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, *CYP2H1* and *CYP2H2*. These two genes have homologous coding regions but divergent 3' noncoding regions. We previously identified in the chicken *CYP2H1* 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 *CYP2H1* 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 *CYP2H1* 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

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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 dehydogenase
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*-doixin
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

Figure 1.1 Summary of the chemical reactions catalysed by cytochrome P450s.

This table lists the reactions and representative substrates catalysed 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.

REACTIONS CATALYSED BY CYTOCHROME P450

Type of reaction	Example of substrates	Products
Aliphatic hydroxylation	Fatty acids, *n*-alkanes, phenobarbital, cyclohexane, hexobarbital, testosterone	Hydroxylated derivatives; dependent on the cytochrome P450 isozyme and substrate, oxygenation can occur at different sites on the molecule.
Aromatic oxidation	Halogenated benzenes, biphenyls, polycyclic aromatic hydrocarbons	Hydroxylation or epoxidation at different sites
Alkene epoxidation	Aflatoxin B_1 benzo[*a*]pyrene-7,8-dihydrodiol Aldrin	Aflatoxin B_1-2,3-oxide benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide Dieldrin
N-Dealkylation	Benzphetamine Aminopyrine Ethylmorphine	Norbenzphetamine, formaldehyde Monomethyl-4-aminoantipyrine, formaldehyde Norethylmorphine, formaldehyde
Oxidative deamination	Amphetamine	Phenylacetone, ammonia
O-Dealkylation	7-Ethoxycoumarin Phenacetin	7-Hydroxycoumarin, acetaldehyde Acetaminophen, acetaldehyde
N-Oxidation	2-Acetylaminofluorene Phenacetin Phentermine	*N*-Hydroxy-2-acetylaminofluorene *N*-Hydroxyphenacetin *N*-Hydroxyphentermine
Oxidative desulfuration	Parathion Carbon disulfide	Paraoxon Carbonyl sulfide, sulfur
Sulfoxidation	Chlorpromazine	Chlorpromazine sulfoxide
Oxidative dehalogenation	Dibromomethane Chloroform	Carbon monoxide, hydrogen bromide Phosgene, hydrogen chloride
Oxidative denitrification	2-Nitropropane	Acetone, nitrite
Oxidative denitrosation	*N*-Nitrosodimethylamine	Nitrite, methylamine, formaldehyde
Nitro reduction	*p*-Nitrobenzoic acid Nitrobenzene	*p*-Aminobenzoic acid Aniline
Azo reduction	Prontosil Azobenzene	Sulfanilamide, triaminobenzene Aniline
Tertiary amine *N*-oxide reduction	Imipramine *N*-oxide *N*, *N*-Dimethylaniline *N*-oxide	Imipramine *N*, *N*-Dimethylaniline
Arene oxide reduction	Benzo[*a*]pyrene-4,5-oxide	Benzo[*a*]pyrene
Reductive dehalogenation	Carbon tetrachloride	Chloroform
Chromate reduction	Chromate (VI)	Chromium (III)

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 λ_{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 ecosanoid biosynthesis (100).

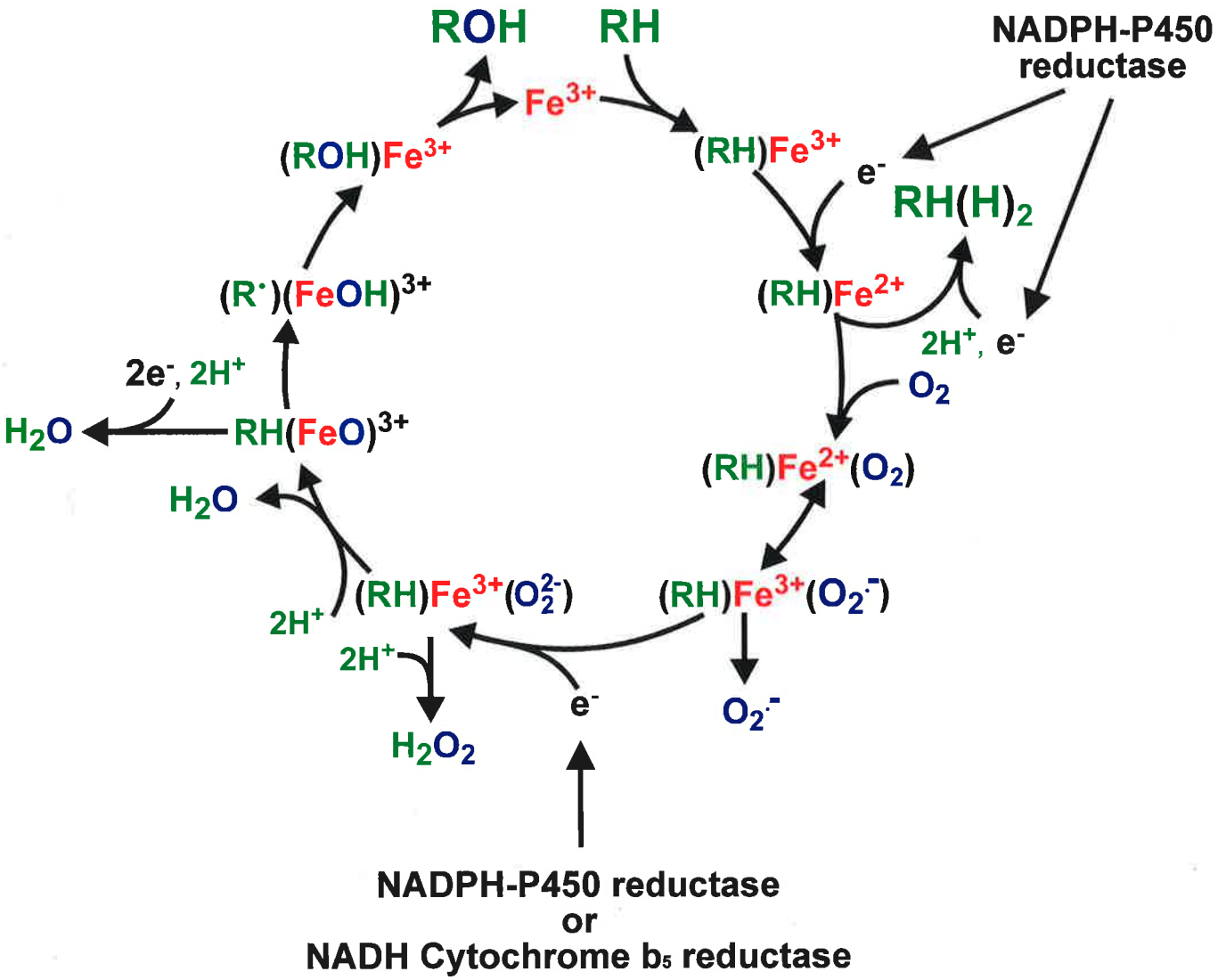
1.3 Chemical Reaction of Cytochrome P450s

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, $Fe^{3+}(O_2^-)$, with substrate still present. Transfer of the second electron then occurs, with the possible involvement of cytochrome b_5 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 H_2O (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.

$$RH + O_2 + NADPH + H^+ \longrightarrow ROH + H_20 + NADP^+$$



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

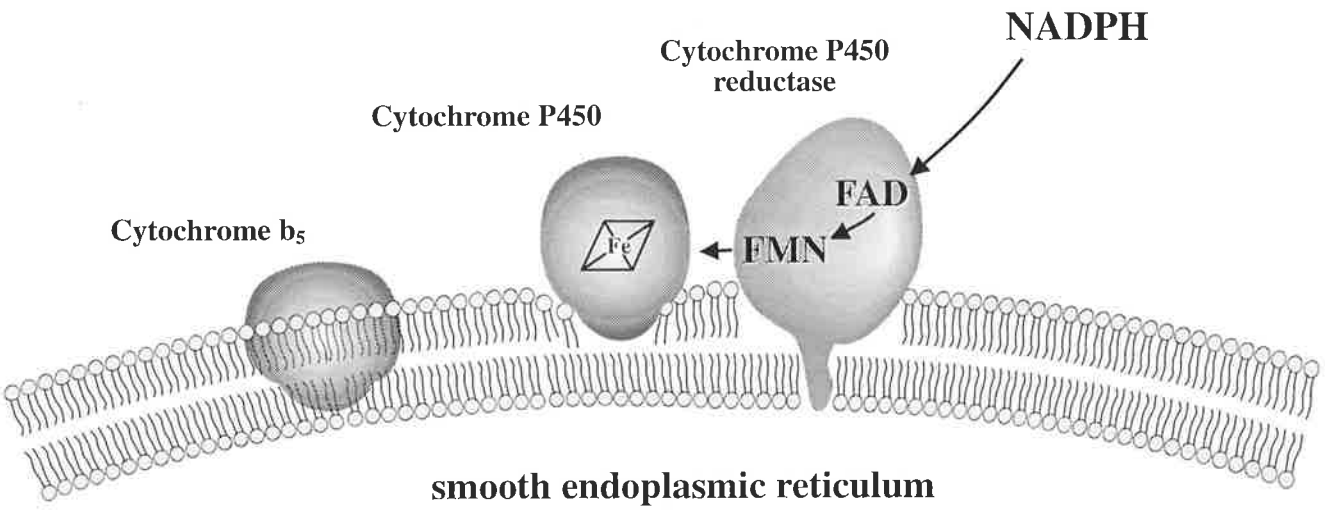
In the endoplasmic reticulum, NADPH donates electrons to NADPH-cytochrome P450 reductase (Fig. 1.3A). In mammals this enzyme contains both flavin adenine dinucleotide (FAD) and flavin mononucleotides (FMN) as prosthetic groups. The FAD serves as the entry point for electrons from NADPH, and FMN serves 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.

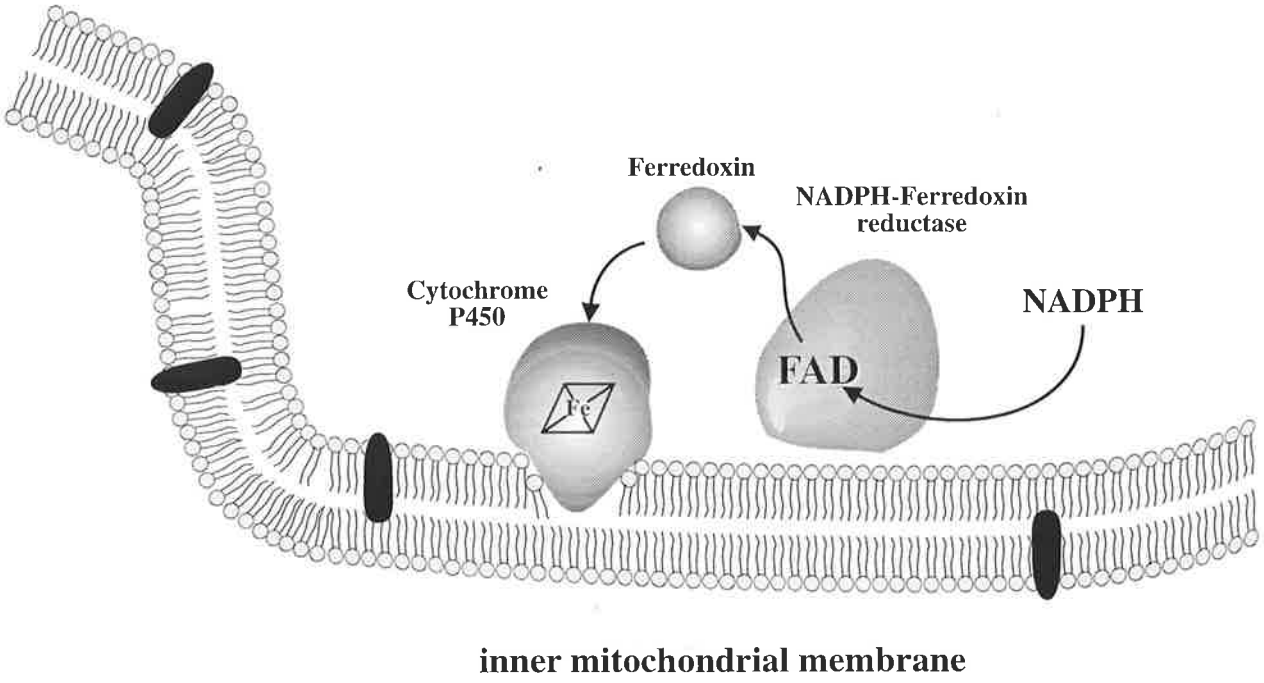
A. The components of the microsomal cytochrome P450 system. NADPH-cytochrome P450 reductase is membrane bound by its hydrophobic tail to the cytosolic surface of the smooth endoplasmic reticulum whereas cytochrome P450 is deeply embedded in the membrane. Also shown is cytochrome b_5, which participates 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-ferreodoxin reductase and ferredoxin are peripheral proteins and are not embedded in the membrane but are loosely associated with the inner mitochondrial matrix.

A


NADPH
Cytochrome P450 reductase
Cytochrome P450
FAD
FMN
Fe
Cytochrome b_5
smooth endoplasmic reticulum

B


Ferredoxin
NADPH-Ferredoxin reductase
Cytochrome P450
NADPH
FAD
Fe
inner mitochondrial membrane

reduced either by NADPH-cytochrome P450 reductase or another microsome-bound flavoprotein, NADH-cytochrome b_5 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).

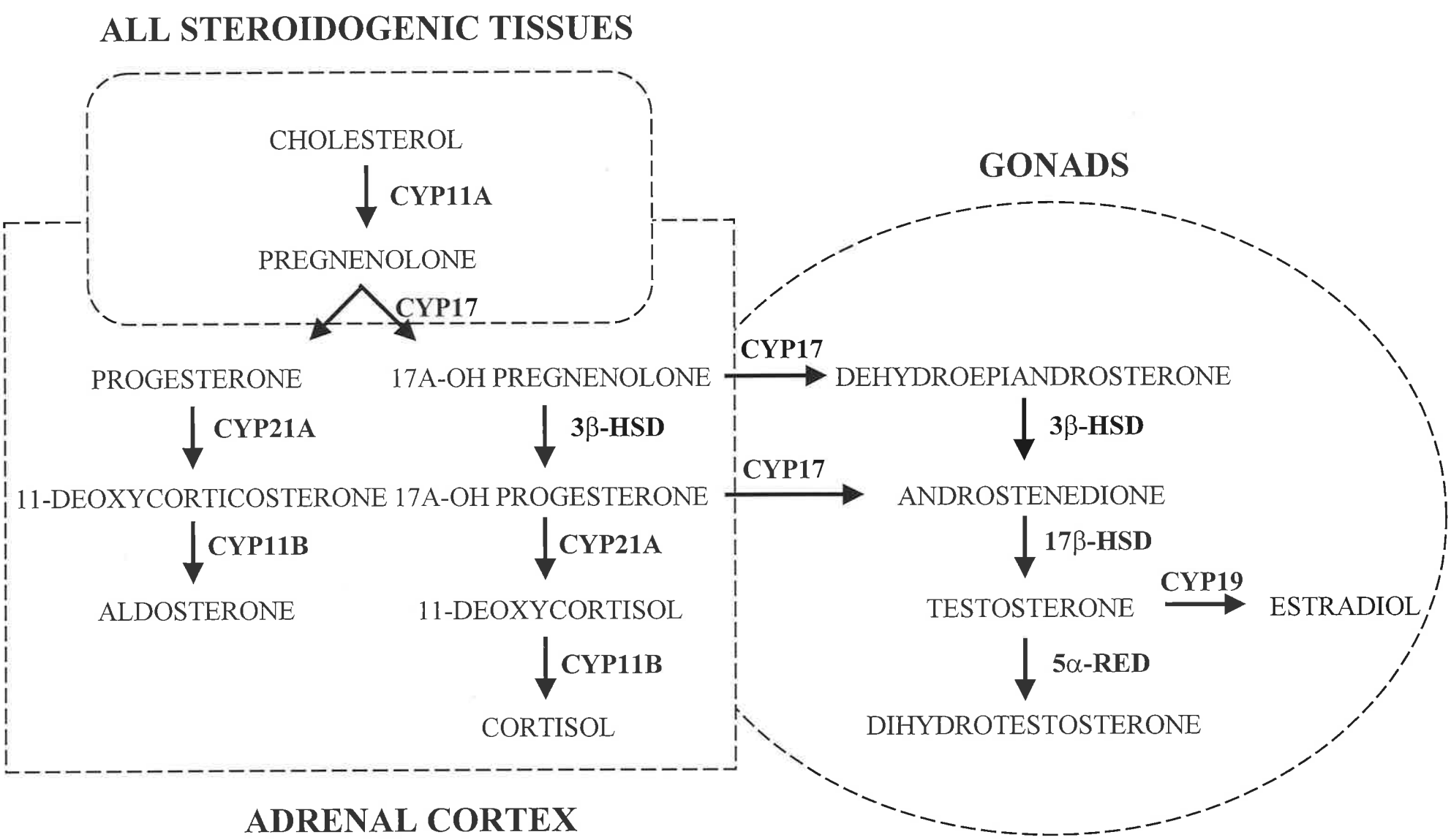
1.4 Cytochrome P450 Metabolism of Endogenous Substrates

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 (eg., 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).


ALL STEROIDOGENIC TISSUES
CHOLESTEROL
CYP11A
PREGNENOLONE
CYP17
PROGESTERONE
17A-OH PREGNENOLONE
CYP21A
3β-HSD
11-DEOXYCORTICOSTERONE
17A-OH PROGESTERONE
CYP11B
CYP21A
ALDOSTERONE
11-DEOXYCORTISOL
CYP11B
CORTISOL
ADRENAL CORTEX
GONADS
CYP17
DEHYDROEPIANDROSTERONE
3β-HSD
CYP17
ANDROSTENEDIONE
17β-HSD
CYP19
TESTOSTERONE
ESTRADIOL
5α-RED
DIHYDROTESTOSTERONE

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 ($P450_{scc}$ 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 *CYP21* 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 *CYP11B*1 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 *CYP21* 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 D_3 and subsequently by kidney mitochondrial cytochrome P-450 to 1,25-dihydroxyvitamin D_3, 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

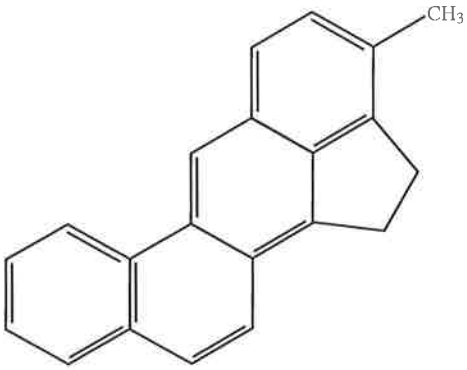
Induction of CYP enzymes occurs predominantly at the level of transcription (66, 100, 247). A notable exception is the ethanol-inducible *CYP2E1* gene, the induction of which involves a posttranscriptional mechanism (100, 293, 340). Induction of CYPs generally occurs at the sites of exposure or excretion 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

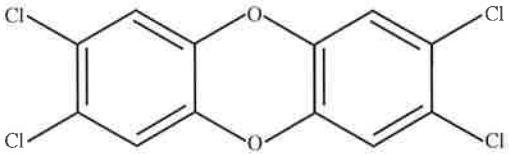
The polycyclic aromatic hydrocarbon-inducible P450 gene family (*CYP1)* contains *CYP1A1*, *CYP1A2* and *CYP1B1*. These genes encode proteins that are similar in their amino acid sequence but differ in their substrate specificity (238). CYP genes in this class are transcriptionally activated by polycyclic 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

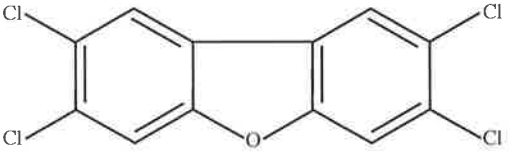
Figure 1.5 Chemical structure of the polycyclic aromatic hydrocarbon compounds.
The aromatic hydrocarbon compounds are related structurally in that they all contain benzene rings and are essentially planer molecules.



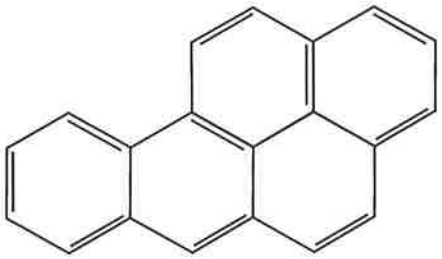
3-Methylcholanthrene



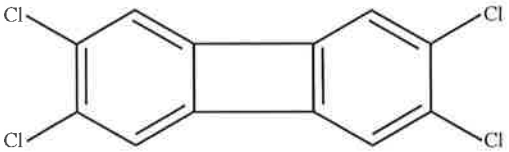
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin



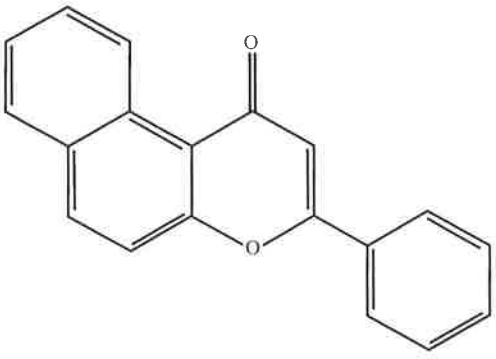
2,3,6,7-Tetrachlorobibenzofuran



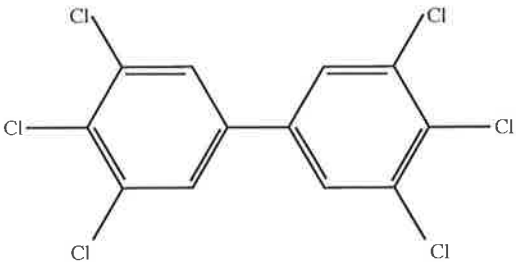
Benzo[a]pyrene



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).

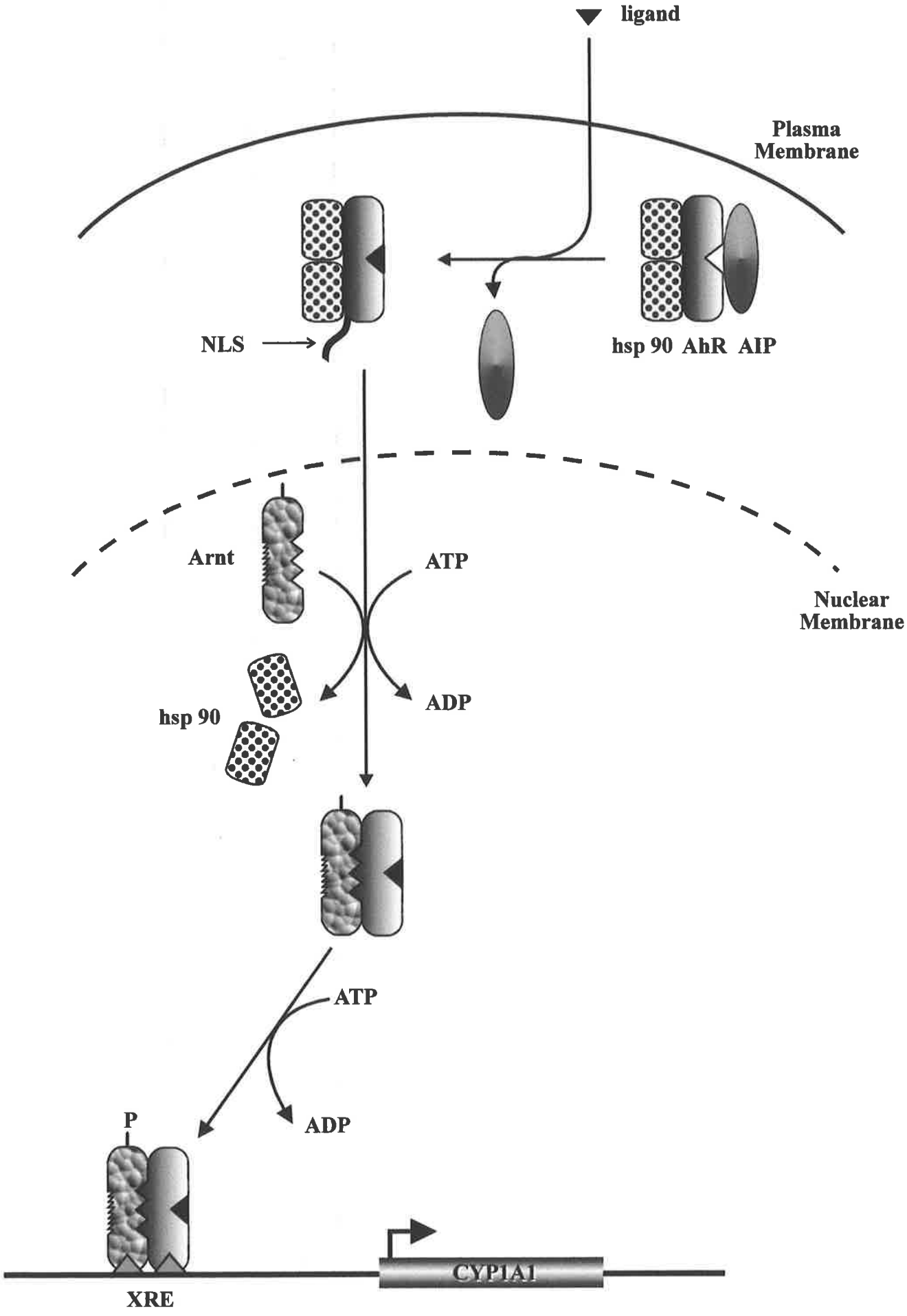
The cytosolic Ah receptor has been cloned and found to be a heteromeric complex of greater than 270 kDa which consists of a 95 kDa ligand binding subunit and at least two molecules of the 90 kDa heat shock protein, hsp90 (36, 176, 273). The association of the Ah receptor with hsp90 results in a non-DNA 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, tetradecanoyl phorbol 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.


ligand
Plasma Membrane
NLS
hsp 90 AhR AIP
Arnt
ATP
Nuclear Membrane
hsp 90
ADP
ATP
P
ADP
CYP1A1
XRE

where they function as classic enhancer regions. *CYP1A1* 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 al 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 *CYP1A1* 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 *CYP1A1* 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 *CYP1A1* 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 Sp1 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 Sp1 transcription factors have been found to invoke a synergistic response. Indeed, the zinc finger domain of Sp1 can interact with both AhR and Arnt via their bHLH/PAS domains (184). These

results support the notion that on a native *CYP1A1* 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 *CYP1A1* promoter (46), creating a transcription factor assembly culminating in strong induction of the *CYP1A1* gene.

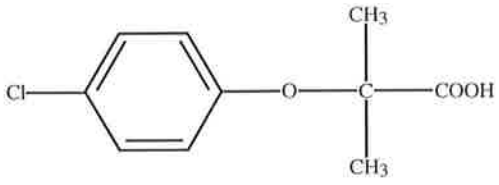
In addition to their role in xenobiotic detoxification, components of the *CYP1* system have other functions. For example, expression of *CYP1B1* has been detected in the testis (304), suggesting that *CYP1B1* may have a role as a steroid hydroxylase (250). This gene has been shown to be cAMP responsive and its basal promoter contains two Sp1 sites and an SF1 (steroidogenic factor 1) site (379). Indeed, *CYP1B1* 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.

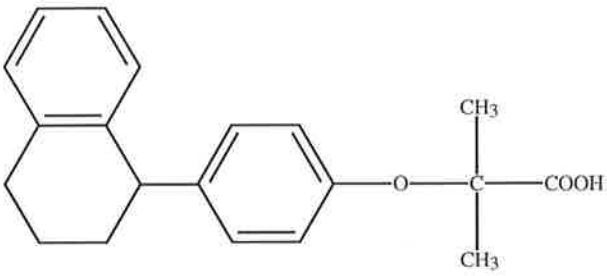
Peroxisome proliferators are a structurally diverse (100) group of compounds of industrial, pharmaceutical and agricultural origin that, when administered to responsive species including rodents and primates, produce a dramatic increase in the size and number of hepatic peroxisomes (315) (Fig 1.7). Some 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.

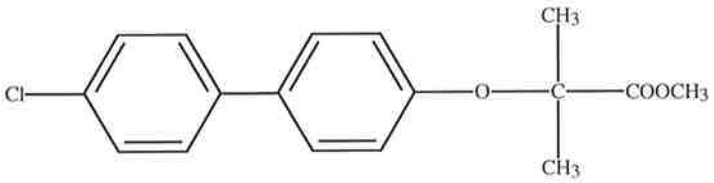
The peroxisome proliferator compounds vary in their chemical structures, but all lead to a proliferation of peroxisomes in liver cells.



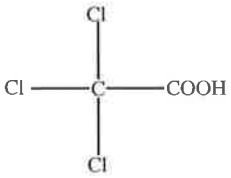
Clofibric Acid



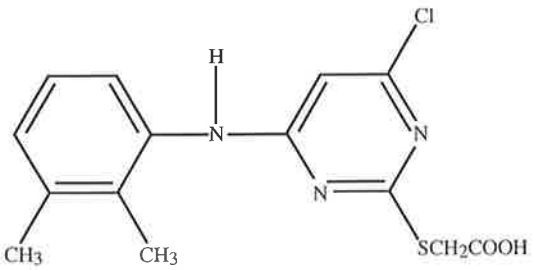
Nafenopin



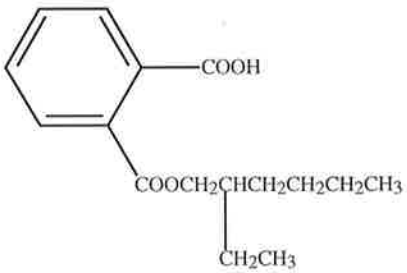
Methylclofenapate



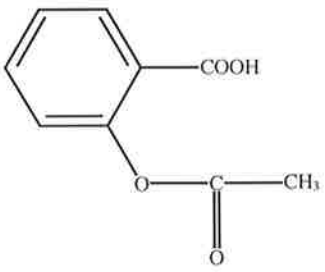
Trichloroacetic Acid



Wy-14,643



Mono-ethylexylphthalate



Aspirin

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* (*CYPLAω*) (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 B_4 (LTB_4) 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 AGTTGA, an imperfect direct repeat of the consensus binding site for the nuclear receptor family (AGGTCA) with a spacing of one nucleotide (DRl; Ref. 258). Deletion of this DRl 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 (4l, 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 DRl motifs (4l, 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 (4l, 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 f*amily induced by this compound have been referred to as the "PCN-inducible" cytochromes P450. Other inducers include dexamethasone, spironolacetone (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

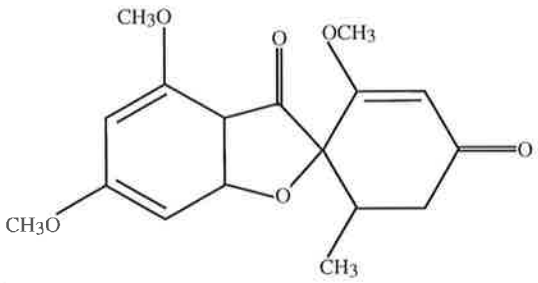
induction and presumably the two distantly located half-sites interact in some way to facilitate glucocorticoid binding.

