Studies on the Regulation of the Barbiturate-Inducible Cytochrome P450 Genes CYP2H1 and CYP2H2

A Thesis Submitted for the Degree of Doctor of Philosophy at the University of Adelaide

by

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Abstract

Currently two phenobarbital-inducible cytochrome P450 genes have been characterised in the chicken, *CYP2HI* and *CYP2H2*. These two genes have homologous coding regions but divergent 3' noncoding regions. We previously identified in the chicken *CYP2HI* gene an upstream enhancer domain (-5900/-1100) that responds to phenobarbital. Deletion and restriction enzyme analyses of this domain have now identified two separate enhancer regions that respond to phenobarbital (from -5900 to -4550 and from -1956 to -1400). The focus here is on the latter region and in particular a resident 240 bp restriction enzyme fragment that retains drug responsiveness. Using deletion analysis and *in vitro* DNase I footprinting, transcription factor binding sites have been located in the 240 bp fragment. The sites identified are an E-box like element, a consensus HNF-1 site, a CCAAT box motif and a novel site. Mutagenesis demonstrated that each site contributed to enhancer activity although there was a weaker contribution from the CCAAT box and that no individual site was critical for responsiveness. In keeping with the tissue restricted expression of the *CYP2HI* gene, gel shift experiments established that the proteins binding to these enhancer sites are enriched in chicken liver, kidney and small intestine. *In vitro* footprint experiments showed a stronger protection with liver nuclear extracts from drug treated chickens compared with control extracts on the E-box like element, the CCAAT box motif and the novel binding site but the basis for this apparent increase in binding remains to be determined.

The early promoter region of the *CYP2HI* gene has previously been isolated and characterised. This region binds multiple ubiquitous and liver-enriched transcription factors and directs a high level of basal expression but does not respond to drug. In this study I isolated a 920 bp proximal promoter segment of the *CYP2H2* gene from a chicken genomic clone. Binding sites for transcription factors were located within the first 160 bp of promoter sequence using promoter deletion experiments and DNase I footprint analysis. Sequence analysis revealed characteristic sites for the liver-enriched transcription factors
of the HNF-1, HNF-3, and C/EBP families (44) and for the ubiquitous factor, USF. A Barbie box-like sequence overlapped the USF element but was not functional. Sequence comparison with the CYP2H1 proximal promoter revealed that, with the exception of the HNF-3 transcription factor binding site, all the transcription factor binding sites necessary for basal expression were totally conserved between the two genes. The HNF-3 binding site contained a base pair mismatch in its core and an 8 bp insertion in the 3'-flanking sequence. Further analysis revealed that this 8 bp insertion contained a second HNF-3 binding site. Protein binding to the adjacent HNF-3 binding sites was not seen in DNase I footprint assays. However, mutation of one or other HNF-3 site restored binding to the other site in these assays. Transient transfection experiments revealed that mutation of one or other HNF-3 site produced an increase in expression of a promoter/CAT reporter gene constructs. This data indicate that the adjacent positioning of the two HNF-3 binding sites prevents protein binding.

RU486, a glucocorticoid antagonist, was used to investigate the mechanism of induction of the CYP2H1 gene. RU486 was shown to inhibit induction of the endogenous CYP2H1 mRNA in chick embryo primary hepatocytes. RU486 was also shown to inhibit induction of transiently transfected constructs containing the CYP2H1 enhancer region. This inhibition was shown to be specific for the CYP2H1 mRNA and did not effect the basal expression of a transiently transfected construct containing the early promoter of the CYP2H1 gene. Dexamethasone was unable to reverse the RU486 mediated inhibition, suggesting that RU486 is not acting through the glucocorticoid receptor. The RU486 responsive region was isolated to -3000/-1950 of the 4.8 kb enhancer region of the CYP2H1 gene.
Declaration

This thesis has been submitted to the Faculty of Science at the University of Adelaide for examination for the degree of Doctor of Philosophy.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for photocopying and loan.

Benjamin Davidson

February, 2000
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Abbreviations

bp, kb  base pair(s), kilobase pair(s)
CAT  Chloramphenicol acetyltransferase
cDNA  complementary DNA
Ci  Curie
CYP  Cytochrome P450(s)
DNA  deoxyribonucleic acid
GAPDH  glyceraldehyde 3-phosphate dehydrogenase
h  hour(s)
kD  kilodalton
mRNA  messenger ribonucleic acid
PB  Phenobarbital
PBS  phosphate buffered saline
poly(A)  polyadenylic acid
RNA  ribonucleic acid
RU486  17β-hydroxy-11β-(4-dimethylaminophenyl)-17α-(pro-1-ynyl)-estra  
        4,9-dien 3-one
SV40  Simian virus 40 promoter
TCDD  2,3,7,8-Tetrachlorodibenzo-p-dioxin
UTR  untranslated region
V  Volt
µF  microFaradays
µg, mg  microgram, milligram
µl, ml  microlitre, millilitre
µM, mM  micromolar, millimolar
Chapter 1. Introduction
1.1 Introduction

Cytochromes P450 (CYPs) are haem containing enzymes that catalyse a biologically ubiquitous oxidative metabolism reaction. These enzymes are remarkable because of their huge number and are unmatched in their multiplicity of isoforms, substrate specificities and catalytic and regulatory mechanisms. To date the cytochrome P450 gene superfamily consists of over 500 genes and pseudogenes (242) encoding more than 400 proteins catalysing more than 60 different chemical reactions from a diverse range of organisms including plants, animals, yeast and bacteria. It is safe to predict that each mammalian species may be found to have up to a hundred CYP isoforms that respond in toto to a thousand or more inducers which metabolise a million or more potential substrates (Fig 1.1).

The CYP catalysed reactions include steroid, fatty acid, bile acid and vitamin metabolism in animals, insecticide resistance and pheromone metabolism in insects, herbicide resistance and flower colouring in plants and environmental bioremediation by microorganisms. Cytochromes P450 also play a vital role in protecting organisms from foreign chemicals including innumerable drugs, procarcinogens, antioxidants, solvents, dyes, anaesthetics, pesticides, petroleum products, alcohols, food additives, odorants, environmental pollutants and plant metabolites (59, 117, 118, 236, 324). CYPs involved in metabolism of endogenous compounds are highly substrate specific, located primarily in extrahepatic tissues and are generally constitutively expressed. However CYPs involved in the metabolism of exogenous compounds have very broad and overlapping substrate specificities, are located predominantly in the liver and are generally inducible (102, 169, 247, 274).

This thesis is involved with the mechanisms of regulation of the inducible cytochromes P450 and particularly the drug inducible CYPs. This review will briefly cover the structure and chemical action of cytochromes P450 and their role in the metabolism of endogenous compounds. The major focus will be on the regulation of the inducible cytochromes P450
Summary of the chemical reactions catalysed by cytochrome P450s.

This table lists the reactions and representative substrates catalyzed by the cytochrome P-450 system. It is clear from this table that substrates are often metabolised at more than one site on the molecules, by either the same or different cytochrome P-450 isoenzymes, to produce multiple products depending on the oxygen tension in the reaction mixtures or in the tissues. In addition, various cytochrome P-450-catalyzed oxidation reactions may involve different chemical mechanisms and do not appear to have a single, identifiable rate-limiting step. Thus, the diversity of the cytochrome P-450 function is characterised not only by the presence of multiple cytochrome P-450 isoenzymes, but also by the ability of the enzyme system to catalyse the metabolism of numerous substrates via different types of reactions involving different chemical mechanisms and rate-limiting steps.
<table>
<thead>
<tr>
<th>Type of reaction</th>
<th>Example of substrates</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aliphatic hydroxylation</td>
<td>Fatty acids, n-alkanes, phenobarbital, cyclohexane, hexobarbital, testosterone</td>
<td>Hydroxylated derivatives; dependent on the cytochrome P450 isozyme and substrate, oxygenation can occur at different sites on the molecule.</td>
</tr>
<tr>
<td>Aromatic oxidation</td>
<td>Halogenated benzenes, biphenyls, polycyclic aromatic hydrocarbons</td>
<td>Hydroxylation or epoxidation at different sites</td>
</tr>
<tr>
<td>Alkene epoxidation</td>
<td>Aflatoxin B1, benzo[a]pyrene-7,8-dihydrodiol, Aldrin</td>
<td>Aflatoxin B1-2,3-oxide, benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide, Dieldrin</td>
</tr>
<tr>
<td>N-Dealkylation</td>
<td>Benzphetamine, Aminopyrine, Ethylmorphine</td>
<td>Norbenzphetamine, formaldehyde, Monomethyl-4-aminoantipyrine, formaldehyde, Norethylmorphine, formaldehyde</td>
</tr>
<tr>
<td>Oxidative deamination</td>
<td>Amphetamine</td>
<td>Phenylacetone, ammonia</td>
</tr>
<tr>
<td>O-Dealkylation</td>
<td>7-Ethoxycoumarin, Phenacetin</td>
<td>7-Hydroxycoumarin, acetaldehyde, Acetaminophen, acetaldehyde</td>
</tr>
<tr>
<td>N-Oxidation</td>
<td>2-Acetylanthracene, Phenacetin, Phentermine</td>
<td>N-Hydroxy-2-acetylanthracene, N-Hydroxyphenacetin, N-Hydroxyphentermine</td>
</tr>
<tr>
<td>Oxidative desulfuration</td>
<td>Parathion, Carbon disulfide</td>
<td>Paraoxon, Carbonyl sulfide, sulfur</td>
</tr>
<tr>
<td>Sulfoxidation</td>
<td>Chlorpromazine</td>
<td>Chlorpromazine sulfoxide</td>
</tr>
<tr>
<td>Oxidative dehalogenation</td>
<td>Dibromomethane, Chloroform</td>
<td>Carbon monoxide, hydrogen bromide, Phosgene, hydrogen chloride</td>
</tr>
<tr>
<td>Oxidative denitrification</td>
<td>2-Nitropropane</td>
<td>Acetone, nitrite</td>
</tr>
<tr>
<td>Oxidative denitrosation</td>
<td>N-Nitrosodimethylamine</td>
<td>Nitrite, methylamine, formaldehyde</td>
</tr>
<tr>
<td>Nitro reduction</td>
<td>p-Nitrobenzoic acid, Nitrobenzene</td>
<td>p-Aminobenzoic acid, Aniline</td>
</tr>
<tr>
<td>Azo reduction</td>
<td>Prontosil, Azobenzene</td>
<td>Sulfanilamide, triaminobenzene, Aniline</td>
</tr>
<tr>
<td>Tertiary amine N-oxide reduction</td>
<td>Imipramine N-oxide, N, N-Dimethylaniline N-oxide</td>
<td>Imipramine, N, N-Dimethylaniline</td>
</tr>
<tr>
<td>Arene oxide reduction</td>
<td>Benzo[a]pyrene-4,5-oxide</td>
<td>Benzo[a]pyrene</td>
</tr>
<tr>
<td>Reductive dehalogenation</td>
<td>Carbon tetrachloride</td>
<td>Chloroform</td>
</tr>
<tr>
<td>Chromate reduction</td>
<td>Chromate (VI)</td>
<td>Chromium (III)</td>
</tr>
</tbody>
</table>
by exogenous compounds with emphasis on the PB (phenobarbital) inducible cytochrome P450 genes.

1.2 Nomenclature of Cytochrome P450s

The designation of a protein as a cytochrome P450 originated from its spectral properties before its catalytic function was known. In 1958, Garfinkel (98) first identified a reduced pigment that had an absorption band with a $\lambda_{\text{max}}$ at 450 nm after binding to carbon monoxide. A system of classification based on amino acid sequence homology has been developed that groups P450 genes into families, subfamilies and individual genes within subfamilies (240). P450 proteins within a gene family are defined as having > 40% amino acid sequence similarity and > 55% similarity to be classified within the same gene subfamily. For example, CYP2H2 is the cytochrome P450 gene belonging to family 2, subfamily H and was the second member of this subfamily identified.

The cytochrome P450 gene superfamily currently consists of 27 gene families found in organisms ranging from bacteria to mammals and also in plants (242). Mammals contain at least 17 distinct P450 gene families that together code for an estimated 50-60 individual P450 genes in any given species (241). Four of these P450 gene families designated CYP1-4 code for liver-expressed enzymes that metabolise foreign compounds and endogenous lipophilic substrates (59). The remaining 13 mammalian P450 gene families typically do not metabolise foreign chemicals but are involved in metabolism of endogenous substrates along physiologically important pathways including steroid, fatty acid, bile acid and eicosanoid biosynthesis (100).

1.3 Chemical Reaction of Cytochrome P450s

Cytochrome P450s catalyse many oxidative reactions with hydroxylation of substrates commonly observed. The reaction is referred to as mono-oxygenation and the cytochrome P450 enzyme as a mono-oxygenase, as only one of the two oxygen atoms is incorporated into the substrate. Figure 1.2 shows the chemical reaction catalysed by cytochrome P450s.
Figure 1.2  Scheme for the mechanism of action of P450.

The first step in the reaction cycle is substrate binding, which perturbs the spin state equilibrium of the cytochrome and facilitates uptake of the first electron. Substrates that undergo reduction rather than oxygenation, such as epoxides, N-oxides, nitro and azo compounds, and lipid hydroperoxides, accept two electrons in a stepwise fashion as shown, to give RH(H)₂. To initiate the oxidative reactions, molecular oxygen is bound to the ferrous P450 with coordination to iron trans to thiolate (355). This intermediate can also be written as the resonance form, Fe³⁺(O₂⁻), with substrate still present. Transfer of the second electron then occurs, with the possible involvement of cytochrome b₅ as an additional electron donor in mammalian microsomal systems (272). The next step is not well understood but involves splitting of the oxygen-oxygen bond with the uptake of two protons at some stage and the generation of an “activated oxygen,” perhaps an iron-oxene species, and the release of H₂O (354). Several resonance forms are possible for the active oxygen intermediate, considering the redox possibilities with the sulfur, iron, and oxygen atoms. Oxygen insertion into the substrate is believed to involve hydrogen abstraction from the substrate and recombination of the resulting transient hydroxyl and carbon radicals to give the product. Dissociation of ROH then restores the P450 to the starting ferric state. (115) Fe represents the haem iron atom at the active site, RH the substrate, RH(H)₂ a reduction product and ROH a monooxygenation product.
RH + O₂ + NADPH + H⁺ → ROH + H₂O + NADP⁺
The cytochrome P450 protein contains a single iron protoporphyrin IX prosthetic group (haem) that is required for the binding of molecular oxygen. For the cytochrome P450 catalysed hydroxylation reaction to occur, the haem iron must first be reduced from the ferric (Fe\(^{3+}\)) to its ferrous (Fe\(^{2+}\)) state (115). Two electrons, donated by NADPH, are required for the monooxygenation reaction and are transferred to the cytochrome P450 molecule individually (156). The first electron reduces the haem iron allowing oxygen to bind. Donation of the second electron allows cleavage of the oxygen molecule to generate the active oxygen species for hydroxylation of the substrate (272).

NADPH is a two electron donor while cytochrome P450, with its single haem prosthetic group can only accept one electron at a time. This potential problem is overcome by the presence of a NADPH-dependent flavoprotein reductase, which accepts the two electrons from NADPH simultaneously but transfers the electrons to the cytochrome P450 directly or via an intermediate iron-sulphur protein (306). The mode of electron donation appears to be specific for each individual P450 but generally microsomal electron transfer involves an enzyme called NADPH-cytochrome P450 reductase while in the mitochondrial and bacterial systems electron transfer involves a ferredoxin reductase and a nonhaem iron protein (354).

1.3.1 The Microsomal Cytochrome P450 System
In the endoplasmic reticulum, NADPH donates electrons to NADPH-cytochrome P450 reductase (Fig. 1.3A). In mammals this enzyme contains both flavin adenine dinucleotide (FAD) and flavin mononucleotides (FMN) as prosthetic groups. The FAD serves as the entry point for electrons from NADPH, and FMN serves as the exit point, transferring electrons individually to cytochrome P450 (95). In certain reactions catalysed by the microsomal P450, the second electron may not be donated directly to the cytochrome P450 from NADPH-cytochrome P450 reductase, but may be donated from cytochrome b\(_5\), a small haem protein also present in the endoplasmic reticulum (157). Cytochrome b\(_5\) is
Figure 1.3  Diagrammatic representation of the cytochrome P450 system.

A. The components of the microsomal cytochrome P450 system. NADPH-cytochrome P450 reductase is membrane bound by its hydrophobic tail to the cytosolic surface of the smooth endoplasmic reticulum whereas cytochrome P450 is deeply embedded in the membrane. Also shown is cytochrome b5, which participates in selected cytochrome P450-mediated reactions.

B. The components of the mitochondrial cytochrome P450 system. Cytochrome P450 is an integral protein of the inner mitochondrial membrane. NADPH-ferrodoxin reductase and ferrodoxin are peripheral proteins and are not embedded in the membrane but are loosely associated with the inner mitochondrial matrix.
A

Cytochrome b<sub>5</sub>

Cytochrome P450

Cytochrome P450 reductase

FAD

FMN

NADPH

smooth endoplasmic reticulum

B

Cytochrome P450

Ferredoxin

NADPH-Ferredoxin reductase

NADPH

inner mitochondrial membrane
reduced either by NADPH-cytochrome P450 reductase or another microsome-bound flavoprotein, NADH-cytochrome b5 reductase (307).

1.3.2 The Mitochondrial Cytochrome P450 System
In mitochondria, NADPH-ferredoxin reductase acts as the electron acceptor from NADPH (Fig. 1.3B) and is only weakly associated with the inner mitochondrial membrane (226). This reductase cannot directly transfer either the first or second electron to the haem iron of cytochrome P450 but uses ferredoxin as an intermediate (192). Ferredoxin contains two iron-sulphur clusters that serve as redox centres and functions as an electron shuttle between the ferredoxin reductase and the mitochondrial cytochrome P450. Ferredoxin receives an electron from its mitochondrial flavoprotein reductase and then interacts with the cytochrome P450 protein embedded in the inner mitochondrial membrane to transfer this electron to the haem iron (253).

1.4 Cytochrome P450 Metabolism of Endogenous Substrates
Cytochromes P450 function as the essential components in steroidogenesis. This includes production of glucocorticoids and mineralcorticoids by the adrenal cortex as well as androgen and estrogen synthesis in the gonads (80, 113, 329). The brain also catalyses many or all of the reactions found in the better known steroidogenic tissues, as does the placenta (141). Estrogen production also occurs in adipose tissue and certain members of the steroidogenic pathways are found in other tissues (e.g., retina and stomach) suggesting that additional sites of steroid hormone production are yet to be uncovered (343). Herein, however, attention will be focused on activities in the traditional steroidogenic tissues as shown in Figure 1.4.

In the adrenal gland, CYP11A, CYP11B and CYP21 are responsible for several steps in the synthesis of aldosterone, a mineralocorticoid involved in regulating salt and water balance, and cortisol, the glucocorticoid that governs protein, carbohydrate and lipid metabolism. Also in the adrenal gland, CYP21 mediates the production of small quantities of dehydroepiandrosterone and androstenedione, precursors for both oestrogen and
Figure 1.4  
Generic steroidogenic pathways.
The enzymes are: CYP11A, cholesterol side chain cleavage cytochrome P450; CYP17, 17α-hydroxylase cytochrome P450; CYP21A, 21-hydroxylase cytochrome P450; CYP11B, 11β-hydroxylase cytochrome P450; CYP19 aromatase cytochrome P450; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; 5α-RED, 5α-reductase (adapted from Ref. 169).
testosterone. In the testis CYP11A and CYP17 participate in the synthesis of testosterone and dihydrotestosterone from cholesterol. The ovary and placenta contain CYP11A, CYP17 and CYP19 allowing the production of estrogen and progesterone (80, 113, 329).

In addition to steroids, cytochromes P450 are involved in the metabolism of ω-3 fatty acids, such as arachidonic acid, into bioactive eicosanoids including leukotrienes and prostanoids. This family of P450 genes (CYP4A) are also involved in xenobiotic metabolism and so their regulation will be discussed in section 1.5.1.1.

1.4.1 Regulation of Steroidogenic Cytochromes P450

The conversion of cholesterol to pregnenolone is the initial step in steroidogenic pathways (169). This reaction takes place in the mitochondrion and is catalysed by an integral membrane protein of the inner mitochondrial membrane, cholesterol side chain cleavage cytochrome P450 (P450scc or CYP11A; Reference 328). However, the rate limiting step in steroidogenesis is not the production of pregnenolone but rather the mobilisation of cholesterol to the vicinity of CYP11A in the inner mitochondrial membrane from lipid stores outside the mitochondrion (159). Recently a protein called steroidogenic acute regulatory (STAR) protein has been identified and appears to be important in this mobilisation (55). The primary trigger of this mobilisation is adrenocorticotropin (ACTH), a peptide hormone derived from the inner pituitary (329). ACTH binds to its cell surface receptor which activates adenylate cyclase leading to elevated levels of intracellular cAMP which in turn induces steroid hydroxylase activities at the transcriptional level (162). Interestingly, it takes several hours for enhanced transcription to be observed suggesting that the CRE/CREB system may not be involved since it responds much more rapidly to cAMP (296). Investigation of the biochemistry of cAMP-dependent transcriptional regulation of steroid hydroxylase pathways has been carried out primarily in three species: bovine, human and mouse. Analysis of the 5'-flanking regions of the bovine adrenocortical steroid hydroxylase and adrenodoxin genes coupled to reporter genes reveals that each gene contains its own distinct cAMP-responsive elements. In addition adrenal 4 binding protein (Ad4BP, also called steroidogenic factor 1, SF-1) binding sites important in
developmental and tissue specific expression are found in each of these gene promoters (126, 231). The CRS (cAMP responsive sequence) in bovine CYP2I contains overlapping binding sites for two nuclear proteins, Sp1 and adrenal-specific nuclear protein or ASP, in addition to SF-1 (167, 168, 377). The binding of ASP, not Sp1, is required for cAMP responsiveness of this gene. Conversely the bovine CYP11A gene contains the same overlapping binding sites but requires Sp1 binding, not ASP, for cAMP responsiveness (228).

The bovine CYP17 gene contains two distinct CRS elements each binding its own group of nuclear proteins including Ad4BP, COUP-TF and two homeodomain proteins of the PBX family of genes (214). In primary cultures of adrenocortical cells maintained in the absence of ACTH, CYP17 protein disappears indicating that CYP17 level is strictly dependent on cAMP (381). Bovine CYP11A contains two CRSs in the promoter region, both of which bind Sp1 and enhance transcription of reporter genes in response to elevated levels of cAMP (5, 228). Bovine CYP11B is regulated by cAMP through a near consensus CRE sequence which binds the leucine zipper containing transcription factor, CREB (142). In addition transcription mediated by this CRE is strongly enhanced by cooperation of an upstream Ad4BP binding site.

In summary, it is evident that each of the genes encoding bovine adrenocortical steroid hydroxylases utilises a different cAMP-responsive system for maintenance of optimal steroid hydroxylase levels. cAMP responsive mechanisms for steroid hydroxylases in other species show both similarities and differences with the bovine genes. The mouse CYP11B1 gene does not contain a CRE-like sequence and responds slowly to cAMP (290) while the CYP11B2 gene does contain such a sequence and responds rapidly to cAMP (76). The two Sp1 binding sites near the TATA box in the bovine CYP11A gene are also present in the human, mouse and rat genes and have been shown to confer cAMP responsiveness (107, 254, 291). The mouse CYP17 gene contains a CRS that has no sequence homology to CRS elements in either the human of bovine CYP17 genes (374). The mouse CYP2I and CYP11 genes contain multiple elements involved in cAMP responsiveness (260). One such
element binds Ad4BP and the transcription factor NGF1-B (363). The human CYP21 gene contains an ASP binding site identical to that in bovine CYP21 (167) and participates in cAMP-dependent transcription, in addition to a CRE-like sequence far upstream of the promoter (349).

In contrast to cAMP-dependent regulation discussed above there are numerous reports of regulation of steroid hydroxylase genes by factors independent of cAMP; examples include insulin-like growth factor, epidermal growth factor, interferons, calcium, angiotensin II, phorbol esters, salt, androgens and transforming growth factor-β (8, 15, 31, 88, 142, 199). Waterman and Simpson (350) have shown that in the absence of ACTH primary cultures of bovine adrenocortical cells maintain approximately 50% of their normal level of steroid hydroxylases, except for CYP17. Hence it can be expected that cAMP-independent regulation provides a basal level of transcription of steroid hydroxylase genes in steroidogenic tissues on which cAMP-responsive transcriptional regulation is superimposed.

Cytochrome P450 enzymes, closely related to the steroidogenic CYPs, are involved in the conversion of a provitamin to an active vitamin. Vitamin D, the hormone involved in calcium homeostasis, is first metabolised by liver microsomal cytochrome P450 to 25-hydroxyvitamin D₃ and subsequently by kidney mitochondrial cytochrome P-450 to 1,25-dihydroxyvitamin D₃, the active form of vitamin D (64, 136). A cytochrome P450 is also involved in vitamin D degradation. The genes for these enzymes are controlled by vitamin D, through the vitamin D receptor, in a reciprocal manner (341).

1.5 Cytochrome P450 Metabolism of Exogenous Substrates
Microsomal cytochromes P450 are key detoxification enzymes that catalyse the first step in the biotransformation of xenobiotics. These enzymes convert lipid-soluble compounds into highly water soluble products that can be eliminated from the body in the urine or bile. This usually requires two different types of reactions termed "phase I" or functionalisation and "phase II" or conjugation (263). Phase I reactions involve the production of a
functional group (commonly an OH group by hydroxylation) while the phase II reactions which follow, involve the use of the newly formed functional group for attachment of a highly polar moiety such as glucuronic acid, glutathione, glucose, cysteine or sulphate hence increasing the water solubility of the product. The phase I reactions are catalysed by cytochromes P450 whereas the phase II reactions require enzymes such as the UDP glucuronosyltransferases and glutathione S-transferases (217, 269).

Cytochromes P450 enzymes responsible for xenobiotic metabolism are generally present at very low levels but are induced in response to specific xenobiotics, in some cases by orders of magnitude. This induction phenomena was first recognised because it produced alterations in pharmacological responses to drugs in animals. For example, rats, rabbits and dogs chronically exposed to barbiturates become tolerant to the hypnotic effects of these drugs, because they induce the cytochromes P450 responsible for their own metabolism (288). Similarly, the induction of cytochrome P450 enzymes reduced the incidence of neoplasia in animals exposed to chemical carcinogens (57). These examples illustrate the elegance of the cytochrome P450 detoxification system. The inducers are often substrates for the induced enzymes thus enzyme activity increases only as needed.

In addition to its main involvement in the elimination of exogenous compounds, cytochromes P450 are also involved in the detoxification of various compounds by altering their structure and so reducing or abolishing their pharmacological activity or toxicity (292). With certain compounds, however, this defence mechanism can go astray converting foreign compounds into highly toxic molecules that can damage DNA, RNA and proteins leading to mutations, cell transformations and cell death (166, 364). The best example of this is the conversion of benzo[a]pyrene to a number of metabolites which are able to bind covalently to DNA and have been shown to be carcinogenic in animals (59). Similarly, high concentrations of the analgesic paracetamol (such as those obtained after a suicide attempt) saturate detoxification pathways, leading to reactions via cytochromes P450 that generate reactive electrophiles, which bind to cellular macromolecules and produce hepatic necrosis (336). In addition, some xenobiotics induce the phase I enzymes but not the phase
II enzymes (117). In this instance the xenobiotic is hydroxylated but not conjugated and hence not excreted resulting in an accumulation of the xenobiotic with in the cell. This can lead to cell transformation or cell death.

1.5.1 Regulation of Xenobiotic-inducible Cytochromes P450

Induction of CYP enzymes occurs predominantly at the level of transcription (66, 100, 247). A notable exception is the ethanol-inducible CYP2El gene, the induction of which involves a posttranscriptional mechanism (100, 293, 340). Induction of CYPs generally occurs at the sites of exposure or excretion of xenobiotics such as the liver, lung, skin, gastrointestinal tract and brain (71, 110, 187, 370). Depending on the chemical nature of the xenobiotic, CYP isozymes belonging to a particular sub-family are predominantly induced. Xenobiotic-inducible CYPs fall into four gene families, CYP1-4 (100). Each of these families will be discussed, with emphasis on the CYP1 gene family, the mechanism of induction of which is best understood, and also on the CYP2 phenobarbital-inducible gene family which is the subject of the present study.

1.5.1.1 Polycyclic Aromatic Hydrocarbon-Inducible Cytochrome P450s

The polycyclic aromatic hydrocarbon-inducible P450 gene family (CYP1) contains CYP1A1, CYP1A2 and CYP1B1. These genes encode proteins that are similar in their amino acid sequence but differ in their substrate specificity (238). CYP genes in this class are transcriptionally activated by polycyclic aromatic hydrocarbons, chlorinated dioxins (from paper bleaching), benzopyrenes and anthrenes (from combustion) as well as steroids including estradiol and progesterone (198, 362). In their chemical structure all these inducers are similar and contain aromatic rings that are essentially planar (Fig. 1.5).

These chemical inducers, being lipophilic, are thought to enter the cell by passive diffusion where they bind with high affinity to the cytosolic aromatic hydrocarbon receptor (AhR). Ligand-receptor binding activates the AhR which then becomes a potent transcription factor (reviewed in 96, 239, 248, 359). This activated form of the receptor induces transcription of the CYP1 genes, subsequently increasing the concentration of aryl
Figure 1.5  Chemical structure of the polycyclic aromatic hydrocarbon compounds. The aromatic hydrocarbon compounds are related structurally in that they all contain benzene rings and are essentially planer molecules.
3-Methylcholanthrene

2,3,7,8-Tetrachlorodibenzo-p-dioxin

2,3,6,7-Tetrachlorobenzofuran

2,3,6,7-Tetrachlorobiphenylene

β-Napthoflavone

3,3',4,4',5',5'-Hexachlorobiphenyl
hydrocarbon hydroxylase enzyme (360, 361), possibly aided by mRNA stabilisation (326). Increased aryl hydrocarbon hydroxylase enzyme activity leads to oxidation of the inducer and in the majority of cases, elimination of the chemical by the phase II reaction. However, as mentioned previously this enzyme activity can occasionally result in the generation of toxic products. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a contaminant of the herbicide "Agent Orange" and the most potent inducer of this CYP family, has been shown to cause birth defects and cancer in animals (271, 299). This is thought to result from an aberrant genetic program initiated by the activated AhR (123, 270). In addition, the aryl hydrocarbon hydroxylase activity of the CYP1A1 enzyme has been shown to convert polycyclic aromatic hydrocarbons in smog and cigarette smoke into carcinogenic intermediates (100).

