# REGULATION OF CHICKEN CYTOCHROME P450s AND 5-AMINOLEVULINIC ACID SYNTHASE

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BY

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## CONTENTS

## THESIS SUMMARY

The work in this thesis was aimed at understanding at the molecular level the way in which foreign compounds such as pharmaceutical drugs induce, in the liver, the synthesis of the phenobarbital-inducible class of cytochrome P450s (CYPs) and the house-keeping form of aminolevulinic acid synthase (ALAS) that is required for haem formation. Important in this study was an understanding of the role that the end-product haem plays in regulating ALAS, the first enzyme of the haem biosynthetic pathway. A problem in the past, has been the availability of a suitable culture system in which to study this regulation at the gene level. A study was undertaken in primary hepatocyte cultures, derived from the livers of 17-18 day old chick embryos and, in these, phenobarbital was shown to induce CYP and ALAS mRNA. This culture system was therefore used as a model system for the study of the regulation of the *CYP* and ALAS genes.

In the liver of 17-18 day old chick embryos, the drugs phenobarbital and 2-allyl-2-isopropylacetamide (AIA) were known to increase the transcription rates of the *CYP* and ALAS genes, but the effect of haem at this level was not known. An important question was whether drugs (specifically phenobarbital) increased ALAS transcription as a result of haem depletion by induced apoCYP synthesis. This was studied in chick embryo liver and primary hepatocytes. Phenobarbital was shown to rapidly induce both hepatic ALAS and CYPs at a transcriptional level. Haemin or ALA administration did not significantly affect the phenobarbital-induced rates of transcription for ALAS or for *CYPs* in these systems. This finding was in marked contrast to that found by others in rat liver where haemin inhibited transcription of both ALAS and *CYP* genes. However, both haemin and ALA specifically reduced the levels of chicken hepatic ALAS mRNA, implying that in chick embryo liver, haem acts post-transcriptionally, possibly to destabilise ALAS mRNA. These findings led to the proposal of a model for the regulation of ALAS and CYP synthesis in chick embryo liver.

Studies were undertaken on the mechanism by which phenobarbital and structurally dissimilar drugs induce transcription of the phenobarbital-inducible *CYP2H1* and ALAS genes of the chicken. To do this, the 5' flanking region of these chicken genes was fused to the chloramphenicol acetyltransferase (CAT) reporter gene and transient expression studies

investigated in chick embryo primary hepatocytes in the presence and absence of phenobarbital. The first 1700 bp of the ALAS promoter was capable of promoter activity, but failed to show any response to phenobarbital. However, a 4.8 kb *Bam* HI fragment at position -5.9 to -1.1 kb of the *CYP2H1* gene, when fused to an enhancerless SV40 promoter, was able to confer responsiveness to phenobarbital in either orientation and at different distances from this promoter. This region was therefore called a "phenobarbital-responsive enhancer domain". Further analysis of this region revealed smaller regulatory regions or modules, some of which acted as basal enhancers and two of which were able to confer a response to phenobarbital. The smallest of these latter regions, a 238 bp region, was sequenced and this was compared to the known promoter sequences of other phenobarbital-inducible genes. No obvious common sequence, which may indicate a putative phenobarbital-inducible element, was identified. In the future, further analysis of the two phenobarbital-responsive regions should enable the identification of a phenobarbital-responsive element and lead to an understanding of the mechanism(s) by which phenobarbital and phenobarbital-like drugs exert their effect.

## DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University.

In all of the experiments described in this thesis, the author was involved as the principal worker and organiser. However, some of the work reported in Chapter 5 and 6 was performed in collaboration with Dr Antony J. Hansen and in Chapter 7 with Dr Satish Dogra, and due reference is given in the appropriate sections.

To the best of my knowledge and belief, the thesis contains no material previously published or written by any other person, except where due reference is made in the text of the thesis.

Christopher Norman Hahn

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

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## ABBREVIATIONS

| | |
|---|---|
| AIA | 2-allyl-2-isopropylacetamide |
| ALA | aminolevulinic acid |
| ALAD | aminolevulinic acid dehydratase |
| ALAS | aminolevulinic acid synthase |
| bp | base pair(s) |
| BSA | bovine serum albumin |
| CAT | chloramphenicol acetyltransferase |
| Ci | Curie |
| CYP | cytochrome P450 |
| DDC | 3,5-diethoxycarbonyl-1,4-dihydrocollidine |
| DRE | dioxin-responsive element |
| DTT | dithiothreitol |
| x g | times the force of gravity |
| GRE | glucocorticoid-responsive element |
| kb | kilobase(s) |
| $Me_2SO$ | dimethylsulfoxide |
| µF | microFaradays |
| $NaDodSO_4$ | sodium dodecyl sulfate |
| nt | nucleotide(s) |
| PBS | phosphate buffered saline |
| PCN | pregnenolone-16α-carbonitrile |
| rpm | revolutions per minute |
| TCA | trichloroacetic acid |
| TCDD | 2,3,7,8-tetrachlorodibenzo-*p* -dioxin |
| V | volts |
| XRE | xenobiotic-responsive element |

# CHAPTER 1

## INTRODUCTION

## 1.1. GENERAL INTRODUCTION

The cytochrome P450s (CYPs) constitute a large family (superfamily) of enzymes. In animals, the majority of these enzymes are located in the liver and are involved in the oxidative metabolism and subsequent removal from the body of lipophilic exogenous compounds such as drugs, environmental pollutants and some plant constituents, and endogenous compounds including steroids, prostaglandins and fatty acids. The synthesis of many of these CYPs are induced by the compounds which they metabolise. In fact, more than 300 chemicals have been described which induce their own metabolism or the metabolism of compounds with chemically related structures (Conney, 1967). A feature of the hepatic CYPs is their ability to accommodate a broad range of substrates. While the majority of CYPs are located in the liver and are involved in metabolism of foreign compounds, CYPs are also present in other tissues, notably the gonads and adrenal cortex. In these latter tissues, CYPs catalyse specific steps in the synthesis of steroids.

CYPs are enzymes that require haem as a prosthetic group for activity and therefore the availability of haem is an important factor in the synthesis of functional CYPs. The production of haem is regulated by the level of 5-aminolevulinic acid synthase (ALAS) which is the first enzyme in the haem biosynthetic pathway (May *et al.*, 1986) and which, in the liver and possibly in most tissues, catalyses the rate-limiting step of this pathway (Kappas *et al.*, 1983; Granick, 1966). Haem is able to repress the synthesis of functional ALAS and so regulate its own production (Granick, 1966). It has been shown that the level of ALAS enzyme increases upon induction of many of the members of the CYP families (Granick, 1966; Rifkind *et al.*, 1973; Granick *et al.*, 1975; Witting *et al.*, 1977; Althaus *et al.*, 1979; May *et al.*, 1986) and the increased ALAS activity is located in those tissues in which the relevant CYPs are induced (reviewed by May *et al.*, 1986). For example, the exogenous inducer phenobarbital induces ALAS in the liver, where members of the CYP2 family are also induced, but not in the adrenals. In addition, the pituitary hormone ACTH induces ALAS in the adrenal cortex along with the steroid hydroxylases CYP11, CYP17 and CYP21, but none of these enzymes is induced in the liver (Condie *et al.*, 1976; Beaumont *et al.*, 1984; John *et al.*, 1986). A similar specific induction of ALAS occurs in the testis and

ovary by specific pituitary derived hormones which induce steroidogenic CYPs (Tofilon and Piper, 1980; Cook *et al.*, 1982). Analysis of these results has led to the proposal of a model for the co-ordinated induction of specific CYPs and ALAS which is as follows. Inducing compounds or hormones act directly to induce the synthesis of apocytochrome P450s (apoCYPs), which sequester available haem within the cell. In this way haem repression of ALAS synthesis is removed leading to an increase in the level of ALAS and subsequently an increase in the level of cellular "free" haem. Once sufficient haem has been produced to accommodate the increased level of apoCYP, "free" haem rises within the cell to a level where the synthesis of ALAS is once again repressed. In this model, which will be referred to as the "haem repression model", the predominant regulator of ALAS production is a haem-dependent repressor protein. An alternative model is one in which the levels of ALAS and CYPs are directly controlled by drugs irrespective of haem levels. In this model, any endogenous or exogenous compound which induces CYP synthesis must also, in the same tissue, induce ALAS synthesis. This might imply that the regulation of ALAS is complex.

To facilitate a better understanding of this proposed inter-relationship between ALAS and CYPs, an overview of the current knowledge on the regulation of CYPs and a brief review on the control of ALAS expression will be given.

## 1.2. THE CYTOCHROME P450 GENE SUPERFAMILY.

### 1.2.A. Nomenclature and complexity

At present the CYP superfamily consists of at least 154 functional genes for which full-length or nearly full-length cDNA and/or derived amino acid sequences are known, and 7 putative pseudogenes (Nebert *et al.*, 1991). To simplify referral to, and classification of, these CYPs, a nomenclature system was proposed in 1987 (Nebert *et al.*, 1987) and has been updated biennially (Nebert *et al.*, 1989; 1991). Based on the similarities between the amino acid sequences of the CYPs, this system groups *CYP* genes into families, subfamilies and individual genes within a subfamily. For instance, *CYP1A2* relates to the cytochrome P450 belonging to family "1" (Arabic Numeral), subfamily "A" (capital letter) and was the second (Arabic numeral) member of this subfamily identified. When referring

to the gene or cDNA the name is italicised (*e.g. CYP1A2* ) and when referring to the mRNA or protein, it is not italicised (*e.g.* CYP1A2). To positively identify a particular CYP, the species name is also given (*e.g.* rat *CYP1A2* or human *CYP1A2* ), except for the mouse CYPs which are named using lower case letters and a hyphen following the subfamily classification (*e.g. Cyp1a-2* for gene and cDNA and Cyp1a-2 for mRNA or protein).

CYP proteins within a gene family are defined as having > 40% amino acid sequence similarity whereas > 55% amino acid sequence similarity is required for CYP proteins to be classified within the same gene subfamily. Finally, CYP proteins having > 97% sequence similarity are considered to be allelic variants of the same gene. There are exceptions to these rules and for more details the reader is referred to Nebert *et al* (1991). The recommended CYP nomenclature has been used throughout this thesis.

This arbitrary form of classification has been useful since all human and mouse CYPs within a subfamily so far investigated, have been shown to reside in clusters on the respective genomes, suggesting relatedness. The *CYP* gene superfamily currently consists of 27 gene families which are found in a total of 23 eucaryote and 6 procaryote species. Ten of these families exist in mammals, six code for CYPs involved in steroid metabolism while the remaining four families are involved primarily in catabolism of endogenous steroids and fatty acids, in addition to exogenous drugs, plant metabolites, food additives, carcinogens and many environmental pollutants. The reactions catalysed by the latter four families of enzymes occur primarily in the liver (Nebert *et al.*, 1991).

### 1.2.B. Cytochrome P450 enzymatic activities and their role in detoxification

A major role of the drug metabolising enzymes is to convert lipid-soluble compounds into highly water-soluble products that can be eliminated from the body in the urine or bile. This usually requires at least two different types of reactions. In the past, these two reactions have been termed "phase I" or functionalisation and "phase II" or conjugation (Parke, 1968). 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 sulfate to increase water-solubility of the product. The majority of phase I reactions are catalysed by CYPs whereas the phase II reactions require enzymes such as the UDP glucuronosyltransferases and glutathione S-transferases.

As well as involvement in the elimination of exogenous compounds, the CYPs are also directly involved in detoxification of such compounds by altering their chemical structure and hence, abolishing their biological activity (Riviere and Cabanne, 1987). Conversely, CYPs may also activate the biological activity of certain compounds. The best example of this is the conversion of benzo(a)pyrene into a number of metabolites which are able to covalently bind to DNA and are thought to be potentially carcinogenic (Levine *et al.*, 1982).

As mentioned, the CYP enzymes are haemoproteins, being synthesised as apoproteins which bind haem as a prosthetic group and subsequently require co-ordinate binding of a thiolate anion and oxygen for activity (Riviere and Cabanne, 1987). In untreated rats, CYP is the most abundant membrane-bound protein of the liver microsomes and administration of xenobiotics further increases the level of CYP (Adesnik *et al.*, 1981). By far the most common enzymatic activity of CYPs is that of oxidation of lipophilic substrates (*i.e.* monooxygenase activity), although some CYPs act as oxidases, reductases or peroxidases which result in a myriad of chemical reactions including C-, N- and S-hydroxylations, O- and N-dealkylations, deaminations, dehalogenations, reductions, oxidations and the formation of sulfoxides and N-oxides (Riviere and Cabanne, 1987; Gonzalez and Nebert, 1990). For monooxygenase activity, CYPs are supplied with electrons by NADPH-cytochrome P450 reductase, a flavoprotein which receives electrons from NADPH or NADH. The majority of CYPs are microsomal-bound and function in this way, but the CYPs of mitochondria, in addition to a flavoprotein also require an iron-sulphur protein such as adrenodoxin (Estabrook *et al.*, 1979). These CYPs catalyse important steps in the anabolism and catabolism of endogenous steroids and fatty acids (usually hydroxylations) and aid in the detoxification of lipophilic foreign compounds such as drugs, environmental pollutants, certain plant products, food additives and carcinogens.

### 1.2.C. Regulation of the synthesis of Cytochrome P450s

The *CYP* gene superfamily contains many members which differ quite substantially in their nucleic acid sequence and hence in the encoded protein amino acid sequence (see section 1.2.A for the differences which occur between families of CYPs) and it is known that different catalytic properties may result from even subtle differences in CYP amino acid sequence. Different mechanisms of regulation are employed by the many *CYP* genes, with the majority of these genes being expressed in a highly tissue-specific fashion and, in many instances, with expression being controlled by endogenous or exogenous molecules. To date, 154 *CYP* genes have been classified and presumably many more *CYP* genes remain to be identified. Within such a large family of proteins, it is to be expected that the regulatory mechanisms, in some cases, will be quite complex and diverse.

In the eucaryote cell, DNA is contained within the nucleus in the form of chromatin, where it is bound by histone and non-histone proteins. Within a specific tissue, certain genes are in the activated state or are able to be activated, while others are held in a configuration which does not enable them to be expressed. Activated genes are transcribed by RNA polymerase II producing primary transcripts which contain both exonic and intronic sequences. The intron sequences are then removed by the nuclear splicing apparatus and individual exons joined, to produce the mature mRNA molecule which is transported from the nucleus to the cytoplasm. Here translation occurs to produce a protein which is folded and often modified in some way and transported to its functional site within the cell. Further modifications to the protein may occur once it has reached its functional site.

To date, the study of the mechanisms of gene control in many systems has demonstrated that regulation of gene expression may occur at virtually any of the stages mentioned above. This thesis is largely involved with the mechanisms of transcriptional regulation of genes, in particular, one class of inducible CYPs - the phenobarbital-inducible CYPs. This section on regulation of CYP synthesis will be divided into two subsections; transcriptional regulation and mechanisms of regulation other than transcriptional. Due to the large size and complexity of the *CYP* gene superfamily, only those members of *CYPs* which are known to be inducible will be dealt with in some detail in the transcriptional regulation section. These include *CYPs* that are induced by drugs and also by cAMP. The

section on mechanisms of regulation at levels other than transcription will, however, involve a general overview of the CYP field.

### *1.2.C.1. Transcriptional regulation of CYPs*

In this section, an initial brief summary of general transcriptional regulatory mechanisms of genes will be followed by short reviews of the current knowledge on transcriptional control for specific classes of inducible *CYP* genes.

#### a. General transcriptional regulatory mechanisms of genes

By far the greatest amount of research effort has been directed at the mechanisms of transcriptional control of genes. In general, the rate at which a gene is transcribed by RNA polymerase II depends on two factors. These are firstly, the sequence-specific *cis* -acting DNA elements which are most commonly located in the 5' flanking region of the gene or less frequently within the gene or in the 3' flanking region and secondly, the *trans* -acting factors which bind to these DNA elements. *Trans* -acting factors are also referred to as *trans* -activators or transcription factors. Many *cis* -acting elements are reported to be functionally important in gene expression and have been shown to bind specifically one or, in some cases, a number of *trans* -acting factors. An extensive list of such sequences has been reported by Locker and Buzard (1990), but such is the growing number of known elements that by the time a review of this kind is published, it is already out dated.

Transcriptional activation requires the complex assembly of the transcriptional apparatus including RNA polymerase II at or near the transcription start site (*i.e.* initiation, reviewed by Saltzmann and Weinmann, 1989). The rate at which this complex forms, appears to be modulated by *trans* -acting factors bound to gene control regions termed promoters (located within approximately the first 200 bp upstream of the transcription start site) and enhancers (more distant regulatory regions). Promoters and enhancers consist of a combinatorial modular array of *cis* -acting elements and both may contain similar or quite different elements.

It is now generally accepted that via a process of "looping", the *trans* -acting factors bound to enhancers are able to associate with those bound to the promoter to increase the rate

of transcription (reviewed by Muller and Schaffner, 1990). There is substantial evidence for the existence of other proteins unable to bind to DNA directly ("coactivators" or "transcriptional adaptors"), which are apparently important in communication between promoters, enhancers and the transcriptional apparatus assembly (Berger *et al.*, 1990; Herendeen *et al.*, 1990; Pugh and Tjian, 1990; reviewed by Lewin, 1990).

The *trans* -acting factors are the ultimate effectors in a wide variety of regulatory cascades controlling gene expression. They may be initially present in the nucleus or cytoplasm and may be activated, for example, by ligand binding (*e.g.* drugs or hormones), by phosphorylation (*i.e.* second messenger signalling systems) or by metabolite induced dimerisation or dissociation (Johnson *et al.*, 1987) prior to exerting their effect in the nucleus. Induced synthesis of *trans* -acting factors may also be a mechanism by which gene expression is controlled. Many *trans* -acting factors have been shown to belong to a family of functionally similar proteins, the individual members of which may differ slightly in their DNA-binding specificities. Examples of such families are the homeobox proteins (Levine and Hoey, 1988), the CCAAT box-binding proteins (Santoro *et al.*, 1988; Short, 1988) and the nuclear receptor superfamily (Martinez *et al.*, 1991). Often these factors function as dimers or multimers and one can envisage that the formation of heterodimers may greatly increase the diversity of transcriptional responses obtainable from a specific DNA *cis* -acting element.

For further information, the reader is referred to reviews on the regulation of inducible and tissue-specific gene expression (Maniatis *et al.*, 1987), transcriptional activation and suppression (Robertson, 1988), enhancer and promoter composition (Schatt *et al.*, 1990) transcriptional repression (Levine and Manley, 1989; Renkawitz, 1990) and *trans* -activation of enhancers or transvection (Muller and Schaffner, 1990).

Transcriptional regulation has been established for several *CYP* genes. The best studied example of a mechanism of transcriptional control is the induction process of the *CYP1A1* gene (a TCDD-inducible *CYP* gene) by aromatic hydrocarbons. It is also known that the phenobarbital-inducible, glucocorticoid-inducible, and peroxisome proliferator-inducible *CYP* genes are activated transcriptionally by the various classes of drugs. In

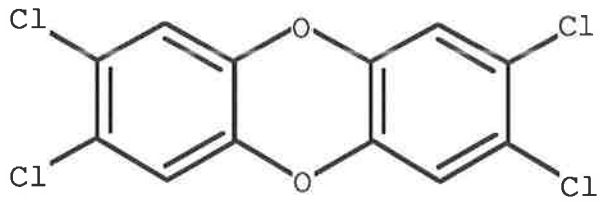
addition, the steroid hydroxylase *CYP* genes are transcriptionally induced by hormones via cAMP. Each of these inducible classes of *CYPs* will now be discussed in some detail.

### b. Transcriptional regulation of the aromatic hydrocarbon-inducible CYPs.

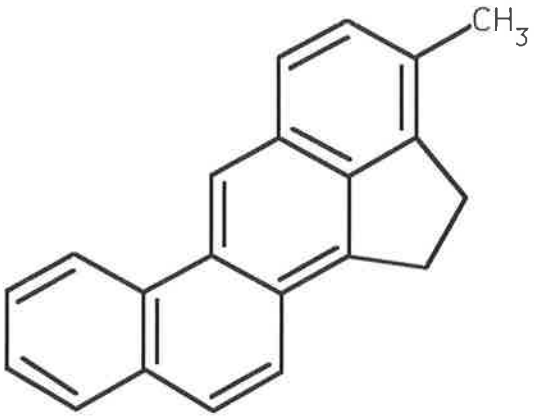
The *CYP1* gene family to date consists of only two genes designated *CYP1A1* and *CYP1A2* both of which have been shown to exist in the human, rat, mouse, rabbit, hamster, dog and monkey, while the trout has only an orthologue to *CYP1A1* (Nebert *et al.*, 1991). These genes encode proteins that are similar in amino acid sequence, but differ in their substrate specificity (Nebert and Gonzalez, 1987, and references therein). They are also inducible by aromatic hydrocarbons such as polycyclic aromatic hydrocarbons and some benzoflavones. These molecules are structurally similar containing aromatic rings that are essentially planar (see Fig. 1.1). 2,3,7,8-Tetrachlorodibenzo-*p* -dioxin (TCDD) being the most potent inducer has become the prototype for this class of molecules and therefore the members of this family of proteins are often referred to as the TCDD-inducible CYPs. TCDD was found to be a contaminant of the herbicide "Agent Orange" which was used as a defoliant in the Vietnam War, and has been shown to cause birth defects and cancer in animal experiments. The effect of TCDD on humans is unclear (Poland and Knutson, 1982), although a recent extensive report has correlated the extent of exposure to TCDD with elevated levels of the compound in the serum (even after 15 to 37 years) and an increased risk of developing certain cancers (Fingerhut *et al.*, 1991). Of all the CYPs, most work at the molecular level has been performed on the members of this family of enzymes. One reason for this is the apparent link between the metabolism of certain aromatic hydrocarbons by these CYPs with the formation of mutagens and carcinogens in animals (Gonzalez, 1989). The expression of these proteins, based mainly on catalytic activities, have also been linked to the onset of cancer in humans (Gelboin, 1983). The aryl hydrocarbon hydroxylase activity of the CYP1A1 enzyme plays a major role in the conversion of polycyclic aromatic hydrocarbons found in smog and cigarette smoke, into carcinogenic intermediates (Gonzalez, 1989). CYP1A2 is the principal enzyme involved in the activation of certain heterocylic amines that constitute the major mutagens found in cooked meat (Butler *et al.*,

**Figure 1.1. Chemical structures of aryl hydrocarbon compounds**

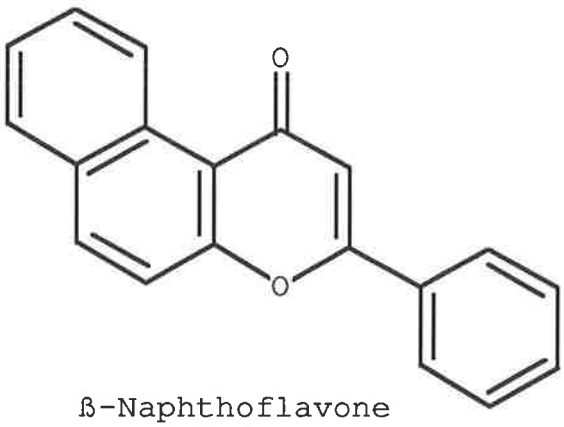
The aryl hydrocarbon compounds are related structurally in that they all contain benzene rings and are essentially planar molecules.



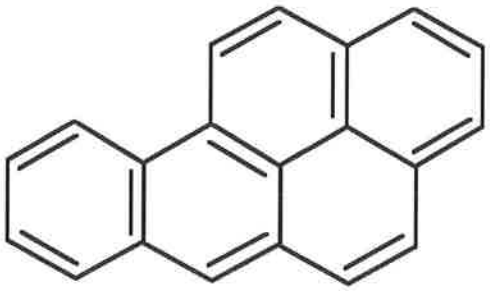

2,3,7,8-tetrachlorodibenzodioxin
(TCDD)




20-Methylcholanthrene
(3-Methylcholanthrene)




ß-Naphthoflavone
(ß-NF)



Benzo(a)pyrene

1989). The best evidence for this is the metabolism of benzo(a)pyrene to form several products which are potentially more carcinogenic than the parent compound (Levine *et al.*, 1982).

In mouse and rat, CYP1A1 protein is expressed in the liver as well as many extrahepatic tissues, while expression of CYP1A2 occurs predominantly in the liver and appears to be restricted to fewer extrahepatic tissues (reviewed by Silver and Krauter, 1988). Although both genes are inducible by aromatic hydrocarbons in mammalian liver, expression of the *CYP1A2* gene is quickly extinguished in organ cultures and in most cases is silent in tissue culture cells (Kimura *et al.*, 1987; Nebert and Gonzalez, 1987; Pasco *et al.*, 1988; Silver and Krauter, 1988).

Three groups of researchers have been instrumental in the bulk of the study performed on the regulation of the first of the genes in this family, *CYP1A1*. Whitlock and coworkers have concentrated on the study of the regulation of the mouse gene, Nebert and coworkers on the mouse and human orthologous genes and Fujii-Kuriyama and coworkers on the rat orthologous gene. For comprehensive reviews on this work, the reader is referred to Whitlock (1987, 1989) and Fujii-Kuriyama *et al* (1989). Studies performed in various naturally occurring and inbred strains of mice, and in the hepatoma cell lines of mouse, rat and human, and using various molecular and genetic approaches, have provided evidence for a model of inducible regulation of the *CYP1A1* gene by TCDD and other structurally similar aromatic hydrocarbons. Briefly, in this model, TCDD enters the cell by diffusion and binds to a cytosolic receptor. The TCDD-receptor complex then translocates from the cytoplasm into the nucleus where it binds to specific *cis* -acting sequences in the regulatory regions of the *CYP1A1* gene and this in turn results in an increase in the rate of transcription of the gene. This increase in transcription is reflected by increased synthesis of the protein product of the gene as measured by aryl hydrocarbon hydroxylase (AHH) activity. The following is a more detailed summary of the current knowledge of this induction process.

Being lipophilic, TCDD is thought to enter the cell by passive diffusion where it binds saturably and with high affinity to the cytosolic soluble receptor which has been designated the "Ah receptor" as it mediates the response to aromatic hydrocarbons. The affinity with which the Ah receptor binds TCDD and other aromatic hydrocarbon ligands

correlates well with the degree of activation of the Ah receptor (*i.e.* the ability to bind DNA) *in vitro*, and with the relative potencies of these compounds to induce target gene transcription *in vivo* (Cuthill *et al.*, 1991). The binding process requires reduced sulfhydryl groups (Denison *et al.*, 1987; Kester and Gasiewicz, 1987) and ATP as an energy source (Gudas and Hankinson, 1986). Wilhelmsson *et al* (1990) have demonstrated that the inactive form of the Ah receptor which does not bind to DNA, is complexed with the 90 kD heat shock protein (hsp 90). The hsp90 is however required for efficient binding of TCDD to the receptor, and subsequent dissociation of hsp90 from the TCDD-Ah receptor complex is sufficient to activate the liganded receptor enabling it to bind to specific DNA sequences termed dioxin-responsive elements (DREs) or xenobiotic-responsive elements (XREs). The hsp90 is therefore thought to inhibit in some way, the DNA binding capacity of the Ah receptor. A temperature-dependent process is involved following the formation of the liganded Ah receptor complex which increases its affinity for DNA binding, but the exact nature of this process is not understood. Possible suggestions that have been put forward include an enzymic modification of the complex, a conformational change, or interactions with other intracellular molecules (Whitlock, 1989). The dissociation of the hsp90 from the liganded receptor complex may be a candidate for the latter of these suggestions.

A number of classes of mutants of the mouse hepatoma Hepa-1 cell line were discovered by Hankinson (1983), which were shown by genetic techniques to represent different genes involved in the Cyp1a-1 induction pathway. Class A variants (designated $P_1^-$ mutants) contained mutations in the *Cyp1a-1* gene itself. Class B variants (designated $r^-$) contained < 10% of the levels of the Ah receptor compared with wild-type Hepa-1 cells and Class C variants (designated $nt^-$) displayed an inability to translocate the liganded receptor complex from the cytosol to the nucleus and hence such complexes were unable to bind to the DNA. The existence of this latter class of variant cells (*i.e.* nuclear translocation defective cells) together with evidence showing that radio-labelled TCDD rapidly moves into the nucleus of wild-type cells, led to the proposal of a specific nuclear translocator protein which was essential for a response to TCDD. More recently however, the inactive non-liganded Ah receptor-hsp90 complex and hsp90 itself, as well as the hsp90-free TCDD-Ah receptor complex have been detected within the nucleus, suggesting the possibility that the

components involved in this signal transduction pathway are able to freely diffuse within the cytosolic and nuclear compartments; in this case, the apparent nuclear translocation may simply represent a "trapping" within the nucleus of the activated complex which is the only component able to bind to the DNA (Whitlock and Galeazzi, 1984; Wilhelmsson *et al.*, 1990). However, in contrast to this, evidence for the existence of a specific translocator protein comes from the recent report of the cloning of a human cDNA for a factor that is essential in the functioning of the Ah receptor (Hoffman *et al.*, 1990). In this study, the expressed 87 kD protein does not bind ligand and has been called the Ah receptor nuclear translocator (ARNT) protein, because it was isolated by rescuing inducibility of aryl hydrocarbon hydroxylase activity (one enzyme activity of CYP1A1) in a Class C mutant mouse Hepa-1 cell line considered to be deficient in nuclear translocation (Hankinson, 1983). Whether this protein acts as a specific transporter of the liganded Ah receptor complex from the cytoplasm to the nucleus remains to be proven.

Elferink *et al* (1990) have provided evidence that the activated form (*i.e.* DNA binding form) of the liganded Ah receptor complex consists of two biochemically different proteins. A 100 kD protein has been shown to be involved in binding of the ligand (*e.g.* TCDD) while a 110 kD protein is thought to confer strong DNA binding to the liganded Ah receptor complex. While both the 110 kD and 100 kD proteins appear to bind DNA as detected by protein-DNA cross-linking experiments, the 110 kD protein cross-links to the DNA recognition motif more quickly and it has been speculated that this larger component of the Ah receptor heterodimer binds to the DNA, inducing it to bend towards the smaller ligand-binding component, thereby enabling contact of this 100 kD component with the DNA to be more favourable. Evidence for bending of the DNA upon binding of the liganded Ah receptor to its recognition motif has been published by Elferink and Whitlock (1990). The DNA motifs to which the liganded Ah receptor complex have been shown to bind have been named dioxin-responsive elements (DREs) in the mouse and human *CYP1A1* genes and xenobiotic-responsive elements (XREs) in the rat *CYP1A1* gene. The core consensus sequence for the DRE motif is TNGCGTG (Fisher *et al.*, 1990) and for the XRE motif is $C^{G}/_{C}T^{G}/_{C}C/_{T}T^{G}/_{C}TCACGC^{T}/_{A}A$ (Fujisawa-Sehara *et al.*, 1987). The underlined bases show an identical core sequence common to both, but in opposite

orientation. The presence of these sequences (including several neighbouring bases) upstream of a heterologous promoter, are able to confer TCDD-inducibility to that promoter in transient expression studies (Fujisawa-Sehara *et al.*, 1987; Denison *et al.*, 1988). The position of the XRE motifs in the mouse, human and rat *CYP1A1* genes are remarkably similar - two being located approximately 1 kb upstream of the transcription start site and divergently orientated, and another positioned approximately 0.5 kb upstream (Nebert and Jones, 1989). Of interest are the findings of Rushmore *et al* (1990) who have reported the presence of two xenobiotic regulatory regions in the glutathione S-transferase Ya subunit promoter, both of which are able to activate transcription in response to the readily metabolisable aromatic hydrocarbons 3-methylcholanthrene and ß-naphthoflavone. However, only the more distal region (-908 to -899 bp) which contains a DNA sequence with identity to the XRE sequence is able to confer inducibility with the poorly metabolisable TCDD. The proximal xenobiotic-responsive region (-722 to -682 bp) has been designated the "antioxidant-responsive element" (ARE) since it responds to phenolic antioxidants such as those formed upon metabolism of 3-methylcholanthrene, ß-naphthoflavone and other aromatic hydrocarbons.

It is becoming clear, that the liganded Ah receptor is not the only factor which binds to the core consensus DRE motif. Puga and Nebert (1990) have isolated a partial cDNA clone encoding poly(ADP-ribose) polymerase which binds to a catenated DRE sequence. Specific inhibition of the activity of this enzyme led to a decrease in the magnitude of induction of Cyp1a-1 mRNA by TCDD. The role of poly(ADP-ribose) polymerase in the basal and drug-induced expression of TCDD-inducible genes awaits investigation. Furthermore, Saatcioglu *et al* (1990) have demonstrated the presence of two factors termed XF1 and XF2 which constitutively bind to the rat XRE core consensus sequence, and which do not respond to TCDD or other aromatic hydrocarbons. Evidence is given to support the idea that these two proteins may act as negative regulators and that the binding of the liganded Ah receptor is a competitive process. In this way, the degree of induction with TCDD may differ in various tissues depending on the relative abundances of the Ah receptor, XF1 and XF2. Genes also may differ in their response to TCDD depending on the respective affinities of the Ah receptor, XF1 and XF2 for specific XRE sequences

remembering that sequences surrounding the core XRE consensus sequence affect receptor binding and response to TCDD (Fujisawa-Sehara *et al.*, 1987). These results are in keeping with those of Poellinger *et al* (1990) who have reported the presence of a DNase I hypersensitivity site in the vicinity of the two XRE motifs (approximately 1 kb upstream of the *CYP1A1* gene transcription start site), which does alter upon treatment of the rat with TCDD. A constitutive XRE-specific factor(s) has been suggested to be important in holding this region of the promoter in an open configuration to allow for a rapid response to drug, but whether this DNase I hypersensitivity site is caused by binding of the constitutively expressed XF1 and/or XF2 remains to be seen.

The exact mechanism by which binding of the liganded Ah receptor complex to the specific DNA motifs activates transcription is not known. It does however appear that multiple sequences or regions other than the XRE or DRE sequences are of importance. For instance, in the mouse *Cyp1a-1* gene 5' flanking region, a dioxin-responsive enhancer (-1302 to -820 bp) consisting of four DREs contains within it a GC box known to bind the ubiquitous transcription factor Sp1. Using transient expression studies, this GC box appears not only important in constitutive expression, but also synergistically increases TCDD-induced expression (Fisher *et al.*, 1990). Further regions within the *CYP1A1* gene 5' flanking sequence are important in basal expression and the process of induction by TCDD and other aromatic hydrocarbons. One of these is an element approximately 200 bp upstream of the transcription start site which is absolutely essential for constitutive and TCDD-inducible *Cyp1a-1* expression, but in the absence of the DREs cannot alone confer TCDD-inducibility (Neuhold *et al.*, 1989). Yet another upstream element is involved in the negative autoregulatory loop in which the Cyp1a-1 enzyme itself leads to a reduction in the endogenous Cyp1a-1 mRNA levels (Puga *et al.*, 1990; Raychaudhuri *et al.*, 1990). For a more detailed review of the regulation of the *Cyp1a-1* gene the reader is referred to the following reviews (Whitlock, 1987; Nebert and Jones, 1989; Whitlock *et al.*, 1989).

It now appears that the actual mechanism of induction of the *CYP1A1* gene by TCDD and other aromatic hydrocarbons is a complex one incorporating many possible players, both in the signal transduction pathway and in the mechanism of transcriptional activation. This is made even more interesting by reports suggesting that while the levels of

CYP1A1 in rat liver are induced by TCDD or 3-methylcholanthrene predominantly by a transcriptional mechanism, the levels of CYP1A2 are regulated primarily at a post-transcriptional level (Pasco *et al.*, 1988; Silver and Krauter, 1988). Studies by Silver and Krauter (1990) provide evidence that in rat primary hepatocyte cultures the liganded Ah receptor complex interacts in some as yet unknown way with either the *CYP1A2* gene or primary transcript resulting in stabilisation of this transcript. This stabilisation may take the form of a more favourable structure for efficient splicing or 3' end formation or may involve protection of the primary transcript from rapid nuclear degradation, but as yet there is no evidence for either of these suggestions. In addition, in this system, dexamethasone has been shown to potentiate the 3-methylcholanthrene-induced accumulation of CYP1A2 mRNA at a post-transcriptional level, but appears to have no such effect alone (Silver *et al.*, 1990). Conversely, from work by Quattrochi and Tukey (1989) and Tukey and Okino (1990) in mouse liver, the conclusion was made that TCDD-dependent Cyp1a-1 and Cyp1a-2 mRNA accumulation could be completely attributed to transcriptional induction of the respective genes. The reason for this discrepancy is not clear.

To add to the complexity of regulation by TCDD, a domain containing the DREs in the mouse *Cyp1a-1* gene 5' flanking region (position -1100 to -896 bp), in addition to its role as a transcriptional enhancer, has been implicated in providing an important post-transcriptional function (Puga *et al.*, 1990). The mechanism of this action is at present unknown.

Apart from initiation as a stage for transcriptional control, the process of elongation of transcripts may also be important. In this regard, haem has been reported to positively regulate transcription elongation of the *CYP1A1/1A2* genes in rat liver (Padmanaban *et al.*, 1990).

Overall then, the control of the members of the *CYP1* gene family is very interesting. Induction by structurally similar aromatic hydrocarbons appears to be extremely complex. In addition to the differences in the mechanism of induction displayed between CYP1A1 and CYP1A2, regulation of a TCDD-inducible rat liver aldehyde dehydrogenase differs yet again from that of CYP1A1 with respect to dose-response relationship, induction kinetics, and tissue specificity (Dunn *et al.*, 1988). This implies that TCDD may be able to

modulate specific gene expression via pathways in addition to the classical Ah receptor mechanism. A clearer understanding of the mechanism of drug induced increase in transcription rates will be possible with the isolation of genomic and cDNA clones for the Ah receptor component(s) and the production of specific antibodies against these components.

### c. Transcriptional regulation of the phenobarbital-inducible CYPs

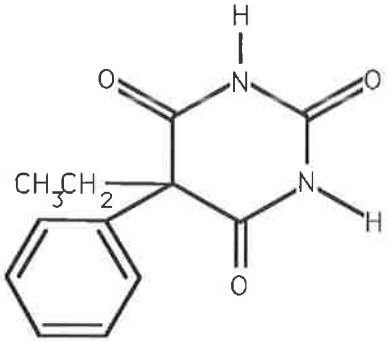
Historically, members of the *CYP2* gene family were referred to as the "phenobarbital-inducible" CYPs. However, with the continual addition of new members to this family, it has become clear that many of the members are constitutively expressed and are not known to be inducible by drugs. The majority of mammalian phenobarbital-inducible CYPs belong to the subfamilies *CYP2B* and *CYP2C*. When rats are administered phenobarbital, there is an increase in the synthesis of two predominant CYPs designated CYP2B1 and CYP2B2. Increased amounts of two rat hepatic CYP mRNA species following phenobarbital-treatment, have been shown by nuclear transcription run-on assays to occur as a result of an increase in the transcription of the *CYP2B1* and *CYP2B2* genes (previously designated P450b and P450e), respectively (Hardwick *et al.*, 1983). In the rabbit, three *CYP* genes (*CYP2C1*, *CYP2C2* and *CYP2C4* ) have been shown in a similar way to be induced transcriptionally by phenobarbital (Zhao *et al.*, 1990). The major glucocorticoid-inducible CYP in rat liver (see also section 1.2.C.1.d) designated CYP3A1 (P450p) is also induced by phenobarbital and this has been shown to be due to increased mRNA levels (Burger *et al.*, 1990; Schuetz *et al.*, 1990). Whether this increase is derived at the transcriptional level is not known. The prokaryote, *Bacillus megaterium* ATCC 14581, contains three reported *CYP* genes, one of which, *CYP102* (previously $P450_{BM-3}$), is strongly induced transcriptionally by pentobarbital and other barbiturates (Wen *et al.*, 1989). To date, the only reported phenobarbital-inducible CYP (designated CYP6) in insects has been isolated from the housefly (*Musca domestica* ), the mRNA levels of which are increased by phenobarbital (Feyereisen *et al.*, 1989). It is hoped that the study of this CYP will be an important tool in the understanding of the molecular genetics of insecticide resistance.

Of importance in this thesis are the two phenobarbital-inducible *CYPs* genes that have been shown to exist in the chicken (Mattschoss *et al.*, 1986). One of these genes, *CYP2H1* (previously designated gene A), encodes a 3.5 kb mRNA whilst the other gene, *CYP2H2* (previously designated gene B) gives rise to a 2.2 kb mRNA. Both of these mRNAs in liver are highly induced following phenobarbital administration to chick embryos and it has been shown that these increases are a result of enhanced gene transcription and as well a post-transcriptional mechanism (Hansen *et al.*, 1989). A phenobarbital-inducible mRNA of size 2.5 kb is also present, probably due to an alternative splicing event of the RNA transcribed from either *CYP2H1* or *CYP2H2* (Hansen *et al.*, 1989).

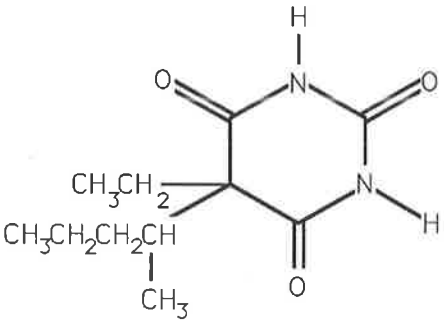
Unlike the TCDD-inducible CYPs, little is known about the mechanism(s) by which phenobarbital exerts its effect. The drugs, like phenobarbital, that are able to induce the same phenobarbital-inducible CYPs are commonly called "phenobarbital-like" drugs although they vary substantially in chemical and structural properties (Elliott *et al.*, 1989; see Fig. 1.2). This is quite different from the Ah receptor situation where the receptor binds to structurally similar, essentially planar aromatic hydrocarbon compounds and subsequently leads to induction of the TCDD-inducible *CYP* genes (refer to section 1.2.C.1.b). A major property therefore of a receptor for phenobarbital and phenobarbital-like drugs would be the ability to bind a broad range of structurally different ligands. It is possible also that several distinct receptors exist which bind different subclasses of compounds, but which evoke the same pleiotropic response (Poland *et al.*, 1981). Another suggestion is that a structurally similar metabolic product may in fact be the active inducer, but to date no such intermediate common metabolic structure has been recognised (Hardwick *et al.*, 1983). The crucial question of whether a receptor exists for phenobarbital and phenobarbital-like drugs remains unanswered. Experiments performed to search for such a receptor using radioactive phenobarbital have not yielded positive evidence (Poland *et al.*, 1980, 1981; Tierney *et al.*, 1981). However, since phenobarbital is not a potent inducer, it may not bind with high affinity to a receptor if one exists. Fonne and Meyer (1987) have shown, in rat, that the CYP enzyme itself appears not to be the receptor. Evidence suggesting the presence of a receptor has come from studies demonstrating that while phenobarbital is able to induce CYP enzymatic activities in the mouse and rat liver, 1,4-bis[2-(3,5-dichloropyridyloxy)]-benzene

**Figure 1.2. Chemical structures of phenobarbital and "phenobarbital-like" compounds**

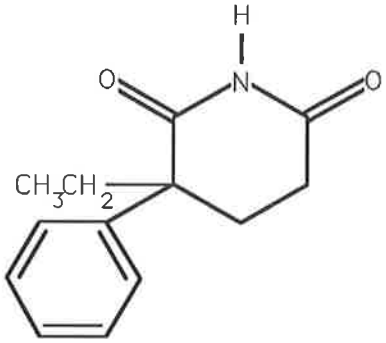
The compounds shown are called "phenobarbital-like" compounds as they, like phenobarbital, all induce the same *CYP* genes. While some of the compounds such as pentobarbital, glutethimide and mephenytoin are similar in structure to phenobarbital, there are a number of compounds which bear no resemblance to phenobarbital or to other compounds in this class of drugs.