1.5.4 Regulation of the Phenobarbital Inducible Cytochromes P450

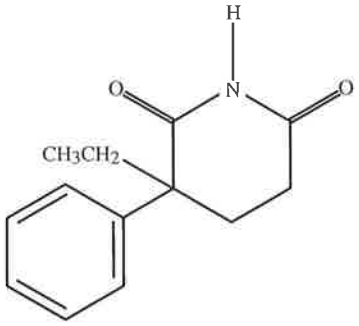
The administration of barbiturates such as phenobarbital to a wide variety of species results in the induction of certain members of the cytochrome P450 2A, 2B and 2C subfamilies (298, 353). The effect of phenobarbital treatment is widespread and includes proliferation of smooth endoplasmic reticulum, 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-glucuronsyltransferase 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 megateruim.*

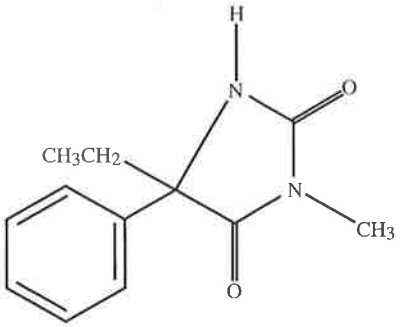
Figure 1.8 Chemical structures of phenobarbital and phenobarbital-like compounds. The compounds shown are called "phenobarbital-like" compounds as they, like phenobarbital, all induce the same cytochrome P450 genes. While some of the compounds such as pentobarbital, glutethimide and mephenytoin are similar 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.



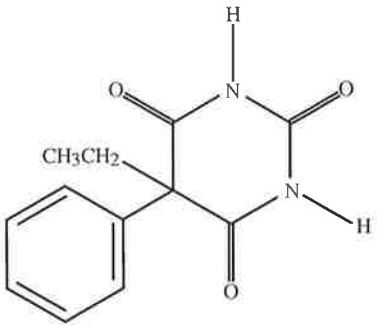
Griseofulvin



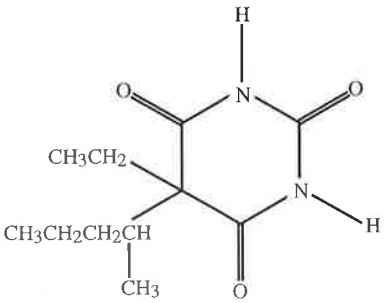
Glutethamide



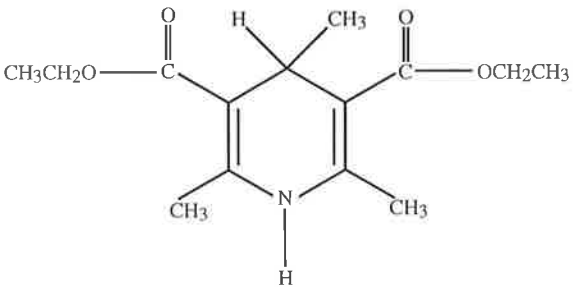
Mephenytoin



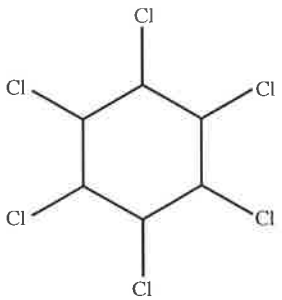
Phenobarbital



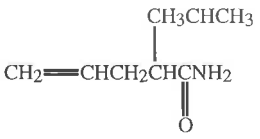
Pentobarbital



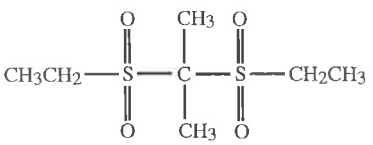
3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)



Lindane



2-Allyl-2-isopropylacetamide (AIA)



Sulphonal

Induction of these bacterial fatty acid monooxygenases (*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 1 (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 *CYP2H1* 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 pr*oteins 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

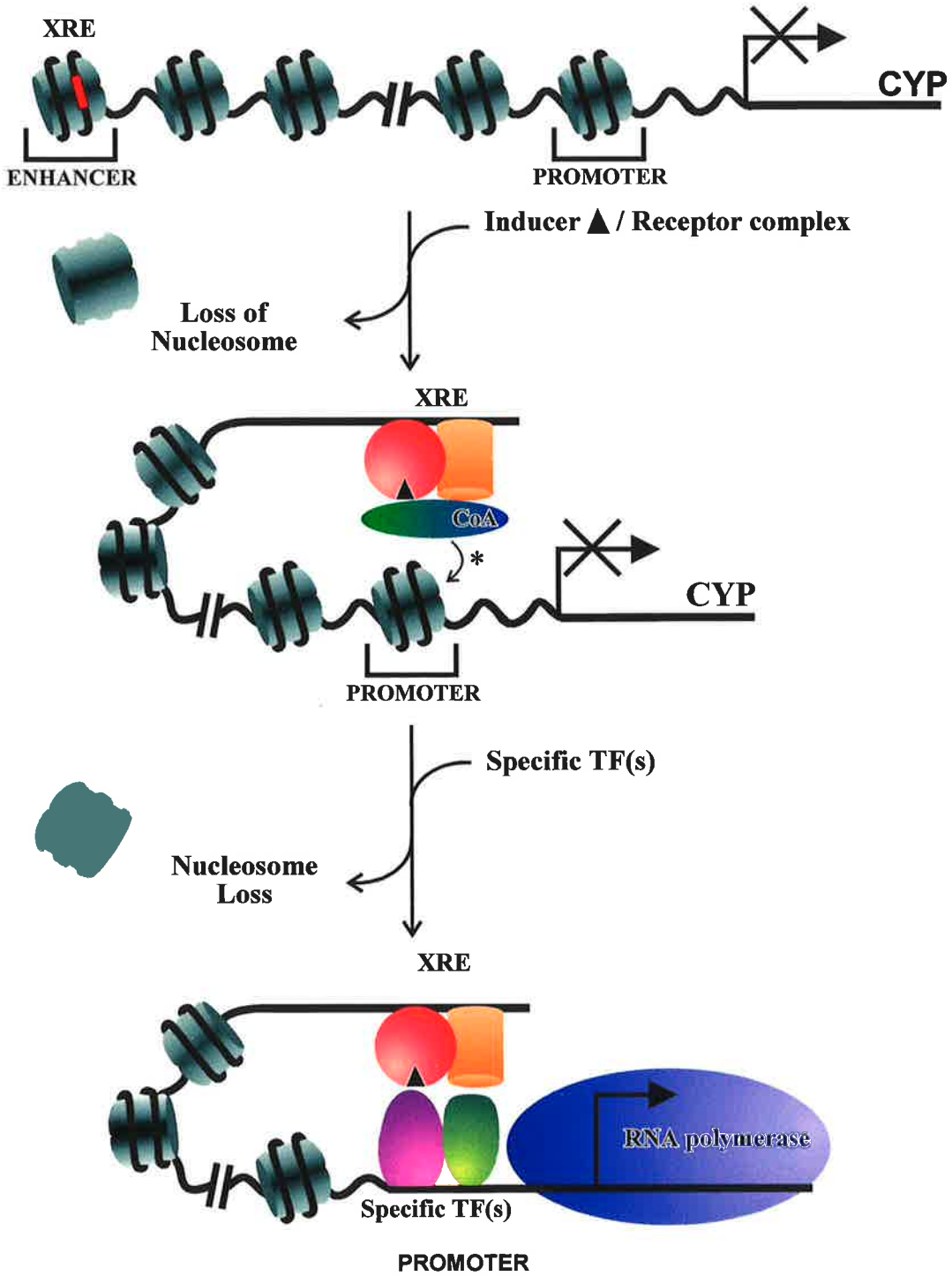
by this promoter. In addition, transcriptional nuclear run-on studies using different segments that spanned the *CYP2H1* cDNA sequence as probes did not show any difference in the density of RNA polymerase II molecules across the gene in either control or phenobarbital-induced chick embryo liver nuclei (our unpublished data). Hence, it is unlikely that the action of phenobarbital on the chicken *CYP2H1* 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 *CYP2H1* gene is normally repressed and that the drug relieves this repression.

We propose that the repression of the native *CYP2H1* 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 *CYP2H1* 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 *CYP2H1* 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).


XRE
CYP
ENHANCER
PROMOTER
Inducer ▲ / Receptor complex
Loss of
Nucleosome
XRE
CoA
*
CYP
PROMOTER
Specific TF(s)
Nucleosome
Loss
XRE
RNA polymerase
Specific TF(s)
PROMOTER

that interacts with the promoter to inhibit transcription. Since the chicken *CYP2H1* 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, *CYP2H1* and *CYP2H2*. Characterisation of the *CYP2H1* 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 *CYP2H2* gene. Firstly, to isolate the enhancer and promoter regions of this gene for comparison with the *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 *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 *CYP2H1* gene.

Glucocorticoids have been implicated the induction of the closely related rat *CYP2B1/2* genes (333). Thus, the third aim was to investigate the role that glucocorticoids play in expression and/or induction of the *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 *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 thiocynate, 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'- tetramethethylethenediamine (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.

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

[γ-^{32}P]dATP (1000 Ci/mmol) and [α-^{33}P]dATP (1000 Ci/mmol) were purchased from Bresatec.

2.1.5 Enzymes

All restriction endonucleases, β-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:

mFP1	-61	GGCATTTCTGCAAT**G**A**G**CT**C**AATCACCTGA	-32
mFP2	-113	GGGAGTTCAGAC**CTGC**A**G**ATTTAACCAAAC	-84
mFP3	-170	GAGAGCAGTTATG**AATTC**GGCCTGGTCCTG	-141
mFP4	-221	TTCAGAGACCG**TCTAG**ATACATAGCAATCT	-192

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'- tcgacGGCATTTCTGCAATCACCTGAg -3'
gCCGTAAAGACGTTAGTGGACTcagct

FP2 5'- tcgaGACACAAATATTTAACCAAACC -3'
CTGTGTTTATAAATTGGTTTGGagct

FP3 5'- cgAGCAGTTATGTCAGTGGCCTat -3
TCGTCAATACAGTCACCGGAtagc

FP4 5'- aattCCAATACATAGCAATCTGTCG -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'
GCTTAAGGTGCACTGCctag (305)

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

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

HNF-5 5'- TAGAACAAACAAGTCC -3'

ATCTTGTTTGTTCAGG (111)

NF1 5'- ATTTTGGCTTGAAGCCAATATG -3'

TAAAACCGAACTTCGGTTATAC (51)

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

Primer 6728 : 5'-dGATACAGACCAGCAAGAGGATTGTG-3'

CAT Primer 5'-dCAACGGTGGTATATCCAGTG-3'

Gel shift oligonucleotides used in chapter 4 are as follows:

USF Primer : 5'-dTCGACAGAACAAAAGCACGTGGC-3'

C/EBP Primer : 5'-dTCGAGCTGATATTGGCTGATTTAAG-3'

HNF-1 Primer : 5'-dTCGAGGCAGATTAATAAGTAACCTGCTGCG-3'

HNF-3 Primers:

4A 5'-dTCGAGCAGATGAAACAATGAGTTCG-3'

4B 5'-dTCGAGCAGAGCAAACAGAGCAAAG-3'

4AB 5'-dTCGACAGAGCAAACAGAGCAAACTTGAG-3'

TTR 5'-dTCGAAGTTGACTAAGTCAATAATCAGAATCAG-3'

CYP2H1 5'-dCGTCTCATTTGTTACTCAAGC-3'

Oligonucleotides used in chapter 4 for site directed mutagenesis are as follows:

d4B 5'-dAGTCAGAGCAAACATTGAGTTCATCCCTAG-3'

m4A 5'-dAGTCAGAGGTCGAGGAGCAAACTTGAGTTCATCCC-3'

m4B 5'-dAGTCAGAGCAAACAGAGGTCGACTGAGTTCATCCC-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 lac^- F'[$proAB^+$ $lacI^q$ lacZ-M15 Tn10 (tet^r)] 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% $MgSO_4.7H_2O$ 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. % (w/v) polyvinylpyrrolidine, 0.1% (w/v) BSA.

SSC : 150mM NaCl, 15mM sodium citrate

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

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

TE : 10mM Tris-HCl pH 7.5, 0.1mM 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 H_2O.

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 $MgCl_2$, 0.1mM $ZnCl_2$, 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 $MgCl_2$, 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 A_{600} 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 $MgCl_2$, 500mM $CaCl_2$ 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 $MgCl_2$ 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 [a^{32}-P] labelled in a 25µl reaction containing 100mM Tris-HCl pH 7.6, 20mM $MgCl_2$, 100mM NaCl, 200µg/ml BSA, 4µM each of unlabelled

dCTP, dGTP and dTTP, 100μCi each of [a^{32}-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 10^8cpm/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 and 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}P labelled at the 5' end using [g-^{32}P] -ATP and T4 polynucleotide kinase. The reaction mixture contained 10mM $MgCl_2$, 50mM Tris-HCl pH 7.4, 5mM DTT, 0.1mM spermidine, 0.1mM EDTA, 100 μCi [g-^{32}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}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 5 x 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 ^{32}P-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 H_2O 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 H_2O, 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 $MgCl_2$, 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 10^7 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 Na_2HPO_4 and 6 mM dextrose (109) at a cell density of 2.5 x 10^7 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 10^7) 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 10^7 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 CO_2/air mix (5:95) for 16 h. The media was changed to remove dead and non-adherent cells land 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-HC1 pH 7.5 containing 1mM EDTA and 150 mM NaC1. 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 5mM 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 130ml of 250mM Tris-HCl, pH 7.6 containing 20-100mg of protein was added 10ml of 10mM acetyl-coenzyme A, 39ml of water and 1ml of [^{14}C] chloramphenicol. The reaction mixture was incubated at 37°C for 1 h after which 10ml of acetyl-coenzyme A was added and the incubation continued for another hour. To stop the reaction, 1ml 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 10ml of ethyl acetate and spotted onto silica plates (Merck). Acetylated [^{14}C] 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 1mg 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: (OD_{420}/mg protein/hour) x 100 = 1.00. CAT activities were then corrected by an equivalent factor.

2.2.11.5 Assay for β-galactosidase activity

β-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°C for 10 min. as was done for CAT activity determinations, as this denatures β-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 x 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% $NaPO_4$) 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-HCl, pH7.9, 10mM KCl, 1mM DTT, 1.5mM $MgCl_2$). The nuclei pellet was then resuspended in 400ml of cold buffer B (50mM Tris-HCl, 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-HCl, 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 [g-^{32}P]-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 ^{32}P-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 $MgCl_2$, 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 KCI, 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 QuikChange 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 SmaI, the 1.5 kb MMTV HindIII/SmaI fragment purified and cloned into pBluescript. This construct was then digested with KpnI and SmaI 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 *CYP2H1* gene

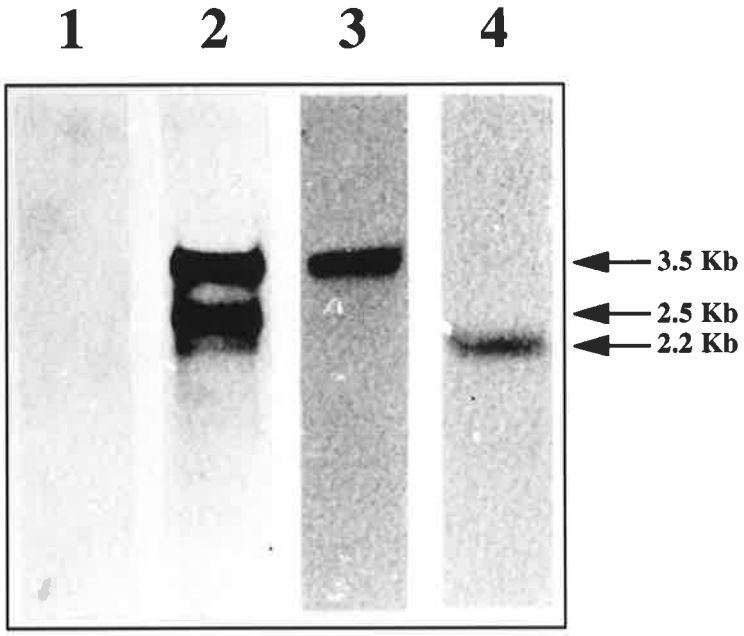
3.1 Introduction

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 *CYP2H1* and *CYP2H2*. Southern blot analysis established that the *CYP2H1* gene encodes the 3.5 kb mRNA while the 2.2 kb mRNA is transcribed from the

Figure 3.1 Comparison of phenobarbital-inducib1e 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 *Acc*I and *Hinc*II (not shown).


1
2
3
4
3.5 Kb
2.5 Kb
2.2 Kb

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 *CYP2H1* gene transcription rate.

An important issue concerns the role of the upstream enhancer domain of the *CYP2H1* gene in the induction mechanism and its relationship to the reported rodent enhancers (145, 147, 262, 333). In this chapter, the *CYP2H1* 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.

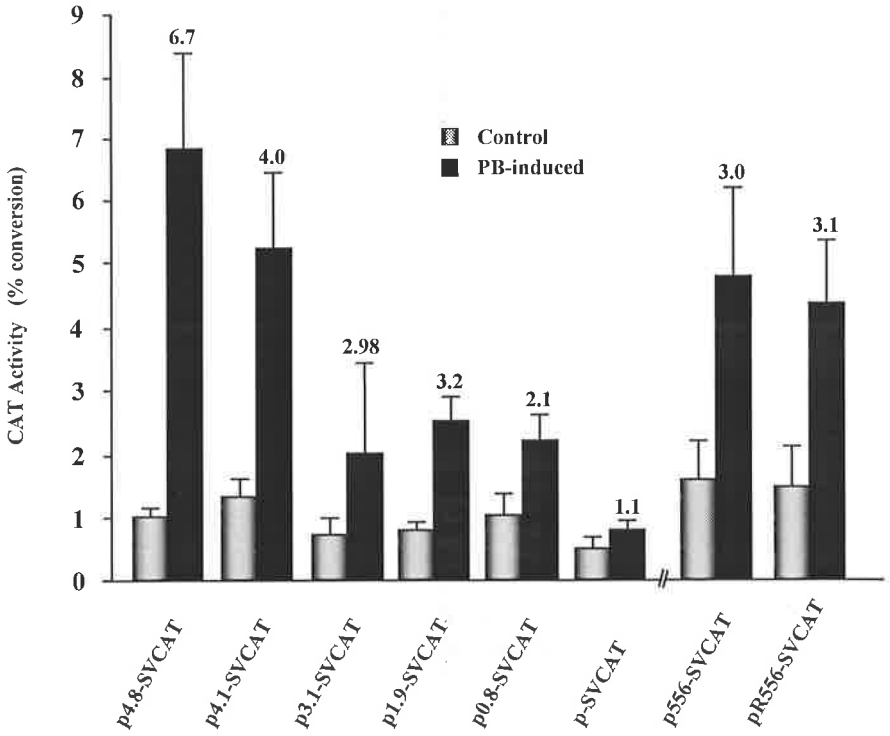
3.2 Results

3.2.1 Progressive deletion of the *CYP2H1* 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 *CYP2H1* 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 *Bam*HI 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 *Bam*HI enhancer domain, *Bam*HI (-5900 to -1100), *Bgl*II/*Xho*I (-1956 to -1400), *Bgl*II/*Stu*I (-1956 to -1640) and *Stu*I/*Xho*I (-1640 to -1400) fragments were blunt ended and cloned in the *Eco*RV 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 [^{14}C]chloramphenicol to acetylated products. Values are the average of three independent experiments ± SD. The fold induction is shown in brackets.


CAT Activity (% conversion)
9
8
7
6
5
4
3
2
1
0
Control
PB-induced
6.7
4.0
2.98
3.2
2.1
1.1
3.0
3.1
p4.8-SVCAT
p4.1-SVCAT
p3.1-SVCAT
p1.9-SVCAT
p0.8-SVCAT
p-SVCAT
p556-SVCAT
pR556-SVCAT

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 *Bam*HI fragment in the forward orientation (p4.8-SVCAT) was used to generate enhancer deletion constructs. This vector was digested with *Kpn*I and *Sal*I and progressive unidirectional deletions from the *Sal*I 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 *Bgl*II/*Xho*I restriction enzyme fragment located near the 3' end of the 4.8 kb enhancer (-1956/-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).

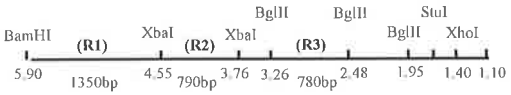
Phenobarbital induction levels in the chick embryo hepatocytes were found to be increased by replacement of Nu serum in the culture media with Serum Supreme, a bovine derived serum, and under these conditions the induction by the 556 bp fragment (pR556-SVCAT) was elevated from 3-fold (Fig 3.2) to 6.7-fold (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

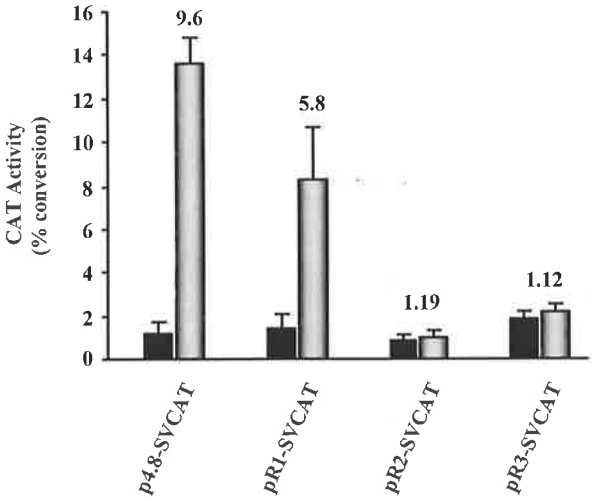
A. Plasmids with restriction enzyme fragments from the 4.8 kb enhancer (R1, R2 and R3) fused to the enhancerless SV40 promoter and a CAT reporter (SVCAT), were transfected into chick embryo hepatocytes and tested as described in Fig. 3.1.

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.

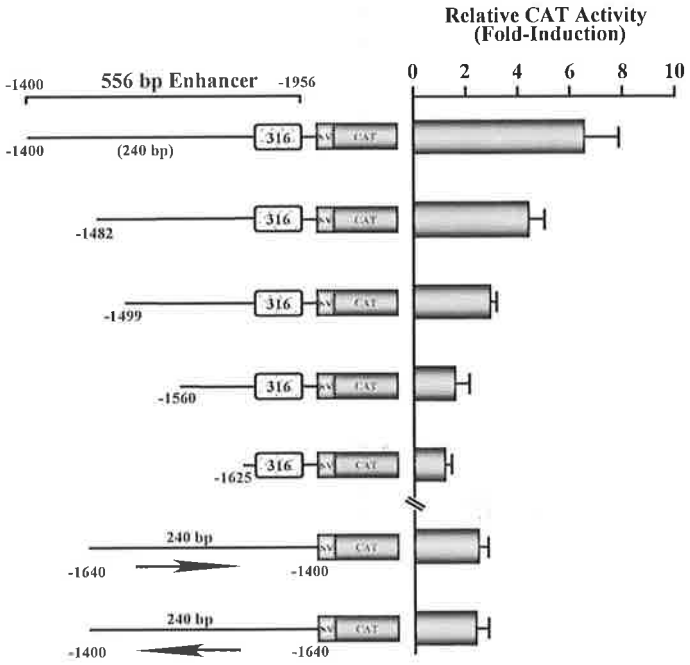
4.8 kb Enhancer



A



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 *Bgl*II/*Stu*I fragment (-1956 to -1640) and a 240 bp *Stu*I/*Xho*I 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 *Kpn*I and *Sal*I and progressive unidirectional deletions from the *Sal*I 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 *Stu*I/*Xho*I 240 bp fragment (-1640 to -1400) was blunt ended and cloned in both orientations into the *Eco*RV site of pBCSVp1. For footprinting, *Kpn*I/*Eco*RI 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

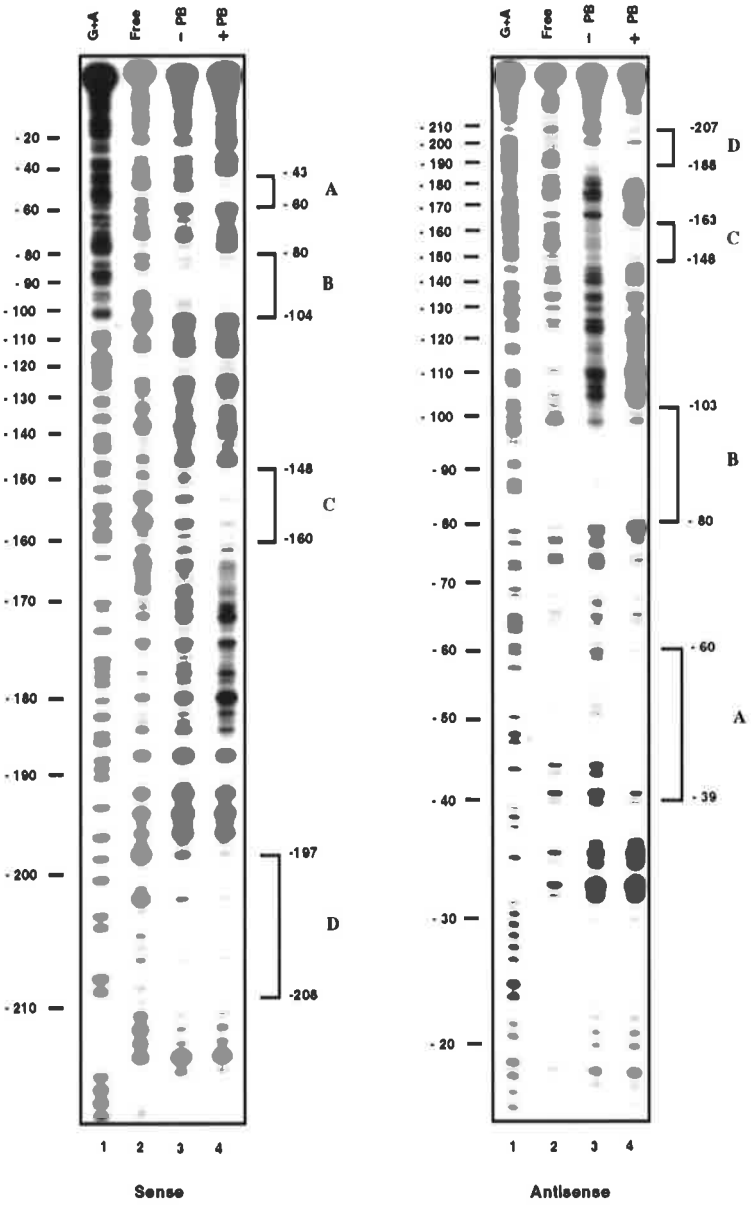
Figure 3.4 DNA sequence of the 240 bp Fragment

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.


C/EBP/NF1
D
-240
GCCTGAACTT CCTTGCCCTT TCAGAGACCG AGCCAATACA TAGCAATCTG
Sp1
C
-190
TCGTACGCCC TAAATGACTG GAGAGCAGTT ATGTCAGTGG CCTGGTCCTG
AP1
H4TF2
HNF5
-140
AGTCAACTGA GTTGTGTTTT GGGTCCTGGG AGTTCAGACA CAAATATTTA
B
A
E-box
-90
ACCAAACCTT TTGTGCTGGC TGGTTAAATG GCATTTCTGC AATCACCTGA
AP1
E-box
-40
ATCACCTGAA AAATAATGAC AAGGTTTGTT ACTTATCTCG

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


G+A
Free
- PB
+ PB
-20
-40
-60
-80
-90
-100
-110
-120
-130
-140
-150
-160
-170
-180
-190
-200
-210
-43
-60
A
-80
-104
B
-148
-160
C
-197
-208
D
1
2
3
4
Sense
G+A
Free
- PB
+ PB
-210
-200
-190
-180
-170
-160
-150
-140
-130
-120
-110
-100
-90
-80
-70
-60
-50
-40
-30
-20
-207
-188
D
-163
-148
C
-103
-80
B
-60
-39
A
1
2
3
4
Antisense

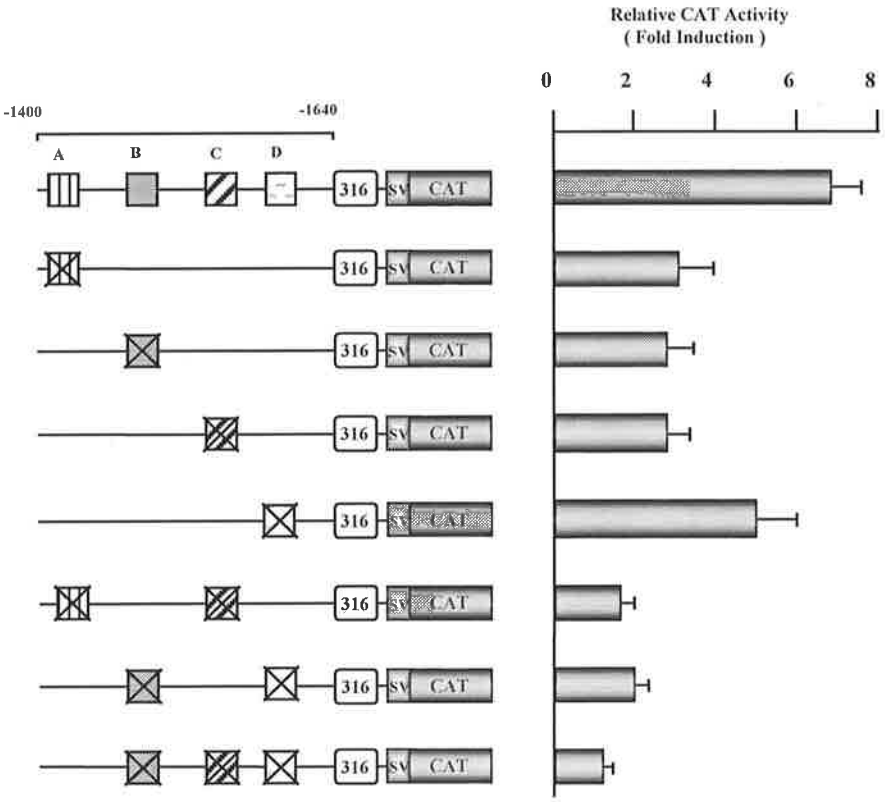
in the *Eco*RV site in the reverse (*Xho*I/*Bgl*II) orientation and this construct (pR556-KS) was used as a template for site-directed mutagenesis. Mutations were introduced in the DNase I footprinted regions and confirmed by sequencing. The mutated fragments were then released from pR556-KS by digestion with *Kpn*I/*Sma*I 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.


