in vitro* and *in*

in vivo. Comparison of the activity obtained for a variety of CYP gene promoters demonstrates that, among those tested, the CYP2D6 promoter exhibits the strongest activity. The order of promoter strength was found to be CYP2D6> CYP1A2> CYP3A4> CYP2C9> CYP2C18> CYP2E1. This order of promoter strength is observed *in vitro* in cell lines transfected with the aid of cationic polymers as well as *in vivo* in mouse liver, kidney, heart, lung, and spleen cells following hydrodynamic transfection.

3.2 RESULTS

3.2.1 Optimization of Transfection Conditions

Although PEI has been successfully used to transfer DNA into various types of cells, its activity varies in different cells (171). The ratio of PEI/DNA required for optimal transfection may vary when a fixed dose of plasmid DNA is used for different cell types. Figure 1 shows the effect of varying the total amount of PEI while keeping the amount of plasmid DNA constant at 1 µg. The peak level of luciferase gene expression was observed at 1.5 µg of PEI for both 293 and BL-6 cells and was greater than 2.5 µg for HepG2.

It was also noted that, although the peak level of luciferase expression was obtained with different amounts of PEI, the absolute amount of luciferase expressed by these three types of cells varied (293> BL-6= HepG2). This suggests different levels of

transfectability of these cells by PEI. Of note is a decrease in the level of luciferase activity when the amount of PEI is more than 5 μ g per well. Increasing the amount of PEI over the optimal amount resulted in cellular toxicity morphologically visible after transfection (data not shown).

The percentage of cells transfected with time was determined using a GFP containing plasmid. Figures 2-4 show the images of selected fields under a fluorescence microscope using a FITC filter or regular light. The estimated percentages of GFP positive cells from an approximately 500 cells counted in randomly selected fields were 75% for 293 cells, 60% for BL-6 cells and greater than 15% for HepG2 cells, suggesting higher transfection efficiency of PEI in 293 cells than the other two cell lines employed.

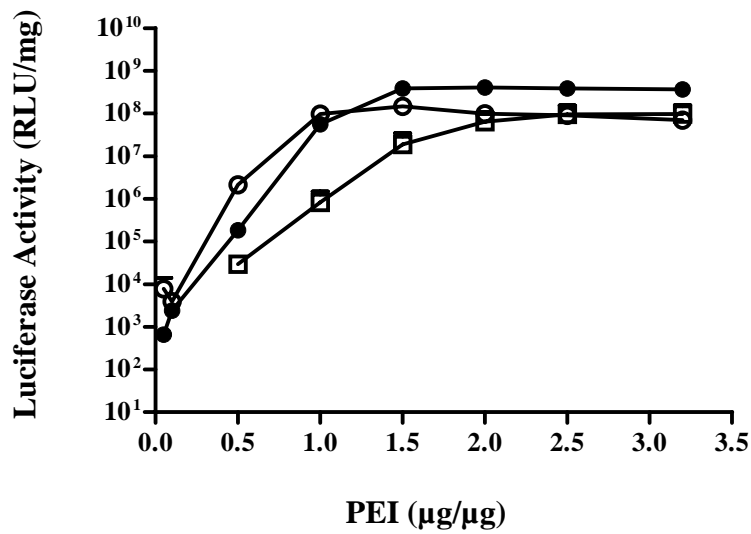


Figure 1. Effect of PEI Amount on Overall Level of Transgene Expression among Various Cell Lines.

Cells (5×10^4 /well) were seeded in each well of a 48-well plate 24 hours prior to transfection with 1 µg of p2D6-luc plasmid mixed with various amounts of PEI. Twenty four hours later, transfection solution was replaced with fresh medium then luciferase activity in each well was determined 48 hours post transfection of 293 (●), BL-6 (○), or HepG2 cells (□). Values represent the mean \pm S.E. of three independent transfections.

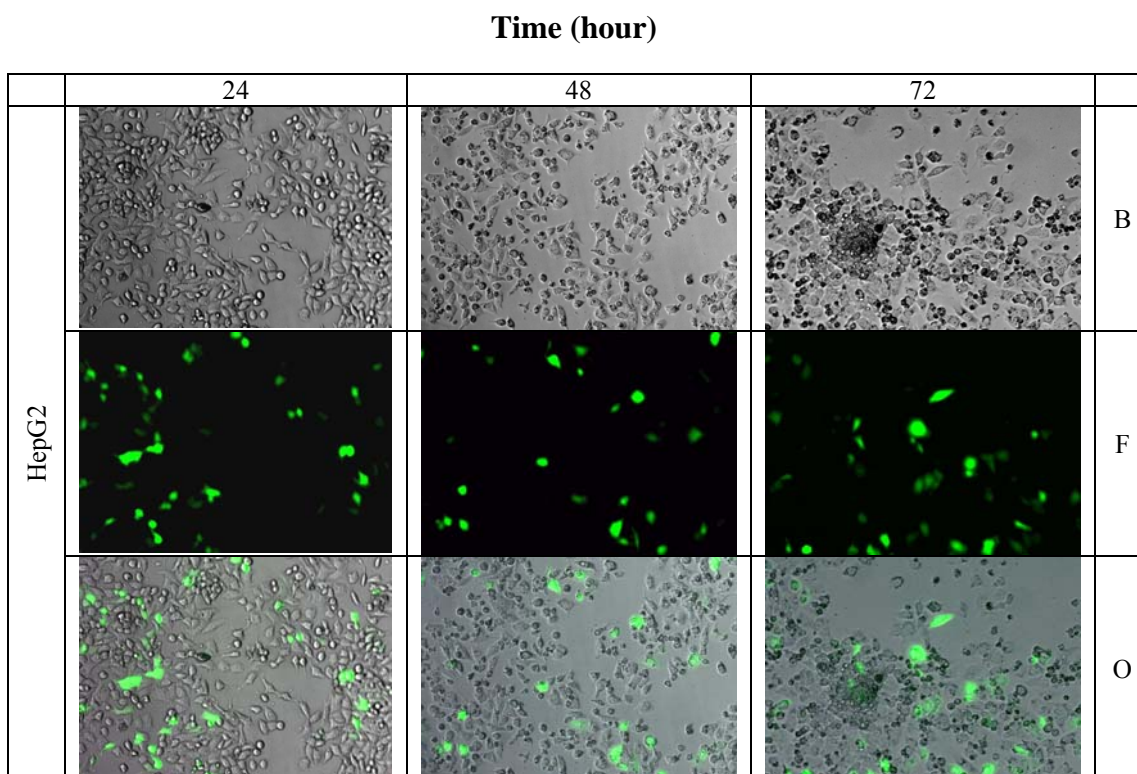


Figure 2. Transfection Efficiency on HepG2 Cells.

HepG2 cells were seeded into a 12-well plate and transfected with 2 μg of pCMV-GFP plasmid using 3.2 $\mu\text{g}/\mu\text{g}$ of PEI. Twenty four, 48, or 72 hours post transfection, GFP positive cells were directly observed under a fluorescence (F) microscope using 488 nm for excitation and 510-530 nm for emission and under the transmitted (B) light. Images were recorded simultaneously and then electronically overlaid (O). Images were a random shot of cells in a Petri dish with transfected cells.

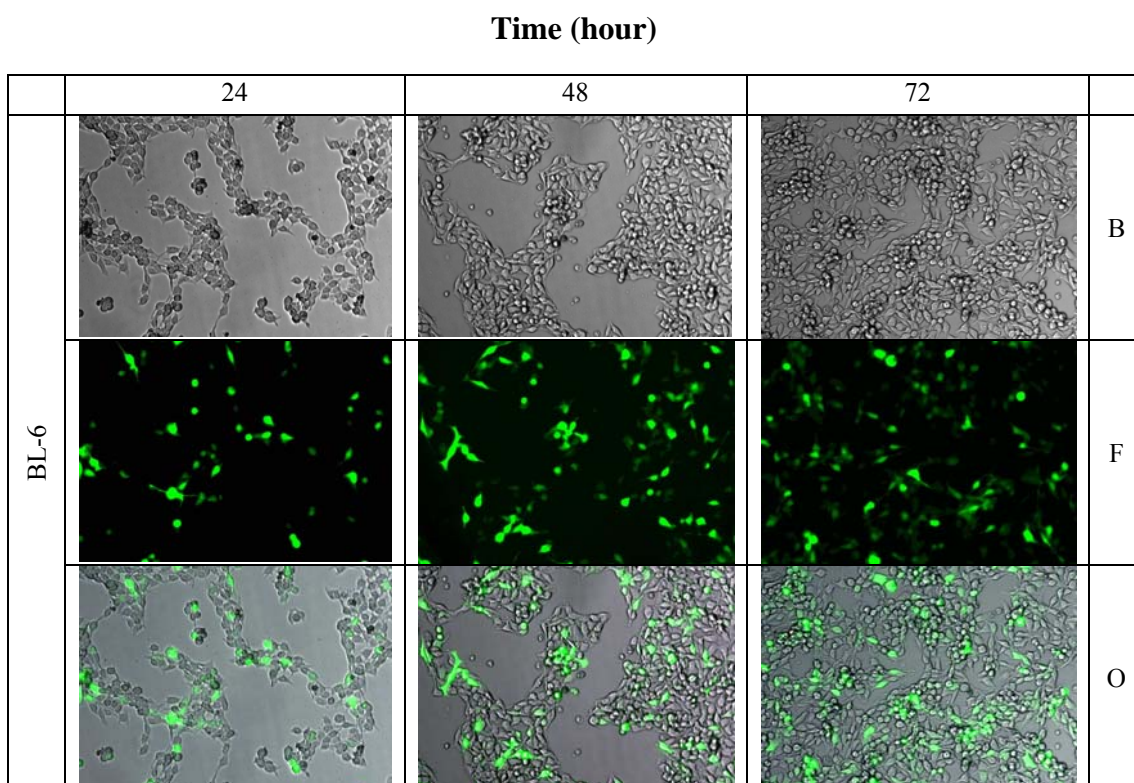


Figure 3. Transfection Efficiency on BL-6 Cells.

BL-6 cells were seeded into a 12-well plate and transfected with 2 μg of pCMV-GFP plasmid using 3.2 $\mu\text{g}/\mu\text{g}$ of PEI. Twenty four, 48, or 72 hours post transfection, GFP positive cells were directly observed under a fluorescence (F) microscope using 488 nm for excitation and 510-530 nm for emission and under the transmitted (B) light. Images were recorded simultaneously and then electronically overlaid (O). Images were a random shot of cells in a Petri dish with transfected cells.

Time (hour)

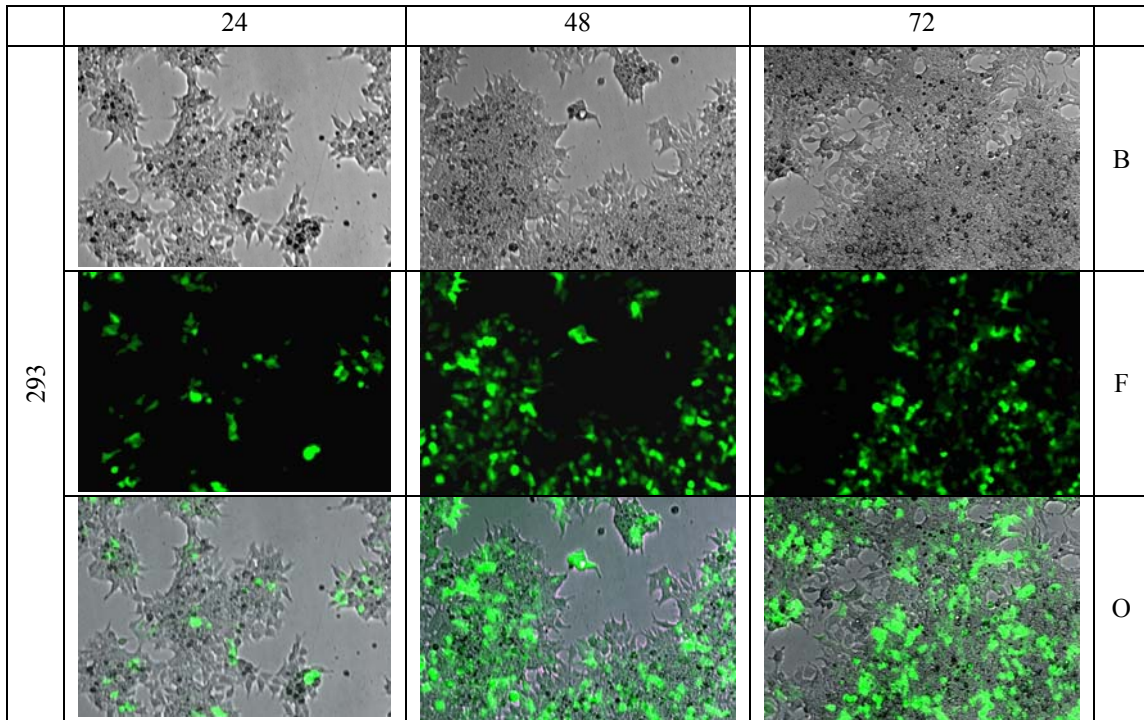


Figure 4. Transfection Efficiency on 293 Cells.

293 cells were seeded into a 12-well plate and transfected with 2 μg of pCMV-GFP plasmid using 3.2 $\mu\text{g}/\mu\text{g}$ of PEI. Twenty four, 48, or 72 hours post transfection, GFP positive cells were directly observed under a fluorescence (F) microscope using 488 nm for excitation and 510-530 nm for emission and under the transmitted (B) light. Images were recorded simultaneously and then electronically overlaid (O). Images were a random shot of cells in a Petri dish with transfected cells.

3.2.2 Comparison of the Relative Strength of CYP Promoters

We compared the transcriptional strength of six CYP promoters in various cell lines using the optimal transfection conditions established in Figure 1. The human serum albumin (SA) gene promoter was used as a reference control. We first determined the relative promoter strength in HepG2 cells. Originating from human liver, HepG2 cells are one of the most commonly used models for studies of transcription regulation of CYP genes (172). Figure 5 shows that the level of luciferase activity seen in p2D6-luc transfected cells is the highest (5×10^8 RLU per mg of protein) and is approximately 6 orders of magnitude higher than that of cells transfected with p2E1-luc and two orders higher than that of pSA-luc. The order of promoter strength observed was CYP2D6 > CYP1A2 > CYP2C9 = CYP3A4 > CYP2C18 > CYP2E1. The strength of the human SA promoter is similar to that of the CYP1A2 promoter.