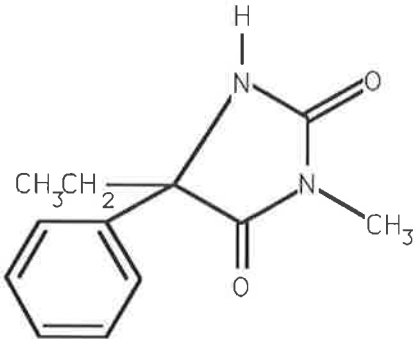
Phenobarbital



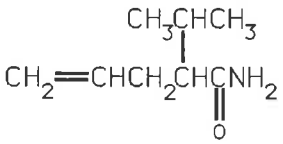
Pentobarbital



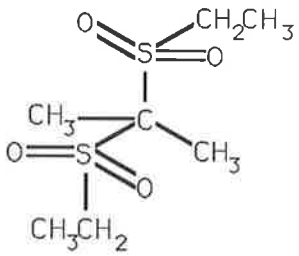
Glutethimide



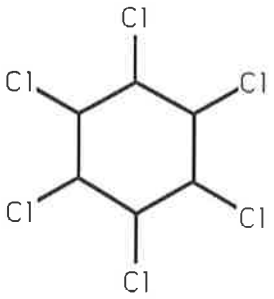
Mephenytoin



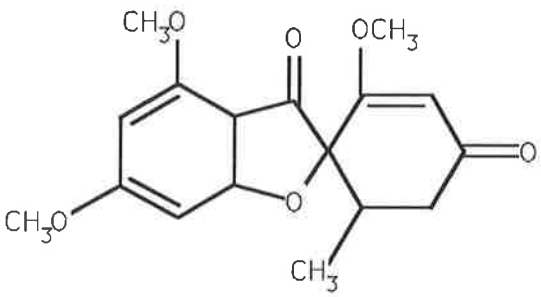
2-Allyl-2-isopropylacetamide
(AIA)



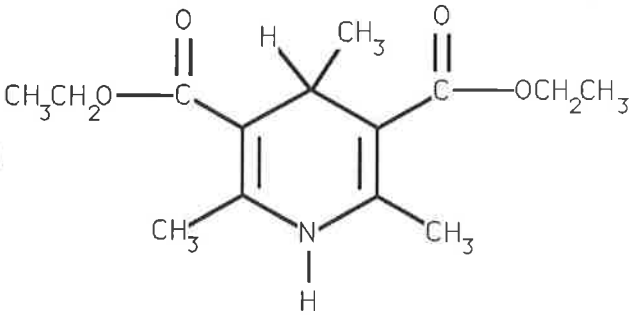
Sulphonal



Lindane



Griseofulvin



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

(TCPOBOP), a phenobarbital-like compound with a potency 650 times that of phenobarbital in mice, induces a strong response in mouse liver, but not in rat liver (Poland *et al.*, 1980; 1981). This result is most easily explained by the loss in rat of a functional specific receptor necessary for activation of CYP2B1 and CYP2B2 by TCPOBOP or an alteration in a single receptor which can no longer bind TCPOBOP, but which retains a phenobarbital-binding capacity.

The concept of the existence of a specific receptor has been questioned. An alternative theory has been based on the fact that all of the inducing drugs in this group are lipophilic and also on the observation that the magnitude of response of such drugs correlates well with the degree of lipophilicity of the drug (DeMatteis, 1967; 1968). Using this information, it has been postulated that the inducing drugs accumulate within the plasma membrane of the cell and in doing so, disrupt the structure of the membrane, resulting somehow in the transduction of a response to cytoplasmic cellular factors and eventually to the specific genes within the nucleus (Poland *et al.*, 1981). In this situation no receptor is necessary. However, this model does not address the issue of drug specificity, as there are many lipophilic drugs which do not induce the phenobarbital-inducible CYPs.

Phenobarbital at high doses has recently been shown to weakly induce aryl hydrocarbon hydroxylase activity in a mouse hepatoma cell line (Hepa 1) by a mechanism apparently involving the Ah receptor (Karenlampi *et al.*, 1989). The physiological significance of this result is not clear.

The response of phenobarbital is tissue-specific, but varies between genes and also species. For instance, of the the most intensively studied major phenobarbital-inducible CYPs in rat, CYP2B1 is constitutively expressed in liver and lung while CYP2B2 is expressed only in liver. Both have been shown to be induced by phenobarbital at the mRNA level in the liver, but not in the kidney, lung, testis, brain or erythroid spleen (Srivastava *et al.*, 1989a). Phenobarbital-induction of the levels of CYP2B1 and CYP2B2 mRNA in the liver is due to an elevation in the rate of gene transcription (Hardwick *et al.*, 1983; Rao *et al.*, 1990; Srivastava *et al.*, 1990). Rabbit CYP2C16 however, is expressed constitutively in liver, lung, testis and kidney and is inducible by phenobarbital in each of these tissues except kidney where mRNA levels are repressed (Hassett and Omiecinski, 1990). Yet again, a

different expression pattern is seen in the adult chicken, where constitutive expression of CYP2H1 and CYP2H1 mRNA occurs in liver, lung, kidney and small intestine and in this case induced expression by phenobarbital is seen in each of these tissues except lung, where mRNA levels are reduced (Hansen *et al.*, 1989).

A number of endogenous and exogenous compounds are known to modulate the effect of phenobarbital. The role of haem in the inductive process of rat liver CYP2B1 and CYP2B2 is at this moment a contentious issue. Studies by Padmanaban and coworkers suggest that haem acts as a positive modulator of phenobarbital-mediated activation of transcription (Ravishankar and Padmanaban, 1985; Dwarki *et al.*, 1987; Bhat and Padmanaban, 1988a; Rangarajan and Padmanaban, 1989). Work on the *CYP2B2* gene promoter by this group of workers has indicated that a fragment of the gene promoter (-179 to +181 bp) is faithfully transcribed *in vitro* in nuclei isolated from livers of phenobarbital treated rats at a level greater than from untreated rats. Phenobarbital increases the amount of a protein binding to a 223 bp fragment located immediately upstream of the translation initiation ATG codon (*i.e.* -179 to +44 bp), and the binding of this protein can be inhibitied by $CoCl_2$ (an inhibitor of haem biosynthesis) and restored by addition of haem (Rangarajan and Padmanaban, 1989). Furthermore, Padmanaban *et al* (1990) have reported that the action of phenobarbital is required to overcome stalling of RNA polymerase II at the exon 1/intron 1 boundary and at a second site near exons 6 and 7 (a potential termination site). In addition, haem has been proposed to be required for correct processing of the CYP2B1/2B2 primary transcripts (Padmanaban *et al.*, 1990). Interestingly, these genes also undergo anti-sense transcription such that the levels of sense and anti-sense transcripts are inversely correlated, and this haem-modulated processing event may be in some way related to the rate of anti-sense transcript formation. In contrast to these results, work from the laboratory of May and coworkers has provided evidence that haem acts rather as a negative regulator of CYP synthesis. They showed that haem suppresses the transcription rates of the *CYP2B1/2B2* genes and lowers the constitutive, phenobarbital-induced, AIA-induced and clofibrate-induced levels of mRNA for CYP2B1 and CYP2B2 (Srivastava *et al.*, 1989b; 1990). This difference in the haem-modulated effect on rat liver CYP2B1/2B2 expression

may not necessarily be incompatible, but may reflect different responses of these genes to various haem levels.

In brief, other factors that have been demonstrated to repress induction of CYP2B1 and CYP2B2 by phenobarbital in rat liver and primary hepatocytes include growth hormone (Yamazoe *et al.*, 1987, Schuetz *et al.*, 1990), L-triiodothyronine (Yamazoe *et al.*, 1989) and the tumour promoting phorbol ester, 12-O-tetradecanoylphorbol-13-acetate which stimulates protein kinase C activity (Steele and Virgo, 1988). The synthetic glucocorticoid, dexamethasone, has been shown to have a number of regulatory influences on phenobarbital-induction of CYP2B1/2B2 (Rao *et al.*, 1990) and these will be dealt with in more detail in the following section (*i.e.* section 1.2.C.1.d).

The possibility exists that phenobarbital and other phenobarbital-like drugs induce CYPs by more than one mechanism. Evidence for this is as follows: Honkakoski and Lang (1989) have demonstrated in mouse liver that phenobarbital and TCPOBOP induce the levels of four CYPs to various extents. For three of these CYPs, TCPOBOP was a more potent inducer than phenobarbital while for one CYP, the reverse situation was true, indicating a difference in the mechanism by which phenobarbital and TCPOBOP act. This idea is further strengthened in rat and guinea pig liver where phenobarbital induces CYP enzymatic activities, but TCPOBOP evokes no response (Poland *et al.*, 1981). Additional indications of multiple mechanisms of induction by phenobarbital and phenobarbital-like drugs comes from the work of Schuetz *et al* (1986) who have studied the effect of polychlorinated biphenyls on the levels of CYP2B1/2B2 and CYP3A1 proteins, both of which are induced by phenobarbital. It was found that biphenyl isomers, with two ortho chlorinated positions, maximally induced CYP2B1/2B2 whereas isomers with three or four ortho chlorines maximally induced CYP3A1. Further support for multiple mechanisms by phenobarbital and phenobarbital-like drugs comes from a report demonstrating in chick embryo primary hepatocytes that while phenobarbital and AIA are both able to induce a 50 kD CYP, only phenobarbital is able to induce a 54.5 kD CYP (Oron and Bar-Nun, 1984). The precise mechanism(s) by which phenobarbital and phenobarbital-like drugs trigger an increase in the synthesis of CYP proteins and other gene products remains to be solved.

### d. Transcriptional regulation of the glucocorticoid-inducible CYPs

The prototypic inducer belonging to the glucocorticoid group of inducers is pregnenolone-16α-carbonitrile (PCN) and classically members of the CYP3 family induced by this compound have been referred to as the "PCN-inducible" CYPs. Another more potent synthetic steroid which functions similarly to induce this class of CYPs is dexamethasone. Not all members of the CYP3 family induce with PCN or dexamethasone. For instance, in rat liver, CYP3A1 is induced by PCN and dexamethasone, but CYP3A2 is not, while both of these enzymes are induced by phenobarbital (Gonzalez *et al.*, 1986). This is another example of CYP proteins with very similar protein sequence, but which are regulated differently within certain tissues.

Little is known about the pathway whereby CYP3A1 and other PCN-inducible CYPs are induced transcriptionally by PCN and other glucocorticoids. What is clear however, is that two distinctly different mechanisms of glucocorticoid-induced gene transcription exist. The mechanism by which PCN induces transcription will be referred to as the PCN-type receptor pathway which differs from that of the classical glucocorticoid receptor pathway (Schuetz *et al.*, 1984; Schuetz and Guzelian, 1984). While dexamethasone is able to act positively via both mechanisms, PCN acts positively via the PCN-type receptor mechanism, but is an antagonist to the classical glucocorticoid receptor mechanism. A dose of dexamethasone approximately 200 times greater than that needed for induction of tyrosine aminotransferase via the glucocorticoid receptor mechanism is required for PCN-type-induction of CYP3A1. This may reflect the difference in avidities of the respective receptors for dexamethasone. *CYP3A1* is the only PCN-inducible gene that has been conclusively shown to be activated transcriptionally by the PCN-type receptor mechanism.

With regard to the classical glucocorticoid receptor pathway, a putative glucocorticoid-responsive element (GRE) has been located in the *CYP2B2* gene 5' flanking region (Jaiswal *et al.*, 1987) and this element in isolation from the rest of the *CYP2B2* promoter confers dexamethasone-responsiveness to a heterologous promoter (Jaiswal *et al.*, 1990). Whether this element is important in the regulation of *CYP2B2* gene transcription remains to be seen. Evidence that this GRE does play some role, comes from reports by Simmons *et al* (1987) and Rao *et al* (1990) who, using a cDNA probe that detects both

CYP2B1 and CYP2B2 transcripts, demonstrated that transcription of these genes was actually decreased by dexamethasone. There have been a number of reports demonstrating that "GRE-like" sequences may confer repression to a promoter rather than activation (Langer and Ostrowski, 1988; Oro *et al.*, 1988; Sakai *et al.*, 1988). It is not known if a glucocorticoid-responsive element exists to regulate the *CYP2B1* gene, which is highly similar to the *CYP2B2* gene both in cDNA sequence and in the patterns of expression (Srivastava *et al.*, 1989a). In the TCDD-inducible *CYP1A1* gene, a glucocorticoid response element, located in the first intron, has been shown by transient transfection studies to be important in potentiating the inductive effect of the TCDD-like drug 1,2-benzanthracene (Mathis *et al.*, 1989).

Dexamethasone has been implicated in the regulation of CYPs and other proteins at many different levels other than that of transcription and for simplicity, these other levels of control will be discussed in this section. For instance, in rat liver, dexamethasone and PCN induce the rate of transcription of the *CYP3A1* gene as mentioned previously, but in addition, these glucocorticoids are involved in stabilisation of the CYP3A1 primary transcripts and mRNA (Simmons *et al.*, 1987). A complex regulation of the "phenobarbital-inducible" CYP2B1/2B2 occurs in rat liver. Reports by Simmons *et al* (1987) and Rao *et al* (1990) demonstrate that dexamethasone results in a decrease in the basal and phenobarbital-induced transcription of *CYP2B1/2B2* as mentioned above. In similar experiments, basal levels of CYP2B1/2B2 mRNA levels were substantially increased by dexamethasone and similarly phenobarbital-induced levels of mRNA were further induced by dexamethasone suggesting a dexamethasone-mediated stabilisation of either primary transcripts or mature mRNA. Rao *et al* (1990) report that increased levels of CYP2B1/2B2 mRNA, mediated by dexamethasone, are due primarily to a stabilisation of primary nuclear transcripts while the results of Simmons *et al* (1987) suggest a stabilisation of polyribosomal mRNAs. These conflicting results remain to be resolved. Finally, in this group of experiments (Simmons *et al.*, 1987; Rao *et al.*, 1990), despite increased mRNA levels, the overall effect of dexamethasone was to slightly decrease both basal and phenobarbital-induced protein levels of at least CYP2B1 and possibly also CYP2B2. This indicates that dexamethasone is either repressing translation or destabilising the proteins of these CYPs. One of the primary

biological effects of glucocorticoids is to stimulate gluconeogenesis at the expense of proteins, and glucocorticoid-induced degradation of rat hepatocyte proteins has been reported (Hopgood *et al.*, 1981). The mechanism involved in this glucocorticoid-modulated degradative process has not been determined. Curiously, in contrast to the decreased levels of CYP2B1 protein following dexamethasone treatment, as measured by immunotechniques in rat liver (Simmons *et al.*, 1987; Rao *et al.*, 1990), dexamethasone alone has been reported to induce CYP2B1/2B2 protein levels by 20 to 100-fold (Yamazoe *et al.*, 1987). In a separate report, Waxman *et al* (1990) demonstrated, in rat primary hepatocytes, that phenobarbital-induced steroid 16ß-hydroxylase activity (a CYP2B1-dependent activity) is potentiated by dexamethasone, while dexamethasone alone is not an effective inducing agent. The reason for these apparently conflicting results remains unclear.

In the expression of CYP1A2, dexamethasone potentiates the 3-methylcholanthrene-induced accumulation of mRNA, but in this case has no effect on mRNA accumulation in the absence of inducing drug (Silver and Krauter, 1990). Dexamethasone has been implicated in mRNA stabilisation of various other proteins influence by glucocorticoids (Brock and Shapiro, 1983; Jefferson *et al.*, 1984; Vannice *et al.*, 1984; Diamond and Goodman, 1985; Wiener *et al.*, 1987).

Overall, glucocorticoids mediate a complex set of primary and secondary responses. These include transcriptional activation (Mathis *et al.*, 1989, and references therein) and repression (Commer *et al.*, 1979), alterations of mRNA turnover (Simmons *et al.*, 1987), mRNA processing (Vannice *et al.*, 1984) and mRNA nucleocytoplasmic transport (Fulton and Birnie, 1985) and alterations in protein processing (Karlksen *et al.*, 1986) and protein trafficking (Sheperd *et al.*, 1985). The exact roles of dexamethasone and other glucocorticoids in expression of CYP protein products have not been fully determined, but it is clear that they involve multiple levels of control and play a very important role.

### e. Transcriptional regulation of the peroxisome proliferator-inducible CYPs

Peroxisome proliferators are a diverse group of compounds including hypolipidaemic drugs, herbicides and plasticisers which, in rodents, lead to a dramatic proliferation of

hepatic peroxisomes (Styles *et al.*, 1988; Gibson, 1990; Lock *et al.*, 1989). Historically, a number of CYPs have been shown to be induced by the hypolipidaemic drug clofibrate and were therefore termed the "clofibrate-inducible" CYPs. These CYPs have since been shown also to be inducible by other peroxisome proliferators. To date, the majority of these CYPs have similar amino acid sequences and belong to the *CYP4* gene family. The most widely studied member of this family is the rat *CYP4A1*, the enzyme which is involved in the hydroxylation of fatty acids including lauric acid and arachidonic acid (Bains *et al.*, 1985). CYP4A1 mRNA levels are induced by clofibrate in rat liver and kidney and this is a result of an increased rate of transcription of the gene occurring within 1 h of administration of the drug (Hardwick *et al.*, 1987).

As stated in the previous section, the phenobarbital-inducible CYPs are induced by a variety of drugs, many of which differ greatly in their structural and chemical properties and hence it is difficult to envisage a single receptor that would accommodate them (although, of course, the CYP enzymes themselves obviously achieve this). Similarly, the peroxisome proliferators differ quite widely in their structural and chemical characteristics (Sharma *et al.*, 1988). One model to explain how these diverse drugs could specifically induce the *CYP4A1* gene was proposed by Lock *et al* (1989). Their proposal was based on work showing that addition of medium chain fatty acids ($C_6$-$C_8$) to cultured hepatocytes, or high fat diets given to rats led to an induction of CYP4A1. In brief, peroxisomal proliferator drugs act directly via one of a number of possible mechanisms to inhibit the action of the mitochondrial ß-oxidation enzyme which acts specifically on medium chain fatty acids. This results, according to the model, in an accumulation of cellular medium chain fatty acids which, in turn, act as the common inducing agent to induce CYP4A1 levels. CYP4A1 enzyme can subsequently oxidise these fatty acids to maintain lipid homeostasis. The model is attractive in that it would be an example of a regulatory system in which a substrate is involved in the control of gene expression in a feed-back type mechanism.

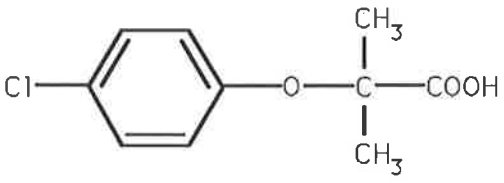
A finding of extreme importance in the understanding of the mechanism of gene activation by peroxisome proliferators has been published recently. Issemann and Green (1990) reported the isolation of a cDNA clone encoding a protein of size 52.4 kD, which has been called the mouse peroxisome proliferator activated receptor (PPAR). Analysis of

the amino acid sequence of this protein clearly demonstrated its similarity to members of the nuclear receptor superfamily (including glucocorticoid receptor, oestrogen receptor, retinoic acid receptor, thyroid hormone receptor and vitamin D3 receptor). It was shown that this protein acts specifically as a receptor for peroxisome proliferator drugs and not for the hormones of the other nuclear receptor superfamily members, or for drugs that induce other classes of CYPs. Furthermore, a correlation has been shown between the rank order of peroxisome proliferator drugs for activation of this receptor protein in transient expression studies and both their degree of carcinogenicity in rodents (Reddy and Lalwani, 1983) and induction of peroxisome proliferator-responsive genes (Mitchell *et al.*, 1984). This evidence strongly points to PPAR as the receptor that mediates the effect of peroxisome proliferators. Whether this receptor can specifically bind this broad range of structurally diverse drugs remains to be seen. It has not been ruled out that these drugs act via an endogenous ligand for this receptor, or via a common alteration of the receptor such as by phosphorylation (Issemann and Green, 1990). Analysis of the protein sequence of PPAR has revealed a region corresponding to the DNA binding domain which is conserved within members of the nuclear receptor superfamily. It has therefore been predicted that the DNA *cis* -acting element to which PPAR is expected to bind will contain, within it, the DNA sequence TGACC (Issemann and Green, 1990).

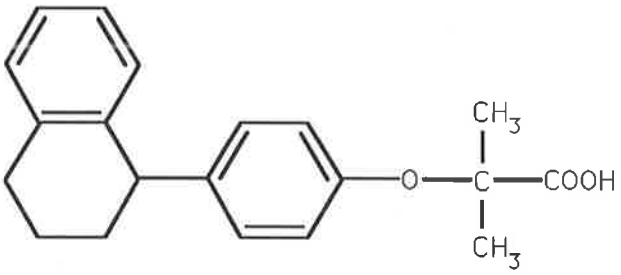
The cloning of the cDNA for the PPAR will be an important step in obtaining a clearer understanding of triglyceride and cholesterol homeostasis, of how peroxisome proliferators act as hypolipidaemic drugs and of the role these drugs play in carcinogenesis in rodents (and possibly humans). The role, if any, of CYPs in these events will also be of interest. Finally, the mechanism by which PPAR is able to be activated by such a broad range of structurally diverse compounds will be of extreme interest, especially in relation to investigation of the mechanism by which phenobarbital and phenobarbital-like drugs (a set of structurally diverse compounds, different from the peroxisome proliferators) are able to induce the phenobarbital-inducible *CYPs* and other phenobarbital-inducible genes (see section 1.2.C.1.c).

**Figure 1.3. Chemical structures of the peroxisome proliferator compounds**

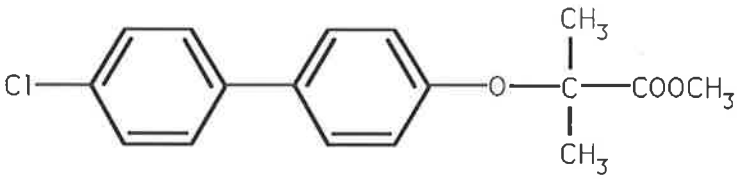
The peroxisome proliferator compounds vary in their chemical structures, but all lead to a proliferation of peroxisomes in liver cells.



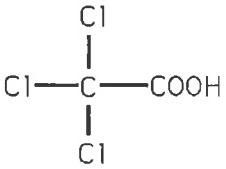
Clofibric Acid



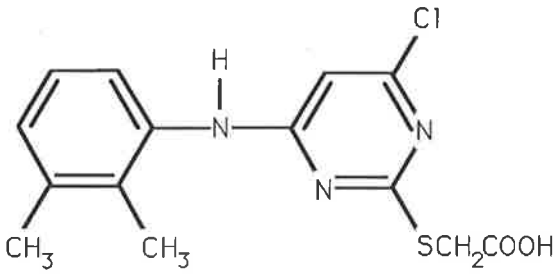
Nafenopin



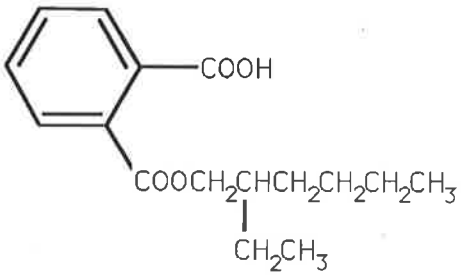
Methylclofenapate



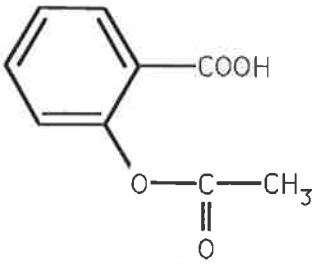
Trichloroacetic Acid
(TCA)



Wy-14,643



Mono-ethylhexylphthalate
(MEHP)



Aspirin

### f. Transcriptional regulation of the cAMP-responsive CYPs

The steroid hydroxylase genes are expressed in steroidogenic tissues and their protein products are CYPs which catalyse hydroxylation reactions in the biosynthesis of steroid hormones including glucocorticoids and mineralocorticoids in the adrenal cortex and sex hormones in the testis and ovary (reviewed by Waterman *et al.*, 1988; 1990a). The four steroid hydroxylase genes, *CYP11A1* ($P450_{scc}$), *CYP11B1* ($P450_{11\beta}$), *CYP17* ($P450_{17\alpha}$) and *CYP21A1* ($P450_{C21}$) are expressed in adrenal cortex, while only *CYP11A1* and *CYP17* are expressed in testis and ovary. CYP11A1 and CYP11B1 are mitochondrial CYPs and therefore also require the flavoprotein adrenodoxin reductase and an iron-sulfur protein adrenodoxin for reducing capacity and hence function. CYP17 and CYP21A1 are microsomal CYPs and subsequently receive reducing equivalents from the microsomal flavoprotein, NADPH-cytochrome P450 reductase.

The levels of all four of these steroid hydroxylases as well as adrenodoxin are increased in adrenal cortex cells by adrenocorticotropic hormone (ACTH). Follicle stimulating hormone (FSH) and luteinising hormone (LH) increase ovary-specific CYP levels and LH and human chorionic gonadotropin (HCG) increase testis-specific CYP levels in their respective tissues. These peptide hormones bind to specific surface receptors on steroidogenic cells and consequently activate adenyl cyclase leading to an increase in intracellular cyclic AMP (cAMP) levels. cAMP has been shown to act via two distinct mechanisms, an acute response and a chronic response. The acute response occurs within minutes and involves increased synthesis of CYP11A1. Interestingly, analysis of the half-lives of the steroid hydroxylase and adrenodoxin mRNAs, revealed that only CYP11A1 showed an increased stability in response to ACTH (Boggaram *et al.*, 1989). This may account for the rapid response of this CYP to ACTH. CYP11A1 catalyses the hydroxylation and therefore the mobilisation of cholesterol. This is the rate-limiting step of the steroid hormone biosynthetic pathway. The chronic response takes longer to manifest itself (hours) and results in increased synthesis of all steroid hydroxylase enzymes and related enzymes such as adrenodoxin (Waterman *et al.*, 1988). Nuclear transcription run-on experiments in adrenal cortex have demonstrated that cAMP acts to increase the rate of transcription of each of these genes (John *et al.*, 1986). Furthermore, experiments involving cycloheximide

treatment, have led to the proposed existence of labile transcription factors, required for steroid hydroxylase gene transcription, and whose levels are responsive to cAMP. These *trans* -acting factors have been collectively referred to as steroid hydroxylase inducing proteins (SHIP) by Waterman and Simpson (1984). Thus cAMP is the common mediator of external hormonal stimuli for each of these steroid hydroxylase genes. The consensus regulatory elements known to be associated with cAMP-dependent gene transcription are the cyclic AMP-responsive element (CRE - TGACGTCA, Montminy *et al.*, 1986), the activator protein 2 (AP-2) binding site (TCCCCANGCG, Imagawa *et al.*, 1987) and to a lesser extent, the activator protein 1 (AP-1) binding site ($TGA^{C}/_{G}TCA$, Bohmann *et al.*, 1987) which differs from CRE by only one base pair.

The mechanisms by which cAMP regulates genes for the steroid hydroxylases and adrenodoxin is proving to be both complex and interesting. Of these genes, only *CYP11B1* contains a sequence similar to the consensus CRE (Rice *et al.*, 1989). The bovine *CYP17* gene contains two regions within the 5' flanking region (-243 to -225 bp and -76 to -40 bp) that confer cAMP-responsiveness to a heterologous promoter; neither of these sequences is similar to the consensus CRE, but the most distal binds a 47 kD nuclear protein that is competed by oligonucleotides containing a consensus CRE from the human chorionic gonadotropin α (HCGα) gene (Lund *et al.*, 1990). While the region -243 to -225 bp of the *CYP17* gene binds a single 47 kD nuclear protein, the HCGα consensus CRE has been shown to bind three adrenal nuclear proteins of size 68 kD, 47 kD (presumably the same protein as binds to the region -243 to -225 bp in the *CYP17* gene promoter) and 43 kD (presumably the CRE binding protein - CREB, Montminy and Bilezikjian, 1987). Thus multiple proteins are able to bind to sequences which are known to confer responsiveness to cAMP. Whether all of these proteins confer the response to cAMP is not clear. The most proximal region of the *CYP17* gene promoter (-76 to -40 bp), is quite unrelated to the distal region in sequence and protein-binding properties and appears to bind two distinct proteins for optimal response to cAMP (Waterman *et al.*, 1990b).

The bovine *CYP11A1* gene contains one region responsive to cAMP (-183 to -83 bp). While there are no sequences similar to the consensus CRE, this region does contain two motifs with a 6 out of 7 match to the binding site for AP-1 (Ahlgren *et al.*, 1990).

Whether these, or other sequences, are important for cAMP-dependent activation remains to be seen. A 14 bp sequence (-126 to -113 bp) of the human *CYP21A1* gene 5' flanking region has been isolated as a cAMP-responsive region and bears no similarity to any of the known cAMP-responsive elements (Kagawa and Waterman, 1990). An adrenal-specific nuclear protein binds this region, which contains no similarity to the other cAMP-reponsive regions of the *CYP11A1* or *CYP17* genes. The presence of a consensus GC box sequence which binds the transcription factor Sp1 (Kadonaga *et al.*, 1987), may play some role in the response to cAMP conferred by this region. Finally, the bovine adrenodoxin gene contains two cAMP-responsive regions, one in the 5' flanking region (-111 to -43 bp) and the other in intron 1 (+687 to +745 bp). Neither of these sequences appears to bear significant similarity to either the CRE, AP-2 binding site, AP-1 binding site, or the cAMP-responsive regions of any of the steroid hydroxylase genes (Waterman *et al.*, 1990b). Interestingly however, the intronic sequence contains three putative Sp1 binding sites. A possible regulatory correlation between these GC boxes and the GC box of the *CYP21A1* gene cAMP-responsive region remains to be investigated.

From this minireview of cAMP-mediated regulation of the steroid hydroxylase genes, it appears that cAMP acts via an increase in the synthesis of numerous transcription factors (steroid hydroxylase inducing proteins - SHIP) as well as via the classic CRE-mediated pathway. A clearer picture of the mechanism by which the cAMP-responsive CYPs (*i.e.* steroid hydroxylases) are induced by cAMP awaits the purification of the specific SHIP and an understanding of their regulatory mechanisms.

### *1.2.C.2. Mechanisms of regulation of CYPs at levels other than transcription*

#### a. Alternative expression of CYPs due to gene polymorphism

An inherited mutation within a gene may lead to an altered or non-functional protein. Mutations within the regulatory regions of a gene may however, result in altered levels of basal or induced transcription rates and hence altered protein levels. These inherited differences between individuals are called polymorphisms. In rodents, polymorphisms in the Ah receptor locus have been correlated with variations in inducibility of *CYP1A1*.

Polymorphisms have been shown to exist in humans for the *CYP2C*, *CYP2D*, *CYP3A* and *CYP21A* subfamilies (Kleinbloesem *et al.*, 1984; Inaba *et al.*, 1986; Knodell *et al.*, 1987; Donohoue *et al.*, 1988; Gonzalez *et al.*, 1988). The most studied of the *CYP* polymorphisms is that associated with *CYP2D6* and the hypotensive ß-adrenergic blocker, debrisoquine. Most individuals have the ability to hydroxylate debrisoquine and have been called "extensive metabolisers" (EM phenotype), while certain individuals who have markedly reduced capacity for debrisoquine hydroxylation are called "poor metabolisers" (PM phenotype). This defective polymorphism has been shown to result from various small deletions and insertions of the gene resulting in frame shifts, some of which disrupt the coding sequence and some which lead to a premature stop codon (Gough *et al.*, 1990). Almost a complete absence of CYP2D6 protein in the liver has been reported in some individuals (Gonzalez *et al.*, 1988). This polymorphism is of importance as the prescribed dosage of at least 26 clinically used drugs differs for EM and PM individuals (Meyer *et al.*, 1990). The EM phenotype has also been linked to a higher incidence of lung cancer in smokers (Caporaso *et al.*, 1989) and also bladder cancer (Kaisary *et al.*, 1987).

### b. Regulation of CYPs by gene activation

Gene activation is the process which occurs upon commitment and differentiation of cells during development of an organism and involves the decondensation of inactive chromatin to form active euchromatin. The mechanism(s) by which this takes place is by no means fully understood, but a combination of gene-specific or locus-specific cytosine demethylation events and the selective binding of activating proteins to the DNA appears to be important (reviewed by Cedar, 1988; Solter, 1988). This process is also known as genomic imprinting. In general, genes which do not express within a cell are hypermethylated and those which do, are markedly demethylated. Many liver-specific genes are hypermethylated in the foetal stage, and after birth become demethylated, which coincides with activation of these genes. The *CYP2E1* gene (inducible by ethanol, acetone and imidazole) follows this pattern of coincidental demethylation and developmental stage-specific gene activation (Umeno *et al.*, 1988).

More recently, convincing evidence relating demethylation with gene activation has been published by Matsunaga and Gonzalez (1990). In rat, the *CYP2D* subfamily consists of five tandemly arranged genes. Measurement of mRNA levels reveals that one gene *CYP2D5* becomes active within the first week following birth and another *CYP2D3* only becomes active after 2 weeks of age. Methylation studies have shown that while both *CYP2D5* and *CYP2D3* genes are hypermethylated at birth, they become demethylated approximately 1 week and 2 weeks after birth, respectively. Furthermore, only the 5' flanking regions of these genes have been shown to become demethylated, implying the involvement of demethylation in regulation of the gene. It is quite probable that tissue-specific expression and developmental-stage-specific gene expression of other CYPs will also be controlled at this level.

### c. Regulation of CYPs by post-transcriptional mechanisms

Post-transcriptional regulation includes those mechanisms involved in the splicing and stability of primary transcripts, and the transport, stability and translatability of mature mRNA. The mechanisms of control at these levels are not well characterised.

Messenger RNAs with a different coding sequence, stability or translatability can be derived from the same primary transcripts by alternative splicing. The primary transcript of the peroxisome proliferator-inducible *CYP4A1* gene appears to be alternatively spliced in the 3' non-coding region. The result of this effect is not known, other than that both mRNA forms are induced by the appropriate drugs. A phenobarbital-inducible *CYP* gene in rat, *CYP2C6*, produces a primary transcript which is alternatively spliced at the eighth exon (*i.e.* the exon containing coding sequence for the haem binding site) producing identical mRNAs, except for a region of 159 nt. One of these mRNAs encodes CYP2C6 apoprotein, while the other encodes a CYP-like protein lacking the haem binding site and hence would not function as a monooxygenase. The CYP2C6 and aberrant mRNA, and the proteins derived from them, are regulated temporally and both proteins are found bound to microsomal membranes (Kimura *et al.*, 1989). Possible functions, if any, of the aberrant protein are not known. The only reported case of a CYP mRNA which is alternatively spliced within the coding region and that is known to form a possibly functional product is that derived from

the rat phenobarbital-inducible *CYP2B2* gene (Lacroix *et al.*, 1990). In this case, an alternative splicing event inserts an extra 24 nt at the junction between exons 5 and 6. This would lead to a protein with an insertion of 8 amino acids. Using computer assisted modelling, the additional amino acids are predicted not to affect the catalytic site of this CYP although the substrate specificity may be changed. In this regard, a report by Lindberg and Negishi (1989) shows that the substitution of a single amino acid dramatically changes the substrate specificity of the CYP.

Stabilisation of mRNA is a common level of control in CYP expression. As mentioned earlier, dexamethasone has been implicated in the stabilisation of primary transcripts for CYP2B1/2B2 and CYP3A1 and of mRNA for CYP3A1 (for more details, see section 1.2.C.1.d). Dexamethasone and troleandomycin appear important for CYP3A2 mRNA stabilisation (Schuetz *et al.*, 1988). The liganded Ah receptor complex has been implicated in the stabilisation and/or correct processing of the CYP1A2 primary transcript (Silver and Krauter, 1990) while phenobarbital and AIA appear important at a post-transcriptional level for induction of CYP2H1 and CYP2H2 mRNA levels in chick embryo liver (Hansen *et al.*, 1989). Rats induced to become diabetic with streptozotocin display increased CYP2E1 mRNA levels due to an increased stability of the mRNA; treatment of these rats with insulin or testosterone destabilises the CYP2E1 mRNA (Goldfarb, 1990).

The male-specific CYP2C11 ($P450_{16\alpha}$) and female-specific CYP2C12 ($P450_{15\beta}$) are positively regulated by growth hormone in the liver at two levels, the transcriptional level and at a stage preceding translation, but following transcription - presumably at the mRNA stabilisation level. By an as yet unknown mechanism, intermittent administration of growth hormone to hypophysectomised rats (*i.e.* mimicking the male secretory pattern) induces only CYP2C11 mRNA while continuous administration of growth hormone (*i.e.* mimicking the female secretory pattern) induces only CYP2C12 mRNA levels (Mode *et al.*, 1989).

In rat primary hepatocyte cultures, liver-specific mRNAs were shown to be stabilised in serum-free cell culture medium, but not in the presence of serum (Jefferson *et al.*, 1984). Silver and Krauter (1988) have reported rat CYP1A1 mRNA levels to be stabilised to a greater extent in primary hepatocyte cultures than in the rat liver *in vivo*. The different components of the culture medium have also been shown to be important in the levels of

expression of various other CYPs (Steward *et al.*, 1985; Turner *et al.*, 1988; Turner and Pitot, 1989). Whether mRNA stabilisation plays a role in expression of these CYPs *in vivo* remains to be proven. Overall, the mechanisms involved in the stabilisation and destabilisation events of primary transcripts or mRNAs are not well characterised.

To date, there have been no reports of CYPs being regulated at the translation level. However, as mentioned earlier, steroids are involved in the regulation of a number of CYPs at levels including transcriptional activation, primary transcript stabilisation and mRNA stabilisation. Recently, a 9 nt segment (*i.e.* AGAAGACCC) in the 5' untranslated region of the myelin basic protein mRNA has been shown to increase translation of this mRNA in the presence of the steroid, hydrocortisone and to confer to a heterologous mRNA, responsiveness to steroid-induced translation (Verdi and Campagnoni, 1990). Whether the mRNAs of CYPs, known to be regulated by steroids, are also translationally controlled in this way remains to be seen.

### d. Regulation of CYPs by post-translational mechanisms

Control at post-translational levels is becoming more widely recognised as being important for regulation of gene expression. Such levels of control include modifications to alter the activity of proteins, transportation of proteins to functional sites, protein-protein interactions (*e.g.* dimerisation) and protein stabilisation. The following are examples of CYPs whose synthesis is controlled by post-transcriptional mechanisms.

As CYPs are haemoproteins, the availability of haem is clearly an important factor in the production of functional CYP enzymes and as discussed earlier (see section 1.1), induction of CYPs by drugs or hormone-induced cAMP levels results in an increase in haem synthesis, presumably to supply the extra demand for haem by newly synthesised apocytochrome P450s (apoCYPs). Haem also appears necessary to stabilise nascent apoCYP2B1 in response to phenobarbital (Dwarki *et al.*, 1987).

The clearest example of CYP protein stabilisation is found with the rat ethanol-inducible CYP2E1 protein. In rat hepatocyte cultures, glucagon (acting via generation of cAMP and indirectly by activation of protein kinase C) or the cAMP analogue, 8-bromo-cAMP, stimulate phosphorylation of CYP2E1 through cAMP-dependent protein kinase

resulting in the degradation of this CYP protein. Interestingly, the CYP2E1 enzyme is protected from phosphorylation and hence subsequent degradation by ethanol and imidazole (these are inducers and substrates for CYP2E1). The phenobarbital-inducible CYP2B1 and CYP2B2 are similarly phosphorylated by cAMP-dependent protein kinase and at least for CYP2B1, phosphorylation is known to cause a loss of monooxygenase activity (Koch and Waxman, 1989). Addition of phenobarbital to hepatocyte cultures protects CYP2B1 from phosphorylation (Eliasson *et al.*, 1990). Importantly, ethanol and imidazole specifically protect CYP2E1 but not CYP2B1, while phenobarbital specifically protects CYP2B1 but not CYP2E1. Hence, a substrate-specific mechanism exists for regulation of CYP activity and degradation. Other CYPs that are phosphorylated by cAMP-dependent protein kinase include human CYP2C8 and CYP2C9. Phosphorylation by cAMP-dependent protein kinase is competitively inhibited by cytochrome $b_5$, which may be an important factor in maintaining functional CYP enzymes (Epstein *et al.*, 1988).

### 1.2.D. Conclusion

From this review of CYP regulation, it is clear that there are many different strategies for regulating levels of CYP proteins. It is also clear that this superfamily is very complex, containing constitutively expressing genes and inducible genes, many of which are induced by a broad range of compounds. In addition, the CYP enzymes vary greatly in their substrate-specificity; certain CYPs are highly specific, while others have broad and overlapping specificities. While extremely important in detoxification of foreign compounds and in endogenous metabolism of steroids and fatty acids, CYPs are gaining more and more interest with respect to their role in carcinogenesis (see reviews by Guengerich, 1988; Ioannides, 1990).

## 1.3. 5-AMINOLEVULINIC ACID SYNTHASE

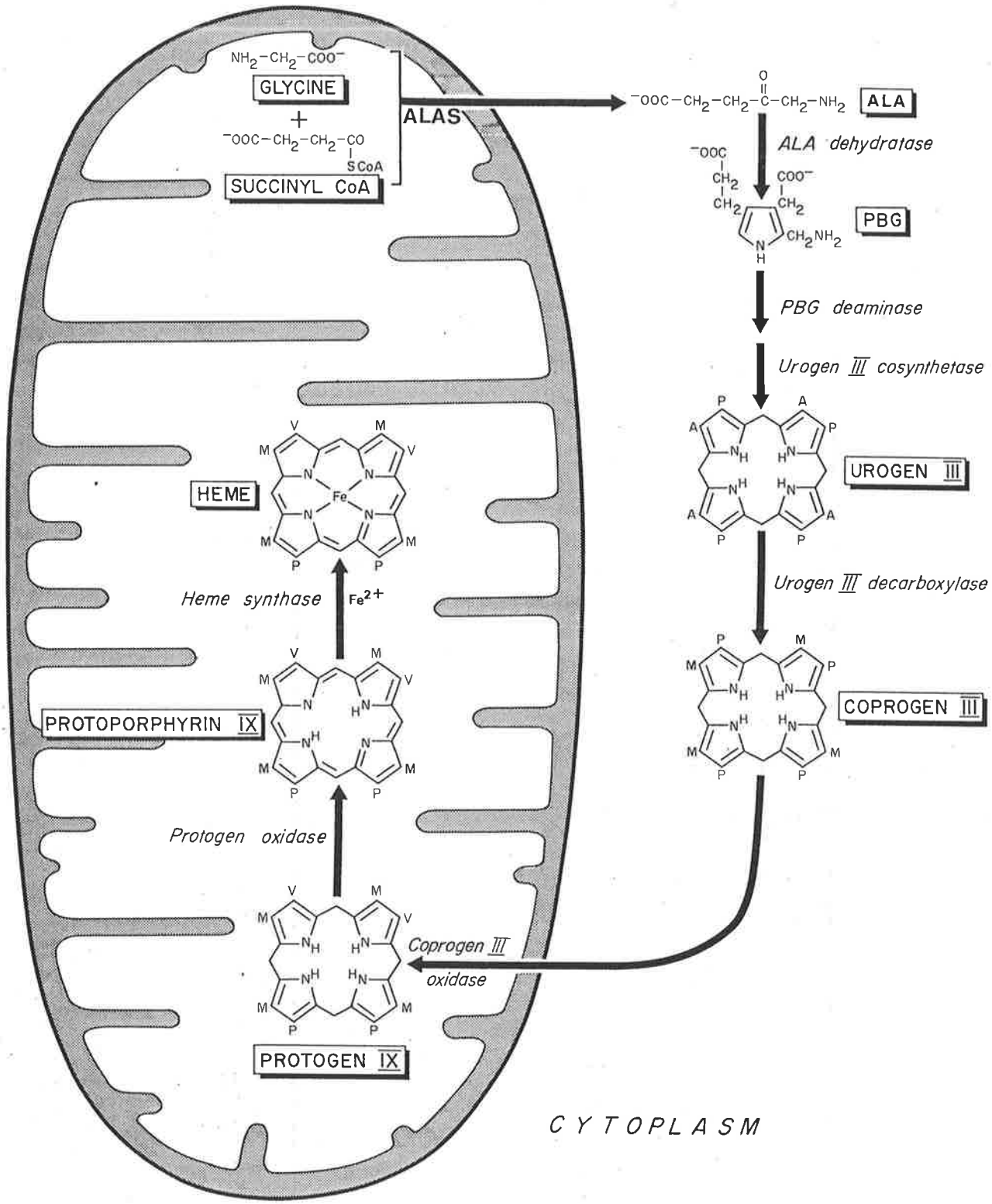
### 1.3.A. Introduction

5-Aminolevulinic acid synthase (ALAS; EC 2.3.1.37) catalyses the first of eight enzymic reactions in the synthesis of haem (see Fig. 1.4). Until recently, it was thought that

**Figure 1.4. The heme biosynthetic pathway**

This flow diagram shows the steps involved in the biosynthesis of heme and the the site in the cell in which each step occurs. The following abbreviations have been used: ALA, 5-aminolevulinic acid; PBG, porphobilinogen; UROGEN, uroporphyrinogen; COPROGEN, coproporphyrinogen; PROTOGEN, protoporphyrinogen; A, acetate; M, methyl; P, propionate; V, vinyl. Heme synthase is often also called ferrochelatase. In this figure, taken from May *et al* (1986), heme is equivalent to haem (used throughout this thesis).