1.5.1.1.1 The Aromatic Hydrocarbon Receptor

The AhR belongs to the basic helix-loop-helix/PAS (bHLH/PAS) transcription factor family (36, 84), a subclass of bHLH proteins that contain a region of homology with the proteins Per, a circadian rhythm factor in Drosophila, Arnt, the AhR nuclear translocator protein, and Sim, a Drosophila neurogenic factor. The AhR is unique among bHLH proteins in that it requires a ligand for activation although another bHLH/PAS protein, the Hypoxia Inducible Factor (HIF-1α), may also be transformed from a latent to an active state in response to an environmental stress-related signal, that of low oxygen tension (348).

The cytosolic Ah receptor has been cloned and found to be a heteromeric complex of greater than 270 kDa which consists of a 95 kDa ligand binding subunit and at least two molecules of the 90 kDa heat shock protein, hsp90 (36, 176, 273). The association of the Ah receptor with hsp90 results in a non-DNA binding form (276) possibly by masking a nuclear localisation signal within the receptor. In yeast model systems, low levels of hsp90 almost totally abolish ligand activation of the AhR (43, 358). Geldanamycin, a drug that inhibits hsp90 binding, was shown to decrease AhR levels by 80 % within one hour of exposure (49). These results indicate that hsp90 may stabilise the AhR and maintain it in
the ligand-binding configuration (9, 62). The current signalling model suggests that ligand binding induces a conformational change in the receptor exposing the nuclear localisation signal and subsequent nuclear translocation (Fig 1.6). Recent evidence suggests that phosphorylation via protein kinase C (PKC) is involved in this process as PKC inhibitors block AhR mediated signal transduction (209). In vitro dephosphorylation experiments have indicated that both heterodimerisation between the AhR and Arnt and DNA binding of the dimeric complex are dependent upon phosphorylation (21). Consistent with this model, tetradeanoxyphorol acetate-induced down regulation of PKC activity in vivo inhibits both DNA binding of the ligand stimulated receptor (249) and activity of an XRE-driven reporter gene (21), suggesting a critical role for PKC in receptor function. In addition to hsp90, another protein, AIP (AhR-interacting protein) has been shown to associate with AhR (216). AIP is structurally related to the immunophilin family of proteins (224), which function as molecular chaperones in steroid receptor signalling (256). AIP plays a positive role in AhR mediated signalling possibly by assisting targeting of ligand bound AhR to the nucleus.

Once the AhR has been transported into the nucleus, dimerisation with a specific bHLH/PAS partner protein, termed the Ah receptor nuclear translocation protein (Arnt), occurs (135). This dimerisation process, as with all other bHLH proteins, occurs via the Helix-Loop-Helix domains, while the basic regions contact the DNA. The PAS domain also participates in dimerisation and possibly assists in partner selection (206). The PAS domain of AhR also contains the ligand and hsp90 binding regions (356). In contrast, Arnt does not interact with hsp90. While transcription activating domains have been shown to localise to the C terminus of both proteins (154, 331, 357), the Arnt transactivation domain participates minimally in activation of CYP1A1 (77, 181, 287)

The point at which hsp90 dissociates from the AhR is unknown, however, as Arnt is a nuclear protein, it is possible that hsp90 release is a nuclear event. As a heterodimer with Arnt, the AhR is able to bind to the XRE and activate transcription (82). These XRE sequences are commonly found in upstream of the genes induced by the activated AhR,
Figure 1.6. Scheme depicting current knowledge on mode of action of the Ah receptor.

AhR is bound to hsp90 and AIP (AhR-interacting protein) in the cytoplasm in the absence of ligand. Ligand enters the cell via diffusion and binds to the AhR, displacing AIP and exposing a nuclear localisation signal (NLS) on the AhR. The AhR-hsp90 complex then translocates into the nucleus where hsp90 dissociates from the AhR exposing the Arnt binding site. Arnt binds to the AhR-ligand complex whereupon the complex becomes a transcriptional activator. This complex binds to the AhRE in the promoter of the CYP1A1 gene and induces expression of this gene possibly involving coactivator recruitment and subsequent chromatin disruption.
where they function as classic enhancer regions. *CYPIA1* has six XRE sequences approximately 1 kb upstream (65). These XREs are arranged in an irregular pattern which may reflect constraints imposed by chromatin structure. For example, as the DNA helix wraps around the histone core of the nucleosome, the major groove (which contains the XRE) is periodically accessible and inaccessible. Therefore, increasing the number of binding sites at irregular intervals along the enhancer increases the probability that at least one site will be accessible, even when the DNA is nucleosomal. In addition, the receptor heterodimer contacts a relatively short (6 bp) DNA segment increasing the probability that the entire binding site will be accessible in the nucleosome. Thus, the multiplicity, irregular distribution and small size of the binding sites may have evolved as a mechanism for overcoming the steric constraint imposed by the nucleosomal organisation of the enhancer in vivo. The recently discovered *CYP1B1* has 9 core XRE motifs within a 2.5 kb region 5' of the gene (24) and at least 3 of these XREs appear to be functional in mediating dioxin induced transcription of the *CYP1B1* gene (335). The XREs do not appear to be the only response elements involved in *CYP1* induction. Phenobarbital induces the *CYP1A2* gene expression in mice the absence of AhR via a pretranslational mechanism (60, 300, 376) while metyrapone and dexamethasone in combination induce *CYPIA1* gene expression, possibly via the glucocorticoid receptor not the AhR (128). These results suggest other elements, possibly GRE-like, are present in the enhancer regions of these genes. These elements are possibly involved in mediating hormonal and regulation of these genes as observed by the sexual dimorphism and developmental variation of CYP1 expression (207, 219, 353).

1.5.1.1.2 *CYPIA1* gene induction and chromatin structure

DNase I footprinting studies have revealed that binding of the receptor complex to the upstream XRE enhancer sequence alters the proximal promoter region to allow binding of other transcription-activating proteins (250, 294). Thus, it has been proposed that the major role of the AhR/Arnt heterodimer is to disrupt the chromatin structure of the *CYPIA1* promoter and to allow access to a series of transcription factors that play a more direct role in harnessing RNA polymerase II. In support of this model, it has been demonstrated that
inhibition of topoisomerase I, a DNA relaxing enzyme that modifies the topology of supercoiled DNA, abolishes AhR-ligand induction of CYP1A1 and CYP1B1 genes (108). Importantly, inhibition of topoisomerase I had no detrimental effect on the DNA binding ability of AhR/Arnt and did not reduce expression of CYP genes if inhibition occurred after the CYP genes had already been activated. These results are consistent with ligand induction of the CYP genes being dependent upon chromatin modifications. In further agreement with a model where AhR/Arnt complexes propagate an altered chromatin structure, deletion of the AhR transactivation domain has been found to result in the removal of ligand-induced DNase I footprints on the CYP1A1 proximal promoter in vivo (182). Therefore, a transcriptionally inactive AhR/Arnt complex, while maintaining XRE binding activity, lacks the ability to induce changes in chromatin structure, implying the mode of gene regulation by the receptor complex involves interaction with proximal transcription factors, as discussed further below. Interestingly, removal of the Arnt transactivation domain, which does not seem to be functional on the CYP1A1 promoter, does not interfere with the ability of the heterodimer to disrupt chromatin structure (181). It appears that Arnt may play another role. Yeast and mammalian two hybrid systems revealed that the coactivator CBP/p300 interacted with the transactivation domain of Arnt, but not with that of AhR, via the CREB-binding domain (183). Considering these results and that Arnt functions as a common partner in the formation of transcriptional regulators with other bHLH/PAS (a conserved domain among Per, Arnt and Sim) transcription factors such as HIF-1α (348) and HLF (85), it is reasonable to conclude that CBP/p300 is a common coactivator for a group of bHLH/PAS transcription factors.

The proximal promoter regions of several AhR-regulated genes contain GC boxes, which bind the common Spl transcription factor. The CYP1A1 gene contains a GC box termed the BTE (basic transcription element) sequence, immediately upstream of the TATA box and this is essential for the full activity of the CYP1A1 gene (151). On model promoters containing XRE and BTE recognition sequences, the AhR/Arnt and Spl transcription factors have been found to invoke a synergistic response. Indeed, the zinc finger domain of Spl can interact with both AhR and Arnt via their bHLH/PAS domains (184).
results support the notion that on a native CYPIAI promoter, the AhR/Arnt heterodimer located at 1 kb may interact with Sp1, which recognises the BTE close to the TATA box. Such a protein-protein interaction may be an important trigger in disrupting chromatin structure to allow anchoring of transcription factors to the proximal CYPIAI promoter (46), creating a transcription factor assembly culminating in strong induction of the CYPIAI gene.

In addition to their role in xenobiotic detoxification, components of the CYPI system have other functions. For example, expression of CYP1BI has been detected in the testis (304), suggesting that CYP1BI may have a role as a steroid hydroxylase (250). This gene has been shown to be cAMP responsive and its basal promoter contains two Sp1 sites and an SF1 (steroidogenic factor 1) site (379). Indeed, CYP1BI has been shown to convert 17β-oestradiol to its 4-hydroxy metabolite, a derivative implicated as a breast tissue carcinogen (130). Targeted disruption of the AhR has been reported to produce impaired liver development (90, 91) and hepatic fibrosis (308) due to accelerated rates of apoptosis (101). Recently, the AhR has been implicated in cell cycle control in a signalling pathway involving retinoic acid and TGFβ (86, 215, 375). The AhR has also been suggested to have a developmental role due to its constitutive pattern of expression over long periods in early embryonic development (66, 265), and has been implicated as an early regulator of adipocyte differentiation (7). Recently the haem degradation products bilirubin and biliverdin have been shown to be AhR ligands which can regulate the AhR-dependent gene expression pathway (268).

1.5.2 Regulation of the Peroxisome Proliferator Inducible Cytochrome P450s.

Peroxisome proliferators are a structurally diverse (100) group of compounds of industrial, pharmaceutical and agricultural origin that, when administered to responsive species including rodents and primates, produce a dramatic increase in the size and number of hepatic peroxisomes (315) (Fig 1.7). Some of the more potent inducers of proliferation are hypolipidaemic drugs that belong to the fibrate class of compounds, such as clofibrate, and have attracted attention for use in the prevention of coronary heart disease (286).
Figure 1.7  Chemical structures of the peroxisome proliferator compounds.
The peroxisome proliferator compounds vary in their chemical structures, but all lead to a proliferation of peroxisomes in liver cells.
Environmental contaminants recognised as peroxisome proliferators include phthalate ester plasticisers such as DEHP, (di-(2)-ethylhexyl phthalate) as well as industrial solvents and herbicides (208, 286). Exposure to peroxisome proliferators leads to increased oxidation of medium and long chain fatty acids and prostaglandins through microsomal ω-oxidation and peroxisomal β-oxidation pathways (112, 163, 208, 285, 286). The microsomal ω-oxidation pathway reflects induction of microsomal CYP enzymes that belong to the CYP4A subfamily and have fatty acid ω-hydroxylase activity (10). Enzymes of the β-oxidation pathway are also induced, in particular acyl-coenzyme A oxidase (345), the rate-limiting enzyme, and the two pathways probably act in concert, with the β-hydroxylated fatty acids, after conversion to dicarboxylic fatty acids, preferentially entering the peroxisomal ω-oxidation pathway (163).

Specific but related forms of CYP4A can be induced by peroxisomal proliferators in the livers of animal species (177, 373). Gene regulation studies have focused on the highly inducible genes for rat CYP4A1 (CYPLAα) (47, 127, 176) and rabbit CYP4A6 (232, 373).

1.5.2.1 Isolation of PPARα

Issemann and Green (153) first isolated a clone for peroxisomal proliferator activator receptor (PPAR). This protein is now referred to as PPARα since other isoforms of PPAR (PPARβ and PPARγ) have been identified in mice (113). Until recently, no ligand had been identified for PPARα, which has been classified as an orphan receptor of the nuclear receptor superfamily (69, 218)

Chimeric receptor transactivation assays provided the first evidence that the cDNA clone isolated by Issemann and Green (153) encoded a receptor that could activate gene expression in a peroxisomal proliferator-dependent fashion. A chimeric receptor cDNA plasmid with the oestrogen (or glucocorticoid) receptor DNA-binding domain fused to the putative ligand-binding domain of PPARα was introduced into mammalian cells. The expression of a cotransfected promoter/CAT construct with an oestrogen (or glucocorticoid) response element in the promoter was shown to be activated by this
chimeric receptor in response to added chemical peroxisome proliferators, but was not affected by other compounds tested, including dexamethasone, pregnenolone-16α-carbonitrile and phenobarbital (153). Of particular interest is the structural variation of the peroxisomal proliferators and the question arises as to whether these chemicals bind directly to PPARα.

Recently, Devchand et al. (69) demonstrated that the dihydroxy fatty acid leukotriene B₄ (LTB₄) binds to PPARα. (Leukotriene B₄ modulates the inflammatory response and is metabolised by the ω- and β-oxidation pathways following their induction.) There was also evidence that the hypolipidaemic drug Wy-14,643 is a ligand for PPARα (69). These observations now raise the possibility that all chemical peroxisomal proliferators act as ligands for PPARα and directly activate the receptor. Based on the observation that PPARα can be activated by fatty acids in transactivation assays (173), it may be possible that peroxisome proliferators, perhaps through disrupting normal mitochondrial fatty acid metabolism (285), stimulate the accumulation of an endogenous fatty acid with a strong affinity for PPAR. On this basis, the induction of enzymes by peroxisome proliferators would mimic the cellular response to excess fatty acids. This may be biologically significant as recent evidence has revealed that short-term starvation of PPARα-null mice resulted in hepatic steatosis, myocardial lipid accumulation, hypoglycemia and inadequate ketogenic response (202, 282). In addition, mRNA levels of CYP4 genes were shown to be markedly induced in the livers of fasting mice and that this induction was strictly dependent on functional PPARα (188). These results indicate a critical role for PPARα in lipid metabolism and utilisation.

1.5.2.2 PPARα activation of CYP4A-responsive genes

Chimeric receptor transactivation assays suggested that PPARα was a transcription factor. However, it was important to directly establish that PPARα could transcriptionally activate responsive genes and to identify the peroxisomal proliferator responsive element(s) (PPRE) in the gene promoters. Muerhoff et al (233) first demonstrated, by transient transfection analysis in kidney and hepatoma cell lines, that transcription of rabbit CYP4A6
gene promoter was induced by peroxisome proliferators following cotransfection of an expression plasmid for PPARα. Analysis of the CYP4A6 promoter defined a region (-677 to -644) that was strongly responsive to the inducers and contained the sequence AGGGCA A GTTGA, an imperfect direct repeat of the consensus binding site for the nuclear receptor family (AGGTCA) with a spacing of one nucleotide (DRI; Ref. 258). Deletion of this DRI sequence abolished the response to inducers and identified the sequence as a PPRE (258). However, subsequent studies have shown that for transcriptional functionality, an extended PPRE is required that contains an additional 5’ sequence (41, 257). There is evidence that this 5’ extended sequence may allow for the efficient and selective binding of PPARα in preference to dimers of other competitor nuclear receptors that also have affinity for DRI motifs (41, 257). A functional upstream PPRE has also been identified in the rat CYP4A1 promoter (6, 257). Other PPRE motifs have been located in genes responsive to peroxisomal proliferators, notably acyl CoA oxidase, bifunctional enzyme and other peroxisomal fatty acid beta oxidation genes (41, 173).

The action of PPARα requires RXRα, a member of the RXR (Retinoid X Receptor) family of nuclear receptors, as the accessory partner protein. When both PPARα and RXRα were co-expressed in kidney cells, there was a synergistic increase in CYP4A6 promoter expression in peroxisomal proliferator-treated kidney cells (258). While RXR is essential for DNA binding of this heterodimer it is the activation domain of PPARα, not that of RXR, that produces this synergistic increase in transcription (315). Gel mobility shift assays established that the 5’ extended PPRE sequence strongly bound the PPARα/RXRα complex, but not PPARα alone (258). The data are consistent with a model where inducers mediate their response by enhancing the dimerisation of PPARα and RXRα with the heterodimer binding to the extended PPRE in the responsive promoter and activating transcription. The coactivator p300 may be involved in this activation. Recent evidence has shown that the mouse PPARα interacts with p300 in a ligand dependent manner to enhance transcriptional of the CYP4A6 gene (78). PPAR forms a complex with a member of the heat shock protein 70 (hsp70) family in the absence of ligand. It appears that hsp70
may act as a molecular chaperone to translocate PPAR from the cytoplasm to the nucleus (148). The hsp70 has been shown to be responsible for the translocation of certain other nuclear proteins (322). Although the exact mechanism by which the PPAR activates peroxisomal and CYP4 genes is unknown, the above evidence suggests a similar mechanism to that of the Ah receptor.

While mouse RXRα activated PPRE-containing promoter constructs in cotransfection experiments, there was the possibility that another PPAR isoform was functional in vivo. To investigate this, PPARα was disrupted in the mouse, using gene knockout techniques (197). In the homozygous mouse, peroxisomal proliferation was not observed following treatment with peroxisome proliferators and the genes for the CYP4A proteins and the peroxisomal lipid metabolising enzymes were not activated in the liver (197). These data demonstrated an in vivo requirement for PPARα in the pleiotropic response, a finding that agreed with the predominance of this isoform in the liver (28) and the weak transactivation by other PPAR isoforms in the transient assays (233).

Recently, investigations have focused on PPARγ, which predominates in adipose tissue and plays a central role in the control of adipocyte gene expression and differentiation as well as lipid storage (68). Ligands have been identified for PPARγ. This receptor can directly bind prostaglandins of the J2 series, suggesting that a fatty acid derivative may function as the in vivo adipogenic agent (92, 179). Also, thiazolidinedione derivatives have recently been shown to bind directly to PPARγ (201). Because these compounds are antidiabetic and also induce adipocyte differentiation, a role for PPARγ and its endogenous ligand in adipogenesis and glucose homeostasis is suggested (201). In support of this PPARγ has been shown to interact with steroid receptor co-activating factor 1 (SRC-1), a coactivator important in adipocyte differentiation (245). Thus, the transcription factors PPARα and PPARγ play important roles in fatty acid metabolism and adipogenesis, respectively. In the liver, PPARα regulates expression of CYP4A and peroxisomal enzymes involved in fatty acid metabolism and responds to hypolipidaemic drugs, while
PPARγ expressed in adipocytes regulates adipogenesis and functions in the therapeutic action of thiazolidinedione compounds.

An intriguing question that remains to be answered is whether PPARα in the liver acts indiscriminately to bind all peroxisomal proliferators, as suggested by the finding that LTB4 and Wy-14,643 are ligands for this receptor. The recent crystallisation of the human apo-PPARγ ligand binding domain revealed that its structure contains a large binding pocket, which may explain the ability of PPAR to bind a diversity of ligands (245).

1.5.3 Regulation of the Glucocorticoid Inducible Cytochromes P450

The CYP3A family consists of steroid and drug metabolising enzymes expressed in the liver and intestine (104, 351). CYP3A enzymes metabolise endogenous steroids (testosterone, cortisol and 17β-estradiol), environmental pollutants (benzo[a]pyrene and 1-nitropyrene), dietary xenobiotics (aflatoxin B1) and drugs (cyclosporin A, erythromycin and warfarin) (3, 11, 32, 120, 235, 324). Members of the rat CYP3A subfamily can be induced by several chemically unrelated compounds. The first CYP3A inducer to be identified was pregnenolone 16α-carbonitrile (PCN) (210) and classically members of the CYP3 family induced by this compound have been referred to as the "PCN-inducible" cytochromes P450. Other inducers include dexamethasone, spironolactone (139) as well as nonsteroidal compounds such as phenobarbital and macrolide antibiotics (104, 369).

It appears that two different mechanisms for glucocorticoid-induced gene transcription exist. The mechanism by which dexamethasone induces transcription follows the classical glucocorticoid receptor pathway (277) in which the steroid hormone binds stereospecifically to the glucocorticoid receptor (GR), altering the conformation of this protein and thereby permitting its interaction with glucocorticoid response elements (GREs) located in the promoter, thus increasing the transcription of the responsive genes (358).
The induction by glucocorticoids of the rat CYP3A1 gene, however, appears to act via a different mechanism (139, 311). Induction by this pathway required higher doses of glucocorticoid and exhibits a different order of sensitivity to glucocorticoid agonists (311). In addition, PCN, a glucocorticoid antagonist that typically blocks the induction of glucocorticoid responsive genes was found to induce the CYP3A1 gene (313). A 164 bp region at position -220/-56 upstream of the CYP3A1 gene has been identified that confers dose dependent dexamethasone and PCN responsiveness independent of its orientation (38). DNase I footprint analysis of this fragment revealed two protected regions, one of which was shown to confer dexamethasone and PCN inducibility to a reporter gene (278). However, this fragment does not contain a traditional GRE and did not bind recombinant glucocorticoid receptor protein (278). These observations were extended by Huss and coworkers (150) who investigated the promoter for the rat CYP3A family member CYP3A23, which is related to CYP3A1. A dexamethasone-responsive region was identified in the proximal promoter at position -167/-60 and this region comprised two functional elements. Neither element contained a GRE, but one site contained an imperfect direct repeat of the steroid receptor motif AGGTCA, separated by 4 bp, and the other contained a direct repeat of ATGAACT separated by 2 bp. Quattrochi et al (279) have investigated the latter direct repeat in detail. They found that this element interacts with several proteins of different molecular weights and that both repeats were necessary for dexamethasone or PCN induction of CYP3A23. None of the identified proteins that bound to this element were identified as the glucocorticoid receptor. These results were supported by Huss and Kasper (149) who demonstrated binding of the orphan receptor chicken ovalbumin upstream promoter transcription factor to these two elements. They suggest that induction of CYP3A23 involves members of the nuclear receptor superfamily.

Recently a novel orphan nuclear receptor has been described, pregnane X receptor (PXR), that binds as a heterodimer with RXR to a hormone response element composed of two half sites organised as a direct repeat (180, 213). PXR is activated by endogenous steroids, synthetic glucocorticoids (dexamethasone) and antiglucocorticoids (PCN and RU486). In addition ligand bound PXR was shown to activate the expression of a CYP3A1 promoter
reporter plasmid in CV-1 cells (180). Similarly, PXR was shown to be required for activation of *CYP3A23* by chlordane, PCBs and the anti-androgen, cyproterone acetate (310). Collectively, these results suggest that *CYP3A* genes are activated via a unique pathway involving the binding of a ligand activated nuclear receptor heterodimer (possibly PXR/RXR) to a direct repeat hormone response element located in the promoter region of these genes.

In addition to steroid inducers, *CYP3A* genes are also responsive to non-steroids, such as metyrapone and rifampicin (367). It has been demonstrated that in rat hepatocyte cultures the *CYP3A1* gene is transcriptionally activated by metyrapone (368). Metyrapone does not compete with the binding of dexamethasone for glucocorticoid receptor in soluble liver fractions (368). This indicates that metyrapone is not a ligand for glucocorticoid receptor, but induces *CYP3A1* by a mechanism independent of this receptor. The possible involvement of PXR in activation of this gene by metyrapone has not been investigated.

Glucocorticoids have been implicated in the regulation of cytochromes P450 at levels other than that of transcription. For instance, in rat liver, dexamethasone and PCN not only increase the rate of transcription of *CYP3A1* but also stabilise the *CYP3A1* primary transcript and mRNA (283, 312). Dexamethasone has also been shown to affect the rate of translation and the stability of the cytochrome P450 proteins (327).

### 1.5.3.1 Dexamethasone induction of the human *CYP3A5* gene

A region in the proximal promoter of the human *CYP3A5* gene has been identified that responds to dexamethasone and this induction in cultured cells requires cotransfection of glucocorticoid receptor (314). This region did not contain a classic GRE however, two GRE half-sites, 160 bp apart, were identified. Mutagenesis confirmed that both half-sites were required for the dexamethasone response (314) and that the 5' GRE half-site bound glucocorticoid receptor in gel shift experiments. The data strongly suggest that, unlike the *CYP3A1* and *CYP3A23* genes, glucocorticoid receptor is required for dexamethasone induction of the *CYP3A5* gene.
induction and presumably the two distantly located half-sites interact in some way to facilitate glucocorticoid binding.

1.5.4 Regulation of the Phenobarbital Inducible Cytochromes P450

The administration of barbiturates such as phenobarbital to a wide variety of species results in the induction of certain members of the cytochrome P450 2A, 2B and 2C subfamilies (298, 353). The effect of phenobarbital treatment is widespread and includes proliferation of smooth endoplasmic reticulum, stimulation of liver weight gain, liver tumour promotion, and a general stabilisation of liver microsomal protein (58, 252, 316). A number of other structurally diverse chemicals exhibit barbiturate-like properties in this regard including trans-stilbene oxide, organochlorine pesticides such as DDT and dieldrin, polychlorinated biphenyls, various phenothiazines, 2-allyl-2-isopropylacetamide, and acetylaminofluorene (100, 247, 352) (Fig 1.8). In addition to the cytochrome P450 family, phenobarbital affects several other enzymes that contribute to foreign compound metabolism including aldehyde dehydrogenase, microsomal epoxide hydrolase, NADPH-cytochrome P450 reductase, UDP-glucuronosyltransferase and multiple forms of glutathione S-transferase (81, 211, 217, 269).

Induction of cytochrome P450 enzymes by phenobarbital and phenobarbital-like chemicals occurs in numerous species. Phenobarbital induces the extensively studied rat CYP2B1 and CYP2B2 genes. While these two genes share 97% amino acid sequence identity they have a different spectrum of catalytic activity (298) and exhibit different levels of basal expression where CYP2B1 is at least 5- to 10-fold lower than CYP2B2 (53, 185, 366). Phenobarbital rapidly (within 30 min) increases transcription of these genes which accounts for the subsequent mRNA accumulation and increased CYP2B1/2 enzymatic activity (251). In chick embryo liver, phenobarbital rapidly induces CYP2H1 and CYP2H2 which are closely related structurally to CYP2B1/2 (221, 377). The high levels of inducible expression of these genes (15-50 fold) involves both transcriptional and posttranscriptional mechanisms and occurs in chick embryo hepatocytes in ovo and in primary cell cultures (119). Phenobarbital-inducible P450s have also been observed in Bacillus megaterium.
Figure 1.8  Chemical structures of phenobarbital and phenobarbital-like compounds. The compounds shown are called “phenobarbital-like” compounds as they, like phenobarbital, all induce the same cytochrome P450 genes. While some of the compounds such as pentobarbital, glutethimide and mephenytoin are similar in structure to phenobarbital, there are a number of compounds which bear no resemblance to phenobarbital or to other compounds in this class of drugs such as AIA and sulphonal.
Induction of these bacterial fatty acid monoxygenases (CYP102 and CYP106) by barbiturates is extremely rapid (<5 min), with a maximal rate of synthesis occurring in less than 30 min, compared to 12-24 hr in rat and chicken primary hepatocytes. Induction reflects an increase in the rate of transcription of these genes (97). Taken together these observations imply that phenobarbital acts primarily at the level of transcription to induce cytochrome P450 activity.

Because phenobarbital induces cytochrome P450 in bacteria, birds and mammals, it might be anticipated that the induction mechanism would be conserved. This appears not to be the case. For example, studies in rat hepatocytes using cycloheximide reveal that ongoing protein synthesis is required for induction of CYP2B1/2 by phenobarbital (23, 37). In contrast, inhibition of protein synthesis by cycloheximide synergistically enhances CYP3A mRNA accumulation following exposure of rat hepatocytes to phenobarbital (37). Similar differences occur in other systems. For example, cycloheximide blocks the induction of CYP102 by phenobarbital in B. megaterium, while in chicken hepatocytes, cycloheximide alone induces, and in combination with phenobarbital, superinduces the CYP2H1/2 genes (72, 121). Together, these results demonstrate that regulation of phenobarbital-inducible cytochrome P450s is complex and involves many proteins playing a role in this induction process.

1.5.4.1 A phenobarbital receptor?

The mechanism by which the cell recognises phenobarbital and phenobarbital-like chemicals and the pathway(s) by which the phenobarbital signal activates the transcriptional machinery are unknown. While many have speculated about the presence of a cellular receptor for phenobarbital, the structural diversity among phenobarbital-like inducers is difficult to reconcile with the existence of a specific receptor. In addition, studies using radiolabeled phenobarbital have failed to detect a specific phenobarbital-binding protein (337). However, this does not eliminate receptor-dependent mechanisms. Only ligand-receptor complexes that are abundant or have a high affinity for their ligands are likely to be detected by these methods. Hence, the lack of binding could reflect the low
affinity of phenobarbital for its hypothetical target protein (implied by the relatively high concentrations of phenobarbital needed for induction, Ref. 185).

While it is true that phenobarbital-like inducers both in eukaryotes and prokaryotes include a huge variety of chemicals with no obvious structural similarity, other than their general lipophilicity, this does not preclude receptor-mediated mechanisms. For instance, the insecticide chlordecone and other structurally unrelated chemicals can bind to the oestrogen receptor to elicit hormone-like effects (122). Similarly, diverse drugs and other chemicals that induce CYP4A gene expression and peroxisome proliferation all appear to activate the peroxisome proliferator receptor.

One such model for phenobarbital induction in eukaryote cells proposed by Waxman (352) involves the binding of inducer to a specific intracellular phenobarbital receptor protein which then interacts with the promoter of target genes at one or more drug responsive elements (DRE) to enhance the initiation of gene transcription. Since the phenobarbital-type inducers are diverse in structure, it would be necessary to hypothesise that the active site of the receptor is a sloppy fit or an elastic recognition site (247). Microsomal CYP proteins are known to have a remarkably broad substrate specificity. Waxman, therefore, postulated that such a CYP protein may be the putative sloppy fit receptor. The active site of this CYP enzyme may be a common binding site for the structurally diverse inducers. If this particular CYP metabolises an endogenous inducer to an inactive form, binding of the phenobarbital-type inducers to this CYP would prevent this inactivation and result in an increased concentration of endogenous inducer, which would in turn lead to activation of phenobarbital-inducible cytochrome P450 genes.