Relative CAT Activity
(Fold Induction)
0
2
4
6
8
-1400
-1640
A
B
C
D
316
SV
CAT

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'-TGGTTAAATATTTGTG-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

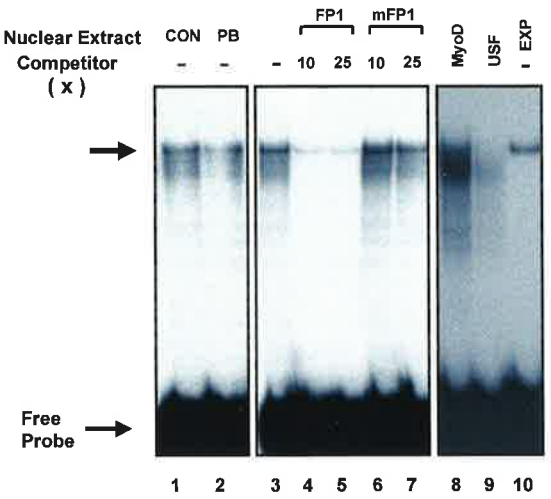
A. Assays were performed with radiolabeled double stranded oligonucleotide FP1 using 3 μg of nuclear extract from control (CON) and phenobarbital induced (PB) hepatocytes (lanes 1 and 2). The major complex is arrowed. In competition experiments, nuclear extracts from control hepatocytes were incubated 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).

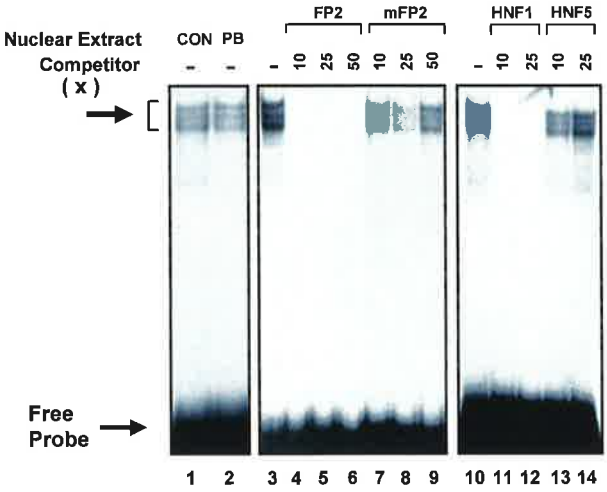
C. Radiolabeled FP3 was incubated with the same nuclear extracts as in Fig. 3.6 (lanes 1-2). Three major complexes are arrowed. In competition experiments, nuclear extracts from control hepatocytes were incubated either without competitor (lane 3) or with a 10 to 50-fold molar excess of FP3 (lanes 4-6)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

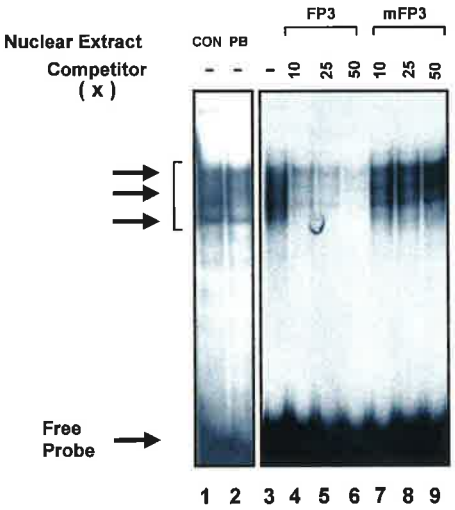
A


Nuclear Extract
Competitor
(x)
CON
PB
FP1
mFP1
MyoD
USF
EXP
Free
Probe
1 2 3 4 5 6 7 8 9 10

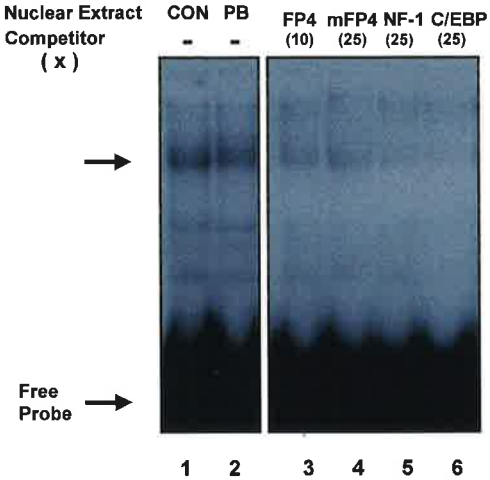
B


Nuclear Extract
Competitor
(x)
CON
PB
FP2
mFP2
HNF1
HNF5
Free
Probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14

C


Nuclear Extract
Competitor
(x)
CON
PB
FP3
mFP3
Free
Probe
1 2 3 4 5 6 7 8 9

D


Nuclear Extract
Competitor
(x)
CON
PB
FP4 (10)
mFP4 (25)
NF-1 (25)
C/EBP (25)
Free
Probe
1 2 3 4 5 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).

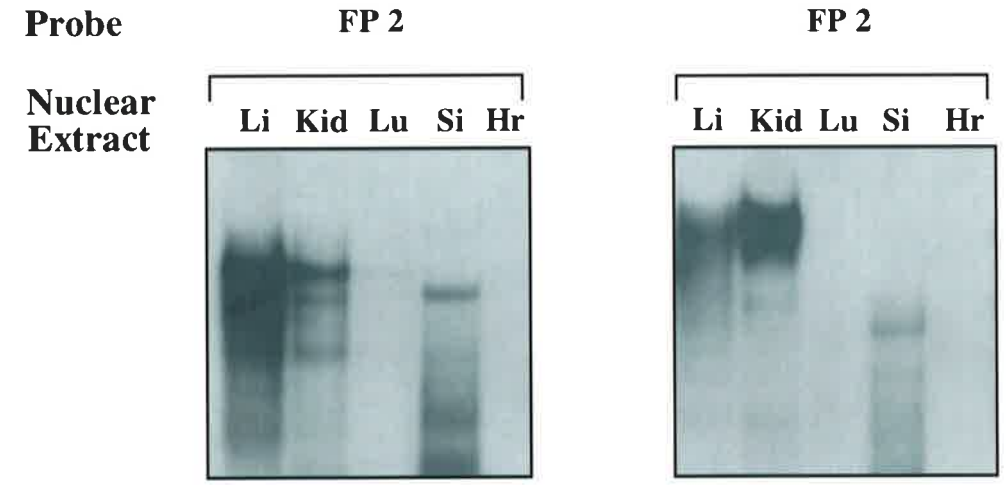
Protein binding to FP4 containing a CCAAT box binding site was very weak; one major protein complex was observed with a similar intensity using control or drug treated nuclear extracts (Fig. 3.7D). Formation of this complex was markedly inhibited in self competition with FP4 (lane 3) and weakly reduced 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).

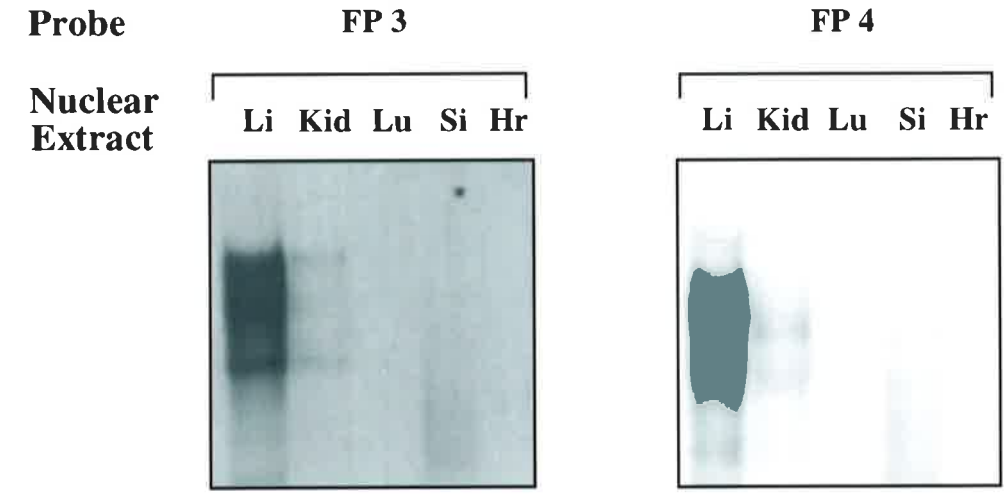
Figure 3.8 Gel mobility shift assays of footprinted regions A-D with nuclear extracts from various tissues

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.

A


Probe
FP 2
FP 2
Nuclear Extract
Li Kid Lu Si Hr
Li Kid Lu Si Hr

B


Probe
FP 3
FP 4
Nuclear Extract
Li Kid Lu Si Hr
Li Kid Lu Si Hr

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 *CYP2H1* 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 *CYP2H1* 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 *CYP2H1* 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 (NR1) 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 *CYP2H1* 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 *CYP2B1/2* (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 *CYP2H1* 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 *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 *CYP2HI* 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

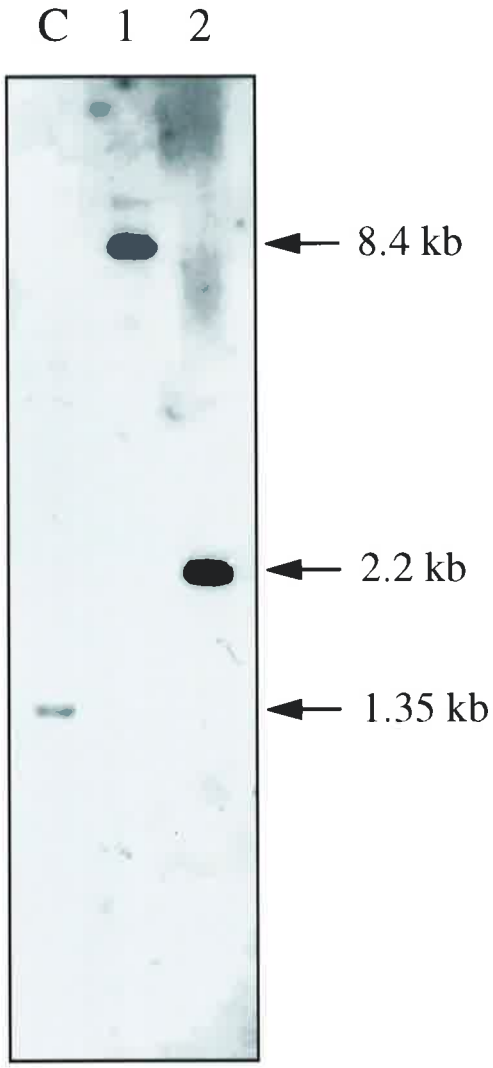
4.2.1 Identification and Cloning of the Promoter Region of *CYP2H2*

Previously in this laboratory, a series of *CYP2H2* genomic clones were isolated from a λ Charon 4A chicken genomic library (221). Clone λ20 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 λ20 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 λ20. 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→T) and -167 (T→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 ^{32}P-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.


C
1
2
8.4 kb
2.2 kb
1.35 kb

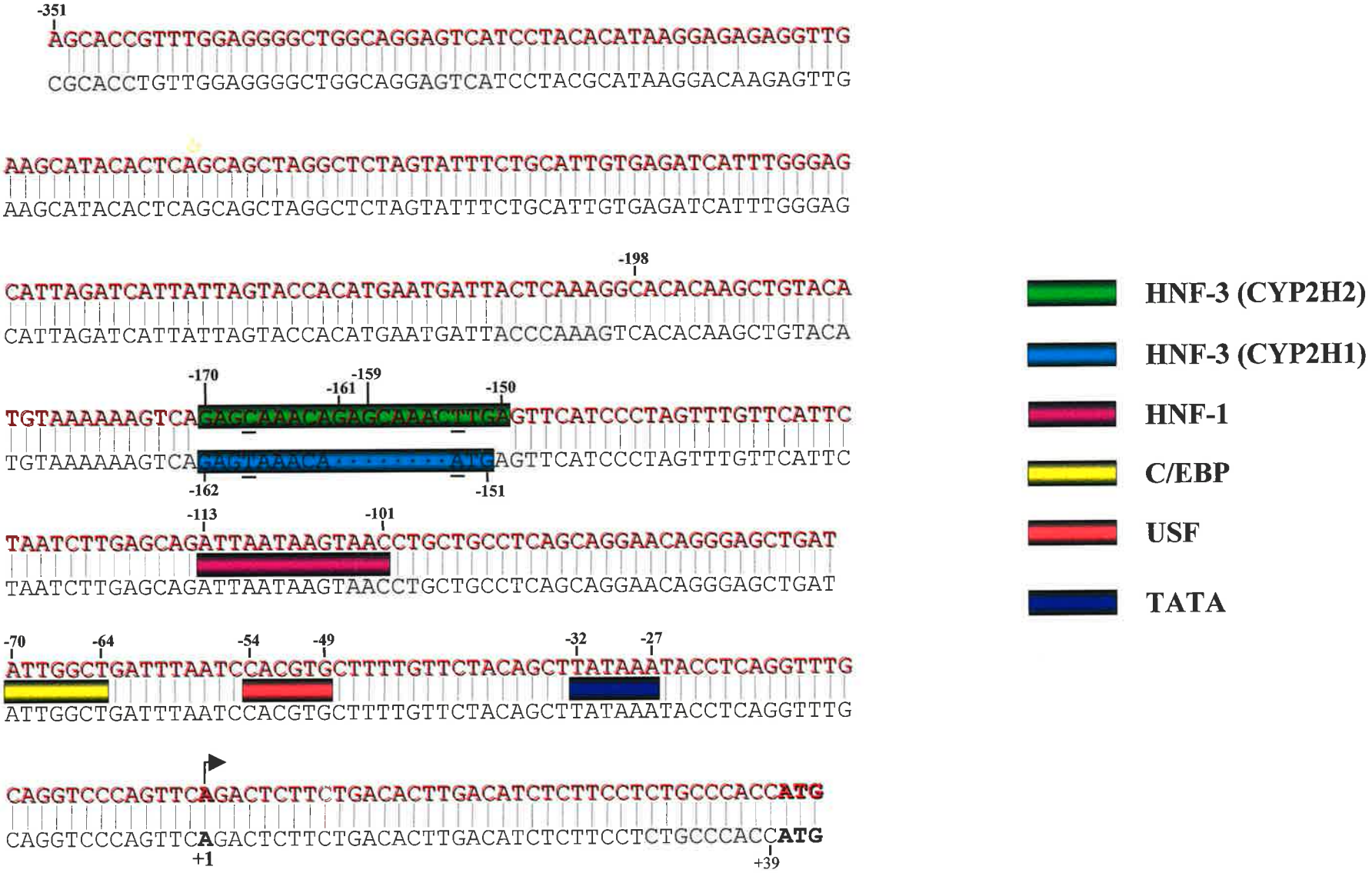
TATA box at -32/-27, and a putative binding site for the ubiquitous upstream stimulatory factor (USF) of sequence 5' CACGTG 3' at -54/-49 (305). Additionally, possible binding sites for three liver-enriched transcription factors were identified. Using the consensus HNF-3 sequence 5' (C/A/G)A(A/T)T(G/A)TT(T/G)(G/A)(C/T)T(C/T) 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' CTCTGTTTGCTC 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' ATCAAAAGCTGGAGG 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 *CYP2H1* (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.


-351
AGCACCGTTTGGAGGGGCTGGCAGGAGTCATCCTACACATAAGGAGAGAGGTTG
CGCACCTGTTGGAGGGGCTGGCAGGAGTCATCCTACGCATAAGGACAAGAGTTG
AAGCATACACTCAGCAGCTAGGCTCTAGTATTTCTGCATTGTGAGATCATTTGGGAG
AAGCATACACTCAGCAGCTAGGCTCTAGTATTTCTGCATTGTGAGATCATTTGGGAG
-198
CATTAGATCATTATTAGTACCACATGAATGATTACTCAAAGGCACACAAGCTGTACA
CATTAGATCATTATTAGTACCACATGAATGATTACCCAAAGTCACACAAGCTGTACA
-170
-161
-159
-150
TGTAAAAAAGTCA
AGTTCATCCCTAGTTTGTTCATTC
TGTAAAAAAGTCA
AGTTCATCCCTAGTTTGTTCATTC
-162
-151
-113
-101
TAATCTTGAGCAGATTAATAAGTAACCTGCTGCCTCAGCAGGAACAGGGAGCTGAT
TAATCTTGAGCAGATTAATAAGTAACCTGCTGCCTCAGCAGGAACAGGGAGCTGAT
-70
-64
-54
-49
-32
-27
ATTGGCTGATTTAATCCACGTGCTTTTGTTCTACAGCTTATAAATACCTCAGGTTTG
ATTGGCTGATTTAATCCACGTGCTTTTGTTCTACAGCTTATAAATACCTCAGGTTTG
CAGGTCCCAGTTCAGACTCTTCTGACACTTGACATCTCTTCCTCTGCCCACCATG
CAGGTCCCAGTTCAGACTCTTCTGACACTTGACATCTCTTCCTCTGCCCACCATG
+1
+39
HNF-3 (CYP2H2)
HNF-3 (CYP2H1)
HNF-1
C/EBP
USF
TATA

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.

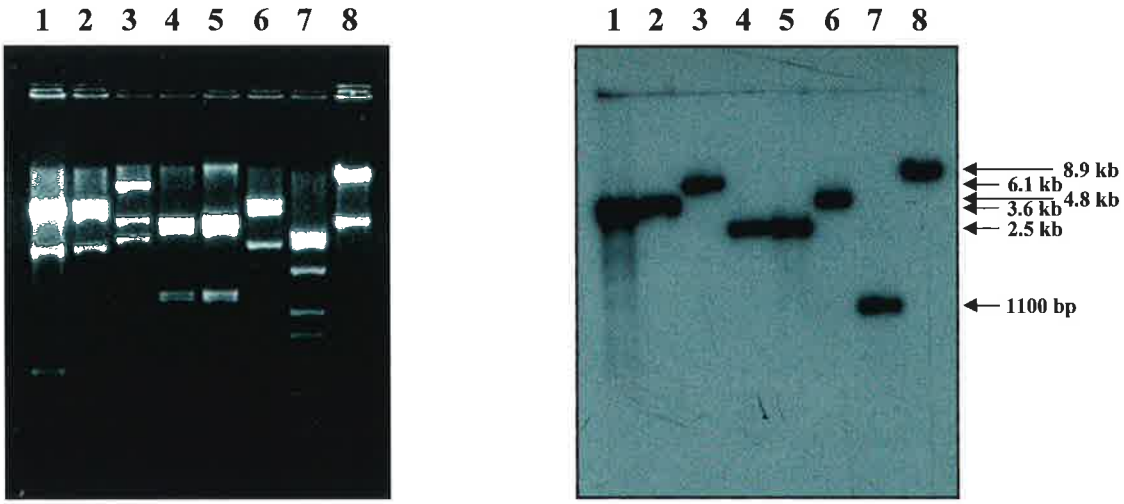
The 1100 bp PvuII fragment from the *CYP2H2* gene was analysed for its ability to confer drug inducibility to a heterologous promoter. The PvuII fragment in the forward and reverse orientation was fused to the enhancerless SV40 promoter driving the CAT reporter gene, and these constructs are referred 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 [^{14}C]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.

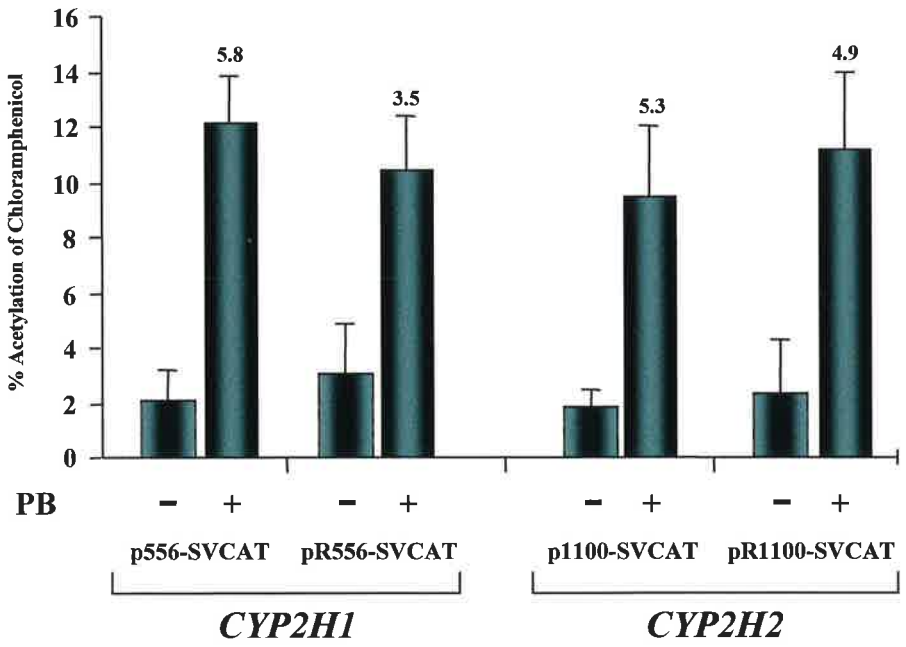
A. DNA (0.1 μg) of the purified 8.4 kb EcoRI/BamHI fragment isolated previously (Section 3.2.2) was digested with the following enzymes: PstI (lane 1), PstI and BamHI (lane 2), SacI and EcoRI (lane 3), HindIII and BamHI (lane 4), HindIII (lane 5), PstI and EcoRI (lane 6), PvuII (lane 7) and EcoRI and 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 (2×10^7) 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 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 [^{14}C]chloramphenicol to acetylated product are shown as the mean ± standard deviation.

A


1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
8.9 kb
6.1 kb
4.8 kb
3.6 kb
2.5 kb
1100 bp

B


% Acetylation of Chloramphenicol
16
14
12
10
8
6
4
2
0
5.8
3.5
5.3
4.9
PB
- + - + - + - +
p556-SVCAT
pR556-SVCAT
p1100-SVCAT
pR1100-SVCAT
CYP2H1
CYP2H2

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.

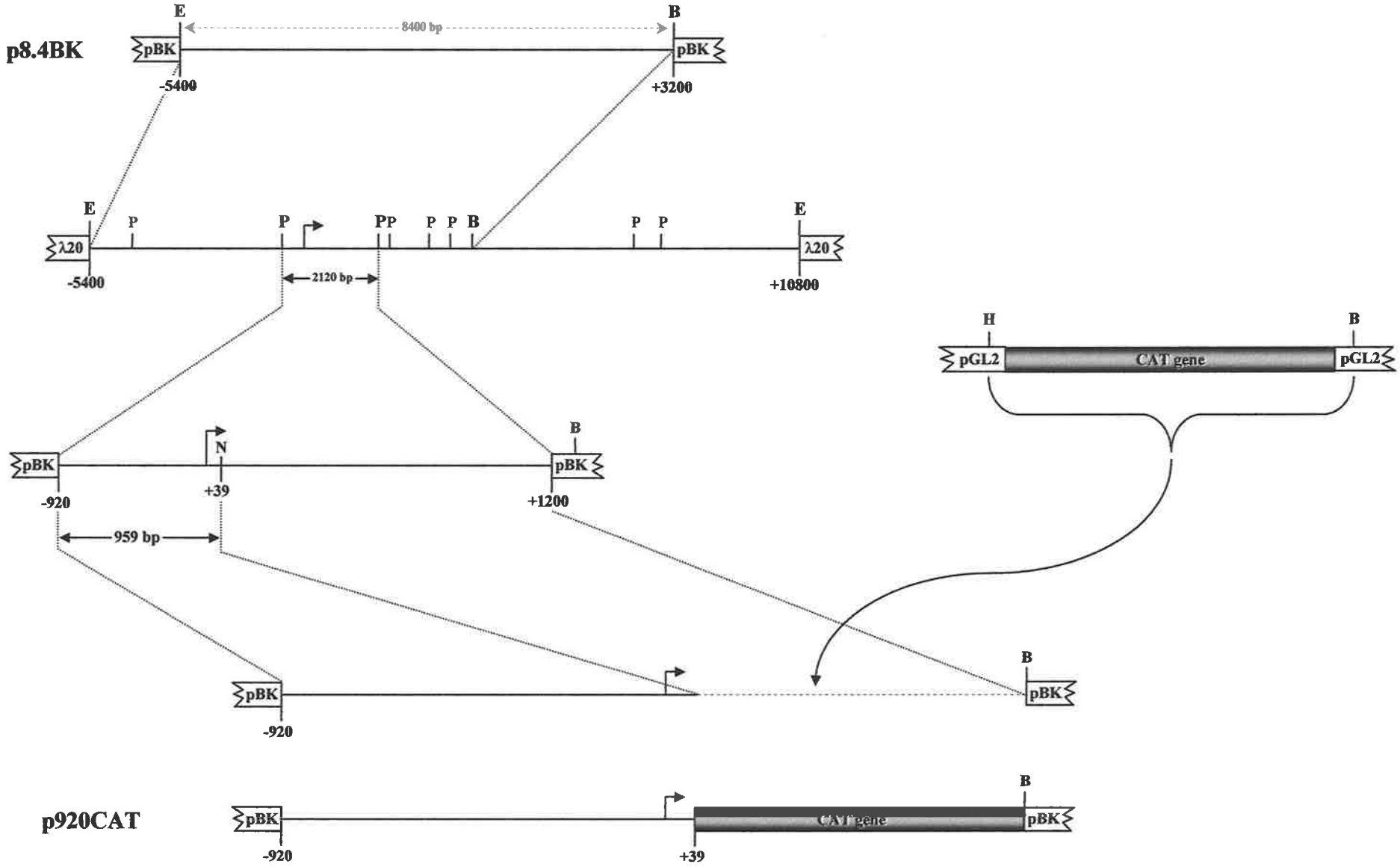
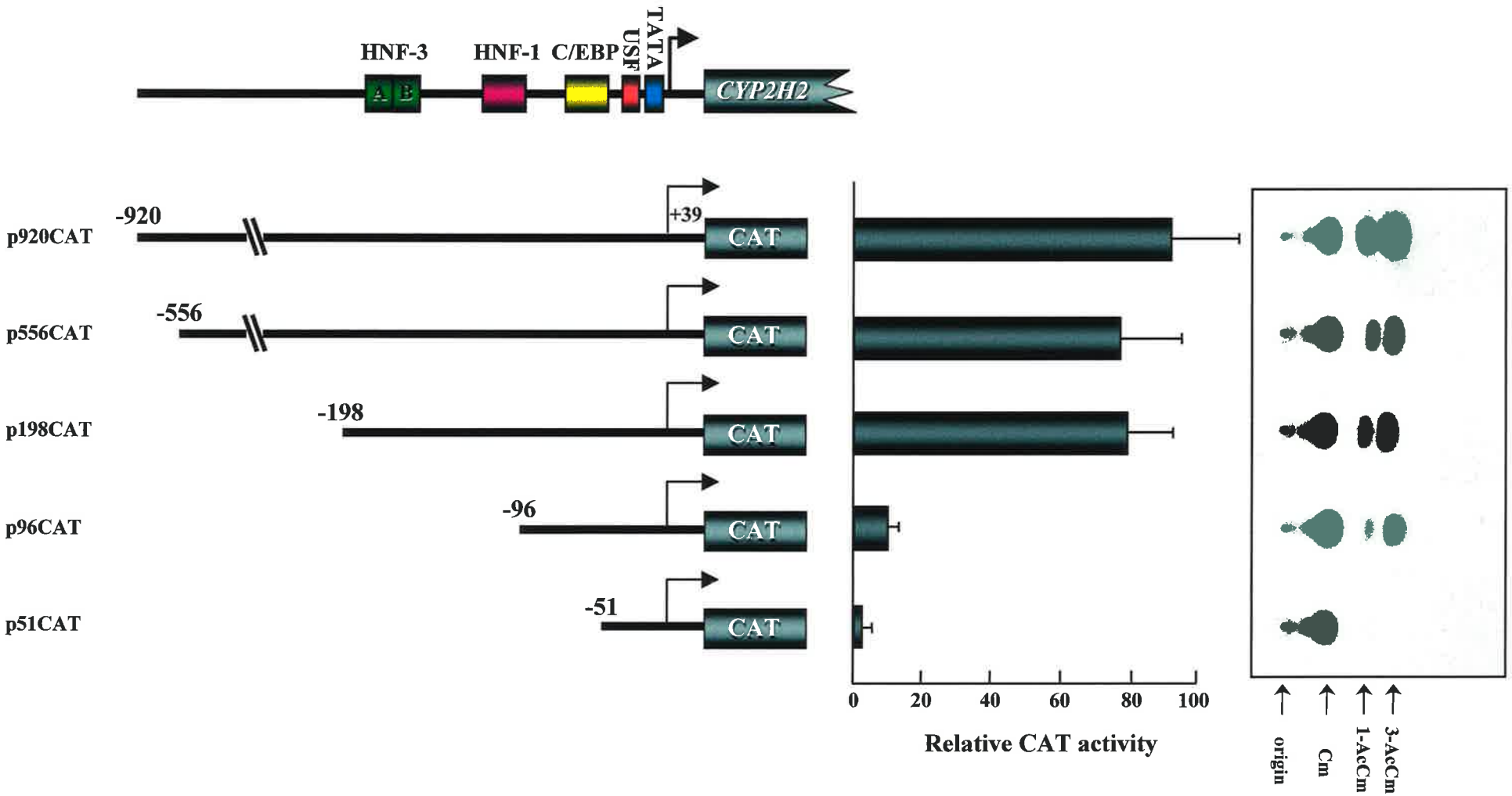

p8.4BK
E
B
8400 bp
pBK
pBK
-5400
+3200
E
P
P
PP
P
P
B
P
P
E
λ20
λ20
-5400
2120 bp
+10800
H
B
pGL2
CAT gene
pGL2
B
N
pBK
pBK
-920
+39
+1200
959 bp
B
pBK
pBK
-920
p920CAT
B
pBK
CAT gene
pBK
-920
+39

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 (2×10^7) 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 [^{14}C]chloramphenicol to acetylated product are shown as the mean ± standard deviation. CAT activities were normalised for transfection efficiency by β-galactosidase activity.