# HEME BIOSYNTHETIC PATHWAY



only one ALAS gene existed and that it was regulated differently in different tissues. However, it is now clear that two genes and two isozymes for ALAS exist, at least in chicken (Borthwick *et al.*, 1985; Riddle *et al.*, 1989), mouse (Schoenhaut and Curtis, 1989; Dierks, 1990) and human (Bawden *et al.*, 1987; Cox *et al.*, 1990). One of these ALAS isozymes is expressed ubiquitously while the other is expressed only in erythroid tissues and these will be referred to as the "house-keeping" form and "erythroid" form of ALAS, respectively. Controlled expression of ALAS is vital in the formation and regulation of haem biosynthesis.

### 1.3.B. Haem and its biosynthesis

Haem consists of a tetrapyrrole ring structure or porphyrin complexed with a centrally located iron atom. Haemoproteins which require haem as an essential prosthetic group for function include the mitochondrial respiratory cytochromes (required for aerobic respiration in animals and plants as well as lower organisms), the superfamily of CYPs (*i.e.* the microsomal monoxygenase system), the oxygen carrying proteins - haemoglobin in the blood system and myoglobin in muscle, and other enzymes such as catalase, peroxidase and tryptophan pyrrolase.

Some of the enzyme-catalysed reactions of the haem biosynthetic pathway as shown in Figure 1.4, occur in mitochondria and others in the cytoplasm. Succinyl CoA and glycine are condensed in the mitochondria by ALAS in the presence of the coenzyme pyridoxal-5'-phosphate, to form 5-aminolevulinic acid (ALA). In the following four reactions which take place in the cytoplasm, two ALA molecules are initially fused by aminolevulinic acid dehydratase (ALAD) to form a pyrrole and then four of these pyrroles are condensed to produce a tetrapyrrole and finally a porphyrin molecule. In the final step, a ferrous ion is inserted into protoporphyrin IX by ferrochelatase (also called haem synthase) to form the functional haem molecule.

In relation to this pathway, the site of action of several compounds used later in this thesis will be mentioned at this stage for ease of reference. 4,6-Dioxoheptanoic acid (or succinyl acetone) is a structural analogue of ALA and upon binding to the reactive site of ALAD (the second enzyme of this pathway), irreversibly inhibits its action (Tschudy *et al.*,

1981). In this way 4,6-dioxoheptanoic acid is able to inhibit haem synthesis. Another inhibitor of haem synthesis is desferrioxamine which specifically and with high avidity chelates $Fe^{3+}$ and, in doing so, inhibits the action of ferrochelatase (Granick *et al.*, 1975). The "phenobarbital-like" drugs (see Fig. 1.2) AIA and DDC may also lead to reduced cellular levels of haem in the liver and in other tissues in which the phenobarbital-inducible CYPs are expressed (reviewed by Ortiz de Montellano and Correia, 1983). In brief, these drugs act as "suicide substrates", binding to the CYP active site and in the process of being metabolised, alkylate the haem moiety of the CYP enzyme. This results in inactivation of the CYP and a release of the alkylated haem. The remaining apoCYP can be functionally reconstituted with haem. However, some of the alkylated forms of haem (*e.g.* N-methylprotoporphyrin; formed by reaction with DDC), being structural analogues of protoporphyrin IX, are known to inhibit the synthesis of haem by competitively inhibiting ferrochelatase. In contrast, to inhibitors of haem synthesis, several compounds result in an increased production of haem. As will be discussed later in this thesis, a multitude of drugs that are known to induce CYPs in the liver and hormones that induce CYPs in the steroidogenic tissues, also result in an increased production of haem via induction of the levels of mitochondrial ALAS. As ALAS is the rate-limiting enzyme of the haem biosynthetic pathway (May *et al.*, 1986), direct addition of exogenous ALA bypasses this rate-limiting step. Administration of ALA to hepatocytes increases the synthesis of haem in a dose-dependent manner (Bonkowsky *et al.*, 1980).

### 1.3.C. Two genes exist for ALAS

The regulation of ALAS is fascinating in that expression of active enzymatic ALAS proteins appears to be closely co-ordinated with expression of various, functionally quite different, genes. As mentioned, two distinct ALAS genes exist, the house-keeping ALAS gene which is expressed in all cells and the erythroid ALAS gene which is specifically expressed in erythroid cells. The house-keeping form of ALAS is involved in the supply of haem for the cytochromes involved in oxidative phosphorylation, which occurs in all respiring cells and for the enzymes catalase, peroxidase and tryptophan pyrrolase. In addition, this enzyme is also required in certain tissues to supply often quite large amounts

of extra haem for CYP production. The erythroid form of ALAS is an important enzyme in the control of haem biosynthesis in erythroid cells. Increased expression is co-ordinated with increased synthesis of the α-globin and the ß-globin family of polypeptide chains, resulting in the formation of various forms of haemoglobin during embryonic, foetal and adult stages of development and during the process of stem cell differentiation in the bone marrow. Regulation of the house-keeping form of ALAS will be dealt with in some detail because of its relevance to the work in this thesis. Although control of expression of the erythroid form of ALAS is not directly related to work in this thesis, a brief overview will be given.

### *1.3.C.1. Regulation of hepatic ALAS synthesis*

It is clear that in the liver, ALAS is the rate-controlling enzyme of the haem biosynthetic pathway and therefore its level reflects the requirement of the cell for haem (May *et al.*, 1986). The liver is the major organ involved in detoxification of foreign compounds, as mentioned earlier, and contains a multitude of CYP enzymes whose expression may be modulated. Hepatic levels of ALAS are known to be increased by administration to animals of drugs and other foreign chemicals which induce CYPs. Presumably this is to supply extra haem for these CYPs. The supply of exogenous haem in the form of haemin (*i.e.* ferriprotoporphyrin) to animals such as rats (Srivastava *et al.*, 1988; Yamamoto *et al.*, 1988) or to chick embryo primary hepatocyte cultures (Granick, 1966; Granick *et al.*, 1975) results in a reduction of the magnitude of drug-induced ALAS levels. It is now generally accepted that haem, the end-product of the haem biosynthetic pathway, exerts a negative feed-back or end-product repression on expression of the first enzyme of the pathway, ALAS. Evidence exists that this repression occurs at several stages in the expression of ALAS (May *et al.*, 1986). The vast majority of work on ALAS regulation has been performed in adult rat liver, chick embryo liver and chick embryo primary hepatocyte cultures.

### a. Transcriptional control of hepatic ALAS

Administration of the drugs AIA and phenobarbital to adult rats results in a marked increase in the rate of transcription of the house-keeping ALAS gene in liver. Injections of haemin or its precursor ALA, leads to a reduction in both basal and drug-induced transcription rates of the ALAS gene (Srivastava *et al.*, 1988; 1990). In chick embryos, as in adult rats, AIA and phenobarbital induce the level of ALAS mRNA and this has been shown to be due almost entirely to an increase in the transcription rate (Hansen *et al.*, 1989). The effect of haem on transcription has not been directly studied in chick embryos.

Little is known about the mechanisms by which drugs enhance transcription rates and by which haem represses them. It remains to be determined whether drugs and haem act via completely different *cis* -acting sequences or whether haem is able to negatively modulate the inductive process of drugs in a direct fashion or via a process involving a haem-dependent protein.

### b. Post-transcriptional control of hepatic ALAS

As mentioned earlier, post-transcriptional regulation can include mechanisms involving splicing and stability of primary transcripts as well as transport, stablility and translatability of mature mRNA. Very little is known about control at these stages of ALAS expression. In adult rat liver, Yamamoto *et al* (1988) have determined the half-life of ALAS mRNA to be 20 min in the presence or absence of administered haemin. Since the commencement of work on this thesis, Drew and Ades (1989) have reported that in primary cultures of chick embryo hepatocytes ALAS mRNA has a half-life of 220 min, which is reduced to only 80 min upon addition of haemin to the culture medium. Hence in chick embryo primary hepatocyte cultures, but not in adult rat liver, ALAS mRNA is apparently destabilised by the presence of haem. The mechanism of this destabilising effect and the difference between the regulation of these two systems remains to be investigated.

There is no evidence for translational control of the house-keeping form of ALAS, although the erythroid form of ALAS appears to be critically regulated at this stage (see section 1.3.B.3).

### c. Post-translational control of hepatic ALAS

ALAS is encoded by a nuclear gene and translated on cytoplasmic polysomes to produce a 74 kD precursor. The enzyme functions within the mitochondrial matrix as a 68 kD enzyme (Srivastava *et al.*, 1983; Borthwick *et al.*, 1985). Hayashi *et al* (1972) first postulated that haem blocks the transfer of ALAS into mitochondria. Later, Ades (1983) showed in chick embryo primary hepatocytes that haem blocks the processing of the ALAS precursor. It is now known, at least in chick embryo liver, that haem inhibits the transfer of the ALAS precursor from the cytosol into the mitochondrial matrix and that this block is specific for ALAS and is not a general effect on the mitochondrial transport mechanism (Hayashi *et al.*, 1983; Srivastava *et al.*, 1983). The mechanism by which haem inhibits mitochondrial transport of the ALAS precursor is unknown. There is however, evidence that mitochondrial proteins are required to unfold, at least partially, during transport through the mitochondrial membrane for assembly in the matrix (Eilers and Schatz, 1988; Silver and Goodson, 1989) and that this unfolding process is facilitated by either a cytosolic or mitochondrial protein factor (Pfanner *et al.*, 1987). One can speculate then, that haem either directly or through a haem-modulated factor specifically inhibits the precursor of ALAS from unfolding and/or from binding to the mitochondrial membrane. The exact mechanism awaits clarification.

The role of haem in modulating the activity of the ALAS enzyme has been proposed (Scholnick *et al.*, 1972; Hayashi *et al.*, 1983). However, Pirola *et al* (1984) have clearly demonstrated that the activity of ALAS enzyme, purified to homogeneity, is not inhibited by the addition of haem or haemin. Despite this result, it is still possible that haem in conjunction with other mitochondrial components can affect the catalytic rate of mitochondrial ALAS *in vivo*.

The half-life of the mature mitochondrial ALAS protein in adult rat liver and chick embryo liver has been reported to be 35 min (Hayashi *et al.*, 1980) and 120 min (Ades *et al.*, 1983), respectively, which is considerably shorter than the average estimated half-life of the majority of mitochondrial proteins of several days (Druyan *et al.*, 1969). The reason for this instability of ALAS active enzyme and also for that of the ALAS mRNA is presumably to ensure a rapid response to negative regulatory signals that are required to maintain tight

control of the production of haem (Pirola *et al.*, 1984). To date, there is no evidence for haem regulating the rate of ALAS protein degradation.

### *1.3.C.2. Regulation of extra-hepatic ALAS synthesis*

In this section, the regulation of ALAS will be discussed in extra-hepatic tissues excluding erythroid tissues which are dealt with in the next section (see section 1.3.B.3).

Srivastava *et al* (1988, 1989a) have shown in the adult rat, that mRNA for the house-keeping form of ALAS is detectable not only in liver, but also in kidney, heart, lung, anaemic spleen, testis and brain. Of these tissues, the drugs phenobarbital and AIA were able to induce ALAS mRNA levels only in liver and kidney, which correlated with induction of the phenobarbital-inducible CYPs, CYP2B1 and CYP2B2 (Srivastava *et al.*, 1989a). Administration of the haem precursor ALA, resulted in a repression of the basal levels of ALAS mRNA in all tissues examined and a reduction of the drug-induced ALAS mRNA levels in liver and kidney. It therefore appears that end-product repression by haem on ALAS is a mechanism which occurs in many different tissues of the rat.

Experiments performed in the adult chicken revealed that AIA induces the level of ALAS mRNA in liver, kidney and small intestine and, curiously, reduces the ALAS mRNA level in lung (Hansen *et al.*, 1989). In a manner similar to that in the rat, the AIA-modulated levels of mRNA for the chicken phenobarbital-inducible CYPs, CYP2H1 and CYP2H2, correlated with those for ALAS. It remains to be determined whether drugs and haem act at the transcriptional level in these extrahepatic tissues.

In the steroidogenic tissues such as adrenal cortex, ovary and testis, hormones are known to increase cellular cAMP levels resulting either directly or indirectly in a transcriptional induction of the steroid hydroxylase *CYP* genes (for more details, see section 1.2.C.1.f). For example, adrenocorticotropic hormone (ACTH) and human chorionic gonadotropin (HCG) regulate the biosynthesis of specific CYPs which are required for the synthesis of the appropriate steroid hormones in the adrenal cortex and testis, respectively (May *et al.*, 1990). Levels of activity of ALAS, believed to be the rate-controlling haem biosynthetic enzyme in endocrine tissues, are similarly increased by ACTH and HCG specifically in rat adrenal cortex and rat testis, respectively (Piper, 1988). Srivastava *et al*

(1988) have shown that the increase in ALAS activity in rat testis by HCG reflects an increase in ALAS mRNA levels specifically in this tissue and that basal and HCG-induced ALAS mRNA levels are repressed by haem. It remains to be seen whether hormone-stimulated induction of ALAS in steroidogenic tissues results from the direct stimulation of the ALAS gene by cAMP-induced transcription factors, as has been proposed for the induction of the steroid hydroxylase CYPs. Alternatively, this induction of ALAS activity may be due to a derepression of ALAS brought about by reduced cellular haem levels as a result of increased production of steroid hydroxylase CYPs.

### *1.3.C.3. Regulation of erythroid ALAS synthesis*

In the human body, approximately 80% of all haem synthesis occurs in erythroid cells with the majority of the remaining 20% occurring in the liver for the supply of the CYPs. Erythroid cells undergo a process of proliferation and differentiation from stem cells containing no detectable haemoglobin, through to fully differentiated erythrocytes in which greater than 95% of the total protein is haemoglobin (Harris and Kellermeyer, 1970; Marks and Rifkind, 1972). In mammals, erythropoietin synthesised in the kidney in response to low oxygen tension in the blood, stimulates erythropoiesis in the bone marrow (Goldberg *et al.*, 1988). As erythroid cells progressively differentiate, the levels of the haem biosynthetic pathway enzymes including ALAS, aminolevulinic acid dehydratase (ALAD), porphobilinogen deaminase (PBGD), uroporphyrinogen synthase (previously called uroporphyrinogen III cosynthetase) and uroporphyrinogen III decarboxylase, are significantly induced (Beaumont *et al.*, 1984; Beru and Goldwasser, 1985). At least for ALAS (Elferink *et al.*, 1988), PBGD (Mignotte *et al.*, 1989) and for uroporphyrinogen III decarboxylase (Romeo *et al.*, 1986), this increase has been shown to be due to transcriptional activation of the respective genes. The onset of induced haem synthesis is necessary for induction of globin synthesis and subsequently haemoglobin formation (Beru *et al.*, 1983).

A study of the regulation of erythroid ALAS expression in differentiating mouse erythroleukaemic cells (MEL cells) has revealed that during differentiation, the increase in the rate of transcription of the ALAS gene and the induced levels of ALAS mRNA are not

subject to end-product repression by haem (Elferink *et al.*, 1988; May and Bawden, 1989). This is in contrast to the situation found with the house-keeping ALAS gene in the liver. However, the amount of ALAS (measured as enzyme activity) was markedly affected by haem. Inhibition of haem synthesis using 4,6-dioxoheptanoic acid (succinyl acetone) caused induction of ALAS activity and this induction was prevented by addition of exogenous haemin (Beaumont *et al.*, 1984; Elferink *et al.*, 1988). Since haem does not affect ALAS mRNA levels (Elferink *et al.*, 1988), and since there is neither evidence that haem inhibits transport of ALAS precursor into the mitochondria of erythroid cells (Elferink *et al.*, 1988; May and Bawden, 1989) nor affects enzymic activity of erythroid ALAS (Bottomley and Smithee, 1968; Ponka and Schulman, 1985), it has been proposed that haem negatively regulates the synthesis of ALAS at the translational level (May and Bawden, 1989). In this way haem, via a negative feed-back mechanism is able to control its own synthesis.

Recent reports from different laboratories have suggested that the 5' untranslated sequence of the human erythroid ALAS mRNA (Cox *et al.*, 1991) and mouse erythroid ALAS mRNA (Dierks *et al.*, 1990) forms a hairpin loop structure with similarity to the iron-responsive elements (IREs) present in the 5' untranslated region of the intracellular iron storage protein, ferritin and in the 3' untranslated region of the cellular iron uptake transporter, transferrin receptor. The IREs of ferritin and transferrin receptor have been shown under conditions of low iron to bind a cytosolic repressor protein (Rouault *et al.*, 1988; Koeller *et al.*, 1989) which inhibits translation of ferritin mRNA (Hentze *et al.*, 1987) and inhibits degradation of transferrin receptor mRNA (Mullner *et al.*, 1989) without affecting its translation. In the presence of elevated iron levels the IRE repressor protein is displaced enabling increased translation of ferritin mRNA and a destabilisation of transferrin receptor mRNA. With regard to erythroid ALAS regulation, the presence of an "IRE-like" domain in the 5' untranslated region, makes it highly probable that, like ferritin mRNA, ALAS will be translationally controlled by cellular iron (May *et al.*, 1990). An IRE-like domain is not found in the rat, chicken or human house-keeping ALAS mRNAs (May *et al.*, 1990).

Exactly how haem negatively regulates its own synthesis through control of ALAS activity levels is not totally clear. There is however, evidence in reticulocytes that haem

inhibits the release of iron from the internalised transferrin/iron complex (Ponka *et al.*, 1988). This would result in reduced "free" cellular iron and increased binding of IRE repressor protein to the putative ALAS IRE with subsequent inhibition of translation of ALAS mRNA. The ultimate outcome would be a reduction in haem biosynthesis (May *et al.*, 1990). Of interest in the latter stage of erythroid differentiation will be the regulatory processes which enable vast amounts of haem and globin to be made until finally, as mentioned earlier, greater than 95% of the protein in the mature erythrocyte is haemoglobin.

For more details on the regulation of expression of the house-keeping and erythroid forms of ALAS, the reader is referred to reviews by May *et al* (1986; 1990) and Dierks (1990).

### 1.3.D. Disorders of haem biosynthesis

An understanding of the regulation of expression of both of the ALAS isozymes is of fundamental as well as medical importance. From a mechanistic viewpoint, the regulation of haem biosynthesis is directly related to the porphyria diseases and the disorder sideroblastic anaemia.

Porphyrias constitute a group of diseases in which the normal biosynthesis of haem has been disturbed by a specific defect or deficiency in one of the enzymes of the pathway beyond ALAS. There are therefore seven different types of porphyria; each type being characterised by a defect of one of the seven enzymes. Of these, three acute porphyrias, namely, acute intermittent porphyria (AIP), hereditary coproporphyria (HCP) and variegate porphyria (VP) are most common and are inherited via an autosomal dominant genetic mechanism. Clinically, the acute porphyrias are characterised by neurological and psychiatric abnormalities. Common symptoms include abdominal pain, vomiting, tachycardia, hypertension and urinary retention. Cutaneous photosensitivity is also a major problem for sufferers of HCP and VP, but not AIP and it is due to an accumulation of porphyrins in the skin (Poh-Fitzpatrick, 1982). In these three disorders, latent periods with no clinical symptoms may be disrupted by acute porphyric attacks, being precipitated by one or more metabolic or environmental factors including systemic illness, certain drugs, steroid

hormones, stress, starvation and menstruation (Kappas *et al.*, 1983; Bottomley and Muller-Eberhard, 1988).

In each of the acute hepatic porphyrias, the activity of the deficient enzyme is approximately 50% of that in the normal situation (McColl *et al.*, 1981). The enzyme deficiency is found in all cells examined, but manifestation of the defect by an accumulation of haem precursors seems to be confined to the liver. Presumably, in the latent phase of the disease, ALAS levels are low and despite an enzyme deficiency further along the pathway, there remains enough activity to cope with the required conversion rate of ALA to haem. However, at least in the case of certain precipitating factors such as drugs, CYPs are induced especially in the liver and, as a result, there is an elevation in the level of ALAS activity. The defective enzyme then becomes the rate-limiting step of the haem biosynthetic pathway resulting in an accumulation of haem-precursors prior to this enzymic block. Elevated levels of porphyrins and other haem precursors, occuring within the liver, are thought to evoke the cellular disturbances and the toxic manifestations of these diseases. Thus, in the porphyrias, while ALAS is not the primary defect of the disease, increased levels of ALAS activity appear to be a major cause of the disease (Yamamoto *et al.*, 1985). It is for this reason that a clear understanding of the regulation of ALAS enzyme levels is of extreme importance in the maintenance and treatment of this group of diseases. For further information on porphyrias, the reader is referred to extensive reviews by Rimington (1985), Hindmarsh (1986), Bottomley and Muller-Eberhard (1988) and Moore (1990).

Sideroblastic anaemia refers to a group of diseases of the erythropoietic system characterised by reduced haemoglobin formation (Bottomley and Muller-Eberhard, 1988). In many cases, the patients have reduced levels of ALAS activity in the bone marrow (Fitzsimmons *et al.*, 1988). For this and other reasons, defective haem biosynthesis is thought to be the major disturbance underlying these forms of anaemia. An interesting correlation has been found between the congenital form of sideroblastic anaemia, which follows an X-linked manner of inheritance (Bottomley, 1980) and the recent localisation of the human erythroid ALAS gene to the X-chromosome at position Xp11.2 (Cox *et al.*, 1990). Whether a defective ALAS gene, leading to a structurally altered protein or a

deficiency of the protein, is the principle cause of the sideroblastic anaemias remains to be seen.

## 1.4. AIMS OF THIS THESIS

One of the major aims of the work in this laboratory is to develop an understanding, at the molecular level, of how CYPs and the house-keeping form of ALAS are induced by drugs, hormones and other stimuli. The mechanism of this control is of fundamental interest and may be medically important in the understanding and management of porphyrias.

To investigate the induction of CYPs and ALAS, the chick embryo system was chosen. This system is attractive since the levels of specific CYPs and ALAS are significantly induced by phenobarbital and "phenobarbital-like" drugs in the liver of chick embryos. In addition, cDNA and genomic clones had been previously isolated for ALAS and two phenobarbital-inducible *CYPs* designated *CYP2H1* and *CYP2H2*. At the time of commencement of this work, cultures of chick embryo primary hepatocytes were the only available *in vitro* culture sytem in which CYP and ALAS protein levels responded to phenobarbital in a manner similar to the *in vivo* situation. This provided the opportunity to investigate, at the gene level, the molecular mechanism of phenobarbital-induction of CYPs and ALAS in a truly homologous system both *in vivo* and *in vitro*.

A focus of the work reported here was an investigation into the level(s) at which phenobarbital (and phenobarbital-like drugs) and haem regulate the synthesis of CYPs and ALAS. Inherent in this study was the measurement of *CYP* and ALAS gene transcription rates and mRNA levels in the chick embryo liver and in primary hepatocyte cultures. An additional aim was to utilise chick embryo primary hepatocytes as a homologous culture system in which to study the regulation of the ALAS gene and the phenobarbital-inducible *CYP2H1* gene. This involved transient expression of chimeric gene constructs containing the ALAS gene promoter or *CYP2H1* gene promoter fused to the bacterial chloramphenicol acetyltransferase (CAT) gene in the primary hepatocyte cultures. Of importance in this work was the search for a *cis* -acting DNA region(s) involved in the transcriptional activation of these genes by phenobarbital. Ultimately, such sequences may be important in unravelling the story of how drugs, like phenobarbital, are able to induce expression of CYPs and

ALAS, proteins which are distinctly different in structure and location in the cell, but which are part of a common biological response.

# CHAPTER 2

## MATERIALS AND METHODS

## 2.1. MATERIALS

### 2.1.A. Chemicals

The following chemicals were obtained from Sigma:

Acrylamide, agarose (type 1), aminolevulinic acid, ampicillin, bisacrylamide (N,N'-methylene-bis-acrylamide), bovine serum albumin (BSA), 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (BCIG), chloramphenicol, coenzyme A, deoxyribonucleotide triphosphates (dNTPs), 4,6-dioxoheptanoic acid (succinyl acetone), dithioerythritol (DTE), dithiothreitol (DTT), DNA (Type III from Salmon testes), ethidium bromide, ethylenediaminetetraacetic acid (EDTA), [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (Hepes), o-nitrophenyl-ß-D-galactopyranoside (ONPG), phenylmethylsulfonyl fluoride (PMSF), ribonucleotide triphosphates (rNTPs), salmon sperm DNA, sodium dodecyl sulfate ($NaDodSO_4$), spermidine trihydrochloride, spermine tetrahydrochloride, Tris-base.

Other reagents were obtained as follows:

Agarose (low melting temperature) and *E.coli* tRNA from Bethesda Research Laboratories (BRL); phenol and polyethylene glycol 6000 from BDH Chemicals; tetracycline from Upjohn Pty. Ltd.; trichloroacetic acid from Univar Pty. Ltd.; N,N,N',N'-tetramethylethenediamine (TEMED) from Tokyo Kasei; RNasin from Promega, Madison, WI, USA; Hemin from Porphyrin Products, Inc., Logan, Utah, U.S.A.

All other chemicals used were of analytical reagent grade or of the highest available purity.

### 2.1.B. Drugs

Sodium phenobarbital was obtained from May and Baker, Ltd. 2-allyl-2-isopropylacetamide (AIA) was a generous gift from Roche, Australia. Dexamethasone (9α-fluoro-16α-methylprednisolone) was purchased from Sigma. Porcine growth hormone was kindly donated by Bresatec Ltd., South Australia.

### 2.1.C. Kits

Kits for nick translation of DNA, oligo-labelling of DNA, dideoxy sequencing of DNA, RNA transcription *in vitro* using SP6 RNA polymerase and 5'-terminal kinasing were purchased from Bresatec Ltd., South Australia.

### 2.1.D. Radionucleotides

[$\alpha$-$^{32}$P]dATP (1800Ci/mmol), [$\alpha$-$^{32}$P]dCTP (1800Ci/mmol), [$\gamma$-$^{32}$P]ATP (2000Ci/mmol) and [$\alpha$-$^{32}$P]UTP (1500Ci/mmol) were purchased from Bresatec Ltd., South Australia. [$^{14}$C]Acetyl Coenzyme A and D-*threo* -[dichloroacetyl-1-$^{14}$C]Chloramphenicol was purchased from Amersham Australia, Sydney, Australia. [$^{3}$H]acetate was purchased from NEN Research Products.

### 2.1.E. Enzymes

All restriction endonucleases, T4 DNA ligase, *E.coli* DNA polymerase I, Klenow fragment of *E.coli* DNA polymerase I , SP6 RNA polymerase, T7 RNA polymerase and T4 polynucleotide kinase were purchased from Bresatec Ltd., South Australia. Other enzymes were purchased from the following sources: collagenase (type IV), DNase I (RNase-free); ß-galactosidase (grade VI), lysozyme, ribonuclease A and ribonuclease T1 from Sigma; calf intestinal alkaline phosphatase and proteinase K from Boeringher Mannheim; *E.coli* chloramphenicol acetyltransferase (CAT) and restriction endonuclease *Stu* I from Pharmacia.

### 2.1.F. DNA vectors, constructs and cDNA clones

pSV2CAT and pBRCAT were kindly supplied by Dr R. Crawford, Howard Florey Institute, Melbourne; pIBI76 was obtained from Integrated Sciences Pty. Ltd., Sydney, Australia; pGEM1 and pCAT™-promoter were purchased from Promega, Madison, WI, USA; pBCSVp-1 was a generous gift from K. Docherty, Department of Medicine, University of Birmingham, Birmingham, UK (Clark *et al.*, 1989); pRSVCAT was kindly provided by G.R. MacGregor, Baylor College of Medicine, Houston, Texas, USA (MacGregor *et al.*, 1987); a chicken ß-actin cDNA clone was a generous gift from S. Dalton, Department of Biochemistry, University of Adelaide, Australia; chicken *CYP2H1* cDNA

clones, pCHP3 and pCHPB15 and the ALAS cDNA clone have been previously described (Borthwick *et al.*, 1984; Hobbs *et al.*, 1986); a rat cDNA clone for *CYP1A1* was generously supplied by S. Dogra, Department of Biochemistry, University of Adelaide, Australia and *CYP3A1* was kindly supplied by F. Gonzalez, National Institutes of Health, Bethesda, Maryland, U.S.A.

### 2.1.G. Bacterial Strains

The following *E.coli* K12 strains were used as hosts for recombinant plasmids:

*E.coli* ED8799 : hsdS($r_{k-}$,$m_{k-}$), metB7, supE (glnV)44, supF (tyrT)58, lacZΔM15 (a gift from Dr S. Clarke, Biotechnology Australia).

*E.coli* BB4 : hsdR514, metB1, supE (glnV)44, supF (tyrT)58, galK2, galT22, trpR55, tonA, ΔlacU169, F'[proAB$^+$, lacI$^q$, lacZΔM15,Tn10 (tet$^r$ )].

*E.coli* XL1-blue : hsdR17, supE (glnV)44, recA1, endA1, gyrA46, thi, relA1, lac$^-$, F'[proAB$^+$, lacI$^q$, lacZΔM15,Tn10 (tet$^r$ )].

For explanation of terms used, see a review on the linkage map of *E.coli* K12 by Bachmann (1983).

### 2.1.H. Bacterial Culture Media

All buffers and solutions were prepared in Milli-Q water in glassware carefully rinsed to ensure removal of all detergents. Media and solutions were sterilised by autoclaving.

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 and adjusted to pH7.0 with NaOH. LB agar plates were prepared by the addition of 1.5%(w/v) Bacto-agar (Difco) to the broth. When necessary, agar was supplemented with ampicillin (50 μg/ml) or tetracycline (10 μg/ml) after cooling to 55°C and prior to pouring of the plates.

*E.coli* strain ED8799 was cultured in LB medium while *E.coli* strains BB4 and XL1-blue were cultured in LB medium containing 10 μg/ml tetracycline. Once transformed by plasmids containing the ampicillin resistance gene, all three strains were grown in LB

medium containing 50 µg/ml ampicillin (N.B. in the presence of ampicillin and tetracycline, transformed *E.coli* strains BB4 and XL1-blue grew poorly).

### 2.1.I. Tissue Culture Media

All tissue culture media were made up in water filtered through a Milli-Q Reagent Water System fitted with a Millistak-GS filter and in glassware washed and rinsed to ensure complete removal of detergents. All media were prepared according to the manufacturers instructions and sterilised by filtration through 0.45 µm filters. William's E medium (Williams *et al.*, 1971), Dulbecco's modified eagle's medium (DMEM), Ham's F12 medium and Opti-MEM I were purchased from GIBCO~BRL, Life Technologies, Inc., U.S.A. (N.B. DMEM was modified to contain 20 mM Hepes, 0.45% (w/v) glucose and 0.011% (w/v) sodium pyruvate). Nu-Serum was purchased from Integrated Sciences Pty. Ltd., Sydney, Australia and is a consistent formulation of epidermal growth factor, endothelial cell growth supplement, insulin, transferrin, triiodothyronine, progesterone, oestradiol-17ß, testosterone, hydrocortisone, selenium, *o* -phosphorylethanolamine, glucose, amino acids, vitamins, other nutrients and 25% (v/v) newborn calf serum. ITS$^+$ medium supplement and Matrigel were obtained from Flow Laboratories Australisia Pty. Ltd., Sydney, Australia. Matrigel (200 µl) was spread at 4°C to cover the bottom of a 60 mm diameter petri dish and allowed to gel at room temperature for 30 min. Chick embryo primary hepatocytes were then cultured in medium upon this connective tissue sub-stratum. Gentamicin was purchased from Schering Corp., U.S.A and used at a concentration of 40 µg/ml. Dulbecco's phosphate buffered saline (PBS) was made without magnesium and calcium.

### 2.1.J. 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 10°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.K. General Solutions

SSC - 150 mM NaCl, 15 mM Na citrate, pH7.0

SSPE - 180 mM NaCl, 10 mM $NaH_2PO_4$, 1 mM EDTA pH7.7

TE - 10 mM Tris-HCl pH8.0, 1 mM EDTA

NET - 20 mM Tris-HCl pH7.4, 2 mM EDTA, 200 mM NaCl

Denhardt's solution - 0.2% (w/v) Ficoll, 0.2% (w/v) polyvinylpyrrolidone, 0.2% (w/v) bovine serum albumin (Fraction V)

TBE - 130 mM Tris-HCl pH 8.0, 45 mM boric acid, 2.5 mM EDTA

TAE - 40 mM Tris-HCl pH8.2, 20 mM Na acetate, 1 mM EDTA

3x urea loading buffer - 4.0 M urea, 50% sucrose, 50 mM EDTA, 0.1% bromophenol blue

### 2.1.L. Miscellaneous

Nitrocellulose (BA85) filters were purchased from Schleicher and Schuell; thin layer chromatography silica gel 60 $F_{254}$ sheets from E. Merck, Darmstadt, FRG; GF/A glass fibre filter discs from Whatman Ltd.; OptiScint 'HiSafe' scintillation fluid from Pharmacia; QIAGEN columns from DIAGEN, Dusseldorf, FRG; HETS (high efficiency transfer solution) from Cinna/Biotecx Laboratories Inc., Houston, Texas U.S.A.

Densitometry measurements were performed using a Molecular Dynamics computing densitometer Model 300A and ImageQuant software version 2.0. Spectrophotometric measurements were performed using a Shimadzu (Model UV-160A) UV-Visible Recording Spectrophotometer. Counting of radioactive isotopes was performed using an LKB (Wallac) 1214 Rackbeta Liquid Scintillation Counter. Homogenisation of chick embryo livers was performed using an Ultra-tarrax homogeniser, Janke and Kunkel IKA WERK obtained from John Morris Pty. Ltd. Scientific Instruments, Adelaide, Australia.

## 2.2. GENERAL METHODS

Unless stated otherwise, all recombinant DNA techniques and enzymatic manipulations used in this thesis were performed according to standard procedures as described by Maniatis *et al* (1982).

## 2.3. ISOLATION, ANALYSIS AND MANIPULATION OF DNA

### 2.3.A. Isolation of plasmid DNA

1. Plasmid DNA for analytical restriction digests was prepared by the alkaline lysis method of Birnboim and Doly (1979) from 1.3 ml of overnight culture. For larger amounts of plasmid DNA, the method was scaled up to accommodate 500 ml of culture.

2. Plasmid DNA for use either as an immobilised template in nuclear run-on analysis, as a probe following radiolabelling or in transient transfection studies was prepared by CsCl centrifugation. In brief, plasmid DNA was isolated by the rapid alkaline hydrolysis procedure (see above) followed by two CsCl/ethidium bromide equilibrium density gradients, dialysis with TE buffer and concentration by ethanol precipitation.

3. Plasmid DNA for use in transient transfection studies was also 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.

The CsCl centrifugation and QIAGEN column procedures both resulted in plasmid DNA, the majority of which was supercoiled, and free of detectable RNA, as shown by agarose gel electrophoresis followed by staining with ethidium bromide.

### 2.3.B. Quantification of DNA and RNA

DNA and RNA samples were quantified by spectrophotometry analysis at a wavelength of 260 nm. The concentrations of DNA and RNA in solution were calculated assuming that one absorbance unit corresponds to 50 μg/ml and 40 μg/ml, respectively. As a measure of purity, the $A_{260}/A_{280}$ ratio was calculated and consistently fell within an acceptable range of 1.6-2.0.

The concentration of plasmid DNA for use in transient transfection studies was firstly determined spectrophotometrically and then checked by electrophoresis on a 1% agarose gel followed by ethidium bromide staining. A quantity of 100 ng of each DNA preparation was

analysed to confirm the spectrophotometric result and to ensure a consistent proportion of supercoiled DNA between preparations. Similarly, 1 μg of each DNA preparation was also analysed to check for the presence of contaminating RNA which would lead to a falsely elevated estimation of DNA concentration by spectrophotometric analysis. Only preparations free of visible RNA, similar in quantity as seen on a gel and similar in degree of supercoiling were used for transfections.

## 2.3.C. Cloning and sub-cloning techniques

### *2.3.C.1. Restriction endonuclease digestion of DNA*

DNA (1-2 μg) prepared as outlined in 2.3.A.1, was digested with 2 units of enzyme/restriction site/μg of DNA for 1-2 h using conditions as described by the manufacturer. In the most recent work in this thesis, "Super Duper" buffer consisting of 33 mM Tris-acetate pH7.8, 62.5 mM K acetate, 10 mM Mg acetate, 4 mM spermidine and 0.5 mM dithioerythritol (Dr R. Saint, personal communication) proved suitable for efficient functioning of all restriction endonucleases used.

### *2.3.C.2. Dephosphorylation of vector DNA*

Vectors containing compatible "sticky" ends or "blunt" ends were dephosphorylated with calf intestinal alkaline phosphatase as described by Maniatis *et al* (1982).

### *2.3.C.3. Purification of digested DNA fragments*

All linearised vectors and fragments for cloning were separated on agarose gels in 1x TAE or TBE buffer. The agarose concentration was adjusted to allow for efficient separation of various size fragments. An approximate guide is as follows: 0.7-0.8% agarose for fragments > 10,000 bp; 1.0% agarose for fragments 500-10,000 bp; 2.0% agarose for fragments 200-800 bp; 3.0% agarose for fragments 50-500 bp. Following staining in ethidium bromide solution (1 μg/ml) and illumination with long wavelength UV light, appropriate bands were excised from the gel using a scalpel blade and placed into Eppendorf

tubes. One of two methods was then used for extraction and purification of the DNA from the gel slices.

a. Phenol extraction - This method required separation of the appropriate digested fragments in low melting temperature agarose and is described by Maniatis *et al* (1982) with the exception that gel slices were initially placed into 1 volume of NET buffer instead of 5 volumes of 20 mM Tris-HCl pH8.0 containing 1 mM EDTA.

b. GENECLEAN KIT - GENECLEAN™ and GENECLEAN™II kits were used for extraction and purification of DNA from agarose gels run in TAE and TBE buffer, respectively. The manufacturer's instructions were followed carefully.

Recoveries of >80% were routinely obtained using both procedures and the resultant DNA was amenable to further enzymatic manipulations.

### *2.3.C.4. Quantification of small amounts of DNA following purification*

Following purification by the phenol extraction or either of the GENECLEAN™ methods, a portion of each preparation of DNA was run in parallel with a known amount of SPP1 (*Eco* RI restricted) size markers on a 1% agarose gel. Following staining with ethidium bromide, an approximation of the amount (ng) of purified DNA was made by comparison of the staining intensity with that of the marker bands.

### *2.3.C.5. Ligation of DNA fragments*

Ligation of DNA fragments was performed using T4 DNA ligase in a buffer comprising 50 mM Tris-HCl pH7.6, 10 mM $MgCl_2$, 1 mM DTT and 1 mM ATP in a final volume of 20 µl at 4°C overnight. Ligations containing several vector:insert ratios (ranging from 1:3 to 3:1) were routinely performed to increase the probability of obtaining a favourable ratio for the particular vector and insert. The addition of 5% (w/v) polyethylene glycol 6000 to the ligation reaction (BRL Focus 8.1, 1986), in most cases, greatly increased the efficiency of ligation.

### *2.3.C.6. Transformation of* E.coli *with recombinant plasmids*

*E.coli* strains were transformed by a rapid, modified $CaCl_2$ procedure. Overnight cultures of the appropriate *E.coli* strain were diluted 1/200 (v/v) into fresh medium and incubated with aeration until an $A_{600}$ of 0.3-0.6 was obtained. The culture was then placed on ice for 5 min and the bacteria pelleted by centrifugation at 2000 x g for 5 min at 4°C. The bacterial pellet was resuspended in 1/40 volume of ice cold 50 mM $CaCl_2$, 20 mM $MgCl_2$ and left on ice for at least 60 min to form competent cells. Plasmid DNA (~5 ng) or ligation mix (10 µl) was added to 200 µl of competent cells and incubated on ice for a further 40 min. The cells were heat shocked at 42°C for 2 min and placed immediately back on ice for a further 2 min. LB broth (900 µl) containing 20 mM glucose was added and the cells were then incubated with aeration at 37°C for 45 min. The bacteria were pelleted by centrifugation at 2000 x g for 2 min, resuspended in 150 µl of LB broth and spread using a glass spreader onto LB agar plates containing 50 µl/ml ampicillin. After the media had completely soaked into the agar, the plates were inverted and incubated at 37°C overnight.

### *2.3.C.7. Screening of recombinant clones*

Routinely, 6-12 colonies were chosen for each cloning event. Colonies were removed with a tooth-pick, placed into 2 ml of LB broth containing 50 µg/ml ampicillin and incubated at 37°C with aeration overnight. Plasmid DNA was prepared from 1.3 ml of culture as described in 2.3.A.1.

### *2.3.C.8. Storage of recombinant clones*

Overnight culture (400 µl) was placed into a 1.5 ml eppendorf tube and an equal volume of sterile 80% glycerol was added. Following complete mixing by vortexing, the clones were stored at -80°C.

## 2.3.D. Preparation of $^{32}$P-DNA probes

### *2.3.D.1. Nick translation and oligo-labelling of DNA*

Template DNA (200-500 ng) was labelled with either a nick translation or oligo-labelling kit obtained from Bresatec, Ltd. Following incorporation of [$\alpha$-$^{32}$P]ATP and [$\alpha$-$^{32}$P]CTP, the reactions were stopped by the addition of 5 µl of 0.5 M EDTA pH7.0 and 5 µl of 10% $NaDodSO_4$. To precipitate the $^{32}$P-DNA probe, 50 µl of NET buffer, 5 µl of 10 mg/ml tRNA, 125 µl of 4 M ammonium acetate and 600 µl of ethanol were added. After 10 min at -80°C, the $^{32}$P-DNA probe was pelleted in a Microfuge (12,000 x g) for 15 min. The pellet was washed with 800 µl of 70% ethanol, dried *in vacuo* and redissolved in 194 µl of TE buffer. Immediately prior to hybridisation, the probe DNA was denatured by the addition of 6 µl of 10 M NaOH and incubation at 95°C for 10 min. The probe was then placed on ice for 30 s, neutralised with 200 µl of 4 M anmmonium acetate and immediately added to the hybridisation buffer.

### *2.3.D.2. Labelling of 5' ends of synthetic DNA oligonucleotides*

Synthetic oligonucleotides were labelled at the 5' end in a reaction containing 50 mM Tris-HCl pH7.4, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA, 100 µCi [$\gamma$-$^{32}$P]ATP and 2 units of T4 polynucleotide kinase in a final volume of 20 µl. After incubation at 37°C for 1 h, the reaction mix was electrophoresed on a 20% polyacrylamide gel to separate incorporated from unincorporated label. The band was located by autoradiography for 1 min, excised and the labelled oligonucleotide eluted from the gel in a solution of 0.5 M ammonium acetate, 1 mM EDTA and 0.1% $NaDodSO_4$ at 65°C for 2 h. The probe was used directly in hybridisation reactions.