Enriched expression of CYP genes in the liver has been known for many years. The reason for the high level of CYP enzymes in the liver has been attributed to unique sequences called liver specific promoters (or tissue specific promoters in general). We decided to examine whether our CYP promoters are active in cells of non-hepatic origin by performing similar transfection experiments in 293 and BL-6 cells. Neither of these cell lines have an obvious similarity morphologically or functionally to HepG2 cells. As evidenced in Figures 6 and 7, all constructs containing CYP gene promoters are active in these non-hepatic cells. In fact, the amount of luciferase protein expressed in these cells is generally higher than that in HepG2 cells due to the fact that these cells are more transfectable under the experimental conditions.

Importantly, the relative promoter strength among the CYP gene promoters examined is very similar to that seen in HepG2 cells, indicating a versatile nature of these CYP promoters in driving gene expression in different cells.

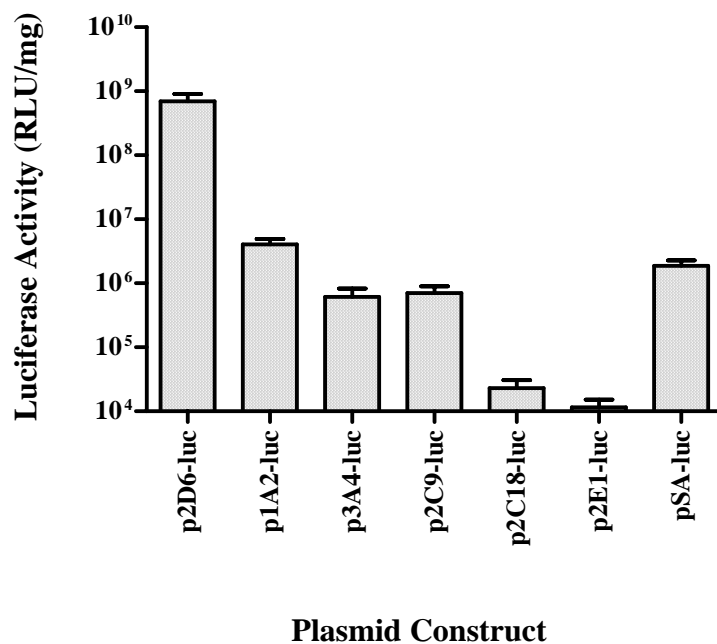


Figure 5. Activity of CYP Promoters in HepG2 Cells.

HepG2 cells were seeded on a 48-well plate (5×10^4 /well) 24 hours prior transfection with $1 \mu\text{g}$ of the indicated CYP plasmid using $2.5 \mu\text{g}$ of PEI. Twenty four hours post-transfection, the transfection solution was replaced with fresh medium and luciferase activity evaluated 48 hours later. Values represent the mean \pm S.E. of six independent transfections.

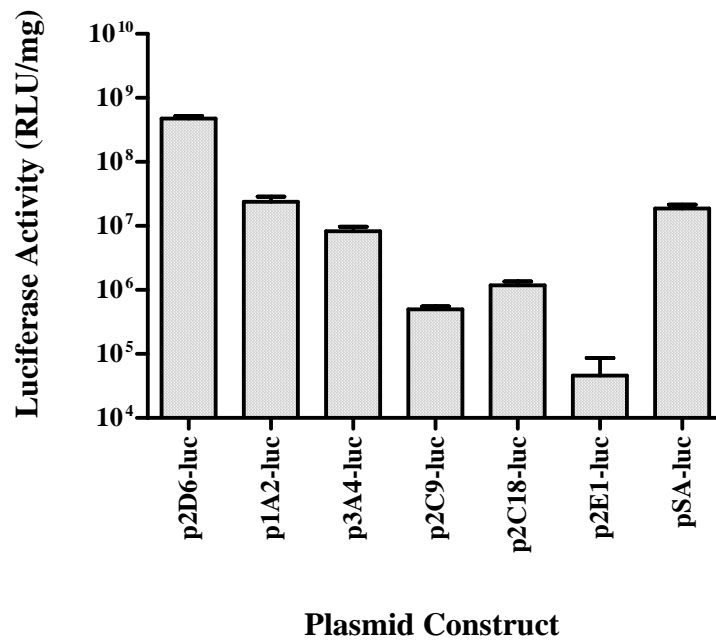


Figure 6. Activity of CYP Promoters in BL-6 Cells.

BL-6 cells were seeded on a 48-well plate (5×10^4 /well) 24 hours prior transfection with $1 \mu\text{g}$ of the indicated CYP plasmid using $2.5 \mu\text{g}$ of PEI. Twenty four hours post-transfection, the transfection solution was replaced with fresh medium and luciferase activity evaluated 48 hours later. Values represent the mean \pm S.E. of six independent transfections.

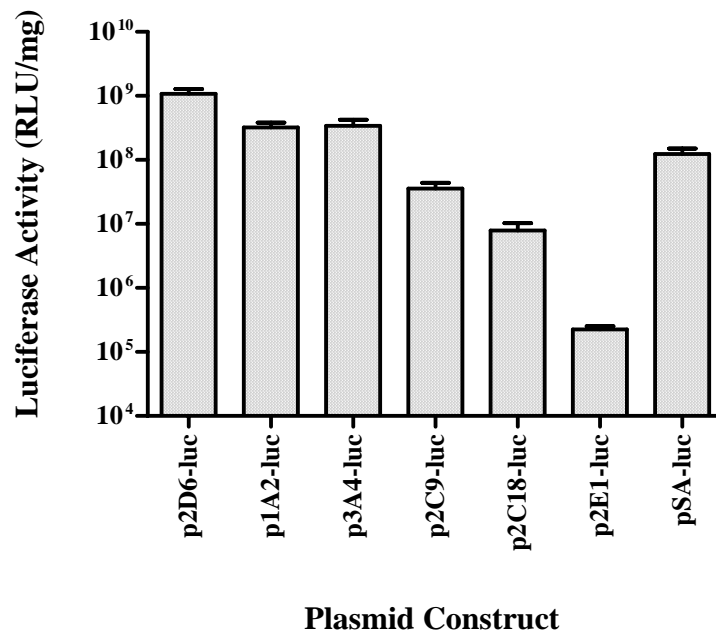


Figure 7. Activity of CYP Promoters in 293 Cells.

293 cells were seeded on a 48-well plate (5×10^4 /well) 24 hours prior transfection with $1 \mu\text{g}$ of the indicated CYP plasmid using $2.5 \mu\text{g}$ of PEI. Twenty four hours post-transfection, the transfection solution was replaced with fresh medium and luciferase activity evaluated 48 hours later. Values represent the mean \pm S.E. of six independent transfections.

We extended this *in vitro* study to whole animals and examined whether a similar order of promoter strength would be obtained in various normal cells under physiological conditions. Toward this end, we employed the hydrodynamics-based procedure previously developed in our laboratory (160). Hydrodynamic delivery uses the hydrodynamic pressure to disrupt the blood vessel endothelium and generate transient pores in the plasma membrane of parenchymal cells surrounding the vascular capillary (173). We have previously shown that in mice a high level of transgene expression in the liver, heart, lung, kidney and spleen can be achieved by a rapid tail vein injection of a large volume of DNA solution (160, 174). A mechanistic study on hydrodynamic delivery revealed that, with the highest level of transgene expression observed, the liver is the most sensitive organ for this technique (160). Figure 8 shows that there 30-40% of hepatocytes are transfected by a hydrodynamic injection of 10 μg of pCMV-GFP plasmid DNA. The percentage of GFP positive cells decreases with time, indicating the transient nature of GFP expression in mouse liver.

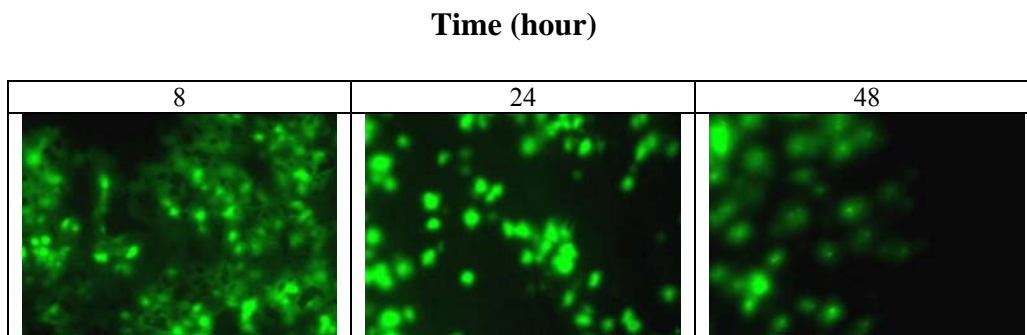


Figure 8. Transfection Efficiency on Mouse Hepatocytes.

Mouse hepatocytes were transfected with pCMV-GFP plasmid DNA via tail vein injection of 1.8 ml saline containing 10 μ g of DNA within 3-5 seconds. At the indicated time, animals were sacrificed, the liver dissected out, and immediately frozen on dry ice. Liver sections (10 μ m) were made in a Cryostat and immediately observed under a fluorescence microscope using 488 nm for excitation and 510-530 nm for emission.

The levels of luciferase expression by CYP constructs in transfected animals are shown in Figure 9. In all internal organs examined, the highest luciferase gene expression was seen in the liver and lower similar levels were seen in the heart, lung, kidney, and spleen. The approximately 3 orders of magnitude higher luciferase expression in the liver is largely due to the relative liver specificity of the hydrodynamic delivery. Importantly, the order of promoter strength for CYP constructs in the liver is very similar to the pattern seen *in vitro* experiments with p2D6-luc the strongest seen followed by p1A2-luc, p3A4-luc, p2C9-luc, p2C18-luc, and p2E1-luc. Compared to the CYP promoters, the human SA promoter exhibits relatively low activity in mice.

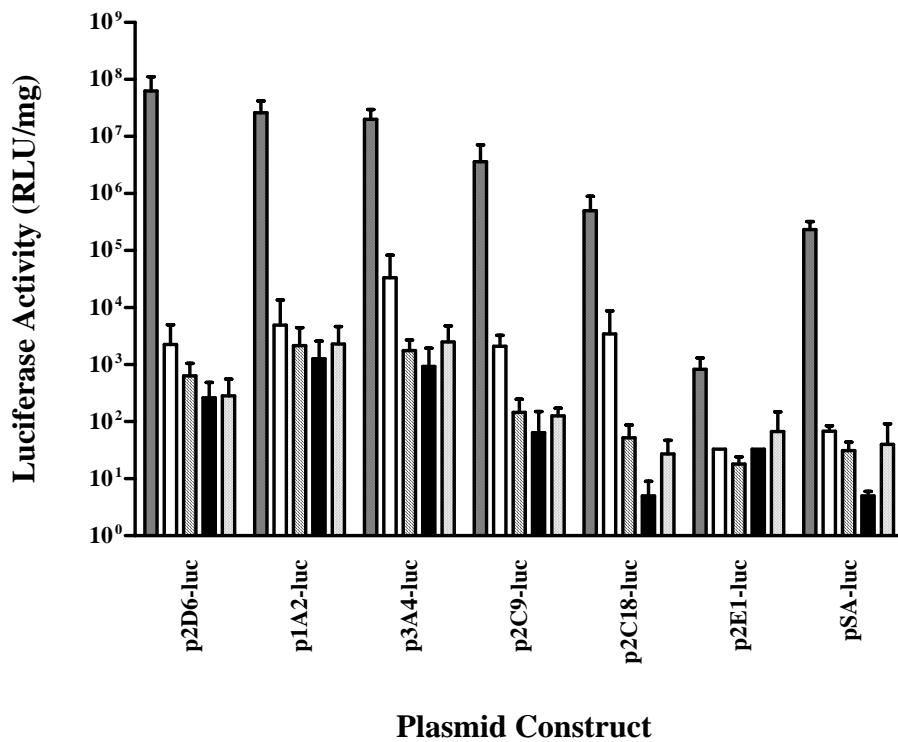


Figure 9. Activity of CYP Promoters in Various Mouse Organs.

Animals were transfected with plasmid DNA via tail vein injection of 1.8 ml saline containing 10 μ g of the indicated plasmid within 3-5 seconds. Luciferase activity was determined 8 hours post transfection in the liver (the first bar), kidney (the second), spleen (the third), lung (the fourth), and heart (the fifth). Values represent the mean \pm S.E. of 3-5 independent transfections.

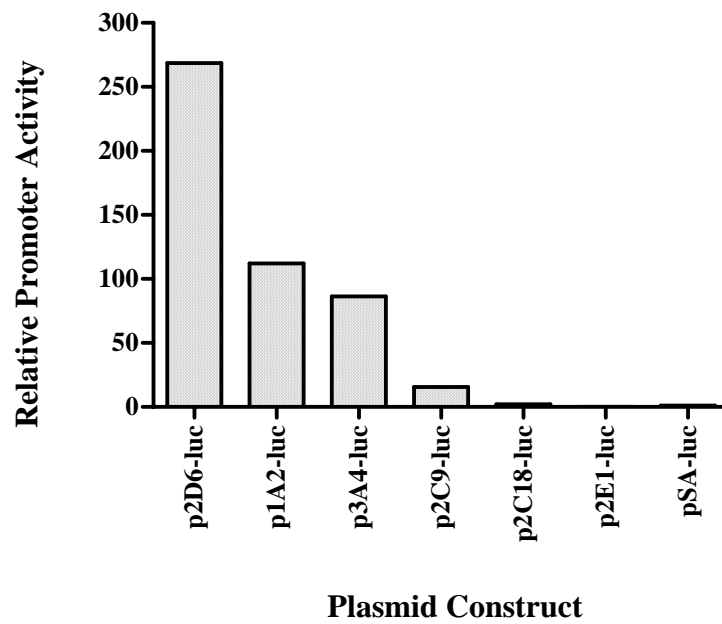


Figure 10. Relative Strength of CYP Promoters in Mouse Liver.

Luciferase activity of mouse livers transfected with various CYP plasmids were normalized by that of pSA-luc activity as one.

3.3 DISCUSSION

Efficient transfection resulting in a high level of reporter gene expression in both cell culture and mice has been demonstrated. PEI has been shown previously to be one of the most effective polymers in transferring plasmid DNA into cells *in vitro* and *in vivo* (175-177). The *in vitro* transfection results agree with those of previous studies in which the ratio of PEI and DNA at 1.2-3.5:1 was found to be optimal (154, 171). The excess of PEI in the DNA/PEI complexes enhances their binding to the negatively charged cell surface resulting in higher level of DNA internalization (154, 178). Transfection of mice using hydrodynamic delivery proved to be very effective with 30-40% of hepatocytes being transfected. This level of transfection efficiency in mice is similar to that of the previous study using the β -galactosidase gene as a reporter (160).