1.5.4.2 Induction of genes in Bacillus megaterium and the Barbie box.
In B. megaterium, phenobarbital induces CYP102 and CYP106 genes (97). Of particular interest has been the identification in the proximal promoters, of a 15 bp consensus sequence, with an AAAG core, designated the Barbie box element that appeared to be important for phenobarbital induction (97, 132, 204, 205). Liang and Fulco (204) have
cloned and characterised a repressor protein (Bm3R1) that binds to the Barbie box and also to operator sequences in the CYP102 promoter which then prevents transcription of this gene. In addition, peroxisome proliferators have been shown to induce CYP102 by causing dissociation of Bm3R1 from its operator sequence (87). Hence, barbiturates function as inducers to prevent binding of the Bm3R1 repressor to the Barbie box (203). The in vivo potency of barbiturates as inducers of CYP102 strongly correlates with their ability to inhibit interaction of Bm3R1 with its operator DNA in vitro. Three other proteins have been characterised (Bm1P1, Bm1P2 and Bm1P3) that have been proposed to act as positive regulatory proteins involved in the expression of CYP106 by interfering with the binding of Bm3R1 to the regulatory regions of CYP106 (132). However, recent work by Shaw et al (319) disputes this. They found that disruption of Bm1P1 did not affect barbiturate-mediated induction of CYP106 expression, while deletion of the Barbie box did not effect pentobarbital-induced expression of a CYP106 promoter-reporter gene. Hence, unlike CYP102, the mechanism by which barbiturates induce CYP106 remains unclear.

1.5.4.3 Induction of Rat CYP2B1/B2 genes

1.5.4.3.1 Proximal phenobarbital-responsive regulatory elements

The rat CYP2B1 and CYP2B2 genes are both highly inducible by phenobarbital. The promoters of these genes contain a Barbie box element (205) and an interesting issue is whether this sequence plays a role in the induction of these genes as inferred from the studies in B. megaterium (204). Padmanaban and coworkers used in vitro transcription and in vivo gene transfer to show that the region of -179/+1 bp in rat CYP2B2 gene is sufficient to enhance transcription in response to phenobarbital (275). Within this DNA region there is a positive element (PE, at -98/-69 bp; Reference 342) that includes a Barbie box site, and a negative element (NE, at -160/-127 bp; Reference 280). Subsequently, a protein of MW 26-28 kDa that binds to both the positive and negative elements in the promoter was purified and phosphorylation of this protein was shown to be increased substantially following phenobarbital treatment of rats (275). Prabhu and coworkers propose that in the absence of drug, the 28 kd protein, in a dephosphorylated form, binds to the negative element and represses gene expression. However, in the presence of drug, the protein is
phosphorylated and now binds preferentially to the positive element thus attracting other transcription factors and increasing gene transcription.

There is abundant evidence to suggest that the Barbie box may not be involved in CYP2B expression. Hashimoto et al. (129) sequenced 800 bp of 5'-flanking DNA from both CYP2B1 and CYP2B2 genes from a mutant Sprague-Dawley rat strain. In this mutant strain, both genes have a low basal expression, but only the CYP2B1 gene is induced by phenobarbital. In spite of this different response, both CYP2B1 and CYP2B2 proximal promoter sequences including the barbie box are identical to their counterparts in the wild-type strain (129). In the mouse phenobarbital-inducible Cyp2b10 gene, the Barbie box is disrupted by a 42 bp DNA insertion (143). The nuclear protein binding to the PE and Barbie box sequences has not been detected by other laboratories working with CYP2B1 and CYP2B2 (212, 261, 281, 321, 332) or Cyp2b10 genes (143). Finally, the deletion or mutation of Barbie box sequences had no effect on the transcription of Cyp2b10 or CYP2B2 reporter genes in hepatic cells (143, 262). It may be concluded that the Barbie box does not play a role in phenobarbital-inducible transcription of the mammalian CYP genes. More direct support for this conclusion comes from studies on the regulatory elements in the distal regions of the CYP2B and CYP2H genes, as discussed below.

1.5.4.3.2 Distal phenobarbital-responsive regulatory elements
A study by Ramsden et al (281) presents evidence that phenobarbital-inducible transcription is regulated by DNA elements in distal regions of the CYP2B genes. Transgenic mouse strains were developed incorporating the rat CYP2B2 gene. Expression in mouse tissues was analysed for two series of rat CYP2B2 gene constructs, each containing the entire coding region, introns, and 3'-flanking sequences of CYP2B2, but differing in the respective lengths of 5'-flanking sequences. One group of mice, whose transgene included the complete CYP2B2 gene but only 800 bp of 5'-proximal sequence, was not phenobarbital inducible in mouse liver or in any extrahepatic tissue. Rather, these genes were expressed at very high levels constitutively and selectively in only liver and kidney. A second group of mice with an identical transgene, except for the presence of an
additional 19 kb of 5'-flanking sequence, expressed CYP2B2 only in the liver and at high levels only after phenobarbital treatment. This is analogous to the expression pattern observed for the endogenous CYP2B2 gene in the rat. Barring any position-dependent effects, these results indicate that DNA elements regulating the phenobarbital induction should be located upstream from the -0.8 kb region. Moreover, the high basal transcription activity of the proximal promoter must be repressed by DNA elements residing in the upstream region in order to be appropriately regulated.

Consistent with these findings, a strong negative activity was located within -971/-775 bp of the Cyp2b10 gene identified by 5'-deletion assays in primary hepatocytes (143). In addition to this putative negative regulatory element, three other regulatory elements were reported to be present around -1.4/-1.2 kb of the rat and mouse CYP2B genes. Jaiswal et al. (155) reported the presence of a functional glucocorticoid response element at position -1357 bp in the CYP2B2 gene that may play a role in the well established dexamethasone dependency of phenobarbital induction (352). Roe et al (295) found that phenobarbital treatment for 18 hours increased protein binding to an AP-1 site at -1441 bp in the CYP2B2 gene. Shaw et al (320) showed that -1.4 kb CYP2B2 promoter activity was increased three fold by phenobarbital in rat hepatoma cells. In line with this report, a minor phenobarbital-responsive enhancer activity has been located in the Cyp2b10 gene at position -1404/-971 bp (143, 145). This region contained a protected 25 bp DNA fragment with a nuclear receptor (NR) binding motif and a high identity to a portion of the DNA element at position -2.3 kb that is responsible for the major phenobarbital-inducible enhancer activity (145).

In rat primary hepatocytes, Anderson and colleagues were first to demonstrate that a DNA fragment at -2318/-2155 bp in the CYP2B2 gene confers phenobarbital-responsive enhancer activity to a heterologous thymidine kinase (tk) promoter (338). Subsequently, this enhancer activity was independently confirmed by using in situ transfection of CYP2B2 promoter-luciferase constructs into rat livers (275). Consistent with previous findings, the enhancer activity was not affected by mutation of the Barbie box sequence in
the promoter region of the construct (262). The corresponding region in the mouse Cyp2b10 gene is located at -2426/-2250 bp. This DNA fragment also elicited phenobarbital-inducible enhancer activity in mouse primary hepatocytes (145). Hence, the enhancer activity of the distal DNA element at -2.3 kb has been independently demonstrated by three different laboratories using three different methodologies, providing compelling evidence for this region to be the enhancer in vivo. This DNA fragment has been named the phenobarbital-responsive element (PBRE, in CYP2B2, Ref. 338) and phenobarbital-responsive enhancer module (PBREM, in Cyp2b10, Ref. 145). Notably, PBRE and PBREM do not contain any Barbie box sequences.

The sequence of PBREM has recently been functionally dissected (145). DNase I footprinting assays were used to define 6 protein binding regions. Various deletions of these regions were linked to the tk promoter-CAT plasmids, and their phenobarbital-responsive enhancer activities determined in mouse primary hepatocytes. A minimal phenobarbital-responsive element was identified at -2365/-2297 bp that gave 3 fold induction in the presence of phenobarbital. Analysis revealed that the sequence consisted of a nuclear receptor (NR) binding motif (AGGTCA) and a nuclear factor I (NF1) binding site. Mutations in either of these sites abolished enhancer activity. Honkakoski et al (144) have recently extended this sequence to 51 bp (-2339/-2289) to include a second NR binding site that was shown to be functionally active by specific mutation. This 51 bp region gave 11 fold induction in the presence of phenobarbital and in addition was shown to be responsive to 16 other structurally unrelated phenobarbital-type inducers.

Significantly, Honkakoski and colleagues have shown that a heterodimeric complex of RXR and the nuclear orphan receptor CAR (Constitutive Androstane Receptor, Ref. 14) bind to the NR1 sites of PBREM in response to phenobarbital-induction and that PBREM can be activated by this orphan receptor in HepG2 and HEK293 cell lines (147). It was recently demonstrated that CAR is localised in the cytoplasm of the livers of untreated mice, while PB treatment results in translocation of CAR into the nucleus and this translocation can be prevented by the protein phosphatase inhibitor okadaic acid. Nuclear
accumulation of CAR perfectly correlated with an increase of CAR binding to PBREM (172). Thus, the CAR-mediated transactivation of PBREM in vivo becomes PB responsive through an okadaic acid-sensitive nuclear translocation process. Further, stable transfection of a mouse CAR expression vector into human HepG2 hepatoblastoma cells, that do not normally express CAR, led to the constitutive activation of the PB-inducible human CYP2B6 gene (334). It was shown that androstanes can repress this CAR-mediated activation of both the endogenous CYP2B6 gene and a transiently transfected reporter plasmid containing the Cyp2b10 PBREM. This repression was overcome by treatment with PB and PB-like inducers (334). Thus a model can be proposed in which endogenous inhibitory steroids related to androstanol and androstenol bind to CAR and maintain it in an inactive state. In the presence of PB or PB-like inducers, however, the binding of inhibitory androstanes to CAR is abolished and receptor activity is thereby derepressed. The intrinsic constitutive activity of CAR would thereby become manifest, leading to transactivation of CYP2B and other PBRE-regulated target genes. The observation that CAR can directly transactivate a PBRE-linked reporter gene in transfected cells in the absence of PB inducers (147) could thus be explained by the receptor's constitutive activity and by the absence of inhibitory androstanes in the HepG2 cell transfection system.

It is important to note that the NR1 site of the mouse Cyp2b10 gene, to which CAR binds, is critical for maximal PB response (144), however, when this site is mutated in the rat CYP2B2 gene there is only a partial loss of PB-response (333). In addition, CAR does not appear essential for PB-responsiveness of the chicken CYP2HI gene, as discussed in chapter 3. Therefore, CAR may only regulate a subset of PB-inducible genes in mammals. These data further reinforce the fact that phenobarbital responsiveness is mediated by different factors between species.

From these studies, the following conclusions emerge. Firstly, the proximal promoters of the CYP2B genes are inherently very active. Secondly, this high proximal promoter activity must be suppressed either by negative elements located upstream or by the formation of a
transcriptionally inactive chromatin structure (174). Thirdly, phenobarbital induction and tissue-specific control requires interaction of regulatory elements far upstream of the core CYP2B2 promoter region and upstream of the motifs indicated previously as determinants of phenobarbital responsiveness.

The identification of proteins that bind to the upstream enhancer sequence will be important and should shed some light on the induction mechanism. Overall, the studies from both bacteria and mammals suggest that a depression event is important with the drug overcoming the action of a repressor protein that inhibits gene transcription. A similar mechanism emerges from studies on the chicken CYP2H1 gene.

1.5.4.4 Drug induction of Chicken cytochromes P450

When phenobarbital is injected into chick embryos, mRNAs for three related CYP proteins are induced in the liver (125) and similar levels of induction are observed following drug treatment of hepatocyte cultures. This laboratory is are examining the molecular regulation of the CYP2H1 and CYP2H2 genes that encode mRNAs of sizes 3.5 and 2.2 kb respectively.

Experiments carried out on the proximal promoter of the CYP2H1 gene using transient transfection of chick embryo primary hepatocytes revealed that the first 160 bp of this promoter can direct very strong basal expression of this gene and that this expression is independent of drug inducer (75, 119). This result was unexpected since the basal level of endogenous CYP2H1 mRNA is almost undetectable. This suggested that a repression mechanism which acted on endogenously was not operating in the transient assays as proposed for the rat CYP2B2 gene (281). This promoter region of 160 bp does not respond to phenobarbital in the transient assays. However, a distal region between -5.9 and -1.1 kb was identified as a drug responsive domain. When this domain was fused to a weak heterologous enhancerless SV40 promoter substantial induction was observed with the domain in either orientation (119). The domain however conferred only a weak induction response when fused to the -160 bp promoter because of the high basal expression driven
by this promoter. In addition, transcriptional nuclear run-on studies using different segments that spanned the CYP2HI cDNA sequence as probes did not show any difference in the density of RNA polymerase II molecules across the gene in either control or phenobarbital-induced chick embryo liver nuclei (our unpublished data). Hence, it is unlikely that the action of phenobarbital on the chicken CYP2HI gene is to relieve either stalled RNA polymerase II molecules or, alternatively, a repressor protein bound in the first 8.9 kb of 5'-flanking region. These data suggests the inherently strong basal expression of the native CYP2HI gene is normally repressed and that the drug relieves this repression.

We propose that the repression of the native CYP2HI gene may be due to assembled nucleosomes on the promoter and distal enhancer. In the presence of drug, the distal nucleosome is removed upon binding of a drug-receptor complex to one or more DREs together with the binding of other transcription factors. The enhancer protein complex in turn leads to removal of the nucleosome over the proximal promoter, possibly by recruitment of coactivator and/or histone acetyl transferase proteins, with subsequent binding of transcription factors to the promoter (Fig 1.9). In this model, derepression of the strong proximal promoter is chiefly responsible for induced transcription rate. In this model the CYP2HI gene would behave in a similar manner to the CYP1A1/2 genes where it has been demonstrated that AhR-Arnt binding alters local chromatin structure over the CYP1A1/2 enhancer and that AhR then facilitates the alteration of chromatin structure over the promoter from a distance without affecting the intervening chromatin (181, 230, 250).

The protein kinase inhibitor 2-aminopurine is a potent inhibitor of induction of the CYP2HI gene in chick embryo hepatocytes (74), indicating a vital role for phosphorylation in derepression of the CYP2H genes in a similar manner to the CYP1A1 (21), CYP2B1/2 (275) and CYP2b10 (146) genes. The general concept of this model is in keeping with the findings of others for the regulation of the rat CYP2B2 gene (262) and the mouse Cyp2b10 gene (143) as well as the model for AhR mediated drug induction. While we suggest that chromatin configuration is important for repression, a repressor protein could be involved
Figure 1.9  Model for the involvement of nucleosomes and the chromatin structure in the induction of CYP genes by specific chemicals.

The inducer/receptor complex binds to the enhancer region, initiating nucleosome loss from the promoter, possibly by coactivator/histone acetyl transferase recruitment (CoA) and subsequent histone acetylation (*), although the precise mechanistic details are unclear. RNA polymerase II is then attracted to the promoter. XRE, xenobiotic-responsive element; specific TF(s), specific transcription factor(s).
that interacts with the promoter to inhibit transcription. Since the chicken CYP2HI promoter constructs tested extend to 8.9 kb and all give a high basal level of expression such a repressor protein binding site would presumably lie further upstream than 8.9 kb or even downstream of the transcription start site.

1.5.4.5 Summary of the models for phenobarbital induction

From the studies on the phenobarbital induction of CYPs in bacteria, mammals and chickens and general model is emerging in which the inducer elevates the transcription of CYP genes through a derepression mechanism. Several critical questions remain to be answered. Is there a specific receptor protein that binds all inducers or is there a common receptor that binds an endogenous inducer and is CAR this receptor? If phosphorylation of transcription factors is the key event in induction, do drugs activate a protein kinase and by what mechanism?

1.6 Aims of this Thesis

Regulation of gene expression and in particular inducibility of expression, is of fundamental interest. This laboratory has an excellent system to investigate this area, namely phenobarbital-inducible CYP genes of the chicken, CYP2HI and CYP2H2. Characterisation of the CYP2HI gene has revealed two important regions required for regulation, a distal phenobarbital responsive region and a strong early promoter region that is not drug responsive.

The first aim of this thesis was to characterise the large (4.8 kb) distal phenobarbital responsive region in an attempt to isolate a minimal region that could confer drug responsiveness to a heterologous promoter. This involved transient expression of deletion and restriction enzyme fragments of the 4.8 kb drug responsive region fused to the SV40 enhancerless promoter/CAT reporter gene vector in chick embryo hepatocytes. Of importance in this work was the identification of cis-acing elements within the minimal enhancer and characterisation of the transcription factors that bind to these elements. Further, it was of interest to establish the tissue distribution of these transcription factors.
and the effect of phenobarbital treatment on the binding of these factors to the minimal drug-responsive element.

The second aim of this thesis was to characterise the little studied \textit{CYP2H2} gene. Firstly, to isolate the enhancer and promoter regions of this gene for comparison with the \textit{CYP2H1} gene to increase our understanding of the molecular mechanism of induction. Secondly, to determine why this gene is induced by drug to a level 10 fold below that of the highly homologous \textit{CYP2H1} gene. This initially involved Southern blot analysis to identify the minimal enhancer and promoter regions prior to cloning and sequencing of these regions. Expression constructs containing these regions were then created to enable comparison of their relative activity with the corresponding regions from the \textit{CYP2H1} gene.

Glucocorticoids have been implicated the induction of the closely related rat \textit{CYP2B1/2} genes (333). Thus, the third aim was to investigate the role that glucocorticoids play in expression and/or induction of the \textit{CYP2H1/2} genes. This involved employing a glucocorticoid antagonist to block the action of the glucocorticoid receptor. The effect of this antagonist was determined on both the endogenous \textit{CYP2H1} gene as well as its effect on transiently transfected constructs containing various lengths of the 4.8 kb enhancer region of this gene.
Chapter 2. Materials and Methods
2.1 Materials

2.1.1 Chemicals and reagents.
The following were obtained from Sigma Chemical Co: acrylamide, agarose (Type 1), ampicillin, bis-acrylamide (N,N'-methylene-bis-acrylamide), bovine serum albumin (BSA), chloramphenicol, dithiothreitol (DTT), ethidium bromide, ethylenediaminetetra-acetic acid (EDTA), Salmon sperm DNA, sodium dodecyl sulphate (SDS), spermidine trihydrochloride, spermine tetrahydrochloride, Tris-base, transfer RNA, 2β-mercaptoethanol, guanidinium thiocyanate, and cesium chloride. Sources of other important reagents were as follows; deoxyribonucleotide triphosphates (dNTPs): Biotechnology Research Enterprises of South Australia (Bresatec); phenol: BDH chemicals; polyethylene glycol 6000: BDH chemicals; N,N,N',N'- tetramethylethenediamine (TEMED): Tokyo Kasei; sodium phenobarbital was obtained from Fauldings, Australia. RU486 was a generous gift from Roussel Uclaf, France. TSA, Wako BioProducts, USA. General chemicals not listed above were obtained from one of the following suppliers: Ajax Chemicals Pty. Ltd., BDH Chemicals Pty. Ltd., Merck, Pharmacia or Sigma Chemical Co. All chemicals and reagents were of analytical grade or of the highest purity available.

2.1.2 Kits
The kits for oligo labelling and 5'-terminal kinasing of DNA were purchased from Pharmacia. Sequenase Version 2.0 sequencing kit was purchased from United States Biochemical Corporation.

2.1.3 Antibodies
Antibodies against rat HNF-3α and HNF-3β were kindly provided by Dr. J. Darnell (Rockefeller University, New York).

2.1.4 Radiochemicals.
[3H] acetyl coenzyme A, and D-threo-[dichloroacetyl-1-14C]chloramphenicol were purchased from Amersham. [α-32P]dATP (2000 Ci/mmol), [α-32P]dCTP (1800 Ci/mmol).
[\gamma^{-32P}]dATP (1000 Ci/mmol) and [\alpha^{-33P}]dATP (1000 Ci/mmol) were purchased from Bresatec.

2.1.5 Enzymes
All restriction endonucleases, \(\beta\)-galactosidase, chloramphenicol acetyltransferase, Proteinase K and Mung Bean Nuclease were purchased from Pharmacia. Calf intestinal alkaline phosphatase was purchased from Boehringer Mannheim. Deoxyribonuclease I, Klenow fragment of \(E.\ coli\) DNA polymerase I, ribonuclease A, T4 DNA ligase, T4 Polynucleotide Kinase and \(Taq\) DNA polymerase were purchased from Bresatec. AMV reverse transcriptase was purchased from Molecular Genetic Resources.

2.1.6 Plasmid vectors
pGL2-CAT and pBluescript KS\(^+\) were obtained from Chris Hahn. Chicken \(CYP2H1\) cDNA clones pCHP3, pCHPB15 and pCHP7-AB1 clones have been described previously (27, 125, 140).

2.1.7 Synthetic oligonucleotides.
Synthetic DNA primers were synthesized by Bresatec. The primer sequences are listed below.

The primers employed in chapter 3 for site directed mutagenesis of the DNase I footprinted regions are as follows with mutations shown in bold letters and underlined:

\[
\begin{align*}
\text{mFP1} & \quad -61 \quad \text{GGCATTTCTGCAATGAGCTCAATCACCTGA} \quad -32 \\
\text{mFP2} & \quad -113 \quad \text{GGGAGTTCCAGACCTGCAGATTTACCAAC} \quad -84 \\
\text{mFP3} & \quad -170 \quad \text{GAGAGCAGTTATGATACATAGCAATCT} \quad -141 \\
\text{mFP4} & \quad -221 \quad \text{TTCAGAGACCG\underline{TCTAGATACATAGCAATCT}} \quad -192
\end{align*}
\]
The DNA sequences of the double-stranded oligonucleotides encompassing footprinted regions A-D and used as probes in gel mobility shift assays in chapter 3 are given below with added overhangs shown in lower case.

FP1  
5'-tcgacGGCATTTCTGCAATCACCTGA-3'
gCCGTAAGACGTTAGTGGA

FP2  
5'-tcgAGACAATATTTAAACCAACC-3'
CTGTGTTTATAAAATTGGTTGG

FP3  
5'-cgAGCAGTTATGTCAGTGCCCTat-3
TCGTCAATACAGTACCAGGAtagc

FP4  
5'-aattCCAATACATAGCAATCTGTGTC-3'
GGTTATGTATCGTTAGACAGCttaa

The double stranded oligonucleotide competitors used for gel shift analysis in chapter 3 are as follows:

MyoD  
5'-ACCCAGACATGTGGCTGCCC-3'
TGGGTCTGTACACCGACGGG (194)

USF  
5'-gatcCGAATTCCACGTGACG-3'
GCTTAAGGTGCACGTGctag (305)

HNF-1  
5'-TCGAGTGTGGTTAATGATCTACAGTTA-3'
AGCTCACACCAATTACTAGATGTAAT (45)

C/EBP  
5'-aattCAATTTGGCAATCAGG-3
GTTAACCCGTTAGTCCttaa (193)

36
HNF-5  
5'-TAGAACAACAAAGTCC -3'  
ATCTTGTTTGTCAGG  

NF1  
5'-ATTTTGGCTTTGCAAGCCATATG -3'  
TAAAACCGAACCTCGGTTATAC

Oligonucleotides used for double strands sequencing in chapter 4 are as follows:

Primer 6728 :  5'-dGATACAGACCAGCAAGAGGATTGTG-3'
CAT Primer 5'-dCAACGGTGTTATATCCAGTG-3'

Gel shift oligonucleotides used in chapter 4 are as follows:

USF Primer :  5'-dTGCAGATCGACTTCATATGAGTTCG-3'
C/EBP Primer :  5'-dTGCAGCTGATATTGGCTGATTTAAG-3'
HNF-1 Primer :  5'-dTCAAGGACGATTAAATAAGTAACCTGCTGC-3'
HNF-3 Primers:
4A  5'-dTGACTACAGACCAGCAAGAGGATTGTG-3'
4B  5'-dTGAGCTGATATTGGCTGATTTAAG-3'
4AB  5'-dTGACTACAGACCAGCAAGAGGATTGTG-3'
TTR  5'-TGCTCGATTTGACTAAGTCAATAATCAGAATCAG-3'
CYP2H1  5'-dGTCTCCATTTGGTTACTCAAGGC-3'

Oligonucleotides used in chapter 4 for site directed mutagenesis are as follows:
d4B  5'-dAGTCAGAGGATCGACTTCATATGAGGATTGTG-3'
m4A  5'-dAGTCAGAGGATCGACTTCATATGAGGATTGTG-3'
m4B  5'-dAGTCAGAGGATCGACTTCATATGAGGATTGTG-3'

2.1.8 Bacterial strains.
The following *E. coli* strains were used. Glycerol stocks of these strains were obtained from the Department of Biochemistry, University of Adelaide.
(1) *E. coli* DH5α: supE44 -lacU169 (p80 lacZ-M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 host for recombinant plasmids.

(2) *E. coli* XL1-Blue : supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lacF' [ proAB+ lacI9^ lacZ-M15 Tn10 (tet^)] host for recombinant plasmids.

(3) *E. coli* LE392. F' hsdR514, (r-Km-K), sup E44, sup F58 lac Y1. Host for bacteriophage λ propagation.

### 2.1.9 Bacterial growth media.

Growth media were prepared in double-distilled water and sterilised by autoclaving, antibiotics and other labile chemicals were added after the solution had cooled to 50°C. Bacteria were cultured in Luria Bertani (LB) broth containing 1% (w/v) Bacto-tryptone (Difco), 0.5% (w/v) yeast extract (Difco), 1% (w/v) NaCl, adjusted to pH 7.0 with NaOH. Agar plates were prepared by the addition 1.5% (w/v) Bacto-agar (Difco) to the LB broth. Ampicillin (50 μg/ml) or tetracycline (10 μg/ml) were added where appropriate for growth of transformed bacteria, to maintain selective pressure for the plasmid.

*E. coli* strains DH5α and XL1-Blue were cultured in LB medium containing 50 μg/ml of ampicillin or 10 μg/ml of tetracycline respectively. *E. coli* LE392, used for the propagation of λ bacteriophage, was cultured in NZCYM broth containing 1% NZ amine A, 0.5% yeast extract, 0.1% Casamino acids 0.5% NaCl 0.25% MgSO4.7H2O and 0.2% maltose, adjusted to pH 7.5 with NaOH. NZCYM agar plates and soft overlay contained NZCYM medium supplemented with 1.5% and 0.7% Bacto-agar.

### 2.1.10 Chicken Embryos

White Leghorn fertilised eggs were purchased from Parafield Poultry Research Centre, Parafield Gardens, South Australia. The eggs were obtained the day after being laid and were kept at 5°C for 5-8 days before being placed into a humidified incubator (85%) at 37°C. The eggs were tilted by 90° twice a day and the embryos were allowed to develop for 17-18 days before being used for experimentation.
2.1.11 Buffers.

Denhardt's solution: 0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) BSA.

SSC: 150 mM NaCl, 15 mM sodium citrate

TAE: 40 mM Tris-acetate, 20 mM sodium acetate, 1 mM EDTA, pH 8.2

TBE: 90 mM Tris, 90 mM boric acid, 2.5 mM EDTA, pH 8.3

TE: 10 mM Tris-Cl pH 7.5, 0.1 mM EDTA

3x Urea loading buffer: 4 M Urea (pH 7.2), 50% sucrose (w/v), 50 mM EDTA, 0.1% (w/v) bromophenol blue

Solution D: 125 g Guanidinium isothiocyanate, 8.9 ml sodium citrate, 13.2 ml Sarcosyl, 146.5 ml H2O.

All buffers were sterilised by autoclaving or, where necessary, by filtration through a Sartorius™ Minisart NML 0.2µm filter.

2.1.12 Miscellaneous.

DNA Markers from Bresatec

3MM paper: Whatman Ltd

Kodak Diagnostic film X-Omat AR, USA

Nitrocellulose (BA 85) and Nytran 0.45µm: Schleicher and Schuell

X-ray film: Fuji Photo Film Co. Ltd, Tokyo, Japan

QIAGEN columns from DIAGEN, Dusseldorf, FRG.

Poly(A) Quik® push columns from Stratagene.

2.2 General Methods

The following methods were performed essentially as described in Maniatis et al. (220);

Growth, maintenance and preservation of bacteria; quantitation of DNA and RNA;

autoradiography; agarose and polyacrylamide gel electrophoresis; DNA and RNA precipitations; phenol/chloroform extractions; end-filling or end-labelling of DNA fragments using the Klenow fragment of E. coli DNA polymerase I.

2.2.1 Plasmid DNA preparation.
The rapid alkaline hydrolysis procedure of Birnboim and Doly (25) was used for the isolation of plasmid DNA from 2 ml overnight cultures for analytical restriction digests. For larger amounts of plasmid DNA, the method was scaled up to accommodate 100 ml of culture. Plasmid DNA for use in transient transfection studies was prepared by the QIAGEN column procedure according to the manufacturers protocol. In brief, a modified alkaline lysis procedure was used to isolate DNA and cellular RNA, an anion-exchange column was then used to separate plasmid DNA from chromosomal DNA and cellular RNA. Following elution the plasmid DNA was concentrated by successive isopropanol and ethanol precipitations.

2.2.2. Cloning and subcloning techniques

2.2.2.1 Restriction enzyme digestions of DNA.

DNA (1-2μg) prepared as outlined in 2.2.1, was digested with 2 units of enzyme/restriction site/μg of DNA for 1-2 h using conditions specified by the manufacturer. "Super Duper" buffer consisting of 33 mM Tris-acetate pH 7.8, 62.5 mM potassium acetate (KAc), 10 mM magnesium acetate (MgAc), 4mM spermidine and 0.5 mM DTT proved suitable for efficient functioning of all restriction endonucleases.