## 2.3.E. Southern blot analysis

Southern blot analysis was carried out essentially as describe by Maniatis *et al* (1982). Digests of DNA were run in a 1% agarose gel using 1x TBE buffer. Following separation of the DNA fragments and staining with ethidium bromide, the gel was photographed. The gel was then immersed twice in 0.25 M HCl for 10 min, twice in a

solution containing 0.5 M NaOH and 1.0 M NaCl for 10 min, and twice in a solution containing 1.0 M ammonium acetate and 20 mM NaOH for 10 min. Between immersion in the different solutions, the gel was rinsed briefly in water. Transfer of DNA fragments from the gel to nitrocellulose was performed by the method of Southern (1975) in a solution containing 1.0 M ammonium acetate and 20 mM NaOH overnight. After transfer, the filter was allowed to air dry for 30 min and baked *in vacuo* at 80°C for 60 min.

Filters were prehybridised at 52°C for approximately 4 h in a solution containing 5x Denhardt's solution, 5x SSPE pH7.4, 0.1% $NaDodSO_4$, 0.05% sodium pyrophosphate and 200 µg/ml salmon sperm DNA. Hybridisation was carried out under the same conditions for 16-20 h with the inclusion of $^{32}P$-labelled probe (10 ng/ml of solution at a specific activity of 1-2 x $10^8$ cpm/µg). Post-hybridisation washes were carried out using 2x SSC and 0.1% $NaDodSO_4$. An initial wash at room temperature for 30 min was followed by a final wash at 52°C for 30 min. Filters were blotted with Whatman 3MM paper and sealed in polythene bags while still moist. Autoradiography was routinely performed with the aid of one intensifying screen at -80°C until a suitable exposure was obtained.

### 2.3.F. DNA sequencing

DNA sequencing was performed essentially by the dideoxy-sequencing method of Sanger *et al* (1977) using a sequencing kit containing Sequenase Version 2.0, developed by the United States Biochemical Corporation, Cleveland, Ohio, U.S.A., and according to the manufacturers protocol. To sequence double stranded DNA, approximately 2 µg of template was denatured in 0.4 M NaOH at room temperature for 10 min, followed by addition of ammonium acetate to a final concentration of 0.5 M and 5 volumes of ethanol. Following ethanol precipitation, the template DNA was redissolved in 7 µl of water and the annealing and sequencing reactions performed as described in the manufacturers protocol. Two different primers were used in separate reactions to maximise the sequence obtained in pBluescript $KS^+$. The Reverse Primer (AACAGCTATGACCATG) was used to accurately read sequence close to the primer hybridisation site while the KS Primer (CGAGGTCGACGGTATCG; Stratagene) which hybridises within 11 nt of the inserted DNA, was used to derive extended sequence. Sequencing reactions were electrophoresed

on a standard 6% polyacrylamide gel containing 7 M urea. Following this, the gel was fixed with 500 ml of 10% acetic acid, washed with 1 litre of 20% ethanol and dried in a 100°C oven for 40 min. Autoradiography was generally carried out at room temperature overnight.

### 2.3.G. Isolation of single stranded DNA

Single stranded DNA was prepared from pBluescript in the following way. pBluescript clones, transformed in *E.coli* XL1-blue, were grown to a density of approximately $A_{600}$ = 0.5-0.8 at 37°C in LB broth supplemented with 0.001% thiamine and 150 µg/ml ampicillin. A 2 ml aliquot of this culture was then infected with the M13KO7 helper phage at a multiplicity of infection of about 10 (*i.e.* approximately 1.5 x $10^{10}$ plaque forming units). After a 1 h incubation, 400 µl of infected cell culture was transferred into 10 ml of fresh medium containing 70 µg/ml kanamycin and incubated at 37°C for a further 16 h with good aeration. Single stranded DNA was isolated and purified as described for M13 single stranded DNA by Maniatis *et al* (1982).

### 2.3.H. Complementation Test (C-Test)

This test was performed on either approximately 200 ng of purified M13 clone or pBluescript clone single stranded DNA, or directly on an equivalent amount of PEG precipitated M13 clone phage (*i.e.* approximately 1/30 of a 1.2 ml culture of phage). Similar amounts of either single stranded DNA or phage from the two M13 clones to be tested were incubated in 50 mM Tris-HCl pH8.0 containing 0.3 M NaCl, 40 mM EDTA, 10% glycerol, 0.2% $NaDodSO_4$ and 0.004% bromophenol blue in a final volume of 10 µl at 65°C for 45 min. The entire reaction mix was then cooled and run in a 1% agarose gel between lanes containing DNA from each separate clone as markers. Clones of fragments inserted into M13 or pBluescript in the same orientation do not hybridise and run similarly to the markers. Clones of fragments inserted into M13 or pBluescript in the opposite orientation hybridise to form dimers which migrates through the agarose gel more slowly than do the momomeric markers.

## 2.4. ISOLATION AND ANALYSIS OF RNA

### 2.4.A. Isolation of RNA

1. Total RNA from chick embryo primary hepatocytes was isolated by the guanidinium isothiocyanate procedure of Chomczynski and Sacchi (1987). In the initial steps, to prevent RNA degradation, media was decanted from plates containing primary hepatocytes and the denaturing "solution D" added directly to the petri dish. Denatured cell lysates were then harvested by scraping with a rubber policeman. Total RNA was isolated from 2 x $10^7$ hepatocytes using 300 µl of solution D. In this way all steps could be performed in 1.5 ml eppendorf tubes. Yields were typically 80-100 µg of RNA.

2. Total RNA was isolated from 17-18 day old chick embryos by a modified guanidine hydrochloride procedure (Brooker *et al.*, 1980). The chick embryos were removed from the egg, decapitated and dissected. Each liver was perfused, via cannulation of the heart, with 10 ml of 0.9% NaCl containing 2 mM EDTA to remove blood cells. For each drug treatment, three embryo livers were pooled prior to homogenisation. The livers were homogenised in 10 volumes of a solution containing 6 M guanidine-HCl, 20 mM sodium acetate pH5.0 and 2.8 mM 2-mercaptoethanol using an Ultra-tarrex homogeniser for 30 s at half speed. Following the addition of a half volume of ethanol and incubation at -20°C for 30 min, RNA was pelleted by centrifugation in an HB-4 rotor at 6,000 rpm (6,000 x g) for 15 min. The RNA pellet was redissolved in 6 M guanidine-HCl, 20 mM EDTA pH7.0 and 2.8 mM 2-mercaptoethanol and ethanol precipitated as described above with a half volume of ethanol. The RNA pellet was then dissolved and homogenised in a solution consisting of 7 M urea, 100 mM Tris-HCl pH8.5, 0.1 mM EDTA and 1.0% $NaDodSO_4$ using a glass Dounce homogeniser. To the homogenate was added a half volume of phenol and a half volume of chloroform and contaminating proteins extracted. $NaDodSO_4$ was then added to the supernatant to a final concentration of 1.0% and a phenol/chloroform extraction was repeated. RNA was finally precipitated overnight at -20°C after addition of 1/10 volume of 3.0 M sodium acetate pH5.2 and 2.5 volumes of ethanol. Following centrifugation in an HB-4 rotor at 10,000 rpm (16,000 x g) for 30 min, the pellet was washed with 70% ethanol, centrifuged again and dried in a Speedivac. For removal of trace amounts of

contaminating protein including RNases, the RNA was dissolved in 400 µl of a solution of 10 mM Tris-HCl pH7.6, 1 mMEDTA, 0.5% $NaDodSO_4$ and 50 µg/ml proteinase K and incubated at 37°C for 30 min. This was then phenol/chloroform extracted twice, and ethanol precipitated, washed and dried as described above. The final purified RNA pellet was dissolved in 400 µl of 0.1 mM EDTA or 0.5% $NaDodSO_4$. Yields of 0.7 to 1.0 mg of total RNA/liver were typically obtained.

Although RNA samples were generally analysed within one week of being purified, they have been shown to be stable when stored in 0.1 mM EDTA or 0.5% $NaDodSO_4$ at -20°C for at least one year. An alternative method of storage is to leave the RNA under ethanol at -20°C until required.

### 2.4.B. Northern Blot analysis

Northern blot analysis was carried out as previously described (Hansen *et al.*, 1989) with minor alterations. In brief, 20 µg of total RNA per track was run on a 1% agarose gel in 10 mM $NaHPO_4$ pH 7.4 containing 1.1 M formaldehyde. Fractionated RNA was transferred via capillary action to nitrocellulose or Nytran in 20x SSPE or to Zeta probe in 1x HETS (high efficiency transfer solution). RNA was then bound to the nitrocellulose and Nytran filters by either baking *in vacuo* at 80°C for 60 min or cross-linking with 120,000 µjoules using a UV Stratalinker™ 1800 (Stratagene). No further treatment was necessary for Zeta probe filters. All gels were stained with ethidium bromide to verify visually that similar amounts of RNA had been loaded in each lane. For those experiments in which HETS was used for transfer, the RNA on the filter could be visualised directly, without staining, under ultra-violet light.

Filters were prehybridised at 42°C for approximately 4 h in 50% formamide, 5x Denhardt's solution, 5x SSPE, 0.1% $NaDodSO_4$ and 200 µg/ml salmon sperm DNA. Hybridisation was carried out under the same conditions for 16-20 h with the inclusion of $^{32}P$-labelled probe (10 ng/ml of solution at a specific activity of 1-2 x $10^8$ cpm/µg). Post-hybridisation washes were carried out using 2x SSC, 0.1% $NaDodSO_4$ and 0.05% Na pyrophosphate. An initial two washes at room temperature for 10 min each were followed

by a final wash at 60°C for 40 min. Filters were blotted with Whatman 3MM paper and sealed in polythene bags while still moist. Autoradiography was routinely performed with the aid of one intensifying screen at -80°C.

### 2.4.C. RNase protection analysis

RNase protection assays were performed on total RNA using the method of Krieg and Melton (1987). In summary, labelled probe RNA was synthesised from 1-2 μg of linearised template DNA by incorporation of [α-$^{32}$P]UTP into nascent transcripts using an SP6 RNA polymerase transcription kit (Bresatec Ltd.). RNasin at a concentration of 1 U/μl was added to prevent RNA degradation. Full length transcripts were purified following DNase I treatment by separation on a 6% polyacrylamide, 7 M urea, Tris-borate standard sequencing gel, excision of the full length major band, and elution in 10 mM Tris-HCl pH8.0 containing 1 mM EDTA and 0.1% $NaDodSO_4$ at room temperature overnight. Approximately 60,000 cpm of labelled probe RNA was hybridised with 8-20 μg of total RNA, isolated from chick embryo primary hepatocytes as described in section 2.4.A.1. Digestion of single stranded RNA was then performed in a solution containing 10 mM Tris-HCl pH7.5, 5 mM EDTA, 300 mM NaCl, 4 μg/ml RNase A and 0.2 μg/ml RNase T1 at 37°C for 30 min. To this was added Proteinase K (125 μg/ml) and $NaDodSO_4$ (0.5%) and incubation continued at 37°C for a further 15 min. Following phenol/chloroform extraction and ethanol precipitation, the RNA pellet was dissolved in a denaturing solution containing 95% formamide, 10 mM EDTA and 0.02% bromophenol blue, heated at 85°C for 2 min, and loaded onto a 6% polyacrylamide gel made up in 1x TBE buffer, 7 M urea, 0.06% ammonium persulphate and 0.1% TEMED. The gel was run for approximately 90 min at 1750 V, 25 mA. The gel was treated as for a sequencing gel (Maniatis *et al.*, 1982), dried in an oven, wrapped with "Vitafilm" and autoradiographed. To ensure that protected RNA fragments were a quantitative measure of the amount of mRNA species present, controls in which double the amount of radioactive probe was added to the assay were performed. These revealed that the probe was in excess. To further check the linearity of this system, increasing amounts of RNA were assayed. An appropriate increase in intensity of protected fragment was observed (results not shown). In order to size the protected RNA fragments,

sequence derived from M13mp18 using the universal sequencing primer was run in adjacent tracks. In addition, because of the difference in mobility of DNA and RNA fragments, a control transcript of known size (189 nt) provided with the SP6 RNA transcription kit was simultaneously run and the mobility of the M13mp18 sequence markers adjusted appropriately to enable accurate size determination of RNA fragments.

## 2.5. PREPARATION OF CHICK EMBRYO PRIMARY HEPATOCYTE CULTURES

Hepatocytes were prepared from 17-18 day old chick embryos by the method of Giger and Meyer (1981), but with minor variations. 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 2 mM EDTA to remove blood cells followed by 4 ml of 0.05% collagenase in Hank's balanced salts solution (HBSS) to initiate digestion of intercellular 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 into fresh 0.05% collagenase - HBSS, cut into small pieces (1-2 mm) with fine tipped scissors and incubated at 37°C for 30 min with gentle shaking every 5 min and gentle pipetting, up and down, using a 10 ml glass pipette every 10 min to aid in separation of the cells. Collagenase was removed by washing with HBSS (2 ml/liver). Contaminating erythrocytes were lysed in an ammonium chloride solution as described by Sassa and Kappas (1977). Following two washes with William's E medium (2 ml/liver) to remove cell debris and hemoglobin from lysed erythrocytes, the hepatocytes were resuspended in William's E medium (1 ml/liver) and the yield determined. Typically $2 \times 10^7$ cells/liver were obtained. All of the above procedures were performed aseptically using sterile solutions and instruments, and working within a biohazard safety hood.

## 2.6. ISOLATION AND TRANSCRIPTIONAL ANALYSIS OF NUCLEI

### 2.6.A. Isolation of nuclei

All steps were performed at 4°C, cells and all solutions were kept on ice, and equipment was precooled.

Nuclei from chick embryo primary hepatocyte cultures were isolated by a modification of the method of Clayton and Darnell (1983) as outlined by Schuetz *et al* (1990). In summary, culture media was decanted and the hepatocytes harvested in 150 mM NaCl, 40 mM Tris-HCl pH7.5, 1 mM EDTA by scraping with a rubber policeman. Cells were pelleted by centrifugation for 1 min at 1000 x g (3000 rpm) using an SS34 rotor in a Sorvall RC-5B refrigerated superspeed centrifuge and then resuspended in 3 ml of RSB buffer (10 mM Tris-HCl pH7.4, 35 mM $MgCl_2$, 20 mM HEPES, 2 mM $CaCl_2$, 0.1 mM EDTA, 2 mM NaCl, 0.5 mM spermidine, 14 mM 2-mercaptoethanol). After 5 min, Triton X-100 was added to a final concentration of 0.2% and the cells vortexed gently. The cells were transferred to a glass dounce homogeniser and lysed using 10 strokes of the pestle. KCl was added to a final concentration of 30 mM and the nuclei pelleted by centrifugation for 5 min at 1000 x g in an SS34 rotor. The pellet containing nuclei and cell debris was resuspended in 6 ml of CRB buffer (10 mM Tris-HCl pH8.0, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 140 mM KCl, 20% glycerol, 0.5 mM spermidine, 14 mM 2-mercaptoethanol) and passed through four layers of gauze to remove the cell debris. The nuclei were pelleted by centrifugation for 5 min at 1000 x g using an SS34 rotor, resuspended in 10 ml of CRB buffer and once again pelleted. The nuclei were finally resuspended in 50 µl of "storage buffer" (20 mM Tris-HCl pH7.9, 75 mM NaCl, 0.5 mM EDTA, 1 mM DTT and 50% glycerol). A well mixed nuclei suspension was diluted 1/400 in storage buffer and counted on a haemocytometer. Additional storage buffer was added to obtain a density of $3 \times 10^8$ nuclei/ml and 50 µl aliquots (*i.e.* $1.5 \times 10^7$ nuclei) were frozen on dry ice and stored at -80°C until required. In this laboratory, nuclei over 2 years old have been shown to be transcriptionally active with a loss of less than 10% total activity upon storage.

Nuclei from chick embryo livers were isolated using the buffer conditions of Hewish and Burgoyne (1973) as modified by Schibler *et al* (1983) and the procedure of Panyim *et al*

(1978) as a guide for sucrose cushion concentrations. This protocol was developed by Dr A.J. Hansen of this laboratory and is as follows. Chick embryos of age 17-18 days were treated as desired (see figure legends) and decapitated. The livers were exposed by dissection and perfused with 10 ml of ice cold saline via canulation of the heart using a 10 ml syringe and 0.5 x 25 mm needle. The livers were removed, immediately placed into a 1.5 M sucrose solution containing 15 mM Hepes pH7.5, 0.5 mM EGTA, 2 mM EDTA, 60 mM KCl, 15 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine and 5 mM DTT, and homogenised with 10 strokes of an 18 mm diameter power-driven glass/teflon Dounce homogeniser at medium speed. Following filtration through 4 layers of gauze, 9.5 ml of homogenate was layered onto 2.5 ml of a 2.0 M sucrose solution containing 15 mM Hepes pH7.5, 0.1 mM EGTA, 0.1 mM EDTA, 60 mM KCl, 15 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine and 5 mM DTT, and this was centrifuged in an SW41 rotor at 27,000 rpm (*i.e.* 130,000 x g) at 4°C for 60 min. The supernatant was removed and discarded, and the nuclei pellet rinsed with 1 ml of ice cold water. Nuclei were then resuspended in 1 ml of storage buffer (same as for primary hepatocytes), gently homogenised by 3 strokes in a 5.5 mm diameter glass/glass Dounce homogeniser and centrifuged in an Eppendorf Microfuge at 4°C for 30 seconds. The pelleted nuclei were resuspended in 200 µl of storage buffer, and counted, diluted and stored as described above for chick embryo primary hepatocytes.

### 2.6.B. *In vitro* nuclear transcription run-on reactions

*In vitro* transcription reactions on chick embryo primary hepatocytes were performed using 1.5 x $10^7$ nuclei in 150 µl of 100 mM Tris-HCl pH7.9 containing 50 mM NaCl, 4 mM $MgCl_2$, 0.5 mM $MnCl_2$, 4 mM EDTA, 1 mM DTT, 50 µM phenylmethylsulfonyl fluoride, 40% glycerol, 1 U/µl RNasin, 1 mM each of ATP, CTP and GTP, 2 µM UTP, and 100 µCi [$\alpha$-$^{32}$P]UTP (3000 Ci/mmol) at 26°C for 10 min. TCA-precipitable radioactivity of 1 µl aliquots of the reaction mix were measured in triplicate at time = 0 min and at time = 10 min to monitor incorporation of [$^{32}$P]UTP into nascent RNA transcripts (see section 2.6.D). The rate of incorporation of [$^{32}$P]UTP into RNA by nuclei isolated from hepatocyte cultures both untreated and drug-treated have been shown to be linear for 15 min (see Chapter 4).

Nuclei from chick embryo livers were treated as for primary hepatocyte nuclei with the exception that *in vitro* transcription reactions were allowed to proceed at 26°C for 20 min since the rate of incorporation of [$^{32}$P]UTP into RNA was shown to be linear for at least this period of time (see Chapter 4).

### 2.6.C. Purification of nascent nuclear $^{32}$P-RNA transcripts

$^{32}$P-RNA transcripts were isolated and purified by the method of Wagner *et al* (1967). Immediately following incubation, 100 µg of *E.coli* tRNA was added as carrier to the run-on reaction mix and the reaction was then stopped with 750 µl of 0.5% $NaDodSO_4$ and 900 µl of 100 mM sodium acetate pH5.0 containing 20 mM EDTA. Proteins and chromosomal DNA were then extracted with one volume of water saturated phenol, after which the remaining RNA was precipitated by addition of 1/15 volume of 3.0 M sodium acetate pH5.2 and 2.5 volumes of ethanol. The RNA was pelleted by centrifugation in a HB-4 rotor at 10,000 rpm (16,000 x g) and at 4°C for 30 min. The pellet was washed with 100% ethanol, centrifuged as above for 10 min, dried and redissolved in 200 µl of 0.1% $NaDodSO_4$. TCA-precipitable radioactivity was determined in triplicate on 2 µl of the purified $^{32}$P-RNA transcripts (see section 2.6.D).

### 2.6.D. Measurement of $^{32}$P-RNA by TCA precipitation

Estimation of the incorporation of [$^{32}$P]UTP into total RNA was performed essentially by the TCA precipitation method of Marzluff (1978). Aliquots of 1-2 µl of run-on reaction mix were added to 100 µl of 1% $NaDodSO_4$ containing 10 mM EDTA in a 1.5 ml eppendorf tube to terminate the transcription reaction. For measurement of purified RNA transcripts, 1-2 µl was added to 100 µl of water containing 100 µg of *E.coli* tRNA as carrier. Ice cold 5% TCA and 1% sodium pyrophosphate was then added and the RNA was allowed to precipitate on ice for 30 min, after which the samples were filtered onto Whatman GF/A glass fibre discs. A further 10 ml of 5% TCA and 1% sodium pyrophosphate was used to wash the filters to remove unincorporated [$^{32}$P]UTP and this was followed by a wash with 10 ml of ethanol. The filters were dried under a 500 W heat lamp and counted in 3 ml of OptiScint 'HiSafe' scintillation fluid.

To maximise precision with this method, it was found that the run-on reaction mix must be well mixed prior to sampling, as the nuclei tend to settle during incubation. A slow filtration rate (~1 ml/30 s) of TCA-precipitated samples through Whatman GF/A glass fibre discs also helped in achieving precise and accurate results.

### 2.6.E. Detection and quantification of nascent $^{32}$P-labelled RNA transcripts

The binding of specific recombinant plasmid cDNA clones to nitrocellulose filters, hybridisation of $^{32}$P-RNA to the filter-bound target cDNA clones, and washing of filters following hybridisation were performed as described by Hansen *et al* (1989), but with the following modifications:

a. 2 µg of target cDNAs were applied in duplicate to nitrocellulose filters (in these studies, 2 µg/slot of plasmid cDNA clone was shown to be saturating for each of the $^{32}$P-RNA transcripts examined under basal and phenobarbital-induced conditions).

b. filters were prehybridised at 52°C for 18 h in 50% formamide, 10 mM Tris-HCl pH7.6, 1 mM EDTA, 0.1% sodium pyrophosphate, 10x Denhardt's solution, 5x SSC, 0.1% $NaDodSO_4$ and 100 µg/ml of *E.coli* tRNA.

c. hybridisations were also performed in this solution at 52°C for 72 h.

Following washing, the filters were dried and placed in a cassette for autoradiography with one intensifying screen, for a number of different periods of time. In this way an exposure was obtained which gave signals in the linear range of the film used (Kodak X-OMAT™AR). Autoradiographs were quantified using a computing densitometer and background signal from hybridisation of $^{32}$P-RNA non-specifically with pBR322 was subtracted.

## 2.7. TRANSIENT EXPRESSION OF RECOMBINANT DNA IN CHICK EMBRYO PRIMARY HEPATOCYTES

### 2.7.A. 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 pH7.05 containing 137 mM NaCl, 5 mM KCl, 0.7 mM $Na_2HPO_4$ and 6 mM dextrose (Graham and van der Eb, 1973) at a cell density of 2.5 x $10^7$ cells/ml and sheared salmon sperm DNA 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 a 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 Nu-Serum to a final concentration of 10% and containing 40 µg/ml gentamicin. 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 and the remaining healthy cells were treated as follows. To one of each pair of plates was added an appropriate amount of drug stock solution ("drug treated" - see figure legends) and to the other, an equivalent volume of solvent only ("control"). The cultures were further incubated for 48 h prior to harvesting.

### 2.7.B. Harvesting of chick embryo primary hepatocytes

Culture media was decanted and replaced with 1 ml of 40 mM Tris-HCl pH7.5 containing 1mM EDTA and 150 mM NaCl. The cells were dislodged by scraping with a rubber policeman, transferred into an eppendorf tube and pelleted in an Eppendorf Microfuge for 10 seconds. The supernatant was then removed and the pelleted cells were stored at -80°C until required.

### 2.7.C. Assay for CAT activity

Hepatocytes were lysed in 200 µl of 0.25 M Tris-HCl pH7.6 by three freeze/thaw cycles and centrifuged for 10 min in an Eppendorf Microfuge to pellet cell debris. Protein estimation was performed on 5 µl of the supernatant fraction by the method of Bradford (1976) and all cell lysates were heated at 65°C for 10 min to inactivate an inhibitor of CAT activity (Fromm *et al.*, 1985; Sleigh, 1986). It was noted that lysates not heated revealed reduced CAT activity. The heated cell lysates were then centrifuged for 1 min in an Eppendorf Microfuge to pellet denatured proteins prior to assaying of the supernatant for CAT activity (although this final step was routinely performed, the presence of denatured proteins did not affect the assay for CAT activity).

#### *2.7.C.1. CAT assay (Gorman* et al., *1982)*

CAT assays were performed essentially by the method of Gorman *et al* (1982). Assays were performed in 1.5 ml eppendorf centrifuge tubes in 0.25 M Tris-HCl pH7.6 containing 0.8 mM acetyl CoA and 1 µCi of D-*threo* -[dichloroacetyl-1-$^{14}$C]chloramphenicol in a final volume of 180 µl. Protein extract (5 µg) was assayed for 30 min for the CYP2H1CAT and pRSVCAT constructs, while 50 µg of protein extract was assayed for 120 min for the ALASCAT, pCAT™-promoter, pBCSVp-1 and pSV2CAT chimeric constructs. The reaction was stopped by extraction with 1 ml of ice cold ethyl acetate. The ethyl acetate phase was removed following centrifugation for 2 min in a Microfuge, placed into a clean eppendorf tube and dried in a Speedi-Vac. The contents of the tube was then resuspended in 15 µl of ethyl acetate by carefully ensuring that all inner surfaces up to the 1 ml mark of the tube had been "wetted" with the solvent. The contents were immediately centrifuged in a Microfuge for 10 seconds and spotted onto silica gel thin layer chromatography (TLC) plates. A hair dryer on "cool" setting was used to aid in evaporation of the solvent (*N.B.* use of a 500 watt lamp to aid in evaporation of the solvent led to breakdown products of the chloramphenicol). The TLC plates were run vertically with a chloroform/methanol solvent (95:5). Following autoradiography, CAT assays were quantified by cutting out the spots from TLC plates, placing them into scintillation vials containing 1 ml of OptiScint 'HiSafe' scintillation fluid and counting acetylated and

unacetylated [$^{14}$C]chloramphenicol fractions. Results were expressed as a percentage of chloramphenicol acetylated. In this system CAT assays were linear to 60% conversion of chloramphenicol to the acetylated forms and all assays were performed within this range. It should be noted that within an experiment, triplicate assays of CAT activity did not vary by more than 15% of the mean.

*2.7.C.2. CAT assay (Nordeen* et al., *1985)*

CAT assays were also performed as described by Nordeen *et al* (1985) with minor alterations. Essentially in this method two enzymatic reactions occur simultaneously. Firstly, S-acetyl CoA synthetase is used to continuously supply [$^{3}$H]acetyl CoA from the substrates [$^{3}$H]acetate and Coenzyme A. Secondly, [$^{3}$H]acetyl CoA and chloramphenicol act as substrates for the CAT enzyme to form [$^{3}$H]acetylchloramphenicol or [$^{3}$H]diacetylchloramphenicol. The reactions were set up as described and incubated for 120 min. At 60 min and 120 min, 100 µl samples were taken, extracted with 100 µl of ice cold ethyl acetate and then the aqueous phase re-extracted with another 100 µl of ethyl acetate. The two ethyl acetate extracts from each time point were pooled and counted in 3 ml of scintillation fluid. The linearity of the assay was gauged by comparison of the counts from the 60 min and 120 min time points. A major advantage of this technique is that the radioactive substrate [$^{3}$H]acetate is much cheaper than [$^{14}$C]chloramphenicol used in the method of Gorman *et al* (1982) or [$^{14}$C]acetyl CoA used in the method of Sleigh (1986).

*2.7.C.3. CAT assay (Sleigh, 1986)*

CAT assays were performed as described by Sleigh (1986). This method is essentially the same as that of Nordeen *et al* (1985) as described above, with the exception that [$^{14}$C]acetyl CoA is added as one of the substrates rather than being synthesised.

### 2.7.D. Assay for ß-galactosidase activity

ß-galactosidase activity was determined by the method of Herbomel *et al* (1984) on freeze/thawed cell lysates (see section 2.7.C). It was important <u>not</u> to heat the cell lysates at

65°C for 10 min, as was done for CAT activity determinations, as this denatures ß-galactosidase and destroys its activity.

### 2.7.E. Chemical synthesis of acetyl Coenzyme A

Due to the expense of purchasing acetyl Coenzyme A (acetyl CoA), a rapid, simple and reliable method for its synthesis from Coenzyme A and acetic anhydride was employed to produce the quantities necessary for use in CAT assays at approximately 30% of the retail price. The method is described by Simon and Shemin (1953). The final product was spectrophotometrically quantified by absorption at 260 nm and a check for completion of the reaction was performed by determining the $A_{232}/A_{260}$ ratio. The calculated ratio was consistently near the expected value of 0.543 (for pure acetyl CoA).

## 2.8. TREATMENT OF CHICK EMBRYOS WITH DRUGS

17-18 day old chick embryos were administered with the drug or compound of choice (see figure legends) dissolved in 100 µl of dimethylsulfoxide by injection with a 0.5 x 25 mm needle through a small hole made in the top of the egg. The drug was either placed on top of the allantoic membrane in the air sac cavity or injected through this membrane into the fluid surrounding the embryo. Following injection, the hole was closed with a piece of sticky tape and the embryos were incubated for the appropriate amount of time.

## 2.9. REGULATIONS AND CONTAINMENT FACILITIES

All manipulations involving recombinant DNA were performed in accordance with the regulations and approval of the Genetics Manipulations Advisory Committee (GMAC).

All work with radioactive isotopes and all animal experiments were performed in accordance with the regulations of the Biohazards and Animal Ethics Committee, University of Adelaide.

## 2.10. COMPUTER PROGRAMMES

Two different computer software packages were used for analysis of DNA sequence. The first package was written by Staden (1982) and certain programmes adapted by Dr A.

Sivaprasad of the Department of Biochemistry, University of Adelaide. The programmes called "Repeat" and "Inverted repeat" were used to search for directly and inverted repeated DNA sequences, respectively. The second package is termed "GCG Sequence Analysis Software Package" (Devereux *et al.*, 1984) and was written by the Genetics Computer Group of the University of Wisconsin, Biotechnology Center, Madison, Wisconsin, U.S.A. In particular, the following programmes were used. "BESTFIT" is a programme which searches two sequences for a region of greatest similarity while "GAP" aligns two sequences to give the greatest number of matches. "FIND" enables the user to compile a list of specific sequences, such as the consensus sequences to which bind *trans* -acting factors, and then to search any known sequence for each of the listed specific sequences.

This thesis was written with the use of an Apple Macintosh SE/30 computer. Microsoft Word version 4.0 was used for compilation of the text and MacDraw II 1.1 and Cricket Graph version 1.3.1 were used in the figures for writing figure legends, drawing diagrams and constructing graphs. The text and figures were printed using a LaserWriter II NT, Apple Computer, Inc.

# CHAPTER 3

## CHARACTERISATION OF THE EFFECT OF DRUGS AND HAEM ON CYPS AND ALAS AT THE TRANSCRIPTIONAL AND mRNA LEVEL IN CHICK EMBRYO LIVER AND PRIMARY HEPATOCYTES

## 3.1. INTRODUCTION

It has been known for some time that in the chick embryo liver and primary hepatocyte cultures, phenobarbital and "phenobarbital-like" drugs cause a marked increase in both the level of some hepatic CYPs and the level of the house-keeping ALAS (Granick, 1966; Rifkind *et al.*, 1973; Granick *et al.*, 1975; Witting *et al.*, 1977; Althaus *et al.*, 1979). A co-ordinated regulation of these enzymes seems desirable since additional haem would be required by the increased synthesis of hepatic apoCYP following drug treatment. Since ALAS is rate-limiting in the haem biosynthetic pathway, increased synthesis of ALAS would fill this need. The molecular mechanisms by which such drugs induce CYPs and ALAS, in an apparently co-ordinated fashion, are not understood and are of great interest.

Several models have been proposed to explain such a co-ordinate regulation. The most widely accepted model, which I have termed the "haem repressor model", was first proposed by Granick (1966) and has been modified by other workers in this field (De Matteis, 1978; May *et al.*, 1986). In brief, May *et al* (1986) proposed that the level of ALAS is predominantly controlled by a haem-modulated repressor protein which acts at the transcriptional level. It is further proposed that drugs, in some way, increase the levels of CYP apoprotein, most likely through a transcriptional mechanism. The increased amount of apoCYP results in haem sequestration and hence the lowering of inhibiting haem levels with consequent derepression of the ALAS gene; conversely, elevated haem would repress ALAS synthesis. In this model, structurally different drugs enhance specific *CYP* gene transcription and the possible mechanisms by which this occurs is discussed later.

In this laboratory, we are particularly interested in understanding how the genes for *CYPs* and ALAS are controlled by phenobarbital and phenobarbital-like drugs and we have chosen to examine induction in chick embryos and chick embryo primary hepatocytes. There were several reasons for this. Firstly, it was known that both CYP and ALAS levels are induced in chick embryo liver *in ovo* following administration of porphyrinogenic drugs such as phenobarbital, AIA or DDC (Brooker *et al.*, 1983; McCluskey *et al.*, 1986; Lorr and Bloom, 1987), while similar inductions were observed in primary hepatocytes following drug addition (Racz and Marks, 1969; Rifkind *et al.*, 1973; Giger and Meyer, 1981;

Shedlofsky *et al.*, 1987). Additionally, haem was known to repress activity levels of ALAS in primary hepatocytes. By contrast, cultures of adult rat primary hepatocytes were refractory to drug-induction with phenobarbital, and only recently have suitable culturing conditions been derived which permit induction (Schuetz *et al.*, 1988, 1990; Waxman *et al.*, 1990).

While previous work had established that drugs and haem act to affect levels of CYP and ALAS, as revealed by enzymic or immunological techniques, there was no information at the gene level about the mechanisms by which these changes occurred. Studies had established that the chicken haploid genome contained one gene for house-keeping ALAS (Elferink *et al.*, 1987) and probably only two genes for phenobarbital-inducible CYPs (Mattschoss *et al.*, 1986), which have been designated *CYP2H1* and *CYP2H2* (Nebert *et al.*, 1991). This latter finding was in marked contrast to rats, where at least six *CYP* genes with significant similarity to the major phenobarbital-inducible *CYP* genes were present (Kumar *et al.*, 1983). In this laboratory, genomic and cDNA clones for the house-keeping ALAS and a phenobarbital-inducible *CYP* designated *CYP2H1*, were available. A full-length cDNA clone designated p105B1 (Borthwick *et al.*, 1985) and several genomic clones for the house-keeping form of ALAS had been previously isolated and sequenced in this laboratory (Maguire *et al.*, 1986). Similarly, a partial cDNA clone corresponding to the CYP2H1 mRNA designated pCHP3 (containing 2712 bp of an estimated full length 3500 bp) had been isolated and sequenced, and approximately 3800 bp of a genomic clone spanning the *CYP2H1* gene transcription start site had also been sequenced in this laboratory (Mattschoss, 1987). Using these clones, a study was therefore undertaken to investigate how *CYP* and ALAS genes are controlled by drugs and haem in an apparently co-ordinated fashion.

This chapter deals with characterisation of the control of the ALAS and the *CYP2H1* genes in chick embryo liver and primary hepatocytes in response to phenobarbital and haem by measurement of steady state mRNA levels and the rates of gene transcription.

## 3.2. RESULTS

### 3.2.A. Effect of phenobarbital and haem on ALAS and CYP mRNA levels in chick embryo liver

Previous studies in chick embryos demonstrated that phenobarbital and AIA were able to increase CYP and ALAS mRNA levels in the liver (Hansen *et al.*, 1989). The increase in the level of ALAS mRNA was shown to be primarily due to transcriptional induction whereas elevated CYP2H1 and CYP2H2 mRNAs, although partly due to transcriptional activation, were predominantly a result of a post-transcriptional mechanism. Of interest was the role of haem on the rate of transcription and the levels of mRNA for ALAS and CYPs.

To investigate the effect of haem on ALAS and CYP in chick embryos, haemin was administered by injection through a hole in the egg and layering on top of the allantoic membrane. However, haemin was found to precipitate to form an insoluble black mass upon injection, which presumably significantly reduces the amount absorbed by the embryo. To circumvent this problem, the water soluble precursor of haem, 5-aminolevulinic acid (ALA), was utilised in place of haemin. It has been shown that ALA, which by-passes the rate-controlling ALAS enzymatic step of haem biosynthesis, is converted almost entirely to haem in the liver (Anderson *et al.*, 1981). In later experiments, haemin was injected into chick embryos and the effect compared with that of ALA (see section 3.2.F).

In this experiment, three groups of chick embryos were injected with 6 mg of phenobarbital. Two of these groups also received 20 mg of ALA, one group via injection into the fluid surrounding the embryo and the other via layering on top of the allantoic membrane. A fourth group of embryos received solvent only. Four hours after the injections, the embryos were sacrificed, the livers removed and total RNA prepared. Following separation on a 1% agarose-formaldehyde gel, the RNA was transferred to Nytran filters and probed for CYP mRNA with the cDNA clone designated pCHP3. This probe is known to hybridise to at least three CYP mRNAs (Hansen *et al.*, 1989). For detection of ALAS mRNA, a cDNA clone corresponding to the chicken house-keeping ALAS mRNA and designated p105B1 was used as probe; for ß-actin mRNA, a cDNA

clone corresponding to chicken ß-actin mRNA was used. The resultant autoradiographs are shown in Figure 3.1. Phenobarbital greatly increased the levels of three CYP mRNAs of size 3.5, 2.5 and 2.2 kb, as expected from the work of Hansen *et al* (1989); these mRNAs are not clearly discernable in Fig. 3.1, but were visible in the original autoradiograph. ALA administration appeared to have no effect on the phenobarbital-induced levels of these mRNAs. ALAS mRNA was also induced by phenobarbital, but ALA injection markedly reduced this drug-induced level. The level of ß-actin mRNA was not affected by phenobarbital or ALA injections as indicated from several experiments; the reduced ß-actin mRNA in lanes 3 and 4 in this experiment is not typical and the reason for this is not clear.

From these results, it appears that haem negatively regulates the levels of ALAS mRNA, but has no effect on CYP mRNA levels. To determine whether this repressive effect of haem on ALAS mRNA is at the transcriptional level, nuclear transcription run-on experiments were performed (see section 3.2.F).

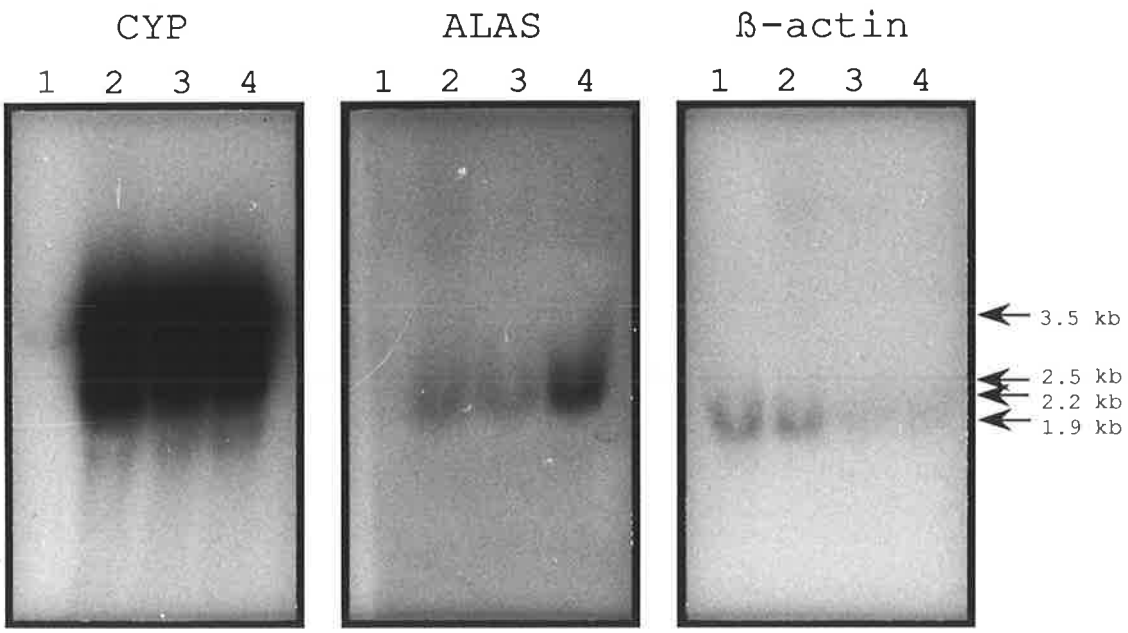
### 3.2.B. Comparison of phenobarbital-inducible CYP mRNAs in chick embryo primary hepatocytes and chick embryo liver.

As already mentioned, phenobarbital and AIA induce at least three CYP mRNAs of size 3.5, 2.5 and 2.2 kb in chick embryo liver. It was of importance then to determine whether the same mRNAs were induced by phenobarbital in chick embryo primary hepatocytes, since these cultures were to be used for transient expression studies.

Chick embryo primary hepatocytes were prepared essentially by the method of Giger and Meyer (1981) as outlined in section 2.5. Briefly, chick embryos were decapitated and the liver was perfused via cannulation of the heart. The hepatocytes were dispersed using collagenase and then cultured in 60 mm diameter petri dishes in 5 ml of William's E medium at a density of 1 x $10^7$ cells/plate. William's E medium is a totally defined medium that does not contain foetal calf serum and hence no exogenous haem. In this experiment, phenobarbital, to a final concentration of 500 µM, was added to the culture medium while control plates received solvent only. After 4 h, total RNA was isolated from the hepatocytes. For comparison, chick embryos were injected with 4 mg of phenobarbital and incubated for 4 h prior to decapitation, removal of the liver and preparation of total RNA.

**Figure 3.1. Effect of exogenous 5-aminolevulinic acid upon CYP and ALAS mRNAs in control and phenobarbital-induced chick embryos**

Twenty eggs containing chick embryos of age 17 days old were placed into four groups of five. The embryos within "Group 1" (control) were administered via injection, 100 µl of the solvent dimethylsulfoxide while those from "Group 2, 3 and 4" received 100 µl of dimethylsulfoxide in which had been dissolved 6 mg of phenobarbital. 5-Aminolevulinic acid (ALA - 20 mg in 100 µl of water) was also administered at this time in two ways. "Group 2" embryos received ALA layered within the air sac on top of the allantoic membrane while "Group 3" embryos received ALA by injection through this membrane into the fluid surrounding the embryo. "Group 1 and 4" also received 100 µl of water injected into the fluid surrounding the embryo. All embryos were then returned to the humidified incubator for 4 h prior to decapitation. Livers from each group of embryos were removed, pooled and total RNA was prepared. RNA samples (50 µg/track) were run on a 1% agarose-formaldehyde gel, transferred to Nytran filters and probed for ALAS, CYPs and ß-actin with p105B1, pCHP3 and a chicken ß-actin cDNA clone. The numbers of each lane correspond to pooled RNA from the groups mentioned above.
Although not clear in the photograph, three discrete CYP mRNAs of size 3.5, 2.5 and 2.2 kb were visible in the phenobarbital-induced lanes on the original autoradiograph. In addition, although the amounts of ß-actin mRNA in lanes 3 and 4 appear reduced, in other similar experiments, ß-actin mRNA levels did not change in the presence of phenobarbital or ALA.