The major interest in pursuing these methods of highly efficient DNA transfer into various cells was to enable the direct comparison of the transcription activity of a variety of CYP promoters. This is important for those interested in drug metabolism, as it is essential to estimate the basal level of CYP gene expression. Toward this end, we have cloned into pGL3-Basic vectors the promoters of six CYP genes that are responsible for metabolism of over 90% of commonly prescribing drugs (34). The results we obtained from both *in vitro* and *in vivo* studies suggest that the CYP2D6 promoter has the strongest promoting activity among those studied and is stronger than that of the human SA gene promoter. To provide a relative promoting value for each promoter, we have normalized the strength of each CYP gene promoter by

dividing the luciferase activity seen in mouse liver by that of pSA-luc transfected animals. Considering the human SA promoter strength as 1, the relative promoter strength is 269 for CYP2D6, 112 for CYP1A2, 86 for CYP3A4, 16 for CYP2C9, 2 for CYP2C18 and less than 1 for CYP2E1 (Figure 10).

The fact that promoter strength determines the basal level of CYP gene expression suggests that mutations in the promoter region can certainly affect the overall CYP protein level. Subsequently, sequence variation in the promoter region or promoter polymorphism could be one of the major causes for inter-individual variability in drug response. Compared to efforts in the study of polymorphisms of the coding regions of CYP genes, efforts in studying polymorphic nature of the regulatory sequences of CYP genes are lacking. Identification of single nucleotide polymorphisms in regulatory elements (promoters, enhancing sequences, insulators, suppressors and introns) that control CYP gene expression thus becomes an increasingly important consideration in pharmacogenomics.

The versatile nature of the CYP promoters driving gene expression in different cells has not been fully realized. Data presented in Figures 5-7 and 9 appear to suggest that CYP promoter activity is not limited to hepatocytes. In contrast to a common belief that tissue specific gene expression is controlled by the promoter sequence (the so-called tissue specific promoter) our data suggests that these sequences do not play a dominant role in determining tissue specific activity. For example, all of the promoters examined in this study, including the human SA promoter, have been considered to be liver specific, although some show relatively low activities in other

organs such as the intestines, kidneys, and lungs. While these promoters exhibited good activity in driving reporter gene expression in hepatoma cell (HepG2), we observed similar activity in human embryonic kidney fibroblasts (293 cells), murine melanoma (BL-6 cells), and in mouse liver, heart, lung, kidney and spleen cells. These results indicate that all these cells possess transcription factors essential in recognizing promoter sequences and driving reporter gene expression. The liver specificity of the promoters could be due to upstream sequences not subcloned into the vector, or due to sequences in the introns or the 3'-flanking region of the genes.

Compared to promoter sequences in genomic DNA that may exist in a highly folded chromatin structure, the promoter in a plasmid is readily accessible for transcription due to the lack of a high degree of folding. Thus, the tissue or cell specific gene expression associated with genomic DNA but not with transfected cells is likely determined by promoter accessibility. Under this assumption, whether a gene will be expressed in a given tissue is determined by its location in the chromatin structure. Genes whose promoter sites are readily accessible are transcribed while those that are wrapped inside the chromatin structure will stay inactive.

It is important to point out that, although a promoter sequence is essential for transcription, transcription efficiency is largely controlled by transcription factors through various regulatory elements in DNA. For example, our attempt to correlate the promoter strength as shown in this study to protein level of various P450 enzymes in human liver did not result in a direct correlation. The lack of a direct correlation between the promoter strength of the CYP genes and their protein level could be due

to difference in CYP enzyme stability, stability of their mRNA, post transcriptional and/or translational modification, and the degree of transcription regulation. Obviously, details in mechanisms underlying gene expression regulation are yet to be fully investigated.

Although different levels of reporter gene expression are obtained with various CYP promoters, at present we do not know if the promoter sequence is acting alone to modulate transcription efficiency or if there are any cooperative/antagonistic interactions between the various transcription factors. In addition, it is possible that transcription factors involved in regulating transgene expression under different CYP promoters are also different in cell lines and in mice.

With respect to the CYP2D6 promoter that exhibited consistently high activity in this study, we speculate that there must be some unique sequence in this promoter region that would enhance RNA polymerase binding. Previous studies using deletion mutation analysis have identified an HNF-4 binding site at the -429/-80 region (80). Cairns *et al* (179) showed that the critical sequence of the 2D6 promoter is between -392 and -18. To see if there is a co-relationship between the HNF element in the promoter region and promoter strength, we have performed a promoter analysis using MatInspector software (180) available at Genomatix web site (www.genomatix.de). In the sequences employed in this study, we identified 2 HNF sites in the 1A2 promoter, 17 in 2C9, 1 in 2C18, 7 in 2E1, and 1 in 3A4. Only 2 HNF sites are found in 2D6 promoter region. Obviously, the number of HNF binding sites in the promoter region does not determine the promoter strength since CYP 2C9 promoter with 17 HNF sites

did not exhibit better promoter activity than the 3A4 promoter that contains only one such site. Evidently, additional work is needed to gain a full understanding of the mechanisms through which transcription is regulated.

In summary, optimized procedures for *in vitro* and *in vivo* transfection were employed to evaluate the relative strength of promoters for 6 CYP genes that are responsible for metabolism of over 90% of prescription drugs. We demonstrated that, among the 6 CYP promoters examined, the CYP2D6 promoter exhibits the strongest activity followed by the 1A2, 3A4, 2C9, 2C18 and 2E1 promoters. All of these promoters were found to be active in driving reporter gene expression in established cell lines (HepG2, 293, BL-6) as well as in cells in mouse liver, heart, lung, kidneys and spleen. Our findings indicate that the *in vitro* and *in vivo* systems explored here are useful tools for studying transcription regulation of various CYP genes.

EVALUATION OF THE ACTIVITIES OF CYTOCHROME P450 PROMOTERS IN SUSTAINING TRANSGENE EXPRESSION *IN VIVO*

4.1 INTRODUCTION

Cytochrome P450 (CYP) enzymes are mainly expressed in the liver, where most of drug metabolism processes takes place. It is well known that hepatic levels of CYP enzymes are subjected to regulation by many factors mostly at the transcription level. The activity of CYP promoters have been the subject of many studies, however, most of those studies aimed to investigate the role of CYP promoter sequences in xenobiotic mediated induction using *in vitro* systems.

In the previous study, we evaluated the relative strength of many human CYP promoters under optimized conditions *in vitro* and *in vivo*. In a continuation of that study, we aimed here to *in vivo* investigate the activities of many CYP promoters in sustaining levels of gene expression under the physiological conditions. Here, we have also tested the activities of many other non-CYP promoters to compare CYP promoter activities to them. Fifteen promoters were examined and categorized into four groups. The hepatic group included human CYP1A2, CYP2C9, CYP2C18, CYP2D6, CYP3A4, AAT (alpha 1-antitrypsin), SA (serum albumin), and mouse *cyp2b10* promoters. The viral promoters group included CMV (cytomegalovirus), CMV-EBV (EBNA1/OriP sequence of the Epstein-Barr virus under the CMV

promoter), and RSV (Rous sarcoma virus) promoters. The non-hepatic promoters included human APP (amyloid precursor protein) and chicken beta actin gene promoters (ACT). Promoters known to be sensitive to stress stimulation including NFkB (nuclear factor kappa B) and HS (heat shock protein 70) promoters were also included. All promoters were constructed in expression vectors containing the luciferase gene as a reporter.

The activity of each promoter in sustaining luciferase gene expression was assessed in mouse livers utilizing the hydrodynamic procedure. These comparative studies demonstrated that among the tested CYP promoters, the CYP2D6 showed the highest activity in sustaining transgene expression, and its activity was comparable to that of known strong promoters.

4.2 RESULTS

4.2.1 Activity of CYP Promoters in Sustaining Transgene Expression

To examine the activities of the various promoters in maintaining levels of luciferase gene expression, 10 µg of each plasmid construct was transfected into mouse hepatocytes using the hydrodynamic procedure. We found that eight hours post transfection, among the tested CYP promoters, the CYP2D6 showed the highest activity in driving luciferase gene expression whereas the CYP2C18 was the lowest (Figure 11). The superiority of CYP2D6 promoter activity over the rest of CYP

promoters was also confirmed by using the hAAT reporter gene, where we found that among the tested CYP promoters (CYP1A2, CYP2C9, CYP2D6, and CYP3A4), the CYP2D6 promoter showed highest activity (Figure 12). At this time point, the viral promoter pCMV-luc and the hepatic promoter pAAT-luc exhibited the highest levels of gene expression compared to all promoters whereas the hepatic promoter pSA-luc exhibited the lowest (Figures 11 and 13). According to the overall order of activity, some CYP promoters can be categorized as strong promoters in driving luciferase gene expression, with the CYP2D6 promoter being two orders of magnitude less active than the strong viral CMV promoter. Activities of the tested promoters were evaluated for period of 2 weeks. By the end of the second week, the activities of CYP promoters had declined significantly but at detectable levels. The same pattern of decline was observed with the non-CYP promoters (Figure 14). The p2D6-luc was able to sustain a high level of luciferase and hAAT gene expression for 3-4 and 7 days, respectively. The viral pCMV-EBV-luc was the most active promoter as it sustained transgene expression at a high level for more than a week. By the end of the second week, the pAAT-luc exhibited the highest activity among the rest of all the tested promoters whereas the stress sensitive promoters (pNFkB-luc and pHS-luc) exhibited sharp declines in their activities a few days post transfection (Figure 15).

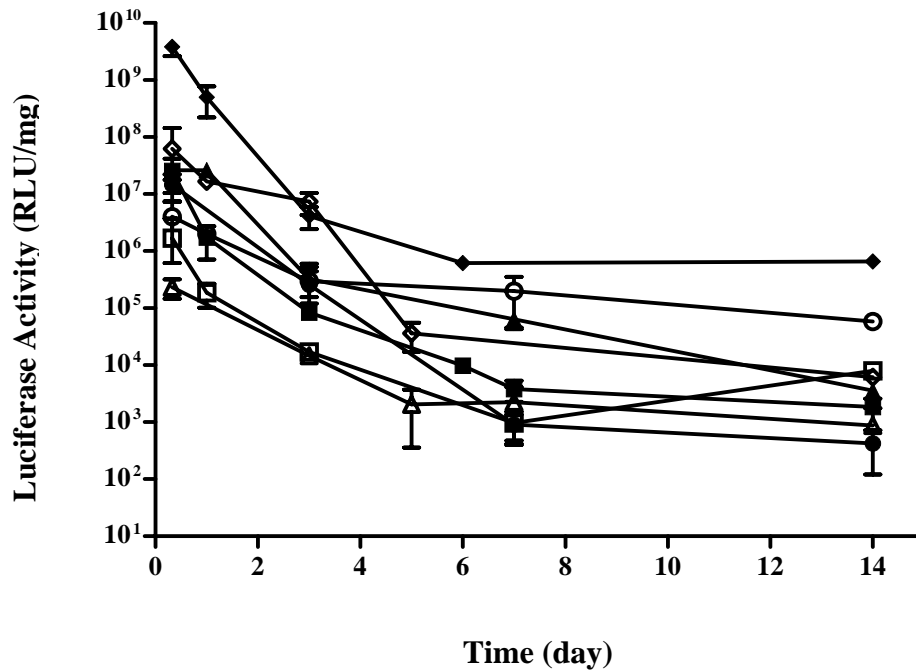


Figure 11. Activity of the Hepatic Promoters in Sustaining Luciferase Gene Expression.

Animals were transfected with plasmid DNA via tail vein injection of 1.8 ml saline containing 10 μ g of pAAT-luc (◆), p2D6-luc (◇), p3A4-luc (■), p2C18-luc (□), p2C9-luc (○), p2b10-luc (●), pSA-luc (△), or p1A2-luc (▲) plasmid DNA within 3-5 seconds. At the indicated time post transfection, luciferase activities were determined in the liver. Values represent the mean \pm S.E. of 3 independent transfections.

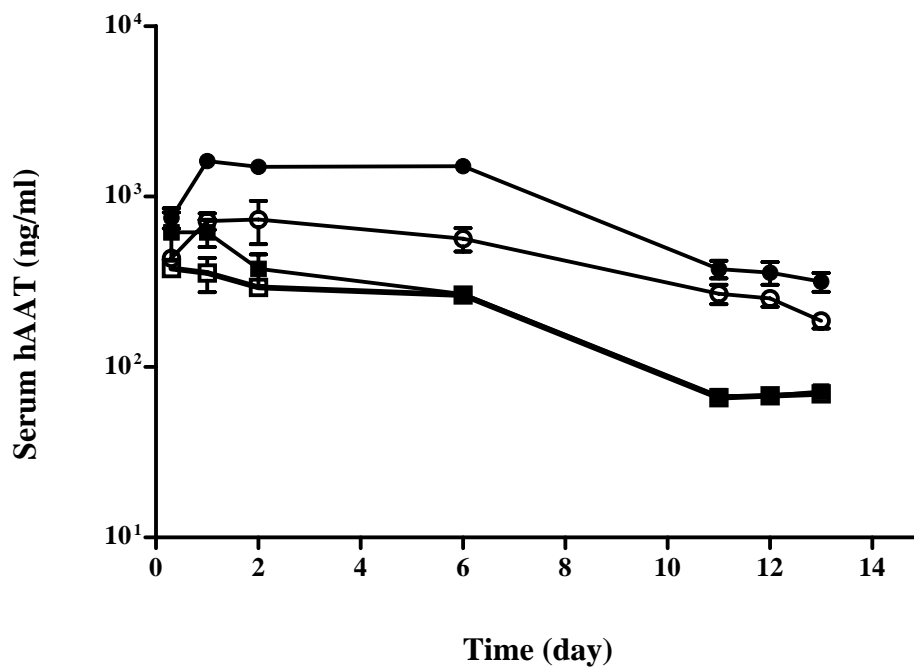


Figure 12. Activity of the CYP Promoters in Sustaining hAAT Gene Expression.

Animals were transfected with plasmid DNA via tail vein injection of 1.8 ml saline containing 10 μ g of p2D6-hAAT (●), p2C9-hAAT (○), p3A4-hAAT (■), or p1A2-hAAT (□) plasmid DNA within 3-5 seconds. Blood samples were collected at the indicated time post transfection, and serum concentration of hAAT was determined. Values represent the mean \pm S.E. of 3 independent transfections.