HNF-3
HNF-1
C/EBP
USF
TATA
A
B
CYP2H2
p920CAT
-920
+39
CAT
p556CAT
-556
CAT
p198CAT
-198
CAT
p96CAT
-96
CAT
p51CAT
-51
CAT
0
20
40
60
80
100
Relative CAT activity
origin
Cm
1-AcCm
3-AcCm

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' GAGCAAACAGAG 3') while the HNF-3B site contains three bases that do not conform to the consensus sequence (5' GAGCAAACTTGA 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' GAGCAAACAGAG 3') and 5 bases in the case of HNF-3B (5' GAGCAAACTTGA 3').

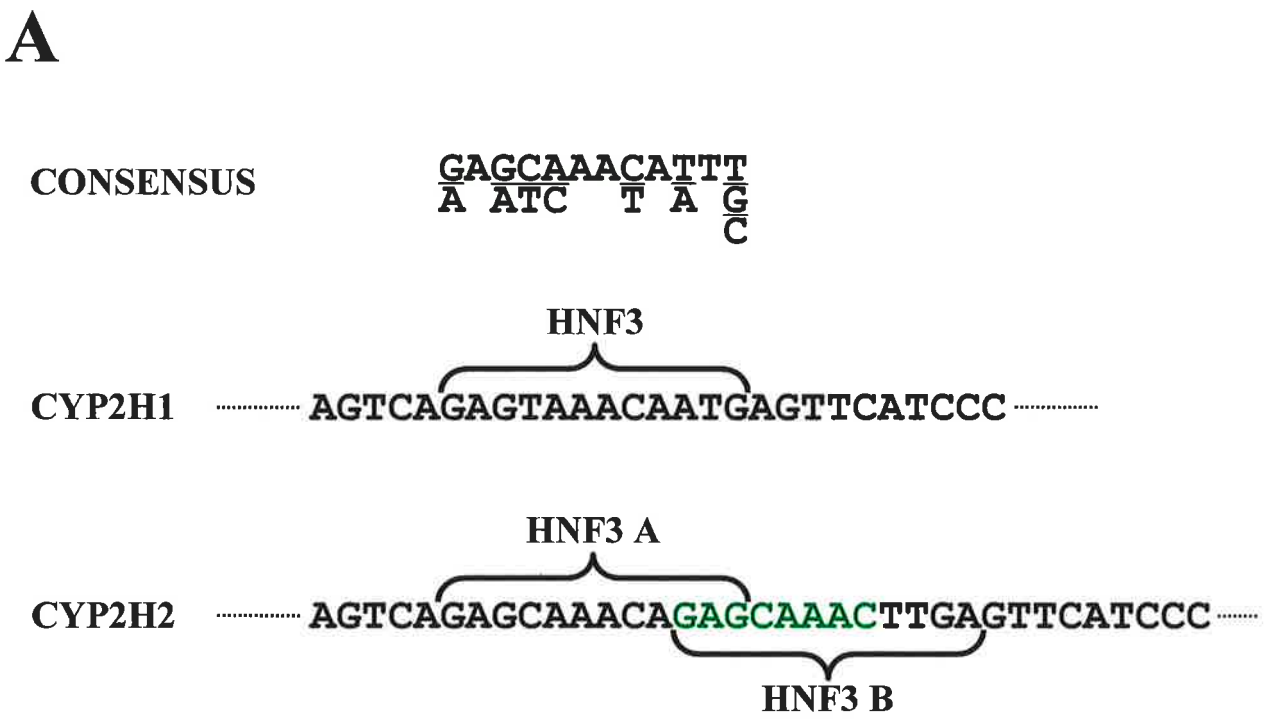
4.2.4 DNase I footprinting analysis of the *CYP2H2* promoter

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
GAGCAAACATTT
A ATC T A G
C
HNF3
CYP2H1
AGTCAGAGTAAACAATGAGTTCATCCC
HNF3 A
CYP2H2
AGTCAGAGCAAACAGAGCAAACTTGAGTTCATCCC
HNF3 B

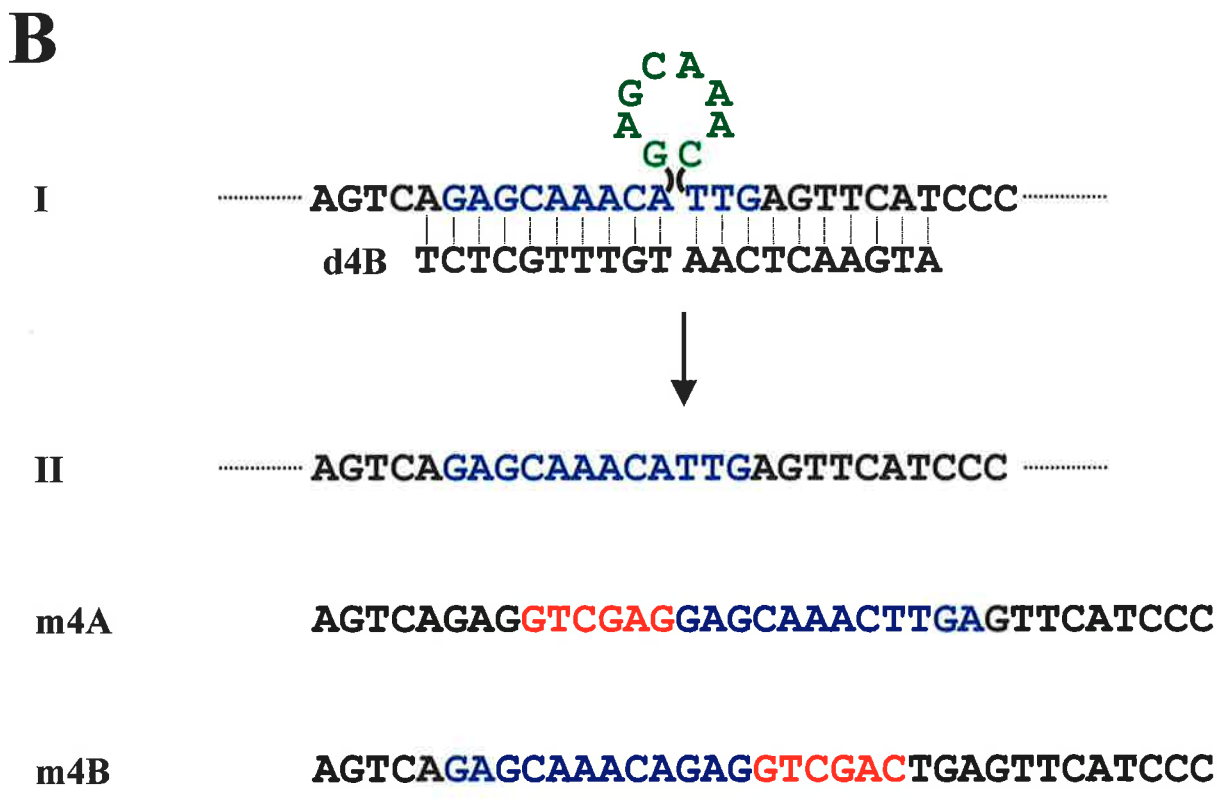

B
C A
G A
A A
G C
I
AGTCAGAGCAAACATTGAGTTCATCCC
d4B TCTCGTTTGT AACTCAAGTA
II
AGTCAGAGCAAACATTGAGTTCATCCC
m4A
AGTCAGAGGTCGAGGAGCAAACTTGAGTTCATCCC
m4B
AGTCAGAGCAAACAGAGGTCGACTGAGTTCATCCC

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.

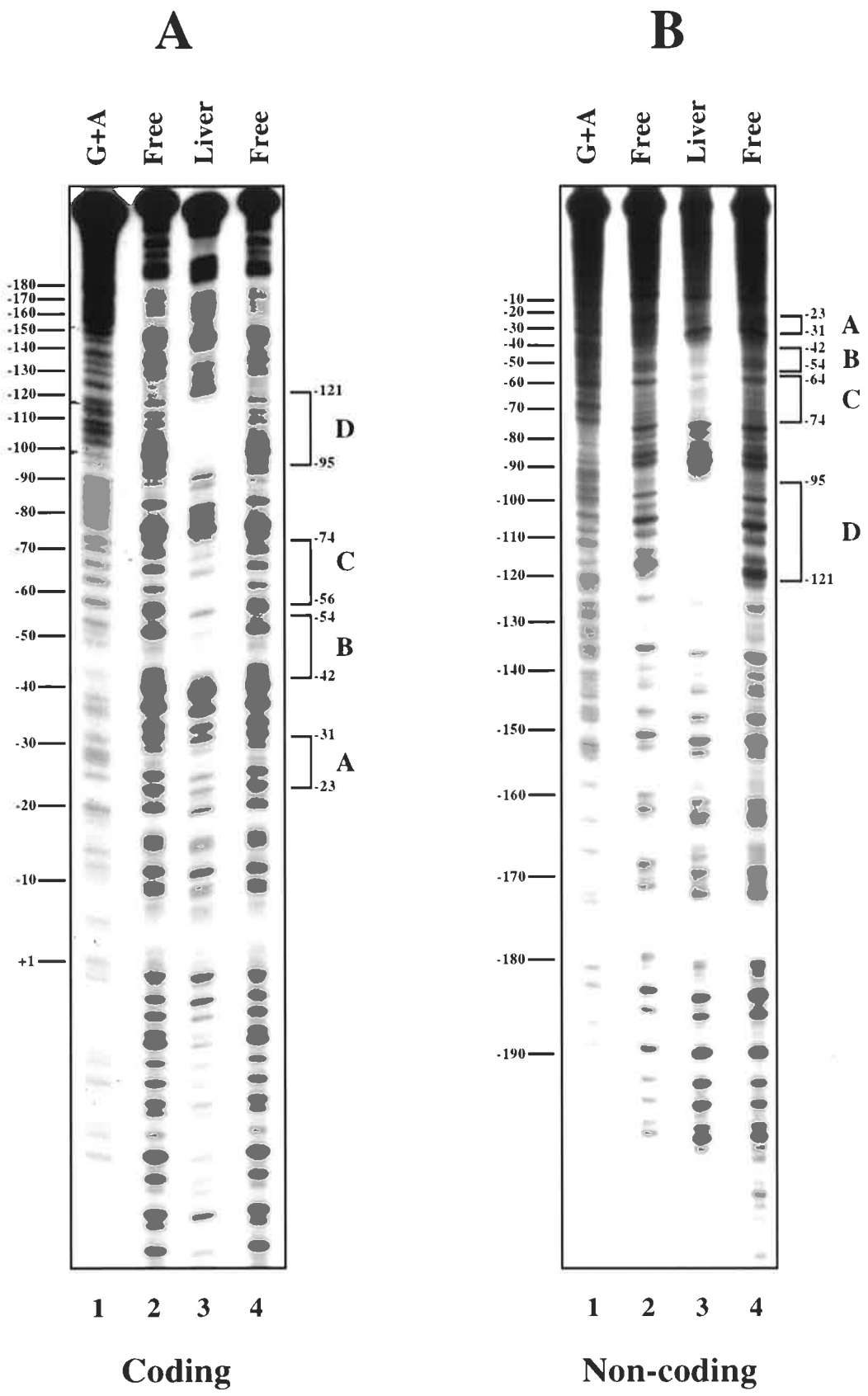
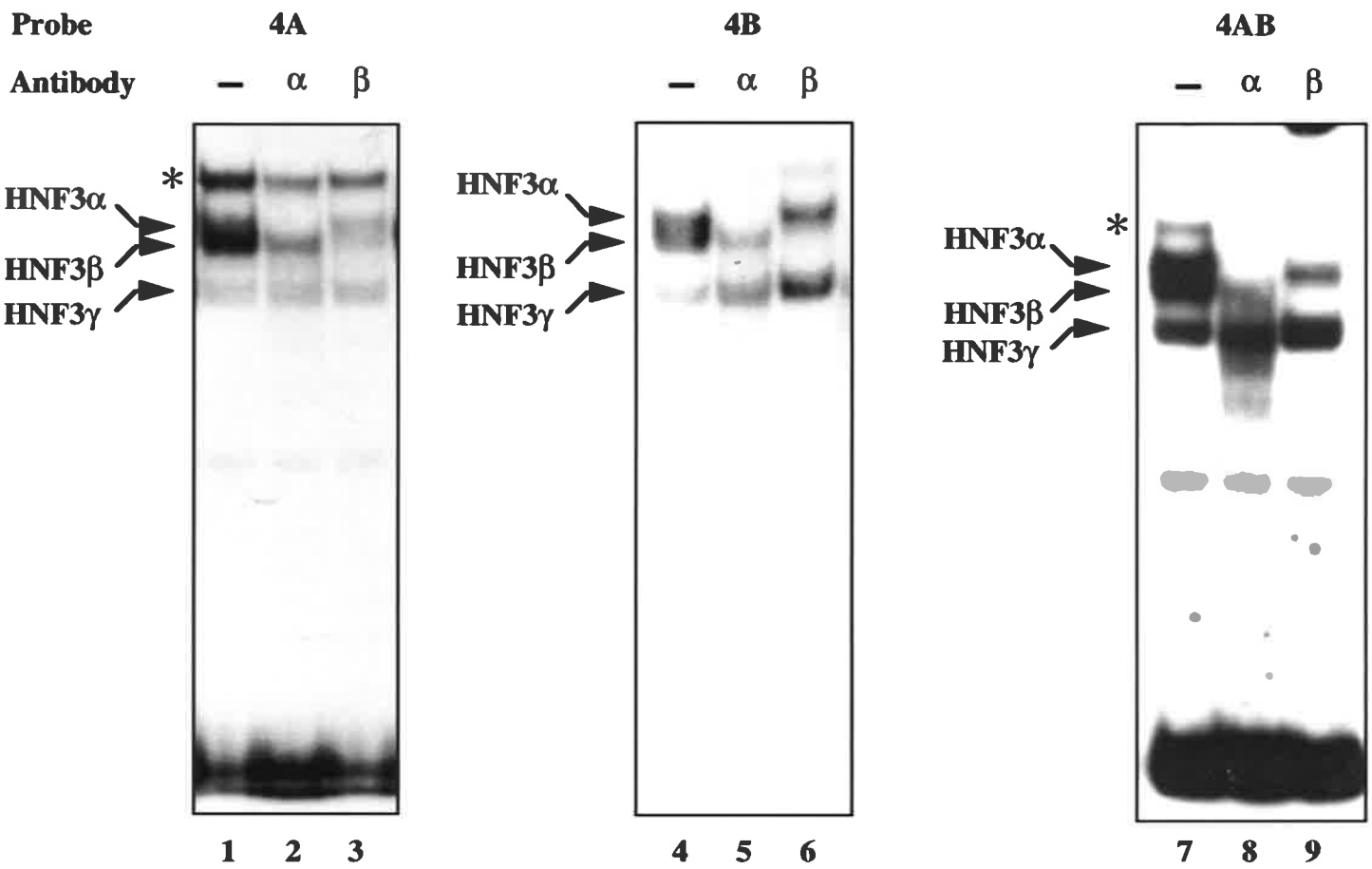

A
G+A
Free
Liver
Free
-180
-170
-160
-150
-140
-130
-120
-110
-100
-90
-80
-70
-60
-50
-40
-30
-20
-10
+1
-121
D
-95
-74
C
-56
-54
B
-42
-31
A
-23
1
2
3
4
Coding
B
G+A
Free
Liver
Free
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
-110
-120
-130
-140
-150
-160
-170
-180
-190
-23
-31
A
-42
B
-54
-64
C
-74
-95
D
-121
1
2
3
4
Non-coding

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.


Probe
4A
4B
4AB
Antibody
–
α
β
HNF3α
HNF3β
HNF3γ
*
1
2
3
4
5
6
7
8
9

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-1 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-3A 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-3 α and HNF-3 β 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.

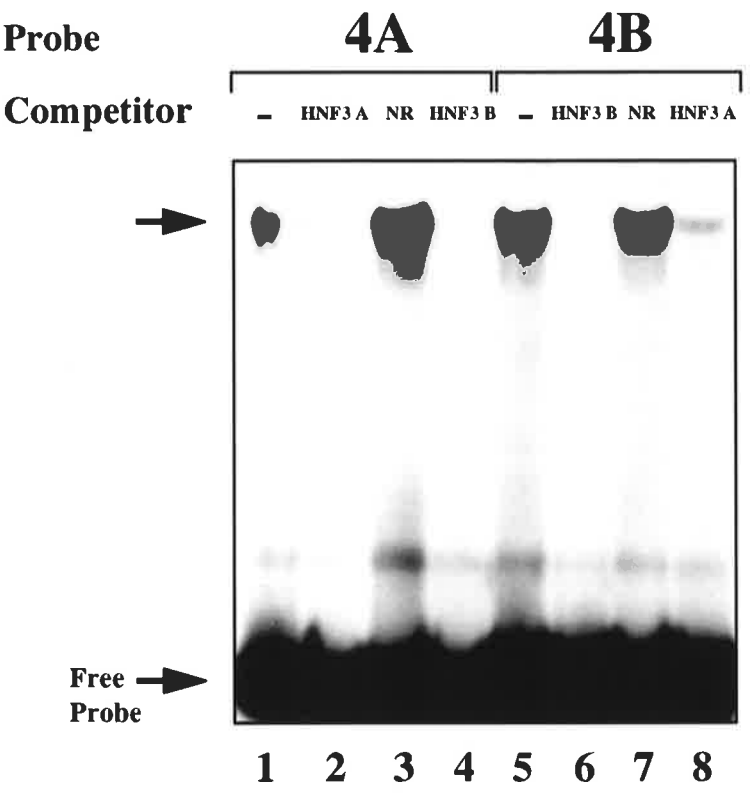
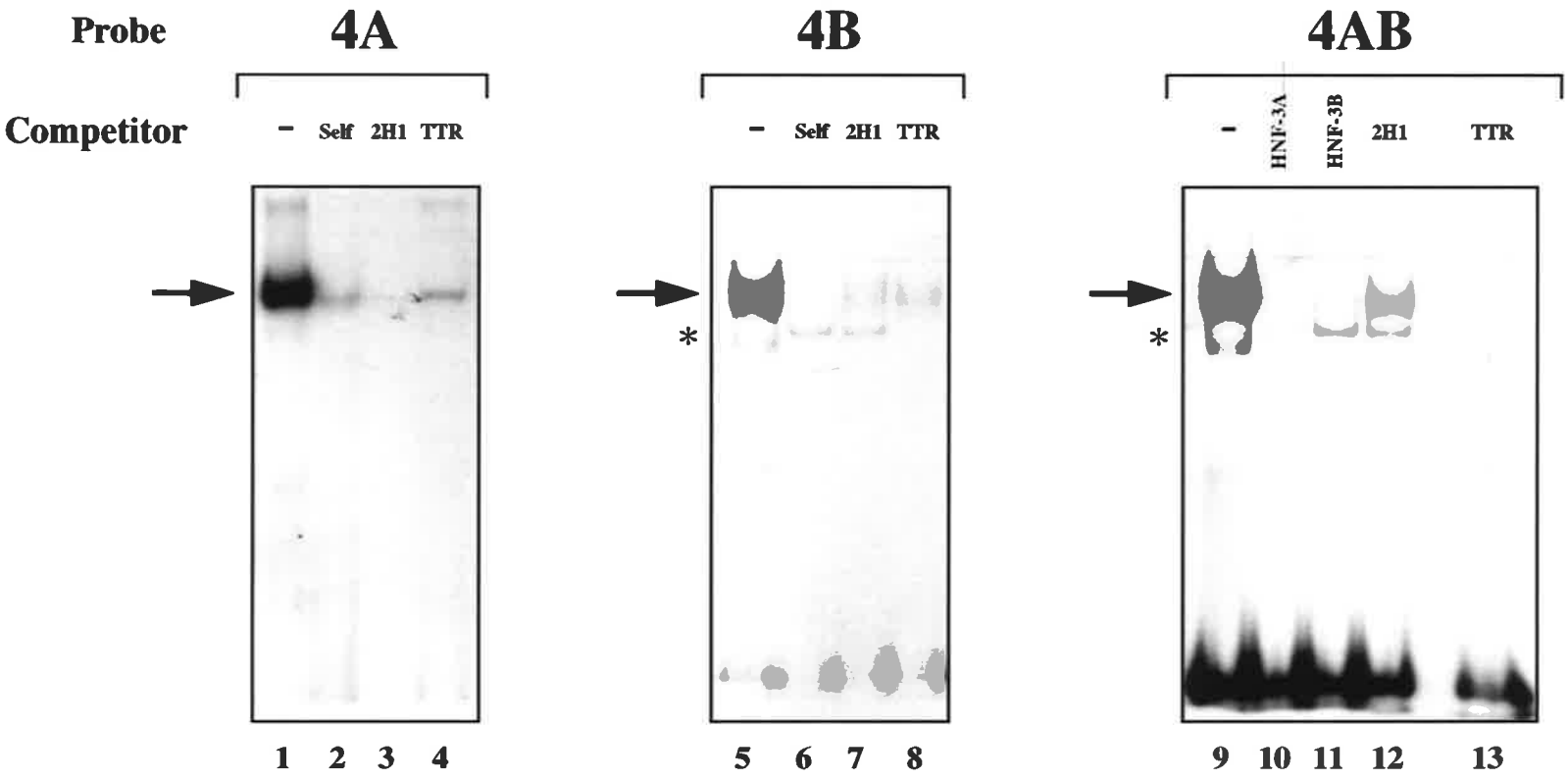

Probe
4A
4B
Competitor
-
HNF3 A
NR
HNF3 B
-
HNF3 B
NR
HNF3 A
Free
Probe
1
2
3
4
5
6
7
8

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 (lane10) 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.


Probe
4A
4B
4AB
Competitor
-
Self
2H1
TTR
-
Self
2H1
TTR
-
HNF-3A
HNF-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.

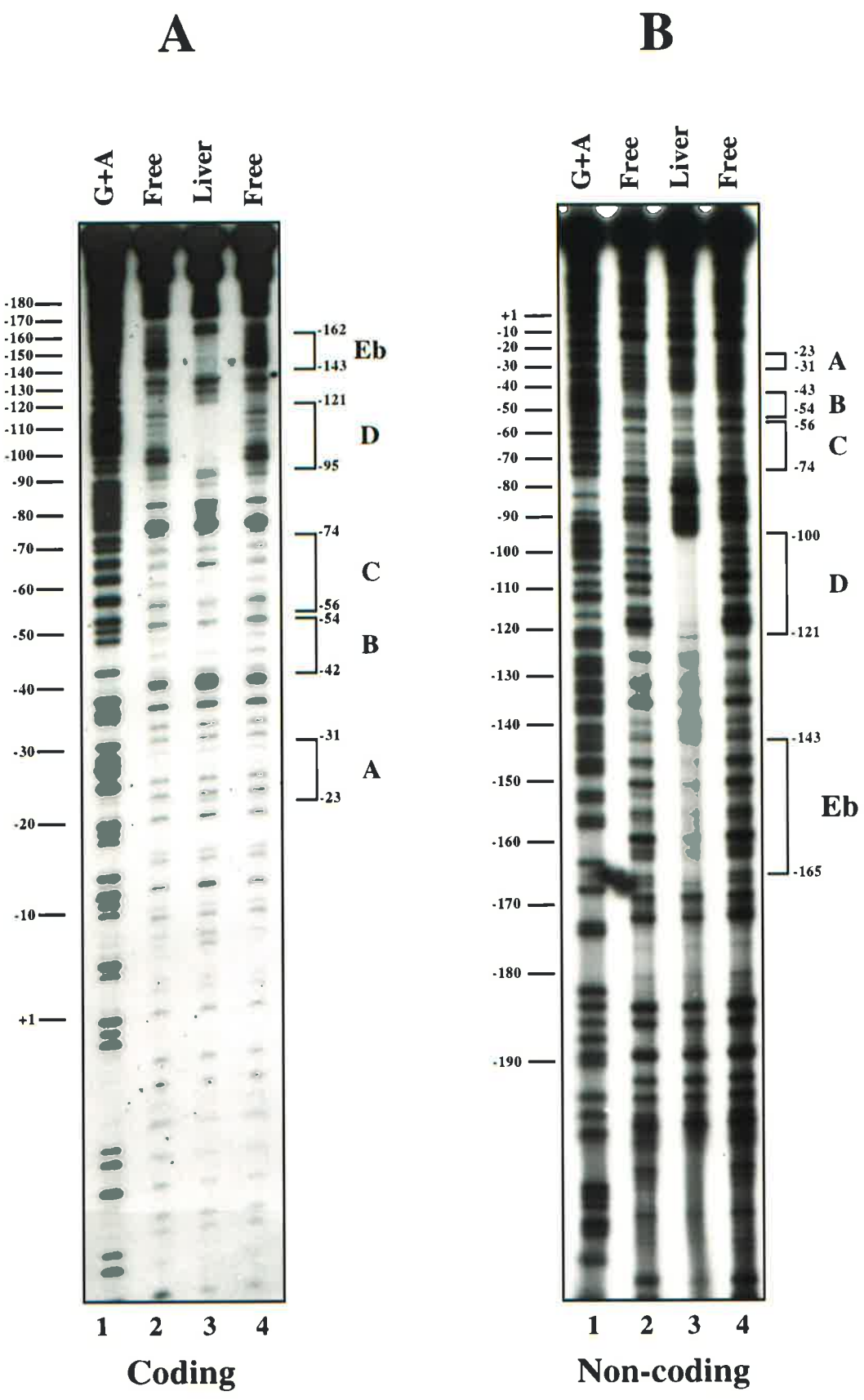
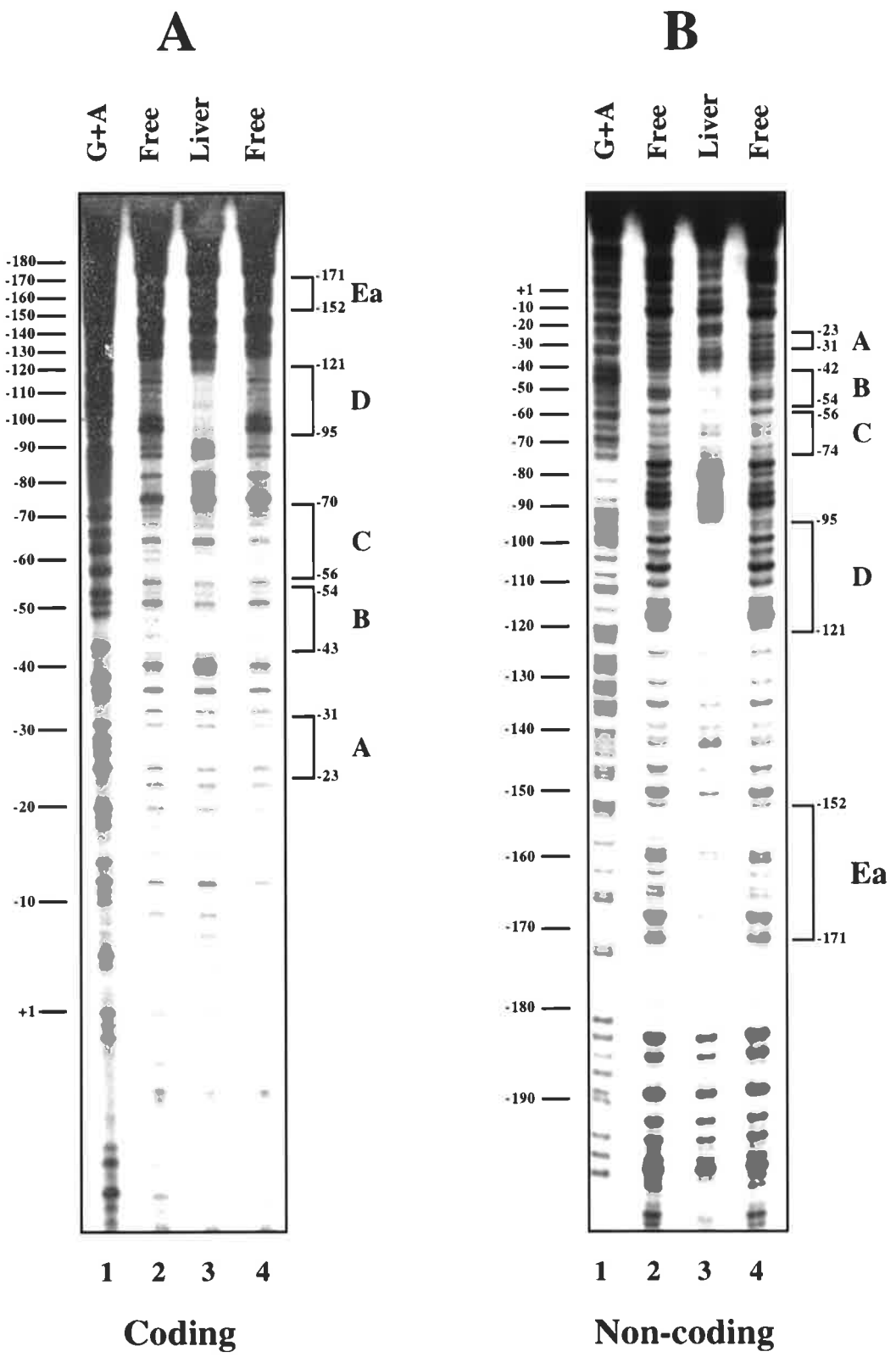

A
G+A
Free
Liver
Free
-180
-170
-160
-150
-140
-130
-120
-110
-100
-90
-80
-70
-60
-50
-40
-30
-20
-10
+1
-162
-143
Eb
-121
-95
D
-74
-56
-54
-42
C
B
-31
-23
A
1
2
3
4
Coding
B
G+A
Free
Liver
Free
+1
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
-110
-120
-130
-140
-150
-160
-170
-180
-190
-23
-31
A
-43
-54
B
-56
-74
C
-100
-121
D
-143
-165
Eb
1
2
3
4
Non-coding

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.