CYP
1 2 3 4
ALAS
1 2 3 4
ß-actin
1 2 3 4
3.5 kb
2.5 kb
2.2 kb
1.9 kb

The total RNA from primary hepatocytes and liver was run on a 1% agarose-formaldehyde gel, transferred to nitrocellulose and probed with pCHP3 (Hansen *et al.*, 1989). The results of autoradiographic exposure are shown in Figure 3.2. Phenobarbital induces the same three CYP mRNAs of size 3.5, 2.5 and 2.2 kb in primary hepatocytes as it does in the liver *in vivo*. Higher basal levels of each CYP mRNA are seen in the primary hepatocytes compared with liver (Fig. 3.2; compare lane 3 with lane 1).

The cDNA probes designated pCHPB15 and pCHP7-AB1 that specifically detect the 3.5 kb mRNA and 2.2 kb mRNA, respectively, from phenobarbital-induced chick embryo liver (Hansen *et al.*, 1989) also specifically detected these mRNAs in drug-induced primary hepatocytes (Fig. 3.2; lanes 5 and 6).

These results demonstrate that CYP mRNA levels in the cultured chick embryo primary hepatocytes respond to phenobarbital, in a similar fashion to that in chick embryo liver, further supporting the notion that these cell cultures closely resemble the *in ovo* situation (see section 3.1).

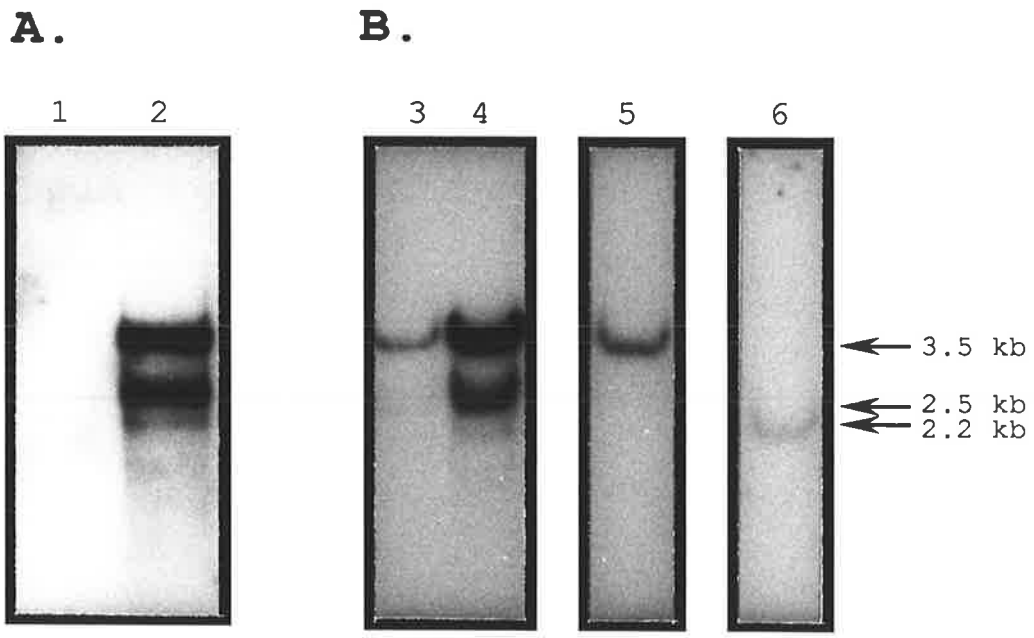
### 3.2.C. Effect of drugs and haem on ALAS and CYP mRNA levels in chick embryo primary hepatocytes cultured in different media

Chick embryo primary hepatocytes cultured in William's E medium, as described in the previous section, were slow to attach to the culture plates taking approximately 12 h for about 80% of the cells to attach (results not shown). Reports in the literature indicated that addition of hormones or combinations of them including insulin, tri-iodo-thyronine ($T_3$), glucagon, growth hormone, epidermal growth factor, prolactin and dexamethasone were beneficial to the survival and maintenance of differentiation of primary hepatocyte cultures (Ades, 1983; Jefferson *et al.*, 1984; Shedlofsky *et al.*, 1987). This is an important consideration since dedifferentiation of hepatocytes leads to a rapid loss of liver-specific functions of which phenobarbital-inducibility of CYPs is one (Guzelian *et al.*, 1977; Sirica and Pitot, 1980; Steward *et al.*, 1985). The mechanism by which certain liver-specific functions are reduced or silenced is not known. Clayton *et al* (1985) have suggested that while soluble components such as nutrients, hormones and other factors play some role, it is

**Figure 3.2. Comparison of phenobarbital-inducible CYP mRNAs in chick embryo primary hepatocytes and chick embryo liver**

**A.** Three 17 day old chick embryos were each treated with 4 mg of phenobarbital in PBS by injection through the allantoic membrane and into the fluid surrounding the embryo. Three control embryos received an equal volume of PBS. After 4 h, the embryos were decapitated, the livers removed and the appropriate ones pooled. Total RNA was isolated and 20 μg run 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).

**B.** Primary hepatocytes were prepared and cultured for 16 h in William's E medium. Phenobarbital was then added to a final concentration of 500 μM while control plates received an equal volume of PBS. After 4 h, total RNA was isolated and treated as described above in **A.** Filters were then probed for phenobarbital-inducible CYP mRNAs with pCHP3 (lane 3, control; lane 4, phenobarbital-treated) or with the 3' partial cDNAs for the 3.5 kb mRNA designated pCHPB15 (lane 5, phenobarbital-treated) or the 2.2 kb mRNA designated pCHP7-AB1 (lane 6, phenobarbital-treated). Molecular weight markers were generated by digestion of pBR322 with *Acc* I and *Hinc* II (not shown).


A.
B.
1
2
3
4
5
6
3.5 kb
2.5 kb
2.2 kb

the cell-cell contact and/or cell architecture that is of major importance in maintaining the correct level of expression of liver-specific genes.

The above reports prompted a trial of different culture media as well as different cell plating densities. The effect of the latter is discussed in section 4.2.B.3. Initially, chick embryo primary hepatocytes were cultured under various conditions as described below and the effect of phenobarbital and haem on mRNA levels were analysed. The media used were William's E medium alone, William's E medium supplemented with 10 μg/ml insulin and 300 ng/ml dexamethasone, William's E medium supplemented with 10% Nu-Serum (which replaces foetal calf serum and contains various growth factors, hormones, amino acids, vitamins and 25% (v/v) newborn calf serum; for details, see section 2.1.I), Dulbecco's modified eagle's medium (DMEM) supplemented with 10% Nu-Serum, DMEM/Ham's F12 (1:1) supplemented with 1% ITS$^+$ (which contains insulin, transferrin, selenious acid, bovine serum albumin and linoleic acid), and Opti-MEM I supplemented with 1% ITS$^+$. Apart from Nu-Serum, which contains 25% newborn calf serum, none of the other media or media supplements contain newborn or foetal calf serum. This was done to ensure that unknown amounts of haem, present in serum, were not being added to the cells. The cells were plated on standard plastic petri-dishes. Hepatocytes in William's E medium supplemented with insulin and dexamethasone were also plated on petri-dishes precoated with Matrigel, a connective-tissue like matrix (Schuetz *et al.*, 1988). Phenobarbital and/or haem were added to the appropriate plates and the cells were incubated for a further 4 h prior to harvesting and isolation of total RNA. The total RNA (20 μg) was fractionated on a 1% agarose-formaldehyde gel and transferred to nitrocellulose. Figure 3.3 shows the results of probing Northern blots for CYP, ALAS and ß-actin using the probes described in section 3.2.A.

In all of the cultures, basal levels of ALAS mRNA were reduced by haem until barely detectable and induced significantly with phenobarbital by varying amounts. The levels of phenobarbital-inducible CYP mRNAs were substantially increased above basal levels by phenobarbital added to cultures containing William's E medium. No repression by haem was seen in control or phenobarbital-induced cultures. When the intensities for CYP mRNA levels are standardised to the corresponding ß-actin levels, a mild induction with

**Figure 3.3. Effect of media on the response of CYP2H1 and ALAS mRNA levels to phenobarbital and haem**

Primary hepatocytes ($1 \times 10^7$) were plated in duplicate into 60 mm diameter culture dishes containing the following media:

**A.** William's E medium supplemented with 10 µg/ml insulin and 300 ng/ml dexamethasone (culture dishes precoated with Matrigel)

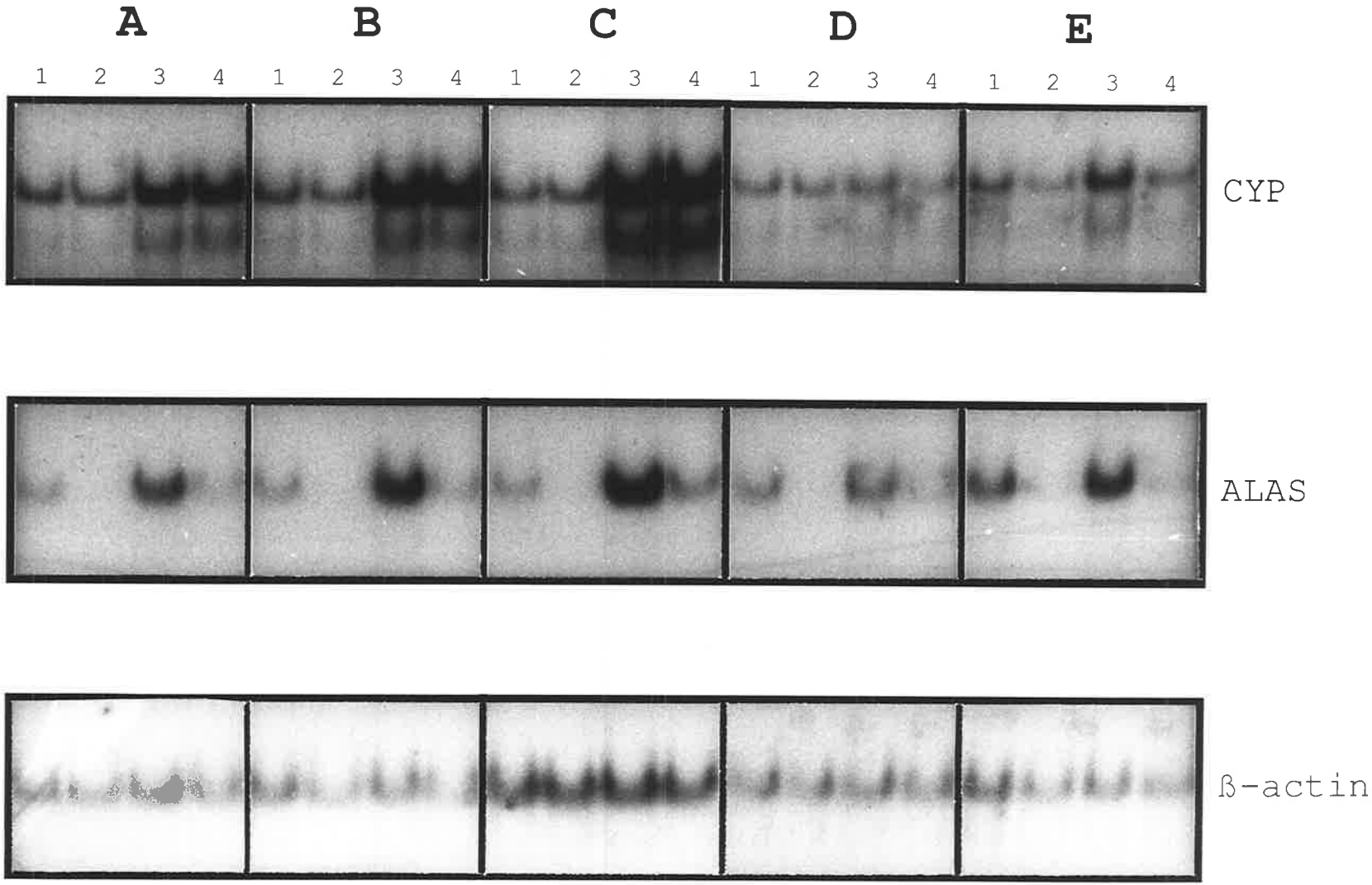
**B.** William's E medium supplemented with 10 µg/ml insulin and 300 ng/ml dexamethasone

**C.** William's E medium supplemented with 10% Nu-Serum

**D.** DMEM/Ham's F12 (1:1) supplemented with 1% ITS$^+$

**E.** Opti-MEM I supplemented with 1% ITS$^+$

All plates were incubated for 16 h to allow for the attachment of hepatocytes to the culture dishes. Phenobarbital and/or haem was then added to a final concentration of 500 µM and 10 µM, respectively. The cells were incubated for a further 4 h prior to harvesting and isolation of total RNA. Total RNA (20 µg) was fractionated on a 1% agarose-formaldehyde gel and transferred to nitrocellulose. The amounts of three CYP mRNAs of size 3.5, 2.5 and 2.2 kb were examined using the cDNA probe pCHP3, hepatic ALAS mRNA (2.2 kb) using the cDNA probe p105B1 and ß-actin mRNA (1.9 kb) using a chicken ß-actin cDNA probe. The resultant autoradiograph is shown (lane 1, control; lane 2, haem; lane 3, phenobarbital; lane 4, phenobarbital and haem).


A
B
C
D
E
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
CYP
ALAS
ß-actin

phenobarbital in Opti-MEM I supplemented with 1% ITS$^+$ is seen. Although there appears to be a slight decrease with haem in this experiment, a repeat experiment revealed unequivocally that this medium behaves in a manner similar to that of William's E medium supplemented with insulin and dexamethasone, where haem has no significant effect (results not shown). It is difficult to explain the CYP mRNA results for DMEM/Ham's F12 (1:1) supplemented with 1% ITS$^+$ in this experiment, where the response to drug while poor for ALAS mRNA, was undetectable for CYP mRNAs. Work using this medium was not pursued any further.

Careful analysis of the ß-actin mRNA levels revealed very slightly lower amounts in those samples that had been treated with haem. This minor decrease may reflect a general effect on the cells due to the relatively high concentration of haem present (*i.e.* 10 µM). It should be noted however, that hepatocytes cultured in William's E medium supplemented with 10% Nu-Serum (see Fig. 3.3.C) did not show this decrease in ß-actin mRNA, although ALAS mRNA levels did decrease. In this medium, the presence of serum proteins (Nu-Serum contains 25% Newborn Calf Serum) that bind haem such as haemopexin, may effectively lower haem levels below that which affects ß-actin by some general or specific mechanism, but not below the levels required for efficient repression of ALAS mRNA.

William's E medium supplemented with 10% Nu-serum was chosen for further studies for the following reasons. The levels of the drug-induced CYP mRNAs were consistently higher in this medium compared with the other media and repression of both basal and drug-induced ALAS mRNA by haem mimicked the pattern seen in chick embryo liver (see Fig. 3.1). In addition, cell attachment to the culture plates was found to be more rapid and extensive in this medium; almost 100% attachment was achieved within 1-3 h after plating compared with, for instance, approximately 80% attachment in 12 h for William's E medium alone or William's E medium supplemented with insulin and dexamethasone (results not shown). With the eventual aim of using the chick embryo primary hepatocyte cultures for transient expression studies, another important consideration in the choice of culture conditions was the greater percentage of hepatocytes surviving in this medium following electroporation. This was shown in an experiment in which hepatocytes were mock-electroporated (*i.e.* no DNA was transfected) under identical conditions and then

cultured in William's E medium with or without 10% Nu-Serum. A 5-fold greater recovery of surviving cells, as determined by protein estimation of freeze/thaw total cell lysates, was obtained in William's E medium supplemented with 10% Nu-Serum (results not shown). This suggests that components of Nu-Serum help the recovery of the electrically damaged cells; that is, those cells most likely to contain transfected DNA. This finding should result in an increased efficiency of transfection of constructs into primary hepatocytes. William's E medium supplemented with 10% Nu-Serum has therefore been employed for transient expression studies in Chapters 5 and 6. However, in Chapters 3 and 4, William's E medium alone has been used since the studies in these chapters were carried out prior to the discovery of the beneficial effect of 10% Nu-Serum.

### 3.2.D. Time courses of CYP2H1 and ALAS mRNA levels in response to phenobarbital in chick embryo primary hepatocytes

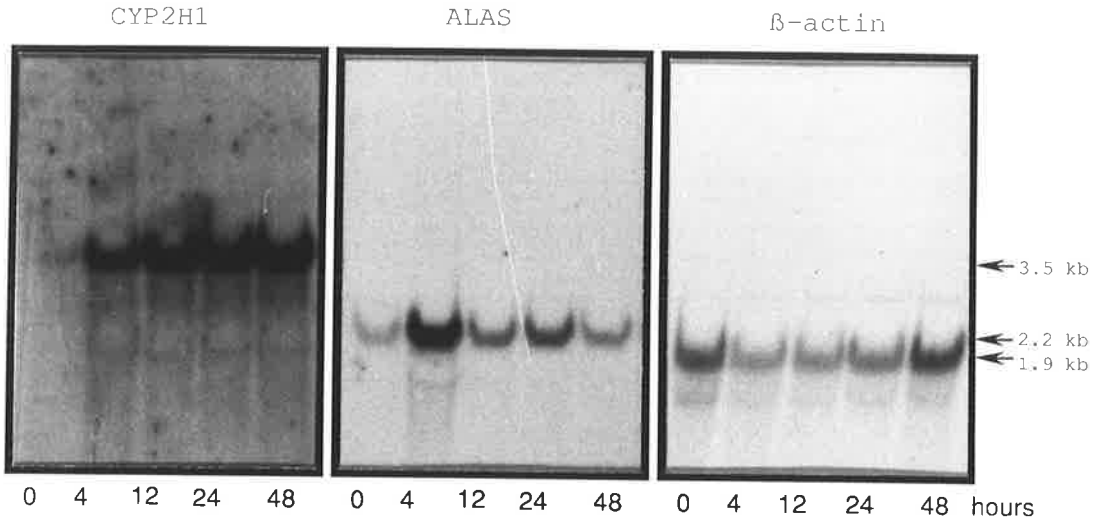
To study the kinetics of induction of CYP and ALAS mRNA levels in response to phenobarbital, primary hepatocytes were prepared and cultured overnight. Phenobarbital was then added to a final concentration of 500 μM and hepatocytes were harvested after 0, 4, 12, 24 and 48 h. Northern blot analysis was performed on total RNA from each time point probing specifically for CYP2H1, ALAS and ß-actin mRNAs. Autoradiographs of the resultant filters are shown in Figure 3.4.A. A graphical representation of the time course for CYP2H1 and ALAS mRNA, standardised to ß-actin mRNA at each time point, is shown in Figure 3.4.B. CYP2H1 mRNA levels increased quite substantially reaching a maximum of a 40-fold increase at 12 h, and then declined gradually to 21-fold at 48 h. In another experiment, this response to phenobarbital was shown to continue for at least 72 h (results not shown). ALAS mRNA levels increased by almost 7-fold after 4 h of phenobarbital treatment, then decreased to levels approximately 2.5-fold above the non-induced control after 12 h and by 48 h had almost returned to control levels. A comparison of the time courses for CYP2H1 and ALAS mRNA levels in response to phenobarbital revealed a larger and more prolonged response of CYP2H1 mRNA. In this and other experiments, the level of ß-actin mRNA remained largely unaffected in the presence of phenobarbital at all time periods.

**Figure 3.4. Time courses of CYP2H1 and ALAS mRNA levels following phenobarbital treatment of chick embryo primary hepatocytes**

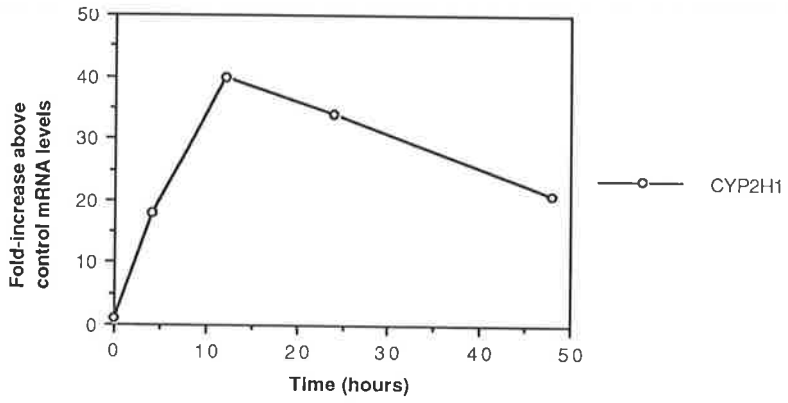
**A.** Primary hepatocytes ($1 \times 10^7$) were cultured in the presence of 500 µM phenobarbital for 0, 4, 12, 24 and 48 h. Total RNA was isolated and 20 µg run on a 1% agarose-formaldehyde gel. The RNA was transferred to nitrocellulose, probed for CYP2H1 mRNA with pCHPB15, for ALAS mRNA with p105B1 and for ß-actin with a chicken ß-actin cDNA and autoradiographed. Molecular weight markers were generated by digestion of pBR322 with *Acc* I and *Hinc* II (not shown).

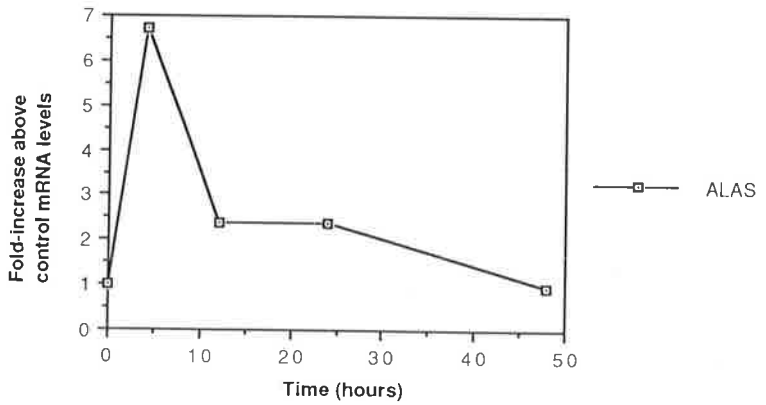
**B.** Autoradiographs were quantified using a computing densitometer. The level of the CYP2H1 and ALAS mRNA were standardised to the level of ß-actin mRNA at the corresponding time point and the fold-increase in CYP2H1 or ALAS mRNA compared to the zero time point was plotted. The basal levels of CYP2H1 and ALAS mRNA did not vary significantly in the control cultures (*i.e.* without phenobarbital) throughout the 48 h period (results not shown).

**A.**


CYP2H1
ALAS
ß-actin
3.5 kb
2.2 kb
1.9 kb
0 4 12 24 48 0 4 12 24 48 0 4 12 24 48 hours

**B.**


Fold-increase above control mRNA levels
0 10 20 30 40 50
Time (hours)
CYP2H1


Fold-increase above control mRNA levels
0 1 2 3 4 5 6 7
0 10 20 30 40 50
Time (hours)
ALAS

## 3.2.E. Time courses of the rates of transcription of the *CYP2H1* and ALAS genes in response to phenobarbital in chick embryo primary hepatocytes

In the previous section, the levels of CYP2H1 and ALAS mRNA were shown to increase in chick embryo primary hepatocytes in response to phenobarbital. However, from these results alone, one cannot distinguish between regulation at a transcriptional or post-transcriptional level. Hansen *et al* (1989) showed in chick embryo livers *in ovo*, that while phenobarbital and AIA increased the level of ALAS mRNA predominantly at the transcriptional level, induction of CYP2H1 mRNA was the result of both transcriptional and post-transcriptional mechanisms. It was of importance therefore to determine the levels of regulation involved in the CYP2H1 and ALAS mRNA response to phenobarbital in cultured chick embryo primary hepatocytes. To do this, nuclear transcription run-on experiments were performed on primary hepatocyte nuclei at various times after administration of phenobarbital.

In order to accurately measure the relative transcription rates of specific genes, it was necessary to determine the period of incubation of the run-on reaction required for optimal production of transcripts and to demonstrate that hybridisation of $^{32}$P-RNA to filter-bound cDNA probes was quantitative.

### *3.2.E.1. Synthesis of $^{32}$P-RNA transcripts using nuclear transcription run-on assays*

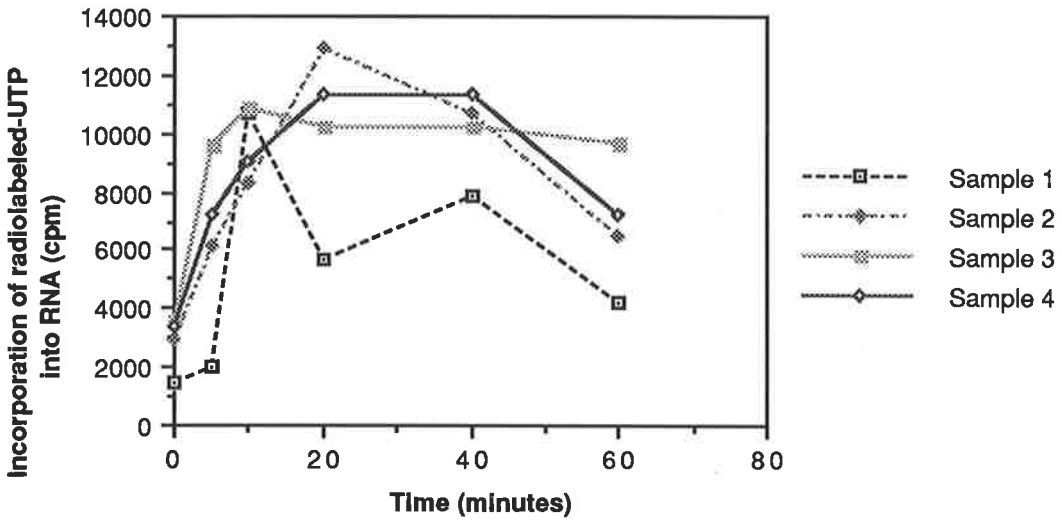
Nuclei from chick embryo primary hepatocytes were isolated by a modification of the procedure employed by Schuetz *et al* (1990) for isolation of rat primary hepatocytes. Using this rapid and simple procedure, approximately 50% recovery of nuclei from cells was routinely obtained. These nuclei were examined for their transcriptional activity by determination of the rate of incorporation of [$\alpha$-$^{32}$P]UTP into total TCA-precipitable nascent RNA transcripts. This involved a nuclear transcription "run-on" reaction in which [$\alpha$-$^{32}$P]UTP was allowed to incorporate into RNA, TCA precipitation of total RNA isolated from the nuclei and measurement of the total radioactivity precipitated (see sections 2.6.A to 2.6.D). This initial test for general incorporation was performed on all preparations of nuclei to ensure that, following the isolation procedure, they maintained the capacity to

transcribe *in vitro*. Only nuclei ($1.5 \times 10^7$) that incorporated at least 2000 cpm/µl of reaction mix were used for determination of the transcription rates of specific mRNAs. Since data on the transcriptional activity of nuclei from chick embryo primary hepatocytes was not available, a comparison was made with that of the previously described chick embryo liver nuclei (Hansen *et al.*, 1989). Chick embryo liver nuclei were made using an alternative procedure involving purification of nuclei through a sucrose density gradient (Hansen *et al.*, 1989). The results of typical incorporation profiles of nuclei from chick embryo primary hepatocytes and chick embryo liver are shown in Figure 3.5. Three aspects are of note when comparing the incorporation rates of [$\alpha$-$^{32}$P]UTP into RNA by these nuclei. Firstly, the total amount of radioactivity incorporated is generally higher for chick embryo liver nuclei, suggesting that they are transcriptionally more active. The observation that nuclei from cultured cells and cell lines are less transcriptionally active than the corresponding nuclei from parent tissue, has been seen by others in this laboratory (H. Healy and G. Bradiotti, personal communication). The reason for this is not known. Secondly, transcription in nuclei from chick embryo livers continues for at least 60 min although it begins to slow after 20-30 min, whereas in nuclei from primary hepatocytes, transcription appears to stop after 10-20 min. Thirdly, the total amount of newly transcribed RNA in nuclei from chick embryo primary hepatocytes begins to decrease after 15-20 min indicating the possible presence of an RNase activity. Investigation into this degradation revealed that the RNase inhibitor RNasin, which was used in the above run-on reactions, provided partial protection since, in its absence, a greater rate of degradation of TCA-precipitable $^{32}$P-RNA was seen. Interestingly, no significant decrease in RNA was apparent in nuclei from chick embryo livers even after 60 min of incubation in either the presence or absence of RNasin (results not shown). It is possible that all three of the above observations are related. For instance, increased RNase activity in primary hepatocyte nuclei may account for the lower total amount of radioactivity incorporated into RNA. Also, as the rate of incorporation of radionucleotides into RNA decreases, a point is reached where the rate of degradation of RNA exceeds the rate of transcription. This results in a graph with a peak as seen in Figure 3.5.A. It is not clear why the apparent RNase activity appears in nuclei from primary hepatocytes, but not from liver. One possibility is that the nuclei from the former contain

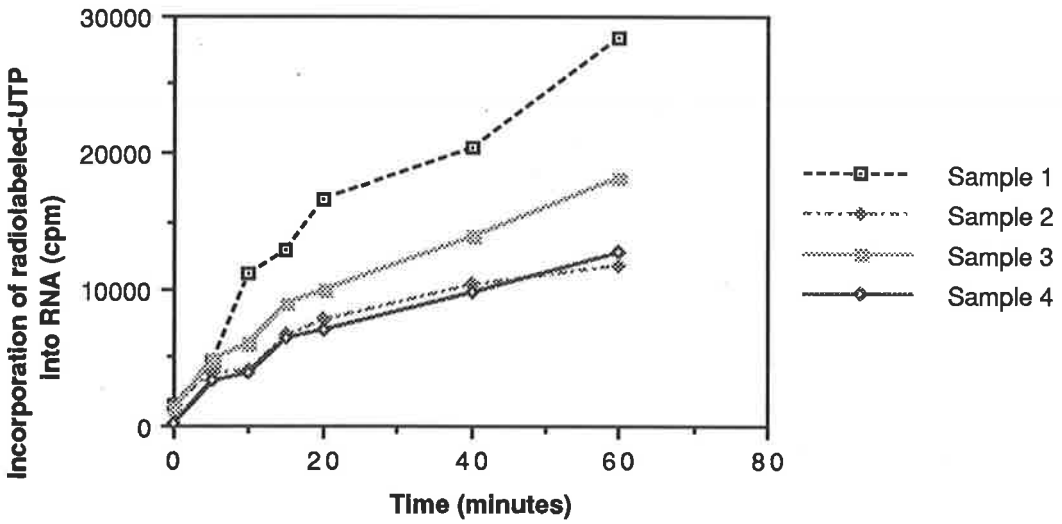
**Figure 3.5. Time course of incorporation of radiolabelled UTP into RNA in nuclei from chick embryo primary hepatocytes and liver**

Nuclei were isolated from either primary hepatocytes or the livers of chick embryos and the incorporation of radiolabelled UTP into $^{32}$P-RNA was determined by TCA precipitation at various time points during the run-on reaction. The incorporation profiles for four different samples of nuclei isolated from primary hepatocytes (**A**) and chick embryo livers (**B**) are shown.

A.


Incorporation of radiolabeled-UTP into RNA (cpm)
14000
12000
10000
8000
6000
4000
2000
0
0
20
40
60
80
Time (minutes)
Sample 1
Sample 2
Sample 3
Sample 4

B.


Incorporation of radiolabeled-UTP into RNA (cpm)
30000
20000
10000
0
0
20
40
60
80
Time (minutes)
Sample 1
Sample 2
Sample 3
Sample 4

more contaminating RNases than do liver nuclei due to the difference in procedures employed for nuclei isolation (see section 2.6.A). In light of these results, nuclear transcription run-on assays were carried out on nuclei isolated from chick embryo primary hepatocytes and liver using incubation times of 10 and 20 min, respectively, during which time the rate of incorporation of [$\alpha$-$^{32}$P]UTP into RNA was linear.

### *3.2.E.2. Quantitative hybridisation of $^{32}$P-RNA transcripts to filter-bound cDNA probes*

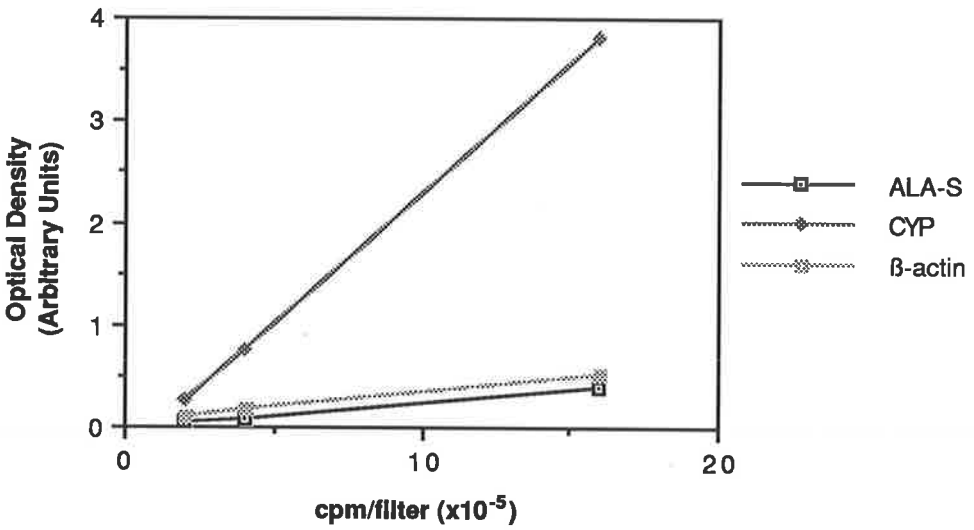
Important in the accurate measurement of the transcription rates of specific genes using nuclear transcription run-on assays is the demonstration that the quantitative detection of nascent $^{32}$P-RNA is obtained. To demonstrate this, increasing amounts of $^{32}$P-RNA, isolated from nuclei of primary hepatocytes treated with phenobarbital, were hybridised to filters containing a fixed amount (2 $\mu$g) of the specific cDNAs corresponding to CYP2H1, ALAS and ß-actin mRNAs. The results as shown in Figure 3.6, clearly show a linear relationship between total radioactivity added to the filters and densitometric quantification of mRNAs. The linear response occurs to at least 1.6 x $10^6$ cpm/filter of $^{32}$P-RNA from nuclei of phenobarbital-induced cultures. Based on these results, subsequent nuclear transcription run-on assays were performed using less than 1.6 x $10^6$ cpm/filter. Using the hybridisation conditions described in section 2.6.E, over 90% of the specific RNA transcripts have been shown to hybridise to the filter-bound cDNA probes (Hansen, 1989). Further examination of the data in Figure 3.6, revealed a linear response range of almost 100-fold, with slots varying in optical density from 0.04 to 3.8 arbitrary units. To ensure accurate quantification of autoradiographs by densitometry in future experiments, exposure times were adjusted to fall within this range of optical densities.

### *3.2.E.3. Analysis of the transcription rates of the* CYP2H1 *and ALAS genes in chick embryo primary hepatocytes in response to phenobarbital*

Primary hepatocytes were prepared and treated with phenobarbital as described in section 3.2.D and nuclei were isolated at times 0, 4, 12, 24 and 48 h after phenobarbital treatment. Nuclear transcription run-on assays were then performed, probing for

**Figure 3.6. Quantitative hybridisation of $^{32}$P-RNA synthesised in nuclei from chick embryo primary hepatocytes**

Primary hepatocytes were treated with phenobarbital for 4 h prior to isolation of nuclei. The nuclei were allowed to run-on in the presence of [$^{32}$P]-UTP and the synthesised $^{32}$P-RNA was purified. 2 x $10^5$, 4 x $10^5$ and 16 x $10^5$ cpm of TCA precipitable radioactivity was hybridised to separate filters each with slots containing 2 µg each of p105B1, pCHP3 and a ß-actin cDNA clone for detection of ALAS, CYP and ß-actin transcripts, respectively. Exposed slots on the resultant autoradiograph were quantified using a computing densitometer and the values plotted.


Optical Density
(Arbitrary Units)
4
3
2
1
0
0
10
20
cpm/filter (x10-5)
ALA-S
CYP
ß-actin

CYP2H1, ALAS and ß-actin transcripts. The resultant autoradiographs of this time course are shown in Figure 3.7.A. The slots were quantified using a computing densitometer and the relative rates of transcription for *CYP2H1* and ALAS standardised to ß-actin. The degree of non-specific hybridisation was determined using immobilised pBR322 plasmid DNA, the vector into which the cDNAs for *CYP2H1*, ALAS and ß-actin had been cloned. It can be seen from Figure 3.7.B that a low level of transcription of the *CYP2H1* gene was observed in nuclei isolated from untreated primary hepatocytes and that treatment of these hepatocytes with phenobarbital for 4 h, resulted in an increase in this transcription rate of approximately 10-fold. This rate increased further to a maximum of 36-fold measured 12 h after phenobarbital addition, but gradually declined to 9-fold at 48 h, indicating that the *CYP2H1* gene remains transcriptionally activated by phenobarbital 48 h after the initial addition of the drug. This time course of the rate of transcription of the *CYP2H1* gene following addition of phenobarbital closely resembles the increase in mRNA levels (compare Fig. 3.7.B with Fig. 3.4.B) where a maximal increase of 40-fold was obtained 12 h after phenobarbital administration. The results therefore indicate that the increase in the amount of the 3.5 kb CYP2H1 mRNA following drug-induction of hepatocytes can be accounted for predominantly by enhanced *CYP2H1* gene transcription.

A time course of the rate of transcription of the ALAS gene following addition of phenobarbital is shown in Figure 3.7.B. A low basal rate of transcription increased almost 5-fold after 4 h in the presence of drug and to a maximum of just over 6-fold after 12 h. The phenobarbital-induced increase in ALAS gene transcription then gradually decreased with time, returning almost to the basal rate after 48 h. A comparison of this time course of transcription rates with those of the ALAS mRNA levels in response to phenobarbital (see Fig. 3.4.B) revealed some interesting points. The rate of transcription of the ALAS gene reached a maximum level of 5-fold at 4 h, which is comparable to the almost 7-fold increase in ALAS mRNA observed at this time. This suggests that the amount of ALAS mRNA present at this time arises predominantly as a result of increased transcription. However, 12 h after phenobarbital administration, the rate of ALAS gene transcription peaked at approximately 6-fold above the basal rate, while the level of ALAS mRNA had dropped dramatically to only 2.5-fold above basal levels (see Fig. 3.4.B), suggesting that ALAS

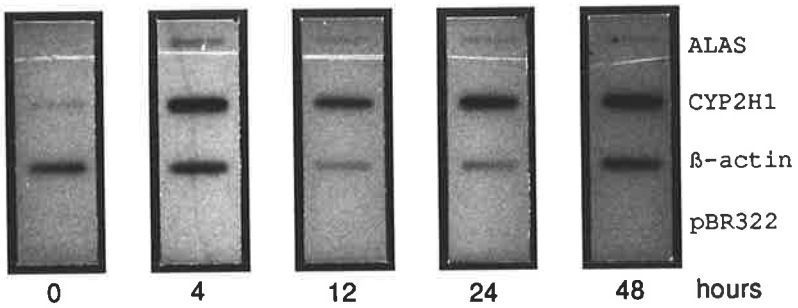
**Figure 3.7. Time courses of the rates of transcription of the *CYP2H1* and ALAS genes following phenobarbital treatment of chick embryo primary hepatocytes**

**A.** Primary hepatocytes ($1 \times 10^7$) were cultured in the presence of 500 μM phenobarbital for 0, 4, 12, 24 and 48 h. Nuclei were isolated, allowed to run-on in the presence of [α-$^{32}$P]UTP, and the nascent $^{32}$P-RNA transcripts were hybridised to slots of filter-bound p105B1, pCHPB15 and a ß-actin cDNA for detection of ALAS, CYP2H1 and ß-actin transcripts, respectively. pBR322 DNA was also present on the filter as a negative control. The resultant autoradiograph is shown.

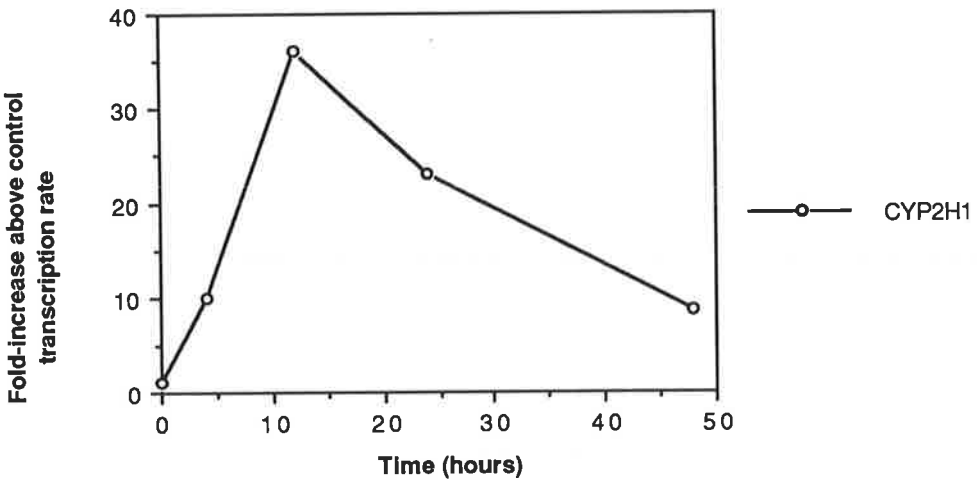
**B.** The transcription rates were quantified from the exposed slots using a computing densitometer. The rate of transcription of the *CYP2H1* and ALAS genes were standardised to that of the ß-actin gene and the fold-increase in this rate compared to the zero time point was plotted. Non-specific hybridisation to pBR322 was undetectable.

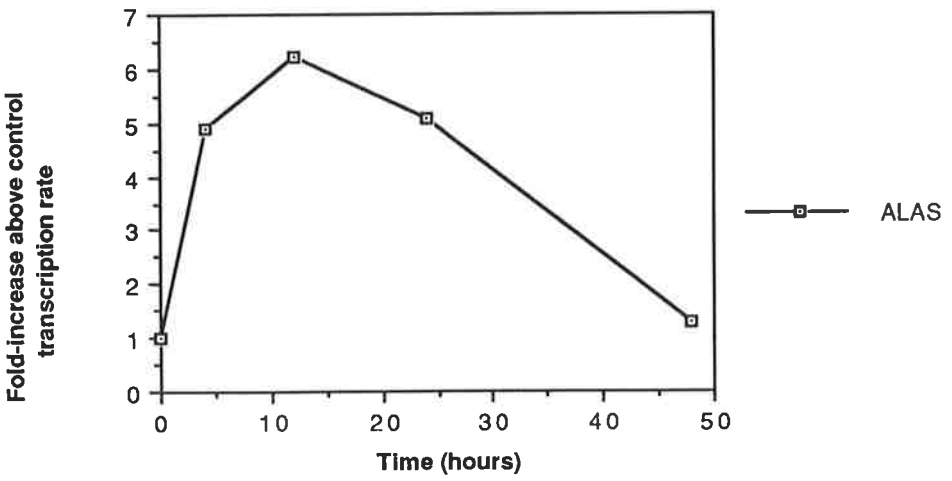
This experiment was repeated and yielded essentially similar results (not shown).

A.


ALAS
CYP2H1
ß-actin
pBR322
0
4
12
24
48
hours

B.


Fold-increase above control transcription rate
40
30
20
10
0
0
10
20
30
40
50
Time (hours)
CYP2H1


Fold-increase above control transcription rate
7
6
5
4
3
2
1
0
0
10
20
30
40
50
Time (hours)
ALAS

mRNA is being destroyed by a post-transcriptional mechanism at the later times. This may be a result of haem accumulation and this is dealt with in the discussion.

Another interesting observation is that the time courses of transcription rates of the *CYP2H1* and ALAS genes in response to phenobarbital follow a similar trend, peaking at 12 h and then decreasing gradually. A difference however, exists in the magnitude of induction by phenobarbital. The *CYP2H1* gene transcription rate increased maximally by 36-fold compared with only 6-fold for the ALAS gene. In addition, 48 h after phenobarbital administration, the rate of transcription of the *CYP2H1* gene was still 10-fold above the basal rate while that for the ALAS gene had almost returned to the basal rate. The reason for these differences is not clear at this stage.