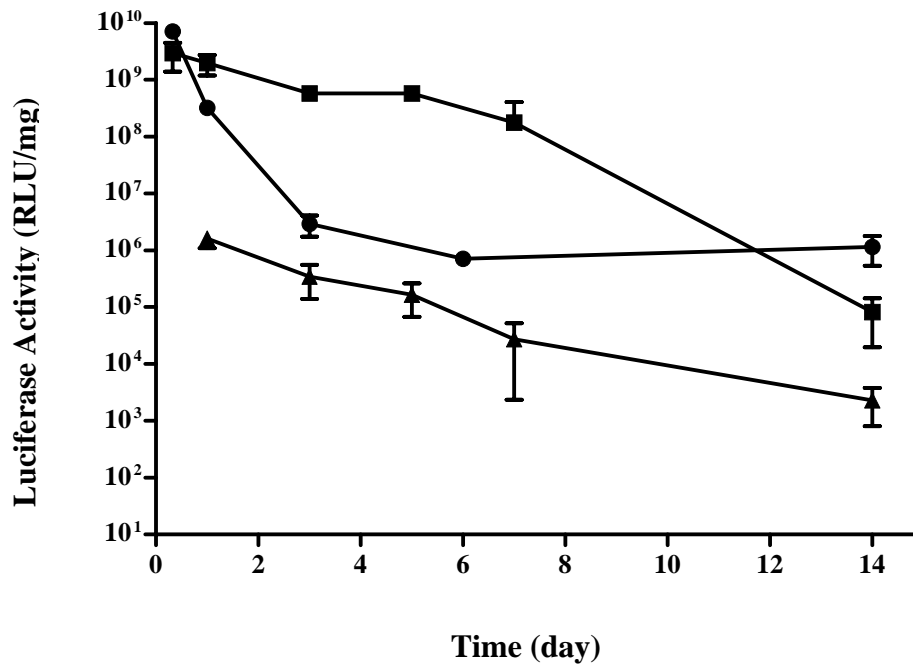


Figure 13. Activity of the Viral Promoters in Sustaining Luciferase Gene Expression.

Animals were transfected with plasmid DNA via tail vein injection of 1.8 ml saline containing 10 μ g of pCMV-EBV-luc (■), pCMV-luc (●), or pRSV-luc (▲) plasmid DNA within 3-5 seconds. Luciferase activities were determined in the liver at the indicated time post transfection. Values represent the mean \pm S.E. of 3 independent transfections.

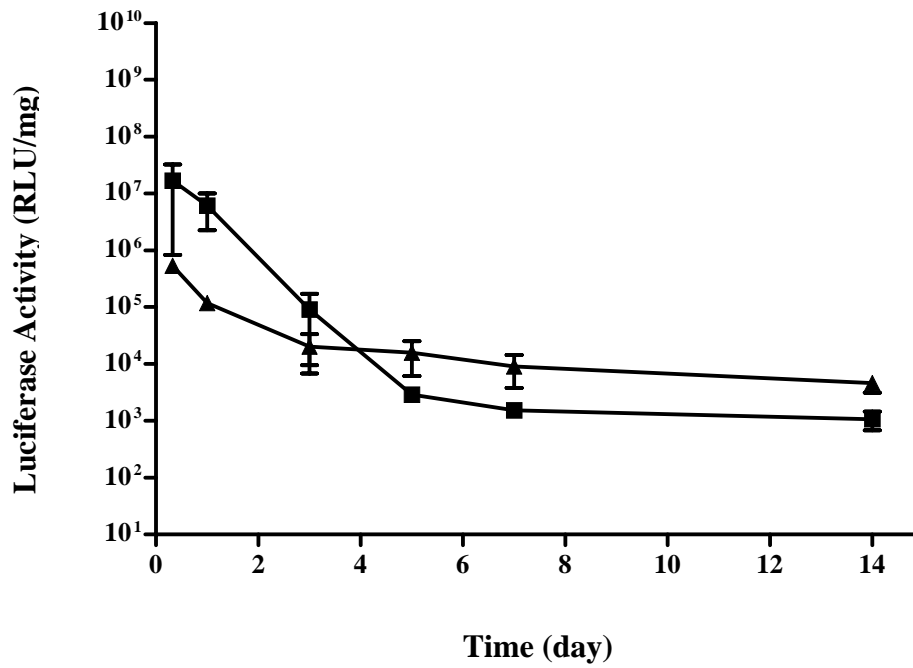


Figure 14. Activities of the Non-hepatic Promoters in Sustaining Luciferase Gene Expression.

Animals were transfected with plasmid DNA via tail vein injection of 1.8 ml saline containing 10 μ g of pAPP-luc (■) or pACT-luc (▲) plasmid DNA within 3-5 seconds. Luciferase activities were determined in the liver at the indicated time post transfection. Values represent the mean \pm S.E. of 3 independent transfections.

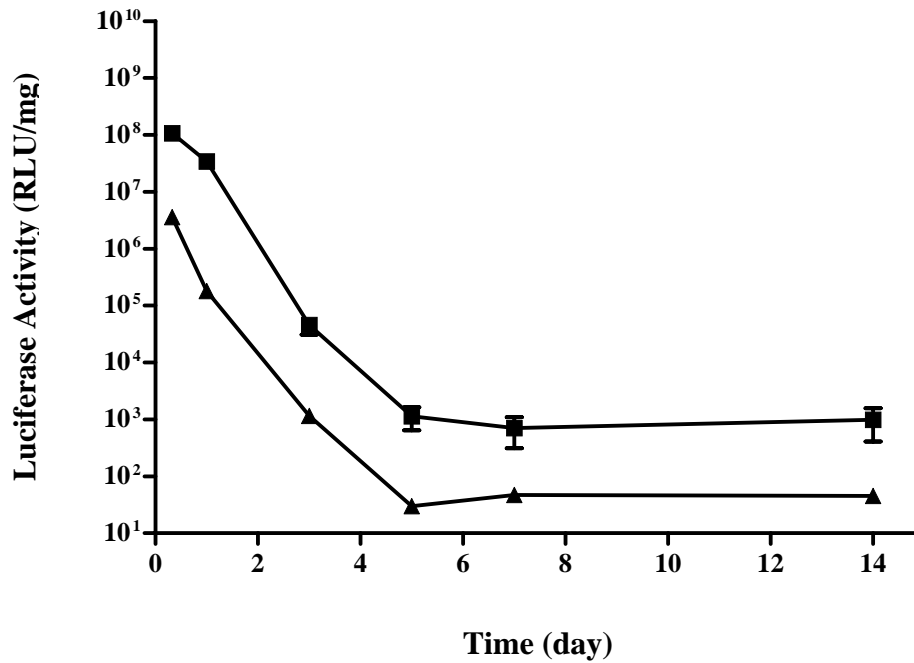


Figure 15. Activities of the Stress-Responsive Promoters in Sustaining Luciferase Gene Expression.

Animals were transfected with plasmid DNA via tail vein injection of 1.8 ml saline containing 10 μ g of pHS-luc (■) or pNFkB-luc (▲) plasmid DNA within 3-5 seconds. Luciferase activities were determined in the liver at the indicated time post transfection. Values represent the mean \pm S.E. of 3 independent transfections.

4.2.2 Persistence of Plasmid DNA in Mouse Liver

The activity pattern of rapid initial decline followed by a slow decay to a lower level was shared by all tested promoters. This pattern might be explained by rapid loss of the plasmid DNA in the transfected liver cells. To examine that, plasmid constructs of pCMV-EBV-luc, pRSV-luc, pACT-luc, p2D6-luc, p3A4-luc, and p2C9-luc were injected to the animals and the levels of their plasmid DNA as function of time were evaluated using Southern blot analysis. We found that regardless the origin of the injected promoter construct, all plasmids stayed in mouse liver in the episomal form for more than 2 weeks. However, some plasmids (pRSV-luc, p2D6-luc, p3A4-luc, and p2C9-luc) were characterized by loss of some of their initial DNA between the first and the third days (Figure 16).

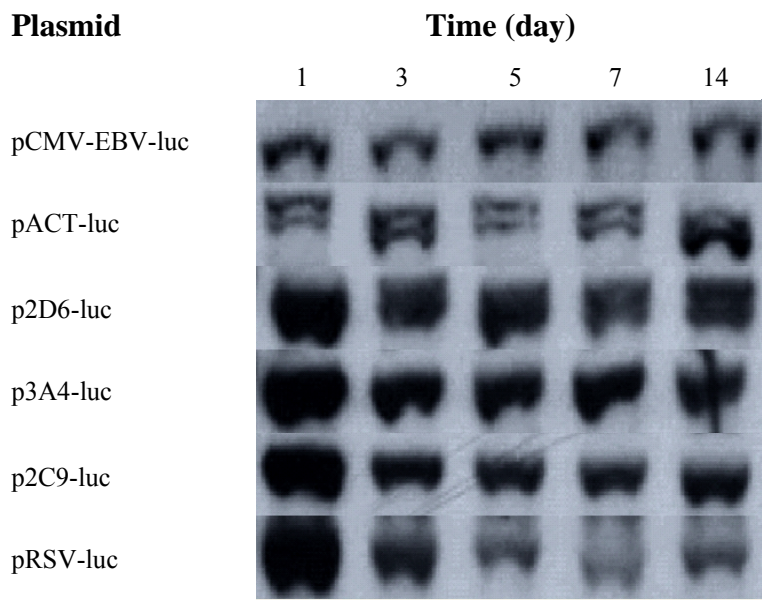


Figure 16. Southern Blot Analysis of Plasmid DNA in Transfected Mouse Liver.

Total DNA from the liver was prepared and analyzed from each mouse at the indicated time points (1, 3, 5, 7, or 14 days) post transfection and after injection of 10 μg of (starting from the top) pCMV-EBV-luc, pACT-luc, p2D6-luc, p3A4-luc, p2C9-luc, or pRSV-luc plasmid DNA. Each band represents the average of 3 liver samples from independent transfections.

4.2.3 Analysis of Transgene mRNA Levels of Plasmid Constructs in Mouse Liver

To evaluate the transgene mRNA levels in transfected mouse livers this study was done. Plasmids were injected to animals and at the indicated time points, livers obtained, and transgene mRNA analyzed. We found that the mRNA level of the reporter gene fell below the detection level one day post transfection for all of plasmid constructs with exception of the plasmid containing EBV viral sequences which maintained a high detectable level of mRNA for more than a week. The levels of mRNA for some plasmid constructs (pRSV-luc, pSA-luc, p3A4-luc, p2D6-luc, p2C18-luc, p2C9-luc, and p1A2-luc) were analyzed in earlier time point, and found that eight p2D6-luc, p3A4-luc, and pRSV-luc mRNA levels could be detected (Figure 17).

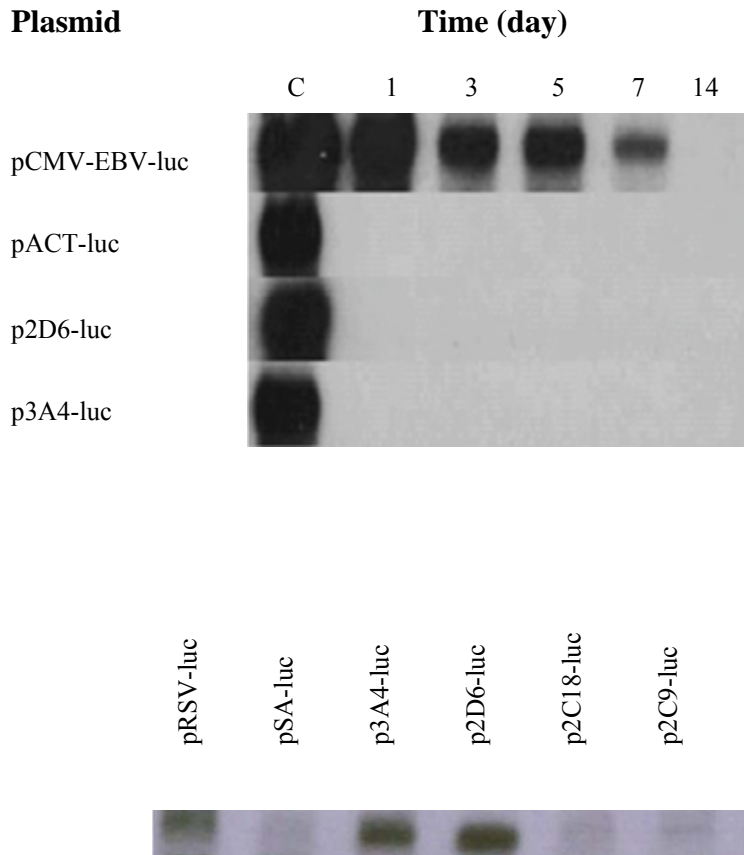


Figure 17. Transgene mRNA Analysis in Mouse Liver.

(Upper) Animals were injected with 10 μ g of (starting from the top) pCMV-EBV-luc, pACT-luc, p2D6-luc, or p3A4-luc; at the indicated time (1, 3, 5, 7, or 14 days) the livers were obtained and the total RNA extracted and subjected to Northern blot analysis. The lane 1 (C) represents the positive control (mouse injected with 20 μ g of pCMV-luc and analyzed eight hours post transfection) and each band represents the average of three liver samples from independent transfections. **(Lower)** Animals were injected with 10 μ g of (starting from the left) pRSV-luc, pSA-luc, p3A4-luc, p2D6-luc, p2C18-luc, p2C9-luc, or p1A2-luc plasmid DNA, and eight hours post transfection liver samples were collected and subjected to Northern blot analysis. Each band represents the average of three liver samples from independent transfections.

4.3 DISCUSSION

The promoter region of any gene is known to play the major role in initiation of the transcription process due to its content of essential elements required by RNA polymerase, nuclear receptors, and transcriptional factors. In the current study, we aimed to investigate the role of CYP promoters in controlling gene expression. We used a reporter assay to achieve this goal by inserting of different CYP 5'-FRs into expression vectors containing luciferase or hAAT as reporter genes.

The study was carried out *in vivo* under the physiological conditions. Promoters of human CYP1A2, CYP2C9, CYP2C18, CYP2D6, and CYP3A4 as well as mouse *cyp2b10* were included this study. The common pattern of all tested promoters was high initial gene expression eight hours post transfection followed by rapid decline and slow decay to a lower level. Unlike the others, the CYP2D6 promoter exhibited a different pattern by maintaining luciferase gene expression sustained at a high level for up to 3 days and hAAT gene expression for 7 days. This promoter was the most active among the tested CYP promoters (Figures 11 and 12).

The activities of CYP promoters versus other hepatic promoters, pAAT-luc and pSA-luc were also compared. Human alpha 1-antitrypsin and albumin are two of the abundant proteins in the serum that synthesized mainly in hepatocytes and their synthesis is regulated at the transcriptional level (181, 182). The human alpha 1-antitrypsin promoter showed higher activity than the CYP promoters and was one of the most active promoters tested in the entire study. It was more active than the

CYP2D6 promoter whereas the albumin promoter showed lower activity than all of the tested CYP promoters (Figure 11).