2.2.2.2 Preparation of cloning vectors.

Plasmids were linearised with the appropriate restriction enzyme(s). To prevent self-ligation of the vector, 5' terminal phosphate groups were removed by incubation in 50mM Tris-HCl pH 9.0, 1mM MgCl₂, 0.1mM ZnCl₂, with 0.5 units of calf intestinal phosphatase (CIP), in a final volume of 50ml for 1 hr at 37°C. The vector DNA was isolated after electrophoresis on a 1.0% agarose TAE gel using a QIAEX DNA extraction kit according to the manufacturers instructions. The DNA was resuspended at a concentration of 20-50 ng/μl, for use in ligation reactions.

2.2.2.3 Preparation of DNA restriction fragments

DNA was incubated with the appropriate restriction enzyme(s) as described above (see 2.2.2.1) and restriction fragments were isolated from either a horizontal 0.8%-2.0%
agarose gel or a vertical 8% polyacrylamide gel, depending on the size of the DNA restriction fragment(s). Bands representing restriction fragments were visualised under UV light following staining with ethidium bromide, and the appropriate fragment(s) excised from the gel. DNA fragments from agarose gels were isolated using the QIAEX DNA extraction kit according to the manufacturers instructions. Fragments isolated from polyacrylamide gels were eluted from the gel slice by incubation in 400μl of 0.5M ammonium acetate, 0.1% SDS, at 37°C for 16 h. The DNA was precipitated by the addition of 2.5 volumes of 100% ethanol, washed in 70% ethanol, air dried and resuspended in 10-20 μl of 0.1mM EDTA.

2.2.2.4 Ligation of DNA fragments.
A 10μl reaction contained 20-50ng of vector DNA, the insert DNA, 50mM Tris-HCl pH 7.4, 10mM MgCl₂, 1mM DTT, 1mM ATP, and 1-2 units of T4 DNA ligase. For cloning into plasmid vectors, a 2-3 molar excess of restriction fragment insert to vector DNA was used. The reactions were incubated for either 4 hours at 26°C, or overnight at 4°C. A control ligation with vector only was set up and included in the subsequent transformation to determine background levels of uncut or recircularised vector DNA.

2.2.2.5 Transformation procedure of *E. coli* with recombinant plasmids.
A single colony of the *E. coli* host strain was inoculated into 5ml of L-broth (where appropriate the L-broth was supplemented with an antibiotic) and the culture incubated overnight at 37°C with continuous shaking. The overnight culture was then diluted 100 fold into 50ml of L-broth (plus antibiotic) and the incubation continued at 37°C, with shaking, until the culture reached an absorbance at A₆₀₀ of 0.6-0.8. The cells were then pelleted by centrifugation at 2,000 x g for 5 minutes, resuspended in 2.5ml of ice cold 200mM MgCl₂, 500mM CaCl₂ and left on ice for 60 minutes. 200μl of this cell suspension was mixed with 2-5μl of the DNA ligation reaction mix (see 2.2.2.4) and left on ice for 40 minutes. The cells were then heat shocked at 42°C for 2 minutes, L-broth containing 20mM glucose was added and the cells were incubated at 37°C for at 20-30 minutes. The transformed cells were then plated onto L-agar containing 50μg/ml of ampicillin by
spreading with a glass spreader. The agar plates were routinely incubated at 37°C overnight.

### 2.2.3 Dideoxy-chain sequencing analysis.

Single stranded template DNA (8μl) was annealed in 10mM Tris-HCl pH 8.0, 1mM MgCl₂ with 5-7 ng of the appropriate primer (1μl) in a final volume of 10μl. The mixture was heated at 95°C for 3 minutes, and incubated at 50°C for at least 15 minutes. Sequencing was performed by the Sanger (302) dideoxy method, using the sequencing reagents supplied in the Sequenase Version 2.0 sequencing kit. The sequencing reactions were performed in accordance with the protocol accompanying the kits.

Double stranded sequencing was performed using plasmid DNA purified by cesium chloride density gradient centrifugation. 2-4μg of plasmid was used per reaction. The DNA was denatured in 0.2M NaOH, 2mM EDTA for 15 minutes at 37°C. The mixture was then neutralised by the addition of 0.1 volumes of 3M sodium acetate pH 4.6, and the DNA precipitated with 3 volumes of ethanol. The DNA pellet was collected by centrifugation, washed in 70% ethanol, and resuspended in 7μl of 0.1mM EDTA. The sequencing reactions were carried out as for single stranded DNA, using a Sequenase® version 2.0 kit in accordance with the protocol accompanying the kit.

### 2.2.4 Gel electrophoresis of DNA for sequence analysis.

Sequencing reactions (1μl) were electrophoresed on 6% polyacrylamide gels containing 7M urea in 1 x TBE buffer at 1800 V. After electrophoresis, gels were transferred to Whatman 3MM paper and dried down. The gels were then autoradiographed for 4 to 16 hours at room temperature.

### 2.2.5 Preparation of [³²-P]-Labelled DNA probes.

#### 2.2.5.1 Oligo-Labelling of DNA.

In all experiments, a Pharmacia kit was used for the oligolabelling of recombinant plasmids. 0.1-0.5 μg of DNA was [³²-P] labelled in a 25μl reaction containing 100mM Tris-HCl pH 7.6, 20mM MgCl₂, 100mM NaCl, 200μg/ml BSA, 4μM each of unlabelled
dCTP, dGTP and dTTP, 100μCi each of \([\alpha^{32}\text{-P}]\) dATP, and 5.0 units of the large Klenow fragment of *E. coli* DNA polymerase I.

The reaction was incubated at 37°C for 30 minutes and then stopped by the addition of 5μl of 0.5 M EDTA, pH 8.0 and 5μl of 10% SDS. 10μl of tRNA (10 mg/ml), 50μl of NET buffer and 125μl of 4M ammonium acetate were then added and following the addition of 400μl of ethanol, the DNA was precipitated at either -80°C for 30 minutes or at -20°C overnight. The DNA was pelleted by centrifugation for 30 minutes at 12,000 x g, washed with 1 ml of ice-cold 70% ethanol, air dried, and resuspended in 388 μl of TE buffer.

Determination of TCA-precipitable radioactivity using 1μl aliquots demonstrated that over 90% of total radioactivity in the ethanol precipitate was TCA-precipitable. The specific activity of probes was generally 1-2x 10⁸ cpm/μg. Just prior to the addition of the probe to the hybridisation mix, NaOH was added to a final concentration of 300mM and the oligolabelled DNA was denatured by incubation at 100°C for 10 minutes. The probe was then snap cooled on ice and neutralised by the addition of an equal volume of 4M ammonium acetate.

### 2.2.5.2 5' End-labelling of synthetic DNA oligonucleotides.

The synthetic DNA oligonucleotides used as probes were \(^{32}\text{P}\) labelled at the 5' end using \(^{32}\text{P}\) -ATP and T4 polynucleotide kinase. The reaction mixture contained 10mM MgCl₂, 50mM Tris-HCl pH 7.4, 5mM DTT, 0.1mM spermidine, 0.1mM EDTA, 100 μCi \(^{32}\text{P}\) ATP and 2 units of T4 polynucleotide kinase in a final volume of 10μl. This was incubated at 37°C for 30 minutes. Following the addition of 10 μl formamide loading buffer, the reaction was run on a 20% polyacrylamide gel at 22 mA for 60 minutes to separate the \(^{32}\text{P}\)-labelled oligomer from unincorporated label. The labelled oligomer was localised by autoradiography, excised from the gel and eluted in TE buffer at 37°C for 16 h. This solution was then used directly for hybridisation.

### 2.2.6 Colony screening.
Colonies were picked onto three replica plates containing nylon membrane stamped with a numbered grid, and grown at 37°C overnight. One plate was used as the master plate and stored at 4°C. The nitrocellulose filters were removed from the other two plates and layered onto a sheet of Whatman 3MM paper saturated in 5% SDS/2x SSC for 5 min after which they were transferred to a microwave oven with a rotating turn table and treated for 2.5 minutes at full setting (650 watts) thereby lysing cells, denaturing and fixing the bacterial DNA to the membrane. The filters were prehybridised in 10ml of 5x SSPE, 5 x Denhardt's solution (Section 2.1.10), 1 % SDS, 0.05 % sodium pyrophosphate and 100 μg/ml of heat denatured sonicated salmon sperm DNA, at 42°C for 2 h. 5 ng of 32p-labelled primer, kinased as described in Section 2.2.5.2, was added per ml of prehybridisation mixture and incubated at 42°C for 16-24 h.

After hybridisation the filters were washed once in 200ml of 2 x SSC/0.1% SDS for 15 min at 60°C and then once in 0.2x SSC/0.1% SDS for 15 min at 60°C. The filters were autoradiographed overnight at -80°C. Positive colonies were identified by aligning the developed autoradiogram and the nylon filters on the master plate. Plasmid DNA was prepared from the putative positive colonies. The presence of the an insert was verified by restriction enzyme analysis.

2.2.7 Deletion analysis

The p920CAT construct containing a 920pb proximal promoter segment of CYP2H2 vector cloned into the PstI site of pBluescript KS⁺ (Stratagene, La Jolla, USA) was digested with KpnI and XhoI and progressive unidirectional deletions from the KpnI site (5'-end) were created using an erase-a-base kit (Promega, Madison, WI, USA). Promoter-CAT plasmids starting at -556, -198, -96 and -51 bp were generated. All constructs were verified by restriction mapping and DNA sequence analysis.

2.2.8 Southern analysis of DNA

A 6 μl sample of the DNA to be analysed was electrophoresed on a 1% agarose gel, the gel was then stained with ethidium bromide for 10 min and photographed under UV light in
the presence of a ruler to measure the migration of band. The gel was washed twice in 0.25 M HCl for 10 min then washed twice in a solution composed of 0.5 M NaOH and 1.5 M NaCl for 15 min. The gel was then washed in a solution containing 1 M ammonium acetate and 200 mM NaOH, before and after each wash the gel was rinsed briefly in water. The cDNA was transferred onto a Nytran filter using a vacuum-blotter for 1 h in a solution containing 1 M ammonium acetate and 20 mM NaOH. Following transfer the filter was placed nucleic side up on two pieces of Whatman 3MM moistened with 2x SSC. The cDNA was irreversibly bound onto the filter by UV-crosslinking using a UV-stratalinker 1800 (Stratagene). The filter was then placed in a pre-hybridisation solution containing 50 % formamide, 5 x Denhardt's solution, 0.5 % (w/v) Na pyrophosphate, 5 x SSPE, 0.1 % SDS and 200 µg/µl heat denatured salmon sperm DNA, for 2 heat 42 °C. The [α-32P]-labelled probe was then added to the pre-hybridisation solution and incubated at 42°C overnight. The filter was then washed in a solution containing 2 x SSC and 1 % SDS at room temperature for 10 min. After which the filter was washed at 65°C in a solution composed of 0.5 x SSC, 1 % SDS for 10 min. The filter was then placed nucleic acid side up onto paper towelettes to remove excess moisture, sealed in Vita-film and either autoradiographed for 5 days using X-OMAT AR X-ray film or placed in a phosphoimager screen for 3 days.

2.2.9 Method for cDNA synthesis
2.2.9.1 Preparation of total RNA
Total RNA is prepared from the PB induced livers of 18 day old chicken embryos by the method described in Chirgwin et al. (50). Briefly, 2g of chicken liver is removed into 20 ml guanidine solution (52) prior to homogenisation and addition of 0.1 g CsCl/ml of solution. This solution is then layered over 5.7 M CsCl prior to centrifugation at 113000xg at 22°C for 12 h. The RNA pellet is washed prior to dissolution in DEPC treated MQ water.

2.2.9.2 Preparation of polyA⁺ RNA
Total RNA (2 mg) from 2.2.9.1 is prepared by heat denaturing at 65°C for 5 min and by adding and equal volume of 10 x sample buffer (10 mM Tris-HCL (pH 7.5), 1 mM EDTA, 5M NaCl). The oligo dT cellulose column is prepared by rinsing twice with 200µl of high salt buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5 M NaCl). The RNA sample is applied to the column, the RNA eluant collected and reapplied to the column. The column is washed twice with 200 µl of high salt buffer and then washed three times with 200 µl of low salt buffer (0.1 M NaCl). The mRNA is eluted from the column with four 200 µl aliquots of preheated (65°C) TE buffer.

2.2.9.3 cDNA synthesis

Initially 1.1 µl of 0.1 M methyl mercury was added to 10 µg of polyA+ in 10 µl of H2O and incubated at RT for 10 min. The following were then added: 6µl of 5x RT buffer, 2 µl of 0.7 M mercaptoethanol and incubated for 5 min at RT then 4.9 µl of H2O, 100 ng of cDNA synthesis primer, 60 U of RNase inhibitor, 3 µl of 10 mM dNTP mix and 100 U of MMLV reverse transcriptase were added and incubated at 30°C for 1 h. The RNA was then hydrolysed by the addition of 2 of 6M NaOH and incubating at 65°C for 30 min prior to neutralisation by 2 µl of 6M acetic acid. cDNA was purified using a QIAEX Kit (Qiagen, Germany). Alterations to this standard procedure are described in Chapter 6.

2.2.9.4 PCR amplification of cDNA

PCR amplification was preformed using a Perkin Elmer Thermocycler. 5 ml of cDNA from 2.2.9.3, 100 ng of each amplification primer, 4 µl of 5mM dNTP mix 5 µl of 10x PCR buffer 2.5 U Taq DNA polymerase in a final volume of 50 µl was incubated at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min for 30 cycles. 10x PCR buffer contains 100 mM Tris-HCL (pH8.8), 15 mM MgCl2, 500 mM KCl and 0.01% (w/v) gelatin. Alterations to this standard procedure are described in Chapter 6.

2.2.10 Methods for isolation and analysis of RNA

2.2.10.1 Preparation of total RNA from chicken liver tissue.
Total RNA was extracted from 2.5 g of tissue using the guanidinium isothiocyanate procedure described by Chomczynski and Sacchi (52), modified in the following way. Firstly, the volumes used in the published extraction procedure were scaled up. Secondly, when RNA is prepared from liver, glycogen tends to co-purify with the RNA and this was removed by precipitation of the RNA with 3 volumes of 4M Na acetate at 0°C overnight. The RNA was recovered by centrifugation at 8000 x g for 15 minutes at 4°C, and resuspended in 0.1mM EDTA.

2.2.10.2 Northern hybridisation analysis of RNA.

Northern hybridisation analysis of total RNA was carried out by denaturation on 1% agarose gels containing 1.1M formaldehyde, and transfer onto either BA85 nitrocellulose or Nytran filters (Schliecher and Schuell). Following transfer, the filters were irradiated with 120 μjoules of UV radiation in a Stratagene UV Stratalinker-1800 which results in the RNA being covalently crosslinked to the filter (manufacturer's instruction manual). Filters were pre-hybridised for 4-16 hours at 42°C in 50% formamide, 5 x SSC, 5 x Denhardt's solution, 0.1% SDS, 0.05% sodium pyrophosphate, and 200 μg/ml of sonicated salmon sperm DNA. Hybridisations were carried out for 18-24 h under exactly the same conditions, except for the addition of radiolabelled probe (1-5 x 10^8 cpm/μg). Filters were washed in 2 x SSC, 0.1% SDS at room temperature for 5 and 20 minutes, followed by one wash in 2 x SSC, 0.1% SDS at 60°C for 40 minutes.

2.2.11 Transient expression of recombinant DNA in chick embryo hepatocytes

2.2.11.1 Preparation of chicken embryo primary hepatocyte cultures

Hepatocytes were prepared from 17-18 day old chicken embryos by the method of Giger and Meyer (99). In brief, chick embryos were removed from the egg, decapitated and dissected to expose the heart and liver. The liver was perfused, via cannulation of the heart, with 10 ml of 0.9% NaCl containing 2mM EDTA to remove blood cells followed by 4 ml of 0.05% collagenase in Hank's balanced salts solution (HBSS) to initiate digestion of intracellular collagen. Each liver was then removed and placed into HBSS until all the livers had been perfused and collected. The pooled livers were transferred into 0.05%
collagenase-HBSS (2 ml/liver) and incubated at 37°C for 20 min. The livers were then transferred to fresh 0.05% collagenase-HBSS, cut into small pieces and incubated at 37°C for 30 min with gentle shaking and pipetting. Collagenase was removed by washing with HBSS. Contaminating erythrocytes were lysed in an ammonium chloride solution. Following two washes with William's E medium to remove cell debris and haemoglobin from lysed erythrocytes, the hepatocytes were resuspended in William's E medium (1 ml/liver) and the yield determined. Typically 1x 10⁷ cells/liver were obtained. All of the above procedures were performed aseptically using sterile solutions and instruments, and working within a biohazard safety hood.

2.2.11.2 Transfection of hepatocytes and cultivation conditions

Transfection of DNA into chick embryo primary hepatocytes was performed by electroporation. Hepatocytes were resuspended in ice cold 20 mM HEPES pH 7.4 containing 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄ and 6 mM dextrose (109) at a cell density of 2.5 x 10⁷ cells/ml. Sheared salmon sperm DNA was added as carrier to a final concentration of 500 µg/ml (salmon sperm DNA was made as a 10 mg/ml solution which was sheared by passage three times through a French Pressure Cell at 12,000 pounds per square inch). Hepatocytes (2 x 10⁷) and 1.0 pmol of construct DNA (ie approx. 4 µg of 6 kb plasmid) unless otherwise stated were electroporated at 250 V, 960 µF using a Bio-Rad Gene Pulser with Capacitance Extender. Following electroporation, samples were placed on ice for 10 min. Each sample was split so that approximately 1 x 10⁷ cells were transferred to each of two 60 mm diameter petri dishes containing 5 ml of William's E medium supplemented with Serum Supreme (BioWhittaker, USA) to a final concentration of 10% and containing 40 µg/ml gentamicin. Serum Supreme is bovine serum derived, the contents of which are not released by the supplier. In early experiments, Nu serum (Flow Laboratories, USA) at 10% was used instead of Serum Supreme. Hepatocytes were allowed to adhere to the petri dish and to recover at 37°C, under a CO₂/air mix (5:95) for 16 h. The media was changed to remove dead and non-adherent cells and the remaining healthy cells were treated as follows. To one of each pair of plates was added an
appropriate amount of drug stock solution and to the other, an equivalent volume of solvent only ("control"). The cultures were further incubated for 48 h prior to harvesting.

2.2.11.3. Harvesting of chick embryo primary hepatocytes

Culture media was decanted and replaced with 1 ml of 40 mM Tris-HCl pH 7.5 containing 1 mM EDTA and 150 mM NaCl. The cells were dislodged by scraping with a rubber policeman, transferred into an eppendorf tube and pelleted in an Eppendorf Microfuge for 10 seconds. The supernatant was then removed and the pelleted cells were stored at -80°C until required.

2.2.11.4 Assay for chloramphenicol acetyltransferase (CAT) activity

Cells were harvested and lysed as described above. To the cell lysate was added EDTA to a concentration of 5 mM prior to incubation at 65°C for 10 minutes and then centrifugation for 5 minutes to remove deacetylase activity. CAT activity in the supernatant was then assayed by the procedure of Gorman et. al. (105) was utilised. To 130 ml of 250 mM Tris-HCl, pH 7.6 containing 20-100 mg of protein was added 10 ml of 10 mM acetyl-coenzyme A, 39 ml of water and 1 ml of [14C] chloramphenicol. The reaction mixture was incubated at 37°C for 1 h after which 10 ml of acetyl-coenzyme A was added and the incubation continued for another hour. To stop the reaction, 1 ml of ethyl acetate was added and the solution vortexed to extract the chloramphenicol. The upper, organic phase was transferred to a clean Eppendorf tube and the ethyl acetate was evaporated. The residue was dissolved in 10 ml of ethyl acetate and spotted onto silica plates (Merck). Acetylated [14C] chloramphenicol was resolved by thin layer chromatography in a solvent of chloroform:methanol (9.5:0.5 v/v). The silica plates were air dried and autoradiographed at -80°C for 16 hours. After autoradiography, the spots corresponding to acetylated chloramphenicol were cut out and the amount of radioactivity quantitated by liquid scintillation counting. CAT activity was expressed as the amount of chloramphenicol acetylated by 1 mg of protein extract in one hour. To normalise for equal transfection efficiency, a correction factor was determined by adjusting the β-galactosidase activities to
1 unit of enzyme activity defined as: \((\text{OD}_{420}/\text{mg protein/hour}) \times 100 = 1.00\). CAT activities were then corrected by an equivalent factor.

2.2.11.5 Assay for \(\beta\)-galactosidase activity

\(\beta\)-galactosidase activity was determined by the method of Herbomel et al (137) on freeze/thawed cell lysates. It was important not to heat the cell lysates at \(65^\circ\text{C}\) for 10 min. as was done for CAT activity determinations, as this denatures \(\beta\)-galactosidase and destroys its activity.

2.2.11.6 Bradford protein assay

The protein content of the cell extracts was determined using 5ml of cell extract and the BIO-Rad protein microassay procedure according to the manufacturer's instructions. Bovine serum albumin was used as the protein standard.

2.2.12 Methods for gel shift assays

2.2.12.1 Preparation of nuclear protein extract

Nuclei from cells were isolated by a modification of the procedure described by Schreiber et al. (309). Typically \(10^6\) cells from tissue culture or 0.5g of homogenised liver tissue was collected, washed in 10ml of PBS and pelleted by centrifugation at 1500 \(\times\) g for 5 minutes. The pellet was resuspended in 1ml of PBS, transferred into an Eppendorf tube and pelleted again by spinning for 10 sec in a microfuge. The PBS was removed and the cell pellet was resuspended in 400ml of ice cold lysis buffer (10mM Tris-Cl, pH7.9, 10mM KCl, 1mM DTT, 1.5mM MgCl\(_2\), 0.5% NaP0\(_4\)) by vortexing for 10 sec. The cells were allowed to swell and lyse on ice for 15 minutes. Nuclei were then pelleted by centrifugation for 1 minute and washed in 400ml of cold buffer A (10mM Tris-Cl, pH7.9, 10mM KCl, 1mM DTT, 1.5mM MgCl\(_2\)). The nuclei pellet was then resuspended in 400ml of cold buffer B (50mM Tris-Cl, pH7.5, 10% sucrose, 0.5M KCl, 5mM MgCl\(_2\), 0.1mM EDTA, 20% glycerol, 2mM DTT). Nuclear proteins were extracted by constant agitation for 1 hour at 4°C. Following centrifugation at 4°C for 15 minutes the supernatant was dialysed against two changes of TM-1 buffer (25mM Tris-Cl, pH7.6, 5mM MgCl\(_2\),
0.5mM EDTA, 0.5mM DTT, 10% glycerol) containing 100mM KCl. The nuclear extract was centrifuged for 5 minutes in a microfuge at 4°C and the supernatant frozen in aliquots at -80°C. Protein concentration was determined using the Bradford protein assay (Bio-Rad) as described in Section 2.2.11.6.

All buffers were supplemented with a cocktail of phosphatase and protease inhibitors consisting of a 1/100 dilution of the following: leupeptin (2mg/ml), aprotinin (1mg/ml), pepstatin A (10mg/ml), benzamide (50mM), bestatin (5mg/ml), β-glycerol phosphate (1M) and PMSF (10mM). Inhibitors were diluted and added to buffers just before use.

2.2.12.2 Preparation of radiolabelled annealed oligonucleotide probes
To generate a radiolabelled probe for gel shift assays, one of the complementary oligonucleotides was end-labelled with [γ-32P]-dATP and polynucleotide kinase as described in Section 2.2.10 and purified from a 20% polyacrylamide gel. To anneal the complementary oligonucleotide, 10ng of 32P-labelled oligonucleotide was combined with 100ng of unlabelled complementary oligonucleotide in 25mM Tris-Cl, pH7.6/100mM NaCl. The mixture was heated to 100°C for 3 minutes followed by 70°C for 10 minutes and then cooled to room temperature for 45 minutes. Unlabelled oligonucleotides were also annealed as described above to give a final concentration of 10ng/ml. These were used as specific competitors in the binding reactions.

2.2.12.3 Gel shift assay
Binding reactions contained 0.1ng of radiolabelled probe (5000-10000cpm) and 5-15 g of nuclear protein extract in a final volume of 20ml containing 25mM Tris-HCl, pH 7.6, 6.25mM MgCl2, 0.5mM EDTA, 0.5mM DTT, 10% (v/v) glycerol and 50-150mM KCl. Non-specific competitor DNA, poly(dI/dC) (0.5-3 µg), was used in all reactions. Specific competitors were added to each reaction mixture as described in individual experiments. The reaction mixtures were analysed on 5-10% polyacrylamide gels in low ionic strength buffers, typically either 0.5 x TBE or Tris-glycine, pH8.5. The gels were pre-electrophoresed at 350-500 Volts for 1 hour and electrophoresed at the same voltage for 1-
2 hours at 4°C. Following electrophoresis, the gels were dried and autoradiographed either for 2-4 hours or overnight.

2.2.13 DNaseI in vitro footprinting assay

2.2.13.1 Preparation of nuclear extracts

Nuclear extracts were prepared by the methods of Gorski et al. (106). Nuclear extract used in the footprinting the CYP2H1 enhancer (chapter 3) was prepared from the livers of 8 week-old chickens that were untreated or injected IP with phenobarbital (40 mg/kg in 0.5 ml dimethyl sulfoxide) in the morning and evening the day before sacrifice. Nuclear extract used in the footprinting of the CYP2H2 promoter (chapter 4) was prepared from the livers of 18 day-old chick embryos that were untreated or injected through the shell onto the allantoic membrane with phenobarbital (40 mg/kg in 0.5 ml dimethyl sulfoxide) 6 hours before sacrifice. Extracts prepared from 8 week-old chickens performed identically to those prepared from chick embryo hepatocytes in preliminary experiments. The latter were used due to their ease of preparation.

2.2.13.2 DNaseI footprinting

For footprinting, a Sau3A/PstI fragment from p198CAT that contained 198 bp of promoter and 39 bp of 5' untranslated region of the CYP2H2 gene was cloned into the corresponding sites in the vector pBluescript KS+ (Stratagene, La Jolla, USA) and digested with KpnI/XbaI or PstI/EcoRI. To radiolabel the coding or noncoding strands of these fragments, the XbaI and EcoRI ends were end-filled with [α\(^{32}\)P]dATP and [α\(^{32}\)P]dCTP using Klenow and the radiolabelled fragments purified by polyacrylamide gel electrophoresis. The DNase I footprinting reaction consisted of the following components in a final volume of 50 μl; 20 mM HEPES (pH 7.9) with 60 mM KCl, 60 mM EDTA, 0.6 mM dithiothreitol, 2 mM spermidine, 10% glycerol, 2 μg poly(dI-dC), and 40 μg of nuclear protein extract from chicken liver. After incubation on ice for 10 min, the probe (25000 cpm) was added and incubation continued at 25°C for an additional 20 min. DNase I digestion and purification of the DNA was adapted from the method of Cereghini et al (45). The DNA products were analyzed on an 8% polyacrylamide sequencing gel.
Fragments partially cleaved by G+A reactions of the Maxam and Gilbert sequencing procedure (222) were run as markers. The DNase I digestion pattern in the absence of nuclear extracts was obtained using one tenth as much DNase I in the reaction as in the presence of nuclear extract (0.05-0.1 units).

2.2.14 Site directed mutagenesis

Site directed mutagenesis was carried out using the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, USA). Briefly, two complimentary oligonucleotides containing the desired mutation were synthesised and gel purified (220). Reactions containing 50 ng dsDNA template, 125 ng of each oligonucleotide, 1 µl of 5mM dNTP mix, 5 µl of 10x reaction buffer 1 µl of PfuTurbo DNA polymerase in a final volume of 50 µl were incubated in a Perkin-Elmer PCR machine at 95°C for 30 sec, 55°C for 60 sec and 68°C for 2 min/kb plasmid length for 18 cycles. Samples were then digested with DpnI at 37°C for 1 h and then transformation into DH5α by electroporation at 1700 V, 960Ω and 25µF prior to selection on ampicillin containing LB agar plates. All mutations were verified by DNA sequence and/or restriction analysis.

2.2.15 Construction of an MMTV luciferase reporter construct

The plasmid pMSG (Pharmacia) that contains the mouse mammary tumor virus long terminal repeat (MMTV LTR) was digested with HindIII and Smal, the 1.5 kb MMTV HindIII/Smal fragment purified and cloned into pBluescript. This construct was then digested with KpnI and Smal to release MMTV which was again purified before being cloned into the multiple cloning site of pGL3-Basic vector (Promega, USA).

2.2.16 Miscellaneous methods

2.2.16.1 Densitometric quantitation of bands on autoradiographs

Quantitations were performed on phosphorimager files using the ImageQuant Software.

2.2.16.2 Computer programs
Sequences were screened using the Sequence Analysis Software Package from Genetics Computing Group of the University of Wisconsin located at http://www.angis.org.au/WebANGIS (39). Putative transcription factor binding sites were located using the National Institutes of Health “TRANSFAC” data base at http://bimas.cit.nih.gov/molbio/signal/index.html (365).
Chapter 3. Characterisation of a drug responsive enhancer from the \textit{CYP2H1} gene
3.1 Introduction

The PB-inducible P450s constitute the largest family within the P450 gene superfamily. The mechanism by which PB and other drugs induce transcription of the PB-inducible P450 genes is currently under intensive investigation in several laboratories. The PB class of inducers are hydrophobic but exhibit no obvious structural similarity and so it is of great interest to know how this diverse range of compounds induce the transcription of the same genes. In this laboratory we are studying the expression of the avian PB-inducible P450s using the chick embryo as a model system. This system is attractive since treatment of the embryo in ovo with drugs such as PB, 2-allyl-2-isopropylacetamide (AIA) and 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) results in a dramatic induction of hepatic P450 gene transcription and also similar levels of induction are observed with a chicken embryo primary hepatocyte culture system derived from chick embryo livers (119).