In contrast to the *CYP2H1* and ALAS genes, the transcription rate of the ß-actin gene remained relatively unchanged over the 48 h period of drug treatment. Although in Figure 3.7.A, there is an apparent reduction in the transcription rate of the ß-actin gene at 12 and 24 h, this was not observed in repeated experiments. These results therefore demonstrate that there is not a general effect of phenobarbital on gene transcription.

### 3.2.F. Effect of haem on the rates of transcription of the *CYP* and ALAS genes in chick embryo liver and primary hepatocytes

As shown earlier in section 3.2.A and 3.2.C, ALA or haemin markedly reduced phenobarbital-induced ALAS mRNA amounts after 4 h in chick embryo liver and in primary hepatocytes while not affecting CYP mRNA levels. It was important then to determine whether haem repressed ALAS gene transcription in these systems.

Chick embryos and cultures of primary hepatocytes were treated in the following way prior to isolation of nuclei. Batches of six 18 day old chick embryos were treated by injection into the air sac and layering on top of the allantoic membrane with the following; dimethylsulfoxide (solvent), phenobarbital, phenobarbital and haemin, or phenobarbital and ALA. After 4 h incubation, the chick embryos were sacrificed, the livers removed and pooled for each experiment, and nuclei isolated as in section 2.6.A. Primary hepatocytes were treated with the same compounds and combinations of compounds as described above,

and similarly incubated for 4 h to enable the compounds to take effect. Hepatocytes were then harvested and nuclei isolated (see section 2.6.A).

The nuclei isolated from chick embryo livers and primary hepatocytes were used in nuclear transcription run-on experiments. The specific transcripts for ALAS, CYP and ß-actin were hybridised to the appropriate filter-bound cDNAs and the amounts of each transcript detected using autoradiography (for detailed methods, see sections 2.6.B to 2.6.E). In this set of experiments, the probe pCHP3 was used and therefore the results for CYP refer to a composite of the three phenobarbital-inducible CYP mRNAs. As the 3.5 kb CYP2H1 mRNA is predominant, representing over 50% of the total mRNA hybridisable to pCHP3 (see Fig. 3.2 and Fig. 3.3), the results can be approximated to those expected for the *CYP2H1* gene. However, these results will be referred to as those for the "phenobarbital-inducible CYPs" or "CYPs" for simplicity. Autoradiographs were quantified by densitometry and the results for ALAS and CYPs standardised to that of ß-actin in each experiment. The results of two separate experiments in chick embryo liver and 2 separate experiments in primary hepatocytes for both the ALAS and *CYP* genes are shown in Table 3.1. They have not been averaged because there was variation in the magnitude of the response to phenobarbital obtained in different experiments. This variation from experiment to experiment is commonly seen in chick embryo liver and to a lesser extent in primary hepatocytes, but the precise reason for this is not clear. From the data in Table 3.1, its is clear that phenobarbital caused an increase in the rate of transcription of the ALAS and *CYP2H1* genes in both primary hepatocytes and liver nuclei, as expected from previous work. The fold induction of both genes was generally higher in liver nuclei, as found previously. Somewhat unexpectedly, ALA and haem did not repress the phenobarbital-induced rate of transcription of the ALAS gene in either liver or primary hepatocytes. Indeed, it appears that haem may slightly stimulate ALAS gene transcription. As expected, haem and ALA exerted no significant effect on the rate of transcription of *CYP* genes. From these findings, it therefore appears that the significant and specific reduction in ALAS mRNA amounts following haemin or ALA treatment, occurs by a post-transcriptional mechanism in both chick embryo liver and primary hepatocytes.

**Table 3.1. Effect of haem on the rates of transcription of the ALAS and *CYP* genes in chick embryo liver and primary hepatocytes**

Chick embryos (17 days old) were injected in triplicate with either phenobarbital (8 mg), phenobarbital (8 mg) and haemin (4.9 mg), phenobarbital (8 mg) and ALA (10 mg) or with dimethylsulfoxide as control. The compounds were administered in a final volume of 200 µl of dimethylsulfoxide by injection into the air sac and layering on top of the allantoic membrane. After 4 h incubation, the embryos were sacrificed, the livers removed and appropriate ones pooled and nuclei isolated. Primary hepatocytes were similarly treated for 4 h with either phenobarbital (500 µM), phenobarbital (500 µM) and haemin (10 µM), phenobarbital (500 µM) and ALA (40 µM) or PBS as control prior to isolation of nuclei. Following run-on assays, autoradiographs were quantified using a computing densitometer and the values obtained for ALAS and CYP were standardised to those of ß-actin. These results have been expressed in relation to the control in each experiment, which consequently has a value of 1.0. The results of two separate nuclear transcription run-on experiments for ALAS and *CYP* in both chick embryo liver and primary hepatocytes are shown.

# Table 3.1

| Condition | Gene transcription rate for ALAS | | | | Gene transcription rate for CYP | | | |
|---|---|---|---|---|---|---|---|---|
| | liver | | primary hepatocytes | | liver | | primary hepatocytes | |
| | Expt 1 | Expt 2 | Expt 1 | Expt 2 | Expt 3 | Expt 4 | Expt 3 | Expt 4 |
| Control | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| PB | 9.5 | 24.4 | 1.8 | 2.1 | 45.7 | 5.7 | 5.4 | 7.9 |
| PB + haemin | 14.6 | - | 1.5 | 3.7 | 38.5 | - | 3.6 | 10.4 |
| PB + ALA | 14.5 | 30.6 | - | 4.3 | 40.3 | 8.3 | - | 10.1 |

In summary, phenobarbital markedly induced the transcription rates of the ALAS and *CYP* genes within 4 h of administration, but haemin or ALA administration did not inhibit these rates. No further attempt was made to study the role of haem in the regulation of ALAS expression. Instead, the clear transcriptional response of the ALAS and *CYP2H1* genes to phenobarbital was pursued (see Chapter 4, 5 and 6).

## 3.3. DISCUSSION

Granick (1966) first showed that in cultures of chick embryo primary hepatocytes porphyrinogenic drugs, known to induce hepatic CYPs, significantly increased the level of ALAS enzyme activity while haemin addition prevented this induction. As described earlier, when work in this thesis was initiated, it was known that haem inhibited the transport of precursor ALAS into mitochondria (Hayashi *et al.*, 1972; Srivastava *et al.*, 1983) and it was assumed that haem also repressed transcription of the ALAS gene (May *et al.*, 1986). In addition, drugs such as phenobarbital were assumed to enhance ALAS levels by inducing *CYP* gene transcription with the resulting CYP apoprotein sequestering haem and lowering inhibitory levels. In this chapter, the regulation of ALAS and CYP has been investigated both in chick embryo and chick embryo primary hepatocytes by determining mRNA levels and gene transcription rates and as a result of this data, a new model for the regulation of ALAS in chick embryos is proposed as described later.

It has been established that ALAS mRNA and the three CYP mRNAs induced by drugs *in ovo* in chick embryo liver (Hansen *et al.*, 1989) are also markedly increased in chick embryo primary hepatocytes following drug addition. It was of importance to compare the rates of transcription and amounts of mRNA for the ALAS and *CYP* genes both in chick embryo primary hepatocytes and in chick embryo liver following drug treatment. It has been shown that in chick embryo primary hepatocytes treated with phenobarbital, an early increase in the amount of ALAS mRNA was a consequence of enhanced ALAS gene transcription. However, at later times (*i.e.* between 4 and 12 h) there was a noticeable post-transcriptional effect resulting in a significantly decreased level of ALAS mRNA despite a continued elevated rate of ALAS gene transcription. A possible explanation for this is given later in this discussion. These results differed from those

reported in chick embryo liver where the induction of ALAS mRNA was accounted for entirely by enhanced transcription at all times examined (Hansen *et al.*, 1989).

With respect to CYP2H1 mRNA levels, the magnitude of response to phenobarbital was similar in chick embryo liver and primary hepatocytes where the levels were increased maximally by 50-fold and 40-fold, respectively. The rate of transcription of the *CYP2H1* gene in primary hepatocytes increased maximally after 12 h by 36-fold in response to phenobarbital, which corresponded to the 40-fold increase in mRNA levels. This implied that induction of CYP2H1 mRNA by phenobarbital occurs predominantly at the transcriptional level. *In ovo*, however, a maximal increase in the rate of *CYP2H1* gene transcription of only 6-fold was reported, suggesting that a marked post-transcriptional mechanism is responsible for the 50-fold increase in CYP2H1 mRNA amounts (Hansen *et al.*, 1989). Studies of gene expression in mouse (Clayton *et al.*, 1985b) and rat (Jefferson *et al.*, 1984) primary hepatocyte cultures and hepatoma cell lines have demonstrated that mRNA levels of several hepatocyte-specific genes are regulated in a post-transcriptional manner, whilst the same mRNAs are regulated solely by transcription *in vivo*. Similarly, the *CYP1A1* (P450c) gene has been shown to be under a far greater degree of transcriptional control by TCDD *in vivo* than is the case in primary hepatocyte cell cultures (Pasco *et al.*, 1988). The reverse situation appears to be the case for the response of the chicken *CYP2H1* gene to phenobarbital in chick embryo primary hepatocytes with respect to the *in ovo* situation. Why this difference between liver cells and cultured primary hepatocytes exists is, at present, not clear.

Haemin or ALA clearly lowered levels of the mRNA for ALAS both in chick embryo liver and in primary hepatocytes and others have recently reported similar findings (Hamilton *et al.*, 1988; Ryan and Ades, 1989). However, no evidence was obtained in the present work for haemin or ALA inhibiting the drug-induced transcription of the ALAS gene either in primary hepatocytes or chick embryo liver. The absence of such a repression was entirely unexpected, especially since in adult rats, there is convincing evidence that haemin or ALA injections inhibit transcription of the ALAS gene (Srivastava *et al.*, 1988; 1990). It is intriguing that haem does not affect transcription of the ALAS gene either in chick embryo liver or primary hepatocytes. It is possible that this reflects an embryonic situation in some

unknown way and similar experiments with haemin injections in adult chickens would be of interest. It should also be kept in mind that in these studies, the transcriptional response to haem was investigated only at 4 h after its addition, and hence possible transcriptional regulation of ALAS may occur at later times. A time course measuring the rate of transcription of the ALAS gene, following the addition of haemin, is therefore required.

The fact that the ALAS mRNA amounts decreased in both chick embryo liver and primary hepatocytes following haemin or ALA treatment, without altering ALAS gene transcription, suggests strongly a post-transcriptional mechanism by haem. In this regard, several papers have been recently published showing that the addition of haemin to chick embryo primary hepatocytes reduces the half-life of ALAS mRNA (Drew and Ades, 1989a) and that haem-stimulated degradation of the ALAS mRNA may require a labile protein factor (Ryan and Ades, 1989; 1991). While these results are of great interest, there was no attempt to establish whether haem is specifically enhancing degradation of the ALAS mRNA nor whether the putative labile repressor protein specifically prevents ALAS mRNA degradation. In the present studies it has been shown that there is no effect of haemin or ALA treatment on drug-induced mRNA amounts for CYPs either in chick embryo liver or primary hepatocytes. This finding suggests that haem may selectively enhance degradation of the ALAS mRNA. It will be of interest to determine those features of the ALAS mRNA that are required for this haem-modulated destabilisation. Transfection studies using appropriate recombinant DNA constructs in which regions of the ALAS mRNA are fused to a reporter mRNA could be used to investigate this.

As mentioned earlier in the discussion, phenobarbital treatment of chick embryo primary hepatocytes resulted initially in an increased ALAS mRNA level due predominantly to an enhanced rate of transcription of the ALAS gene, but at later times the ALAS mRNA level declined despite continued transcription of the gene. This suggested that a post-transcriptional mechanism was operating to reduce ALAS mRNA amounts. A possible explanation for this is that following ALAS induction by drug, haem accumulates to a level within the cell in excess of that required by CYP apoproteins, and consequently enhances the degradation of ALAS mRNA. An experiment in which 4,6-dioxoheptanoic acid (*i.e.* succinyl acetone), a specific inhibitor of haem biosynthesis, is administered with drug would

be expected to prevent this post-transcriptional effect, but this experiment has not been undertaken at this time.

Studies in the human hepatoma cell line HepG2 revealed that haemin or ALA treatment did not affect the rate of ALAS transcription, but markedly reduced ALAS mRNA amounts (Healy, 1990) suggesting, as in chick embryo liver and primary hepatocytes, a post-transcriptional effect of haem. It is also known that ALAS mRNA from the livers of rats and chick embryos are very unstable with half-lives of 20 and 220 min, respectively (Yamamoto *et al.*, 1988; Drew and Ades, 1989a). Presumably then, the half-life of the human hepatic ALAS mRNA will also be short. Interestingly, the presence of an AUUUA pentamer sequence is found in the 3' untranslated region of those mRNAs which have extremely short half-lives of 10 to 30 min (Malter, 1989). This sequence is known to be tightly bound by a protein which appears to target the bound mRNA for rapid cytoplasmic degradation. A search was therefore made for AUUUA pentamers within their 3' untranslated regions of the chicken (Borthwick *et al.*, 1985), rat and human (Bawden *et al.*, 1987) house-keeping ALAS cDNA sequences. Although AU-rich regions were identified, no such pentamers were found, raising the possibility that other sequences are important for the instability of these ALAS mRNAs and presumably also for the apparent haem-mediated degradation of the ALAS mRNA. The sequences which regulate the stability of the ALAS mRNA need not necessarily be confined to the 3' untranslated region. For instance, the 5' untranslated region of the chicken vitellogenin mRNA (UUCACCUUCGCU) is necessary for stabilisation of this mRNA by oestrogen (Liang and Jost, 1991) while the mRNAs for c-*fos* and c-*myc* contain sequences within the protein coding region that mediate their selective destabilisation (Shyu *et al.*, 1991; Wisdom and Lee, 1991). In the 5' untranslated region of the chicken, rat and human ALAS mRNAs, there was no obvious common sequence nor was there any strong similarity with those sequences reported to affect stability in other mRNAs.

The finding that haem does not affect CYP mRNA levels in chick embryo liver or primary hepatocytes is in contrast to the findings of Srivastava *et al* (1988; 1990), where the mRNAs for CYPs and several other proteins involved in drug metabolism were found to be lowered in rat liver following haemin injections. However, Padmanaban and co-workers

have evidence, contradictory to this, which indicates that haem is required for the transcriptional activation of *CYP* genes in rat liver (Dwarki *et al.*, 1987; Bhat and Padmanaban, 1988; Rangarajan and Padmanaban, 1989) and further work to clarify this situation in rats is needed.

As haem does not repress transcription of the ALAS gene in chick embryo liver or primary hepatocytes, then the original model proposed for drug-induction of the ALAS and CYP proteins is untenable, at least in this system. In this model (May *et al.*, 1986) it was proposed that CYP induction by drugs was a prerequisite for removal of haem thereby allowing derepression of ALAS gene transcription. An alternative model is one in which drugs activate transcription of the genes for ALAS and *CYPs* through a common mechanism, not involving haem, and haem then feedback controls levels of ALAS by post-transcriptional mechanisms. From current work, it can be predicted that haem functions predominantly at two levels to repress expression of active ALAS enzyme and hence feed-back repress its own synthesis. It is suggested that the first and major effect of haem is to block transport of ALAS precursor protein into the mitochondria (Srivastava *et al.*, 1983). Such a haem-dependent block would very rapidly halt haem synthesis, in view of the short half-life of the ALAS enzyme in the mitochondria (Hayashi *et al.*, 1980; Ades *et al.*, 1983). A second level of regulation, described here, is the haem-modulated reduction in ALAS mRNA most likely due to a decreased in the stability of this mRNA. Whether such a post-transcriptional mechanism would operate at a cellular haem level higher than that needed to inhibit transport of precursor ALAS protein remains to be established. The proposal that haem acts at two levels to repress ALAS synthesis, indicates that it is very important for the cell to tightly control the production of haem and its precursors, since excessive levels of haem precursors are known to be associated with a porphyric attack in humans. However, ALAS synthesis must also be carefully regulated so that sufficient haem is produced for the respiratory cytochromes essential in cellular respiration and hence survival, as well as for the CYPs involved in protection and functioning of the cell.

In view of the present findings, the "haem repression model" must be modified at least for synthesis of CYPs and ALAS in chick embryo liver and primary hepatocytes, and it is now proposed that drugs activate ALAS and *CYP* gene transcription through a common

mechanism, yet to be discovered. The remainder of this thesis concentrates on investigating the mechanism by which phenobarbital exerts its effect at the transcriptional level. Transient expression studies in chick embryo primary hepatocytes of chimeric constructs containing the ALAS and *CYP2H1* promoters fused to the chloramphenicol acetyltransferase reporter gene have been used to search for *cis* -acting regions responsive to phenobarbital.

# CHAPTER 4

# EXPRESSION OF THE CHICKEN HEPATIC ALAS/CAT CONSTRUCTS IN CHICK EMBRYO PRIMARY HEPATOCYTES

## 4.1. INTRODUCTION

In this chapter, the mechanism of transcriptional regulation of the chicken ALAS house-keeping gene in the homologous chick embryo primary hepatocyte culture system has been investigated. Special emphasis has been placed on the mechanism of regulation by phenobarbital and "phenobarbital-like" drugs. Various lengths of the 5' flanking region of the chicken ALAS gene have been fused to the bacterial chloramphenicol acetyltransferase (CAT) gene and transient transfection studies carried out in primary hepatocytes.

## 4.2. RESULTS

### 4.2.A. Preparation of ALAS promoter/CAT chimeric constructs

Previously, in this laboratory, a full length chick embryo hepatic ALAS cDNA clone p105B1 (Borthwick *et al.*, 1985) was used to screen a chicken genomic Charon 4A λ library. A clone designated λcALA-S 1 was isolated and shown to contain the entire chicken hepatic ALAS gene including approximately 7 kb of 5' flanking region and 3 kb of 3' flanking region. The sequence of the entire gene including approximately 1 kb of both 5' and 3' flanking region was determined (Maguire *et al.*, 1986). This clone was the master from which sub-clones were made and various lengths of 5' flanking region obtained in the present work.

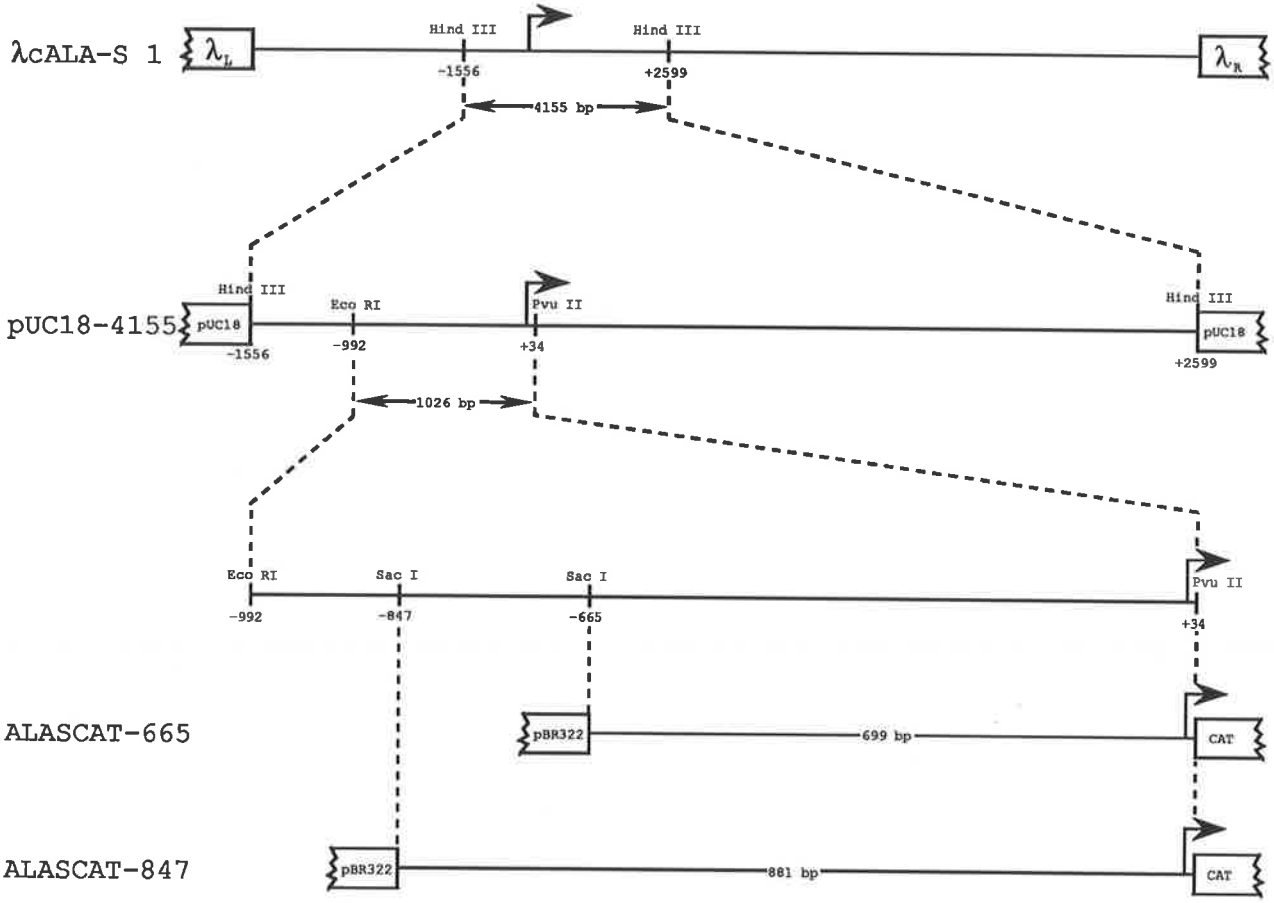
The vector pBRCAT was constructed by force cloning the 1.6 kb *Hind* III/*Bam* HI fragment containing the CAT gene from pSV2CAT, into *Hind* III/*Bam* HI cut pBR322. It was into this vector that various lengths of the ALAS gene 5' flanking region were ligated (see later, Fig. 4.4). ALAS promoter/CAT reporter gene fusion constructs have been designated ALASCAT-X, where "X" refers to the length in base pairs of 5' flanking region fused upstream of the CAT reporter gene. All constructs contained different lengths of 5' flanking region (from -1700 to -163 bp), but have been restricted at a common 3' *Pvu* II site and inserted into the unique *Hind* III site in pBRCAT by blunt-end cloning such that they contain 34 nt of the 5' untranslated region of the ALAS gene.

Constructs ALASCAT-163, -254 and -504 were made by Adrienne Day prior to my commencement on this project, in the following way. Two M13mp19 sub-clones of fragments from λcALA-S 1 were initially made for sequencing purposes (Maguire *et al.*, 1986). One contained a 1924 bp *Bam* HI/*Eco* RI fragment (position -254 to +1670 bp) and the other a 538 bp *Hgi* AI/*Pvu* II fragment (position -504 to +34 bp). ALASCAT-254 was made by digestion of the M13mp19 clone containing the 1924 bp fragment with *Bam* HI and *Pvu* II. A 288 bp fragment was isolated, end-filled and ligated into the blunted *Hind* III site of pBRCAT in a 5' to 3' orientation with respect to the orientation of the CAT gene. ALASCAT-163 was made from ALASCAT-254 by simple digestion with *Sma* I and *Cla* I. This excised a 120 bp fragment which was discarded, and the resultant vector was end-filled and religated. ALASCAT-504 was constructed by restriction of the M13mp19 clone containing the 538 bp *Hgi* AI/*Puv* II fragment with *Eco* RI and *Xba* I, the M13mp19 polylinker sites immediately flanking this fragment. This fragment was then end-filled and ligated into the blunted *Hind* III site in pBRCAT.

The longer ALASCAT constructs ALASCAT-665, -847 and -1700 were made by the author using the following strategy. A 4155 bp *Hind* III fragment (from position -1556 to +2599 bp) obtained by digestion of λcALA-S 1 with *Hind* III (see Fig. 4.1), was sub-cloned into the unique *Hind* III site of pUC18 to create pUC18-4155. This sub-clone was then restricted with *Eco* RI/*Pvu* II and a 1026 bp fragment isolated. Partial *Sac* I digests were performed on this fragment and the desired 699 and 881 bp fragments were isolated (see Fig. 4.1). The 3' overhangs created by *Sac* I digestion were removed by digestion with Klenow fragment as described by Maniatis *et al* (1982) and both fragments were blunt-end cloned into the unique *Hind* III site of pBRCAT, which had been cut with *Hind* III, blunted by end-filling of the 5' overhang with Klenow fragment and dephosphorylated prior to cloning. All clones were screened by restriction mapping with *Eco* RI/*Bam* HI to obtain clones containing the appropriate 5' to 3' orientation of the ALAS promoter with respect to the CAT gene. Clones containing the 699 and 881 bp fragments in the appropriate orientation were designated ALASCAT-665 and ALASCAT-847, respectively. These clones contained 665 and 847 bp of 5' flanking region, respectively in addition to the 34 bp of 5' untranslated region.

**Figure 4.1. Construction of ALASCAT-665 and ALASCAT-847**

A flow diagram of the strategy by which ALASCAT-665 and ALASCAT-847 were constructed is shown. In brief, λcALAS 1 was cut with *Hind* III and a 4155 bp fragment cloned into pUC19 and the resultant clone designated pUC19-4155. A 1026 bp *Eco* RI/*Pvu* II fragment was excised from pUC19-4155, isolated and partially digested with *Sac* I. Two of the resultant fragments of size 699 and 881 bp were isolated, blunted and cloned into the *Hind* III site of pBR322 that had been blunted. These clones were designated ALASCAT-665 and ALASCAT-847. Only the relevant restriction endonuclease sites that are important in the cloning events have been indicated for simplicity.


λcALA-S 1
λL
Hind III
-1556
Hind III
+2599
λR
4155 bp
pUC18-4155
pUC18
Hind III
-1556
Eco RI
-992
Pvu II
+34
Hind III
pUC18
+2599
1026 bp
Eco RI
-992
Sac I
-847
Sac I
-665
Pvu II
+34
ALASCAT-665
pBR322
699 bp
CAT
ALASCAT-847
pBR322
881 bp
CAT

The longest construct, ALASCAT-1700 was made as follows (see Fig. 4.2). The approach taken was to determine and isolate a *Pvu* II fragment whose 3' end resided at position +34 bp of the gene and whose 5' end was known to be beyond position -1556 bp (*Hind* III), the 5'-most limit of known sequence. λcALA-S 1 was initially cut with *Kpn* I and a 26 kb fragment isolated. This was done to remove 22.5 kb of λ arm sequence, for which the number and position of *Pvu* II sites was not known. The 26 kb fragment was then completely digested with *Pvu* II resulting in at least 15 fragments which were subsequently transferred by Southern blotting to nitrocellulose and probed with the *Hind* III/*Bam* HI fragment (-1556 to -254 bp) (see Fig. 4.3). A fragment of approximate size 1730 bp was detected. The 26 kb fragment was once again completely cut with *Pvu* II, the corresponding 1730 bp fragment excised and this was cloned into the previously described dephosphorylated and blunted *Hind* III site of pBRCAT (see Fig. 4.2). The appropriately orientated clone designated ALASCAT-1700 was detected by restriction mapping using *Eco* RI/*Bam* HI digestion. This construct contained 1700 bp of 5' flanking region in addition to 34 bp of 5' untranslated region.

The constructs made therefore covered the first 1700 bp of 5' flanking region of the ALAS gene (see Fig. 4.4). The next phase of this work was to study transient expression of these constructs in chick embryo primary hepatocytes. Since no expression studies had been undertaken previously in these cells, it was necessary to investigate a procedure for introduction of constructs into these primary hepatocytes and to investigate use of the published procedure of Gorman *et al* (1982) for determination of CAT activity.

### 4.2.B. Optimisation for electroporation of chick embryo primary hepatocytes

Reports at the time of the commencement of this work showed that primary rat hepatocytes could not be transfected using the commonly used calcium phosphate precipitation or DEAE-dextran techniques due to the toxicity of some of the transfection components (Tur-Kaspa *et al.*, 1986). In view of this, it was decided to investigate the use of electroporation for introduction of DNA constructs into chick embryo primary hepatocytes. The conditions required for transfection of DNA by electroporation into cells

**Figure 4.2. Construction of ALASCAT-1700**

A flow diagram of the strategy used for the construction of ALASCAT-1700 is shown. In brief, λcALAS 1 was cut with *Kpn* I and a resultant 26 kb fragment isolated. This 26 kb fragment was digested with *Pvu* II to produced at least 15 fragments. The desired *Pvu* II fragment containing the ALAS transcription start site was screened for by Southern blot analysis using the shown *Hind* III/*Bam* HI fragment (position -1556 to -254 bp) as a probe (see Fig. 4.3). A fragment of size 1735 bp was detected, isolated and cloned into the *Hind* III site of pBR322 that had been blunted. This construct was designated ALASCAT-1700. Only the relevant restriction endonuclease sites that are important in the cloning events have been indicated for simplicity.

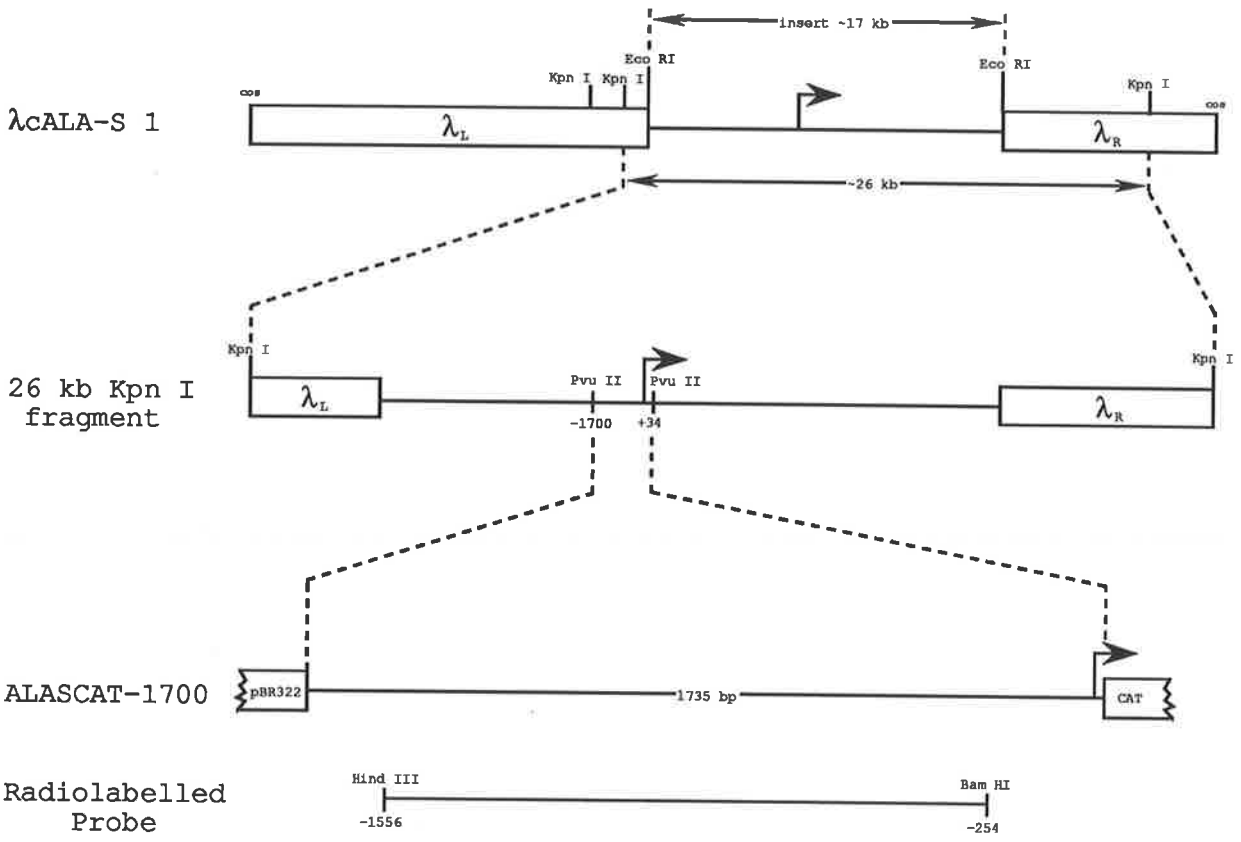

λcALA-S 1
insert ~17 kb
Eco RI
Kpn I
Kpn I
Eco RI
Kpn I
cos
cos
λL
λR
~26 kb
26 kb Kpn I fragment
Kpn I
Pvu II
Pvu II
Kpn I
λL
λR
-1700
+34
ALASCAT-1700
pBR322
1735 bp
CAT
Radiolabelled Probe
Hind III
Bam HI
-1556
-254

**Figure 4.3. Southern Blot Analysis of *Pvu* II cut λcALAS 1 to identify a fragment containing the ALAS gene promoter**

λcALAS 1 DNA was completely digested with *Pvu* II and run in a 1% agarose gel together with *Eco* RI cut SPP-1 marker DNA. Panel **A** shows a photograph of the ethidium bromide stained gel (lane 1, *Eco* RI cut SPP-1; lane 2, *Pvu* II cut λcALAS 1). Following transfer of the DNA fragments to nitrocellulose, the filter was probed with an oligolabelled *Hind* III/*Bam* HI fragment (position -1556 to -254 bp) of the ALAS gene promoter. The resultant overnight autoradiograph is shown in Panel **B** (lane 3, *Eco* RI cut SPP-1; lane 4, *Pvu* II cut λcALAS 1). The appropriate fragment of approximate size 1735 bp is shown.

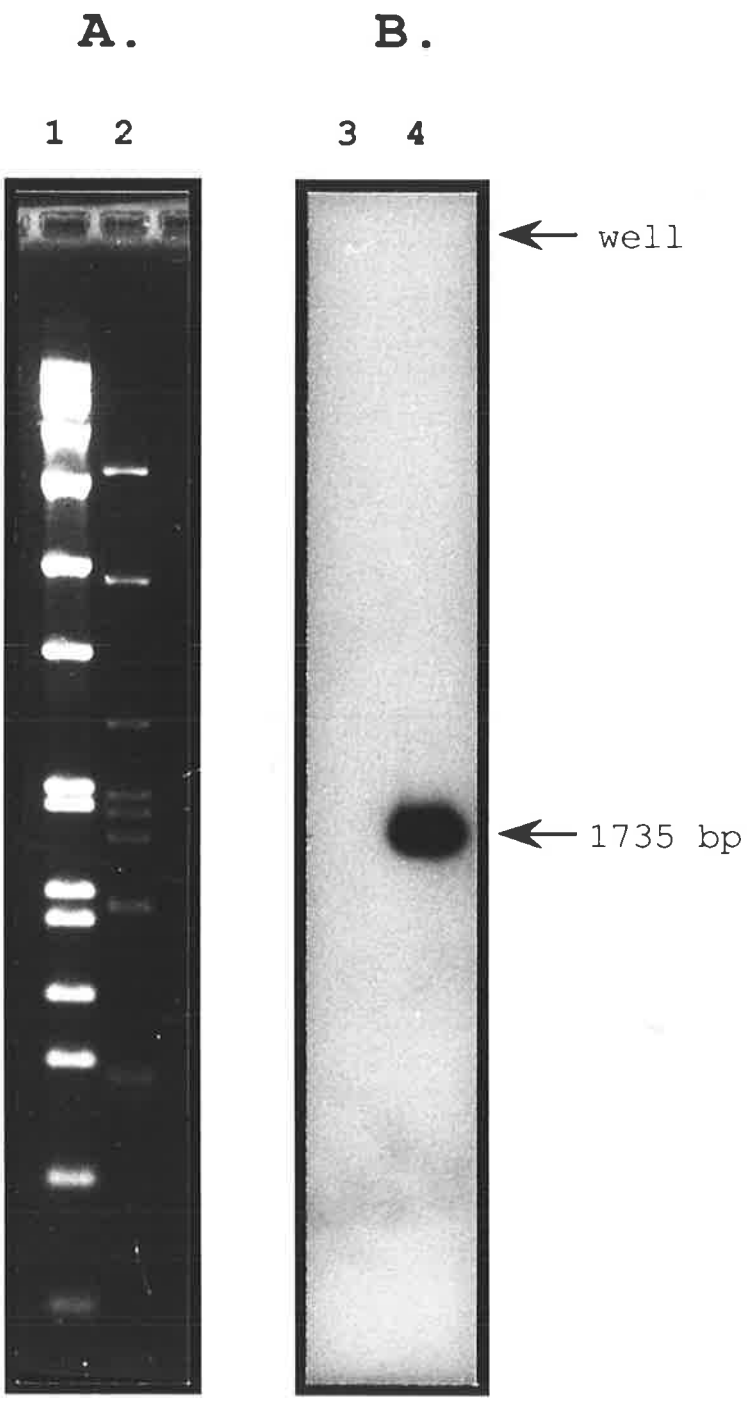
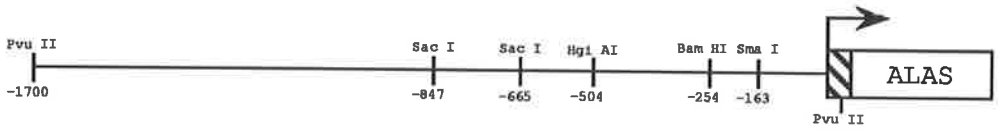

A.
B.
1 2
3 4
well
1735 bp

**Figure 4.4. Diagrammatic representation of the 5' portion of the chicken house-keeping ALAS gene and ALASCAT constructs**

**A.** A diagram of the chicken house-keeping ALAS gene and the 5' flanking region showing the positions of restriction sites important in the production of ALASCAT constructs. The line represents the 5' flanking region, the arrow shows the position of the transcription start site and the hatched region depicts the 5' untranslated region of the ALAS gene. **B.** A diagram of the ALASCAT constructs. The line represents the 5' flanking region of the ALAS gene and the hatched region represents the first 34 bp of the ALAS 5' untranslated region. Although shown as linear molecules, these constructs are circular containing pBR322 as the cloning vector.

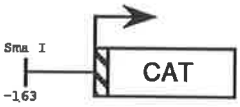
**A.**

ALAS gene

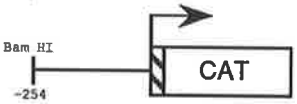



**B.**

ALASCAT-163




ALASCAT-254




ALASCAT-504


ALASCAT-665


ALASCAT-847


ALASCAT-1700

can vary dramatically between different cells and cell lines. Therefore a comprehensive set of experiments were carried out to optimise the conditions necessary for efficient transfection and expression of construct DNA. Electroporation by the method of Chu *et al* (1987) was chosen as a basis upon which initial optimisation experiments were performed.

### *4.2.B.1. Electroporation viability curve*

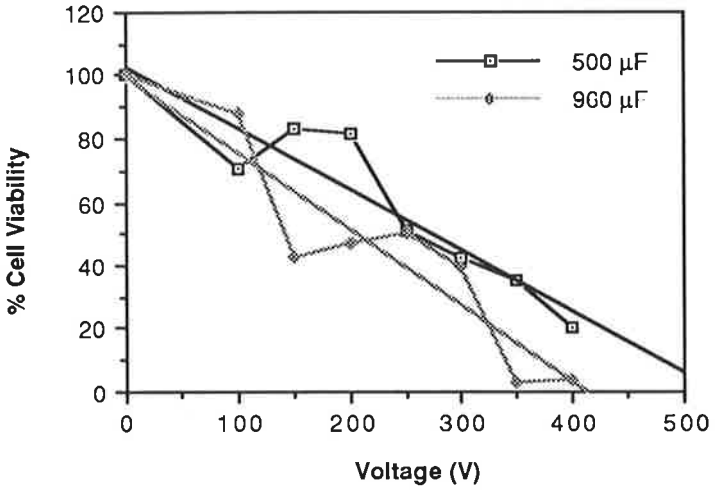
It is generally accepted that optimal transfection of cells using electroporation occurs with a voltage (V) at which approximately 50% of the cells are killed and it has also been reported that increased efficiency of transfection occurs with higher capacitances (Chu *et al.*, 1987). A viability curve relating percentage of cell survival to voltage was therefore performed using the highest capacitances available; these were 500 and 960 microFaradays (μF). Chick embryo primary hepatocytes (3 x $10^6$ cells) were resuspended in 800 μl of electroporation buffer (see section 2.7.A) and a solution of 10 mg/ml of sheared salmon sperm DNA added to a final concentration of 500 μg/ml. These cells were electroporated at voltages ranging from 0 to 400 V at fixed capacitances of 500 or 960 μF and then plated into 60 mm diameter plastic petri dishes containing 5 ml of William's E medium. Following incubation overnight, the hepatocytes were removed by trypsinisation and viable cells counted after staining with trypan blue. The results can be seen in Figure 4.5.A and demonstrate a 50% survival of cells at voltages of approximately 210 and 270 V for capacitances of 960 and 500 μF, respectively. A viability of 100% was assigned to the cells that were not electroporated (*i.e.* 0 V). It should be stressed that this approach is only an approximate guide for the optimisation of conditions for transfection, but it did narrow down the voltage range over which the following experiments were performed. A more accurate determination of optimal voltage is possible by actual transfection of a construct, known to express in the cell of interest, and subsequent measurement of the level of expression with increasing voltage. This approach is described in the next section.

**Figure 4.5. Effect of voltage and capacitance of electroporation on transfection efficiency of chick embryo primary hepatocytes**

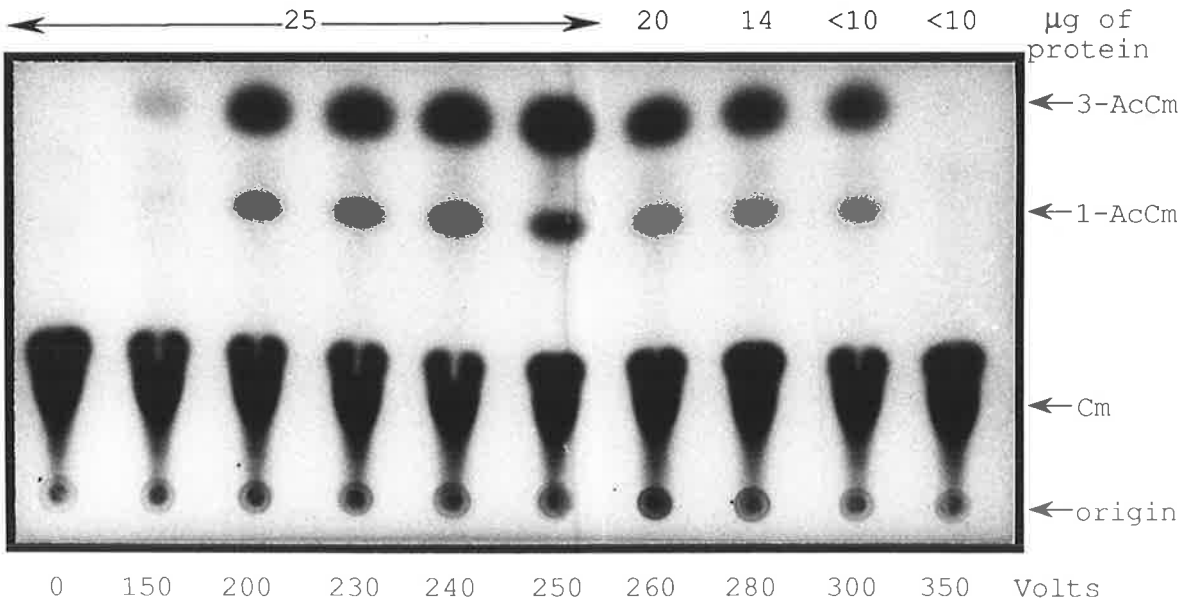
**A.** Electroporation cell viability curve. For experimental details see section 4.2.B.1. The cell viability curves with increasing voltage are shown for capacitances of 500 and 960 μF and the lines of best fit have been determined. Although the plotted results are not expected to be straight line graphs, this approximation is adequate for the purpose required.