We also compared the activity of the hepatic promoters to that of the CMV and RSV viral promoters which are known for their ubiquitously potent activity. The CMV promoter, one of the strongest viral promoters (183), was more active than the CYP2D6 promoter and to a lesser extent than the alpha 1-antitrypsin promoter (Figure 13). In our hands, the RSV promoter was less active than the CMV promoter which was consistent with the findings of Xu *et al* (184). Moreover, activities of CMV, alpha 1-antitrypsin, and serum albumin promoters were consistent with those reported by Kramer *et al* (185). However, the activities of non-hepatic promoters including the promoter of human amyloid precursor protein, mainly expressed in brain and neurons, and the stress-stimulated promoters were lower than many of the tested CYP promoters (Figures 14 and 15).

The kinetics of transgene expression for the tested promoters was similar to that of CYP promoters with a rapid initial decline followed by a slow decay even though plasmid DNA was persistent in the animal liver as shown in Figure 16. This pattern has been considered previously to be a common phenomenon in different organs such as the liver, lungs, or muscles (186-188). However, the plasmid of pCMV-EBV-luc was the exception. This plasmid contains the EBNA1 gene sequence and the oriP elements of the Epstein-Barr virus. These elements are required for the viral properties of retention, replication, nuclear localization, binding to the nuclear matrix of the target cell, and transcriptional up-regulation (189). Figure 17 shows that

pCMV-EBV-luc was the only plasmid kept a high detectable level of mRNA for more than a week, whereas for all the other plasmid constructs mRNA level fell below the detection limit as early as one day post transfection.

Data from mRNA analysis and the observation that pCMV-EBV-luc activity started to significantly decline by the end of the first week, the period at which mRNA was detected, suggest that the major cause of the decline in the activity of the promoters was transcriptional shutdown. For some of the tested promoters, the initial decline could also be partially attributed to the initial loss in the amount of plasmid DNA in mouse liver.

Silencing of the injected plasmid could be due to lack of integration into the chromosomal DNA of the target cell. Integration is the major mechanism by which many viral vectors provide stability and persistence in transgene expression. Despondently, viral vector integration is random and might generate insertional mutation. However, transgene integration is not the only mechanism by which gene silencing can be overcome since many viral vectors are non-integrated but remain extrachromosomal and still provide prolonged transgene expression. Unfortunately, a strong immune response in the host is still the main obstruction that stands in the way of using viral vectors. In recent years, more attention has been paid to establishing non-viral vectors that provide the adequate and prolonged transgene expression required not only for gene therapy but for the study of gene function as well. Different studies have suggested that inclusion of genomic sequences like gene promoters, enhancers, introns, 3'-flanking regions, or binding sites for some transcriptional

factors, dramatically improve the persistence of transgene expression (190, 191). It has been also proposed that the inclusion of certain elements like the ApoE gene hepatic locus control region (HCR), cellular elongation factor 1 α (EF1 α), or ubiquitin C (UbC) prolong transgene expression (187, 190). Moreover, recent studies have suggested that modification in the expression vector by linearization or exclusion of CpG sequences, or the bacterial backbone, significantly reduce gene silencing (186, 192).

Our results suggest that the promoter region does not exhibit tissue or species specificity. Promoters from different species were able to drive transgene expression in mouse liver. These results also suggest that the persistence of transgene expression in the liver is controlled by non-promoter elements. We also can not rule out the possibility that in our expression vectors we missed subcloning of critical promoter or enhancer sequences that might be required for prolonged transgene expression, since it has been reported that distal enhancer elements are crucial for optimizing transgene expression (193). Furthermore, based on our observations that plasmid constructs containing NF κ B or HS promoters exhibited the most transient gene expression, we are suggesting that transgene expression is regulated by components involved in stress-related pathways.

With respect to the activities of CYP promoters in sustaining transgene expression, the CYP2D6 promoter kept luciferase expression at a high level for 3 days. The CYP2C9 promoter also was active and sustained a good level of luciferase for more than 2 weeks. The CYP2C9 promoter started with relatively low activity, but the

interesting feature of this promoter is that it kept its activity stable for prolonged period of time. Whereas many of the tested promoters by the end of the first week lost almost 4-5 order of magnitude of their initial activity, the CYP2C9 only dropped less than 2 orders. In general, we found that the CYP2D6 promoter followed by the CYP2C9 promoter is the most active CYP promoter in sustaining transgene expression. This findings might explain the stability of CYP2D6 and CYP2C9 proteins as suggested by Renwick *et al* and others (194, 195).

We speculated that the CYP2D6 promoter contains elements that enhance the binding of RNA polymerase or transcriptional factors, or lack elements required for down-regulation. To test this hypothesis, we have performed promoter sequence analysis to detect any potential elements using MalInspector software (180). We found that the CYP2D6 promoter contains 2 binding sites for the hepatic nuclear factor 4 (HNF4) and 3 binding sites for the CCAAT/enhancer binding protein (C/EBP) which are known for their up-regulatory role in CYP expression. Compared to the CYP2D6, the CYP2C9 was found to contain 3 sites for the HNF4 and 4 for the C/EBP. We also found that only the CYP2C9 promoter includes 2 potential binding sites for NFkB, known for its down-regulatory role in CYP expression (196), whereas CYP2D6 does not, indicating that repressor elements can dominate CYP promoter activities. Additional work is needed for a complete understanding of the mechanisms through which transcription is regulated.

In summary, we have investigated many CYP promoter sequences for their activities in sustaining the level of transgene expression in mouse liver. We demonstrated that

CYP2D6 was the most active CYP promoter, and its activity was compared relative to many other established promoters.

***IN VIVO* ACTIVATION OF HUMAN CYP2C9 PROMOTER IN MOUSE LIVER AND THE ESSENTIAL ROLE OF CAR AND PXR**

5.1 INTRODUCTION

The human CYP2C subfamily consists of four members, CYP2C8, CYP2C9, CYP2C18, and CYP2C19, whose genes are located in chromosome 10 (45, 197, 198). CYP2C enzymes are predominantly expressed in the liver (199-201), where they account for about 18% of total adult liver CYP content (33) and among them CYP2C9 is the principal element (199). They are also expressed to a lesser extent in duodenum and the kidneys (72). While the enzymes of the CYP2C subfamily account for the metabolism of about 20% of clinically important drugs (202), about 16% out of that are mediated by CYP2C9 alone (203).

Drugs with narrow therapeutic index including the hypoglycemic tolbutamide (204), the anticonvulsant phenytoin (205), and the anticoagulant *S*-warfarin (47) are mainly metabolized by CYP2C9. Therefore, a minor change in CYP2C9 metabolic activity can have a great impact on the pharmacological activity as well as the toxicity of many clinically used drugs. The level of CYP2C9 enzyme is reported to be transcriptionally induced by many chemicals such as dexamethasone, phenobarbital, and rifampicin (105).

The previous studies that investigated the function of the 5'-FR of CYP2C9 were carried out using *in vitro* systems, either in cell lines or in primary cultures of human hepatocytes. Those *in vitro* studies showed some contradictory results regarding CYP2C9 regulation. For example, using human hepatocytes, Runge *et al* proposed that rifampicin and phenobarbital have no inductive role in the expression of CYP2C9 while Raucy *et al* reported that rifampicin and phenobarbital are potent CYP2C9 inducers (195, 206). Moreover, role of the nuclear receptors in CYP2C9 induction has been debated (207).

In our previous studies, we established an *in vivo* animal model using the hydrodynamic gene delivery procedure by which we were able to evaluate the activity of many CYP 5'-FRs. Among the tested CYP promoter, the CYP2C9 promoter showed significant activity. In the current study, we aimed to investigate the validity of the previously established animal model in studying gene induction and regulation using CYP2C9 as a model. Here, we studied the function of the elements of the CYP2C9 5'-FR in regulating both the basal and the induced gene expression.

For this purpose, various 5'-FRs of CYP2C9 were cloned into luciferase containing expression vectors and assessed for their activities in mouse liver. Their activities were also evaluated in presence or absence of CYP-inducers or CYP-activator nuclear receptors. The experiments were also carried out into HepG2 cells to provide an *in vivo* vs. *in vitro* comparison. Our results suggest that PXR and CAR are essential in CYP2C9 induction, and that the elements of 5'-FR residing between -1000 bp and -

2000 bp, upstream of the coding sequence, are crucial for controlling basal gene expression as well as PXR/CAR mediated activation.

5.2 RESULTS

5.2.1 Activity of Various 5'-Flanking Sequences of CYP2C9 in Mouse Liver and in HepG2

To systematically evaluate the function of various segments of 5'-FSs of CYP2C9, we cloned various deletion constructs with part or full 5'-FSs of CYP2C9 beyond the promoter region deletion. Twenty micrograms of plasmids of p2C9-0.2K-luc, p2C9-1K-luc, p2C9-2K-luc, p2C9-3K-luc, p2C9-5K-luc, or p2C9-10K-luc were transfected into mouse livers by the hydrodynamic procedure and twenty four hours later luciferase activities in these livers were determined. The *in vitro* study was carried out by transfection of 1 µg of each of the deletion constructs into HepG2 cells using PEI and luciferase activities were determined forty eight hours post transfection.

We found that all CYP2C9 5'-FSs were functional in driving luciferase gene expression. In mouse liver, the difference in activity between constructs was not significant with the exception of the p2C9-10K-luc which was significantly lower (Figure 18). In HepG2 cells, the activities were similar to what have been seen in liver but with more significant differences (Figure 19). The construct of p2C9-1K-luc led the order and the p2C9-2K-luc exhibited much lower activity compared to the *in vivo*

results. These data suggest that the p2C9-2K-luc includes some transcription elements which were responsive to transcriptional up-regulating factor/s provided by the *in vitro* system. They also suggest that the activity of the p2C9-10K-luc was under control of repressor sequences.

The activity of p2C9-10K-luc, the larger construct, in mouse liver was significantly low compared to the other constructs. Such low activity could be attributed to construct size, since transfection of larger plasmid is more difficult than smaller one and therefore a lower amount of plasmid DNA will be available for gene expression. To examine if the lower amount of the p2C9-10K-luc was the reason for its low activity, we transfected plasmid constructs of different size (p2C9-1K-luc, p2C9-5K-luc, and p2C9-10K-luc) and in different amounts into animal livers. The injected plasmid DNA doses were adjusted based on the size of plasmid, i.e. an identical molar amount of plasmid DNA was injected to each mouse. For example when we injected one group of animals with 100 μg of p2C9-10K-luc, the molar equivalent to p2C9-5K-luc was 68.2 μg , and 39.2 μg for p2C9-1K-luc. As shown in Figure 20, the activity of p2C9-10K-luc tended to be low compared to other constructs at equal molar amount.

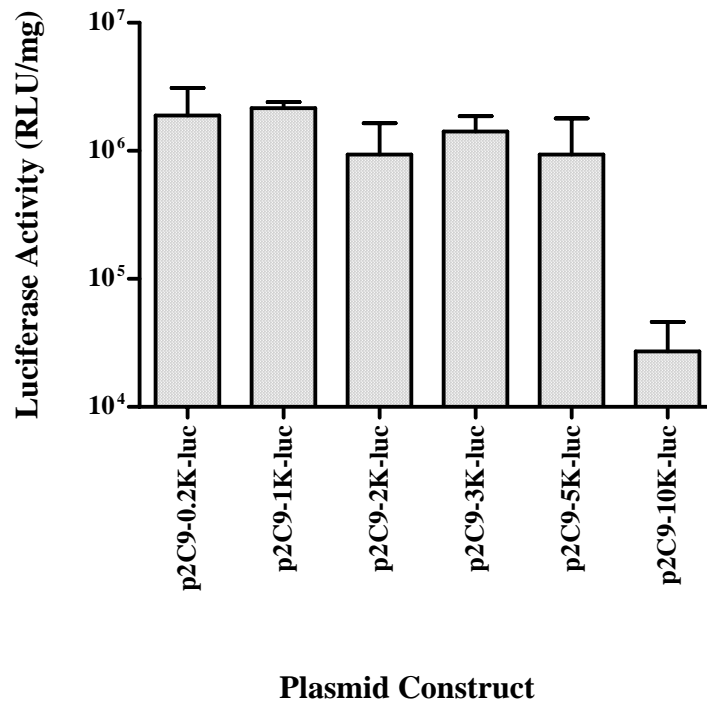


Figure 18. Activity of Various 5'-Flanking Sequences of CYP2C9 in Mouse Liver.

Mice were transfected with plasmid DNA via tail vein injection of 1.8 ml saline containing 20 μ g of CYP2C9 plasmid DNA within 3-5 seconds. Twenty four hours post transfection, luciferase activities in liver were determined. Values represent the mean \pm S.E. of 3 independent transfections.

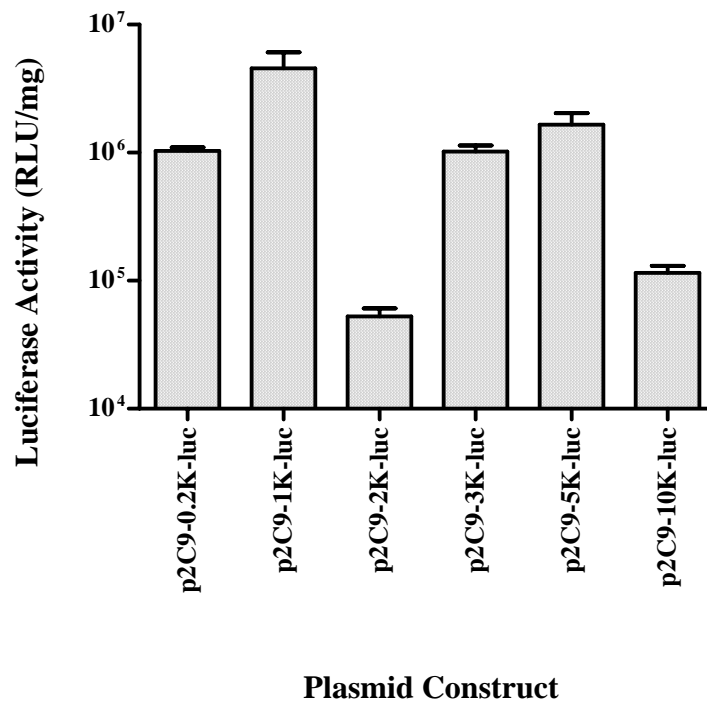


Figure 19. Activity of Various 5'-Flanking Sequences of CYP2C9 in HepG2 Cells.