A
G+A
Free
Liver
Free
-180
-170
-160
-150
-140
-130
-120
-110
-100
-90
-80
-70
-60
-50
-40
-30
-20
-10
+1
-171
-152
Ea
-121
-95
D
-70
-56
C
-54
-43
B
-31
-23
A
1
2
3
4
Coding
B
G+A
Free
Liver
Free
+1
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-30
-40
-50
-60
-70
-80
-90
-100
-110
-120
-130
-140
-150
-160
-170
-180
-190
-23
-31
A
-42
-54
B
-56
-74
C
-95
-121
D
-152
-171
Ea
1
2
3
4
Non-coding

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

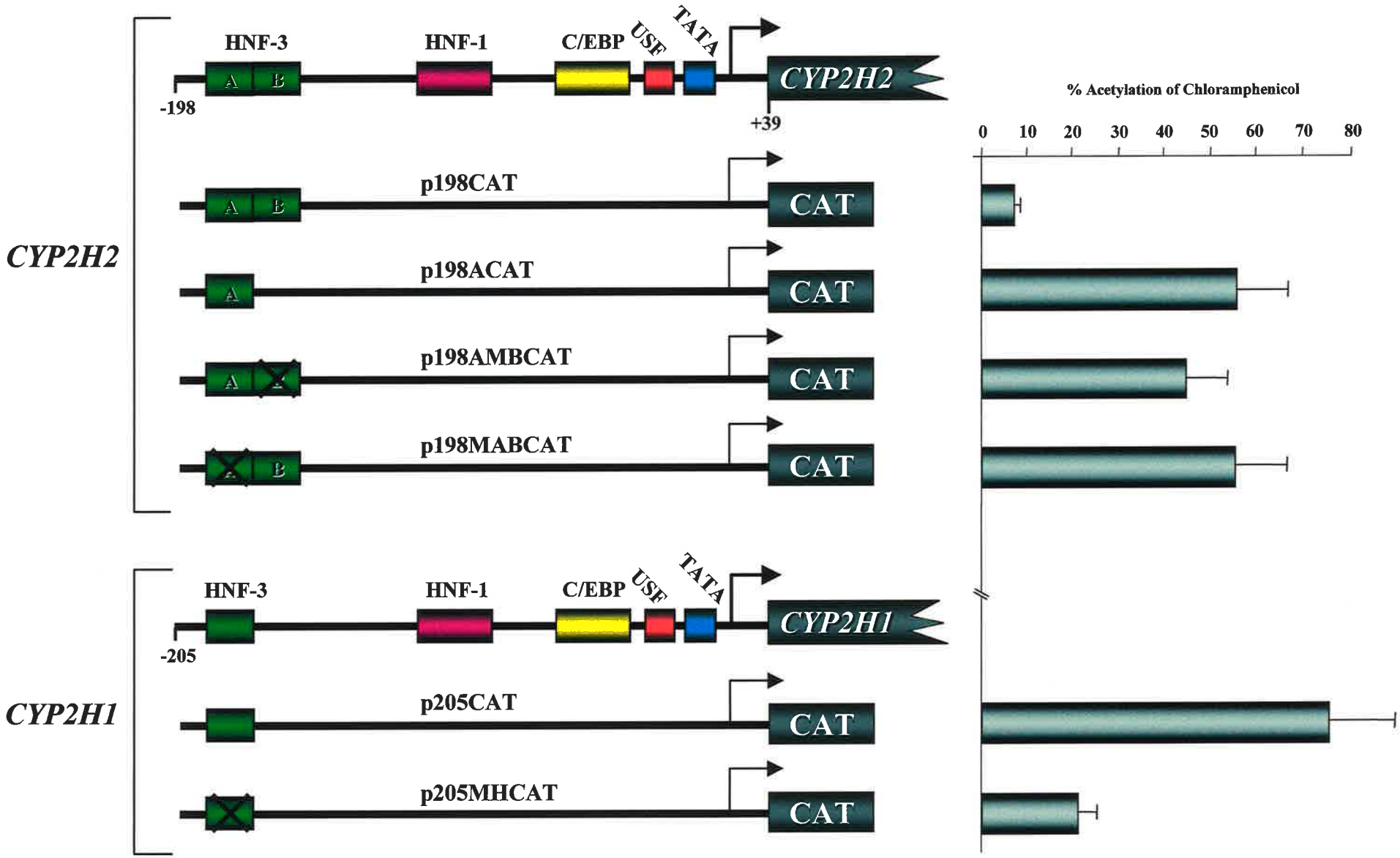
The footprint experiments of wild-type versus mutant HNF-3 sites provided the interesting finding that the wild-type configuration of the HNF-3 binding sites prevents HNF-3 binding to either the A or B site indicating that this may be the reason for the weaker promoter activity of the *CYP2H2* gene. 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 SalI restriction enzyme site into the core region of the HNF-3 sites, changing 5' GAGCAAAC(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 [^{14}C]chloramphenicol to acetylated product are shown as the mean ± standard deviation. CAT activities were normalised to β-galactosidase activity.


CYP2H2
HNF-3
HNF-1
C/EBP
USF
TATA
A
B
-198
+39
CYP2H2
p198CAT
CAT
p198ACAT
CAT
p198AMBCAT
CAT
p198MABCAT
CAT
CYP2H1
HNF-3
HNF-1
C/EBP
USF
TATA
-205
CYP2H1
p205CAT
CAT
p205MHCAT
CAT
% Acetylation of Chloramphenicol
0
10
20
30
40
50
60
70
80

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 *CYP2H1* 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 *CYP2H1* 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-lα (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)TT(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 was 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.

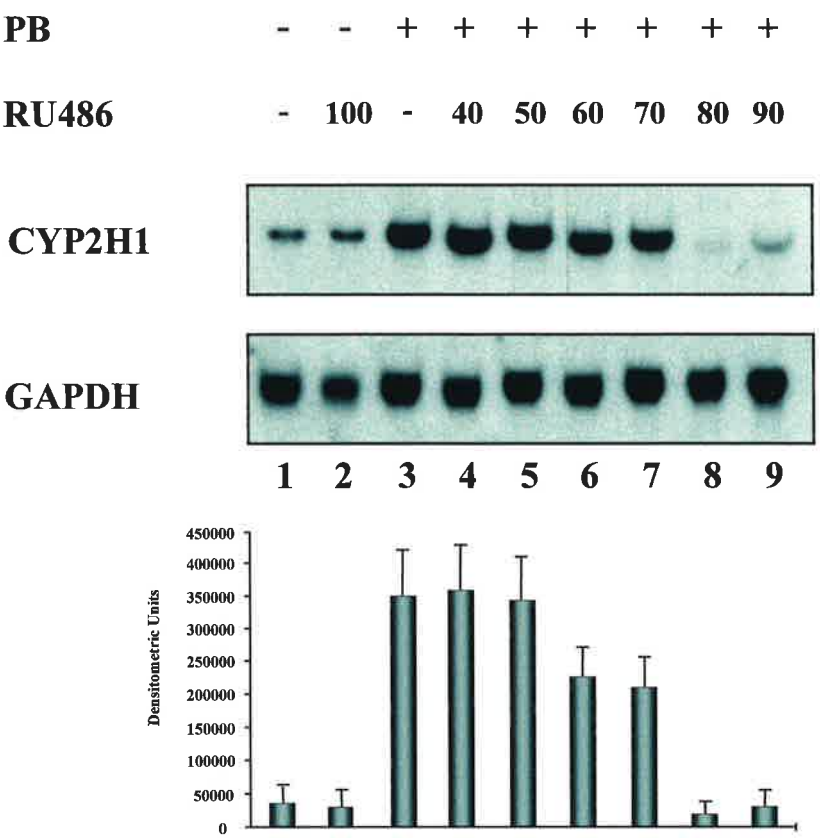
To establish that the inhibition of phenobarbital induction did not result from RU486-mediated cell toxicity or irreversible cell damage the following experiment was performed. Chick embryo primary hepatocytes were treated with either 50 or 100 μM of RU486 for a period of 24 or 48 h. After this treatment 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).

A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RU486 - 100 - 40 50 60 70 80 90
CYP2H1
GAPDH
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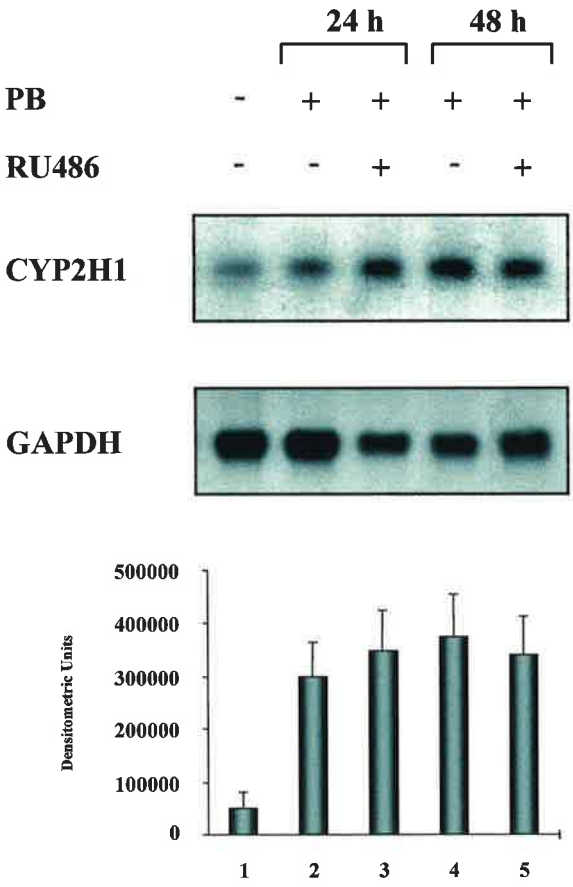
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RU486 - - + - +
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GAPDH
Densitometric Units
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200000
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1 2 3 4 5

concentrations up to 100 μM 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.

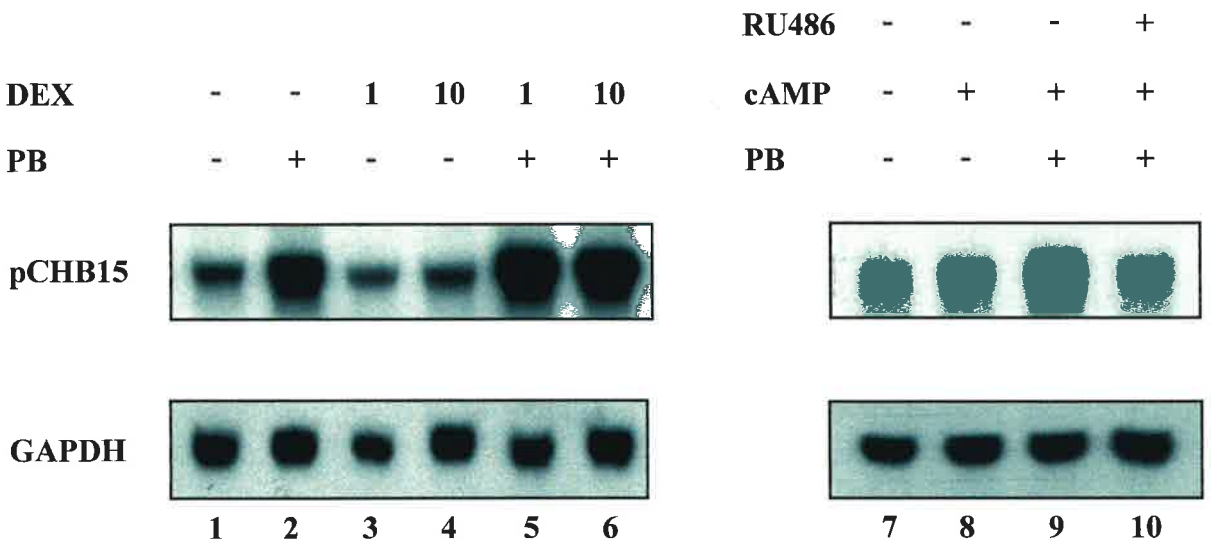
5.2.2 Effect of RU486 and dexamethasone on *CYP2H1* mRNA expression

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 μM of dexamethasone, alone or in combination with 500 μM 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 μM of dexamethasone in combination with 500 μM phenobarbital or 80 μM 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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DEX - - 1 10 1 10
cAMP - + + +
PB - + - - + +
PB - - + +
pCHB15
GAPDH
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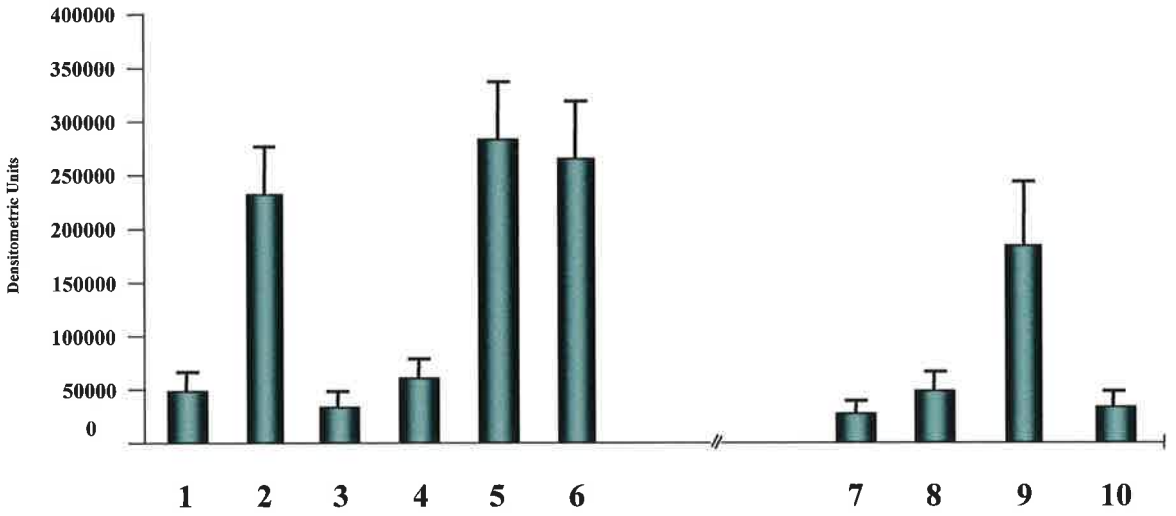
7 8 9 10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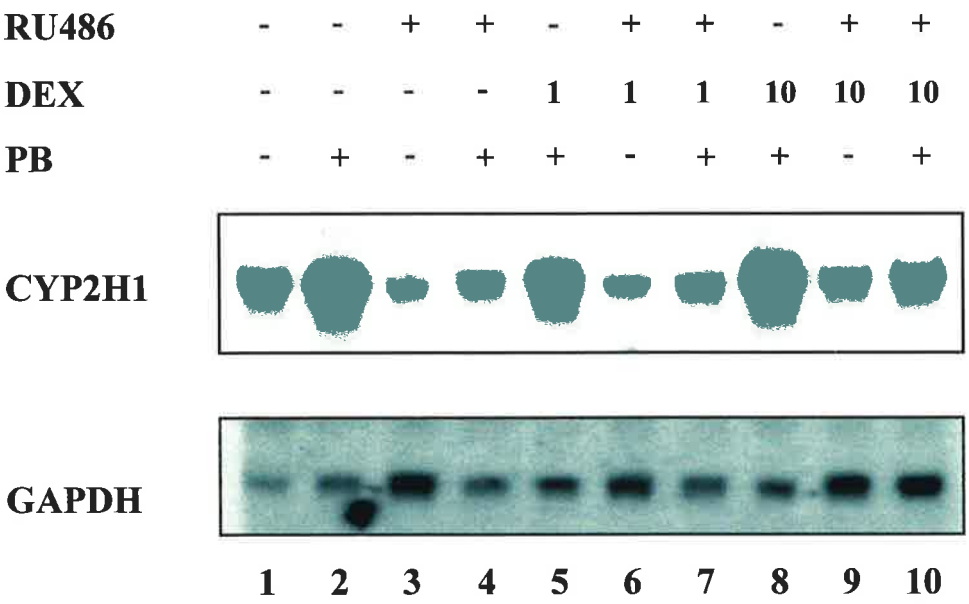
7 8 9 10

Figure 5.3 Effect of Dexamethasone on the RU486 mediated inhibition of induction of *CYP2H1* 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 *CYP2H1* 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.

A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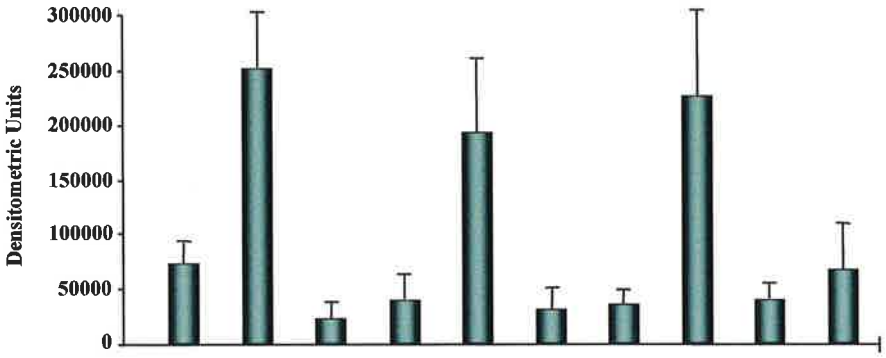
DEX - - - - 1 1 1 10 10 10
PB - + - + + - + + - +
CYP2H1
GAPDH
1 2 3 4 5 6 7 8 9 10


Densitometric Units
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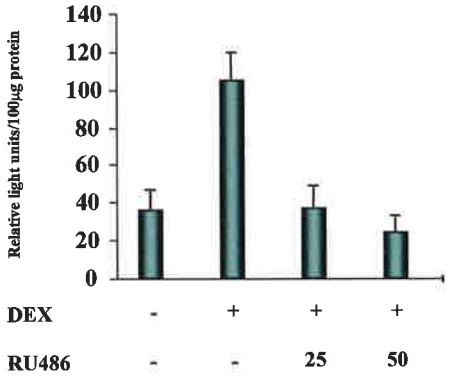
B


Relative light units/100µg protein
140
120
100
80
60
40
20
0
DEX - + + +
RU486 - - 25 50

(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 *CYP2H1* gene.

This data suggest that RU486 may be interfering with a novel pathway involved in the phenobarbital response of the *CYP2H1* gene. One way to isolate this RU486 sensitive pathway would be to identify the sites in the *CYP2H1* 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

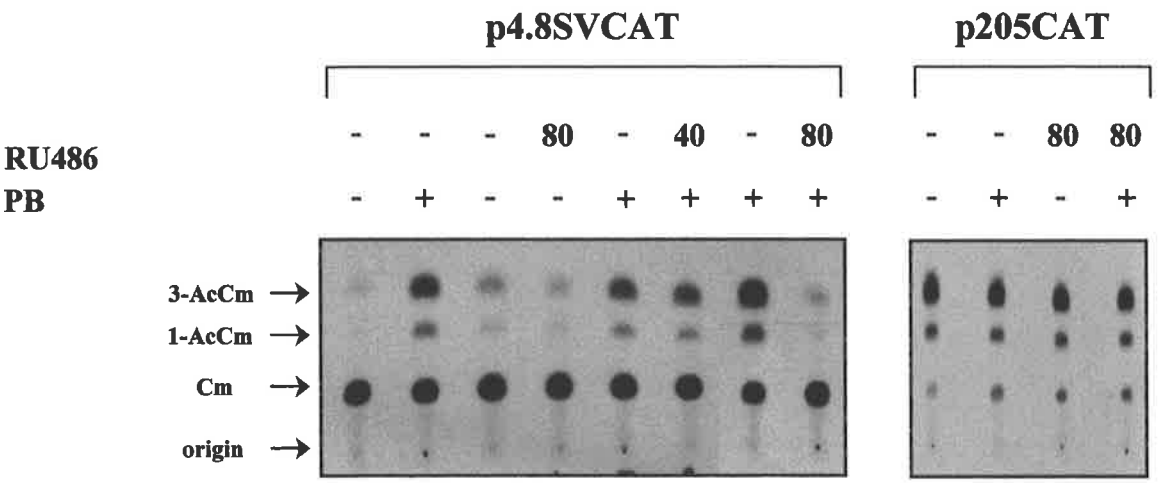
Figure 5.4 Effect of RU486 on the transient expression of p4.8SVCAT and p205CAT.

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 [^{14}C]chloramphenicol to acetylated product is shown. Numbers above columns represent the fold increase in CAT activity compared to untreated (control) activity.

A


p4.8SVCAT
p205CAT
RU486
PB
- - - 80 - 40 - 80
- - 80 80
- + - - + + + +
- + - +
3-AcCm
1-AcCm
Cm
origin

B

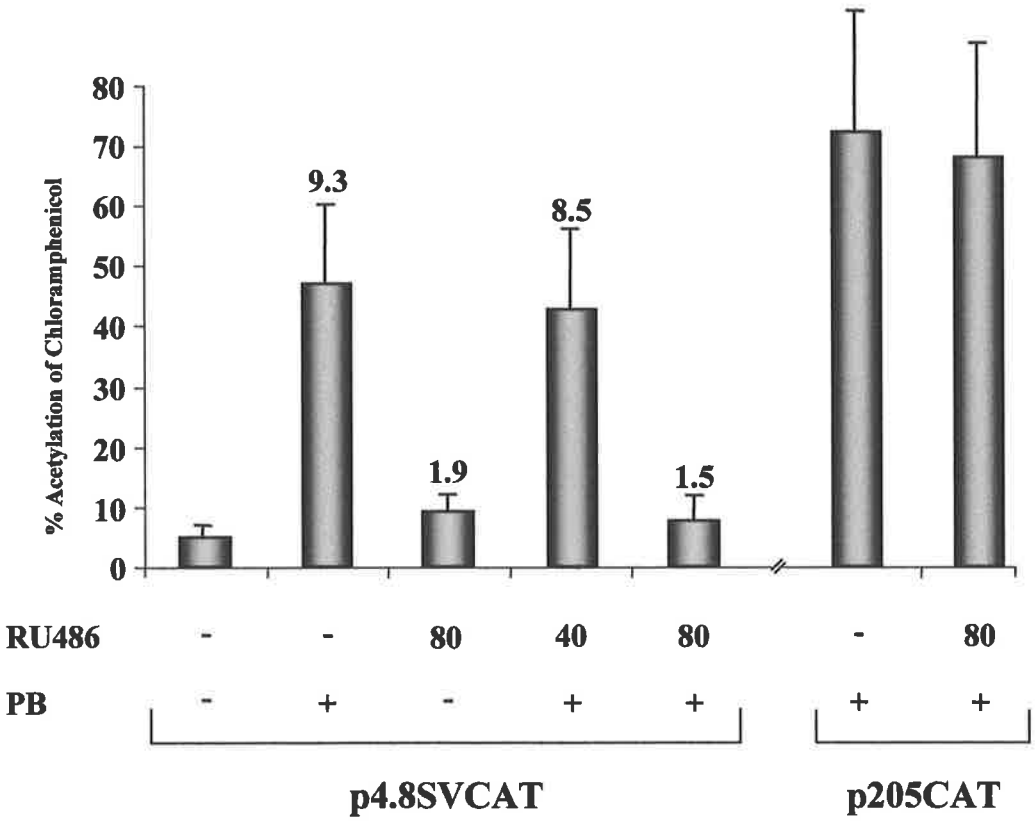
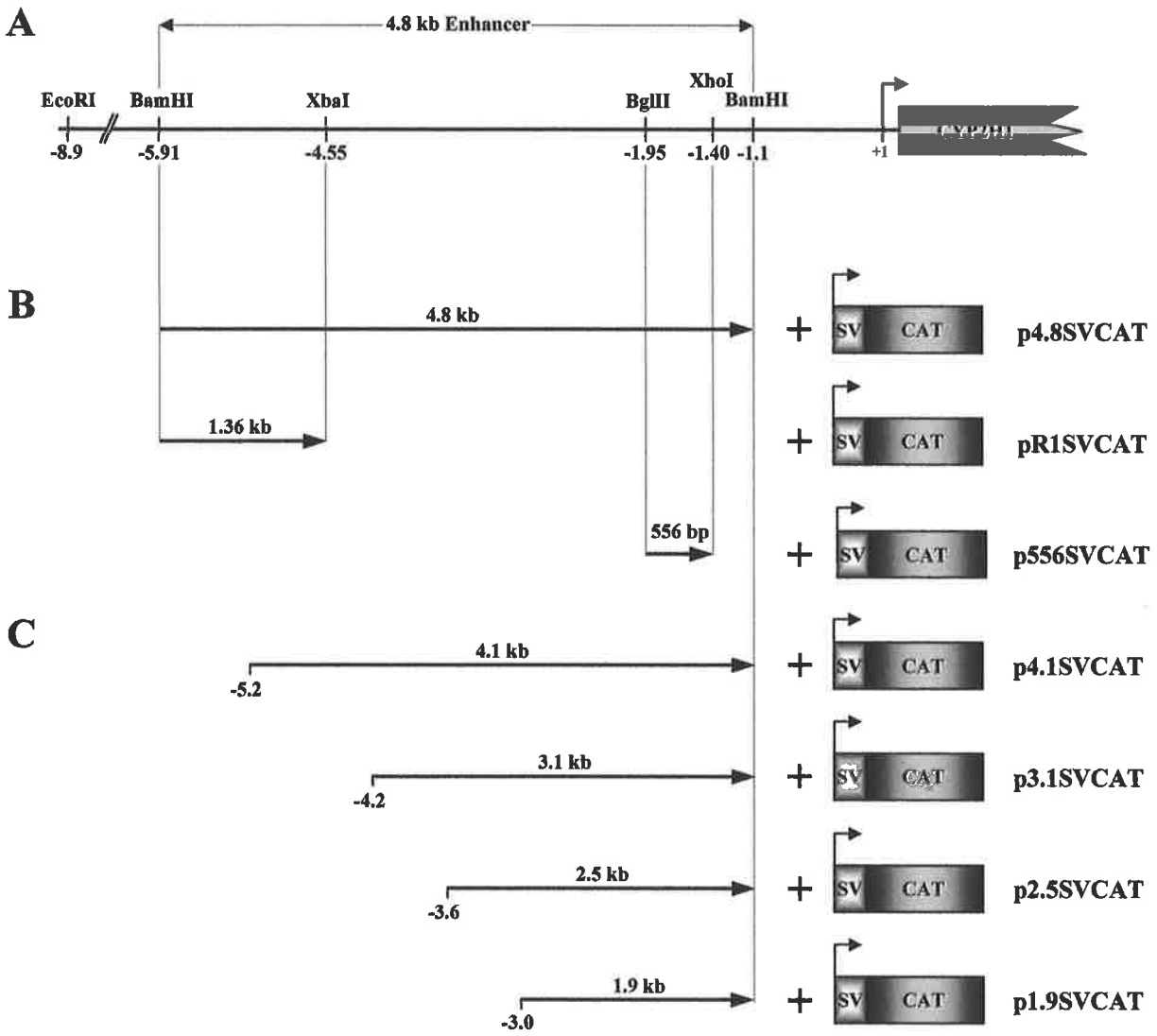

% Acetylation of Chloramphenicol
0
10
20
30
40
50
60
70
80
9.3
1.9
8.5
1.5
RU486 - - 80 40 80 - 80
PB - + - + + + +
p4.8SVCAT
p205CAT

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 *Bam*HI 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 *Bam*HI 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
XhoI
BamHI
-8.9
-5.91
-4.55
-1.95
-1.40
-1.1
+1
B
4.8 kb
1.36 kb
556 bp
SV
CAT
p4.8SVCAT
pR1SVCAT
p556SVCAT
C
4.1 kb
-5.2
3.1 kb
-4.2
2.5 kb
-3.6
1.9 kb
-3.0
p4.1SVCAT
p3.1SVCAT
p2.5SVCAT
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 *CYP2H1* 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 *CYP2H1* 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 [^{14}C]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

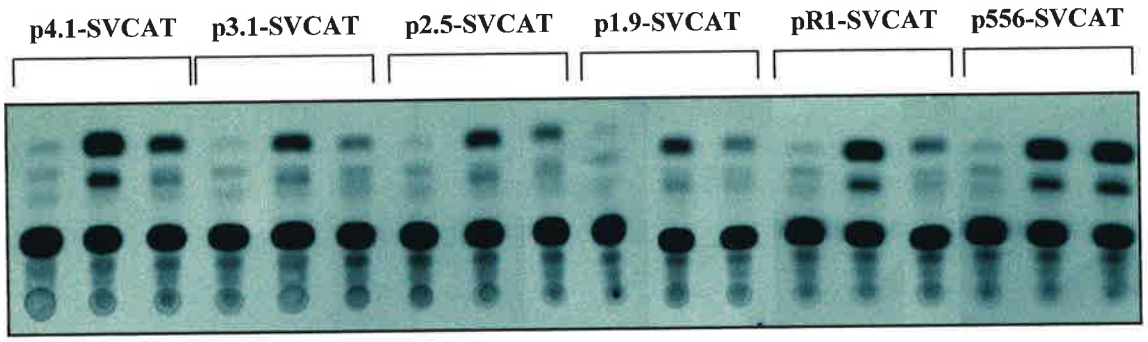
Figure 5.6 Effect of RU486 on expression of SV/CAT constructs containing fragments of the *CYP2H1* 4.8 kb enhancer.

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.