**B.** Transfection efficiency optimisation by measurement of CAT activity. For experimental details, see section 4.2.B.2. Where possible, 25 μg of protein extract was used to assay for CAT activity. Where this was not possible, the total amount of extract obtained was assayed, and this amount is shown above each lane. An autoradiograph of the thin layer chromatography plate and the positions of the origin, unacetylated chloramphenicol (Cm) and the acetylated forms of chloramphenicol (3-AcCm and 1-AcCm) are shown.

**A.**


120
100
80
60
40
20
0
% Cell Viability
0
100
200
300
400
500
Voltage (V)
500 µF
960 µF

**B.**


25
20
14
<10
<10
µg of protein
3-AcCm
1-AcCm
Cm
origin
0
150
200
230
240
250
260
280
300
350
Volts

*4.2.B.2. Optimisation of transfection efficiency with electroporation by measurement of CAT activity*

The construct pSV2CAT contains the viral SV40 promoter/enhancer and was known to express CAT activity in a wide variety of cells, although expression in chick embryo primary hepatocytes had not been investigated. In initial experiments, pSV2CAT was introduced into chick embryo primary hepatocytes by electroporation and incubated in William's E medium for 48 h. The cells were then harvested, lysed by freeze/thawing three times and assayed for CAT activity. These assays were performed as described by Gorman *et al* (1982) such that all results fell within the linear range of the assay. Initially, no attempt was made to optimise the CAT assay, but optimisation is described later (see section 4.2.B.5). Preliminary experiments revealed that pSV2CAT could indeed be transfected and expressed in chick embryo primary hepatocytes and so it was used to further optimise the transfection efficiency of these cells. At this stage, it should be pointed out that several technical factors influenced this work. Firstly, primary hepatocytes from chick embryos were difficult to obtain in large quantities because only a limited number of chick embryos were available at any one time and the number of hepatocytes obtained from an embryonic liver was only about $2 \times 10^7$, thus limiting the numbers of experiments that could be performed simultaneously. Secondly, the ALASCAT constructs, being pBR322-based, were difficult to obtain in large quantities and this limited the amounts that could be transfected. Thirdly, expression from the ALASCAT constructs was expected to be low since only low levels of transcription from the ALAS gene were detected in nuclear run-on assays (see Chapter 3). These three factors were taken into account in the development of conditions for expression studies.

To study the effect of voltage of electroporation on transfection efficiency as measured by expression of CAT activity, the following experiment was performed. Primary hepatocytes ($3 \times 10^6$ cells) were electroporated at voltages ranging from 0 to 350 V with a capacitance of 960 μF in the presence of 500 μg/ml salmon sperm DNA and 10 μg of pSV2CAT plasmid DNA. Following transfection, the cells were plated and incubated in William's E medium for 48 h prior to harvesting and assaying for CAT activity. In the literature, 25 to 100 μg of cellular protein extract was generally used in CAT assays. The

amount chosen for use in this experiment was 25 μg due to difficulty in obtaining larger quantities. Figure 4.5.B demonstrates an optimum of CAT expression at 250 V. This does not really represent a true optimum, but rather the maximum amount of CAT activity obtainable from the designated 25 μg of protein extract assayed under the conditions used. It should be noted that at voltages above 250 V the number of cells that survived electroporation did not, when subsequently lysed, provide the full 25 μg of protein needed for the assay. Since the amount of extract assayed will be of importance in later experiments when comparing expression of different constructs, it is crucial that a consistent amount of extract is available for assay. Thus choosing an optimal voltage includes a balance between disrupting the cell membranes of as many cells as possible to maximise transfection efficiency while ensuring that enough cells survive this ordeal to be experimentally useful. Optimisation in this way can therefore be quite arbitrary. Experimentation revealed that the effect of voltage versus expression of CAT activity followed a similar trend for capacitances of both 500 and 960 μF, except that slightly higher levels of expression were seen with 960 μF (results not shown). On the basis of these results, a capacitance of 960 μF and a voltage of 250 V were chosen for all further work.

### *4.2.B.3. Effect of number of cells electroporated on expression of CAT activity*

A report by Chu *et al* (1987) has suggested that altering the number of cells transfected while keeping the amount of DNA constant, has little effect on transfection efficiency when the results are expressed as CAT activity per cell. This raised the possibility of increasing the overall sensitivity of the assay by using the same amount of DNA to transfect a greater number of cells. As the ALAS promoter was envisaged to function weakly in the non-induced state in chick embryo primary hepatocytes, as determined from nuclear transcription run-on and Northern analysis (Chapter 3), the effect of increasing the number of cells transfected on expression of CAT activity was examined.

To investigate this, a range of $3 \times 10^6$ to $3 \times 10^7$ cells were transfected in the presence of 500 μg/ml salmon sperm DNA with 10 μg of pSV2CAT at 250 V and 960 μF. The cells were then plated in William's E medium, incubated for 48 h, harvested, and 25 μg of protein extract assayed for CAT activity. In contrast to the results of Chu *et al* (1987), a

somewhat surprising result was obtained. From Figure 4.6, it can be seen that transfection efficiency measured as CAT activity per μg of protein extract assayed, increased in a linear fashion to 1 x $10^7$ cells/cuvette and then peaked at 2 x $10^7$ cells/cuvette before declining. It is possible that as the cell density increases, the chance of cells becoming transfected with DNA increases. Alternatively, the percentage of "competent" cells remains constant, but the number of constructs entering each competent cell increases causing an overall increase in CAT activity. These possibilities were not further investigated.

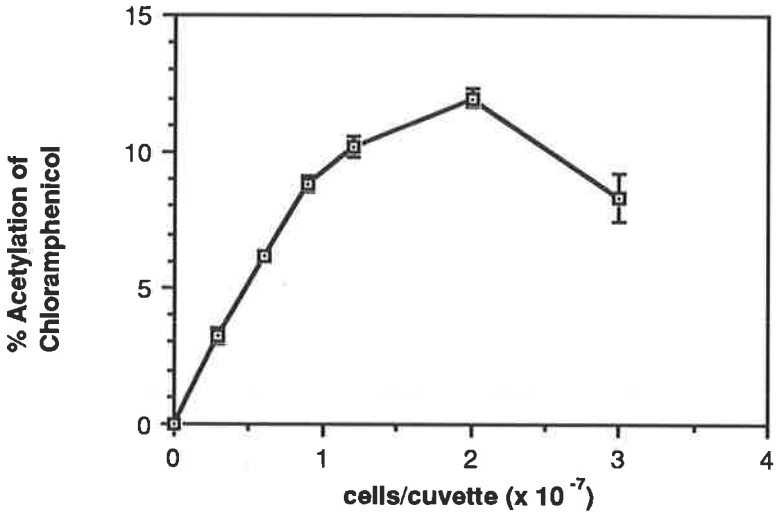
In earlier experiments described in Chapter 3, it was shown that 60 mm diameter petri dishes were capable of accommodating up to approximately 1 x $10^7$ primary hepatocytes without the loss of transcriptional response of the ALAS or *CYP2H1* genes to phenobarbital. As described above, the electroporation conditions employed resulted in approximately 50% cell death. Since it is known that chick embryo primary hepatocytes in culture do not continuously divide, but have the capacity to undergo one cell division, a density of 1 x $10^7$ cells/cuvette was initially chosen for electroporation. Therefore, following electroporation and cell division, approximately 1 x $10^7$ cells/dish would result. Based on preliminary expression studies using pSV2CAT, some variation in efficiency of transfection occurred between cuvettes. To minimise such variation in drug-induction experiments, 2 x $10^7$ cells/cuvette were electroporated and the cell sample divided into parts which were plated onto separate plates. One of these plates acted as a control without inducer and the other contained the drug or compound under test. This technique of splitting the electroporated sample was developed later and is used in Chapters 5 and 6. Using these plating densities, almost 100% cell confluency was obtained after 48 h of culture and this ensured a good yield of cellular protein for assaying CAT activity.

### *4.2.B.4. Effect of the amount of DNA transfected on expression of CAT activity*

The majority of published experiments on transient expression studies using electroporation or calcium phosphate precipitation involve transfection of cells with between 5 and 20 μg of plasmid DNA corresponding to 1.5 - 6.0 pmol of a 5 kb construct. A study was undertaken to determine the effect in chick embryo primary hepatocytes of varying the amount of DNA transfected by electroporation on CAT expression. This was performed

**Figure 4.6. Effect of the number of cells electroporated on transfection efficiency**

Primary hepatocytes ranging in number from 3 x $10^6$ to 3 x $10^7$ were transfected in the presence of 500 µg/ml salmon sperm DNA with 10 µg of pSV2CAT by electroporation at 250 V and 960 µF. The cells were then plated and incubated in William's E medium for 48 h before harvesting and lysis of the cells. CAT assays were determined on 25 µg of protein extract. Following autoradiography of the thin layer chromatography plate, the spots were excised, counted and the percentage of acetylated forms of chloramphenicol calculated. The mean of three determinations is plotted and the bars represent the standard deviation values.


% Acetylation of Chloramphenicol
15
10
5
0
0
1
2
3
4
cells/cuvette (x 10 -7)

with the aim of determining whether efficient expression of plasmid DNA could be obtained with less than the 10 μg of DNA used in previous experiments, since large quantities of the ALASCAT constructs were difficult to obtain.

Primary hepatocytes (1 x $10^7$) were electroporated with varying amounts of pSV2CAT DNA ranging from 0 to 10 μg at 250 V and 960 μF in the presence of 500 μg/ml salmon sperm DNA. These cells were plated into William's E medium and incubated for 48 h prior to harvesting, lysis and assaying of 25 μg of protein extract for CAT activity. As is shown in Figure 4.7.A, increasing the amount of the construct transfected leads to an increase in the overall level of CAT activity in a linear fashion. This may represent a greater percentage of cells transfected or a greater number of constructs transfected per cell or both. To determine which of these was occurring, a similar experiment to that above was performed except that the construct pRSVß-gal, containing the strong Rous Sarcoma Virus (RSV) long terminal repeat promoter/enhancer driving expression of the ß-galactosidase gene, was used. Amounts of construct ranging from 0 to 20 μg were transfected and the transfection efficiency measured by assaying for ß-galactosidase activity in cell extracts as well as *in situ* by staining the cells with X-gal. X-gal is converted into an insoluble blue dye in the presence of ß-galactosidase and molecular oxygen and therefore will stain cells which have been transfected with pRSVß-gal and express ß-galactosidase. This approach enabled the percentage of cells transfected to be determined rather than the level of expression from the cells.

A linear response of ß-galactosidase activity measured by the colourimetric assay on cell extracts, in relation to the amount of pRSVß-gal transfected was obtained (see Fig. 4.7.B) demonstrating that the more DNA used for transfection, the greater the number of constructs transfected and expressed. The percentage of cells transfected as measured by *in situ* X-gal staining also increased in a linear fashion to 5 μg (*i.e.* 1.25 pmol) of pRSVß-gal, but beyond this the increase in the percentage of cells transfected was no longer proportional to the amount of construct DNA transfected (see Fig. 4.7.C). Taken together, the results from Figure 4.7.B and Figure 4.7.C imply that increasing the amount of construct DNA transfected, initially resulted in an increase the number of transformants rather than transfection of more constructs into the same competent cells, but beyond 1.25 pmol of

**Figure 4.7. Effect of the amount of construct DNA transfected on transfection efficiency**

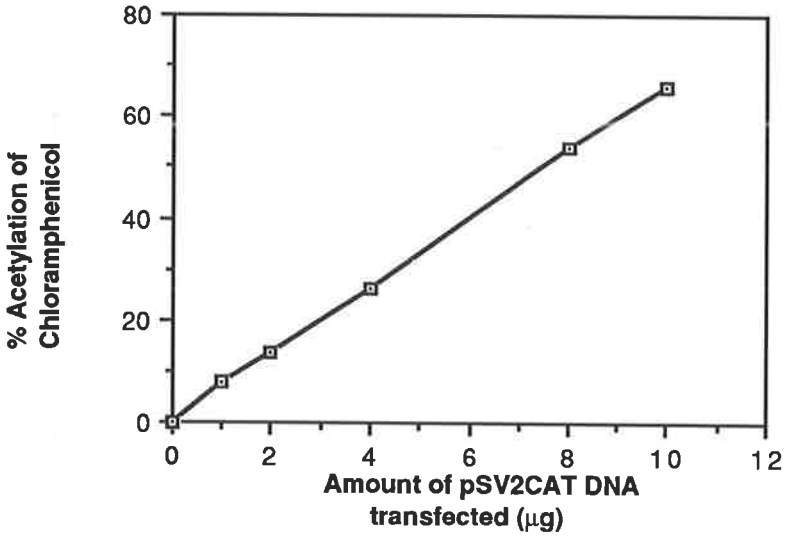
**A.** Assay for CAT activity. Primary hepatocytes ($1 \times 10^7$) were transfected in the presence of 500 µg/ml salmon sperm DNA with various amounts of pSV2CAT DNA, ranging from 0-10 µg. After 48 h, the hepatocytes were harvested and CAT activity was determined on 50 µg of protein extract. The results of duplicate transfections were averaged and plotted.

**B** and **C.** Primary hepatocytes ($2 \times 10^7$) were transfected in duplicate as in part A, except with pRSVß-gal DNA ranging from 0-20 µg. The sample was split and cultured in separate plates. After 48 h, the hepatocytes were analysed for ß-galactosidase activity in two different ways.

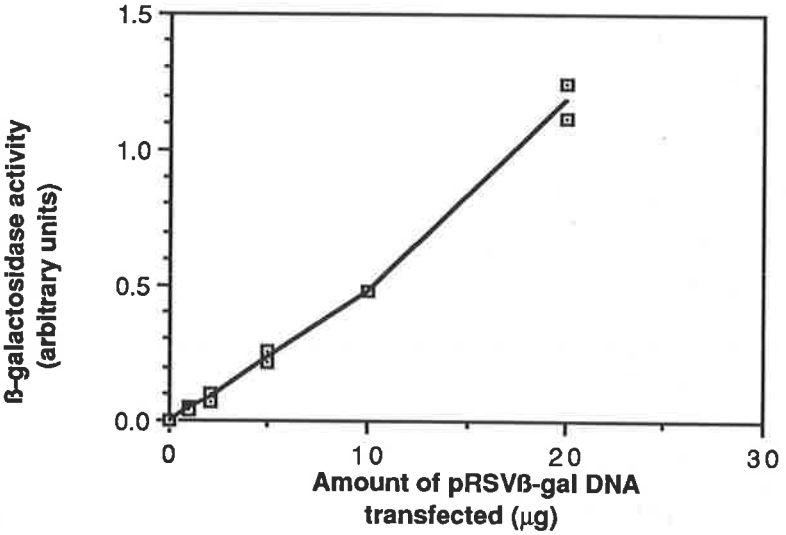
**B.** Assay for ß-galactosidase activity. Hepatocytes from one of each pair of plates were harvested for determination of ß-galactosidase activity. The results of duplicate transfections are shown.

**C.** Determination of the proportion of hepatocytes transfected by *in situ* staining for ß-galactosidase activity. Hepatocytes from the other of each pair of plates were fixed *in situ* and stained for ß-galactosidase activity with X-gal. Following staining, two representative fields of each plate were photographed and the percentage of blue-stained cells determined. The results of duplicate transfections are shown.

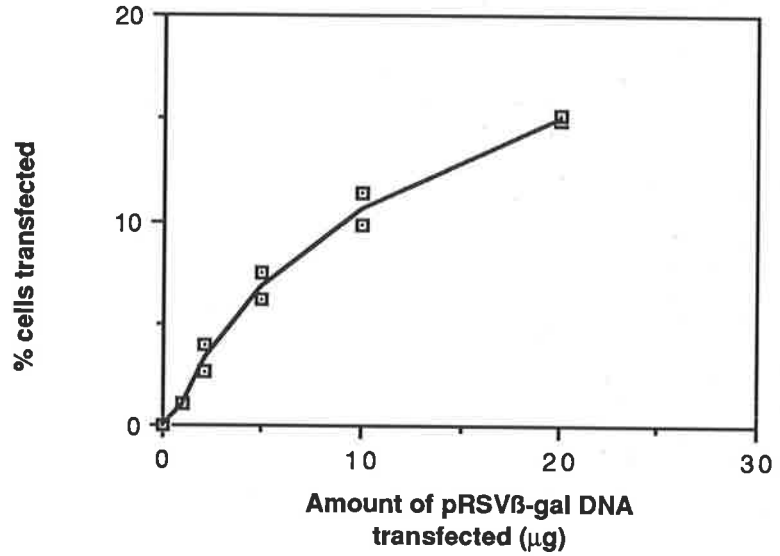
A.


% Acetylation of Chloramphenicol
80
60
40
20
0
0
2
4
6
8
10
12
Amount of pSV2CAT DNA transfected (μg)

B.


ß-galactosidase activity (arbitrary units)
1.5
1.0
0.5
0.0
0
10
20
30
Amount of pRSVß-gal DNA transfected (μg)

C.


% cells transfected
20
10
0
0
10
20
30
Amount of pRSVß-gal DNA transfected (μg)

construct DNA, the number of construct DNA molecules transfected per cell began to increase. With a DNA to cell ratio of approximately 1.5 x $10^5$ : 1 (*i.e.* 10 µg of the ~6 kb pRSVß-gal : 1 x $10^7$ cells), it seems clear that a vast excess of construct DNA is required for efficient transfection. As already stated, these transfection experiments were all performed using salmon sperm DNA as carrier at the final concentration of 500 µg/ml which was found to be optimal by Chu *et al* (1987). Table 4.1 demonstrates the marked effect of salmon sperm DNA on CAT expression in cells transfected with pSV2CAT. The level of CAT expression was increased by 10-fold with carrier DNA. A similar phenomenon was also shown later with expression of the ALASCAT-163 construct which showed an 18-fold increase in CAT expression with carrier (see section 4.2.D and Table 4.1).

The salmon sperm DNA used in these studies was sheared by passing a 10 mg/ml solution of salmon sperm DNA three times through a French Pressure Cell. A method for preparing sheared salmon sperm DNA for Southern blotting by autoclaving was recently published (Reed, 1988). This much simpler method of shearing DNA was considered for use in preparation of carrier. However, autoclaved carrier was found to be considerably less effective than the mechanically sheared carrier DNA (~5-fold) as measured by CAT expression from transfected constructs (results not shown). It was decided therefore that the use of salmon sperm DNA prepared by passage through the "French Pressure Cell" would be employed as carrier for all transfection studies to achieve the highest possible expression from 10 µg of construct DNA.

### *4.2.B.5. Examination of the CAT assay procedure (Gorman* et al., *1982)*

The method of Gorman *et al* (1982) was initially chosen for determination of CAT activity. There are three major components to the enzymatic reaction step of this assay, namely acetyl CoA, [$^{14}$C]chloramphenicol and the cell protein extract containing the CAT enzyme. Experiments were performed to check that under the conditions used, the assay was in the linear response range so that valid comparisons could be made.

The first component of the assay to be tested was acetyl CoA, which was synthesised in the laboratory (see section 2.7.E). To determine whether the assay was linear over 120 min as described by Gorman *et al* (1982), cell lysate (25 µg) from chick embryo

primary hepatocytes transfected with pSV2CAT was added to a reaction containing 1 μCi of [$^{14}$C]chloramphenicol and 0.8 mM acetyl CoA, the reaction incubated at 37°C and samples taken at times 0, 20, 40, 60 and 120 min. Figure 4.8.A demonstrates that a linear response with respect to CAT activity over 120 min was obtained. It is to be noted that after 120 min, only 14% of the [$^{14}$C]chloramphenicol was acetylated. To check whether there was indeed sufficient acetyl CoA in the assay to achieve 100% acetylation of the chloramphenicol, a separate experiment in which a similar time course as above was performed with the exception that 1 unit of highly purified CAT enzyme was used instead of cell lysate. Figure 4.8.B shows that 100% conversion of the [$^{14}$C]chloramphenicol to the acetylated form was achieved. A recent report suggested that acetyl CoA was unstable in the reaction mix and became a limiting factor with time in such assays, and therefore it needed to be added every 45 min to the reaction mix (Heard *et al.*, 1987). However, under the conditions used here, no significant difference in CAT activity was seen between 180 min assays that had a single initial addition of acetyl CoA as compared to those with additions every 45 min (results not shown) suggesting that the amount of acetyl CoA in the reaction mix was not limiting.

Because of the expense of [$^{14}$C]chloramphenicol, 1 μCi was the greatest amount of this substrate used per assay. Under the conditions of Gorman *et al* (1982), [$^{14}$C]chloramphenicol was known to be a limiting substrate and therefore it was of importance to determine the linearity of the assay with respect to the extent of acetylation of the [$^{14}$C]chloramphenicol. From Figure 4.8.B, the assay appeared linear up to approximately 60% acetylation. To accurately determine the linearity of the assay with respect to the percentage of [$^{14}$C]chloramphenicol acetylated, assays containing 1 μCi of [$^{14}$C]chloramphenicol, 0.8 mM acetyl CoA, 0 to 0.2 units of purified CAT enzyme, and 50 μg of cell lysate from untransfected primary hepatocytes (added to mimic the real situation of assaying cell lysates for CAT activity) were performed at 37°C for 120 min. Figure 4.8.C shows clearly that the assay remains linear to at least 60% conversion of [$^{14}$C]chloramphenicol to the acetylated form. Therefore, in all future assays for CAT activity, the reaction time and/or the amount of protein extract assayed were adjusted to ensure that less than 60% conversion of chloramphenicol was obtained.

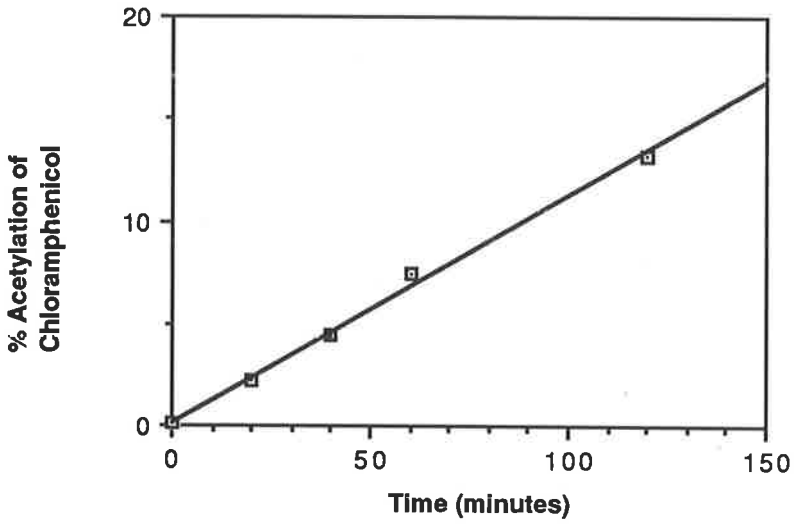
**Figure 4.8. Examination of the linear range of the CAT assay (Gorman *et al.*, 1982)**

**A.** Primary hepatocytes ($1 \times 10^7$) were transfected in the presence of 500 µg/ml salmon sperm DNA with 10 µg of pSV2CAT by electroporation at 250 V and 960 µF. The cells were plated and incubated in William's E medium for 48 h prior to harvesting and lysis of the cells. CAT assays were performed on 25 µg of protein extract for 0, 20, 40, 60 and 120 min. Following autoradiography, the radioactivity in each spot was determined by liquid scintillation counting. From these values, the percentage of acetylated chloramphenicol was calculated.

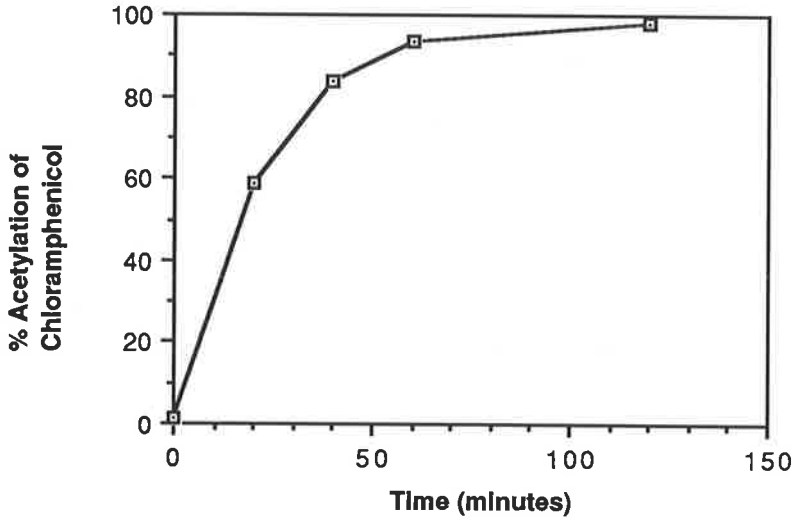
**B.** Assays for CAT activity were performed under identical conditions to those above in part **A**, except that 1 unit of purified CAT enzyme (Pharmacia) was used instead of protein extract.

**C.** CAT assays were performed under identical conditions to those above in part **A**, except that various amounts of purified CAT enzyme ranging from 0 to 0.2 units were used instead of protein extract, and the assay for all reactions was carried out for 120 min.

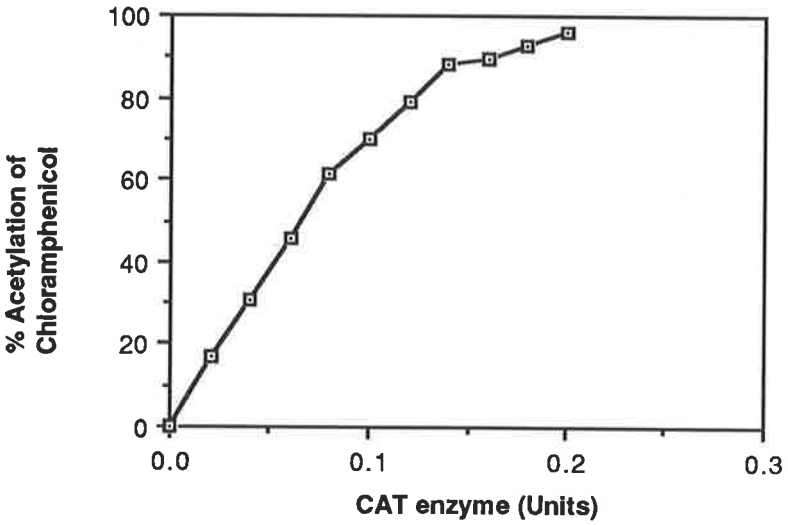
A.


20
10
0
0
50
100
150
% Acetylation of Chloramphenicol
Time (minutes)

B.


100
80
60
40
20
0
0
50
100
150
% Acetylation of Chloramphenicol
Time (minutes)

C.


100
80
60
40
20
0
0.0
0.1
0.2
0.3
% Acetylation of Chloramphenicol
CAT enzyme (Units)

The presence of an unknown heat-labile inhibitor of CAT activity has been reported in cell lysates and this can be destroyed by heating at 65°C for 10 min prior to assaying for CAT activity (Fromm *et al.*, 1985; Sleigh, 1986). Sleigh (1986) has reported that heating of cell lysates from the F9 mouse embryonal carcinoma cell line leads to an increase in CAT activity of 5 to 7-fold, and that the increase in CAT activity following heating varies between cell types. Such heating has been reported not to affect the activity of the CAT enzyme itself (Sleigh, 1986). No data was available on whether this inhibitory activity existed in the lysates prepared from chick embryo primary hepatocytes. On heating lysates from chick embryo primary hepatocytes, previously transfected with pSV2CAT, about 90% of soluble proteins in the cell lysates were precipitated and these were removed by centrifugation (see section 2.7.C) prior to assaying of the supernatants for CAT activity. Protein estimations were performed on cell lysates prior to heating and in all cases a volume of heated-treated cell lysate supernatant, equivalent to 50 μg or less of protein cell lysate (*i.e.* prior to heat-treatment), was assayed. When CAT activity was determined following heat-treatment, a 2-fold increase in CAT activity was seen. It was important to determine whether any inhibitory activity remained in the supernatants of heat-treated cell lysates. To do this, primary hepatocytes were mock-electroporated in the absence of construct DNA and various amounts of a heat-denatured cell lysate supernatant, up to 50 μg, were added to assays containing a constant amount of purified CAT enzyme. Within this range, the amount of cell lysate supernatant added had no effect on the activity of the CAT enzyme (results not shown) indicating that the heat-treatment had completely removed the inhibitory activity. For all future experiments involving the determination of CAT activity, the amount of lysate assayed is quoted as the amount of protein present prior to heating. This was done to avoid any variation that might occur during the protein heat-denaturation process, and to enable comparison with the results of others in the literature.

In summay, under the conditions used here, the assay for CAT activity is linear up to 60% acetylation of [$^{14}$C]chloramphenicol over a time period of at least 120 min. In addition, heating of the cell lysates prior to assaying for CAT activity results in an approximately 2-fold greater detection of CAT activity.

*4.2.B.6. Examination of alternative assay procedures for CAT activity (Nordeen* et al., *1985, Sleigh, 1986)*

The methods of Nordeen *et al* (1985) and Sleigh (1986) which employ [$^{3}$H]acetate and [$^{14}$C]acetyl Coenzyme A, respectively, as radioactive substrates were also investigated for determination of CAT activity since these procedures are simple, rapid and more sensitive when compared with the method of Gorman *et al* (1982) which uses [$^{14}$C]chloramphenicol as the radioactive substrate. To investigate these alternative methods, heated cell lysates containing either low or high levels of CAT activity, as determined by the method of Gorman *et al* (1982), were assayed by these two methods. Both procedures were shown to be linear over a greater range of CAT activity than found for the method of Gorman *et al* (1982). However, at the other end of the scale, unacceptably high background levels were obtained with these two procedures when heated cell lysates containing low levels of CAT activity were assayed. Hence, the sensitivity of these alternative assays was considerably less than that for the method of Gorman *et al* (1982) and this may be due to a lack of specificity, since they will detect any activity that can convert the radiolabelled acetate or radiolabelled acetyl CoA into an organic-soluble form, while the method of Gorman *et al* (1982) detects only acetylation of chloramphenicol. In this regard, it is interesting that Nordeen *et al* (1985) reported significant levels in lysates from primary hepatocytes, but not from other cell types of an activity other than CAT, which converted labelled acetate or labelled acetyl CoA into an organic-soluble form. Because of the lack of sensitivity of these alternative methods, the method of Gorman *et al* (1982) was used in all further work.

## 4.2.C. Effect of electroporation of chick embryo primary hepatocytes on the levels of endogenous ALAS and CYP mRNAs

An important consideration was whether electroporation *per se* affected the levels of endogenous mRNA for ALAS and CYPs. It is possible that electroporation evokes some "shock" response such as the "heat shock" or "SOS"-type response which may affect the levels of cellular mRNAs. To investigate this, the following experiment was performed. Primary hepatocytes (1 x $10^{7}$ cells) in the presence of 500 μg/ml salmon sperm DNA were

placed into electroporation cuvettes. Half of the cuvettes were electroporated while the others were not. No exogenous construct DNA was used in this experiment. The cells were plated in William's E medium and incubated at 37°C overnight. Phenobarbital, haemin and/or 4,6-dioxoheptanoic acid were then added to separate cultures in the following combinations: solvent alone; haemin alone; phenobarbital alone; 4,6-dioxoheptanoic acid alone; phenobarbital and 4,6-dioxoheptanoic acid; and haemin, phenobarbital and 4,6-dioxoheptanoic acid. The hepatocytes were cultured for a further 48 h prior to harvesting and preparation of total RNA. Figure 4.9 shows the autoradiographs of Northern blots of total RNA isolated from electroporated and non-electroporated cells that have been probed for ALAS, CYPs and ß-actin mRNAs. Comparison of the amounts of mRNAs between electroporated and non-electroporated hepatocytes revealed no significant difference after 48 h of the various drug treatments, although responses at earlier times following electroporation cannot be ruled out. These results indicate that the cell repair processes following transfection by this method do not significantly affect the responses of interest in these cells such as drug-induction and haem repression.

From Figure 4.9 it can be seen that neither haemin nor 4,6-dioxoheptanoic acid (a specific inhibitor of haem biosynthesis) had any noticeable effect on CYP mRNA levels, confirming the results obtained in Chapter 3 that haem is not involved in regulation of CYP mRNA. As expected however, phenobarbital caused a marked induction in the levels of three CYP mRNAs of size 3.5, 2.5 and 2.2 kb. Also as reported in Chapter 3, haemin treatment resulted in an approximate 50% reduction in the amount of ALAS mRNA present in either untreated or phenobarbital-treated cultures and this is probably due to a specific destabilising effect of haem on the mRNA. 4,6-Dioxoheptanoic acid alone slightly increased basal ALAS mRNA levels, but when tested with phenobarbital, there was a synergistic response. These results imply, firstly, that 4,6-dioxoheptanoic acid is significantly inhibiting haem biosynthesis and that cellular haem levels are subsequently reduced for a small effect to be observed on basal levels of ALAS mRNA. However, following treatment with both 4,6-dioxoheptanoic acid and phenobarbital, it is possible that 4,6-dioxoheptanoic acid inhibits haem biosynthesis while the drug-induction of CYP apoprotein synthesis would lead to a substantial decrease in cellular haem levels. This result is also in keeping with the

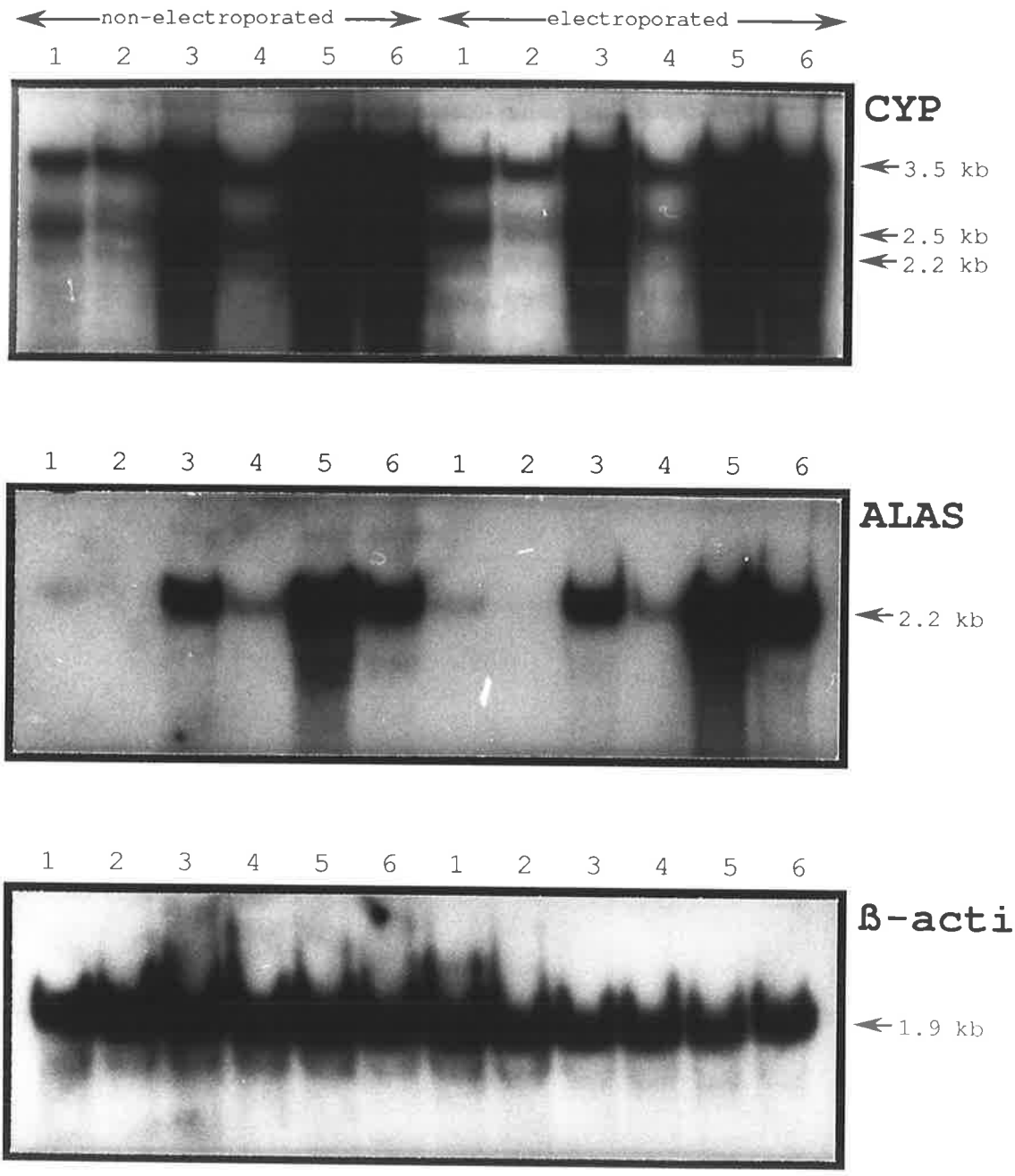
**Figure 4.9. Effect of electroporation on endogenous ALAS and CYP mRNA levels in chick embryo primary hepatocytes**

Chick embryo primary hepatocytes were isolated and prepared for electroporation by resuspension in electroporation buffer and addition of salmon sperm DNA to a final concentration of 500 µg/ml. Twelve aliquots of 1 x $10^7$ cells each were placed into electroporation cuvettes, six of which were electroporated and six of which were not. The hepatocytes were plated and cultured in William's E medium for 20 h. The medium was then replaced with fresh medium and phenobarbital, haemin and/or 4,6-dioxoheptanoic acid were added simultaneously in the combinations described below. Each compound was made as a 100x stock solution and added to give a final concentration of 10 µM haemin, 2 mM phenobarbital and 1 mM 4,6-dioxoheptanoic acid. After a further 48 h incubation, the hepatocytes were harvested, total RNA purified and 20 µg of each sample was run in a 1% agarose-formaldehyde gel. Fractionated RNA was transferred to Nytran filters and probed for CYP, ALAS and ß-actin mRNAs using the probes pCHP3, p105B1 and a chicken ß-actin cDNA clone, respectively. Autoradiographs allowed to expose for 4, 8 and 2 days for CYPs, ALAS and ß-actin, respectively, are shown.

The combinations of compounds added were as follows:

lane 1, PBS (control)
lane 2, haemin
lane 3, phenobarbital
lane 4, 4,6-dioxoheptanoic acid
lane 5, phenobarbital and 4,6-dioxoheptanoic acid
lane 6, haemin, phenobarbital and 4,6-dioxoheptanoic acid

Although not clear from the photograph, three discrete CYP mRNAs of size 3.5, 2.5 and 2.2 kb were visible in lanes 3, 5 and 6. The intensities of these bands are similar in lanes 5 and 6. Other minor bands can be seen in the phenobarbital-induced lanes when probed for CYPs or ALAS. The significance or identity of these is not known.


non-electroporated
electroporated
1 2 3 4 5 6 1 2 3 4 5 6
CYP
3.5 kb
2.5 kb
2.2 kb
1 2 3 4 5 6 1 2 3 4 5 6
ALAS
2.2 kb
1 2 3 4 5 6 1 2 3 4 5 6
ß-actin
1.9 kb

studies of Schoenfeld *et al* (1982) who showed in chick embryo primary hepatocytes that a 98% inhibition of total cellular ALAD activity by 4,6-dioxoheptanoic acid alone had only a minor effect on lowering cellular haem levels, but the simultaneous addition of the "phenobarbital-like" drug AIA together with 4,6-dioxoheptanoic acid led to, at least, a 50% decrease in cellular haem.

### 4.2.D. Transfection of ALASCAT constructs in chick embryo primary hepatocytes

The above work on transfection optimisation, now permitted a study of ALASCAT construct expression in chick embryo primary hepatocytes. Up until this stage, the preliminary studies on transfection had been performed using the pSV2CAT plasmid. Experiments were now carried out using the longest and shortest construct, ALASCAT-1700 and ALASCAT-163 to determine their level of basal expression in these cells. Although salmon sperm DNA had been previously shown to be important in efficient transfection of pSV2CAT, initial experiments were performed to determine the level at which these constructs expressed in the presence and absence of carrier. Electroporation of 1 x $10^7$ primary hepatocytes with 10 μg and 50 μg of ALASCAT construct DNA and without salmon sperm DNA was performed at 250 V and 960 μF. The control plasmid pRSVCAT (10 μg) was also introduced into hepatocytes in a similar way. To check for the effect of carrier DNA, hepatocytes were transfected with 10 μg of ALASCAT-163 as above, but in the presence of 500 μg/ml salmon sperm DNA. Following transfection, the cells were plated in William's E medium and incubated for 48 h. To determine the level of expression of the constructs, the cells were harvested, lysed and 50 μg of protein extract was assayed for CAT activity for 2 h. The amount of protein assayed and the length of assay were chosen to maximise sensitivity. Table 4.1 and 4.2 show the data obtained in this experiment. The level of expression of CAT activity from ALASCAT-163 increased by almost 18-fold in the presence of carrier DNA (see Table 4.1). This result compares with that obtained following transfection of hepatocytes with pSV2CAT, where carrier DNA markedly increased expression of CAT activity by 10-fold (see section 4.2.B.4 and Table

**Table 4.1. Effect of salmon sperm DNA on transfection efficiency**

Primary hepatocytes ($3 \times 10^6$) were transfected either in the presence or absence of salmon sperm DNA with 10 µg of pSV2CAT by electroporation at 250 V and 960 µF. The cells were plated and incubated in William's E medium for 48 h prior to harvesting and cell lysis. To measure CAT activity, 50 µg of protein extract was assayed for 2 h. Following autoradiography the spots were excised and the amount of radioactivity determined by liquid scintillation counting. CAT activity is expressed as the percent acetylation of chloramphenicol produced in 2 h per 50 µg of protein extract.

A similar experiment was performed on a different day using ALASCAT-163. The only alterations in experimental detail include electroporation of $1 \times 10^7$ primary hepatocytes and culture in William's E medium supplemented with 10% Nu-Serum. The results are shown including calculation of the fold-increase in CAT activity obtained in the presence of salmon sperm DNA as compared to that without carrier DNA.

**Table 4.2. Effect of increasing the amount of transfected construct DNA on CAT activity in the absence of salmon sperm DNA**

Primary hepatocytes ($1 \times 10^7$) were transfected by electroporation at 250 V and 960 µF with either 10 or 50 µg of ALASCAT-163 or ALASCAT-1700 or with 10 µg of pRSVCAT. No salmon sperm DNA was used as carrier in this experiment. The cells were plated and incubated in William's E medium supplemented with 10% Nu-Serum for 48 h prior to harvesting and cell lysis. To measure CAT activity, 50 µg of protein extract was assayed for 2 h. Following autoradiography the spots were excised and the amount of radioactivity determined by liquid scintillation counting. CAT activity is expressed as the the percent acetylation of chloramphenicol produced in 2 h per 50 µg of protein extract. **a.** The Ratio (50 µg/10 µg) represents the fold-increase in CAT activity obtained by increasing the amount of construct DNA transfected by 5-fold from 10 µg to 50 µg. **b.** Because of the strong expression of CAT activity from the pRSVCAT construct in these cells, to obtain a percent acetylation within the linear range of the assay, required that only 5 µg of protein extract be assayed for 1 h. The value given is one which has been calculated from the experimentally obtained value to adjust it to an assay of 50 µg of protein extract for 2 h.