HepG2 cells were seeded on a 48-well plate, 5×10^4 cells/well, prior to transfection with 1 μ g of plasmid DNA using 3.2 μ g of PEI. Twenty four hours post transfection, the transfection solution was replaced with fresh medium and luciferase activities were determined 24 hours later. Values represent the mean \pm S.E. of 3 independent transfections.

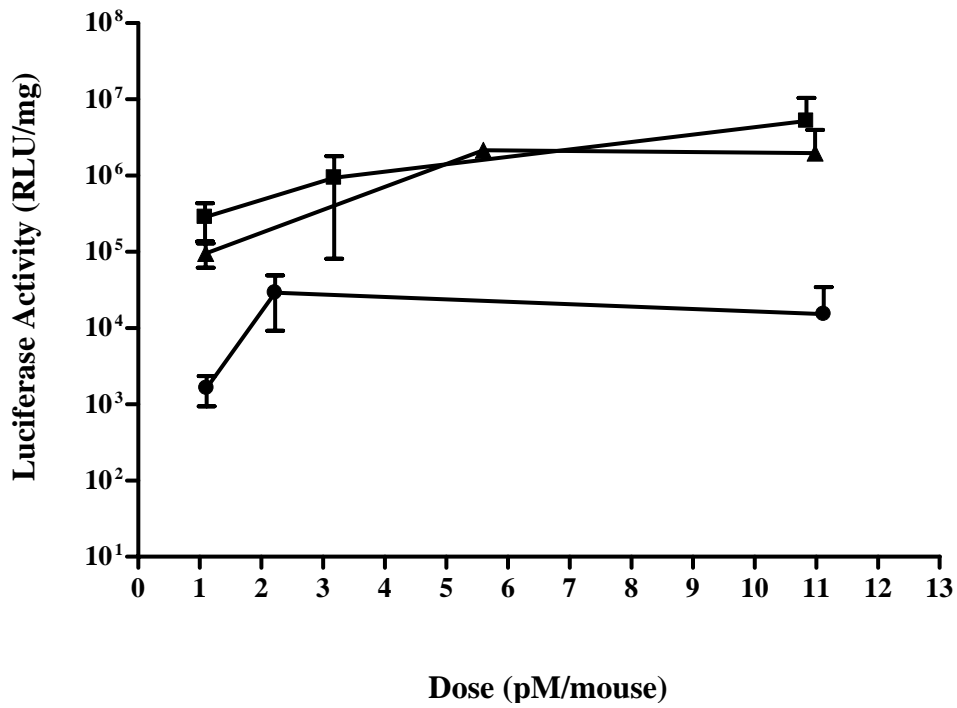


Figure 20. Effect of Plasmid Amount and Size on Promoter Activity in Mouse Liver.

Mice were transfected with plasmid DNA via tail vein injection of 1.8 ml saline containing various doses of p2C9-10K-luc (●) (100, 20, or 10 μ g), p2C9-5K-luc (■) (68.4, 20, or 6.8 μ g), or p2C9-1K-luc (▲) (43, 20, or 4.3 μ g). Twenty four hours post transfection, luciferase activities in liver were determined. Values represent the mean \pm S.E. of 3 independent transfections.

5.2.2 Activation of CYP2C9 5'-Flanking Region by CYP Inducers and the Major Role of PXR and CAR

Previous studies have shown that many transcriptional factors and nuclear receptors, including CAR, PXR, PPAR, HNF4 α , and many others, are tightly involved in CYP induction (208). Here we tested the effect of some of these on the activity of 5'-FR of CYP2C9. Such studies would enhance our understanding of the molecular mechanisms underlying CYP induction.

To examine whether PXR or CAR can enhance the promoter activity of CYP2C9 in mouse liver, mice were co-injected via tail vein with p2C9-5K-luc and pCMX-SXR, the expression vector of PXR, or pCMX-CAR, the expression vector of CAR. Two hours later, the animals were injected with a single dose of rifampicin (a strong activator of human PXR, 200 mg/kg), PCN (strong activator of rodent PXR, 200 mg/kg), phenobarbital (CAR activator, 200 mg/kg), or TCPOBOP (strong activator of mouse CAR, 10 mg/kg) dissolved in DMSO (100 μ l). Twenty four hours post transfection, liver luciferase activities were determined. For the *in vitro* studies, HepG2 cells were co-transfected with 500 ng of p2C9-5K-luc and 100 ng of pCMX-SXR or pCMX-CAR using PEI. Twenty four hours post transfection, cells were treated with rifampicin or TCPOBOP. Luciferase activities were then determined 48 hours post transfection.

We found that PXR itself enhanced the activity of the p2C9-5K-luc in mouse liver by 2 to 3fold. When an animal was challenged with rifampicin or PCN, PXR enhanced the activity of the CYP2C9 5'-FR by 71 or 22fold, respectively (Figure 21). In HepG2

cells, we found that PXR activation of the p2C9-5K-luc was totally dependent on rifampicin treatment in dose dependent manner (Figure 22). When the expression vector of the activated PXR (pCMX-VPSXR) was used, PXR by itself was able to enhance p2C9-5K-luc activity by 16 fold (Figure 27).

With regard to CAR, we found that CAR enhanced the activity of the p2C9-5K-luc in mouse liver by 2fold, whereas when the animals were challenged with phenobarbital or TCPOBOP, CAR significantly enhanced the CYP2C9 5'-FR activity by 10 or 57fold, respectively (Figure 23). In HepG2, CAR enhanced the activity of the p2C9-5K-luc by 7 fold only when TCPOBOP was added to the culturing medium. Similar to PXR, CAR activation of the p2C9-5K-luc was totally dependent on xenobiotic activation with dose dependent manner (Figure 24). When the expression vector of the activated CAR (pCMX-VPCAR) was used, CAR itself induced the activity of the p2C9-5K-luc by 31 fold (Figure 29).

Roles of PPAR and HNF4 α were also investigated (data not shown). We found that co-transfection of PPAR α , γ , or δ did not affect the activity of the p2C9-5K-luc in mouse liver. Likewise, co-transfection of HNF4 α with various 5'-FSs of CYP2C9 did not significantly enhance their activities in HepG2 cells. These data indicated the crucial role of PXR and CAR in CYP2C9 induction.

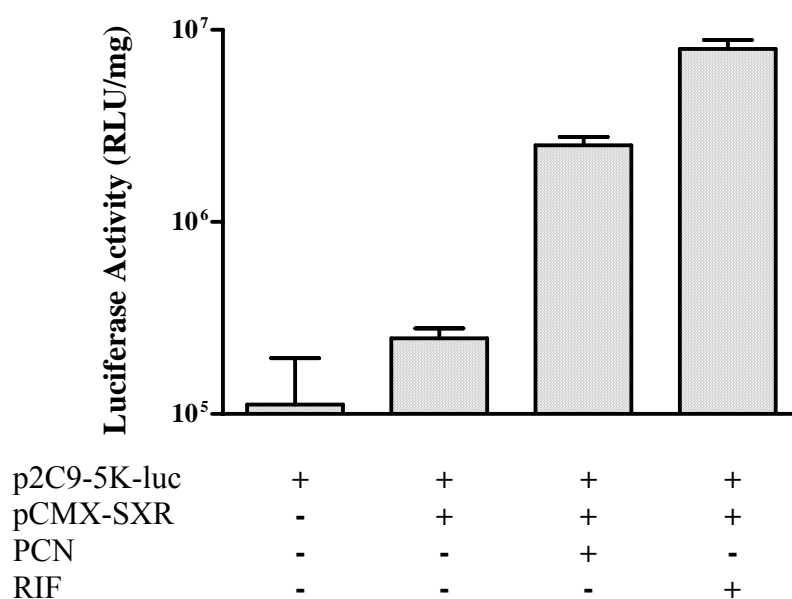


Figure 21. Effect of PXR Expression on the Activity of CYP2C9 5'-FR in Mouse Liver.

Mice were transfected with plasmid DNA via tail vein injection of 1.8 ml saline containing 2 µg of p2C9-5K-luc and 5 µg of pCMX-SXR within 3-5 seconds. Two hours post transfection, the animals whose co-transfected with pCMX-SXR, if needed, were treated with PCN (200 mg/kg) or rifampicin (RIF, 200 mg/kg) dissolved in 100µl of DMSO. Twenty four hours post transfection, luciferase activities in the livers were determined. Values represent the mean ± S.E. of 3 independent transfections.

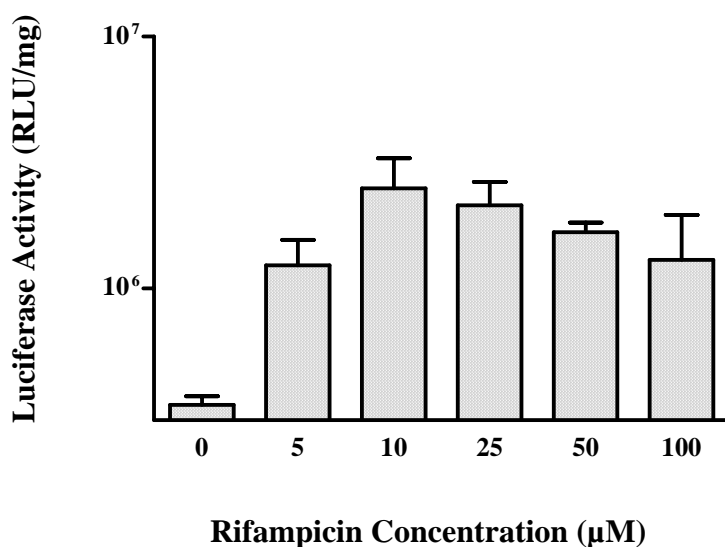


Figure 22. Effect of PXR Expression on the Activity of CYP2C9 5'FR in HepG2 Cells.

HepG2 cells (5×10^4 /well) were co-transfected with 500 ng of p2C9-5K-luc and 100 ng of pCMX-SXR using $3.2 \mu\text{g}/\mu\text{g}$ of PEI. Twenty four hours post transfection, transfection solution was replaced with a fresh medium containing various doses of rifampicin (0, 5, 10, 25, 50, or 100 μM). Luciferase activities in each well were then evaluated 48 hours post transfection. Values represent the mean \pm S.E. of 3 independent transfections.

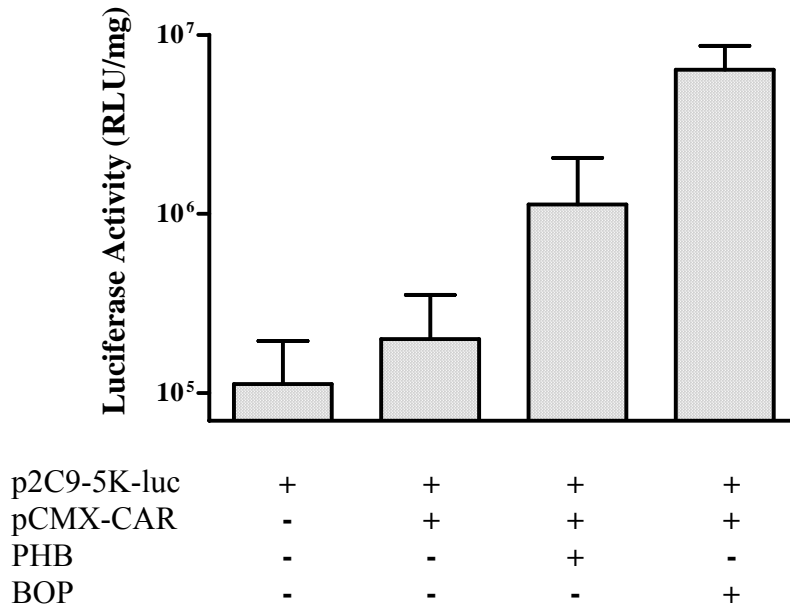


Figure 23. Effect of CAR Expression on the Activity of CYP2C9 5'FR in Mouse Liver.

Mice were co-transfected with plasmid DNA via tail vein injection of 1.8 ml saline containing 2 μg of p2C9-5K-luc and 5 μg of pCMX-CAR within 3-5 seconds. Two hours later, the animals whose co-transfected with pCMX-CAR, if needed, were injected with phenobarbital (PHB, 200 mg/kg) or TCPOBOP (BOP, 10 mg/kg) dissolved in 100 μl of DMSO. Twenty four hours post transfection, luciferase activities in the livers were determined. Values represent the mean \pm S.E. of 3 independent transfections.

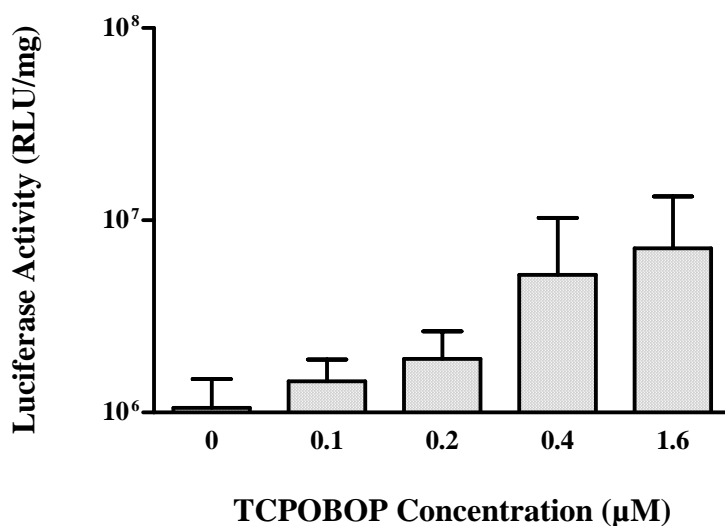


Figure 24. Effect of CAR Expression on the Activity of CYP2C9 5'-FR in HepG2 Cells

HepG2 cells (5×10^4 /well) were co-transfected with 500 ng of p2C9-5K-luc and 100 ng of pCMX-CAR using $3.2 \mu\text{g}/\mu\text{g}$ of PEI. Twenty four hours later, transfection solution was replaced with a fresh medium containing different doses of TCPOBOP (0, 0.1, 0.2, 0.4, or 1.6 μM). Luciferase activities in each well were then evaluated 48 hours post transfection. Values represent the mean \pm S.E. of 3 independent transfections.

5.2.3 Activity of CYP2C9 5'-Flanking Region in Sustaining the Transgene Expression

We previously studied the activity of CYP2C9 5'-FR in sustaining transgene expression (chapter 4). In the current study, we investigated the effect of CAR/PXR co-transfection as well as new distal elements of 5'-FS on the sustaining activity of CYP2C9 5'FR. Two groups of animals were co-transfected with 2 µg of p2C9-5K-luc and with either 2 µg of pCMX-PXR or pCMX-CAR. Two hours later, animals were treated with rifampicin (200 mg/kg) or TCPOBOP (10 mg/kg) and 22 hours later liver luciferase activities were determined.