Previously in this laboratory, two chicken P450 cDNA clones were isolated from a library prepared using RNA from AIA/DDC-induced chicken embryo livers (33, 140). These cDNA clones, designated pCHP3 and pCHP7, were approximately 2.7 and 2.2 kb in length respectively and represented two separate mRNA species. These cDNA clones show 95% nucleotide sequence similarity in the coding region. While their 5' non-coding regions are identical in length and sequence, their 3' non-coding regions diverge markedly, with only 41% sequence similarity (125). When pCHP3 was employed as a probe for Northern blot analysis of PB-induced chick embryo liver RNA, it was shown to hybridise to three P450 mRNA species of approximately 3.5, 2.5 and 2.2 kb in size (Fig. 3.1, lane 2). Northern blot analysis using probes that correspond to the divergent 3' non-coding regions of pCHP3 and pCHP7 demonstrated that pCHP3 was derived from the 3.5 kb mRNA species while pCHP7 was derived from the 2.2 kb mRNA species. The 2.5 kb mRNA species did not hybridise with either of these specific probes (Fig. 3.1). The pCHP3 clone was employed as a probe to screen a chicken genomic lambda phage library and resulted in the isolation of two genes designated \textit{CYP2HI} and \textit{CYP2H2}. Southern blot analysis established that the \textit{CYP2HI} gene encodes the 3.5 kb mRNA while the 2.2 kb mRNA is transcribed from the
Figure 3.1  Comparison of phenobarbital-inducible CYP mRNAs in chick embryo liver.

Three 17 day old chick embryos were treated with 6 mg of phenobarbital in 100 µl of DMSO by injection through the allantoic membrane and into the fluid surrounding the embryo. Three control embryos received an equal volume of DMSO. After 4 h, the embryos were decapitated, the livers removed and pooled. Total RNA was isolated and 20 µg electrophoresed on a 1% agarose-formaldehyde gel. The RNA was transferred to nitrocellulose and probed for CYP mRNAs with nick-translated pCHP3 (lane 1, control; lane 2, phenobarbital-treated), or probes corresponding to the divergent 3’ regions of CYP2H1 (lane 3, phenobarbital-treated) or CYP2H2 (lane 4, phenobarbital-treated). Molecular weight markers were generated by digestion of pBR322 with AccI and HincII (not shown).
CYP2H2 gene (221). The origin of the 2.5 kb mRNA is unknown but is possibly an alternate splice product transcribed from either the CYP2H1 or CYP2H2 gene.

Isolation of the CYP2H1 gene has lead to its analysis for tissue-specific and drug responsive expression in chick embryo hepatocyte cultures. Promoter studies of the CYP2H1 gene were carried out using reporter gene fusion constructs containing various lengths of the CYP2H1 gene 5'-flanking region fused to the CAT gene (119). In transient transfection assays the first 205 bp of this promoter produced a strong basal level of activity, but was not phenobarbital inducible. This high basal level of expression was also observed when 8.9 kb of 5'-flanking region was used indicating that the first 205 bp of the promoter contains all of the functional elements required for basal expression (119). Several putative cis-acting DNA regulatory elements are located in this region including a consensus TATA box and a number of potential binding sites for liver specific factors including C/EBP, HNF-1 and HNF-3 (94, 378).

The high basal expression observed from the 205 bp promoter construct in transient transfection assays contrasts markedly with the low level of transcription normally observed for the endogenous genes in the absence of drug (Fig. 3.1, lane 1). A distal region located at -5.9/-1.1 kb was identified as a drug responsive domain (119). This domain produced a 6-8 fold level of drug induction when fused to the weak enhancerless SV40 promoter. However, only a weak induction response is observed with the entire 8.9 kb 5' flanking region including the early promoter. This is presumably due to the high basal expression driven by the early promoter. It is therefore proposed that the CYP2H1 gene is normally repressed and that drug relieves this repression (Fig. 1.9). Drug action may result in an increase in the affinity of binding factors at the drug-responsive enhancer region possibly through phosphorylation and this overcomes repression. A second model of repression, favoured by this laboratory, involves nucleosome assembly on the promoter and enhancer. Perhaps in the presence of drug, nucleosomes are removed from the enhancer upon binding of a drug-receptor complex, thus allowing the binding of transcription factors that in turn result in the loss of a nucleosome at the promoter through
recruitment of a coactivator with HAT (histone acetyltransferase) activity (34). In this model, derepression of the strong proximal promoter is chiefly responsible for the induced CYP2HI gene transcription rate.

An important issue concerns the role of the upstream enhancer domain of the CYP2HI gene in the induction mechanism and its relationship to the reported rodent enhancers (145, 147, 262, 333). In this chapter, the CYP2HI enhancer domain was analysed and a region that responds to phenobarbital identified. The transcription factors that bind to this region were characterised by in vitro footprinting and gel shift analysis and the implications of these findings on the mechanism of phenobarbital induction of CYP genes in eukaryotes are discussed.

The work described in this chapter has been presented in reference 73. The deletion and expression studies presented in section 3.2.1 and the DNase I footprint analysis presented in section 3.2.2 were carried out in conjunction with Dr. Satish Dogra.

3.2 Results

3.2.1 Progressive deletion of the CYP2HI 5' flanking region

In a previous study, CAT reporter gene constructs containing 0.5 to 8.9 kb of 5'-flanking sequence of the chicken CYP2HI gene were transiently expressed in chick embryo hepatocytes (119). Maximal phenobarbital induction was observed with 8.9 kb of 5'-flanking sequence but inducibility was lost when this was reduced to 1.1 kb (119). Removal of a 4.8 kb BamHI fragment (-5900 to -1100) from within the 8.9 kb sequence completely eliminated the drug response. This 4.8 kb fragment was subsequently shown to behave as a drug responsive enhancer and to markedly increase the expression of the weak enhancerless SV40 promoter in transfected chick embryo hepatocytes (119). To locate the phenobarbital responsive region in the 4.8 kb BamHI enhancer domain, BamHI (-5900 to -1100), BglII/XhoI (-1956 to -1400), BglII/Stul (-1956 to -1640) and Stul/XhoI (-1640 to -1400) fragments were blunt ended and cloned in the EcoRV site of the pBCSVp1 vector.
Figure 3.2  **Deletion analysis of the 4.8 kb enhancer domain**

Plasmids with various lengths of enhancer from 4.8 kb to 0.8 kb fused to the enhancerless SV40 promoter and a CAT reporter (SVCAT), were transfected into chick embryo hepatocytes. p-SVCAT lacks enhancer sequence. p556-SVCAT contains 556 bp enhancer sequence from the 3' end of the 4.8kb enhancer in the 5'→3' and pR556-SVCAT in the 3'→5' orientation. Following transfection, each sample of the transfected hepatocytes was halved and phenobarbital at 500 µM added to one dish (PB-induced) and PBS to the other control dish. CAT activities were determined in cell lysates (50 µg of protein) after 48 h and expressed as a percentage conversion of [14C]chloramphenicol to acetylated products. Values are the average of three independent experiments ± SD. The fold induction is shown in brackets.
Control
PB-induced

CAT Activity (% conversion)
This vector has been described (54) and contains the SV40 enhancerless promoter fused to the CAT gene. The chimeric enhancer-pBCSVp1 vectors, containing the 4.8 kb BamHI fragment in the forward orientation (p4.8-SVCAT) was used to generate enhancer deletion constructs. This vector was digested with KpnI and SalI and progressive unidirectional deletions from the SalI end were created. Deletion constructs from p4.8-SVCAT retained 4.1, 3.1, 1.9 and 0.8 kb of the 4.8 kb enhancer in the pBCSVp1 vector. 5'-End deletions of the 4.8 kb enhancer fused to the SV40 promoter/CAT reporter plasmid were introduced into chick embryo hepatocytes

From the 4.8 kb domain other constructs were prepared containing restriction enzyme fragments: pR1-SVCAT vector (BamHI/XbaI), pR2-SVCAT (XbaI/XbaI) and pR3-SVCAT (BglII/BglII) as shown in Fig 3.3A. These fragments were blunt ended and cloned into the EcoRV site of pBCSVp1 vector upstream of SVCAT. All constructs were verified by restriction mapping and DNA sequence analysis. As shown in Fig 3.2, progressive deletions from -4.8 to 0.8 kb (ie. -5900 to -1900) did not substantially alter basal levels of CAT activity but drug induction was reduced from about 7-fold (p4.8-SVCAT) to a final level of about 2-fold (p0.8-SVCAT). A 556 bp BglII/XhoI restriction enzyme fragment located near the 3' end of the 4.8 kb enhancer (-19561/-1400) was also tested. This fragment (p556-SVCAT) conferred about a 3-fold increase in the level of drug induction and a similar result was obtained with the fragment in the reverse orientation in pR556-SVCAT (Fig 3.2).

Phenobarbital induction levels in the chick embryo hepatocytes were found to be increased by replacement of Nu serum in the culture media with Serum Supreme, a bovine derived serum, and under these conditions the induction by the 556 bp fragment (pR556-SVCAT) was elevated from 3-fold (Fig 3.2) to 6.7-fold (Fig. 3.3B). Serum Supreme was employed in all subsequent experiments. Three restriction enzyme fragments located within the 4.8 kb enhancer and designated R1, R2 and R3 were fused to SVCAT and analysed by transient transfection analysis (Fig. 3.3A). The fragment R1 (-5900/-4550 in pR1-SVCAT) induced CAT activity by 5.8 fold whereas the other two fragments R2 (-4550/-3760) and
Figure 3.3  Analysis of DNA fragments from the 4.8 kp enhancer and deletion analysis of the 556 bp sequence

A. Plasmids with restriction enzyme fragments from the 4.8 kb enhancer (R1, R2 and R3) fused to the enhancerless SV40 promoter and a CAT reporter (SVCAT), were transfected into chick embryo hepatocytes and tested as described in Fig. 3.1.

B. Unidirectional deletions were generated in the 240 bp region of pR556-SVCAT. Plasmids with various lengths fused to the enhancerless SV40 promoter and CAT reporter (SVCAT) were tested as in Fig. 3.1. The 240 bp fragment alone, cloned in pSVCAT in both the forward (→) and reverse (←) orientations was also tested for drug response. CAT activities determined in cell extracts (100 µg protein) were expressed as fold increase in CAT activity following phenobarbital treatment and are an average of three independent experiments ± SD.
**4.8 kb Enhancer**

![Bar chart showing CAT activity](Diagram A)

**Relative CAT Activity**

![Bar chart showing relative CAT activity](Diagram B)
R3 (-3260/-2480) did not show any response to phenobarbital. These results demonstrated that there are two independent drug responsive regions in the 4.8 kb enhancer domain. By comparison with the deletion data in Fig. 3.2, it appears that the phenobarbital response observed with p3.1-SVCAT and p1.9-SVCAT is mainly contributed by the 556 bp enhancer region in p556-SVCAT.

To further narrow down the drug responsive elements in the 556 bp enhancer region, two contiguous fragments were isolated, a 316 bp BgIll/StuI fragment (-1956 to -1640) and a 240 bp StuI/XhoI fragment (-1640 to -1400) and each inserted into the expression plasmid pBCSVp1 in both orientations. While the 556 bp region gave a 6.7-fold level of drug induction, the 240 bp fragment in either orientation resulted in a 2 to 3-fold increase (Fig. 3.3B, last two constructs) and the 316 bp fragment did not respond to phenobarbital in either orientation (data not shown). Hence the 240 bp fragment contains sequence(s) responsive to phenobarbital while other sequence(s) in the 316 bp fragment stimulates the level of this response.

The 240 bp fragment within the 556 bp enhancer was more precisely mapped by 5' deletion analysis using the pR556-SVCAT plasmid (Fig. 3.3B). This vector was digested with KpnI and SalI and progressive unidirectional deletions from the SalI end were created. Deletion constructs generated from pR556-SVCAT retained 205, 158, 141, 80 and 15 bp of the 240 bp enhancer region. These fragments were blunt ended and cloned into the EcoRV site of pBCSVp1 vector upstream of SVCAT. All constructs were verified by restriction mapping and DNA sequence analysis. Deletion of sequence from -1400 to -1435 bp did not affect the response to phenobarbital (data not shown) but deleting sequence to -1482 resulted in a lowered level of induction (6.7 to 4.5-fold). Reduction to -1499 further lowered induced activity (to 3.0-fold) while continued deletion to -1560 substantially lowered induction. Essentially no induction was observed with the -1625 deletion construct. This data indicated that multiple regulatory elements located from -1435 to -1625 contribute to drug responsiveness. Computer sequence analysis of the 240 bp fragment revealed a number of possible binding sites: a CCAAT-box binding site at -208/-204 for either C/EBP (193) or
nuclear factor 1 (NF1) (51), an Sp1 site at -186/-181, two AP1 sites at -142/-136 and -43/-37, an H4TF2 site (63) at -119/-115, a site for the liver enriched hepatocyte nuclear factor (HNF5) (111) at -96/-90, and two E-box like elements (194) at -47/-42 and -38/-33 (Fig. 3.4). No sequence was identified with similarity to the Barbie box element implicated in the phenobarbital mechanism in bacteria (131).

3.2.2 DNase I footprint analysis of the 240 bp fragment
Protein binding sites in the 240 bp fragment were investigated by *in vitro* DNase I footprint analysis using nuclear extracts prepared from the livers of phenobarbital treated and control chickens. The *SstI/XhoI* 240 bp fragment (-1640 to -1400) was blunt ended and cloned in both orientations into the *EcoRV* site of pBCSV p1. For footprinting, *KpnI/EcoRI* fragments, representing the 5'→3' and 3'→5' directions of the 240 bp fragment were obtained. Four separate protected regions (A-D) were detected on both strands (Fig. 3.5A). While region A (-60 to -43) was very weakly protected with control nuclear extracts, strong protection was observed with extracts from drug treated livers. This region contained one of the two E-box like sequences (see Fig. 3.4). Region B (-104 to -80) was protected by nuclear extracts from both phenobarbital treated and control livers and as shown in Fig. 3.4 encompassed a possible binding site for HNF5 (111). Footprint C (-160 to -148), as with footprint A, was substantially stronger with extracts from phenobarbital treated liver. Computer sequence analysis, however, did not reveal a binding site in region C for any known transcription factor. The protection of footprint D (-208 to -197) was slightly greater with extracts from drug treated livers and this region contained a possible CCAAT box motif. It was noted that with nuclear extracts from drug treated livers, there was no extension of the footprint pattern on any of the regions compared with control extracts. No footprint was detected over the putative Sp1, AP1 or H4TF2 sites.

3.2.3 Functional role of the protein binding sites
To evaluate the function of the protein binding sites identified from footprinting the 240 bp fragment, the sites were mutated singly or in combination in pR556-SVcat (Fig. 3.6). The 556 bp enhancer fragment was blunt ended and cloned into the pBluescript KS+ vector.
Figure 3.4  DNA sequence of the 240 bp Fragment

Protein binding sites as predicted by computer analysis are shown in bold reverse text (white) and numbered arbitrarily from the 5'-end of the fragment in its native orientation: C/EBP/NF1 (-208/-204), Sp1 (-186/-181), AP1 (-142/-136) and (-43/-37), H4TF2 (-119/-115), HNF-5 (-96/-90), and two E-box like binding sites (-47/-42) and (-38/-33). The four footprinted sequences protected from DNase I cleavage are boxed and marked as A-D.
-240  GCCGAACTT CCTGCCCTT TCAGAGACCG

-190  TCGACGCCCT AAAATGACTG GAGGCCAGTT

-140  ACTCAACTGA GTTGTGTTTT GCTTCTGGG AGTTTGAGACA CAAAATTTTA

-90   ATCAACCTTT TTTGCTGGGC TGGTTAATG

-40   ATTCACCTCG AAAAAATGAC AAGTTTGTG ACTGATCTCG

C/EBP/NF1  D

Sp1

-190  TCGACGCCCT AAAATGACTG GAGGCCAGTT

C

-140  ACTCAACTGA GTTGTGTTTT GCTTCTGGG AGTTTGAGACA CAAAATTTTA

HNF5

B

E-box

AP1

E-box

AP1

E-box
Figure 3.5  DNase I footprint analysis of the 240 bp fragment

The 240 bp fragment was radiolabeled on the sense and antisense strands and footprint analysis performed. The radiolabeled probe was incubated with liver nuclear extracts from control (lane 3) or phenobarbital treated (lane 4) chickens or without nuclear extract addition (lane 2). The fragment was partially cleaved at G and A residues as a marker for the sequence (lane 1). The DNase I protected regions (A-D) are bracketed
in the EcoRV site in the reverse (XhoI/BglII) orientation and this construct (pR556-KS) was used as a template for site-directed mutagenesis. Mutations were introduced in the DNase I footprinted regions and confirmed by sequencing. The mutated fragments were then released from pR556-KS by digestion with KpnI/Smal and cloned into pBCSVp1 at the corresponding sites to generate mutated enhancer-CAT plasmids. The expression of CAT activity by wild type pR556-SVCAT was increased 6.9-fold in the presence of phenobarbital. Mutagenesis of the E-box motif in footprint region A (5'-CACCTG-3' to 5'-GAGCTC-3') substantially decreased enhancer activity to 3.1-fold. Footprint region B was mutated at two different sites. The first mutation located at the 3' end of the footprint (5'-ACCAAA-3' to 5'-GAGCTC-3') did not affect enhancer activity (result not shown). A second mutation (5'-ACAAAT-3' to 5'-CTGCAG-3') which altered the first base of the putative HNF5 binding site (T to G) significantly lowered enhancer activity to 2.8-fold (Fig. 3.6). Mutagenesis of footprint region C (5'-GTCAGT-3' to 5'-GAATTC-3') also decreased enhancer activity to 2.9-fold while alteration of footprint region D sequence (5'-AGCCAA-3' to 5'-TCTAGA-3') encompassing the putative C/EBP/NF1 site only moderately reduced enhancer activity (5.2-fold). When regions A and C (or regions B and D) were mutated in combination (Fig. 3.6), drug induction was substantially reduced although some residual activity remained (1.7-2.0 fold). Also when regions B, C and D were mutated together, enhancer activity was almost completely lost (Fig. 3.6). These mutagenesis results established that all four protein binding sites identified by footprinting are required for maximal enhancer activity of the 556 bp enhancer region and that no single binding site is critically required.

3.2.4 Gel mobility shift analysis of the footprinted regions

Gel mobility shift assays were employed to characterise the proteins that bound to the footprinted regions. Double-stranded oligonucleotides (FP1-FP4) corresponding to the protected regions A-D were used in binding reactions with nuclear extracts prepared from untreated chick embryo hepatocytes and from phenobarbital-induced hepatocytes (75). The mutated oligonucleotides for FP1-FP4 (mFP1-mFP4) were also employed and contained
Figure 3.6  Functional role of the DNase I protected regions (A-D)

Mutations were introduced into potential transcription factor binding sites in the DNase I protected regions A-D (crosses) in pR556-SVCAT. Constructs (4 pmol) containing the 556 bp enhancer fused to the SV40 promoter and CAT reporter (SVCAT) were transfected into chick embryo hepatocytes. The sample was divided into two and phenobarbital (500 μM final) added to one plate and PBS to the control plate. Hepatocytes were harvested after 48 h. CAT activities were measured in control hepatocytes (without drug) or induced hepatocytes (with drug) and expressed as fold increase in CAT activity following treatment with phenobarbital. CAT values are expressed as an average of three independent experiments ± SD.
the same mutations which were tested in the functional assay of the 556 bp enhancer (see Fig. 3.6).

FP1, which contained an E-box like element, gave one major and two minor protein complexes and these complexes were of about the same intensity using either the drug treated or control nuclear extracts (Fig. 3.7A). In self-competition experiments the formation of these complexes was efficiently prevented at a 10-fold molar excess of unlabelled FP1 but not with the corresponding mutated oligonucleotide thus demonstrating the specificity of formation of the complexes. Binding was not competed (lane 8) with an oligonucleotide containing an E-box motif for MyoD (194) while an oligonucleotide containing an E-box binding site for upstream stimulatory factor (USF) was a relatively strong competitor (lane 9). The E box sequence in FP1 (5'-CACCTG-3') differs from the consensus USF site (5'-CACGTG-3') at one position. FP1 was also found to bind recombinant USF (lane 10) although the extent of binding was considerably weaker than that observed previously for the consensus USF site (75). These results indicate that a member of the E-box family of transcription factors binds to footprint region A which may be USF or a related protein.

FP2, when incubated with nuclear extracts from control and phenobarbital-treated hepatocytes, gave three protein complexes (Fig. 3.7B) the amounts of which were the same with both extracts. In self competition experiments the formation of these complexes was totally inhibited with FP2 at 10-fold molar excess but there was no effect with the mutant oligonucleotide mFP2. A possible HNF5 binding site is located in footprint region B (Fig. 3.4). However, a competitor oligonucleotide containing an authentic HNF5 site (111) did not prevent the formation of the three complexes (lanes 13 & 14). It was noticed that the proposed HNF5 site at -96/-90 (5'-TATTTAA-3') lies within a possible HNF-1 binding site (5'-TGGTAAATATTTTG-3') located on the negative strand and was not identified by computer sequence analysis as shown in Fig. 3.4. Therefore, competition experiments were also carried out with an oligonucleotide containing the functional HNF-1 site from the rat albumin promoter (45). The formation of all three protein complexes was almost totally
Figure 3.7  Gel mobility shift assays of footprinted region A, B, C and D

A. Assays were performed with radiolabeled double stranded oligonucleotide FP1 using 3 µg of nuclear extract from control (CON) and phenobarbital induced (PB) hepatocytes (lanes 1 and 2). The major complex is arrowed. In competition experiments, nuclear extracts from control hepatocytes were incubated without competitor (lane 3) or with 10 and 25-fold molar excess of FP1 (lanes 4 and 5) and mFP1 (lanes 6 and 7). The probe was incubated with nuclear extract from control hepatocytes and competed with 30-fold molar excess MyoD oligonucleotide (lane 8) and 30-fold molar excess USF oligonucleotide (lane 9). Binding of bacterially expressed recombinant human USF (5 ng) is given in lane 10.

B. Radiolabeled FP2 was incubated with the same nuclear extracts as in Fig. 3.6 (lanes 1-2). Three major complexes are arrowed. In competition experiments, nuclear extracts from control hepatocytes were incubated without competitor (lane 3), 10 to 50-fold molar excess of FP2 (lanes 4-6), or mFP2 (lanes 7-9). The probe was incubated with nuclear extracts from control hepatocytes in the presence of either no competitor (lane 10) or 10 and 25-fold molar excess of an HNF1 oligonucleotide (lanes 11 and 12) and an HNF5 oligonucleotide (lanes 13 and 14).

C. Radiolabeled FP3 was incubated with the same nuclear extracts as in Fig. 3.6 (lanes 1-2). Three major complexes are arrowed. In competition experiments, nuclear extracts from control hepatocytes were incubated either without competitor (lane 3) or with a 10 to 50-fold molar excess of FP3 (lanes 4-6) and mFP3 (lanes 7-9).

D. Radiolabeled FP4 was incubated with the same nuclear extracts as in Fig. 3.6 (lanes 1-2). One major complex is arrowed. In competition experiments, nuclear extracts from control hepatocytes were incubated with a 10-fold molar excess of FP4 (lane 3) or 25-fold molar excess of mFP4 (lane 4), NF1 oligonucleotide (lane 5) and a C/EBP oligonucleotide (lane 6).
inhibited with this competitor at 10-fold molar excess (lane 11). Moreover, the mobility of each of these three protein complexes bound to FP2 are identical to those detected previously with a functional HNF-1 site (75) and correspond to dimers of HNF-1α and HNF-1β isoforms (289). In keeping with these findings, the mutation examined earlier (5'-ACAAAT-3' to 5'-CTGCAG-3') which substantially lowered enhancer activity (see Fig. 3.6) altered three bases in the HNF-1 binding site.

FP3 gave three major protein complexes and a similar intensities of these protein complexes were observed with extracts from either control or drug treated hepatocytes (Fig. 3.7C). The binding of these proteins was efficiently prevented in self competition experiments with FP3 but not mFP3 (lanes 4-6 and 7-9).

Protein binding to FP4 containing a CCAAT box binding site was very weak; one major protein complex was observed with a similar intensity using control or drug treated nuclear extracts (Fig. 3.7D). Formation of this complex was markedly inhibited in self competition with FP4 (lane 3) and weakly reduced with an oligonucleotide containing a known C/EBP binding site (193) (lane 6), but an oligonucleotide containing an authentic NF1 site (51) (lane 5) had little effect.

Gel mobility shift analysis was also employed to examine the tissue distribution and relative abundance of the proteins that bound to FP1-FP4, using nuclear extracts from the liver, kidney, lung, small intestine and heart of untreated chickens (Fig. 3.8). Proteins binding to footprint regions A and B (FP1 and FP2) are relatively enriched in liver, kidney, and small intestine but not detectable in lung and heart (Fig. 3.8A). Regions C and D (FP3 and FP4) strongly bound nuclear proteins from liver and only weakly from kidney and small intestine with no binding from the other tissues (Fig. 3.8B). These studies show that the protein complexes which bind to the enhancer are enriched in those tissues (liver, kidney and small intestine) which are drug responsive (125). The pattern of protein binding to FP1-FP4 oligonucleotides by liver and kidney nuclear extracts was very similar to that observed previously with nuclear extracts from chick embryo hepatocytes (Fig. 3.7A-D).
Figure 3.8  Gel mobility shift assays of footprinted regions A-D with nuclear extracts from various tissues

Nuclear extracts from various untreated chicken tissues were used with radiolabeled probes FP1-FP4. A, FP1 or FP2 were incubated with nuclear extracts (5-8 μg of protein) from liver (Li), kidney (Kid), lung (Lu), small intestine (Si) and heart (Hr). B, FP3 or FP4 were incubated with nuclear extracts from various chicken tissues as in A.
However, the protein binding profile for FP1 and FP2 with the small intestine nuclear extracts differed from that of the liver and kidney reflecting perhaps the presence of tissue specific isoforms and this requires further investigation.

3.3 Discussion
A 556 bp enhancer sequence (-1956/-1400) in the chicken CYP2H1 5'-flanking region that responds to phenobarbital in transiently transfected chick embryo hepatocytes has been characterised. This sequence has been analysed by dissection into two restriction enzyme fragments; a 240 bp fragment that responds to drug and a 316 bp fragment that increases the drug response but does not itself respond to drug. Deletion analysis of the 240 bp sequence (performed within the 556 bp enhancer), showed that several regions contributed to drug responsiveness and DNase I protection assays using liver nuclear extracts from phenobarbital induced chickens identified four protected regions (A-D). Sequence analysis and gel shift assays indicated that footprint region A binds a member of the E-box family of transcription factors which is possibly USF or a related protein (305). Footprint region B binds members of the HNF-1 family (75, 289) while footprint region D binds a CCAAT box binding protein possibly related to C/EBP (47, 67, 297) but which is not NF1. Sequence within region C did not match any known transcription factor binding site and the three major protein complexes which bound to this novel region have yet to be characterised. Site directed mutagenesis of the protein binding sites in regions A-D and transient expression studies established that inactivation of a single site or two sites together (A and C or B and D) reduced but did not abolish induction by the 556 bp enhancer, while mutagenesis of multiple sites (B, C and D) resulted in almost complete loss of induction. These findings demonstrated that sites (A-D) are all necessary for maximal induction and that no site alone is critical. In vitro footprint analysis of the 316 bp fragment has revealed binding sites for HNF-1 and HNF-4 transcription factors. The data therefore show that more than one element in the 556 bp enhancer is required for drug response with the other elements contributing to the increased level of induction but not being drug responsive.
A promoter-located 17 bp Barbie box sequence has been implicated in the phenobarbital mediated induction of the bacteria CYP102 and CYP106 genes (131, 318) and also in the induction of the rat CYP2B1 gene (275). However, inactivation of the corresponding site in the promoter of the rat CYP2B2 gene (262, 333, 338) and mouse Cyp2b10 gene (145) did not affect drug induction. Moreover, recent evidence suggests that the Barbie box of the bacterial CYP106 gene may not be required for barbiturate-mediated induction (319). In the present work, a Barbie box was not identified in the 556 bp enhancer or the promoter (75) of the CYP2HI gene. This sequence is also absent from the recently reported upstream phenobarbital responsive enhancer regions of the mouse Cyp2b10 gene (145) and rat CYP2B1/2 genes (262, 333). Overall, a general role for the Barbie box in the drug induction mechanism in both eukaryotes and prokaryotes appears unlikely.

The phenobarbital enhancer region for the Cyp2b10 gene (145) and CYP2B1/2 genes (174, 262, 333) is located upstream at approximately -2 kb. Multiple sites which contribute to phenobarbital responsiveness have been identified by in vitro footprint analysis and mutagenesis within the mouse 132 bp enhancer and the corresponding rat enhancer region and include a putative glucocorticoid responsive element and a nuclear factor 1 (NF1) site (145, 333). There is evidence (333) that phenobarbital responsiveness of the CYP2B2 enhancer is directed by interactions between multiple proteins on the enhancer and as mentioned, a similar finding has been made in the present study with the chicken CYP2HI enhancer. A recent report has defined a 51 bp element in the 132 bp enhancer of the mouse Cyp2b10 gene that independently responds to phenobarbital and other phenobarbital-type inducers and this element contains the NF1 site and is flanked by apparent novel nuclear receptor binding sites (NR1 and NR2) composed of direct half sites with a spacing of 4 bp (144).

The nuclear receptor CAR has been shown to bind to the NR1 site and is critical in regulating the phenobarbital responsiveness of the Cyp2b10 gene (147). Whether CAR is critical in the regulation of other PB-inducible genes is not clear as experiments indicate that this receptor may not play a role in the response of the CYP2HI gene to phenobarbital.
pR1-SVCAT and p556SVCAT were transfected into HepG2 cells along with a murine CAR expression plasmid in the absence of phenobarbital. Activity of p556SVCAT increased by 9.3 fold in the presence of CAR while pR1-SVCAT activity was unaffected. Further, gel shift analysis using a mouse CAR binding site (NRI) showed a weak retarded complex that had the same intensity when either untreated or PB treated chicken embryo nuclear extracts were employed (1). In addition, competition experiments employing the four footprinted regions of p556SVCAT protected in PB-treated chicken liver nuclear extracts showed that none of the four footprint regions contained a CAR binding site (S. Dogra, Personal Communication). The p556SVCAT constructs containing mutations in one of the four DNase I protected regions were transfected into HepG2 cells along with the CAR expression clone. Mutations in any of the four sites did not affect the ability of CAR to increase activity of these constructs (S. Dogra, Personal Communication). These findings indicate that firstly, there is very little CAR in chick embryo hepatocytes. Secondly, the binding sites identified by footprint analysis within the 556 bp enhancer do not bind CAR. Thirdly, phenobarbital treatment appears to have no effect on the translocation of CAR into the nucleus of chick embryo hepatocytes. It is possible that CAR was not observed in DNase I footprint assays of the 556 enhancer due to its very low abundance in chick embryo hepatocytes. This data indicate that CAR may not be the nuclear receptor that mediates PB-inducibility of all the CYP2 gene subfamilies. Whether CAR plays a role in PB-responsiveness of the 556 bp enhancer will require identification of the binding site (eg, using purified CAR and footprint analysis) and subsequent mutagenesis of this site within the enhancer.