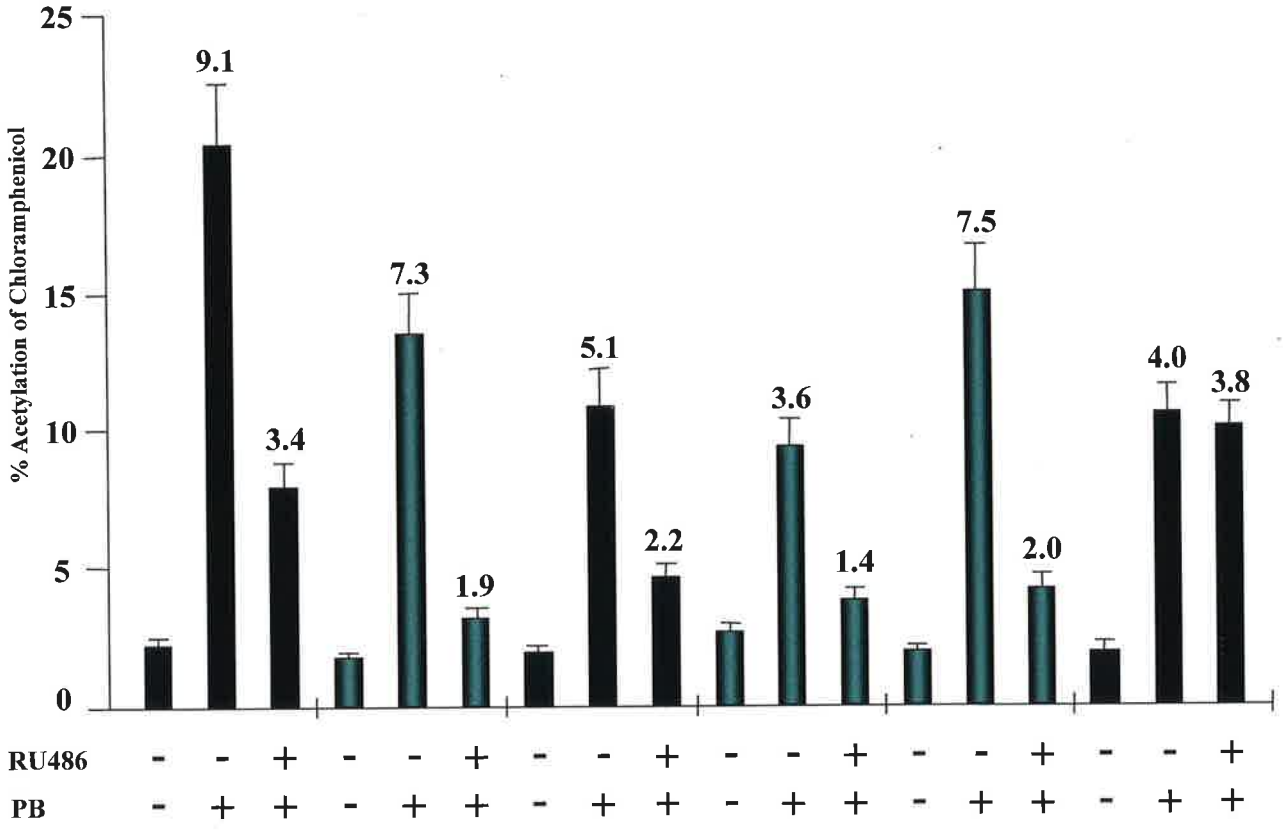
A. A typical CAT assay of one such experiment is shown.

B. The average of three independent experiments, repeated in duplicate and represented as a percentage of conversion of [^{14}C]chloramphenicol to acetylated product is shown.

A


p4.1-SVCAT
p3.1-SVCAT
p2.5-SVCAT
p1.9-SVCAT
pR1-SVCAT
p556-SVCAT

B


% Acetylation of Chloramphenicol
25
20
15
10
5
0
9.1
3.4
7.3
1.9
5.1
2.2
3.6
1.4
7.5
2.0
4.0
3.8
RU486 - - + - - + - - + - - + - - + - - +
PB - + + - + + - + + - + + - + + - + +

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 *CYP1A1* gene (372).

Shaw and co-workers, who demonstrated RU486 mediated inhibition of PB induction of the rat *CYP2B1/2* 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 *CYP2H1* 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 *CYP2H1* 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 *CYP2H1* and *CYP2H2* genes.

6.2 Isolation and characterisation of a phenobarbital-responsive element within the 5' flanking region of the *CYP2H1* 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, *CYP2H1* and *CYP2H2*, in the chicken genome (221). Two separate regions have been identified within the 5' flanking sequence of the *CYP2H1* 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 *CYP2B1/2* (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

Davidson BP, Dogra SC and May BK. (1996). Liver specific expression: Identification of control elements in the promoters of two chicken cytochrome P450 genes. 18th Annual Conference on the Organisation and Expression of the Genome.

Davidson BP, Dogra SC and May BK. (1997). Hepatocyte nuclear factor 3 (HNF-3) and its role in the differential expression of two chicken cytochrome P450 genes. *FASEB Journal* **11(9)**:A1067

Papers

Dogra SC, Davidson BP and May BK. (1998). Analysis of a phenobarbital-responsive enhancer domain located in the 5' flanking region of the chicken *CYP2H1* gene: identification and characterisation of functional protein binding sites. *Molecular Pharmacology* **55**:14-22

Grover PK, Dogra SC, Davidson BP, Stapleton AMF and Ryall RL. (2000). The prothrombin gene is expressed in the rat kidney. Implications for urolithiasis research. *European Journal of Biochemistry*. **267(1)**:61-67

References

1. Abayratna S. (1999). Characterisation of the phenobarbital-responsive enhancer regions in the chicken *CYP2H1* gene. *Honours Thesis.*

2. Abbot BD, Probst MR and Perdew GH (1994) Immunohistochemical double staining for Ah receptor and Arnt in human embryonic palatal shelves. *Tetarology* **50**:361-6.

3. Abel SM, Back DJ, Maggs JL and Park BK. (1993). Cortisol metabolism *in vitro* II. Species difference. *J. Steroid Biochem. Mol. Biol.* **45**:445-453

4. Agarwal M and Lazer G. (1987). Analysis of the structure and function of steroid receptors with the aid of the antihormone RU486: In: *Receptor mediated antisteroid action.* (Agarwal MK. ed) Walter de Gruyter, New York, pp 43-75

5. Ahlgren R, Simpson ER, Waterman MR and Lund J. (1990). Characterisation of the promoter/regulatory region of the bovine *CYP11A* (P450scc) gene: Basal and cAMP-dependent expression. *J. Biol. Chem.* **265**:3313-3319

6. Aldridge TC, Tugwood JD and Green S. (1995). Identification and characterisation of DNA elements implicated in the regulation of *CYP4A1* transcription. *Biochem. J.* **306**:473-479

7. Alexander DL, Ganem LG, Fernandez-Salguero P, Gonzalez F and Jefcoate CR. (1998). Aryl-hydrocarbon receptor is an inhibitory regulator of lipid synthesis and of commitment to adipogenesis. *J. Cell Sci.* **111**:3311-3322

8. Angel P, Imagawa M, Chiu R, Stein B, Imbra RJ, Rahmsdorf HJ, Jonat C, Herrlich P and Karin M. (1987). Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. *Cell* **49**:729-739

9. Antonsson C, Whitelaw ML, McGuire J, Gustafsson J-A and Poellinger L. (1995). Distinct roles of the molecular chaperone hsp90 in modulating dioxin receptor function via the basic helix-loop-helix and PAS domains. *Mol. Cell. Biol.* **15**:756-765

10. Aoyama T, Hardwick JP, Imaoka S, Funae Y, Gelboin HV and Gonzalez FJ. (1990). Clofibrate-inducible rat hepatic P450s IVA1 and IVA3 catalyse the ω- and (ω-1)-hydroxylation of fatty acids and the ω-hydroxylation of prostaglandins E_1 and $F_{2\alpha}$. *J. Lipid Res.* **31**:1477-1482

11. Aoyama T, Yamano S, Waxman DJ, Lapenson DP, Meyer UA, Fischer V, Tyndale R, Inaba T, Kalow W, Gelboin HV and Gonzalez FJ (1989). Cytochrome P-450 hPCN3, a novel cytochrome P-450 IIIA gene product that is differentially expressed in adult human liver, cDNA and deduced amino acid sequence and distinct specificities of cDNA-expressed hPCN1 and hPCN3 for the metabolism of steroid hormones and cyclosporin. *J. Biol. Chem.* **264**:10388-10395

12. Archer TK, Lefebvre P, Wolford RG and Hager GL. (1992). Transcription factor loading on the MMTV promoter: a bimodal mechanism for promoter activation. *Science* **255**:1573-1576

13. Auge-Gouillou C, Petropoulos I and Zakin MM. (1993). Liver-enriched HNF-3α and ubiquitous factors interact with the human transferrin gene enhancer. *FEBS Lett.* **323**:4-10

14. Baes M, Gulick T, Choi HS, Martinoli MG, Simha D and Moore DD. (1994). A new orphan member of the nuclear hormone receptor superfamily that interacts with a subset of retinoic acid response elements. *Mol. Cell. Biol.* **14**:1544-1552

15. Bakke, M. and Lund J. (1992) A novel cyclic adenosine 3',5'-monophosphate-responsive sequence in the bovine *CYP17* gene is a target of negative regulation by protein kinase C. *Mol. Endocrinol.* **6**:1323-1331

16. Bartsch J, Truss M, Bode J and Beato M. (1996). Moderate increase in histone acetylation activates the mouse mammary tumor virus promoter and remodels its nucleosome structure. *Proc. Natl. Acad. Sci. USA.*. **93**:10741-10746

17. Baulieu EE. (1989). Contragestion and other clinical applications of RU486, an antiprogesterone at the receptor. *Science* **245**:1351-1357

18. Beato M, Chalepakis G, Schauer M and Slater EP. (1989). DNA regulatory elements for steroid hormones. *J. Steroid Biochem.* **32**:737-748

19. Beck CA, Weigel NL, Moyer ML, Nordeen SK and Edwards DP. (1993). The progesterone antagonist RU486 acquires agonist activity upon stimulation of cAMP signalling pathways. *Proc. Natl. Acad. Sci. USA.*. **90**:4441-4445

20. Becker PB, Gloss B, Schmid W, Strahle U and Schutz G. (1986). In vivo protein -DNA interactions in a glucocorticoid response element require the presence of the hormone. *Nature* **324**:686-688

21. Berghard A, Gradin K, Pongratz I, Whitelaw M and Poellinger L. (1993) Cross-coupling of signal transduction pathways: the dioxin receptor mediates induction of cytochrome P-4501A1 expression via a protein kinase C-dependent mechanism. *Mol. Cell. Biol.* **13**:677-689

22. Bertilsson G, Heidrich J, Svensson K, Asman M, Jendeberg L, Sydaw-Backman M, Ohlsson R, Postlind H, Blomqmst P and Berkenstam A. (1998). Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. *Proc. Natl. Acad. Sci USA...* **95**:12208-12213

23. Bhat GJ, Rangarajan PN and Padmanaban G. (1987) Differential effects of cyclohexamide on rat liver cytochrome P-450 gene transcription in the whole animal and hepatocyte cell culture. *Biochem. Biophys. Res. Commun.* **148**:1118-23

21. Bhattacharyya KK, Brake PB, Eltom SE, Otto SA and Jefcoate CR. (1995). Identification of a rat adrenal cytochrome P450 active in polycyclic hydrocarbon metabolism as rat *CYP1B1*. *J. Biol. Chem.* **270**:11595-602

25. Birnboim HC and Doly J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**:1513-1523.

26. Blumberg B, Sabbagh W, Jr, Juguilon H, Bolado J, Jr, van Meter CM, Ong ES and Evans RM. (1998). SXR, a novel steroid and xenobiotic-sensing nuclear receptor. *Genes Dev.* **12**:3195-3205.

27. Borthwick IA, Srivastava G, Hobbs AA, Priola BA, Brooker JD, May BK and Elliott WH. (1985). Molecular cloning of hepatic 5-aminolevulinate synthase. *Eur. J. Biochem.* **150**:481-484.

28. Braissant O, Foufelle F, Scotto C, Dauca M and Wahli W. (1996). Differential expression of peroxisome proliferator-activated receptors (PPARs):Tissue distribution of PPAR-alpha, -beta and -gamma in adult rat. *Endocrinology* **137**:354 366

29. Brake PB, Zhang L and Jefcoate CR. (1998). Aryl hydrocarbon receptor regulation of cytochrome P4501B1 in rat mammary fibroblasts: Evidence for transcriptional repression by glucocorticoids. *Mol. Pharmacol.* **54**:825-833

30. Brennan RG. (1993). The winged-helix DNA-binding motif: another helix-turn-helix takeoff. *Cell* **74**:773-776

31. Brentano ST, Picardo-Leonard J, Mellon SH, Moore CCD and Miller WL. (1990). Tissue-specific, cyclic adenosine 3',5'-monophosphate -induced, and phorbol ester-repressed transcription from the human P450c17 promoter in mouse cells. *Mol. Endocrinol.* **4**:1972-1079

32. Brian WR, Sari M-A, Iwasaki M, Shimada T, Kaminsky LS and Guengerich FP. (1990). Catalytic activities of human liver cytochrome P-450 IIIA4 expressed in *Sacharomyces cereviciae*. *Biochemistry* **29**:11280-11292

33. Brooker JD and O'Connor R. (1982). cDNA cloning and analysis of chick-embryo-liver cytochrome P-450 mRNA induced by porphyrinogenic drugs. *Eur. J. Biochem.* **129**:325-333

34. Brownell, JE and Allis CD. (1996). Special HATs for special occasions: linking histone acetylation to chromatin assembly and gene activation. *Curr. Opin. Genet. Dev.* **6**:176-184

35. Bulla GA, DeSimone V, Cortese R and Fournier RE. (1992). Extinction of alpha 1-antitrypsin gene expression in somatic cell hybrids: evidence for multiple controls. *Genes Dev.* **6**:316-327

36. Burbach KM, Poland A and Bradfield CA. (1992). Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. *Proc. Natl. Acad. Sci. USA.*. **89**:8185-8189

37. Burger H, Schuetz EG, Schuetz JD and Guzelian PS. (1990). Divergent effects of cyclohexamide on the induction of class II and class III cytochrome P450 mRNAs in cultures of adult rat hepatocytes. *Arch. Biochem. Biophys.* **281**:204-211

38. Burger H, Schutez JD, Schutez EG and Guzelian PS. (1992). Paradoxical transcription activation of rat liver cytochrome P-450 3A1 by dexamethasone and antiglucocorticoid pregnenolone 16α-carbonitrile: analysis by transient transfection into primary monolayer cultures of adult rat hepatocytes. *Proc. Natl. Acad. Sci. USA.*. **89**:2145-2149

39. Busch SJ and Sassone-Corsi P (1990). Dimers, leucine zippers and DNA-binding domains. *Trends Genet.* **6**:36-40

40. Cameron EE, Bachman KE, Myohanen S, Herman JG and Baylin SB. (1999). Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat. Genet.* **21**:103-107

43. Carver LA, Jackiw V and Bradfield CA. (1994). The 90 kDa heat shock protein is essential for Ah receptor signalling in a yeast expression system. *J. Biol. Chem.* **269**:30109-30112

44. Cereghini S. (1996). Liver-enriched transcription factors and hepatic differentiation. *FASEB J.* **10**:267-282

45. Cereghini S, Raymondjean AG, Carranca AS, Herbomel P and Yaniv M. (1987). Factors involved in control of tissue-specific expression of albumin gene. *Cell* **50**:627-638

46. Cereghini S and Yaniv M. (1994).Assembly of transfected DNA into chromatin: structural changes in the origin-promoter-enhancer region upon replication. *EMBO J.* **3**:1243-1253

47. Chang C-J, Chen T-T, Lei H-Y, Chen D-S and Lee S-C. (1990) Molecular cloning of a transcription factor, AGP/EBP, that belongs to members of the C/EBP family. *Mol. Cell. Biol.* **10**:6642-6653

48. Chasserot-Golaz S and Beck G. (1992). How the potency of the steroid RU486 is related to P450 activities induced by dexamethasone and phenobarbital in rat hepatoma cells. *J. Steroid Biochem. Mol. Biol.* **41**:653-657

49. Chen HS, Singh SS and Perdew GH. (1997) The AH receptor is a sensitive target of geldanamycin-induced protein turnover. *Arch. Biochem. Biophys.* **348**:190-198

50. Chirgwin JJ, Przbyla AE, MacDonald RJ and Rutter WJ. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294

51. Chodosh LA, Baldwin AS, Carthew RW and Sharp PA (1988) Human CAAT-binding proteins have heterologous subunits. *Cell* **53**:11-24.

52. Chomczynski P and Sacchi N. (1987). Single step method of RNA isolation by acid guanidine thiocynate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156-159

53. Christou M, Wilson NM and Jefcote CR. (1987). Expression and function of three P-450 isozymes in rat hepatic tissues. *Arch. Biochem. Biophys.* **258**:519-534

54. Clark AR, Boam DS and Docherty K. (1989). A new series of CAT expression vectors. *Nucleic Acids Res.* **17**:10130.

55. Clark BJ, Wells J, King SR and Stocco DM. (1994). The purification, cloning, and expression of a novel luteinising hormone-induced mitochondrial protein in MA-10 mouse Leydig tumour cells. *J. Biol. Chem.* **269**:28318-28322.

56. Clark KL, Halay ED, Lai E and Burley SK. (1993). Co-crystal structure of the HNF-3/*fork head* DNA-recognition motif resembles histone H5. *Nature* **364**:412-420

57. Conney AH, Miller EC and Miller AJ. (1956). The metabolism of methylated aminoazo dyes 5. Evidence for induction of enzyme synthesis in the rat by 3-methylcholanthrene. *Cancer Res.* **16**:450-459.

58. Conney AH. (1967). Pharmacological implications of microsomal enzyme induction. *Pharmacol. Rev.* **19**:317-366

59. Conney AH. (1982). Induction of microsomal enzymes by foreign compounds and cancinogenesis by polycyclic aromatic hydrocarbons. *Cancer Res.* **42**:4875-4917

60. Corcos L, Marc N, Wein S, Fautrel A, Guillouzo A and Pineau T. (1998) Phenobarbital induces cytochrome P4501A2 hnRNA, mRNA and protein in the liver of C57BL/6J wild type and aryl hydrocarbon receptor knock-out mice. *FEBS Letters* **425**:293-297

61. Costa RH, Grayson DR and Darnell JE Jr. (1989). Multiple hepatocyte-enriched nuclear factors function in the regulation of the transthyretin and α1-antitrypsin genes. *Mol. Cell. Biol.* **9**:1415-1425

62. Coumailleau P, Poellinger Gustafsson J-A and Whitelaw ML. (1995). Definition of a minimal domain of the dioxin receptor that is associated with hsp90 and maintains wild type ligand binding affinity and specificity. *J. Biol. Chem.* **270**:25921-25930

63. Dailey LS, Roberts B and Heintz N (1988) Purification of the human histone H4 gene-specific transcription factors H4TF-1 and H4TF-2. *Genes Dev* **2:**1700-1712.

64. DeLuca HF. (1982). Metabolism and molecular mechanism of action of vitamin D. *Biochem. Soc. Trans.* **10**:147-154

65. Denison MS, Fisher JM and Whitlock JP Jr. (1989). Protein-DNA interactions at recognition sites for the dioxin-Ah receptor complex. *J. Biol. Chem.* **264**:16478-16482

66. Denison MS and Whitlock JP. (1995). Xenobiotic-inducible transcription of cytochrome P450 genes. *J. Biol. Chem.* **270**:18175-18178

67. Descombes P, Chojkier M, Lichtsteiner S, Falvey E and Schibler U. (1990). LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein. *Genes Dev.* **4**:1541-1551

68. Desvergne B, Ijpenberg A, Devchand PR and Wahli W. (1998) The peroxisome proliferator-activated receptors at the cross-roads of diet and hormone signalling. *J. Ster. Biochem. Mol. Biol.* **65**:65-74

69. Devchand PR, Keller H, Peters JM, Vazquez M, Gonzalez FJ and Wahli W. (1996). The PPARα-leukotriene B_4 pathway to inflammation control. *Nature* **384**:39-43

70. Dickson PW, Howlett GS and Schreiber G. (1985). Rat transthyretin (prealbumin) molecular cloning, nucleotide sequence and gene expression in liver and brain. *J. Biol. Chem.* **260**:8214-8219

71. Ding X and Coon MJ. (1993). Extrahepatic microsomal forms: Olfactory cytochrome P450. In *Cytochrome P450.* (Schenkman JB and Greim H. eds), Springer-Verlag Press, Berlin. pp 351-361

72. Dogra SC, Hahn CN and May BK. (1993). Superinduction by cyclohexamide of cytochrome P4502H1 and 5-aminolevulinate synthase gene transcription in chick embryo liver. *Arch. Biochem. Biophys.* **300**:531-534

73. Dogra SC, Davidson BP and May BK (1999). Analysis of a phenobarbital-responsive enhancer region located in the 5' flanking region of the chicken *CYP2H1* gene: Identification and characterisation of functional protein binding sites. *Mol. Pharmacol.* **55**:14-22

74. Dogra SC and May BK. (1996). Phenobarbital-induced activation of *CYP2H1* and 5-aminolevulinate synthase genes in chick embryo hepatocytes is blocked by an inhibitor of protein phosphorylation. *Arch. Biochem. Biophys.* **327**:271-278

75. Dogra SC and May BK. (1997). Liver-enriched transcription factors HNF-1, HNF-3 and C/EBP are major contributors to the strong activity of the chicken *CYP2H1* promoter in chick embryo hepatocytes. *DNA Cell Biol.* **16**:1407-1418

76. Domalik LJ, Chaplin DD, Kirkman MS, Wu RC, Liu WW, Howard, TA, Seldin MF and Parker KL. (1991). Different isozymes of mouse 11b-hydroxylase produce mineralocorticoids and glucocorticoids. *Mol. Endocrinol.* **5**:1853-1861

77. Dong L, Ma Q and Whitlock JP Jr. (1996). DNA binding by the heteromeric Ah receptor. *J. Biol. Chem.* **271**:7942-7948

78. Dowell P, Ishmael JE, Avram D, Peterson VJ, Nevrivy DJ and Leid M. (1997). P300 functions as a coactivator for the peroxisome proliferator-activated receptor alpha. *J. Biol. Chem.* **272**:33435-33443

79. Dowell P, Ishmael JE, Avram D, Peterson VJ, Nevrivy DJ and Leid M. (1999). Identification of nuclear receptor corepressor as a peroxisome proliferator-activated receptor α interacting protein. *J. Biol. Chem.* **274**:15901-15907

80. Dufau M. (1988). Endocrine regulation and communicating functions of Leydig cells. *Annu. Rev. Physiol.* **50**:483-508

81. Dunn TJ, Koleske AJ, Lindahl R and Pitot HC. (1989). Phenobarbital-inducible aldehyde dehydrogenase in the rat. cDNA sequence and regulation of the mRNA by phenobarbital in responsive rats. *J. Biol. Chem.* **264**:13057-13065

82. Elferink CJ and Whitlock JP. (1994). Dioxin-dependent, DNA sequence-specific binding of a multiprotein complex containing the Ah receptor. *Receptor* **4**:157-173

83. Ellis J, Tan-Un KC, Harper A, Michalovich D, Yannoutsos N, Philipsen S, and Grosveld F. (1996). A dominant chromatin-opening activity in 5' hypersensitive site 3 of the human beta-globin locus control region. *EMBO J.* **15**:562-568

84. Ema M, Sogawa K, Watanabe N, Chujoh Y, Matsushita N, Goto O, Funae Y and Fujii-Kuriyama Y. (1992). CDNA cloning and structure of mouse putative Ah receptor. *Biochem. Biophys. Res. Commun.* **184**:246-253

85. Ema M, Taya S, Yokotani N, Sogowa K, Matsuda Y and Fuji-kuriyama Y. (1997). A novel bHLH/PAS factor with close sequence similarity to hypoxia inducible factor 1 a regulates the VEGF expression and is potentially involved in lung and vascular development. *Proc. Natl. Acad. Sci. USA.*. **94**:4273-4278

86. Enan E, Elsabeawy F, Scott M, Overstreet J and Lasley B. (1998). Alterations in the growth factor signal transduction pathways and modulators of the cell cycle in endocervical cells from Macaques exposed to TCDD. *Tox. Appl. Pharm.* **151**:283-293

87. English N, Hughes V and Wolf CR. (1994). Common pathways of cytochrome P450 gene regulation by peroxisome proliferators and barbiturates in *Bacillus megaterium* ATCC14581. *J. Biol. Chem.* **269**:26836-26841

88. Enyeart JJ, Mlinar B and Enyeart JA. (1993). T-Type Ca^{2+} channels are required for adrenocorticotropin-stimulated cortisol production by bovine adrenal zona fasciculata cells. *Mol. Endocrinol.* **7**:1031-1040

89. Evans RM. (1988). The steroid and thyroid hormone receptor superfamily. *Science* **240**:889-895

90. Fernandez-Salguero P, Pineau T, Hilbert DM, McPhail T, Lee SS, Kimura S, Nebert DW, Rudikoff S, Ward JM and Gonzalez FJ. (1995). Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor. *Science* **268**:722-726

91. Fernandez-Salguero P, Ward JM, Sundberg JP and Gonzalez FJ. (1997). Lesions of aryl hydrocarbon receptor-deficient mice. *Vet. Pathol.* **34**:605-614

92. Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM and Evans RM. (1995). 15-Deoxy-delta 12,14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. *Cell* **83**:803-812

93. Forman BM, Tzameli I, Choi HS, Chen J, Simha D, Seol W, Evans RM and Moore DD. (1998). Androstane metabolites bind to and deactivate the nuclear receptor CAR-β. *Nature* **395**:612-615

94. Frain M, Swart G, Monaci P, Nicosia A, Stampfli S, Frank R and Cortese R. (1989). The liver-specific transcription factor LF-B1 contains a highly diverged homeobox DNA binding domain. *Cell.* **59**:145-157.

95. French JS, Guengerich FP and Coon MJ. (1980). Interactions of cytochrome P-450, NADPH-cytochrome P-450 reductase, phospholipid, and substrate in the reconstituted liver microsomal enzyme system. *J. Biol. Chem.* **255**:4112-4119

96. Fujii-Kuriyama Y, Imataka H, Sogawa K, Yasumoto K and Kikuchi Y. (1992). Regulation of *CYP1A1* expression. *FASEB J.* **6**:706-710

97. Fulco AJ. (1991). $P450_{BM-3}$ and other inducible P450 cytochromes: Biochemistry and regulation. *Annu. Rev. Pharmacol. Toxicol.* **31**:177-203

98. Garfinkle D. (1958). Studies on pig liver microsomes. I. Enzyme and pigment composition of different microsomal fractions. *Arch. Biochem. Biophys.* **77**:493-509

99. Giger U and Meyer UA. (1981). Induction of delta-aminolevulinate synthase and cytochrome P-450 hemoproteins in hepatocyte culture. Effect of glucose and hormones. *J. Biol. Chem.* **256**:11182-11190

100. Gonzalez FJ. (1989). The molecular biology of cytochrome P450s. *Pharmacol. Rev.* **40**:243-288

101. Gonzalez FJ and Fernandez-Salguero P. (1998). The aryl-hydrocarbon receptor - studies using the AHR-null mice. *Drug Metab. Dispos.* **26**:1194-1198

102. Gonazalez FJ and Lee Y-H. (1996). Constitutive expression of hepatic cytochrome P450 genes. *FASEB J.* **10**:1112-1117

103. Gonzalez FJ, Peters JM and Cattley RC. (1998). Mechanism of action of the nongenotoxic peroxisome proliferators: role of the peroxisome proliferator-activator receptor alpha. *J. Natl. Cancer Inst.* **90**:1702-1709

104. Gonzalez FJ, Song BJ and Hardwick JP. (1986). Pregnenolone-16α-carbonitrile-inducible P-450 gene family: Gene conversion and differential regulation. *Mol. Cell. Biol.* **6**:2969-2976

105. Gorman CM, Moffat LF and Howard BH. (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell Biol* **2**:1044-1051.