We found that PXR, upon activation by rifampicin was able to enhance CYP2C9 promoter activity to the maximal peak level 24 hours post transfection, followed by a fast decline to a lower level. The same pattern was seen with CAR and TCPOBOP (Figure 25). In spite of the sharp decline in promoter activity, we found that CYP2C9 5'-FR remained functional, since a second dose of rifampicin (200 mg/kg) at day 3 enhanced the activity of CYP2C9 promoter by 6fold within 24 hours and a second dose of TCPOBOP (10 mg/kg) at day 4 enhanced the activity by about 2fold. These data suggest that CAR or PXR activation and the new included distal elements of CYP2C9 5'-FR did not improve its activity in sustaining transgene levels. They also clearly indicated that even when CYP2C9 5'-FR exhibited low activity in mouse liver, it is still functional.

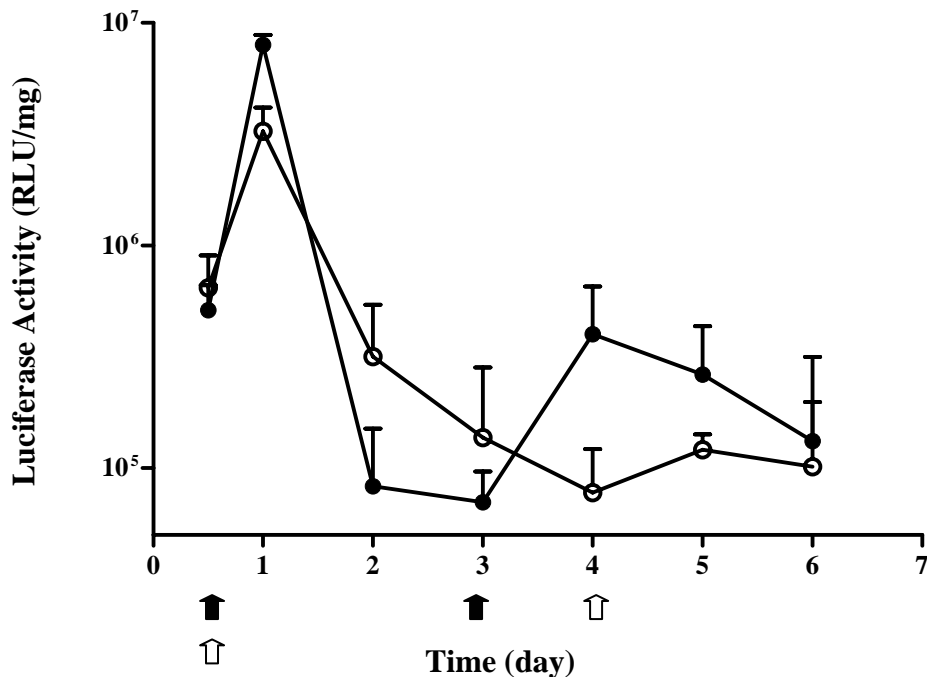


Figure 25. Activity of CYP2C9 5'-FR in Sustaining Transgene Expression in Mouse Liver.

Tow groups of animals were co-transfected with plasmid DNA via tail vein injection of 1.8 ml saline containing 2 μ g of p2C9-5K-luc and 2 μ g of pCMX-SXR (the first group, ●) or pCMX-CAR (the second group, ○) plasmid DNA within 3-5 seconds. Two hours later, animals were injected with 100 μ l of DMSO containing rifampicin (200 mg/kg) (the first group) or TCPOBOP (10 mg/kg) (the second group). At the indicated time post transfection, luciferase activities in the livers were determined. Values represent the mean \pm S.E. of 3 independent transfections. Arrows indicate the time of drug injection.

5.2.4 Identification of the Functional Elements in 5'-Flanking Region of CYP2C9 Required for PXR/CAR-Mediated Induction

In the previous studies we found that PXR and CAR are two essential nuclear receptors that mediate CYP2C9 induction. From our understanding of CAR and PXR activation mechanisms, these nuclear receptors exert their action in gene regulation by binding to certain elements in the 5'-FR of the target gene (99). In this study, we aimed to identify where PXR or CAR essential elements are located in CYP2C9 5'-FR. For this purpose, we co-transfected the animals with various CYP2C9 deletion constructs containing different sequences of CYP2C9 5'-FSs with or without PXR or CAR expression vectors. For the *in vitro* studies, HepG2 cells were co-transfected with various deletion constructs of CYP2C9 5'-FSs in presence or absence of pCMX-VPSXR or pCMX-VPCAR using PEI.

We found that PXR, when activated by rifampicin, enhanced the p2C9-5K-luc by 71fold. It also enhanced the p2C9-3K-luc by 18fold and the p2C9-2K-luc by 39fold. Such significant activation was abolished when the p2C9-1K-luc was used (Figure 26). In HepG2, the same pattern of activity was seen, PXR enhanced the p2C9-5K-luc by 16fold, p2C9-3K-luc by 3fold, and p2C9-2K-luc by 57fold whereas had no effect on the p2C9-1K-luc (Figure 27).

We also found that CAR, when activated by TCPOBOP, enhanced the p2C9-5K-luc, p2C9-3K-luc, and p2C9-2K-luc by 57, 23, and 52fold, respectively. Dissimilarly, such significant induction disappeared when the p2C9-1K-luc was used (Figure 28). In HepG2, the same pattern of activity was seen, CAR enhanced the activities of

p2C9-5K-luc by 31fold, p2C9-3K-luc by 3fold, and p2C9-2K-luc by 52fold but not the p2C9-1K-luc (Figure 29). These data indicate that the p2C9-2K-luc contains the minimal elements required for CAR/PXR activation.

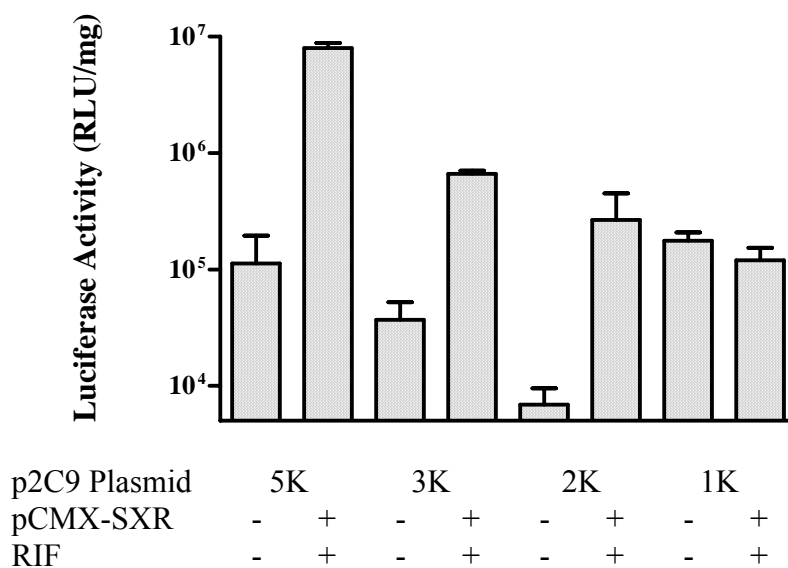


Figure 26. Effect of PXR Expression on the Activity of Various 5'-FSs of CYP2C9 in Mouse Liver.

Animals were transfected with plasmid DNA via tail vein injection of 1.8 saline containing 2 μ g of various deletion constructs of CYP2C9 with (second bar in each pair) or without (first bar in each pair) 2 μ g of pCMX-SXR plasmid DNA within 3-5 seconds. Two hours later, animals were injected with 100 μ l of free DMSO (first bar in each pair) or with DMSO containing rifampicin (200 mg/kg) (second bar in each pair). Twenty four hours post transfection, luciferase activities in the liver were determined. Values represent the mean \pm S.E. of 3 independent transfections. 5K, 3K, 2K, and 1K represent p2C9-5K-luc, p2C9-3K-luc, p2C9-2K-luc, and p2C9-1K-luc, respectively.

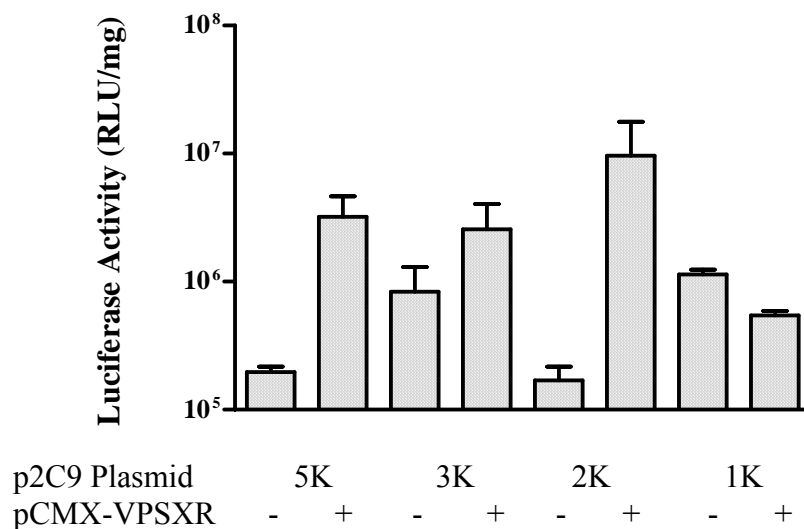


Figure 27. Effect of PXR Expression on the Activity of Various 5'-FSs of CYP2C9 in HepG2 Cells.

HepG2 cells (5×10^4 /well) were co-transfected with 500 ng of various deletion constructs of CYP2C9 with (second bar in each pair) or without (first bar in each pair) 100 ng of pCMX-VPSXR using $3.2 \mu\text{g}/\mu\text{g}$ of PEI. Forty eight hours post transfection, luciferase activities in each well were determined. Values represent the mean \pm S.E. of 3 independent transfections. 5K, 3K, 2K, and 1K represent p2C9-5K-luc, p2C9-3K-luc, p2C9-2K-luc, and p2C9-1K-luc, respectively.

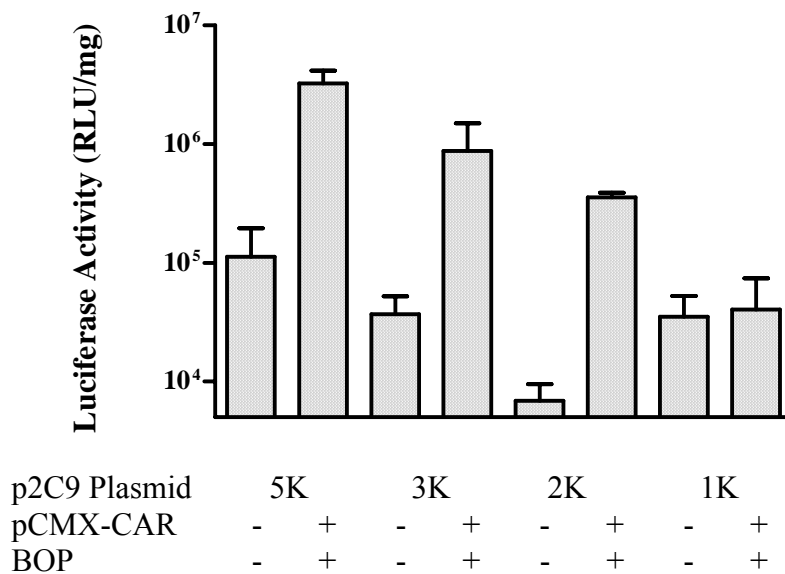


Figure 28. Effect of CAR Expression on the Activity of Various 5'-Flanking Sequences of CYP2C9 in Mouse Liver.

Animals were transfected with plasmid DNA via tail vein injection of 1.8 saline containing 2 µg of various deletion constructs of CYP2C9 with (second bar in each pair) or without (first bar in each pair) 2 µg of pCMX-CAR plasmid DNA within 3-5 seconds. Two hours later, animals were treated with 100 µl of free DMSO (first bar in each pair) or DMSO containing TCPOBOP (10 mg/kg) (second bar in each pair). Twenty four hours post transfection, luciferase activities in the livers were determined. Values represent the mean ± S.E. of 3 independent transfections. 5K, 3K, 2K, and 1K represent p2C9-5K-luc, p2C9-3K-luc, p2C9-2K-luc, and p2C9-1K-luc, respectively.

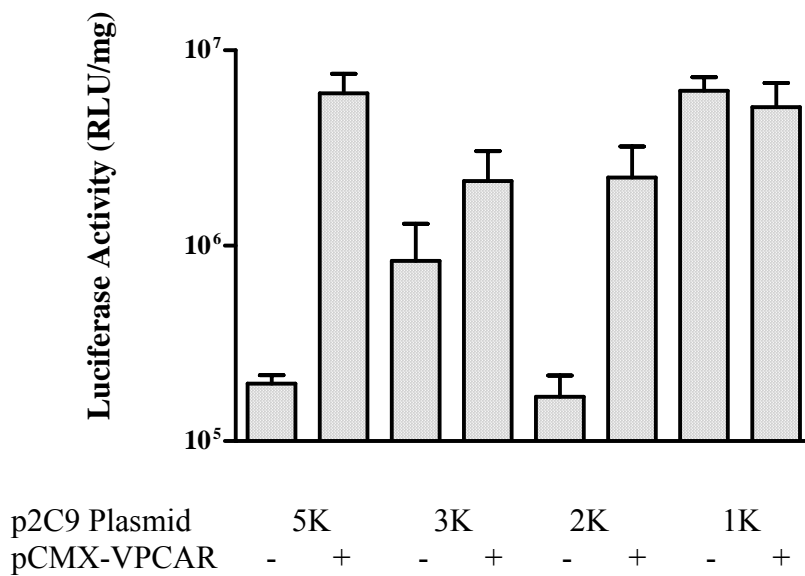


Figure 29. Effect of CAR Expression on the Activities of Various 5'-FSs of CYP2C9 in HepG2 Cells.

HepG2 cells (5×10^4 /well) were co-transfected with 500 ng of various deletion constructs of CYP2C9 with (second bar in each pair) or without (first bar in each pair) 100 ng of pCMX-VPCAR using $3.2 \mu\text{g}/\mu\text{g}$ of PEI. Forty eight hours post transfection, luciferase activities in each well were determined. Values represent the mean \pm S.E. of 3 independent transfections. 5K, 3K, 2K, and 1K represent p2C9-5K-luc, p2C9-3K-luc, p2C9-2K-luc, and p2C9-1K-luc, respectively.