An important finding here is that the proteins which bind to the chicken 556 bp enhancer appear to have no counterparts on the rodent enhancers. A sequence comparison of this region with the rodent enhancer sequences did not reveal any common protein binding sites, in particular a glucocorticoid response element or a CAR binding site. As mentioned there is a functional CCAAT box element but this site binds a protein complex possibly related to C/EBP rather than the ubiquitously expressed NF1. The C/EBP related complex is particularly enriched in nuclear extracts from chicken liver (and to a lesser extent kidney
and small intestine), tissues which are highly responsive to drug (125), but is absent from extracts of the non-responsive tissues lung and heart. The other three footprinted regions in the 240 bp fragment also bound proteins enriched in the liver, kidney and small intestine. As mentioned earlier, the adjacent 316 bp fragment contains sites for the liver enriched transcription factors HNF-1 and HNF-4. Hence, tissue restricted expression of the CYP2HI gene (125) is likely to be directed by transcription factors binding to the 556 bp enhancer and in addition to the early promoter which has functional sites for HNF-1, HNF-3, C/EBP and USF (75, 371).

It is of interest to note that in contrast to the enhancer regions of the rat CYP2Bl/2 (174, 262) and mouse CYP2b10 (145) genes, in vitro footprint analysis of the chicken 240 bp fragment revealed an increase in the binding of nuclear proteins from drug-treated livers. This binding was probably not due to an increase in the amount of these nuclear proteins in response to phenobarbital since in the gel mobility shift studies an increase in binding with drug-treated nuclear extracts was not observed. In control experiments, the same nuclear extracts which revealed increased binding on the 240 bp fragment did not show this with the 316 bp fragment eliminating the possibility that this is a general effect (data not shown). The footprint data suggest that drug action leads to increased binding on the 240 bp fragment through a cooperative interaction between proteins. In keeping with this proposal, footprint analysis using competitor oligonucleotides corresponding to footprint regions A-D showed that depletion of one binding protein could result in a weaker footprint on the other regions (data not shown, Ref. 73). The possibility that in the presence of drug one or more proteins are modified, for example by a phosphorylation event (74), and this results in increased binding to the enhancer in a cooperative fashion, is currently under investigation.

In summary, this chapter details the identification and analysis of a drug responsive enhancer 556 bp region in the CYP2HI gene. Maximum drug responsiveness is dependent upon binding of multiple proteins. Further analysis is required to identify precisely the proteins that bind, the way in which they interact and cooperate and whether drug action
leads to the modification of any of these proteins. In this regard, analysis of various upstream restriction enzyme fragments located in the 4.8 kb enhancer domain of the *CYP2H1* gene, has revealed that, in addition to the 556 bp sequence, there is a second separate region that responds to drug and to about the same extent. It will be of interest to identify the transcription factors that bind to this region. If it eventuates that the transcriptional factors which activate the chicken and rodent phenobarbital responsive enhancers are different, this would imply that either the induction mechanisms are fundamentally different or that there is a common site of action of phenobarbital, yet to be determined, that leads to the modification and activation of multiple transcription factors. Whether phenobarbital and the other phenobarbital-type inducers primarily mediate such action through direct binding to a specific receptor protein or through CAR remains a key issue.
Chapter 4. Characterisation of an enhancer and the promoter of the \textit{CYP2H2} gene
4.1 Introduction

As discussed in the previous chapter, two phenobarbital-inducible genes, CYP2H1 and CYP2H2, have been identified in the chicken (221). Two separate regulatory regions have been identified for the CYP2H1 gene, an upstream enhancer domain that responds to drug (73, 119) and a proximal promoter region that directs strong basal expression but does not respond to drug (75). Chapter 3 describes the isolation and characterisation of the 556 bp enhancer region of the CYP2H1 gene that confers drug responsiveness. The proximal promoter of the CYP2H1 gene has been characterised previously and was shown to be driven by the liver-enriched transcription factors HNF-3, HNF-1, C/EBP together with the ubiquitous factor USF (75). As mentioned, the strong transient expression observed with CYP2H1 promoter constructs in chick embryo hepatocytes is in marked contrast to the low level of expression of the endogenous gene (119, 125). Whereas phenobarbital treatment results in a dramatic increase in the expression of the endogenous gene, the strong transient expression of the CYP2H1 promoter in chick embryo hepatocytes is only moderately increased (119). In view of these findings, we suggest that drug action in vivo substantially reflects derepression of the inherently strong CYP2H1 promoter. While the CYP2H1 gene has been extensively studied, very little is known about the molecular regulation of the CYP2H2 gene. The CYP2H2 gene is highly induced by PB (see Fig 3.1, lane 4), however, the induced steady state mRNA levels of the CYP2H2 gene (lane 4) are on average 8 fold (124) less than that of the CYP2H1 gene (lane 3) as observed in repeated experiments. The model of drug induction suggests that drug relieves repression of a strong promoter resulting in induction. Hence, it is possible that the lower level of CYP2H2 gene expression may be due to a weaker promoter compared with the CYP2H1 gene. Thus it was of interest to isolate both the promoter and enhancer regions of the CYP2H2 gene as this could indicate common transcription factors involved in basal and drug responses and perhaps shed light on the proposed model of drug induction.
4.2 Results

4.2.1 Identification and Cloning of the Promoter Region of CYP2H2

Previously in this laboratory, a series of CYP2H2 genomic clones were isolated from a \(\lambda\) Charon 4A chicken genomic library (221). Clone \(\lambda 20\) was chosen for further analysis of the CYP2H2 gene as preliminary Southern blots indicated that it extended furthest in the 5' direction. In order to identify the promoter region of CYP2H2, an oligonucleotide probe (Primer 6728), complementary to sequence within the 5' end of the coding region of the reported CYP2H2 cDNA clone (125), was used in a Southern blot analysis of \(\lambda 20\) DNA digested with EcoRI and BamHI or PstI (Fig. 4.1, lane 1). This probe hybridised to an 8.4 kb EcoRI/BamHI fragment and a 2.2 kb PstI fragment (lane 2) of \(\lambda 20\). These two restriction fragments were then cloned into pBluescript for further characterisation of drug enhancer and promoter regions in the CYP2H2 gene respectively. The 2.2 kb PstI fragment, designated pBKP2.2, was chosen for sequencing. Sequencing was carried out to confirm that the promoter region had been isolated and to compare the sequence with that of the promoter for the CYP2H1 gene (75) with the aim of identifying conserved sequences that may play a role in basal and tissue-specific expression. Double-stranded sequencing was performed on pBKP2.2 using the primer 6728 and modified T7 DNA polymerase as described in section 2.2.3. Sequencing of the opposite strand using the reverse primer was carried out to confirm the sequence obtained.

The first 351 bp of sequence of the CYP2H2 promoter was compared to that of the promoter of the CYP2H1 gene (Fig 4.2). Overall there was 96% similarity between the two promoters in this region. The region from the CAP site (at +1) to position -152 in the CYP2H2 gene is 100% identical to the corresponding region of the CYP2H1 gene. While the CAP site for the CYP2H2 gene has not been formally determined it seems reasonable to assume that it is at the same position as in the CYP2H1 gene (140). Upstream from position -152 the CYP2H2 sequence contains an 8 bp insertion at position -154/-161 and two single base pair differences at -153 (A\(\rightarrow\)T) and -167 (T\(\rightarrow\)C). In addition there are 6 single base pair differences that occur between -199/-345. Sequence analysis (Fig. 4.2) of the proximal promoter using the Transfac computer program (365) revealed a consensus
Figure 4.1  Southern blot analysis of the genomic clone λ20 using a 5'-specific oligonucleotide probe.

DNA (0.1 μg) from clone λ20 was digested with EcoRI/BamHI (lane 1) or PstI (lane 2) and electrophoresed on a 1% agarose gel. After transfer to a nylon membrane by Southern blot (Section 2.2.8) the filter was probed with kinased ³²P-labelled primer 6728 that is specific for the 5' UTR of CYP2H2. After 12 h hybridisation, the filters were washed under stringent conditions (65°C in 0.1X SSC). The size of hybridised bands was determined by comparison with EcoRI digested SPP-1 phage DNA markers (not shown). A PstI restriction fragment of pCHP3 cDNA corresponding to the first 1350 bp of the coding region of CYP2H1 (lane C) was included as a positive control for primer hybridisation.
CI2

\[ \text{O} + 8.4 \text{kb} + 2.2 \text{kb} + 1.35 \text{kb} \]
TATA box at -32/-27, and a putative binding site for the ubiquitous upstream stimulatory factor (USF) of sequence 5' CACGTG 3' at -54/-49 (305). Additionally, possible binding sites for three liver-enriched transcription factors were identified. Using the consensus HNF-3 sequence 5' (C/A/G)A(A/T)T(G/A)TT(T/G)(G/A)(C/T)T(C/T) 3' compiled by analysis of sites in liver-enriched promoters (255) two overlapping sites for hepatocyte nuclear factor-3 (HNF-3) at -170/-159 (5' CTCTGTCTTGCTC 3') and -161/-150 (5' TCAAGTTTGCTC 3') were located on the non-coding strand (underlined bases indicate variation from the consensus sequence). A hepatocyte nuclear factor-1 (HNF-1) site at -113/-101 (5' ATTAATAAGTAAC 3') and a CCAAT-enhancer binding protein (C/EBP) site at -70/-64 (5' ATTGGCT 3') were also identified. In addition, a sequence resembling the Barbie box element of consensus 5' ATCAJqJAAGCTGGAGG 3', a sequence implicated in PB-induction in rats (131) and bacteria (205) was identified at -51/-39 on the non-coding strand of sequence 5' CCACGTGCTTTTGTT 3'. This element partially overlapped the putative USF site. These five putative cis-acting elements identified in the CYP2H2 early promoter are all observed in the CYP2H1 promoter. Between -351/-170 only a putative AP-1 site at -328/-322 of sequence 5' GGAGTC 3' was identified (not shown in Fig. 4.2). Extensive mutagenesis of the CYP2H1 promoter has shown that the HNF-3 site of sequence 5' CATTGTTTACT 3' at -162/-151 together with the HNF-1, C/EBP and USF sites are all required for maximal basal expression of the promoter in transient transfection experiments using chick embryo hepatocytes (75). The barbie box was shown to have no effect on basal expression of the CYP2H1 promoter (75). The major difference between these two promoters is at position -154/-161 in the CYP2H2 promoter where a duplication of the 8 bp sequence 5' GAGCAAAC 3' creates a second putative HNF-3 binding site leading to two overlapping putative HNF-3 sites.

4.2.2 Isolation of a drug responsive region from the CYP2H2 gene
As discussed in chapter 3, a 556 bp drug responsive sequence located in the 5' flanking region of the CYP2H1 gene can confer approximately a 7 fold drug induction to a heterologous promoter/reporter construct in chick embryo hepatocytes. Multiple and different transcription factor binding sites were identified within this 556 bp region. All of
Figure 4.2  DNA sequence of the *CYP2H2* promoter.

Shown in red is the promoter sequence of *CYP2H2*. This sequence is compared to the promoter sequence of *CYP2HI* (black). The transcription start is arrowed (+1) and the translation start site is in bold (AUG). Potential binding sites for the transcription factors HNF-3 (-170/-159, -161/-150), HNF-1 (-113/-101), C/EBP (-70/-64), USF (-54/-49) are shown together with the TATA box (-32/-27). HNF-3 and C/EBP binding sites are on the noncoding strand. The nucleotide differences at the HNF-3 region are indicated by underlining.
these sites contributed to the maximal drug expression but no single site appeared to be absolutely required for drug induction (73).

As this 556 bp region is vital for drug inducibility of the CYP2H1 gene it was important to investigate the corresponding region in the CYP2H2 gene and to determine its role in the induction process. The 8.4 kb EcoRI/BamHI fragment (p8.4BK) isolated previously (Fig 4.4) was digested with various restriction enzymes, electrophoresed on an agarose gel, transferred to a nylon membrane and probed with the 556 bp region of CYP2H1 (Fig 4.3A). The probe hybridised with one fragment in all 8 digests under stringent wash conditions. The smallest fragment to hybridise with the 556 bp probe, a 1100 bp PvuII restriction fragment, was isolated and cloned into pBluescript. Double stranded DNA sequencing of this fragment revealed that the 556 bp enhancer region is wholly contained within the 1100 bp PvuII fragment and that the sequence of these 556 bp is identical between the two genes. The sequence within the PvuII fragment that lies outside the 556 bp enhancer diverged slightly and showed 96 % homology with the corresponding CYP2H1 sequence (data not shown). Further restriction enzyme mapping is required to locate the PvuII fragment. From this study it appears to be within the first 5.4 kb of 5’ flanking sequence.

The 1100 bp PvuII fragment from the CYP2H2 gene was analysed for its ability to confer drug inducibility to a heterologous promoter. The PvuII fragment in the forward and reverse orientation was fused to the enhancerless SV40 promoter driving the CAT reporter gene, and these constructs are referred to as p1100SVCAT and pR1100SVCAT respectively. After these constructs, along with p556SVCAT and pR556SVCAT containing the 556 bp enhancer from the CYP2H1 gene in both orientations, were transfected into chick embryo hepatocytes by electroporation, each sample was halved and phenobarbital (500 μM) added to one dish and PBS to the control dish. The average of three independent experiments, repeated in duplicate and represented as a percentage conversion of [14C]chloramphenicol to acetylated product is shown in Figure 4.3B. The 1100 bp fragment isolated from the 5’ distal region of CYP2H2 conferred drug inducibility
Figure 4.3  Southern blot analysis and transient transfection analysis of p1100SVCAT and pR1100SVCAT.

A. DNA (0.1 μg) of the purified 8.4 kb EcoRI/BamHI fragment isolated previously (Section 3.2.2) was digested with the following enzymes: PstI (lane 1), PstI and BamHI (lane 2), SacI and EcoRI (lane 3), HindIII and BamHI (lane 4), HindIII (lane 5), PstI and EcoRI (lane 6), PvuII (lane 7) and EcoRI and BamHI (lane 8). After electrophoresis on a 1% agarose gel the digests were transferred to a nylon membrane by Southern blot and probed with radiolabelled 556 bp enhancer element from the CYP2H1 gene. After 12 h hybridisation the filters were washed under stringent conditions (65°C and 0.1X SSC). The sizes of hybridised bands were determined by comparison with EcoRI digested SPP-1 phage DNA markers (not shown).

B. Chick embryo hepatocytes (2 x 10⁷) were transfected in the presence of 500 μg/ml salmon sperm DNA with 2 pmol of each construct by electroporation at 250 V and 960 μF. Each sample was cultured in William’s E medium supplemented with 10% Serum Supreme overnight, at which time fresh media without PB (-) or with 500 μM PB (+) were added and the cells incubated for a further 48 h. CAT assays were performed on 50 μg of cellular protein extract for 2 h. p556SVCAT and pR556SVCAT represent the 556 bp drug responsive element isolated from the 5’ flanking sequence of the CYP2H1 gene fused to the enhancerless SV40 promoter in the forward and reverse (R) orientation. p1100SVCAT and pR1100SVCAT represent the 1100 bp drug responsive element isolated from the 5’ flanking sequence of the CYP2H2 gene fused to the enhancerless SV40 promoter in the forward and reverse (R) orientation. The data represents the average of three independent experiments, repeated in duplicate and represented as a percentage of conversion of [³¹⁴C]chloramphenicol to acetylated product are shown as the mean ± standard deviation.
**A**

![Image](image1.jpg)

**B**

![Image](image2.jpg)

---

**Figure A**

- Lanes 1-8 show different bands, with the following sizes:
  - 8.9 kb
  - 6.1 kb
  - 3.6 kb
  - 2.5 kb
  - 1100 bp

**Figure B**

- Bar graph showing % acetylation of chloramphenicol for different combinations of PB and constructs:
  - PB: - + - +
  - Constructs: p556-SVCAT, pR556-SVCAT, p1100-SVCAT, pR1100-SVCAT

- For CYP2H1:
  - Control (PB-) acetylation: 3.5%
  - PB+ acetylation: 16%

- For CYP2H2:
  - Control (PB-) acetylation: 4.9%
  - PB+ acetylation: 16%
in both orientations to the SV40 enhancerless promoter (5.3 and 4.9 fold respectively). Similar levels of induction were observed for the 556 bp enhancer isolated from the 5' distal region of CYP2H1 (5.8 and 3.5 fold for the forward and reverse orientations respectively).

These results show that the 5' distal 1100 bp region of the CYP2H2 gene contains a drug inducible enhancer that exhibits very similar strength to the 556 bp region of the CYP2H1 enhancer. Since the 1100 bp region contains an identical 556 bp region to that observed in the CYP2H1 gene, it can be concluded that this sequence is required for phenobarbital induction. These data suggest that the difference in the steady state levels of these genes as determined by Northern blot analysis (Fig. 3.1) most likely arises from a difference in the relative strengths of these promoter regions and not their drug responsive enhancers.

4.2.3 Deletion analysis of the 5'-flanking region of CYP2H2

In order to examine the expression of the CYP2H2 promoter, a 959 bp region corresponding to position -920/+39 was cloned in front of the CAT reporter gene. Figure 4.4 shows a diagrammatic representation of the method of construction of p920CAT.

To determine the minimum promoter length required for maximum basal expression of the CYP2H2 gene, a series of deletion constructs were created from p920CAT by exonuclease III digestion. These promoter/CAT deletion constructs (p920CAT, p556CAT, p198CAT, p96CAT and p51CAT) were then electroporated into chick embryo hepatocytes in the absence of phenobarbital and CAT activity determined in cell lysates. Deletion of the promoter sequence from -920 to -198 had no significant effect on the level of expression (Fig. 4.5). However, further deletion of sequence from -198 to -96 markedly reduced CAT activity to less than 10% of the -198 construct. Deletion of the promoter sequence from -96 to -51 virtually abolished expression. Thus the first 198 bp of promoter appears to contain all the elements necessary for basal expression. This region contains all of the conserved regulatory elements (see Fig. 4.2) previously identified as being required for full basal expression of the CYP2H1 promoter (75). It is worth noting that this 198 bp of promoter
Figure 4.4  Strategy for construction of p920CAT.

A flow diagram for the strategy by which p920CAT was constructed is shown. Briefly, λ20 was digested with PstI and a 2120 bp fragment cloned into pBluescript KS+ (pBKP2.2). The pBKP2.2 clones were oriented by digestion with SacI. These clones were then digested with NcoI (NcoI cuts pBKP2.2 once at the ATG initiation codon). Mung Bean nuclease digestion was performed to remove the 5'-overhang thus destroying the initiation site of the CYP2H2 gene. Linearised pBKP2.2 was digested with BamHI and a 3.8 kb fragment containing the vector and the region of CYP2H2 5' to the initiation site was isolated. The CAT gene was prepared from the pGL2CAT vector. This vector was made linear with HindIII and end filled to produce a blunt end. This blunt ended fragment was digested with BamHI to release the CAT gene from the vector. The CAT gene was then isolated (section 2.2.2.3) and cloned into the NcoI(blunt)/BamHI cut pBKP2.2 plasmid. To confirm the cloning of the CAT gene, plasmid DNA was isolated and analytical restriction digests were performed using BamHI/PstI. The resultant construct was designated p920CAT. Also shown is the cloning of the 8.4 kb EcoRI/BamHI fragment into pBluescript. The resultant construct was designated p8.4BK. Only the relevant restriction endonuclease sites that were important in cloning are shown.
Figure 4.5   Deletion analysis of the CYP2H2 promoter region by transient expression in chick embryo hepatocytes.

Different promoter lengths were generated by exonuclease III digestion of p920CAT. Chick embryo hepatocytes (2 x 10⁷) were co-transfected in the presence of 500 µg/ml salmon sperm DNA with 2 pmol of each construct and 5 µg of RSV-βgal by electroporation at 250 V and 960 µF. Hepatocytes were cultured in William’s E medium supplemented with 10% Serum Supreme for 48 h. CAT assays were performed on 50 µg of cellular protein extract for 2 h. The resultant autoradiograph of one such experiment is shown. CAT activities were quantified by liquid scintillation counting and the average of three independent experiments, repeated in duplicate and represented as a percentage of conversion of [¹⁴C]chloramphenicol to acetylated product are shown as the mean ± standard deviation. CAT activities were normalised for transfection efficiency by β-galactosidase activity.
did not to respond to drug (data not shown), a finding also observed with the CYP2H1 promoter (119).

Interestingly, in comparison, transfection of the CYP2H1 construct, p205CAT, containing -205/+39 bp of CYP2H1 promoter (75) and the CYP2H2 construct, p198CAT, containing -198/+39 bp of the CYP2H2 promoter into chick embryo hepatocytes revealed that basal expression from the CYP2H2 promoter was consistently 6 to 8 fold lower than that from the CYP2H1 promoter (see later Fig 4.13). This result was surprising, given the very high sequence homology and conservation of regulatory elements between these two promoters (Fig. 4.2). A similar phenomena is observed in vivo where the endogenous CYP2H2 gene shows approximately 10 fold lower levels of mRNA expression than CYP2H1 following PB induction (Fig. 3.1, 124). This lower level of CYP2H2 mRNA induction following PB treatment may therefore be due to a weaker CYP2H2 promoter. Importantly, the only variation of the CYP2H2 promoter compared with the CYP2H1 promoter within the -198/+39 region lies in the HNF-3 binding site (Fig 4.2 and Fig 4.6A). An 8 bp region (5’ GAGCAAAC 3’) containing the core sequence (5’ GCAAAC 3’) of the HNF-3 binding site is perfectly duplicated in the CYP2H2 promoter generating two partially overlapping putative HNF-3 binding sites. These sites are denoted HNF-3A and HNF-3B. The HNF-3A site contains 2 bases that do not conform to the consensus sequence (5’ GAGCAAAACAGAG 3’) while the HNF-3B site contains three bases that do not conform to the consensus sequence (5’ GAGCAAAACTTGA 3’). In addition, both HNF-3 sites in the CYP2H2 promoter differ from the HNF-3 site (5’ GAGTAAACAATG 3’) in the CYP2H1 promoter by 3 bases in the case of HNF-3A (5’ GAGCAAAACAGAG 3’) and 5 bases in the case of HNF-3B (5’ GAGCAAAACTTGA 3’).

4.2.4 DNase I footprinting analysis of the CYP2H2 promoter

Nuclear proteins binding to the -198/+39 promoter region of the CYP2H2 gene were investigated by in vitro DNase I footprint analysis. Promoter fragments were radiolabelled at either the 5’ or 3’ end and incubated with nuclear extracts prepared from 18 day old chick embryo liver (section 2.2.13.1). Four separate protected regions (designated A to D)
Figure 4.6  Sequence of the HNF-3 DNA binding sites.

A. Shown is the consensus HNF-3 DNA binding site (313), the HNF-3 binding site of CYP2H1 and the two HNF-3 binding sites (A and B) of CYP2H2. Shown in green is the 8 bp duplicated sequence in the CYP2H2 gene.

B. Shown in I is the region of CYP2H2 containing the HNF-3 sites (blue) and the binding of oligonucleotide d4B to this region forcing the 8 bp duplicated sequence (green) to loop out. II shows the resulting sequence of this region after mutagenesis using d4B with only the HNF-3 A site (blue) remaining. Also shown are the oligonucleotides used to mutate the HNF-3A site (m4A) and the HNF-3B site (m4B) of CYP2H2. Bases in red are those mutated. Bases in blue are the HNF-3 binding sites.
A

CONSENSUS

\[
\text{GAGCAAAACATTT} \\
\text{A ATC T A G}
\]

HNF3

CYP2H1

\[
\text{AGTCAGAGTAAACAATGAGTTCATCCC}
\]

HNF3 A

CYP2H2

\[
\text{AGTCAGAGCAAACAAGCAAACCTTGAGTTCATCCC}
\]

HNF3 B

B

I

\[
\text{AGTCAGAGCAAAACAATGAGTTCATCCC}
\]

d4B

\[
\text{TCTCGTTTGT AACTCAAGTA}
\]

II

\[
\text{AGTCAGAGCAAACATTGAGTTCATCCC}
\]

m4A

\[
\text{AGTCAGAGGTGAGGAGCAAACCTTGAGTTCATCCC}
\]

m4B

\[
\text{AGTCAGAGCAAACAGAGGTGACCTGAGTTCATCCC}
\]
Figure 4.7  DNase I footprint analysis of the CYP2H2 promoter.

CYP2H2 promoter fragment from -198 to +39 was radiolabelled on the coding strand (A) and noncoding strand (B) and footprint analysis performed as described in the Materials and Methods with the addition of chick embryo liver nuclear extract (Lane 3) or without this addition (Lanes 2 and 4). The fragment was partially cleaved at G and A residues as a marker for the sequence (Lane 1). The DNase I protected regions (A-D) are bracketed. A represents the putative TATA protein binding site at -31/-23, B the USF protein binding site at -54/-42, C the C/EPB protein binding site at -74/-56 and D the HNF-1 protein binding site at -121/-95. These sites have been previously characterised in the CYP2H1 promoter.
Figure 4.8  Gel mobility shift analysis of the HNF-3 binding sites using HepG2 nuclear extracts.

A radiolabelled double-stranded oligonucleotides spanning the HNF-3A site (Probe 4A, lane 1), the HNF-3B site (Probe 4B, lane 4) or both HNF-3 sites (Probe 4AB, lane 7) were incubated with nuclear extracts from HepG2 cells and gel mobility shift analysis performed as described in the Materials and Methods. The protein complex observed is arrowed. For neutralisation assays, nuclear extracts were incubated with antibodies to rat HNF-3α (lanes 2, 5 and 8) or HNF-3β (lanes 3, 6 and 9) prior to the addition of probe.
on the coding strand and four regions (A-D) on the noncoding strand were detected (Fig 4.7). The protected regions correspond to protein binding sites previously identified (Fig. 4.2) and match those in the CYP2H1 promoter (75). A weak protected region A (-31 to -23) encompasses the TATA box. The USF site, footprint B (-54 to -42), and the C/EBP site, footprint C (-74 to -56), bind close to one another and consistently form a contiguous protected region. Footprint D (-121 to -95), the HNF-I site, produces the largest single protected region and binds with the highest affinity. The protected region extended beyond the consensus core sequences of the TATA, USF, C/EBP and HNF-1 sites. In addition, HNF-1 binding resulted in the production of hypersensitive sites on either side of the protected region. Like the CYP2H1 promoter, no footprint over the barbie box-like region was observed. Interestingly, unlike CYP2H1 (75), no footprint was detected where the HNF-3 binding site occurs (defined as region E in CYP2H1). Thus the weaker activity of the CYP2H2 promoter may be due to a lack of HNF-3 binding to its site. Moreover, this result suggests that no other proteins bind to this region.

4.2.5 Gel shift analysis of the putative HNF-3 binding sites

The HNF-3 binding site in the CYP2H1 promoter has been shown to be functional by mutation analysis and to bind a chicken homologue of the HNF-3 family member HNF-3β, as determined by gel mobility shift analysis using chick embryo hepatocyte nuclear extracts (75). Since the putative HNF-3 sites identified in the CYP2H2 promoter contain the HNF-3 consensus core sequence it was surprising that no protected area was observed by DNase I footprinting in this region. This lack of binding could result from the overlapping of the two putative HNF-3 protein binding sites or from the observed sequence differences between these two sites and the consensus HNF-3 sequence. Therefore, whether HNF-3 can bind to the CYP2H2 HNF-3 binding sites was examined by gel mobility shift analysis.

HNF-3 protein exists as three isoform, HNF-3α, β and γ. To confirm that one of the isoforms of HNF-3 was able to bind to the HNF-3 A and B sites, neutralising antibodies to the α and β isoforms were included in a gel mobility shift assay. Probes containing either
HNF-3α or B were incubated with nuclear extract from HepG2 cells. This extract was used as HepG2 cells are known to contain HNF-3α, β and γ isoforms and in addition, the rat neutralising antibodies employed in this experiment do not cross react with chicken HNF-3 protein. Three major protein complexes are observed with HepG2 nuclear extracts (Fig 4.8, lanes 1 and 4). HNF-3α and HNF-3β proteins migrate very close together due to their similar molecular weights of 48.8 and 48.5 kDa, respectively. They can be distinguished when one or other HNF-3 isoform is inhibited using neutralising antibodies as discussed below. The more weakly binding HNF-3γ migrates further due to its smaller size of 37.7 kDa. Antibodies to this isoform were unavailable at the time of experimentation, however it is assumed that in Figure 4.8 the observed band is HNF-3γ. This binding pattern of HNF-3α, β and γ proteins using HepG2 nuclear extracts and HNF-3 specific probes seen here is very similar to that demonstrated previously (61, 266). The weak slower migrating bands marked with an asterisk (*) are non-specific and of unknown origin and are not consistently observed. Interestingly, a third probe (4AB) containing both HNF-3 sites in their native configuration produced three protein complexes similar to those produced with either HNF-3 site alone (lane 7). Incubation of all three probes with neutralising antibodies specific for the rat HNF-3α and HNF-3β proteins prevented protein complex formation of the upper and middle bands respectively. Collectively these results confirm that HNF-3α and HNF-3β isoforms can bind to the HNF-3 A and B sites. More importantly, HNF-3 is able to bind to one or other of these sites when they are situated in their native overlapping configuration. No complex with decreased mobility, corresponding to both HNF-3 sites being occupied simultaneously, was observed with this probe suggesting that HNF-3 is binding to one or other HNF-3 site and not binding to both sites simultaneously. It is possible that this may be due to the gel shift probe being in vast excess of HNF-3 protein.