106. Gorski K, Carneiro M and Schibler U. (1986). Tissue-specific *in vitro* transcription from the mouse albumin promoter. *Cell* **47**:767-776

107. Gou I-C, Tsai H-M and Chung B-C. (1994). Actions of two different cAMP-responsive sequence and an enhancer of the human *CYP11A*1 (P450scc) gene in adrenal Y1 and placenta JEG-3 cells. *J. Biol. Chem.* **269**:6362-6369

108. Gradin K, Toftgard R and Berghard A. (1995). Differential effects of a topoisomerase I inhibitor on dioxin inducibility and high-level expression of the cytochrome P4501A1 gene. *Mol. Pharmacol.* **48**:610-615

109. Graham FL and van der Eb AJ. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**:456-457

110. Gram TE, Okine LK and Gram RA. (1986). The metabolism of xenobiotics by certain extrahepatic organs and its relation to toxicity. *Annu. Rev. Pharmacol. Toxicol.* **26**:259-292

111. Grange T, Roux J, Rigaud G and Pictet R. (1991). Cell-type specific activity of two glucocorticoid responsive units of rat tyrosine aminotransferase gene is associated with multiple binding sites for C/EBP and a novel liver-specific nuclear factor. *Nucleic Acids Res.* **19**:131-139

112. Green S. (1992). Receptor-mediated mechanisms of peroxisome proliferators. *Biochem. Pharmacol.* **43**:393-401

113. Green S and Wahli W. (1994). Peroxisome proliferator-activated receptors: Finding the orphan a home. *Mol. Cell. Endocrinol.* **100**:149-153

114. Gregori C, Kahn A and Pichard AL. (1993). Competition between transcription factors HNF-1 and HNF-3, and alternative cell-specific activation by DBP and C/EBP contribute to the regulation of the liver-specific aldolase B promoter. *Nucleic Acids Res.* **21**:897-903

115. Groves JT and McClusky GA. (1978). Aliphatic hydroxylation by highly purified liver microsomal cytochrome P-450. Evidence for a carbon radical intermediate. *Biochem. Biophys. Res. Commun.* **81**:154-160

116. Groyer A, Le Bouc Y, Joab I, Radanyi C, Renoir JM, Robel P and Baulieu EE. (1985). Chick oviduct glucocorticoid receptor. Specific binding of RU486 and immunological studies with antibodies to chick oviduct progesterone receptor. *Eur. J. Biochem.* **149**:445-451

117. Guengerich FP. (1988). Roles of cytochrome P-450 enzymes in chemical carcinogenesis and cancer chemotherapy. *Cancer Res.* **48**:2946-2954

118. Guengerich FP. (1991). Reactions and significance of cytochrome P-450 enzymes. *J. Biol. Chem.* **266**:10019-10022

119. Hahn CN, Hansen AJ and May BK. (1991). Transcriptional regulation of the chicken *CYP2H1* gene. *J. Biol. Chem.* **266**:17031-17039

120. Halvorson MR, Safe SH, Parkinson A and Philips TD (1988). Aflatoxin B_1 hydroxylation by the pregnenolone-16α-carbonitrile-inducible form of rat liver microsomal cytochrome P-450. *Carcinogenisis* **9**:2103-2108

121. Hamilton JW, Bement WJ, Sinclair PR, Sinclair JF, Alcedo JA and Wetterhahn KE. (1992). Inhibition of protein synthesis increases the transcription of the phenobarbital-inducible *CYP2H1* and *CYP2H2* genes in chick embryo hepatocytes. *Arch. Biochem. Biophys.* **298**:96-104

122. Hammond B, Katzenellenbogen BS, Krauthammer N and McConnell J. (1979). Estrogenic activity of the insecticide chlordecone (Kepone) and interaction with uterine estrogen receptors. *Proc. Natl. Acad. Sci. USA...* **12**:6641-6645

123. Hankinson O. (1995) The aryl hydrocarbon receptor complex. *Ann. Rev. Pharmacol. Toxicol.* **35**:307-340

124. Hansen AJ. (1989). Regulation of the expression of phenobarbital-inducible P450 genes. *PhD Thesis.*

125. Hansen AJ, May BK and Elferink LA. (1989). Sequence of a chicken phenobarbital-inducible cytochrome P450 cDNA: Regulation of two P450 mRNAs transcribed from different genes [published erratum appears in *DNA* **9**:301]. *DNA* **8**:179-191

126. Hanukoglu I, Feuchtwanger R and Hanukoglu A. (1990). Mechanism of corticotropin and cAMP induction of mitochondrial cytochrome P450 system enzymes in adrenal cortex cells. *J. Biol. Chem.* **265**:20602-20608

127. Hardwick JP, Song B-J, Huberman E and Gonzalez FJ. (1987). Isolation, complementary DNA sequence and regulation of rat hepatic lauric acid ω-hydroxylase (cytochrome P450 LAω). Identification of a new cytochrome P-450 gene family. *J. Biol. Chem.* **262**:801-810

128. Harvey JL, Paine AJ and Wright MC. (1998) Disruption of endogenous regulator homeostasis underlies the mechanism of rat *CYP1A1* mRNA induction by metyrapone. *Biochem. J.* **331**:273-281

129. Hashimoto T, Matsumoto T, Nishizawa M, Kawabata S, Morohashi K, Honda S and Omura T. (1998). A mutant rat strain deficient in induction of a phenobarbital-inducible form of cytochrome P-450 in liver microsomes. *J. Biochem.* **103**:487-492

130. Hayes CL, Spink DC, Spink BC, Cao JQ, Walker NJ and Sutter TR. (1996). 17β-Estradiol hydroxylation catalyzed by human cytochrome P4501B1. *Proc. Natl. Acad. Sci. USA..* **93**:9776-9781

131. He J-S and Fulco AJ. (1991). A barbiturate-regulated protein binding to a common sequence in the cytochrome P450 genes of rodents and bacteria. *J. Biol. Chem.* **266**:7864-7869

132. He J-S, Liang Q and Fulco AJ. (1995). The molecular cloning and characterisation of BMlP1 and BMlP2 proteins, putative positive transcription factors involved in barbiturate-mediated induction of the genes encoding cytochrome P450BM-1 of *Bacillus megaterium*. *J. Biol. Chem.* **270**:18615-18625

133. Heikinheimo O, Ylikorkala O and Lahteenmaki P. (1990). Antiprogesterone RU486 - a drug for non-surgical abortion. *Ann. Med.* **22**:75-84

134. Heinzel T, Lavinsky RM, Mullen TM, Soderstrom M, Laherty CD, Torchia J, Yang WM, Brard G, Ngo SD, Davie JR, Seto E, Eisenman RN, Rose DW, Glass CK and Rosenfeld MG. (1997). A complex containing NCoR, mSin 3 and histone deacetylase mediates transcriptional repression. *Nature* **387**:43-48

135. Henry EC, Rucci G and Gasiewicz TA. (1994). Purification to homogeneity of the heteromeric DNA-binding form of the Aryl hydrocarbon receptor from rat liver. *Mol. Pharmacol.* **46**:1022-1027

136. Henry HL and Norman AW. (1984). Vitamin D: metabolism and biological actions. *Ann. Rev. Nutr.* **4**:493-501

137. Herbomel, P, Bourachot, B, and Yaniv, M. (1984). Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. *Cell* **39**:653-662

138. Herbst RS, Nielsch U, Sladek FM, Lai E, Babiss LE and Darnell JE Jr. (1991). Differential regulation of hepatocyte-enriched transcription factors explains changes in albumin and transthyretin gene expression among hepatoma cells. *New Biol.* **3**:289-296

139. Heuman DM, Gallagher EJ, Barwick JL, Elshourbagy NA and Guzelian PS. (1982). Immunochemical evidence for induction of a common form of hepatic cytochrome P-450 in rats treated with pregnenolone 16α-carbonitrile or other steroidal or non-steroidal agents. *Mol. Pharmacol.* **21**:753-760

140. Hobbs AA, Mattschoss LA, May BK, Williams KE and Elliot WH. (1986). The cDNA and protein sequence of a phenobarbital-induced chicken cytochrome P-450. *J. Biol. Chem.* **261**:9444-9449

141. Hodgson AV, White TB, White JW and Strobel HW. (1993). Expression analysis of the mixed-function oxidase system in rat brain by the polymerase chain reaction. *Mol. Cell. Biochem. 121*:171-174

142. Honda S, Morohashi K and Omura T. (1990). Novel cAMP regulatory elements in the promoter region of bovine P45011β gene. *J. Biochem.* **108**:1042-1049

143. Honkakoski P, Moore R, Gynther J and Negishi M. (1996). Characterisation of phenobarbital-inducible mouse Cyp2b10 gene transcription in primary hepatocytes. *J. Biol. Chem.* **271**:9746-9753

144. Honkakoski P, Moore R, Washburn KA and Negishi M. (1998). Activation by diverse xenochemicals of the 51-base pair phenobarbital-responsive enhancer module in the *CYP2B10* gene. *Mol. Pharmacol.* **53**:597-601

145. Honkakoski P and Negishi M. (1997). Characterisation of a phenobarbital-responsive enhancer module in mouse P450 *Cyp2b10* gene. *J. Biol. Chem.* **272**:14943-14949

146. Honkakoski P and Negishi M. (1998). Protein serine/threonine phosphatase inhibitors suppress phenobarbital-induced *CYP2b10* gene transcription in mouse primary hepatocytes. *Biochem. J.* **330**:889-895

147. Honkakoski P, Zelko I, Sueyoshi T and Negishi M. (1998). The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the *CYP2B* gene. *Mol. Cell. Biol.* **18**:5652-5658

148. Huang Q, Alvares K, Chu R, Bradfield CA and Reddy JK. (1994). Association of peroxisome proliferator-activated receptor and hsp72. *J. Biol. Chem.* **269**:8493-8497

149. Huss JM and Kasper CB. (1998). Nuclear receptor involvement in the regulation of rat cytochrome P450 3A23 expression. *J. Biol. Chem.* **273**:16155-16162

150. Huss JM, Wang SI, Astrom A, McQuiddy P and Kasper CB. (1996). Dexamethasone responsiveness of a major glucocorticoid-inducible *CYP3A* gene is mediated by elements unrelated to a glucocorticoid receptor binding motif. *Proc. Natl. Acad. Sci. USA.*. **93**:4666-4670

151. Imataka H, Sogawa K, Yasumoto K, Kikuchi Y, Sasano K, Kobayashi A, Hayami M and Fujii-Kuriyama Y. (1992). Two regulatory proteins that bind to the basic transcription element (BTE), a GC box sequence in the promoter region of the rat P-4501A1 gene. *EMBO J.* **11**:3663-3671

152. Ip YP, Poon D, Stone D, Granner DK and Chalkley R. (1990). Interaction of a liver-specific factor with an enhancer 4.8 kilobases upstream of the phospnoenolpyruvate carboxykinase gene. *Mol. Cell. Biol.* **10**:3770-3781

153. Issemann I and Green S. (1990). Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature* **347**:645-650

154. Jain S, Dolwick KM, Schmidt JV and Bradfield CA. (1994). Potent transactivation domains of the Ah receptor and the Ah receptor nuclear translocator map to their carboxyl termini. *J. Biol. Chem.* **269**:31518-31524

155. Jaiswal AK Haaparanta T, Lus P-V, Schembri J and Adesnik M. (1990). Glucocorticoid regulation of a phenobarbital-inducible cytochrome P-450 gene: the presence of a functional glucocorticoid response element in the 5'-flanking region of the *CYP2B2* gene. *Nucleic Acids Res.* **18**:4237-4242

156. Jansson I and Schenkman JB. (1981). Possible mechanism of coupled NADPH oxidase and P-450 monooxygenase action. *Adv. Exp. Med. Biol.* **136**:145-163

157. Jansson I and Schenkman JB. (1987). Influence of cytochrome b5 on the stoichiometry of the different oxidative reactions catalyzed by liver microsomal cytochrome P-450. *Drug Metab. Dispos.* **15**:344-348

158. Jarukamjorn K, Sakuma T, Miyaura JI and Nemoto N. (1999). Differential regulation of the expression of mouse hepatic cytochrome P450 2B enzymes by glucocorticoid and phenobarbital. *Arch. Biochem. Biophys.* **369**:89-99

159. Jefcoate CR. McNamara BC, Artemenko I and Yamazaki T. (1992). Regulation of cholesterol movement into mitochondrial cytochrome P450ssc in steroid hormone biosynthesis. *J. Steroid Biochem. Mol. Biol.* **43**:751-767

160. Jenster G, Spencer TE, Burcin MM, Tsai SY, Tsai M-J and O'Malley BW. (1997). Steroid receptor induction of gene transcription: A two step model. *Proc. Natl. Acad. Sci. USA.* **94**:7879-7884

162. John ME, John MC, Boggaram V, Simpson ER and Waterman MR. (1986). Transcriptional regulation of steroid hydroxylase genes by corticotropin. *Proc. Natl. Acad Sci. USA.* **83**:4715-4719

163. Johnson EF, Palmer CNA, Griffin KJ and Hsu M-H. (1996). Role of the peroxisome proliferator-activated receptor in cytochrome P4504A gene regulation. *FASEB J.* **10**:1-8

164. Jung-Testas I and Baulieu EE. (1983). Inhibition of glucocorticoid action in cultured L-929 mouse fibroblasts by RU486, a new anti-glucocorticoid of high affinity for the glucocorticoid receptor. *Exp. Cell Res.* **147**:177-182

165. Jung-Testas I and Baulieu EE. (1984). Antisteroidal action in cultured I-29 mouse fibroblasts. *J. Steroid Biochem.* **20**:301-306

166. Kadlubar FF and Hammons GJ. (1987). The role of cytochrome P-450 in the metabolism of chemical carcinogens. In *Mammalian cytochrome P-450.* (Guengerich FP. ed). CRC Press, Inc. Boca Raton, FL. Vol.1, pp 1-54

167. Kagawa N and Waterman MR. (1991). Evidence that an adrenal-specific nuclear protein regulates cAMP responsiveness of the human Cyp21B (P450c21) gene. *J. Biol. Chem* **266**:11199-11204

168. Kagawa N and Waterman MR. (1992). Purification and characterisation of a transcription factor which appears to regulate cAMP responsiveness on the human *CYP21B* gene. *J. Biol. Chem.* **267**:21213-21219

169. Kagawa N and Waterman MR. (1995). Regulation of steroidogenic and related P450s. In: *Cytochrome P450. Structure, Mechanism, and Biochemistry.* (Ortiz de Montellano PR. ed), Plenum, New York, pp 419-442

170. Kalimi M. (1987). Receptor-mediated antiprogestin action of RU486. In: *Receptor mediated antisteroid action.* (Agarwal MK. ed) Walter de Gruyter, New York, pp 121-137

171. Kalimi M. (1989). Role of antiglucocorticoid RU486 on dexamethasone-induced hypertension in rats. *Am. J. Physiol.* **256**:682-685

172. Kawamoto T. Sueyoshi T, Zelko I, Moore R, Washburn K and Negishi M. (1999). Phenoparbital-responsive nuclear translocation of the receptor CAR in induction of the CYP2B gene. *Mol. Cell. Biol.* **19**:6318-6322

173. Keller H, Dreyer C, Medin J, Mahfoudi A, Ozato K and Wahli W. (1993). Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. *Proc. Natl. Acad. Sci. USA..* **90**:2160-2164

174. Kim J and Kemper B. (1997). Phenobarbital alters protein binding to the *CYP2B1/2* phenobarbital-responsive unit in native chromatin. *J. Biol. Chem.* **272**:29423-29425

175. Kim YG and Chandrasegaran S. (1994). Chimeric restriction endonuclease. *Proc. Natl. Acad. Sci. USA..* **91**:883-887

176. Kimura S, Hanioka N, Matsunaga E and Gonzalez FJ. (1989). The rat clofibrate-inducible *CYP4A* gene subfamily. I. Complete intron and exon sequence of the

CYP4A1 and *CYP4A2* genes, unique exon organisation and identification of a conserved 19 bp upstream element. *DNA* **8**:503-516

177. Kimura S, Hardwick JP, Kozak CA and Gonzalez FJ. (1989). The rat clofibrate-inducible *CYP4A* subfamily. II. cDNA sequence of IVA3, mapping of the Cyp4a locus to mouse chromosome 4 and coordinate and tissue-specific regulation of the *CYP4A* genes. *DNA* **8**:517-525

179. Kliewer SA, Lenhard JM, Willson TM, Patel I, Morris DC and Lehmann JM. (1995). A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. *Cell* **83**:813-819

180. Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterstrom RH, Perlmann T and Lehmann JM. (1998). An orphan nuclear receptor activated by pregnanes defines a novel steroid signalling pathway. *Cell* **92**:73-82

181. Ko HP, Okino ST, Ma Q and Whitlock JP Jr. (1996). Dioxin-induced *CYP1Al* transcription in vivo: The aromatic hydrocarbon receptor mediates transactivation, enhancer-promoter communication, and changes in chromatin structure. *Mol. Cell. Biol.* **16**:430-436.

182. Ko HP, Okino ST, Ma Q and Whitlock JP Jr. (1997). Transactivation domains facilitate promoter occupancy for the dioxin-inducible *CYP1A1* gene in vivo. *Mol. Cell. Biol.* **17**:3497-3507

183. Kobayashi A, Numayama-Tsuruta K, Sogawa K and Fujii-Kuriyama Y. (1997). CBP/p300 functions as a possible transcriptional coactivator of Ah receptor nuclear translocator (Arnt). *J. Biochem.* **122**:703-710

184. Kobayashi A, Sogawa K and Fujii-Kuriyama Y. (1996). Cooperative interaction between AhR-Arnt and Spl for the drug-inducible expression of *CYP1A1* gene. *J. Biol. Chem.* **271**:12310-12316

185. Kocarek TA, Schuetz EG and Guzelian PS. (1990). Differentiated induction of cytochrome P450b/e and P450p mRNA by dose of phenobarbital in primary cultures of adult rat hepatocytes. *Mol. Pharmacol.* **38**:440-444

186. Kocarek TA, Schuetz EG and Guzelian PS. (1994). Biphasic regulation of cytochrome P450 2B1/2 mRNA expression by dexamethasone in primary cultures of adult rat hepatocytes maintained on matrigel. *Biochem. Pharmacol.* **48**:1815-1822

187. Krishna DR and Klotz U. (1994). Extrahepatic metabolism of drugs in humans. *Clin. Pharmacokinet.* **26**:144-160

188. Kroetz DL, Yook P, Costet P, Bianchi P and Pineau T. (1998). Peroxisome proliferator-activated receptor alpha controls the hepatic *CYP4A* induction adaptive response to starvation and diabetes. *J. Biol. Chem.* **273**:31581-31589

189. Kuo CJ, Conley PB, Chen L, Sladek FM, Darnell JB Jr and Crabtree GR. (1992). A transcriptional hierarchy involved in mammalian cell-type specification. *Nature* **355**:457-461

190. Lai E, Clark KL, Burley SK and Darnell JE Jr. (1993). Hepatocyte nuclear factor 3/fork head or "winged helix" proteins: a family of transcription factors of diverse biologic function. *Proc. Natl. Acad. Sci. USA.*. **90**:10421-10423

191. Lai E, Prezioso VR, Smith E, Litvin O, Costa RH and Darnell,J.E. Jr. (1990). HNF-3A, a hepatocyte-enriched transcription factor of novel structure is regulated transcriptionally. Genes Dev. 4:1427-1436

192. Lambeth JD, Seybert DW, Lancaster JR, Salerno JC and Kamin H. (1982). Steroidogenic electron transport in adrenal cortex mitochondria. *Mol. Cell. Biol.* **45**:13-31

193. Landschulz WH, Johnson PF, Adashi EY, Graves BJ and McKnight SL. (1988). Isolation of a recombinant copy of the gene encoding C/EBP. *Genes Dev.* **2**:786-800

194. Lassar AB, Buskin JN, Lockshon D, Davis RL, Apone S, Hauschkaand SD and Weintraub H (1989) MyoD is a sequence-specific DNA binding protein requiring a region of myc homology to bind to the muscle creatine kinase enhancer. *Cell* **58:**823-831.

195. Lee J-S, Lee C-H and Chung JH. (1998). Studying the recruitment of Sp1 to the β-globin promoter with an *in vivo* method: Protein position identification with nuclease tail (PINPOINT). *Proc. Natl. Acad. Sci. USA.*. **95**:969-974

196. Lee PC and Werlin SL. (1995). The induction of hepatic cytochrome P450 3A in rats: Effects of age. *Proc. Soc. Exp. Biol. Med.* **210**:134-139

197. Lee SS, Pineau T, Drago J, Lee EJ, Owens JW, Kroetz DL, Fernandez-Salguero PM, Westphal H and Gonzalez FJ. (1995). Targeted disruption of the α isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. *Mol. Cell. Biol.* **15**:3012-3022

198. Lee SS, Jeong HG and Yang KH. (1998). Effects of estrodial and progesterone on cytochrome P4501A1 expression in 1c1c7 cells. *Biochem. Mol. Biol. Int.* **45**:775-781

199. Lee W, Mitchell P and Tjian R. (1987). Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. *Cell* **49**:741-752

200. Lee Y-H, Yano M, Liu S-Y, Matsunaga E, Johnson PF and Gonzalez FJ. (1994). A novel cis-acting element controlling the rat *CYP2D5* gene and requiring cooperativity between C/EBP and an SP1 factor. *Mol. Cell. Biol.* **14**:1383-1394

201. Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM and Kliewer SA. (1995). An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ). *J. Biol. Chem.* **270**:12953-12956

202. Leone TC, Weinheimer CJ and Kelly DP. (1999). A critical role for the peroxisome proliferator-activated receptor alpha (PPAR alpha) in the cellular fasting response: The PPAR alpha-null mouse as a model of fatty acid oxidation disorders. *Proc. Natl. Acad. Sci. USA.* **96**:7473-7478

203. Liang Q, Chen L and Fulco AJ. (1998). In vivo roles of Bm3R1 repressor in the barbiturate-mediated induction of the cytochrome P450 genes ($P450_{BM-3}$ and $P450_{BM-1}$) of *Bacillus megaterium*. *Biochim. Biophys. Acta* **1308**:183-197

204. Liang Q and Fulco AJ. (1995). Transcriptional regulation of the genes encoding cytochromes $P450_{BM-1}$ and $P450_{BM-3}$ in *Bacillus megaterium* by the binding of Bm3R1 repressor to Barbie box elements and operator sites. *J. Biol. Chem.* **270**:18606-18614

205. Liang Q, He J-S and Fulco AJ. (1995). The role of Barbie box sequences as *cis*-acting elements involved in the barbiturate-mediated induction of cytochromes $P450_{BM-1}$ and $P450_{BM-3}$ in *Bacillus megaterium*. *J. Biol. Chem.* **270**:4438-4450

206. Lindebro MC, Poellinger L and Whitelaw ML. (1995). Protein-protein interaction via PAS domains: role of the PAS domain in positive and negative regulation of the bHLH/PAS dioxin receptor-Arnt transcription factor complex. *EMBO J.* **14**:3528-3539

207. Linder-MW; Prough-RA. (1993). Developmental aspects of glucocorticoid regulation of polycyclic aromatic hydrocarbon-inducible enzymes in rat liver. *Arch. Biochem. Biophys.* **302**:92-102

208. Lock EA, Mitchell AM and Elcome CR. (1989). Biochemical mechanisms of induction of hepatic peroxisome proliferation. *Annu. Rev. Pharmacol. Toxicol.* **29**:145-63

209. Long WP, Praygrant M, Tsai JC and Perdew GH. (1998). Protein kinase C activity is required for aryl hydrocarbon receptor pathway-mediated signal transduction. *Mol Pharmacol.* **53**:691-700

210. Lu AY, Somogyi A, West S, Kuntzman R and Conney AH. (1972). Pregnenolone-16α-carbonitrile: A new type of inducer of drug metabolising enzymes. *Arch. Biochem. Biophys.* **152**:457-462

211. Lubet RA, Dragnev KH, Chauhan DP, Nims RW, Diwan BA, Ward JM, Jones CR, Rice JM and Miller MS. (1992). A pleiotropic response to phenobarbital-type enzymes inducers in the F344/NCr rat. *Biochem. Pharm.* **43**:1067-1078

212. Luc P-VT Adesnick M, Ganguly S and Shaw PM. (1996). Transcriptional regulation of the *CYP2B1* and *CYP2B2* genes by C/EBP-related proteins. *Biochem. Pharmacol.* **51**:345-356

213. Lucas PC and Granner DK. (1992). Hormone response domains in gene transcription. *Annu. Rev. Biochem.* **61**:1131-1173

214. Lund J, Ahlgren R, Wu D, Kagimoto M, Simpson ER and Waterman MR. (1990). Transcriptional regulation of the bovine *CYP17* (P45017α) gene. *J. Biol. Chem.* **265**:3304-3312

215. Ma Q and Whitlock JP Jr (1996). The aromatic hydrocarbon receptor modulates the Hepa 1c1c7 cell cycle and differentiated state independently of dioxin. *Mol. Cell. Biol.* **16**:2144-2150.

216. Ma Q and Whitlock JP Jr. (1997). A novel cytoplasmic protein that interacts with the Ah receptor, contains tetratricopeptide repeat motifs, and augments the transcriptional response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *J. Biol. Chem.* **272**:8878-8884

217. Mackenzie PI. (1990). *Drug Metabolizing Enzymes: Genetics, Regulation and Toxicology* (Ingelman-Sundberg M, Gustafsson J and Orrenius S. eds), *Proc. 8th Int. Symp.* pp 30

218. Mangelsdorf DJ and Evans RM. (1995). The RXR heterodimers and orphan receptors. *Cell* **83**:841-850

219. Mani C, Gelboin HV,Park SS, Pearce R. Parkinson A and Kupfer D. (1993). Metabolism of the antimammary cancer antiestrogenic agent tamoxifen. I. Cytochrome P-450-catalyzed N-demethylation and 4-hydroxylation. *Drug. Metab. Dispos.* **21**: 645-56.

220. Maniatis T, Fritsch EF and Sambrook J. (1989). "Molecular Cloning: A Laboratory Manual", Cold spring Harbour Laboratory, Cold Spring Harbour, New York

221. Mattschoss LA. Hobbs AA, Steggles AW, May BK and Elliot WH. (1986). Isolation and characterisation of genomic clones for two chicken phenobarbital-inducible cytochrome P-450 genes. *J. Biol. Chem.* **261**:9438-9443

222. Maxam AM and Gilbert W. (1980). Sequencing end-labelled DNA with base-specific chemical cleavages. *Meth. Enzymol.* **50**:499-560

223. Meier CA. (1997). Regulation of gene expression by nuclear hormone receptors. *J. Recept. Signal Transduct. Res.* **17**:319-335

224. Meyer BK and Perdew GH. (1999). Characterization of the AhR-hsp90-XAP2 core complex and the role of the immunophilin-related protein XAP2 in AhR stabilization. *Biochemistry* **38**:8907-8917

225. Millonig JH, Emerson JA, Levorse JM and Tilghman SM. (1995). Molecular analysis of the distal enhancer of the mouse alpha-fetoprotein gene. *Mol. Cell. Biol.* **15**:3848-3856

226. Mitani F, Shimizu T, Ueno R, Ishimura Y, Izumi S, Komatsu N and Watanabe K. (1982). Cytochrome P-45011 β and P-450scc in adrenal cortex: zonal distribution and intramitochondrial localization by the horseradish peroxidase-labelled antibody method. *J. Histochem. Cytochem.* **30**, 1066-1074

227. Moguilewsky M and Philibert D. (1984). RU486: a potent antiglucocorticoid activity correlated with strong binding to the cytosolic glucocorticoid receptor followed by impaired activation. *J. Steroid Biochem.* **20**:271-276

228. Momoi K, Waterman MR, Simpson ER and Zanger UM. (1992). 3',5'-Cyclic adenosine monophosphate-dependent transcription of the *CYP11A* (cholesterol side chain cleavage cytochrome P405) gene involves a DNA response element containing a putative binding site for transcription factor Sp1. *Mol. Endocrinol.* **6**:1682-1690

229. Monaghan AP, Kaestner KH, Grau E and Shutz G. (1993). Postimplantation expression patterns indicate a role for the mouse forkhead/HNF-3α, β and γ genes in determination of the definitive endoderm, chordamesoderm and neruoectoderm. *Development* **119**:567-578

230. Morgan JE and Whitlock JP Jr. (1992). Transcription-dependent and transcription-independent nucleosome disruption induced by dioxin. *Proc. Natl. Acad. Sci. USA.* **89**:11622-11626

231. Morohashi K-I and Omura T. (1996). Ad4BP/SF-1, a transcription factor essential for the transcription of steroidogenic cytochrome P450 genes and the establishment of reproductive function. *FASEB J.* **10**:1569-1577

232. Muerhoff AS, Griffin KJ and Johnson EF. (1992). Characterization of a rabbit gene encoding a clofibrate-inducible fatty acid ω-hydroxylase: *CYP4A6. Arch. Biochem. Biophys.* **296**:66-72

233. Muerhoff AS, Griffin KJ and Johnson EF. (1992). The peroxisome proliferator activated receptor mediates the induction of *CYP4A6*, a cytochrome P450 fatty acid ω-hydroxylase, by clofibric acid. *J. Biol. Chem.* **267**:19051-19053

234. Nagy L, Kao HY, Chakravarti D, Lin RJ, Hassig CA, Ayer DE, Schreiber SL and Evans RM (1997). Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. *Cell* **89**:373-380

235. Namkung MJ, Yang HL, Hulla JE and Juchau MR. (1988). On the substrate specificity of cytochrome P450IIIAI. *Mol. Pharmacol.* **34**:628-637

236. Nebert DW. (1990). Growth signal pathways. *Nature* **347**:709-710

237. Nebert DW. (1991). Proposed role of drug-metabolizing enzymes: regulation of steady state levels of the ligands that effect growth, homeostasis, differentiation, and neuroendocrine functions. *Mol. Endocrinol.* **5**:1203-1214

238. Nebert DW and Gonzalez FJ. (1987). P450 genes: Structure, evolution, and regulation. *Annu. Rev. Biochem.* **56**:945-993

239. Nebert DW and Jones JE. (1989). Regulation of the mammalian cytochrome P-450 *CYP1A1* gene. *Int. J. Biochem.* **21**:243-252

240. Nebert DW, Nelson DR, Coon MJ, Estabrook RW, Feyereisen R, Fujii-Kuriyama Y, Gonzalez FJ, Guengerich FP, Gunsalus IR, Johnson EF, Loper JC, Sato R, Waterman MR and Waxman DJ. (1991). The P450 superfamily: update on new sequences, gene mapping, and recommended nomenclature. *DNA Cell Biol.* **10**:1-14

241. Nelson DR. (1999). Cytochrome P450 and the individuality of species. *Arch. Biochem. Biophys.* **369**:1-10

242. Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Gunsalus IC and Nebert DW. (1996). P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* **6**:1-42

243. Nieman L and Loriaux D. (1987). Clinical applications of the glucocorticoid and progestin antagonist RU486. In: *Receptor mediated antisteroid action.* (Agarwal MK. ed) Walter de Gruyter, New York, pp 77-97

244. Nitsch D, Boshart M and Schutz, G. (1993). Activation of the tyrosine aminotransferase gene is dependent on synergy between liver-specific and hormone-responsive elements. *Proc. Natl. Acad. Sci. USA.* **90**:5479-5483

245. Nolte RT, Wisely GB, Westin S, Cob JE, Lambert MH, Kurokawa R, Rosenfeld MG, Willson TM, Glass CK, Milburn MV. (1998). Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-γ. *Nature* **395**:137-143

246. Nordeen SK, Bona BJ and Moyer ML. (1993). Latent agonist activity of the steroid antagonist, RU486, is unmasked in cells treated with activators of protein kinase-A. *Mol. Endocrinol.* **7**:731-742

247. Okey AB. (1990). Enzyme induction in the cytochrome P-450 system. *Pharmacol. Ther.* **45**:241-298

248. Okey AB, Riddick DS and Harper PA. (1994). The Ah receptor: mediator of the toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related compounds. *Toxicol. Lett.* **70**:1-22

249. Okino ST, Pendurthi UR and Tukey RH. (1992). Phorbol esters inhibit the dioxin receptor mediated activation of the mouse CypIa-1 and CypIa-2 genes by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *J. Biol. Chem.* **267**:6991-6998

250. Okino ST and Whitlock JP Jr. (1995). Dioxin induces localized, graded changes in chromatin structure: Implications for CyplA1 gene transcription. *Mol. Cell. Biol.* **15**:3714-3721

251. Omiecinski CJ, Walz FJ Jr and Vlasuk GP. (1985). Phenobarbital induction of rat liver cytochromes P-450b and P-450c: Quantitation of specific RNAs by hybridisation to specific oligonucleotide probes. *J. Biol. Chem.* **260**:3247-3250

252. Omura T. (1980). Cytochrome P-450 linked mixed function oxidase turnover of microsomal components and effects of inducers on the turnover phospholipids, proteins and specific enzymes. *Pharmacol. Ther.* **8**:489-499

253. Omura T and Ito A. (1991). Biosynthesis and intracellular sorting of mitochondrial forms of cytochrome P450. In *Methods in enzymology*, vol. 206. (Waterman MR and Johnson EF. eds) Academic Press, New York, pp 75-81