5.3 DISCUSSION

In the current study we have evaluated the activity of CYP2C9 5'-FR in driving transgene expression *in vivo* and *in vitro*. We also investigated the role of different nuclear receptors and transcriptional factors in regulation of CYP2C9 5'-FR activity and identified the elements of 5'-FR that are required for CYP2C9 gene expression and induction.

Figure 18 shows that all 5'-FSs of CYP2C9 tested were active in driving transgene expression in mouse liver. One of the significant findings in this regard was clear difference between the *in vivo* and *in vitro* activities of the p2C9-2K-luc as seen in Figure 19. This difference can be explained by the presence of some transcription factors in the *in vivo* system that were lost in the *in vitro*. It also can be illustrated by that the p2C9-2K-luc includes binding sites for certain transcription factors that were missed in the other CYP2C9 constructs. Alternatively, the p2C9-2K-luc might also lacks some repressing elements that are included in the other CYP2C9 constructs. From promoter sequence analysis using MalInspector software, we found that the p2C9-2K-luc contains 2 and 4 potential binding sites for HNF4 and C/EBP, these sites were missing in the p2C9-1K-luc. Here we propose that C/EBP is responsible for the *in vivo* enhanced activity of the p2C9-2K-luc, since co-transfection of the expression vector of the HNF4 α did not affect the basal activity of the p2C9-2K-luc in HepG2 cells (data not shown), Although a HNF4 α functional binding site at -155 bp has been reported (209). The previous findings that the C/EBP was able to up-regulate the basal expression of CYP2C9 in cell lines, support such a proposal (144).

Furthermore, a study by Gerbal-Chaloin *et al*, using human hepatocytes, proposed the element at -1839 bp in the CYP2C9 5'-FR as CAR responsive element and the element at -1675 bp as the glucocorticoid receptor-responsive element (114). However unlikely, Chen *et al* , using HepG2 cells, proposed that the element at -1839 bp is primary PXR responsive element (207). Using human hepatocytes, the same element was proposed as vitamin D receptor (VDR) responsive (134). Both elements are included in the p2C9-2K-luc suggesting that GR, CAR, PXR, or VDR were likely mediated its *in vivo* enhanced activity.

Although VDR was suggested to control the basal expression of CYP2 and CYP3 families in absence of xenobiotics, CYP2C9 has not been shown to be involved in metabolism of vitamin D (134). On the other hand, CYP2C9 was described as the primary glucocorticoid receptor-responsive gene and its expression under physiological conditions is maintained at a substantial level through GR direct activation (114). Moreover, GR is known for its constitutive expression and to which stability of CYP2C9 expression was attributed (105, 117, 210). This receptor somehow is involved in induction of many nuclear receptors like PXR, CAR, and VDR (211). This inductive role was recently explained for CAR as involving direct CAR activation through GR-responsive element identified in 5'-FR of CAR gene (212).

The p2C9-10K-luc construct showed a similar pattern of significant low activities in mouse liver and in HepG2. We attributed such weakness to its content of effective repressor sequences. This pattern of low activity with regard to large CYP 5'-FR

construct compared to smaller deletion constructs was also reported by Schuetz *et al* for CYP3A4 (213). Figure 20 shows that the difference in plasmid size and DNA amount were not the reasons for the activity of the p2C9-10K-luc, specially when a much larger construct (about 157 kb) was successfully transfected to mouse liver under the same conditions (214).

A few previous studies, using *in vitro* systems, identified nuclear receptors, including PXR and CAR, as essential factors for CYP2C9 induction (28). These receptors were found to work by directly binding to their responsive elements in CYP2C9 5'-FR. So far, two regulatory elements in the 5'-FR of CYP2C9 have been identified for CAR and PXR. These are the proximal CAR-responsive element (-1839 bp) (114), and the distal CAR-responsive element (-2899 bp) (215). These elements were proposed to be mainly CAR elements.

Under the physiological conditions and by employing a longer segment of CYP2C9 5'-FR, we found that PXR significantly enhanced the activity CYP2C9 primarily post xenobiotic activation. Rifampicin, known for its *in vivo* and *in vitro* induction of CYP2C9 (105, 216), was found to be the most potent inducer for CYP2C9 via PXR activation (Figure 21). In mouse liver, when animals were treated with rifampicin in absence of PXR co-transfection, the activity of CYP2C9 5'-FR did not significantly enhance (data not shown), indicating the crucial role of PXR in the activity of CYP2C9. It also indicated the high selectivity of rifampicin toward the human isoform of PXR even at such a high dose (200 mg/kg). Our results disagree with the finding of Gebral-Chaloin *et al* who proposed that PXR, even when activated by

rifampicin, did not enhance the activity of CYP2C9 (114). Moreover, PCN, a selective rodent PXR activator, was able to induce the PXR-dependent activity of CYP2C9. Here, we propose that PXR is a crucial factor required for CYP2C9 induction, which is consistent with the recent finding of Chen *et al* (207).

In mouse liver, we also found CAR as another essential factor for CYP2C9 induction. Unlike PXR studies, in the CAR related studies we employed the mCAR isoform. This isoform was previously suggested as more sensitive for human CYP2C9 induction studies than the human CAR (114) which their studies exhibited difficulty because of its inherited nuclear localization in cell lines and its high constitutive activity (207). TCPOBOP, a potent selective mouse CAR activator, was able to significantly enhance the activity of CYP2C9 5'-FR via CAR activation (Figure 23). On the other hand, TCPOBOP was partially able to induce CYP2C9 activity even in absence of CAR co-transfection (data not shown), indicating the involvement of the endogenous mouse CAR in the activity of CYP2C9 5'-FR. Phenobarbital, known for its inductive activity toward CYP2C9 (195, 217), also enhanced the activity of CYP2C9 but to a lesser extent. The selectivity of this reagent in activation of CAR has been debated, since it was reported that it can up-regulate some CYP genes via PXR activation (218). However, many studies described the inductive activity of phenobarbital as mCAR selective (215). Our results agree with findings of Ferguston *et al* and Gerbal-Chaloin *et al* with regard to CAR's essential role in CYP2C9 induction.

After we found that PXR and CAR are essential factors for CYP2C9 activity, we proceeded in exploring the mechanisms underlying CYP2C9 induction by analyzing its 5'-FSs and identifying the essential element required for PXR/CAR-mediated activation. Various deletion constructs of CYP2C9 5'-FR, starting from as long as 10 kb to as short as 0.2 kb, were tested. As Figures 26 to 29 show, the p2C9-5K-luc, p2C9-3K-luc, and p2C9-2K-luc plasmid constructs were responsive to CAR and PXR activation in mouse liver and in cell lines as well. The shorter deletion construct, p2C9-1K-luc, showed obvious resistance to CAR/PXR activation, indicating that the p2C9-2K-luc, in its distal 1 kb sequences (from -1000 to -2000 bp), contains the essential elements required for CAR and PXR activation. Our observations are consistent with the findings of Gerbal-Chaloin *et al* and Chen *at al* who proposed the element located at -1839 bp as the primary element required for maximum PXR and CAR activation, at least in the first 10 kb of the CYP2C9 5'-FR (114, 207). Our data also proposed the element at -2899 bp to has a much less significant role if not exhibits some inhibitory effect, since the induction profile of the p2C9-3K-luc was less significant than that of the p2C9-2K-luc particularly under the *in vitro* circumstances.

Even though we agree with some of the previously published studies, our data are characterized by their significant and clear induction profiles. For example, Chen *at al* showed that PXR enhanced CYP2C9 5'-FR activity via rifampicin activation by 3-5fold in HepG2 cells, in our hands, the activity of the same CYP2C9 5'-FR (p2C9-3K-luc) was enhanced 18fold via PXR and rifampicin in mouse liver. The significance of the activity was even more clear when we used a longer sequences

(p2C9-5K-luc), 71fold (Figure 26). The enhanced induction seen with the p2C9-5K-luc can not be referred to sequence elements other than the common PXR element at -1839 bp, therefore, the only factor we feel could contribute to such discrepancy is the *in vivo* environment in which we carried out our studies. Many recent studies realized the significant of such *in vivo* systems and have alternatively employed the hydrodynamic based transfection to study the gene regulation of CYP genes (142, 213, 219, 220).

Furthermore, unlike most of the previously conducted studies that investigated regulation of CYP genes using genomic context-modified CYP 5'-FSs, all plasmid constructs we used in our studies contained intact CYP2C9 5'-FSs, which reflect the real behavior of CYP genes *in vivo*. This is particularly relevant in view of recent findings that revealed that nuclear receptor binding to the target gene 5'-FR element is genomic context-dependent (221).

In summary, here we have demonstrated that the animal model established by the hydrodynamic procedure is suitable for gene regulation studies. This system showed realistic sensitivity to the injected gene sequence. It also showed clear responses toward different inductive gene elements and chemicals. From the applications point view, we have demonstrated that CYP2C9 induction is dependent primarily on CAR and PXR activation, and that these nuclear receptors exhibited their inductive activities via elements that reside between -1000 to -2000 bp of CYP2C9 5'-FR.

SUMMARY AND FUTURE PERSPECTIVE

Cytochrome P450 (CYP) is a superfamily of monooxygenases that mediate biotransformation of wide variety of endogenous and exogenous substances. These enzymes are expressed mainly in the liver and their expression is subjected to regulation. CYP gene expression can be regulated at the levels of transcription, mRNA processing and stabilization, translation, and enzyme stabilization (222). However, CYP induction mediated by xenobiotics mainly occurs at the transcriptional level where RNA polymerase II, cis-acting elements, nuclear receptors, and gene promoter elements are involved.

In this dissertation, I started my studies by establishing an animal model that allows study of the function of CYP 5'-FSs and their roles in regulating gene expression under the physiological conditions. Sequences of 5'-FRs of CYP genes were constructed into expression vectors upstream of the reporter gene. Functions of CYP 5'-FSs were then assessed *in vitro* and *in vivo*. *In vitro* CYP promoter activities were first studied in HepG2, BL-6, and 293 cell lines using optimized transfection of polyethyleneimine as the transfecting agent. The function of CYP 5'-FRs were then evaluated *in vivo* under the optimized conditions in mouse livers using the hydrodynamic procedure. This method allowed transfection of about 40% of mouse hepatocytes by rapid injection of naked DNA in saline. Furthermore, due to its

targeting mainly the liver, this method is ideally suited for study of hepatic genes, such as CYP.

The CYP plasmid constructs used in the first study were later used to evaluate the activity of CYP 5'-FR in sustaining transgene expression in mouse livers. Such a study would help us to further understand the role of CYP promoters on basal CYP gene expression in human liver. This was accomplished by evaluating plasmid DNA and mRNA levels in liver using Southern and Northern blot analysis. In this study, the activity of CYP promoters were assessed and compared to viral, hepatic, and non-hepatic promoters.

Finally, after the establishment of the animal model, I investigated the ability of this system to adapt the gene regulation studies, and studied the function of 5'-FR of CYP2C9 and the molecular mechanisms underlying its activity. Various CYP 2C9 5'-FRs (0.2, 1, 2, 3, 5, or 10 kb) were cloned into expression vectors containing luciferase as a reporter gene and their activities in driving transgene expression were evaluated. The effects of known CYP inducers, transcription factors, and nuclear receptors on CYP2C9 5'-FR activity were also investigated. Via plasmid deletion construction I identified the crucial element in 5'-FR of CYP2C9 that is required for its activation.

Many observations have been made through the course of the study. Among the tested CYP promoters, the CYP2D6 showed the strongest activity followed by CYP1A2, CYP3A4, and CYP2C9. The pattern of activity was similar in different cell lines as

well as in mouse livers. While these promoters were from hepatic origin, they were also functional in other tissues, suggesting that the 5'-FR sequences do not control the tissue specificity of gene expression. With regard to the relationship between the observed promoter activity and the basal CYP expression, a direct correlation was not seen. For example, CYP3A4 is the most abundant CYP enzyme in human liver but its promoter showed lower activity than that of CYP2D6 which has much lower expression level in the human liver. Future studies to investigate whether any other part of the genome sequences, i.e. intron, 3'-flanking region, or distal enhancer, are involved more in determining tissue specificity and the levels of basal gene expression, are indeed necessary. Furthermore, as discussed in chapter 3, lack of chromatin structure of the tested promoter sequences makes the promoter sequence freely accessible for transcription. It is known from the mechanism of gene transcription that the first step required for transcriptional activation is chromatin structure remodeling. Therefore, future studies will be required to assess the effect of the lack of chromatin structure on promoter activity as a basal gene expression determinant.

The activity of the CYP2D6 promoter is interesting, particularly when we know that this gene, unlike most of CYP genes, is resistant to xenobiotic induction. Its strong promoter activity might explain its resistance to xenobiotics activation. The CYP2D6 promoter also showed the highest activity among tested CYP 5'-FRs for their activity in sustaining transgene expression in mouse livers and its initial activity was comparable to known strong promoters such as the viral CMV promoters. These

exciting observations justify the need for studies that could lead to identification of any unique elements responsible for CYP2D6 high promoter activity.

It was observed that the pattern of CYP promoter activity was similar to that of other tested non-CYP promoters with a initial high activity followed by a rapid decline to a lower level. RNA analysis revealed that the main cause for declining was transcriptional shutdown. Future studies should consider gene silencing mechanism and study if inclusion of CYP introns or 3'-FRs could help overcome such dilemma.

With regard to CYP2C9 5'-FR activity, I observed that CYP2C9 5'-FR activity was regulated mainly by PXR and CAR nuclear receptors. It was also demonstrated that , at least in the first 10 kb of the CYP2C9 5'-FR, the element located within the proximal 2 kb sequences (between -1000 and -2000 bp upstream of the coding sequence) was the main element required for PXR and CAR activation and for enhancing basal gene expression. One of the interesting findings was suppressing activity of the p2C9-10K-luc in mouse liver and in cell lines. Investigation of factors that led to this suppression would be a good subject for future studies.

In conclusion, the observations from this dissertation work have enhanced our understanding of the functional role of CYP 5'-FR in gene expression and regulation. They also have provided direct evidence to validate that the animal model established by the hydrodynamic-based injection as a suitable *in vivo* system for gene function studies. The system I established for gene function and induction studies can be modified to adapt gene down-regulation studies under the physiological conditions.

Prolongation of transgene expression at a high level would indeed allow for investigation into the roles of repressor elements, gene down-regulation by cytokines, or polymorphism/mutation in regulatory region of CYP genes, and as a useful tool for studying correlations between gene induction and inhibition. In addition, this system can be used to evaluate newly developed drugs for their activities to induce CYP gene expression, and to help to establish the relationship between CYP gene expression and drug adverse effects.

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