As mentioned, the CYP2H2 promoter contains two HNF-3 sites overlapping one another arising from what appears to be a duplication event (Fig 4.2). Probe 4A that contains the HNF-3 A site of the CYP2H2 promoter was incubated with chick embryo liver nuclear extract and gel shift analysis was performed. As shown in Figure 4.9 (lane 1), a single
Figure 4.9  Gel mobility shift analysis of proteins that bind to the putative HNF-3 binding sites using chick embryo liver nuclear extracts.

Radiolabelled oligonucleotides spanning the HNF-3A site (Probe 4A) or the HNF-3B site (Probe 4B) were incubated with nuclear extracts from chick embryo liver and gel mobility shift analysis performed as described in the Materials and Methods. The protein complex observed is arrowed. For competition experiments, nuclear extracts were incubated with self (lanes 2 and 6), a non-related (NR) oligonucleotide (lanes 3 and 7) or a double-stranded oligonucleotide containing the adjacent HNF-3 site (lanes 4 and 8) at 20-fold molar excess.
Figure 4.10  Gel mobility shift competition analysis of HNF-3 binding sites.

A radiolabelled double-stranded oligonucleotides spanning the HNF-3A site (Probe 4A) or the HNF-3B site (Probe 4B) or containing both HNF-3 sites in the one double stranded oligonucleotide (Probe 4AB) were incubated with nuclear extracts from chick embryo liver and gel mobility shift analysis performed as described in the Materials and Methods. The protein complex observed is arrowed. For competition experiments, nuclear extracts were incubated with unlabelled double stranded oligonucleotides corresponding to self (lanes 2 and 6) or the HNF-3A site (lane 10) or the HNF-3B site (lane 11), the HNF-3 site from the CYP2H1 gene designated 2H1 (lanes 3, 7 and 12) or an authentic HNF-3 site designated TTR (lanes 4, 8 and 13) at 20-fold molar excess.
Probes:

4A

4B

4AB

Competitor

- Self 2H1 TTR

- INF-4A INF-3B 2H1 TTR

1 2 3 4

5 6 7 8

9 10 11 12 13
retarded band was observed. Unlike the HepG2 nuclear extract, only one isoform, HNF-3β, is observed with chick embryo liver nuclear extract (75). Formation of this complex was inhibited by a 20-fold excess of cold self (lane 2) while 20 fold excess of a non specific probe did not inhibit protein complex formation (lane 3). Similarly probe 4B that contains the HNF-3 B site also formed a protein complex when incubated with nuclear extract (lane 5). Again formation of this single complex was inhibited by a 20 fold excess of cold self (lane 6) but not with a 20 fold excess of a non specific probe (lane 7). The two HNF-3 sites can also compete with one another. A 20 fold excess of HNF-3 B is able to prevent protein complex formation with probe 4A (lane 4) while a 20 fold excess of HNF-3 A is able to significantly inhibit protein complex formation with probe 4B (lane 8). Further, protein complex formation with 4A, 4B and 4AB probes was substantially inhibited with a 20 fold excess of a double stranded oligonucleotide containing the CYP2H1 HNF-3 site (Fig. 4.10, lanes 3, 7 and 12) as well as one containing an authentic HNF-3 binding site from the mouse transthyretin gene (lanes 4, 8 and 13)(70). Similarly, protein binding to probe 4AB was significantly inhibited with a 20 fold excess of double stranded oligonucleotides containing either the HNF-3A site (lane 10) or the HNF-3B site (lane 11). The weak faster migrating bands observed with probes 4B and 4AB (marked with *) are non-specific and of unknown origin and are not consistently observed. Taken together these results indicate that both of the HNF-3 sites in the CYP2H2 promoter are capable of binding a specific protein when tested individually or together and that this protein is HNF-3.

4.2.6 Footprint analysis of mutated HNF-3 binding sites

As shown earlier, it was of interest to note that no protected region over the HNF-3 binding sites was observed in footprint analysis of the CYP2H2 promoter but a retarded complex is seen in gel shift experiments using oligonucleotides that contained either or both HNF-3A and B sites. As mentioned this may be due to excess probe employed in gel shift analysis; under such conditions there may not be enough HNF-3 protein to bind both sites simultaneously. However, in footprint analysis the concentration of protein to probe is sufficiently high to allow protein binding to both HNF-3 sites simultaneously. It is proposed that under these conditions, the binding of protein at one site displaces protein
Figure 4.11  DNase I footprint analysis of the HNF-3A mutated CYP2H2 promoter.
CYP2H2 promoter fragment from -198 to +39 in which the HNF-3A site was mutated was radiolabelled on the coding strand (A) and noncoding strand (B) and footprint analysis performed as described in the Materials and Methods with the addition of chicken liver nuclear extract (lane 3) or without this addition (lanes 2 and 4). The fragment was partially cleaved at G and A residues as a marker for the sequence (lane 1). The DNase I protected regions (A-E) are bracketed. A represents the TATA protein binding site at -31/-23, B the USF protein binding site at -54/-42, C the C/EPB protein binding site at -74/-56, D the HNF-1 protein binding site at -121/-95 and Eb the putative HNF-3B site at -165/-143.
Figure 4.12  DNase I footprint analysis of the HNF-3B mutated CYP2H2 promoter.

CYP2H2 promoter fragment from -198 to +39 in which the HNF-3B site was mutated was radiolabelled on the coding strand (A) and noncoding strand (B) and footprint analysis performed as described in the Materials and Methods with the addition of chicken liver nuclear extract (lane 3) or without this addition (lanes 2 and 4). The fragment was partially cleaved at G and A residues as a marker for the sequence (lane 1). The DNase I protected regions (A-E) are bracketed. A represents the TATA protein binding site at -31/-23, B the USF protein binding site at -54/-42, C the C/EPB protein binding site at -74/-56, D the HNF-1 protein binding site at -121/-95 and Ea the putative HNF-3A site at -171/-152.
bound at the adjacent site. This is discussed in more detail later. To investigate whether an arrangement of two overlapping HNF-3 binding sites may prevent HNF-3 binding to any one of the sites within the CYP2H2 promoter, mutations that abolish HNF-3 binding were introduced into one (m4A) or other (m4B) of the HNF-3 binding sites (Fig 4.6B) within the -198/+39 promoter fragment. The base pair mutations introduced were identical to those used to inactivate the HNF-3 A and B sites in the promoter/CAT constructs (see later Fig. 4.13). A DNase I footprint assay was performed with promoter fragments radiolabelled on either the coding strand or the non-coding strand and incubated with 18 day old chick embryo liver nuclear extract. With the HNF-3 A site mutated (m4A) a protected region, designated as E, was observed over the HNF-3 B site (Eb; -165 to -143) on the non-coding strand (Fig 4.11). Similarly, when the HNF-3 B site was mutated (m4A) a protected region was observed over the HNF-3 A site (Ea; -171 to -152) weakly on the coding strand but very clearly on the non-coding strand (Fig 3.13). Hence, in the absence of each overlapping site, HNF-3 can bind to the promoter in vitro. The four protected regions previously identified (A, B, C and D, see Fig. 4.7) were also detected in this experiment.

4.2.7 Transient expression of mutant HNF-3 constructs

The footprint experiments of wild-type versus mutant HNF-3 sites provided the interesting finding that the wild-type configuration of the HNF-3 binding sites prevents HNF-3 binding to either the A or B site indicating that this may be the reason for the weaker promoter activity of the CYP2H2 gene. However, binding was achieved when the overlapping HNF-3 site was inactivated. In the following experiment the two HNF-3 sites were mutated individually in p198CAT and their activity analysed by transient expression assays in chick embryo hepatocytes. The overlapping HNF-3 sites were mutated in two ways. Sequence alignment of the CYP2H2 promoter with the CYP2H1 promoter (Fig. 4.2) reveals that 8 additional nucleotides (5' GAGCAAAG 3') are present in the CYP2H2 promoter. This 8 bp region was removed by site directed mutagenesis (Fig. 4.6B), to examine the possibility that this sequence in some way disrupted functional activity of the adjacent HNF-3A site. The expression of this construct, designated p198ACAT and containing only the HNF-3A site, in chick embryo hepatocytes was approximately 8-fold
higher than the wild-type construct, designated p198CAT (Fig. 4.13). Secondly, mutations that destroy binding of either the HNF-3A site or the HNF-3B site but leave the other HNF-3 site intact were produced. This was achieved by using site directed mutagenesis to introduce a Sall restriction enzyme site into the core region of the HNF-3 sites, changing 5' GAGCAAAAC(A/T) 3' to 5' GAGGTCGAC 3' (Fig. 4.6B). Mutation of these bases within the core region of the HNF-3 binding site has been shown previously to abolish HNF-3 binding by both gel shift analysis (267) and transient transfection analysis (61). Mutation of the HNF-3A site in construct p198MABCAT produced a 7.9-fold increase in expression of this construct when compared with the wild type construct p198CAT. Mutation of the HNF-3B site in construct p198AMBCAT produced approximately 6.4 fold increase in CAT reporter gene expression compared to the wild-type construct. The expression level of these mutant constructs (p198ACAT, p198MABCAT and p198AMBCAT) approached the level of the wild-type CYP2H1 promoter construct, p205CAT. These results demonstrate that removal or mutation of one or other of the HNF-3 sites with in the CYP2H2 promoter markedly increases transactivation of the CYP2H2 reporter constructs, presumably by allowing HNF-3 to bind to the remaining site, as indicated by the in vitro DNase I footprint analysis (Figs. 3.11 and 3.12). The data strongly suggest that HNF-3 is unable to bind to the duplicated HNF-3 region and possible explanations and mechanisms will be outlined in the discussion.

The CYP2H1 promoter construct p205MHCAT in which the HNF-3 site was mutated by introduction of 5 bp changes into the core sequence of the HNF-3 site from 5' GAGTAAACA 3' to 5' GAGTTCGAT 3' shows a 65% decrease in expression compared to wild-type (p205CAT) expression (Fig 4.13). While this is a significant decrease in expression it is approximately 2.5 fold greater than that of the wild-type CYP2H2 construct (compare p205MHCAT with p198CAT, Fig 4.13). It is suggested that the configuration of the HNF-3 sites within the wild-type p198CAT construct prevents HNF-3 protein binding to either HNF-3 site. It was assumed that as both constructs have functional HNF-1, C/EBP, USF and TATA binding sites but non-functional HNF-3 binding sites they should
Figure 4.13  Transient basal expression of wild-type and mutant CYP2H2 promoter constructs in chick embryo hepatocytes.

Mutations were introduced into HNF-3 transcription factor binding sites in the constructs p198CAT and p205CAT. Chick embryo hepatocytes (2 x 10^7) were transfected in the presence of 500 µg/ml salmon sperm DNA with 2 pmol of each construct by electroporation at 250 V and 960 µF. Each sample was cultured in William’s E medium supplemented with 10% Serum Supreme for 48 h. CAT assays were performed on 50 µg of cellular protein. The data represents the average of three independent experiments, repeated in duplicate and represented as a percentage of conversion of [14C]chloramphenicol to acetylated product are shown as the mean ± standard deviation. CAT activities were normalised to β-galactosidase activity.
Acetylation of Chloramphenicol

% Acetylation of Chloramphenicol

0 10 20 30 40 50 60 70 80

CYP2H2

CYP2H1
express at very similar levels. Possible explanations for the observed differences in expression are considered in the discussion.

4.3 Discussion

The purpose of this study was to investigate why the highly homologous and coordinately regulated and drug induced chicken CYP2H1 and CYP2H2 genes exhibit a marked difference in their induced steady state mRNA levels and thus shed light on the mechanism of induction of these genes. A similar expression pattern is seen in related phenobarbital-inducible genes such as the rat CYP2B1/2 genes where the control and induced mRNA levels for the CYP2B2 gene are at least 5 to 10 fold higher than that expressed by the CYP2B1 gene (53). In a similar fashion the induced mRNA level of the CYP2b9 gene was shown to be markedly lower than that of the highly homologous CYP2b10 gene in phenobarbital treated mouse liver (158).

Our repression model for the regulation of the CYP2H1/2 genes suggests that promoter strengths contribute substantially to the steady state levels of expression of these genes and although the two promoters are highly homologous, induced expression levels of the CYP2H2 gene are significantly lower than that of the CYP2H1 gene in vivo. Thus, one possible explanation for this observed difference is that the CYP2H2 promoter is a weaker initiator of transcription than that of the CYP2H1 promoter. The relative contribution of the enhancer region to the overall activity of these genes is unknown but a second possibility is that the enhancer region of the CYP2H2 gene is less active than that of the CYP2H1 gene resulting in decreased drug responsive expression of the CYP2H2 gene.

Initial investigations focused on the enhancer activity of the CYP2H2 gene. It was demonstrated that a 556 bp region at position -1956/-1400 in the CYP2H1 gene confers drug inducibility. To investigate if the enhancer region in the CYP2H2 gene is the same or different to that of CYP2H1, the corresponding region was isolated from the CYP2H2 gene as a 1100 bp PvuII fragment, the location of which remains to be determined. Transient transfection assays demonstrated that this region could confer drug inducibility to a
heterologous promoter to the same extent as does the CYP2H1 enhancer domain. Sequence comparison revealed that the 556 bp CYP2H1 enhancer region was entirely conserved within the CYP2H2 gene. These results suggested that the in vivo expression variation of these two genes is not due to a weaker CYP2H2 drug responsive enhancer region.

Isolation and characterisation of the promoter of the CYP2H2 gene was then carried out. Deletion and transient transfection analysis demonstrated that the minimum region required to drive basal expression of the CYP2H2 gene was -198/+39. Comparison with the corresponding region of the CYP2HI gene revealed a high degree of homology between these two promoters, the major difference being the duplication of 8 bp at position -161/-154 in the CYP2H2 promoter which produced a second HNF-3 transcription factor binding site. In addition to the overall high homology, the three functional cis-acting regulatory elements previously identified in the early promoter of the CYP2HI gene (75), are present in the early promoter of the CYP2H2 gene and are identical in sequence.

DNase I footprinting analysis of the early promoter (-198/+39) of CYP2H2 revealed that, while protected regions were observed over the binding sites for HNF-1, C/EBP, and USF, no binding was observed over the HNF-3 sites, indicating that this may be the reason for the lower expression of CYP2H2. This led to the extensive investigations of the two overlapping HNF-3 sites.

Three isoform of HNF-3 have been reported (190, 191). These isoforms, HNF-3α, HNF-3β and HNF-3γ, belong to family of liver-enriched transcription factors. In hepatocytes, HNF-3 transcription factors function as activators of liver-specific gene expression (344). HNF-3α was originally identified as a protein that binds specifically to sequences proven to be functionally important in the liver-specific expression of the transthyretin (TTR) and α1-antitrypsin genes (35, 61). Other target sites for HNF-3 proteins have been described in the promoter/enhancer region of the genes encoding α-fetoprotein (225), albumin (138), tyrosine aminotransferase (244), phosphoenolpyruvate kinase (152), transferrin (13), and aldolase B (114) and of the transcription factors HNF-1α (189) and HNF-3β (259). HNF-3
has also been implicated in cellular differentiation and gut and neural development (229). HNF-3 binds to DNA as a monomer via a divergent helix-turn-helix motif, referred to as the winged helix motif (30, 190). The winged helix DNA-binding motif is structurally similar to the globular domain of linker histone (56) and HNF-3 has been implicated in organising the nucleosome architecture of the albumin enhancer in hepatocytes (301, 323). Thus, HNF-3 proteins not only contribute to transcriptional activation, but are also required for the establishment of hepatocyte-specific protein accessibility within the regulatory region of these genes.

The HNF-3 sites 5' CTCTGTTTGCTC 3' and 5' TCAAGTTTGCTC 3' located on the non-coding strand of the CYP2H2 promoter are almost identical to the consensus HNF-3 sequence 5' (C/A/G)A(A/T)T(G/A)T(T/G)(G/A)(C/T)T(C/T) 3' compiled by analysis of sites in liver-enriched promoters (255). Both HNF-3 sequences specifically bound one major protein complex in gel mobility shift assays using chick embryo liver nuclear extracts and this complex was competed by an HNF-3 binding site from the mouse transthyretin gene (70) and the CYP2H1 gene (75). Neutralising antibodies specific for the rat HNF-3 α and HNF-3 β proteins prevented protein complex formation with HepG2 nuclear extracts. In addition, binding of HNF-3 was observed when both HNF-3 sites were located in an overlapping configuration as in the native configuration. This is in marked contrast to DNase I footprint assays in which no protein binding was observed. One possible explanation for these conflicting results is the inherent difference between these two assays. In the gel mobility shift assay the amount of probe is vastly in excess of the amount of specific protein able to bind to this probe. Hence, it is unlikely that HNF-3 protein will bind to both of the sites with in the oligonucleotide probe. In the DNase I footprinting assay the opposite situation is occurring: the amount of protein is in excess of the amount of probe. The two HNF-3 sites are very similar in sequence and the binding affinity of HNF-3 for either site is very similar as determined by gel shift analysis (Figs. 4.8-4.10). It is therefore possible that HNF-3, which binds as a monomer (323), is competing for two overlapping binding sites of similar affinity and it is this competition that prevents protein binding to either site. Thus stable protein binding to either HNF-3 site
for sufficient duration for transactivation to occur is never achieved. As far as we are aware, this represents a novel control mechanism, not previously reported in eukaryotic systems.

This conclusion is supported by mutation analysis. DNase I footprint analysis revealed that when the HNF-3A site is mutated, a protected region is observed over the HNF-3B site. Conversely, when the HNF-3B site is mutated a protected region is observed over the HNF-3A site. The conclusion from these data is that HNF-3 protein does not bind to the native CYP2H2 promoter, resulting in weaker overall expression from this promoter. Transient transfection analysis revealed that removal of the HNF-3B site increased expression of the promoter/CAT constructs relative to the wild-type construct and expression of this construct approached that of the wild-type CYP2H1 promoter construct. The same increase in expression was observed when one or other of the HNF-3 sites was mutated, where protein binding to the adjacent site was permitted. This data is consistent with the model where the steady state levels of the CYP2H1/2 genes are substantially determined by the relative promoter strengths, although the enhancer may contribute, and that these promoters are held in a repressed state possibly by chromatin structure (83) and that drug relieves this repression.

As discussed earlier, mutation of the HNF-3 site within the CYP2H1 promoter construct (p205CAT) markedly decreased expression of this construct, although expression of this construct was higher (1.3 fold) than the wild-type CYP2H2 promoter construct (p198CAT). If, as proposed, that the overlapping HNF-3 sites within the CYP2H2 promoter prevent protein binding and the remaining transcription factor binding sites of these promoters are identical as has been demonstrated, then these two constructs (p198CAT and p205MHCAT) should show similar levels of expression. The mutations used to destroy the HNF-3 site within the CYP2H1 promoter may not have completely inactivated this site and it is possible that the mutated CYP2H1 HNF-3 site retains weak protein binding affinity. Co-transfection experiments using the p205MHCAT construct and an HNF-3β expression construct in HeLa cells, that do not express endogenous HNF-3,
revealed a 2 fold increase in expression over that of p205MHCAT alone (75) suggesting that this may be a possible explanation. Other sources of variation include the difference in length of the CYP2H1 construct (-205/+39) compared to the CYP2H2 construct (-198/+39) or possible differences between the CAT reporter gene sequences or vector sequences of the p205CAT and p198CAT constructs. Experiments to eliminate these differences are being undertaken.

It was observed that, while deletion or mutation of one HNF-3 site markedly increased CYP2H2 promoter expression, activity of these constructs was consistently 1.3-1.5 fold lower than that of the wild-type CYP2H1 promoter construct (p205CAT). As discussed previously, both HNF-3 sites within CYP2H2 differ from the consensus sequence, while the HNF-3 site within CYP2H1 does not, suggesting that the CYP2H2 HNF-3 sites may be of lower affinity and this results in slightly weaker activity of the mutant CYP2H2 promoter constructs.

Despite these minor differences these results provide compelling evidence that the two adjacent HNF-3 sites in the CYP2H2 promoter, while able to function as HNF-3 protein binding sites in vitro, are non-functional in vivo and this is due to their overlapping nature. A natural extension of this result is that if HNF-3 protein concentration within the cell is limiting, HNF-3 protein binding may become possible due to a loss of competition between the two HNF-3 sites resulting in increased gene expression. The unusual configuration of these two HNF-3 sites may therefore allow regulation of this gene to be controlled by varying the concentration of cellular HNF-3 protein. It will be fascinating to discover why two functional protein binding sites are not able to achieve stable protein binding in vivo even for sufficient duration to allow gene transactivation.

Experiments are currently under way to further investigate the role of the HNF-3 sites within the CYP2H2 promoter. To determine whether HNF-3 can bind to either HNF-3 site of the CYP2H2 gene in vivo, a PIN POINT (protein position identification with nuclease tail) assay will be performed (195). In this assay an expression vector for a fusion protein
composed of HNF-3β and the nuclease domain of type IIS endonuclease FokI (175) is transiently transfected along with the p198CAT target plasmid into chick embryo hepatocytes. The nuclease domain of FokI lacks sequence specificity and hence the probability of cleavage by the HNF-3β- nuclease fusion protein is determined by HNF-3β. If HNF-3β does in fact bind to either HNF-3 site within the CYP2H2 promoter, the nuclease will cleave the DNA on one side of the recognition sequence. The cleavage site is then determined by primer extension.

To determine whether it is the overlapping nature of the two HNF-3 sites that prevents protein binding, the two sites will be separated by a number of nucleotides and examined for protein binding by transient transfection and DNase I footprinting assays. It is worth noting that the distance between the core sequences of the two HNF-3 sites is 10 bp (see Fig. 4.6). Thus, the postulated competition between the binding of HNF-3 to these sites occurs on the same side of the helix. It will be of interest therefore to create a promoter construct in which a segment of DNA is inserted between the HNF-3 sites such that they are located on opposite sides of the DNA helix. It is possible that in this case both HNF-3 sites may become active.

Experiments to purify HNF-3β to homogeneity are being carried out to allow DNase I footprinting and gel shift assays to be performed in an attempt to determine at what concentration HNF-3 is able to bind to one or other or both HNF-3 sites. In addition, the relative affinities of the two sites compared with each other as well as the HNF-3 site from the CYP2H1 gene will be examined.

In conclusion, an apparently novel mechanism for gene regulation has been identified in which duplication and subsequent overlap of a control element has led to the inactivation of this element. The two genes CYP2H1 and CYP2H2 are predominantly controlled through this difference in promoter activity with the drug responsive regions most likely being identical.
Chapter 5. Studies on the effect of RU486 on the induction the CYP2H1 gene
5.1 Introduction
Traditionally the cytochrome P450 superfamily has been separated into two broad groups, those that metabolise foreign compounds and those that metabolise endogenous compounds. The CYP enzymes involved in metabolism of endogenous substrates are regulated via distinct pathways exclusively dedicated to a particular substrate. Until recently CYP enzymes involved in xenobiotic metabolism were thought to be regulated via similar dedicated pathways. However, there is increasing evidence that the primary role of the regulatory pathways involved in xenobiotic metabolism may be to modulate hepatic P450 activity in response to endogenous dietary or hormonal stimuli and are not exclusively dedicated to metabolism of foreign compounds. This is evidenced by the fact that steroids and members of the steroid receptor/nuclear receptor superfamily (89) have been implicated in xenobiotic induction mechanisms (218, 223). For example, (i) a novel orphan nuclear receptor, termed CAR (see Section 1.5.4.3.2), is implicated in the induction of the CYP2B genes by phenobarbital (PB) and many other “PB-like” lipophilic chemicals (147, 334, 352); (ii) the pregnane nuclear receptor PXR, which activates CYP3A genes in response to diverse chemicals, including certain natural and synthetic steroids (22, 26, 180); and (iii) the peroxisome proliferator-activated receptor PPAR (232), which mediates induction of the fatty acid hydroxylases of the CYP4A family by many acidic chemicals classified as ‘non-genotoxic’ carcinogens and peroxisome proliferators (103).

The discovery of endogenous ligands for CAR (androstanes, which inhibit receptor activity), PXR (certain pregnenolone derivatives and other steroids), and PPAR (certain polyunsaturated long-chain fatty acids and their metabolites) supports the proposed role for these receptors in modulating liver CYP expression in response to endogenous hormonal stimuli (237), in addition to their more obvious role in modulating liver capacity for foreign chemical metabolism by induction of cytochrome P450 enzymes.

The glucocorticoid group of steroid hormones have been implicated in the regulation of several classes of xenobiotic metabolising CYPs. The aromatic hydrocarbon inducible
CYP1A1 gene is induced by dexamethasone and metyrapone in combination and appears to act via the glucocorticoid receptor (128), while a functional glucocorticoid response element has recently been identified in the rat CYP3A1 gene (264). The major phenobarbital inducible rat CYP2B1/2 genes have been shown to contain glucocorticoid response elements in their promoters which are required for maximal phenobarbital responsiveness (333). Similarly, the synthetic glucocorticoid dexamethasone alone was shown to markedly induce the mouse homologues (Cyp2b9 and Cyp2b10) of these rat genes, suggesting involvement of the glucocorticoid hormone in expression of these genes (158).

In support of steroid hormones/glucocorticoids playing a critical role in phenobarbital-induction in mammals, Shaw and coworkers demonstrated that RU486, a glucocorticoid (164, 243) and progesterone antagonist (17, 133), can block phenobarbital induction of the rat CYP2C6 gene in rat hepatoma cells (320). In addition, RU486 was shown to prevent phenobarbital induction of transiently transfected reporter constructs containing a 1.4 kb of 5' flanking region of either the CYP2B1 or CYP2B2 genes (320). Since this work in 1993, there have been no studies on the effect of RU486 on phenobarbital induction of CYP genes. RU486 belongs to the class of 11-β substituted 19-nor-steroids. RU486 has a higher affinity for the progesterone and glucocorticoid receptors than their endogenous ligands (progesterone and glucocorticoid respectively)(4, 170), but displays pure antagonist activity towards both progesterone and glucocorticoid without any agonist activity (116, 165, 227).

The data of Shaw (320) implicate either a progesterone or glucocorticoid sensitive receptor in the phenobarbital induction process. It is possible that the progesterone or glucocorticoid receptor bound by their cognate ligand activate transcription of phenobarbital-inducible genes by binding directly to control elements in these genes and that RU486 competes with the ligand thus inhibiting transactivation. A second indirect model has been proposed (320). In this model, an endogenous inducer is normally inactivated by a specific P450 that is inhibited by phenobarbital. Treatment by phenobarbital would lead to the accumulation
of an endogenous steroid-like compound, which then binds to its specific receptor to form a complex that directly activates CYP gene expression. In this model, RU486 would function as an anti-inducer by competing with the steroid-like compound for the receptor. This chapter examines the effect of RU486 treatment on the phenobarbital-inducibility of the chicken CYP2H1 gene with the aim of further elucidating the mechanism of induction of this gene.

As discussed in chapter 3, our laboratory has previously characterised two phenobarbital-inducible genes, CYP2H1 and CYP2H2 in the chicken (119, 125, 221). Extensive studies using CAT reporter gene constructs containing 0.5 to 8.9 kb of 5'-flanking sequence of the CYP2H1 gene transiently transfected into cultured chick embryo hepatocytes have identified a 4.8 kb BamHI fragment at position -5400/-1100 that contains two drug responsive regions (73). Restriction enzyme analysis of this enhancer domain was further used to map these drug responsive regions to a 556 bp BglII/XhoI restriction fragment and a 1.36 kb fragment at the 5' end of the 4.8 kb enhancer (73). The 556 bp fragment increases CAT activity of an SV40 enhancerless reporter plasmid by 7-fold in the presence of drug (73).

Initially, the effect of RU486 on drug induction was investigated by Northern blot analysis of RNA isolated from chick embryo liver. Once it was established that RU486 could inhibit induction of endogenous CYP2H1 mRNA, transient transfection studies using reporter constructs containing fragments of the phenobarbital-responsive enhancer region were undertaken in an attempt to isolate an RU486 responsive region within this enhancer of the CYP2H1 gene.

5.2 Results

5.2.1 RU486 Inhibits the Drug-Induced Increase in the Levels of CYP2H1 mRNA
To investigate whether RU486 can act as an anti-inducer of the phenobarbital induction mechanism in the avian system, the effect of RU486 on the steady-state levels of mRNA for the CYP2H1 gene in drug-induced chick embryo hepatocyte cultures was examined.
Chick embryo hepatocytes were prepared and cultured as described previously (Section 2.2.11.1-2). These cultures were then treated with RU486 at concentrations ranging from 40 µM to 90 µM for 1 hour prior to the addition of phenobarbital at a concentration of 500 µM. The cells were then incubated for a further 6, 8, 12 or 24 hours. At each of these times, total RNA was isolated using the guanidinium isothiocyanate method (see 2.2.10.1) and the level of CYP2H1 mRNA determined by Northern blot analysis using a probe, specific for CYP2H1 mRNA (125). To control for loading, the filter was then stripped and reprobed for GAPDH mRNA. The results of autoradiographs of these filters are shown in Figure 5.1A. The result shown is for a 6 h incubation with phenobarbital/RU486. The data for the other 8, 12 and 24 h incubation periods are not shown as it was identical to that for the 6 h incubation period. Phenobarbital treatment of chick embryo hepatocytes produced an 8.8 fold increase in mRNA expression (lane 1 versus lane 3) as measured 6 hours after the addition of phenobarbital. RU486 at 100 µM had no effect on basal (non-induced) mRNA levels (lane 2). At concentrations up to 70 µM, RU486 had very little effect on phenobarbital-induced expression of the CYP2H1 mRNA. However, at concentrations of 80 and 90 µM, RU486 almost completely abolished phenobarbital-mediated induction (lanes 8 and 9). This effect was seen in repeated experiments. In addition the level of mRNA expression of GAPDH remained unchanged after treatment with phenobarbital or RU486 either alone or in combination. Hence, RU486 appears to specifically inhibit phenobarbital induction of the CYP2H1 gene.

To establish that the inhibition of phenobarbital induction did not result from RU486-mediated cell toxicity or irreversible cell damage the following experiment was performed. Chick embryo primary hepatocytes were treated with either 50 or 100 µM of RU486 for a period of 24 or 48 h. After this treatment the media was removed, cells washed once with PBS and given fresh media containing 500 µM of phenobarbital and incubated for a further 24 h. Total RNA was extracted and Northern blot analysis performed as described above. An autoradiograph using a CYP2H1 gene specific probe (pCHB15) is shown in Figure 5.1B. Following removal of RU486 from the cells a similar level of induction of CYP2H1 mRNA compared to non-RU486 treated cells was observed indicating that RU486, at
Figure 5.1  Effect of RU486 on induced levels of CYP2H1 mRNA.