254. Oonk RB, Parker KL, Gibson JL and Richards JS. (1990). Rat cholesterol side-chain cleavage cytochrome P450 (P450scc) gene. Structure and regulation by cAMP *in vitro*. *J. Biol. Chem.* **265**:22392-22401

255. Overdier DG, Porcella A and Costa RH. (1994). The DNA-binding specificity of the hepatocyte nuclear factor 3/forkhead domain is influenced by amino acid residues adjacent to the recognition helix. *Mol. Cell. Biol.* **14**:2755-2766

256. Owensgrillo JK. Czar MJ. Hutchison KA. Hoffman K. Perdew GH. Pratt WB. (1996). A model of protein targeting mediated by immunophilins and other proteins that bind to hsp90 via tetratricopeptide repeat domains. *J. Biol. Chem.* **271**:13468-13475

257. Palmer CNA, Hsu M-H, Griffin KJ and Johnson EF. (1995). Novel sequence determinants in peroxisome proliferator signalling. *J. Biol. Chem.* **270**:16114-16121

258. Palmer CNA, Hsu M-H, Muerhoff AS, Griffin KJ and Johnson EF. (1994). Interaction of the peroxisome proliferator-activated receptor with the retinoid X receptor α unmasks a cryptic peroxisome proliferator response element that overlaps an ARP-1 binding site in the *CYP4A6* promoter. *J. Biol. Chem.* **269**:18083-18089

259. Pani L, Qian X, Clevidence DB and Costa RH. (1992). The restricted promoter activity of the liver transcription factor hepatocyte nuclear factor 3β involves a cell-specific factor and positive autoactivation. *Mol. Cell. Biol.* **12**:552-562

260. Parissenti A, Parker KL and Schimmer BP. (1993). Identification of promoter elements in the mouse 21-hydroxylase (Cyp21) gene that require a functional cAMP-dependent protein kinase. *Mol. Endocrinol.* **7**:283-290

261. Park Y and Kemper B. (1996). The *CYP2B1* proximal promoter contains a functional regulatory element. *DNA Cell Biol.* **15**:693-701

262. Park Y, Li H and Kemper B. (1996). Phenobarbital induction mediated by a distal *CYP2B2* sequence in rat liver transiently transfected in situ. *J. Biol. Chem.* **271**:23725-23728

263. Parke DV. (1968). The biochemistry of foreign compounds. Pergamon Press, Oxford, New York.

264. Pereira TM, Carlstedt-Duke J, Lechner MC and Gustafsson J-A. (1998). Identification of a functional glucocorticoid response element in the *CYP3A1/IGC2* gene. *DNA & Cell Biol.* **17**:39-49

265. Peters JM and Wiley LM (1995). Evidence that murine preimplantation embryos express aryl hydrocarbon receptor. *Toxicol. Appl. Pharmacol.* **134**:214-221

266. Peterson RS, Clevidence DE, Ye H and Costa RH. (1997). Hepatocyte nuclear factor-3α promoter regulation involves recognition by cell-specific factors, thyroid transcription factor-1, and autoactivation. *Cell Growth & Differ.* **8**:69-82

267. Petropoulos I, Auge-Gouillou C and Zakin MM. (1991). Charaterisation of the active part of the human transferrin gene enhancer and purification of two liver nuclear factors interacting with the TGTTTGC motif present in this region. *J. Biol. Chem.* **266**:24220-24225

268. Phelan D, Winter GM, Rogers WJ, Lam JC and Denison MS. (1998). Activation of the Ah receptor signal transduction pathway by bilirubin and biliverdin. *Arch. Biochem. Biophys.* **357**:155-163

269. Pickett CB and Lu AY. (1989). Glutathione S-transferases: gene structure, regulation, and biological function. *Ann. Rev. Biochem.* **58**, 743-764

270. Poellinger L. (1995). Mechanism of signal transduction by the basic helix-loop-helix dioxin receptor. In: *Inducible gene expression.* (Beauerle PA. ed), Vol 1. pp 177-205, Birkhauser, Boston

271. Poland A and Knudson JC. (1982). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related aromatic hydrocarbons: examination of the mechanism of toxicity. *Ann. Rev. Pharmacol. Toxicol.* **22**:517-554

272. Pompon D and Coon MJ. (1984). On the mechanism of action of cytochrome P-450. Oxidation and reduction of the ferrous dioxygen complex of liver microsomal cytochrome P-450 by cytochrome b5. *J. Biol. Chem.* **259**:15377-15385

273. Pongratz I, Mason GG and Poellinger L. (1992). Dual roles of the 90-kDa heat shock protein hsp90 in modulating functional activities of the dioxin receptor: evidence that the dioxin receptor functionally belongs to a subclass of nuclear receptors which require hsp90 both for ligand binding activity and repression of intrinsic DNA binding activity. *J. Biol. Chem.* **267**:13728-13734

274. Porter TD and Coon MJ. (1991). Cytochrome P450: Multiplicity of isoforms, substrates and catalytic and regulatory mechanisms. *J. Biol. Chem.* 266:13469-13472

275. Prabhu L, Upadhya P, Ram N, Nirodi CS, Sultana S, Vastala PG Mani SA, Rangarajan PN, Surolia A and Padmanaban G. (1995). A model for the transcriptional regulation of the *CYP2B1/B2* gene in rat liver. *Proc. Natl. Acad. Sci. USA.* **92**:9628-9632

276. Pratt WB. (1990). Interaction of hsp90 with steroid receptors: organizing some diverse observations and presenting the newest concepts. *Mol. Cell. Endocrinol.* **74**:C69-C76

277. Pratt WB. (1993). The role of heat shock proteins in regulating the function, folding, and trafficking of the glucocorticoid receptor. *J. Biol. Chem.* **268**:21455-21458

278. Quattrochi LC, Mills AS, Barwick JL, Yockey CB and Guzelian PS. (1995). A novel *cis*-acting element in a liver cytochrome P450 3A gene confers synergistic induction by glucocorticoids plus antiglucocorticoids. *J. Biol. Chem.* **270**:28917-28923

279. Quattrochi LC, Yockey CB, Barwick JL and Guzelian PS. (1998). Characterisation of DNA-binding proteins required for glucocorticoid induction of *CYP3A23*. *Arch. Biochem. Biophys.* **349**:251-260

280. Ram N, Rao MV, Prabhu L, Nirodi CS, Sultana S, Vastala PG and Padmanaban G. (1995). Characterisation of a negative *cis*-acting DNA element regulating the transcription of *CYP2B1/2* gene in rat liver. *Arch. Biochem. Biophys.* **317**:39-45

281. Ramsden R, Sommer KM and Omiecinski CJ. (1993). Phenobarbital induction and tissue-specific expression of the rat *CYP2B2* gene in transgenic mice. *J. Biol. Chem.* **268**:21722-21726

282. Rao MS and Reddy JK. (1987). Peroxisome proliferation and hepatocarcinogenesis. *Carcinogenesis*. **8**:631-636

283. Rao MV, Rangarajan PN and Padmanaban G. (1990). Dexamethasone negatively regulates phenobarbitone-activated transcription but synergistically enhances cytoplasmic levels of cytochrome P-450b/e messenger RNA. *J. Biol. Chem.* **265**:5617-5622

284. Raux-Demay MC, Pierret T, Bouvier d'Yvoire M, Bertagna X and Girard F. (1990). Transient inhibition of RU486 antiglucocorticoid action by dexamethasone. *J. Clin. Endocrinol. Metab*. **70**:230-233

285. Reddy JK and Mannaerts GP. (1994). Peroxisomal lipid metabolism. *Annu. Rev. Nutr.* **14**:343-370

286. Reddy JK and Rao MS. (1986). Peroxisome proliferators and cancer: Mechanisms and implications. *Trends Pharmacol. Sci*. **7**:438-443

287. Reisz-Porszasz S, Probst MR, Fukunaga BN and Hankinson O. (1994). Identification of functional domains of the Aryl hydrocarbon receptor nuclear translocator protein (ARNT). *Mol. Cell. Biol.* **14**:6075-6086

288. Remmer H and Merker HJ. (1963). Drug-induced changes in the liver endoplasmic reticulum: Association with drug-metabolising enzymes. *Science* **142**:1657-1658

289. Rey-Campos J, Chouard T, Yaniv M and Cereghini S. (1991). vHNF-1 is a homeoprotein that activates transcription and forms heterodimers with HNF-1. *EMBO J* **10:**1445-1457

290. Rice DA, Aitken LD, Vandenbark GR, Mouw AR, Franklin A, Schimmer BP and Parker KL. (1989). A cAMP-responsive element regulates expression of the mouse steroid 11b-hydroxylase gene. *J. Biol. Chem.* **264**:14011-14015

291. Rice DA, Kirkman MS, Aitken LD, Mouw AR, Schimmer BP and Parker KL. (1990). Analysis of the promoter region of the gene encoding mouse cholesterol side-chain cleavage enzyme. *J. Biol. Chem.* **265**:11713-11720

292. Riviere JL and Cabanne F. (1987). Animal and plant cytochrome P-450 systems. *Biochimie*. **69**:743-752

293. Roberts BJ, Song B-J, Soh Y, Park SS and Shoaf SE. (1995). Ethanol induces *CYP2E1* by protein stabilisation. *J. Biol. Chem.* **270**:29632-29635

294. Robertson RW, Zhang L, Pasco DS and Fagan JB. (1994). Aryl hydrocarbon-induced interactions at multiple DNA elements of diverse sequence: A multicomponent mechanism for activation of cytochrome P4501A1 (*CYP1A1*) gene transcription. *Nucleic Acids Res*. **22**:1741-1749

295. Roe AL, Blouin RA and Howard-G. (1996). In vivo phenobarbital treatment increases protein binding to a putative AP-1 site in the *CYP2B2* promoter. *Biochem. Biophys. Res. Commun*. **228**:110-114

296. Roesler WJ, Vandenbar GR and Hanson RW. (1988). Cyclic AMP and the induction of eucaryotic gene transcription. *J. Biol. Chem.* **263**:9063-9066

297. Roman C, Platero JS, Shuman J and Calame K. (1990). Ig/EBP-1: a ubiquitously expressed immunoglobulin enhancer binding protein that is similar to C/EBP and heterodimerizes with C/EBP. *Genes Dev.* **4**:1404-1415

298. Ryan DE and Levin W. (1990). Purification and characterization of hepatic microsomal cytochrome P-450. *Pharmacol. Ther.* **45**:153- 239

299. Safe SH. (1986) .Comparative toxicology and mechanism of action of polychlorinated dibenzo-*p*-dioxins and dibenzofurans. *Annu. Rev. Pharmacol. Toxicol*. **26**:371-399

300. Sakuma T, Ohtake M, Katsurayama Y, Jarukamjorn K and Nemoto N. (1999). Induction of *CYP1A2* by phenobarbital in the livers of aryl hydrocarbon-responsive and non-responsive mice. *Drug Metab. Dispos*. **27**:379-384

301. Samadani U and Costa RH. (1996). The transcriptional activator hepatocyte nuclear factor 6 regulates liver gene expression. *Mol. Cell. Biol*. **16**:6273-6284

302. Sanger F, Nicklen S and Coulson AR. (1977). DNA sequencing with chain terminator inhibitors. *Proc. Natl. Acad. Sci. USA.*. **74**:5463-5467.

303. Sartorius CA, Tung L, Takimoto GS and Horwitz KB. (1993). Antagonist-occupied human progesterone receptors bound to DNA are functionally switched to transcriptional agonists by cAMP. *J. Biol. Chem.* **268**:9262-9266

304. Savas U, Bhattacharyya KK, Christou M, Alexander DL and Jefcoate CR. (1994). Mouse cytochrome P-450EF, representative of a new 1B subfamily of cytochrome P-450s. Cloning, sequence determination, and tissue expression. *J. Biol. Chem.* **269**:14905-14911

305. Sawadago M and Roeder RG. (1985). Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. *Cell* **43**:165-175

306. Schenkman JB and Jansson I. (1975). Interaction between microsomal electron transfer pathways. *Adv. Exp. Med. Biol.* **58**:387-404

307. Schenkman JB, Tamburini PP, Jansson I and Epstein PM. (1987). In *Cytochrome P450: new trends.* (Sato R, Omura T, Imai Y and Fujii-Kuriyama Y. eds), pp 59-64

308. Schmidt JV, Sue GH-T, Reddy JK, Simon MC and Bradfield CA. (1996). Characterisation of a murine Ahr null allele: involvement of the Ah receptor in hepatic growth and development. *Proc Natl. Acad. Sci.* USA. **93**:6731-6736

309. Schreiber E, Matthias P, Muller M and Schaffner W. (1989). Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res.* **17**:6419

310. Schuetz EG, Brimer C and Schuetz JD. (1998). Environmental xenobiotics and the antihormones cyproterone acetate and spironolacetone use the nuclear hormone pregenolone X receptor to activate the *CYP3A23* hormone response element. *Mol. Pharmacol.* **54**:1113-1117

311. Schuetz EG and Guzelian PS. (1984). Induction of cytochrome P450 by glucocorticoids in rat liver. Evidence that glucocorticoids regulate induction of cytochrome P450 by a nonclassical receptor mechanism. *J. Biol. Chem.* **259**:2007-2012

312. Schutez EG, Li D, Omiecinski CJ, Muller-Eberhard U, Kleinman HK, Elswick B and Guzelian PS. (1988). Regulation of gene expression in adult rat hepatocytes cultured on a basement membrane. *J. Cell. Physiol.* **134**:309-323

313. Schuetz EG, Wrighton SA, Barwick JL and Guzelian PS. (1984). Induction of cytochrome P-450 by glucocorticoids in rat liver. I. Evidence that glucocorticoids and pregnenolone 16α-carbonitrile regulate *de novo* synthesis of a common form of cytochrome P-450 in cultures of rat hepatocytes and in the liver *in vivo*. *J. Biol. Chem.* **256**:1999-2006

314. Schuetz JD, Schuetz EG, Thottassery JV, Guzelian PS, Strom S and Sun D. (1996). Identification of a novel dexamethasone responsive enhancer in the human *CYP3A5* gene and its activation in human and rat liver cells. *Mol. Pharmacol.* **49**:63-72

315. Schulman IG, Shao G and Heyman RA. (1998) Transactivation by retinoid X receptor peroxisome proliferator-activated receptor gamma (PPAR-gamma) heterodimers - intermolecular synergy requires only the PPAR-gamma hormone-dependent activation function. *Mol. Cell. Biol.* **18**:3483-3494

316. Schulte-Hermann R. (1974). Induction of liver growth by xenobiotic compounds and other stimuli. *CRC Crit. Rev. Toxicol.* **3**:97-158

318. Shaw G-C and Fulco AJ (1993) Inhibition by barbiturates of the binding of Bm3R1 repressor to its operator site on the barbiturate-inducible cytochrome P450BM-3 gene of *Bacillus megaterium*. *J. Biol. Chem.* **269**:2997-3004

319. Shaw G-C, Sung C-C, Liu C-H and Lin C-H. (1998). Evidence against the Bm1P1 protein as a positive transcription factor for barbiturate-mediated induction of cytochrome $P450_{BM-1}$ in *Bacillus megateruim*. *J. Biol. Chem.* **273**:7996-8002

320. Shaw PM, Adesnick M, Weiss MC and Corcos L. (1993). The phenobarbital-induced transcriptional activation of cytochrome P450 genes is blocked by the glucocorticoid-progesterone antagonist RU486. *Mol. Pharmacol.* **44**:775-783

321. Shephard EA, Forrest LA, Shervington A, Fernandez LM, Ciaramella G and Phillips IR. (1994). Interaction of proteins with a cytochrome P450 *2B2* gene promoter: identification of two DNA sequences that bind proteins that are enriched or activated in response to phenobarbital. *DNA Cell Biol.* **13**:793-804

322. Shi Y and Thomas JO. (1992). The transport of proteins into the nucleus requires the 70-kilodalton heat shock protein of its cytosolic cognate. *Mol. Cell. Biol.* **12**:2186-2192

323. Shim E-Y, Woodcock C and Zaret KS. (1998). Nucleosome positioning by the winged helix transcription factor HNF-3. *Genes Dev.* **12**:5-10

324. Shimada T and Guengerich FP. (1990). Inactivation of 1,3-1,6- and 1,8-dinitropyrene by cytochrome P-450 enzymes in human and rat liver microsomes. *Cancer Res.* **50**:2036-2043

325. Siduh JS and Omiecinski CJ. (1995). Modulation of xenobiotic-inducible cytochrome P450 gene expression be dexamethasone in primary rat hepatocytes. *Pharmacogenetics* **5**:24-36

326. Silver G and Krauter KS. (1990). Aryl hydrocarbon induction of rat cytochrome P-450d results from increased precursor RNA processing. *Mol. Cell. Biol.* **10**:6765-6768

327. Simmons DL, McQuiddy P and Kasper CB. (1987). Induction of the mixed-function oxidase system by synthetic glucocorticoids: transcriptional and post-transcriptional regulation. *J. Biol. Chem.* **262**:326-332

328. Simpson ER. (1979). Cholesterol side-chain cleavage cytochrome P450 and the control of steroidogenesis. *Mol. Cell. Endocrinol.* **13**:213-227

329. Simpson ER and Waterman MR. (1988). Regulation of the synthesis of steroidogenic enzymes in adrenal cortex cells by ACTH. *Annu. Rev. Physiol.* **50**:427-440

330. Sinclair JF, Wood S, Lambrecht L, Gorman N, Mende-Mueller L, Smith L, Hunt J and Sinclair P. (1990). Isolation of four forms of acetone-induced cytochrome *P*-450 in chicken live by h.p.l.c. and their enzymic characterisation. *Biochem. J.* **269**:85-91

331. Sogawa K, Iwabuchi K, Abe H and Fujii-Kuriyama Y. (1995). Transcriptional activation domains of the Ah receptor and Ah receptor nuclear translocator. *J. Cancer Res. Clin. Oncol.* **121**:612-620

332. Sommer KM, Ramsden R, Siduh J, Costa P and Omiecinski CJ. (1996). Promoter region analysis of the rat *CYP2B1* and *CYP2B2* genes. *Pharmacogenetics* **6**:369-374

333. Stoltz C, Vachon M-H, Trottier E, Dubois S, Paquet Y and Anderson A. (1998). The *CYP2B2* phenobarbital responsive unit contains an accessory factor element and a putative glucocorticoid response element essential for conferring maximal phenobarbital responsiveness. *J. Biol. Chem.* **273**:8528-8536

334. Sueyoshi T, Kawanioto T, Zelko I, Honkakoski P and Negishi M. (1999). The repressed nuclear receptor CAR responds to phenobarbital in activating the human

CYP2B6 gene. *J. Biol. Chem.* **274**:6043-6046

335. Tang YM, Wo YYP, Stewart J, Hawkins AL, Griffin CA, Sutter TR and Greenlee WF. (1996). Isolation and characterization of the human cytochrome P450 *CYP1B1* gene. *J. Biol. Chem.* **271**:28324-28330

336. Thomas SHL. (1993). Paracetamol (acetaminophen) poisoning. *Pharmacol. Ther.* **60**:91-120

337. Tieney B and Bresnick E. (1981) Differences in the binding of 3-methylcholanthrene and phenobarbitone to rat liver cytosolic and nuclear protein fractions. *Arch. Biochem. Biophys.* **210**:729-739

338. Trottier E, Belzil A, Stoltz C and Anderson A. (1995). Localisation of a phenobarbital-responsive element (PBRE) in the 5'-flanking region of the rat *CYP2B2* gene. *Gene* **158**:263-268

339. Truss M, Bartsch J and Beato M. (1994). Antiprogestins prevent progesterone receptor binding to hormone response elements in vivo. *Proc. Natl. Acad. Sci. USA.* **91**:1333-1337

340. Ueno T and Gonzalez F. (1990). Transcriptional control of the rat hepatic *CYP2E1* gene. *Mol. Cell. Biol.* **10**:4495-4505

341. Umesono K, Murakami KK, Thompson CC and Evans RM. (1991). Direct repeats as selective response elements for the thyroid hormone, retinoic acid and vitamin D_3 receptors. *Cell* **65**:1255-1266

342. Upadhya P Rao MV, Venkateswara V, Rangarajan PN and Padmanaban G. (1992). Identification and functional characterisation of a *cis*-acting positive DNA element regulating *CYP 2B1/2B2* gene transcription in rat liver. *Nucleic Acids Res.* **20**:557-562

343. Valle LD, Belvedere P, Simontacchi C and Colombo L. (1992). Extraglandular hormonal steroidogenesis in rats. *J. Steroid Biochem. Mol. Biol.* **43**:1095-1098

344. Vallet V, Antoine B, Chafey P, Vandewalle A and Kahn A. (1995). Overproduction of a truncated hepatocyte nuclear factor 3 protein inhibits expression of liver-specific genes in hepatoma cells. *Mol. Cell. Biol.* **15**:5453-5460

345. Varanasi U, Chu R, Huang Q, Castellon R, Yeldandi AV and Reddy JK. (1996). Identification of a peroxisome proliferator-responsive element upstream of the human peroxisomal fatty acyl Coenzyme A oxidase gene. *J. Biol. Chem.* **271**:2147-2155

346. Wagner BL, Norris JD, Knotts TA, Weigel NL and Mcdonnell DP. (1998). The nuclear corepressors NCOR and SMRT are key regulators of both ligand- and 8-bromo-cyclic AMP-dependent transcriptional activity of the human progesterone receptor. *Mol. Cell. Biol.* **18**:1369-1378

347. Walker NJ, Gastel JA, Costa LT, Clark GC, Lucier GW and Sutter TR. (1995). Rat *CYP1B1*: An adrenal cytochrome P450 that exhibits sex-dependent expression in livers and kidneys of TCDD-treated animals. *Carcinogenisis* **16**:1319-1327

348. Wang GL, Jiang B-H, Rue EA and Semenza GL. (1995). Hypoxia-inducible factor 1 is a basic helix-loop-helix PAS heterodimer regulated by cellular O_2 tension. *Proc. Natl. Acad. Sci. USA.* **92**:5510-5514

349. Watanabe N, Kitazume M, Fujisawa J, Yoshida M and Fujii-Kuriyama T. (1993). A novel cAMP-dependant regulatory region including a sequence like the cAMP-responsive element, far upstream of the human *CYP21A2* gene. *Eur. J. Biochem.* **214**:521-531

350. Waterman MR and Simpson ER. (1989). Regulation of steroid hydroxylase gene expression is multifactorial in nature. *Recent Prog. Horm. Res.* **45**:533-566

351. Watkins PB, Wrighton SA, Schuetz EG, Molowa DT and Guzelian PS. (1987). Identification of a glucocorticoid-inducible P-450 in the intestinal mucosa of rats and man. *J. Clin. Invest.* **80**:1029-1036

352. Waxman DJ and Azaroff L. (1992). Phenobarbital induction of cytochrome P450 gene expression. *Biochem. J.* **281**:577-592

353. Waxman DJ, Dannan GA and Guengerich FP. (1985). Regulation of rat hepatic cytochrome P-450: age-dependent expression, hormonal imprinting, and xenobiotic inducibility of sex specific isoenzymes. *Biochemistry* **24**:4409-4417

354. White RE and Coon MJ. (1980). Oxygen activation by cytochrome P-450. *Annu. Rev. Biochem.* **49**:315-356

355. White RE and Coon MJ. (1982). Heme ligand replacement reactions of cytochrome P-450. Characterization of the bonding atom of the axial ligand trans to thiolate as oxygen. *J. Biol. Chem.* **257**:3073-3083

356. Whitelaw ML, Gottlicher M, Gustafsson J-A and Poellinger L. (1993). Definition of a novel ligand binding domain of a nuclear bHLH receptor: Co-localization of ligand and hsp90 binding activities within the regulable inactivation domain of the dioxin receptor. *EMBO J.* **12**:4169-4179

357. Whitelaw ML, Gustafsson J-A and Poellinger L. (1994). Identification of transactivation and repression functions of the dioxin receptor and its basic helix-loop-helix/PAS partner factor Arnt: Inducible versus constitutive modes of regulation. *Mol. Cell. Biol.* **14**:8343-8355

358. Whitelaw ML, McGuire J, Picard D, Gustafsson J-A and Poellinger L. (1995). Heat shock protein hsp90 regulates dioxin receptor function in vivo. *Proc. Natl Acad Sci. USA.* **92**:4437-4441

359. Whitlock JP Jr. (1987). The regulation of gene expression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Pharmacol. Rev.* **39**:147-161

360. Whitlock JP Jr. (1989). The control of cytochrome P-450 gene expression by dioxin. *Trends Pharmacol. Sci.* **10**:285-288

361. Whitlock JP Jr. (1990). Genetic and molecular aspects of 2,3,7,8-tetrachlorodibenzo-p-dioxin action. *Annu. Rev. Pharmacol. Toxicol.* **30**:251-277

362. Whitlock JP Jr, Okino ST, Dong L, Ko HP, Clarke-Katzenberg R, Ma Q and Li H. (1996). Induction of cytochrome P4501A1: A model for analyzing mammalian gene transcription. *FASEB J.* **10**:809-818

363. Wilson T, Mouw AR, Weaver, CA, Millbrandt J and Parker KL. (1993). The orphan nuclear receptor NGF1-B regulates steroid 21-hydroxylase gene expression. *Mol. Cell. Biol.* **13**:861-868

364. Wilson NM, Christou M, Turner CR, Wrighton SA and Jefcoate CR. (1984) Binding and metabolism of benzo(a)pyrene and 7,12-dimethylbenz(a)anthracene by seven purified forms of P-450. *Carcinogenesis* **5**:1475-1483

365. Wingender E, Dietze P, Karas H and Knuppel R. (1996). TRANSFAC: a database on transcription factors and their DNA binding sites. *Nucleic Acids Res.* **24**:238-241

366. Wolf CR, Miles JS, Seiman S, Burke MD, Rosendowski BN, Kelly K and Smith WE. (1988). Evidence that catalytic differences of two structurally homologus forms of cytochrome P450 are related to their heme environment. *Biochemistry* **27**:1597-1603

367. Wright MC, Paine AJ, Skett P and Auld R. (1994). Induction of rat hepatic glucocorticoid-inducible cytochrome P450 3A by metyrapone. *J. Steroid Biochem. Mol. Biol.* **48**:271-276

368. Wright MC, Wang XJ, Pimenta M, Ribeiro V, Paine AJ and Lechner MC. (1996). Glucocorticoid receptor-independent transcriptional induction of cytochrome P450 3Al by metyrapone and its potentiation by glucocorticoid. *Mol. Pharmacol.* **50**:856-863

369. Wrighton SA, Maurel P, Schuetz EG, Watkins PB, Young B and Guzelian PS. (1985). Identification of the cytochrome P-450 induced by macrolide antibiotics in rat liver as the glucocorticoid responsive cytochrome P-450p. *Biochemistry* **24**:2171-2178

370. Wrighton SA and Watkins P. (1991). Nonuniform distribution of cytochrome P4501A2 in liver. *Gastroenterology* **100**:1487-1488

371. Xanthopoulos KG, Prezioso VR, Chen WS, Sladek FM, Cortese R and Darnell JE. (1991). The different tissue transcription patterns of genes for HNF-1, C/EBP, HNF-3, and HNF-4, protein factors that govern liver-specific transcription. *Proc. Natl. Acad. Sci. USA.*. **88**:3807-3811

372. Xiao GH, Pinaire JA, Rodrigues AD and Prough RA. (1995). Regulation of the Ah gene battery via Ah receptor-dependent and independent processes in cultured adult rat hepaotcytes. *Drug Metab. Dispos.* **23**:642-650

373. Yokotani N, Bernhardt R, Sogawa K, Kusunose E, Gotoh O, Kusunose M and Fujii-Kuriyama Y. (1989). Two forms of ω-hydroxylase toward prostaglandin A and laurate. cDNA cloning and their expression. *J. Biol. Chem.* **264**:21665-21669

374. Youngblood GL and Payne AH. (1992). Isolation and characterisation of the mouse P450 17α-hydroxylase/C_{17-20}-lyase gene (*CYP17*): Transcriptional regulation of the gene by cyclic adenosine 3',5'-monophosphate in MA-10 Leydig cells. *Mol. Endocrinol.* **6**:927-934

375. Zaher H, Fernandez-Salguero PM, Letterio J, Sheikh MS, Fornace AJ, Roberts AB and Gonzalez FJ. (1998) The involvement of aryl hydrocarbon receptor in the activation of transforming growth factor-beta and apoptosis. *Mol. Pharmacol.* **54**:313-321

376. Zaher H, Yang TJ, Gelboin HV, Fernandez-Salguero P and Gonzalez FJ. (1998). Effect of phenobarbital on hepatic *CYP1A1* and *CYP1A2* in the AHR-null mouse. *Biochem. Pharmacol.* **55**:235-238

377. Zanger UM, Kagawa N, Lund J and Waterman MR. (1992). Distinct biochemical mechanisms for cAMP-dependent transcription of *CYP17* and *CYP21. FASEB J.* **6**:713-719

378. Zaret KS, Liu JK and DiPersio CM. (1990). Site-directed mutagenesis reveals a liver transcription factor essential for the albumin transcriptional enhancer. *Proc. Natl. Acad. Sci. USA.*. **87**:5469-5473

379. Zhang LY, Savas U, Alexander DL and Jefcote CR. (1998) Characterisation of the moue *CYP1B1* gene - identification of an enhancer region that directs aryl hydrocarbon receptor-mediated constitutive and induced expression. *J. Biol. Chem.* **273**:5174-5183

380. Zhang X, Jeyakumar M, Petukhov S and Bagchi MK. (1998). A nuclear receptor corepressor modulates transcriptional activity of antagonist-occupied steroid hormone receptor. *Mol. Endocrinol.* **12**:513-524

381. Zuber MX, John ME, Olcamura T, Simpson ER and Waterman MR. (1986). Bovine adrenocortical cytochrome P45017α, regulation of gene expression by ACTH and

Erratum

1. Page X. Should read 2,3,7,8-Tetrachlorobenzo-*p*-dioxin
2. Page 1. Should read "each mammalian species may have up to 50 isoforms"
3. Page 14. Reference 250 should read 347.
4. Page 19. Spironolacetone should read Spironolactone.
5. Page 33. Should read "Glucocorticoids have been implicated in the induction ..."
6. Page 55. Should read "The PB-inducible p450s constitute the largest 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".
9. Page 104. Reference 21 should read 24.