A. Representative Northern blot analyses of steady-state levels of mRNAs are shown. Hepatocytes were pretreated with RU486 (40-90 μM), 1 h prior to the addition of phenobarbital (PB) at 500 μM. After a further 6 h incubation, total RNA was isolated and 15 μg analysed by Northern blotting. The filter was hybridised in turn with $^{32}$P-labeled probes specific for CYP2H1 (pCHB15) and GAPDH (as a control). These mRNAs are approximately 3.5 and 1.8 kb in length, respectively. Radiolabelled filters were quantified using a Phosphoimager (Molecular Dynamic Model 300A), the level of CYP2H1 mRNA was standardised to that of GAPDH mRNA and the relative amounts of mRNA plotted for comparison as arbitrary densitometric units. pCHB15 is a cDNA isolated from the divergent 3' UTR of the CYP2H1 gene. This cDNA is specific for the CYP2H1 mRNA only.

B. Reversal of RU486 inhibition of CYP2H1 induction. Hepatocytes were treated with RU486 at 100 μM for 24 or 48 h and the media replaced with media lacking RU486. The cells were left for 18 h at which time fresh media without PB (lane 1) or with 500 μM PB (lanes 2-5) were added and the cells incubated for a further 6 h. Total RNA was isolated and analysed for CYP2H1 and GAPDH mRNAs as described above. Control cells were treated in the same way but were not initially exposed to RU486 or PB (lane 1).
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![Image of CYP2H1 and GAPDH gel with lanes 1 to 9 and corresponding densitometric analysis]

B

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![Image of CYP2H1 and GAPDH gel with lanes labeled 1 to 5 and corresponding densitometric analysis]
concentrations up to 100 \(\mu\)M and incubation times up to 48 h, does not affect the ability of chick embryo hepatocytes to respond to phenobarbital. This data therefore show that RU486 is specifically interfering with the phenobarbital-responsive mechanism and does not affect the basal expression of the \(CYP2H1\) gene.

5.2.2 Effect of RU486 and dexamethasone on \(CYP2H1\) mRNA expression

It is possible that the inhibition of phenobarbital induction by RU486 is caused by its antagonist effect on the glucocorticoid receptor which plays some as yet unknown role in phenobarbital induction of the \(CYP2H1\) gene. Dexamethasone, a potent synthetic glucocorticoid agonist, has been shown previously to activate mammalian phenobarbital-inducible CYP genes at concentrations ranging from \(10^{-9}\) M to \(10^{-5}\) M (186, 320, 325) and to inhibit the antiglucocorticoid action of RU486 (171, 284). Hence, dexamethasone was employed to determine whether the glucocorticoid receptor is involved in the drug-mediated induction of the \(CYP2H1\) gene. Chick embryo primary hepatocytes were treated with either 1 or 10 \(\mu\)M of dexamethasone, alone or in combination with 500 \(\mu\)M of phenobarbital. The results of Northern analysis of total RNA extracted from these cultures are shown in Figure 5.2. In contrast to the rat \(CYP2B1/2\) genes (186, 325), dexamethasone, at similar concentrations, had no effect on either the basal or phenobarbital-induced expression levels of \(CYP2H1\) mRNA. The level of GAPDH mRNA expression remained unchanged. A subsequent experiment investigating the effect of dexamethasone on RU486-mediated inhibition of induction was performed.

Chick embryo primary hepatocytes were treated with either 1 or 10 \(\mu\)M of dexamethasone in combination with 500 \(\mu\)M phenobarbital or 80 \(\mu\)M RU486 or with phenobarbital and RU486 together. Northern analysis of RNA prepared from these cells was performed and autoradiographs using radiolabelled pCHB15 or GAPDH cDNA as probes are shown in Figure 5.3A. RU486 at this concentration, while having no effect on basal expression (lane 3) effectively prevented induction by phenobarbital (lane 4). Dexamethasone did not, in combination with either phenobarbital or RU486, alter the basal or phenobarbital-induced \(CYP2H1\) mRNA levels (lanes 5, 8 and 6, 9) with respect to RU486 or phenobarbital alone.
Figure 5.2  Effect of Dexamethasone or cAMP on expression of CYP2H1 mRNA.
Representative Northern blot analyses of the steady state levels of mRNAs are shown. Chick embryo hepatocytes were pretreated with Dex (1 and 10 μM), cAMP (1 mM) or RU486 (100 μM), 1 h prior to the addition of phenobarbital (PB) at 500 μM. After a further 6 h incubation, total RNA was isolated analysed for CYP2H1 and GAPDH (control) mRNAs as described in Fig. 5.1. Control cells were treated in the same way but were not initially exposed to Dex (lane 1), PB (lane 1 and 7), RU486 (lane 7) or cAMP (lane 7).
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**pCHB15**

![pCHB15 Image]

**GAPDH**

![GAPDH Image]

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Bar graph showing the quantification of pCHB15 and GAPDH expression with different treatments.
Figure 5.3  Effect of Dexamethasone on the RU486 mediated inhibition of induction of CYP2HI mRNA

A. Chick embryo primary hepatocytes were treated with either 1 or 10 µM of dexamethasone in combination with 500 µM phenobarbital or 100 µM RU486 or with phenobarbital and RU486 together. Total RNA was isolated and analysed for CYP2HI and GAPDH mRNAs as described in Fig 5.1. Control cells were treated in the same way but were not initially exposed to Dex (lanes 1 and 2), PB (lane 1 and 3) or RU486 (lanes 1 and 2).

B. A MMTV/luciferase reporter construct was transfected into chick embryo hepatocytes by electroporation, each cuvette was halved and then either Dex (0.1 µM) or Dex and RU486 (25 or 50 µM) added to one dish while PBS was added to the control dish. The average of three independent experiments, repeated in duplicate, is represented as relative light units per 100 µg of protein.
(lanes 2 and 3). Most importantly, dexamethasone, at 1 or 10 μM, was unable to reverse the inhibition of drug induction mediated by RU486 (lanes 7 and 10), suggesting that RU486 does not suppress phenobarbital induction via a mechanism involving the glucocorticoid receptor.

To establish the efficacy of dexamethasone and the concentration of RU486 required to antagonise dexamethasone, a luciferase reporter construct driven by the dexamethasone responsive mouse mammary tumour virus long terminal repeat (Section 2.2.15) was transfected into chick embryo hepatocytes (Fig. 5.3B). Dexamethasone induced this construct (3-fold) above control and importantly RU486 at 25 and 50 μM concentrations significantly repressed induction. These results indicate that dexamethasone can induce gene expression via the glucocorticoid receptor in chick embryo hepatocytes and that RU486 can block this induction at a concentration as low as 25 μM.

In addition to dexamethasone, cAMP has been shown to reverse the antagonistic effect of RU486 on the progesterone (19, 303) and glucocorticoid receptors (246), possibly due in part to a disruption of the interaction between the progesterone or glucocorticoid receptors and the corepressors NCoR and SMRT (346). To determine whether the progesterone of glucocorticoid receptor may be involved in mediating the RU486 suppression of phenobarbital induction, chick embryo hepatocytes were treated with cAMP at a concentration of 1mM either alone, with phenobarbital, with RU486 or with phenobarbital and RU486 together. Treatment of chick embryo hepatocytes with cAMP had no effect on phenobarbital induction, or RU486 mediated inhibition of induction (Fig. 5.2), suggesting that neither the progesterone nor the glucocorticoid receptor is involved in phenobarbital induction of the CYP2HI gene.

This data suggest that RU486 may be interfering with a novel pathway involved in the phenobarbital response of the CYP2HI gene. One way to isolate this RU486 sensitive pathway would be to identify the sites in the CYP2HI gene that RU486 is operating
through. This could in turn lead to identification of proteins involved in the phenobarbital response.

5.2.3 RU486 inhibits drug-induction of CYP2H1/CAT chimeric constructs

In previous transient transfection studies, CAT reporter gene constructs containing 0.5 to 8.9 kb of 5' flanking sequence of the chicken CYP2H1 gene were transiently expressed in chick embryo hepatocytes (119). This study lead to the identification of a 4.8-kb BamHI fragment (-5900 to -1100) which behaved as a drug-responsive enhancer. To determine whether RU486 could also suppress phenobarbital induction from this construct and to localise the RU486 responsiveness to a particular region, the effect of RU486 on the expression of this 4.8 kb phenobarbital-responsive enhancer fused to the enhancerless SV40 promoter/CAT reporter plasmid was investigated. The above construct, designated p4.8SVCAT (see Fig 5.5B), was transfected into chick embryo hepatocytes by electroporation, each sample was halved and either phenobarbital or phenobarbital and RU486 (40 or 80 µM) was added to one dish while PBS was added to the control dish. A typical CAT assay of one such experiment is shown in Figure 5.4A and the average of three independent experiments represented as a percentage conversion of \[^{14}C\]chloramphenicol to acetylated product is shown in Figure 5.4B. As expected, expression of the enhancer construct (p4.8SVCAT) is increased 9.3 fold by phenobarbital treatment. Importantly, RU486 at 80 µM markedly lowered phenobarbital induction (5.9 fold) while RU486 at 40 µM did not antagonise induction by phenobarbital. At a concentration of 80 µM, RU486 did not affect basal expression of this construct. The results from this experiment parallel those obtained previously in which RU486 specifically inhibited phenobarbital induction of the endogenous CYP2H1 gene. Collectively these data indicate that the RU486 responsive region appears to be located within the phenobarbital-responsive region of the CYP2H1 gene.

The effect of RU486 on the expression of the p205CAT construct containing the proximal CYP2H1 promoter was also investigated. As discussed in chapter 3, one model of phenobarbital induction proposes that the phenobarbital-responsive enhancer leads to
Figure 5.4  Effect of RU486 on the transient expression of p4.8SVCAT and p205CAT. The p4.8SVCAT and p205CAT constructs were transfected into chick embryo hepatocytes, each sample halved and phenobarbital (500 μM) or RU486 (40 and 80 μM) added to one dish and PBS to the control dish. When analysing the effect of phenobarbital and RU486 in combination, the control plate received phenobarbital (500 μM) and the other plate received phenobarbital (500 μM) and RU486 (40 and 80 μM).

A. A typical CAT assay of one such experiment, repeated in duplicate is shown.

B. The average of three independent experiments, repeated in duplicate and represented as a percentage of conversion of [\textsuperscript{14}C]chloramphenicol to acetylated product is shown. Numbers above columns represent the fold increase in CAT activity compared to untreated (control) activity.
Figure 5.5  Diagrammatic representation of the 5' portion of the CYP2H1 gene and CYP2H1/CAT constructs.

A. Diagram of the CYP2H1 gene and the 5' flanking region showing the positions of restriction sites important in the production of CYP2H1/CAT constructs. The line represents the 5' flanking region, the arrow shows the position of the transcription start site (+1) and the numbers are positions, in kb, upstream from this site.

B. Diagrammatic representation the 4.8 kb phenobarbital-responsive enhancer domain and SV40 promoter/CAT constructs. Subfragments of the 4.8 kb BamHI fragment were isolated and cloned immediately upstream of the enhancerless SV40 promoter in the vector construct pBCSVp1. The subfragments are indicated by lines containing an arrowhead pointing in the 5' to 3' orientation. The length of each fragment is shown above each arrowed line.

C. 5'-End deletions of the 4.8 kb BamHI enhancer fragment, from 4.1 to 1.9 kb, were fused to the enhancerless SV40 promoter and a CAT reporter gene in the pCAT vector. Shown is the 5' position in kb where the fragments begin. The length of each fragment is shown above each arrowed line.
A

4.8 kb Enhancer

EcoRI BamHI XbaI BglII BamHI

-8.9 -5.91 -4.55 -1.95 -1.40 -1.1

B

4.8 kb

1.36 kb

-5.2

4.1 kb

-4.2

3.1 kb

-3.6

2.5 kb

-3.0

1.9 kb

556 bp

C

SV CAT p4.8SVCAT

SV CAT pR1SVCAT

SV CAT p556SVCAT

SV CAT p4.1SVCAT

SV CAT p3.1SVCAT

SV CAT p2.5SVCAT

SV CAT p1.9SVCAT
derepression of the strong promoter. Thus it is possible that RU486 inhibits drug induction by reducing promoter activity. The p205CAT construct contains 205 bp of the proximal promoter of the CYP2HH gene and has been shown to direct strong basal expression in transient transfection assays but does not respond to phenobarbital (75). Transient transfection analysis of this construct was carried out in an identical manner to that described above. RU486 at 80 μM had no effect on the level of expression (Fig. 5.4). This data suggested that RU486 is acting specifically to inhibit phenobarbital-induced expression of CYP2HH by interfering with the phenobarbital-responsive enhancer region. Furthermore, the finding that the 4.8 kb enhancer is repressed by RU486 allows for the identification of an RU486 responsive region or element.

5.2.4 Isolation of an RU486 responsive region
As described previously, there appears to be at least two independent drug responsive regions within the 4.8 kb enhancer. One contained within a 556 bp BglII/XhoI restriction fragment, located at the 3' end of the enhancer region, and the second localised to a 1360 bp BamHI/XbaI restriction fragment, near the 5' end of the 4.8 kb enhancer region (73). The results discussed previously indicate that RU486 does not mediate repression of drug induction via the glucocorticoid or progesterone receptors. An alternate possibility is that RU486 is interfering directly or indirectly with proteins binding to the phenobarbital-responsive enhancer region or that RU486 may encourage the binding of a repressor to, or inhibit the release of a repressor from, the enhancer region. In an attempt to isolate regions within the 4.8 kb enhancer through which RU486 may be acting, constructs containing either deletions from the 5' end of the 4.8 kb enhancer or the restriction fragments that contain known phenobarbital-responsive regions (Fig. 5.5) were transfected into chick embryo hepatocytes and tested for RU486 responsiveness. After electroporation a single cuvette was halved and either incubated with phenobarbital or phenobarbital and RU486. Figure 5.6A shows a typical result of one such experiment while the average of three independent experiments, represented as a percentage conversion of [14C]chloramphenicol to acetylated product is shown in Figure 5.6B. As described (73), deletions from the 5’ end of the 4.8 kb enhancer caused a gradual decrease in phenobarbital induction from 9.1 fold
Figure 5.6  Effect of RU486 on expression of SV/CAT constructs containing fragments of the CYP2H1 4.8 kb enhancer.
The constructs described in Fig. 5.6 were transfected into chick embryo hepatocytes, halved and treated with RU486 (80 μM) and/or PB (500 μM) as described previously in Fig. 5.5.
A. A typical CAT assay of one such experiment is shown.
B. The average of three independent experiments, repeated in duplicate and represented as a percentage of conversion of [14C]chloramphenicol to acetylated product is shown.
for p4.1SVCAT to 3.6 fold for p1.9SVCAT indicative of multiple phenobarbital-responsive regions/elements with in the 4.8 kb enhancer. Significantly, RU486 reduced phenobarbital induction of all four deletion constructs p4.1SVCAT, p3.1SVCAT, p2.5SVCAT and p1.9SVCAT by between 2 and 4 fold. This result indicated that an RU486 responsive region was localised in p1.9SVCAT. This construct contains 1.9 kb of DNA from the 3’ end of the 4.8 kb enhancer region.

As discussed, two drug responsive regions of 1.36 kb and 556 bp with in the 4.8 kb enhancer have been characterised in our laboratory. The 556 bp region is located near the 3’ end of the smallest deletion construct p1.9SVCAT. Interestingly, RU486 treatment inhibited phenobarbital induction of pR1SVCAT by 3.7 fold, but had little effect on p556SVCAT. Thus these data suggest that there are two RU486 responsive regions, one in pR1SVCAT and the other in the region of p1.9SVCAT that does not include the 556 bp region from p556SVCAT. Therefore it appears that at least for p1.9SVCAT, RU486 does not directly interact with phenobarbital-responsive proteins binding to the 556 bp enhancer region. This data is consistent with RU486 causing binding or modification to proteins outside the minimal phenobarbital-responsive region that then interact with proteins within the 556 bp enhancer to suppress induction.

5.3 Discussion

We have identified in the early promoter of the CYP2H1 gene, the elements that determine basal expression (75) and located upstream, two drug responsive domains (73, 119). The aim of the work in this chapter was to investigate the mechanism of phenobarbital-induction of CYP2H1 gene expression in chick embryo hepatocytes. As discussed previously other phenobarbital-inducible CYP genes are regulated by glucocorticoids (128, 158, 264, 333) and RU486, a glucocorticoid antagonist, has been shown to inhibit phenobarbital induction of the rat CYP2B1/2 genes (320). Thus, the involvement of glucocorticoids in the induction response of CYP2H1 was investigated. RU486 was tested for its effect on the drug induced activation of the CYP2H1 gene. Pretreatment of hepatocytes with RU486 almost completely inhibited the drug induced increase in mRNA
levels of CYP2H1. The inhibitory action of RU486 on drug induction was shown to be reversible. Upon removal of RU486 from the cell culture medium, the addition of phenobarbital was again able to induce the CYP2H1 gene. This finding, together with the fact that the basal rates of expression of the CYP2H1 gene (or the expression of the GAPDH as a control, Figures 4.1-4.5) was not altered by RU486, argued that the inhibitory action of RU486 is specific for drug induction and is not due to general toxicity or irreversible cellular damage.

RU486 has been shown to antagonise both the glucocorticoid and the progesterone hormone receptor pathways. Of most interest in the present study was the discovery that neither dexamethasone nor cAMP had any effect on either basal or induced expression of the CYP2H1 gene and were unable to antagonise the effect of RU486 on drug induction even at high concentrations. These results indicate that the ability of RU486 to inhibit induction of the CYP2H1 gene is not be due to its antagonist activity of the glucocorticoid or progesterone receptors. This was supported by the fact that no glucocorticoid of progesterone response element has been identified within the 4.8 kb enhancer region (data not shown). However, recent experiments have suggested that, in the absence of glucocorticoid response element(s), the glucocorticoid receptor can suppress the action of the AhR/Arnt receptor complex via protein-protein interaction (29). Dexamethasone was shown to inhibit CYP1B1 induction by TCDD, and that these effects are mediated through the glucocorticoid receptor as RU486 was able to relieve this dexamethasone mediated repression (29). The conclusion of this study was that the glucocorticoid receptor suppresses activity of the AhR/Arnt complex in the CYP1B1 enhancer region, even though this region lacks glucocorticoid response element(s) (379). Similarly, RU486 was shown to fully reverse the inhibition by dexamethasone of TCDD induction of the CYP1AI gene (372).

Shaw and co-workers, who demonstrated RU486 mediated inhibition of PB induction of the rat CYP2Bl/2 genes, proposed that RU486 exerts its action by competitively binding to an endogenous steroid receptor and hence preventing its activation (48, 320). The
subsequent discovery of glucocorticoid response elements within the enhancer on the CYP2B1/2 genes and their regulation by the glucocorticoid receptor (280, 333) indicate that the endogenous steroid receptor is the glucocorticoid receptor. Therefore the action of RU486 on these mammalian genes appears to occur via interaction with the glucocorticoid receptor and the classical glucocorticoid activation pathway. This is in contrast to the results presented here, which indicate that the action of RU486 on chicken CYPs occurs by a mechanism independent of the glucocorticoid receptor.

Transient transfection data presented in this chapter have shown that while RU486 does not affect basal expression, phenobarbital induction directed by the upstream domain is sensitive to RU486. All of the enhancer fragments tested demonstrated drug-inducibility and RU486 repression except for the short p556SVCAT construct that showed drug inducibility but was unaffected by RU486. The 556 bp enhancer region is present in the longer p1.9SVCAT construct and this construct was affected by RU486 treatment. Thus the phenobarbital- and RU486-responsive regions are separate. A second phenobarbital-responsive construct pR1SVCAT was also repressed by RU486 but further analysis is required to determine whether the phenobarbital and RU486 responsive elements are the same or separate regions. Thus, it appears that RU486 action is not directly involved in the mediation of phenobarbital induction but may involve a separate system that acts independently of the drug-responsive pathway.

A possible model of RU486 action can be suggested from this data. RU486 treatment leads to the binding of a protein to an element within position -3000/-1950 of the 4.8 kb enhancer region of the CYP2HI gene. Binding of this protein, either directly or through recruitment of other negative acting factors, such as a corepressor, interfere with the 556 bp enhancer binding factors to prevent drug induced activation of the CYP2HI gene. Several questions arise from this model. Firstly, is RU486 acting directly on the unknown protein or is it activating a steroid-like receptor which then promotes protein binding? Secondly, is this mechanism of repression by RU486 physiologically significant? Sexual dimorphism and developmental variation of CYP gene expression has been described
previously (196, 347, 353). Thus, it is possible that the mechanism observed here may be involved in hormonal control of the CYP2H genes during sexual differentiation or development and growth of the organism. Thirdly, what endogenous signal is RU486 mimicking to exert its effect?

In support of the repressor model, it has been shown that the RU486 liganded progesterone receptor, while still able to dimerise and bind DNA, is unable to dissociate from its associated corepressor, NCoR, thus preventing interaction with the basal transcriptional machinery (380). In a similar fashion, PPARα has been shown to interact with NCoR and this interaction suppresses transcription. This repression was reversed by the addition of PPAR agonists and was accompanied by dissociation of NCoR from PPARα (79). It is possible that the antagonist receptor-bound corepressor recruits a histone deacetylase to the target promoter and thereby maintains the chromatin in an inactive state (40, 134, 234). Analysis of chromatin structure by in vivo footprinting of an integrated MMTV gene has indicated that glucocorticoid agonists can induce remodelling of the chromatin structure, leading to receptor binding to DNA and recruitment of transcription factors (12, 16). RU486 was shown to inhibit these chromatin remodelling effects (20, 339).

Experiments to answer these questions are focusing on characterisation of the DNA element responsible for mediating RU486 repression. This will lead to the identification of the protein(s) binding to this element. Other studies are examining the possible involvement of a corepressor through the use of the deacetylase inhibitor trichostatin A (160).

In conclusion, the results presented in this chapter demonstrate that the glucocorticoid or progesterone steroid receptor pathways are not involved in the induction of the chicken phenobarbital-inducible genes. However, an RU486 sensitive pathway has been characterised that is independent of the elements involved in mediating phenobarbital induction, and possibly operates via interaction with a novel protein or receptor and involves recruitment of a corepressor.
Chapter 6. Final discussion
6.1 Introduction
The mechanisms by which genes are turned on and off are of fundamental interest. The phenobarbital-inducible genes of the chicken provide an excellent system for investigating the multiple levels of regulation of gene expression including basal, induced and tissue specific control, receptor signalling and chromatin structure. The major aims of this work were to investigate the regulation, at these various levels, of the \textit{CYP2HI} and \textit{CYP2H2} genes.

6.2 Isolation and characterisation of a phenobarbital-responsive element within the 5' flanking region of the \textit{CYP2HI} gene
Prior to the commencement of this project, the isolation of a number of P450 cDNA clones was reported (140). One of these clones, pCHP3, hybridised strongly to three mRNA species (3.5, 2.5 and 2.2 kb). The larger and smaller of these species were strikingly similar in their coding regions but divergent in their 3' non-coding sequences. Restriction mapping analysis had identified two PB-inducible genes, \textit{CYP2HI} and \textit{CYP2H2}, in the chicken genome (221). Two separate regions have been identified within the 5' flanking sequence of the \textit{CYP2HI} gene, an upstream enhancer domain that responds to drug (73, 119) and a proximal promoter region that strong directs basal expression but does not respond to drug (75). The distal 4.8 kb drug responsive region was dissected by deletion and restriction enzyme analysis. Two separate enhancer elements were identified at -5900/4550 and -1956/-1400. The latter element was selected for further analysis. Within this 556 bp region, a minimal drug responsive element of 240 bp was isolated. DNase I footprinting assays identified four protected regions. Gel shift analysis characterisation proteins that bind to three of the four protected regions. These include a member of the E-box family of transcriptions factors, possibly USF, together with HNF-1 and C/EBP. The fourth protein binding site did not match any known transcription factor binding site and the protein bound to this novel region has yet to be characterised. These four proteins were shown to be enriched in chicken liver, kidney and small intestine. This correlates with the tissue restricted expression of this gene. Mutation analysis of the 556 bp region demonstrated that
all four sites are required for maximal expression from this enhancer and that no one site is critical for drug mediated expression. While multiple sites have also been shown to direct phenobarbital responsiveness of the rat CYP2Bl/2 (174, 262, 333) and mouse Cyp2b10 genes (145) the proteins identified here do not appear to have parallels on the rodent enhancers implying that either the mechanisms are fundamentally different or that there is an as yet unidentified factor that leads to the activation of multiple and different transcription factors. CAR is a possible candidate for this unidentified factor. While CAR does not appear to be the master controller of the chicken CYP2H1/2 genes, it does activate the 556 bp enhancer. It will be interesting to investigate CARs action in primary chick hepatocytes, firstly to isolate the DNA region through which it is acting and secondly, to study the effect of androstanes on CAR activity (93) in these cells.

6.3 Characterisation of the CYP2H2 gene enhancer and promoter regions

A second phenobarbital-inducible gene CYP2H2 exists in the chicken. The induced expression of this gene is on average 10 fold lower than that of the CYP2H1 gene. Our model of drug induction, that drug relieves repression of a strong promoter resulting in induction, suggests that the lower activity of this gene is due to a less active promoter region. Isolation and characterisation of firstly the enhancer and secondly the promoter region of the CYP2H2 gene demonstrated that this is in fact the case.

In the present study the promoter of the CYP2H2 gene was isolated. The sequence of the first 353 bp of the promoter was obtained and shown to be 96% homologous to the corresponding region of the CYP2H1 gene. Functional binding sites for the liver-enriched transcription factors HNF-3, HNF-1 and C/EBP together with ubiquitous factor USF were identified within the CYP2H2 promoter. The HNF-1, C/EBP and USF sites are identical in sequence to those characterised previously for the CYP2H1 promoter. Interestingly, it is the region adjacent to the putative HNF-3 binding site that shows the greatest sequence diversion between the two genes.
The major variation between these two promoters is the duplication of 8 bp at position -161/-154 in the CYP2H2 promoter producing a second HNF-3 transcription factor binding site. Extensive gel shift analysis of these two HNF-3 sites confirmed that both are functional and specifically bind HNF-3 protein. However, no protein binding over either HNF-3 site was observed using DNase I footprint analysis. Mutation of one or other site allowed protein binding to the adjacent site and increased expression of transiently transfected promoter/reporter gene constructs.

The conclusion drawn from these data is that, due to the monomeric nature of the HNF-3 protein (323), the overlapping nature of these cis-acting elements, shown to be functional in vitro, prevents protein binding in vivo, possibly due to competition between the two sites for protein binding. As discussed previously, this promoter architecture may provide another aspect by which gene expression can be controlled. The high degree of homology suggests that these two genes diverged recently possibly from a gene duplication event forced by the emergence of a slightly altered or new xenobiotic into the avian ecosystem. It has been demonstrated that despite the 92% amino acid homology between these two genes, they can be distinguished enzymatically (330). Similarly, the phenobarbital induced rat CYP2B1 and CYP2B2 genes, with 97% amino acid homology, can also be distinguished enzymatically (366). The 8 bp duplication has been shown to decrease the level of induction of this gene. It is possible that this was a desirable outcome if the encoded P450 enzyme, while being required to metabolise the new or altered xenobiotic, also catalysed the production a toxic metabolite or if the new xenobiotic occurs at a low level or has a low toxicity making a highly induced gene energetically wasteful.

6.4 Investigation of CYP2H1/2 gene induction

The second aim of this project focused on the effect of the glucocorticoid-antagonist RU486 in induction of CYP2H1/2. RU486 was shown to markedly inhibit drug induction of both the endogenous CYP2H1 gene and transiently transfected CYP2H1 enhancer constructs. This repression was not caused by general toxicity or irreversible cellular damage and was specific for the avian P450 genes in particular at the level of induction.
While glucocorticoids have been shown to be involved in expression of other phenobarbital-inducible genes (158, 320, 333), the fact that dexamethasone was unable to antagonise the effect of RU486 even at very high concentrations reveals that RU486 inhibition of induction of the CYP2H1 gene may not be due to its antagonist activity of the glucocorticoid receptor.

It is proposed that RU486 treatment results in the binding of a protein which then recruits a repressor which in turn shuts down activation of proteins bound to the adjacent 556 bp enhancer region and thereby inhibits induction of the CYP2H1 gene. This model would not require ongoing protein synthesis or the presence of functional GREs within the enhancer region. The concentration of RU486 required to prevent PB-mediated induction indicates suggests a direct competition between RU486 and PB for the same factor is unlikely. It is possible that RU486 is interacting with an uncharacterised steroid-like orphan receptor via a pathway independent of PB-induction. The role of this RU486 sensitive pathway in the endogenous expression of this gene and its modulation by environmental factor(s) remains to be determined.

6.6 Final Remarks

Thus the drug inducibility of the chicken phenobarbital-inducible P450 genes provide a fascinating system in which to study the many levels of regulation of eukaryotic gene expression as well as providing an excellent system for the study of metabolism of a diverse range of xenobiotics possibly leading to the development of strategies to reduce or prevent adverse drug interactions. While the induction by polycyclic aromatic hydrocarbons is mediated by a relatively simple mechanism, this project highlights the complexity of the induction mechanism of the phenobarbital-inducible genes and reveals the many differences observed between the phenobarbital-inducible genes of different species.
Publications

Abstracts


Papers


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Erratum

1. Page X. Should read 2,3,7,8-Tetrachlorobenzo-\textit{p}-dioxin
2. Page 1. Should read "each mammalian species may have up to 50 isoforms"
5. Page 33. Should read "Glucocorticoids have been implicated in the induction ..."
6. Page 55. Should read "The PB-inducible p450s constitute the largest \textit{group} within the P450 gene superfamily."
7. Page 98. Should read "Gel shift analysis characterised proteins..."
8. Page 101. Paragraph 2, line 5. Delete the word "indicates".