## Table 4.1

| Construct | No. of cells transfected | CAT activity | | Fold-increase with carrier DNA |
|---|---|---|---|---|
| | | - carrier | + carrier | |
| **pSV2CAT** | $3 \times 10^6$ | 0.8 | 8.0 | 10 |
| **ALASCAT-163** | $1 \times 10^7$ | 1.8 | 31.7 | 18 |

## Table 4.2

| Construct | Amount of DNA transfected (μg) | CAT activity | Ratio[a] 50 μg/10 μg |
|---|---|---|---|
| **ALASCAT-163** | 10 | 1.8 | N.A. |
| | 50 | 42.8 | 23.8 |
| **ALASCAT-1700** | 10 | 2.3 | N.A. |
| | 50 | 45.8 | 19.9 |
| **pRSVCAT** | 10 | 225.6[b] | N.A. |

4.1). Thus carrier DNA appears to be very important in efficient transfection and therefore expression of constructs in chick embryo primary hepatocytes.

As shown in Table 4.2, following electroporation of 10 μg of construct DNA, ALASCAT-163 and ALASCAT-1700 expressed at relatively low levels in primary hepatocytes (see Table 4.2). Interestingly, transfection of 50 μg of both of these ALASCAT constructs, instead of 10 μg, caused a 20 to 24-fold increase in expression rather than the expected 5-fold increase. This situation in which CAT expression increases disproportionately with the amount of DNA transfected has also been reported by Satyabhama and Epstein (1988) and may be explained as follows. There may be a limited number of DNase molecules either inside the cell, outside the cell or on the cell surface which degrade foreign DNA. Carrier DNA, which is in vast excess of the construct DNA, would protect against such degradation. Increasing the amount of transfected construct DNA, in the absence of carrier DNA, might also help to overcome the effects of this degradation.

Of interest also is that expression of CAT activity from pRSVCAT is approximately 100 times greater than that from equivalent amounts of ALASCAT-1700 or ALASCAT-163 in these hepatocytes. This initial experiment is the first in which constructs of the chicken house-keeping ALAS gene promoter have been expressed in an homologous cell system. The level of this expression in comparison with the Rous Sarcoma Virus long terminal repeat promoter/enhancer, is very low, but the absolute amount of CAT expressed can be increased by either transfecting larger quantities of construct DNA or by the use of salmon sperm DNA as carrier. Due to the difficulty in obtaining large quantities of ALASCAT constructs, the use of 10 μg of construct DNA together with carrier was chosen for further experiments.

The major aim of the work in this Chapter is to find and isolate a region of the ALAS gene that responds to the drug phenobarbital. From nuclear run-on experiments, the ALAS gene was shown to be induced by only 2 to 3-fold with phenobarbital (Table 3.1). For this reason the "phenobarbital-like" drug 2-allyl-2-isopropylacetamide (AIA), which had been shown in our laboratory to be a more potent inducer of the phenobarbital-inducible CYPs and ALAS mRNA in chick embryo and adult rat liver, was used. Primary hepatocytes (1 x $10^7$ cells/cuvette) were resuspended in the presence of 500 μg/ml salmon sperm DNA and

10 μg of each of the constructs ALASCAT-1700, -847, -665, -504, -254 and -163. These cells were electroporated in duplicate at 250 V and 960 μF. The control constructs pSV2CAT and pBRCAT were also treated similarly. Following electroporation, the cells were plated in William's E medium and incubated overnight. To one plate of each duplicate, the medium was replaced with William's E medium alone and to the other plate, William's E medium containing 100 μg/ml of AIA. After incubation for a further 48 h, the cells were harvested, lysed and CAT activity within each sample was determined. The results are shown in Figure 4.10. For all ALASCAT constructs, basal expression of CAT activity was essentially the same indicating that the first 163 bp of the 5' flanking region is all that is required for this expression. Two repeated experiments confirmed these findings. A possible minor increase in expression from ALASCAT-665 (see Fig. 4.10) was not confirmed in other experiments (results not shown).

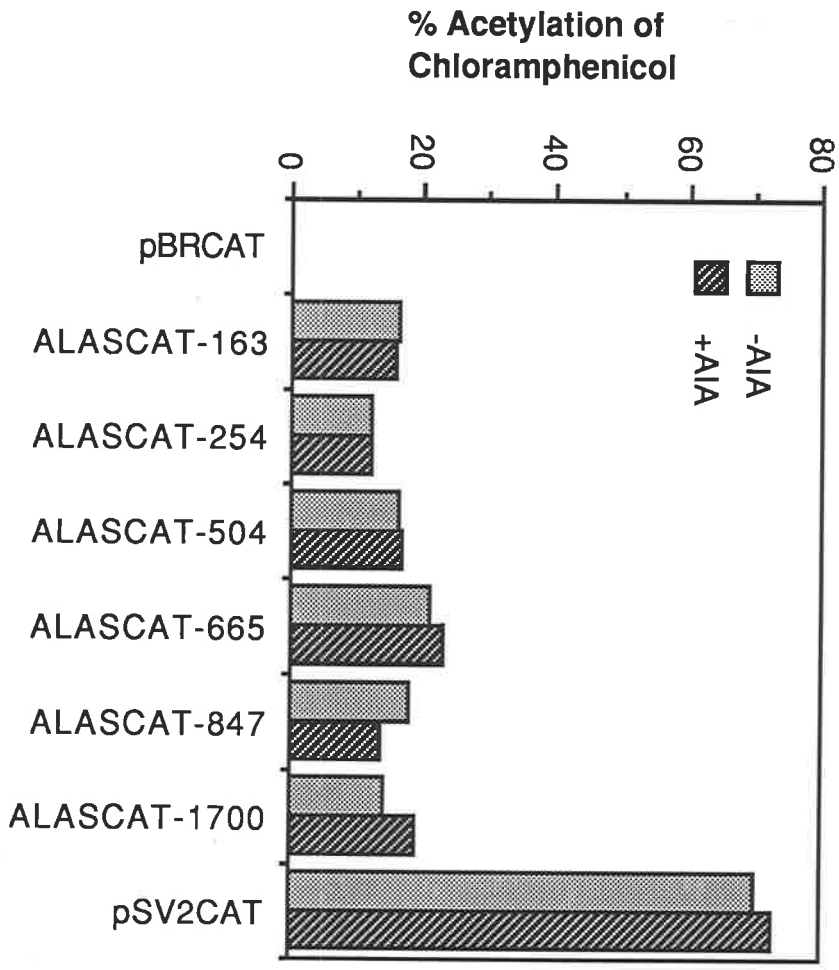
Addition of AIA at a concentration known to induce ALAS mRNA in chick embryo primary hepatocytes cultures, resulted in no significant change in expression of any of the ALASCAT constructs or of pSV2CAT (see Fig. 4.10). Expression of the longest construct ALASCAT-1700 was further examined in response to 2 mM phenobarbital, in a similar experiment to that performed above with AIA, and yielded a similar result showing no effect of drug (results not shown). Thus the first 1700 bp of 5' flanking region appears not to contain the element(s) necessary for the drug response. Such elements must therefore reside further upstream of this region, within the gene itself or downstream of the gene.

## 4.3. DISCUSSION

The question of how phenobarbital regulates ALAS induction at the transcriptional level is an important one. At least in chick embryo liver, it does not appear that phenobarbital induces ALAS gene transcription by the removal of haem repression on ALAS gene transcription, since haemin treatment fails to repress as described in Chapter 3. The implication from this work is that phenobarbital acts via a rapid and more direct induction mechanism or at least one which does not involve haem. A way to approach this problem experimentally was to study expression of the CAT reporter gene under the control of the ALAS gene 5' flanking region. Essential to these studies was the use of a homologous

**Figure 4.10. Effect of 2-allyl-2-isopropylacetamide (AIA) on transient expression of ALASCAT constructs**

Primary hepatocytes ($1 \times 10^7$) were transfected in the presence of 500 µg/ml salmon sperm DNA with 10 µg of construct DNA in duplicate by electroporation at 250 V and 960 µF. The cells were plated and incubated in William's E medium for 20 h. The medium was then replaced with fresh medium and, to one of each duplicate, AIA was added to a final concentration of 100 µg/ml and to the other an equal volume of solvent only. After 48 h of drug treatment, the cells were harvested, lysed and CAT activity determined on 50 µg of protein extract. Following autoradiography, the spots were excised and the amount of radioactivity in each determined by liquid scintillation counting. The results of CAT expression for each construct in the presence and absence of AIA are shown.


% Acetylation of Chloramphenicol
0
20
40
60
80
pBRCAT
ALASCAT-163
ALASCAT-254
ALASCAT-504
ALASCAT-665
ALASCAT-847
ALASCAT-1700
pSV2CAT
-AIA
+AIA

system since heterologous cells may lack the appropriate receptors or regulatory factors necessary for the response to phenobarbital. At this stage, expression of a house-keeping ALAS gene promoter/reporter construct had not been achieved in an homologous system for any species. In this chapter, experiments were performed to examine such constructs for the first time in chick embryo primary hepatocytes.

It was necessary to first establish the appropriate conditions for this study. The introduction of constructs into chick embryo primary hepatocytes by electroporation was substantially increased in the presence of 500 μg/ml salmon sperm DNA at a density of $1 \times 10^7$ to $2 \times 10^7$ cells/cuvette. The use of salmon sperm DNA, as carrier, for electroporation was first employed by Chu *et al* (1987) who found it to significantly increase the efficiency of plasmid transfection into eucaryotic cells. A voltage of 250 V and a capacitance of 960 μF was found to be optimal for primary hepatocytes. Three assays for CAT activity were investigated for use, but the method of Gorman *et al* (1982) was chosen because primary hepatocyte lysates were found to produce unacceptably high backgrounds in the other assays. The chosen CAT assay was shown to be linear up to 60% conversion of [$^{14}$C]chloramphenicol to the acetylated form. Important also for the assay of CAT activity was preheating of the cell lysates to remove an inhibitory factor.

ALASCAT constructs were shown for the first time to express in chick embryo primary hepatocytes albeit at a low, but detectable level. The first 163 bp of the ALAS gene promoter was able to promote basal expression from the CAT gene. Addition of more 5' flanking region to -1700 bp resulted in no alteration in this expression of CAT activity. It is possible however, that regulatory regions do exist between position -1700 and -163 bp, but they are either not utilised under the culture conditions used or are non-functional in these cells tested.

The first 163 bp of the promoter region appears to contain the necessary *cis* -acting elements for basal promotion of the ALAS gene in these cells. A number of putative elements including a TATA box, four GC boxes, a CCAAT box and a motif that binds NRF-1 are contained in this region (for details see later, Fig. 7.1). A study of the contribution to overall chicken ALAS promoter activity of the TATA box, three of the GC boxes and the CCAAT box has been performed in *Xenopus laevis* oocytes (Loveridge *et*

*al.*, 1988). Mutation of these elements individually and in concert revealed that the TATA box was essential for expression and of the others, only the GC box at position -79 to -74 bp contributed significantly to expression (mutation of this GC box lead to a 70% decrease in expression). It remains to be determined precisely which elements in the chicken ALAS promoter are functional in the chick embryo liver and indeed in other tissues.

It is possible that an 8 bp concensus cAMP-responsive element (CRE - TGACGTCA), located in the 5' untranslated region (UTR) of the chicken ALAS promoter at position +40 to +47 bp (see Fig. 7.1), may also be important in expression of this gene. This element was not contained in any of the ALASCAT constructs used in this present study. The role of this element will be of extreme interest in the adrenal cortex, testis and ovary where CYPs involved in steroid metabolism are induced transcriptionally by hormones that bind cell surface receptors and act through cAMP. It would seem logical for ALAS also to be induced by cAMP to supply extra haem demanded by increased apoCYP. If this CRE is functionally important, this will be the first case of a functional CRE located in the 5' UTR of a gene (Montminy *et al.*, 1986; Roesler *et al.*, 1988). However, it should be noted that the presence of an 8 bp CRE consensus sequence within the context of a gene does not necessarily imply function (Montminy *et al.*, 1986; Deutsch *et al.*, 1988) and further work will be necessary to demonstrate the relevance, if any, of this sequence in the ALAS gene 5' untranslated region.

Addition of the porphyrinogenic drug, 2-allyl-2-isopropylacetamide (AIA), to chick embryo primary hepatocytes, resulted in no significant difference in expression of CAT activity from any of the transfected ALASCAT constructs. This raises the probability that some or all of the drug-responsive *cis* -acting elements lie outside of the first 1700 bp of the ALAS gene promoter.

At this stage of the work, following several failed attempts to make constructs containing more than 1700 bp of 5' flanking region, the opportunity to perform similar experiments on drug-induction in the *CYP2H1* gene became available. This gene is more highly responsive to phenobarbital at the transcriptional level (see Table 3.1) and like the ALAS gene provides an opportunity with which to study the mechanism of drug-induction. Chapters 5 and 6 describe the work performed with the *CYP2H1* gene promoter.

In summary, the ALAS house-keeping gene proves to have a fascinating mechanism of control because of the importance of this gene in the supply of haem for a wide range of tissue-specific CYPs, as well as the ubiquitous respiratory cytochromes and the catalase, peroxidase and tryptophan pyrrolase enzymes. This regulation must be tightly controlled to prevent haem depletion of the cells due to underexpression of ALAS and, just as importantly, the onset of a porphyric crisis due to overexpression. Future work to understand the mechanism of basal and phenobarbital-inducible expression of the chicken house-keeping ALAS gene will require more extensive transient transfection studies in an attempt to locate a region responsive to phenobarbital. Constructs containing the putative CRE in the 5' untranslated region will need to be made as well as others containing 5' flanking region beyond -1700 bp and possibly 3' flanking region as well. It may be beneficial to perform DNase I hypersensitivity studies in an attempt to pin-point a region(s) responsive to phenobarbital prior to further transient expression studies. Of interest also will be a comparison of the differences in the mechanisms of transcriptional regulation of the house-keeping ALAS gene in chick embryo liver and in adult rat liver in relation to repression by haem.

# CHAPTER 5

## BASAL AND DRUG-INDUCED EXPRESSION OF CHICKEN *CYP2H1* GENE PROMOTER/CAT CONSTRUCTS IN CHICK EMBRYO PRIMARY HEPATOCYTES.

## 5.1. INTRODUCTION

The chicken *CYP2H1* gene encodes a 3.5 kb mRNA (Hobbs *et al.*, 1986). The levels of this mRNA have been shown to be increased by 2-allyl-2-isopropylacetamide (AIA) in the liver, kidney and small intestine of 12 week old chickens (Hansen *et al.*, 1989), by phenobarbital and AIA in chick embryo primary hepatocytes (see Chapter 3) and by these drugs in the liver of 17-18 day old chick embryos (Hansen *et al.*, 1989). Other drugs such as 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC), mephenytoin and glutethimide have also been shown to increase the 3.5 kb CYP2H1 mRNA levels in chick embryo liver (A.J. Hansen, personal communication). The rate of transcription of the *CYP2H1* gene has been shown to be induced by phenobarbital in chick embryo primary hepatocytes, and by phenobarbital and AIA in chick embryo liver (Chapter 3; Hansen *et al.*, 1989). These two drugs appear structurally quite dissimilar as are many of the other drugs that have been reported to induce the "phenobarbital-inducible" CYPs such as DDC and griseofulvin (see Fig. 1.2). An intriguing problem therefore exists as to how these apparently structurally unrelated molecules are able to induce a common *CYP* gene at the transcriptional level. Two possible explanations have been proposed. Either there exists a broad specificity receptor that accommodates a wide range of structurally different compounds or the various compounds are converted into an as yet unknown metabolic intermediate(s) which is then recognised by a specific receptor. For drugs such as phenobarbital and AIA to exert an effect at the transcriptional level, it seems probable that there is a protein factor, that directly interacts with the gene and/or transcriptional apparatus. This mediator may be a drug-receptor complex or a drug effector protein which upon interaction with the drug becomes activated to confer the drug response, and may act at the initiation, elongation or maturation stage of the transcriptional process.

As described earlier in the introduction to this thesis (see section 1.2.C.1.b), studies on the regulation of transcription of the mouse, rat and human *CYP1A1* genes in response to the drug 2,3,7,8-tetrachlorodibenzo-*p* -dioxin (TCDD) have yielded a wealth of valuable information. In this well characterised system of signal transduction, TCDD and other structurally similar aryl hydrocarbons diffuse into the cell where they bind to a cytosolic aryl

hydrocarbon (Ah) receptor/90 kD heat shock protein complex (Wilhelmsson *et al.*, 1990). Upon binding of drug to the receptor, the heat shock protein is displaced and the drug/receptor complex is specifically transported into the nucleus where it binds to DNA at specific sequences termed dioxin-responsive elements (DREs). Multiple DREs have been located upstream of the *CYP1A1* gene. Upon binding of this drug/receptor complex to the DNA, the rate of initiation of transcription of the *CYP1A1* gene is increased. This has been shown by the construction of chimeric genes in which various lengths of the *CYP1A1* gene upstream region have been fused to the CAT reporter gene and the resultant constructs expressed in hepatoma cell lines and receptor-defective variant hepatoma cells in the presence and absence of TCDD (reviewed by Whitlock *et al.*, 1989). This work established that a dioxin-responsive enhancer system requires both a region of DNA containing DREs and a functional Ah receptor.

Nothing is known about the mechanism of transcriptional induction by phenobarbital and "phenobarbital-like" drugs in the liver. Studies in this area have been hampered because hepatocytes typically lose responsiveness to phenobarbital in culture or upon immortalisation as hepatoma cell lines. Furthermore, attempts to demonstrate the presence of a receptor for phenobarbital have failed (Poland *et al.*, 1980, 1981; Tierney *et al.*, 1981). In this chapter, transcriptional control of the chicken *CYP2H1* gene has been studied by transient transfection of *CYP2H1* promoter/CAT reporter constructs into chick embryo primary hepatocytes. As described in Chapter 3, these cells retain phenobarbital-inducibility of the *CYP2H1* gene and therefore special emphasis has been placed on searching for a region of the 5' flanking region that mediates the response of this gene to phenobarbital.

## 5.2. RESULTS

### 5.2.A. Preparation of *CYP2H1* promoter/CAT reporter chimeric constructs.

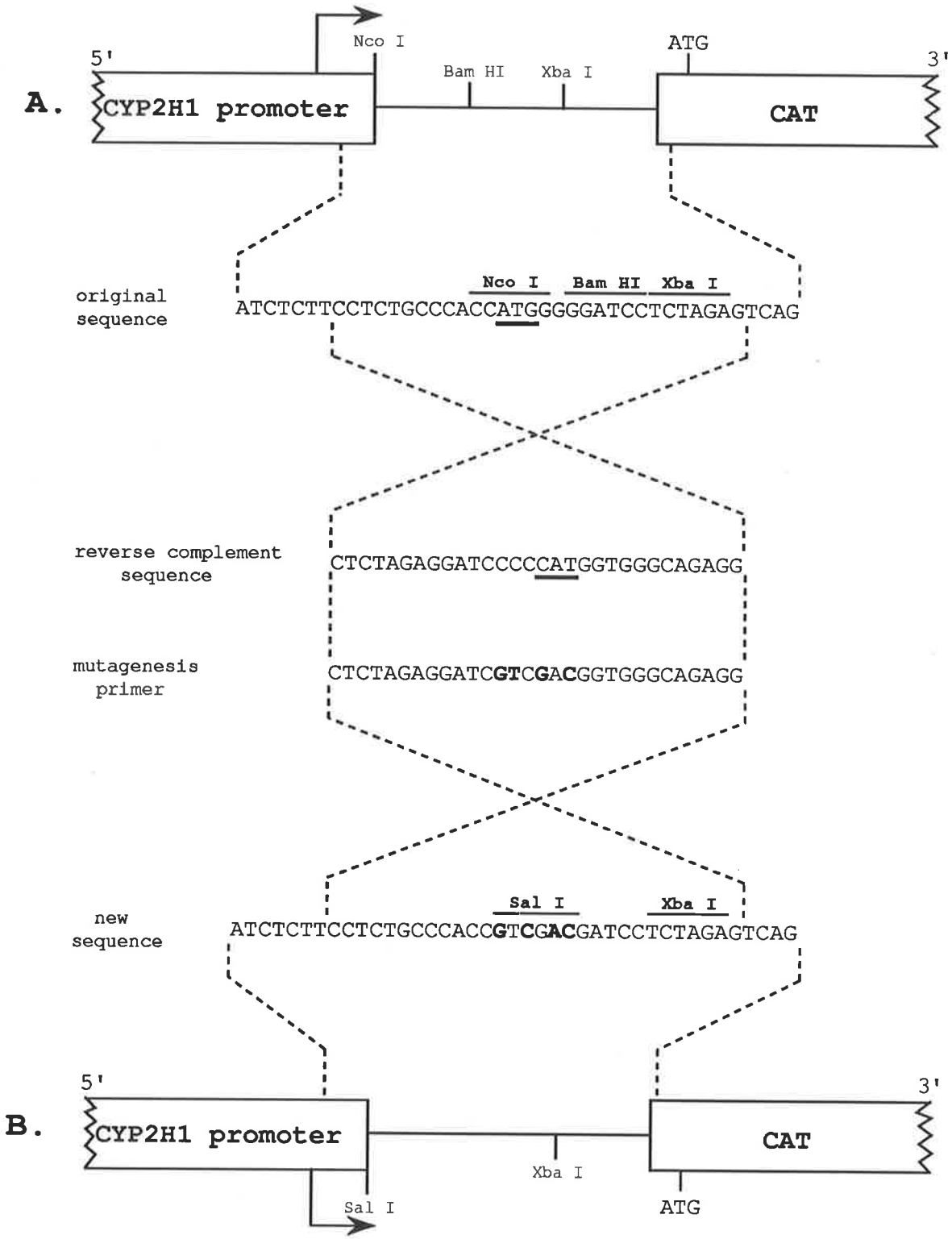
As a first step in the preparation of *CYP2H1* promoter/CAT reporter chimeric constructs, a suitable reporter system was prepared by cloning the CAT gene from pSV2CAT into pIBI76. The CAT gene was removed from pSV2CAT (Gorman *et al.*,

1982) as a *Hind* III/*Bam* HI fragment and end-filled using DNA polymerase I (Klenow Fragment). This fragment was then ligated into the *Hinc* II site of pIBI76 in an orientation that positioned the 5' end of the CAT gene nearest the unique *Sma* I site of the polylinker. This CAT vector was called pIBICAT.

In previous work, a 2.7 kb cDNA clone derived from the 3.5 kb CYP2H1 mRNA designated pCHP3 was used to screen a chicken genomic Charon 4A λ library and a number of overlapping clones for the *CYP2H1* gene were isolated (Mattschoss *et al.*, 1986). One of the genomic clones designated λ3, was shown by restriction mapping and Southern blot hybridisation analyses using pCHP3, to contain approximately 9 kb of 5' flanking sequence (Mattschoss *et al.*, 1986). To locate *cis* -acting regulatory regions that may be involved in both the basal and phenobarbital-induced expression of the *CYP2H1* gene, a series of constructs were made in which various lengths of the 5' flanking region of this gene including the complete 5' untranslated region were cloned into the *Sma* I site of pIBICAT (this part of the work was performed in collaboration with Dr A.J. Hansen). Double digests were performed on clone λ3 using *Nde* I/*Nco* I, *Bam* HI/*Nco* I and *Eco* RI/*Nco* I. Fragments corresponding to the immediate 5' flanking region of the *CYP2H1* gene and of length 0.5, 1.1 and 8.9 kb, respectively were isolated. Each fragment contained a common 3' *Nco* I end corresponding to the ATG translation initiation site of the *CYP2H1* coding sequence. The fragments were then end-filled and ligated individually into the unique *Sma* I site of pIBICAT. All three constructs therefore contained the entire 5' untranslated region of 39 bp as well as the translation initiation ATG codon of the *CYP2H1* gene preserved by end-filling of the *Nco* I site and, in addition, the translation initiation ATG codon of the CAT gene. Analysis of the position of the initiation ATG codon of the *CYP2H1* gene with respect to the initiation ATG codon of the CAT gene in these constructs (see Fig. 5.1) demonstrated that the codons were out of frame. Hence, initiation from the upstream *CYP2H1* initiation codon would lead to the production of a "non-sense" polypeptide. The translation initiation ATG codon of the *CYP2H1* gene, contained in these constructs, was therefore altered by site-directed mutagenesis and replaced with a *Sal* I site, thus ensuring that translation of CAT mRNA would be initiated from the correct ATG codon of the CAT gene (see Fig. 5.1). Using the same mutagenesis primer, a polylinker *Bam* HI site, adjacent

**Figure 5.1. Strategy for mutagenesis to destroy the *CYP2H1* translation initiation ATG codon**

A flow diagram of the strategy used to destroy the *CYP2H1* gene translation initiation ATG codon of the CYP2H1CAT-8.9, CYP2H1CAT-1.1 and CYP2H1CAT-0.5 constructs is shown. **A.** Diagram of the relevant region of the CYP2H1CAT constructs prior to mutagenesis. Mutagenesis was performed using a mutagenesis primer made to the reverse complement sequence of this region, but containing the appropriate sequence alterations (bold type). In destruction of the ATG initiation codon (underlined), a *Sal* I site was introduced to replace the *Nco* I site and a *Bam* HI site was removed. **B.** Diagram of the resultant mutated region of the CYP2H1CAT constructs.


A.
5'
Nco I
ATG
3'
Bam HI
Xba I
CYP2H1 promoter
CAT
original sequence
Nco I
Bam HI
Xba I
ATCTCTTCCTCTGCCCACCATGGGGGATCCTCTAGAGTCAG
reverse complement sequence
CTCTAGAGGATCCCCCATGGTGGGCAGAGG
mutagenesis primer
CTCTAGAGGATCGTCGACGGTGGGCAGAGG
new sequence
Sal I
Xba I
ATCTCTTCCTCTGCCCACCGTCGACGATCCTCTAGAGTCAG
B.
5'
3'
CYP2H1 promoter
CAT
Xba I
Sal I
ATG

to the ATG of the *CYP2H1* was simultaneously mutated to destroy this site; the reason for this will become clear later. This resulted in constructs which were designated CYP2H1CAT-0.5, CYP2H1CAT-1.1 and CYP2H1CAT-8.9 (see Fig. 5.2). Constructs CYP2H1CAT-1.7 and CYP2H1CAT-4.7 were subsequently made by digestion of CYP2H1CAT-8.9 with *Stu* I and *Sac* I respectively, removal of the excised 5' flanking region and recircularisation of the resultant vector (see Fig. 5.2).

### 5.2.B. Preliminary expression of CYP2H1CAT constructs reveals induction with phenobarbital

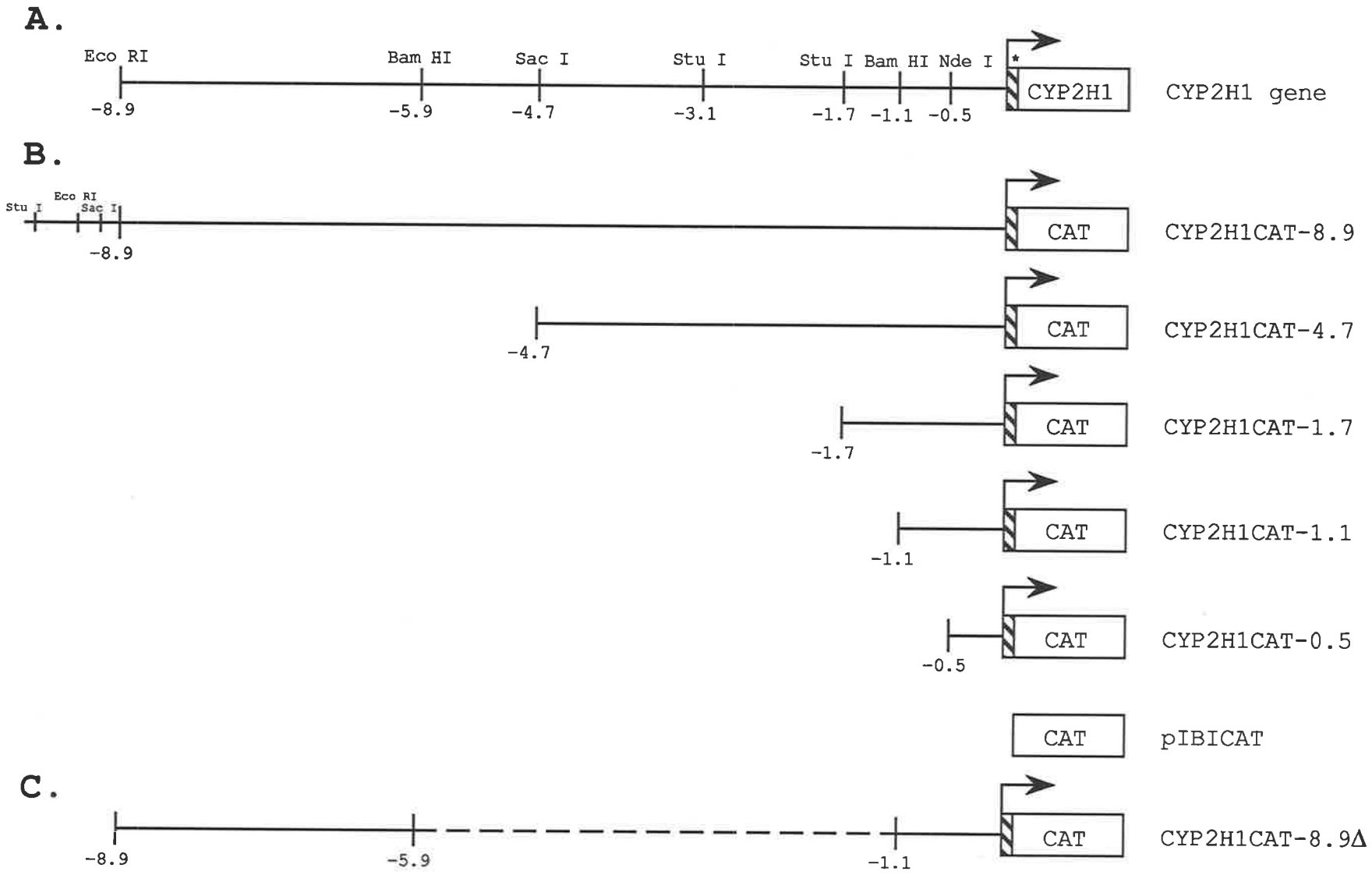
In preliminary experiments, CYP2H1CAT constructs (1.5 pmol) in the presence of 500 μg/ml salmon sperm DNA as carrier were transfected in quadruplicate into 1 x $10^7$ chick embryo primary hepatocytes by electroporation at 250 V and 960 μF, these conditions having been optimised previously as described in Chapter 4. The promoterless/CAT construct, pIBICAT and the strong promoter/CAT construct, pRSVCAT were also transfected in this way. Transfected hepatocytes were then cultured overnight in William's E medium supplemented with 10% Nu-Serum. Phenobarbital was added to a final concentration of 500 μM to two of the plates containing hepatocytes transfected with each construct, and an equal volume of PBS added to the other two plates. After a further 48 h incubation, the cells were harvested, lysed and assayed for CAT activity (for details, see section 2.7.A to 2.7.C). From this and similar preliminary experiments it became clear that the first 0.5 kb of the *CYP2H1* promoter (present in CYP2H1CAT-0.5) was capable of driving strong expression of the CAT gene (results not shown). Of interest was a significant, but variable increase in CAT expression of about 2-fold when hepatocytes transfected with CYP2H1CAT-8.9 and -4.7 were treated with phenobarbital. Results for these early experiments are not shown as there was some variation between CAT activity measurements of duplicate samples and hence also in the effect of phenobarbital upon expression of the constructs. In an attempt to improve reproducibility of the system and to investigate the possibility of increasing the magnitude of response to phenobarbital, the longest and most highly inducible construct CYP2H1CAT-8.9 was used to study the nature of this phenobarbital-induction.

**Figure 5.2. Diagrammatic representation of the 5' portion of the *CYP2H1* gene and CYP2H1CAT constructs**

**A.** Diagram of the *CYP2H1* gene and the 5' flanking region showing the positions of restriction sites important in the production of CYP2H1CAT constructs. The line represents the 5' flanking region, the arrow shows the position of the transcription start site and the hatched region depicts the entire 5' untranslated region of the *CYP2H1* gene. An asterisk marks the translation initiation ATG codon which is present in the gene, but not in the constructs.

**B.** Diagram of CYP2H1CAT constructs containing various lengths of 5' flanking region of the *CYP2H1* gene inserted upstream of the CAT reporter gene. In the diagram of the longest construct, CYP2H1CAT-8.9, the polylinker sites of pIBI76 important in the synthesis of CYP2H1CAT-1.7 and CYP2H1CAT-4.7 are shown. Although depicted as linear molecules, these constructs are circular containing pIBI76 as the cloning vector (not shown).

**C.** Diagram of the CYP2H1CAT-8.9Δ construct. This construct was made from CYP2H1CAT-8.9 by deletion of a 4.8 kb *Bam* HI fragment, and subsequent recircularisation.


A.
Eco RI
Bam HI
Sac I
Stu I
Stu I Bam HI Nde I
-8.9
-5.9
-4.7
-3.1
-1.7 -1.1 -0.5
CYP2H1
CYP2H1 gene
B.
Stu I
Eco RI
Sac I
-8.9
CAT
CYP2H1CAT-8.9
-4.7
CAT
CYP2H1CAT-4.7
-1.7
CAT
CYP2H1CAT-1.7
-1.1
CAT
CYP2H1CAT-1.1
-0.5
CAT
CYP2H1CAT-0.5
CAT
pIBICAT
C.
-8.9
-5.9
-1.1
CAT
CYP2H1CAT-8.9Δ

### 5.2.C. Effect of carrier DNA on level of phenobarbital-induction

The possibility was considered that the high concentration of salmon sperm DNA (500 µg/ml) used as carrier (as described earlier in Chapter 4 section 4.2.D), may sequester some of the *trans* -acting factors required for an optimal phenobarbital response. To investigate this, 1 pmol (*i.e.* 10 µg) of CYP2H1CAT-8.9 was transfected by electroporation into chick embryo primary hepatocytes (1 x $10^7$) in the presence and absence of carrier DNA and the effect of phenobarbital on the expression of CAT activity measured. While expression of CAT activity without carrier DNA, both in the presence and absence of phenobarbital was approximately 10 times less than with carrier, the fold induction with phenobarbital (~2-fold) was similar in both cases (results not shown). Hence, carrier DNA did not seem to affect the levels of induction of expression from this construct by phenobarbital, but rather markedly increased the amount of CAT activity produced following transfection and so increased the sensitivity of this expression system.

### 5.2.D. Time course of CAT expression following transfection of CYP2H1CAT-8.9

Earlier results (see section 3.2.E) showed that the *CYP2H1* gene in chick embryo primary hepatocytes responds transcriptionally to phenobarbital for at least 48 h, with a peak in the transcription rate at 12 h. Thus a time course of expression of CAT activity from CYP2H1CAT-8.9 was performed to determine the time at which maximal induction by phenobarbital occurred. Primary hepatocytes (2 x $10^7$) were transfected by electroporation with 1 pmol (*i.e.* 10 µg) of CYP2H1CAT-8.9. Following transfection, each sample was split and half of the cells were plated into each of two 60 mm diameter petri dishes (*i.e.* pair plates) containing William's E medium supplemented with 10% Nu-Serum. The cells were incubated for 20 h, after which phenobarbital to a concentration of 500 µM was added to one of each pair of plates and an equal volume of PBS was added to the other. The paired cultures were harvested at times ranging from 0 to 72 h after the addition of phenobarbital, the cells lysed and CAT activity determined. The results can be seen in Figure 5.3. Basal levels of CAT activity gradually accumulated over 72 h. Cultures induced with phenobarbital accumulated CAT enzyme at a greater rate, such that over time, the fold

**Figure 5.3. Time course of CAT expression from the CYP2H1CAT-8.9 construct**

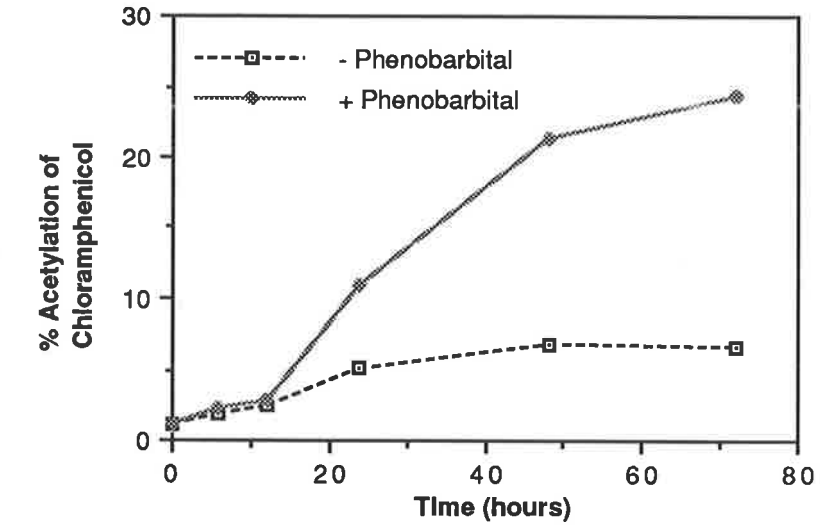
Primary hepatocytes ($2 \times 10^7$) were transfected in the presence of 500 µg/ml salmon sperm DNA with 1 pmol of CYP2H1CAT-8.9 by electroporation at 250 V and 960 µF. Each electroporated sample was then halved and plated in William's E medium supplemented with 10% Nu-Serum for 20 h. The medium was replaced with fresh medium and to one of each half, phenobarbital was added to a final concentration of 500 µM and to the other was added solvent only. Pairs of plates were harvested at times 0, 6, 12, 24, 48 and 72 h after addition of phenobarbital. CAT assays were performed on 50 µg of cellular protein extract. The results of this experiment are expressed in three different ways.

**A.** Time course of CAT expression from CYP2H1CAT-8.9 in the presence and absence of phenobarbital. The level of CAT expression is given as the percent of chloramphenicol acetylated. This demonstrates the difference in expression of CAT activity in response to phenobarbital with time.

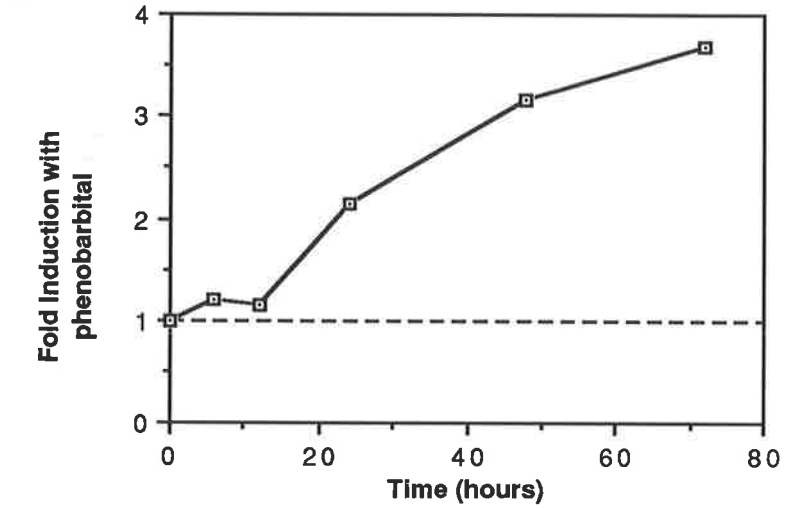
**B.** Time course of the fold induction of CAT expression with phenobarbital. This gives a measure of the magnitude of the effect of phenobarbital with time.

**C.** Time course of the rate of change in fold induction of CAT expression with phenobarbital. This shows the relative rate at which phenobarbital exerts its effect on CAT expression with time.

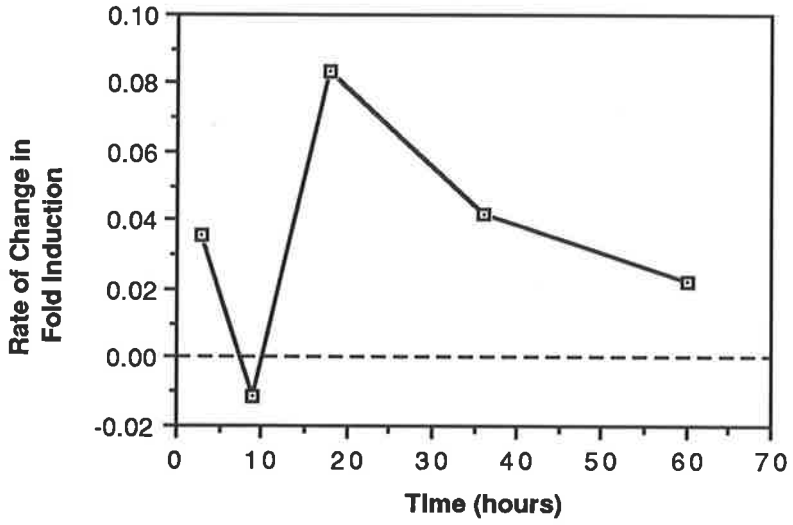
A.


30
20
10
0
- Phenobarbital
+ Phenobarbital
% Acetylation of Chloramphenicol
0
20
40
60
80
TIme (hours)

B.


4
3
2
1
0
Fold Induction with phenobarbital
0
20
40
60
80
Time (hours)

C.


0.10
0.08
0.06
0.04
0.02
0.00
-0.02
Rate of Change in Fold Induction
0
10
20
30
40
50
60
70
TIme (hours)

★ A further attempt to increase precision of the assay for CAT expression was performed by cotransfection of pRSVß-gal as a control plasmid and standardisation of CAT activity to ß-galactosidase activity (results not shown). However, expression of ß-galactosidase activity consistently increased by approximately 1.5-fold upon addition of phenobarbital to the primary hepatocyte cultures. Interestingly, expression from the RSV promoter is not induced by phenobarbital since CAT expression from pRSVCAT is not affected by phenobarbital. This finding implies that ß-galactosidase activity is increased post-transcriptionally by phenobarbital and because of this complication, cotransfection with pRSVß-gal was not pursued.

induction continued to rise. However, the rate of change in fold induction did not appear to be constant, but varied also with time. The graph of the rate of change in fold induction of CAT activity parallels closely that of the endogenously transcribed *CYP2H1* gene, where the greatest increases in CAT activity occurs shortly after peak levels of transcription of the *CYP2H1* gene (compare Fig. 3.7.B with Fig. 5.3.C).

Two important points can be gained from the above results. Firstly, splitting of the cell sample electroporated in one cuvette and treatment of one half with drug and one without, produced results that were more precise (coefficient of variation = 4-12%) compared with samples electroporated in different cuvettes (coefficient of variation = 17-32%). Secondly, the level of induction of CAT activity after 48 h (~3-fold) is lower than that for the endogenous *CYP2H1* gene (>10-fold). The probable explanation for this difference is that part of the regulatory regions necessary for the total response to phenobarbital are not contained in the CYP2H1CAT constructs, and hence a reduced response is seen.

A slight increase in the fold induction of CAT expression between 48 h (3.2-fold) and 72 h (3.7-fold) is shown in Figure 5.3.B. Due to the small size of this increase, little benefit was gained by incubation for an extra 24 h, and so future experiments involving induction with phenobarbital were carried out for 48 h, as had been done previously.
★

### 5.2.E. Effect of amount of CYP2H1CAT-8.9 DNA transfected on induction with phenobarbital

In preliminary experiments, it appeared that all CYP2H1CAT constructs displayed a very high basal level of CAT expression in chick embryo primary hepatocytes (see section 5.2.B). It was reasoned that multiple copies of a strongly expressing construct within a cell following transfection, could lead to a situation where there are not enough relevant available transcription factors to enable full expression of all the constructs within the cell. If this occurred, the cell would reach "saturation" for expression of such a construct and little or no further increase in transcription of the CAT gene would be possible upon addition of phenobarbital because factor availability would be limiting. A reduction in the amount of DNA transfected could therefore remove this limitation and result in a greater increase upon

addition of phenobarbital. To test this hypothesis, various amounts of CYP2H1CAT-8.9 construct DNA ranging from 0.25 to 1.50 pmol were transfected into primary hepatocytes, the sample split and the effect of phenobarbital determined by measurement of CAT activity. A similar fold induction of about 3-fold with phenobarbital was seen for transfection of 0.25 to 1.0 pmol of DNA (see Fig. 5.4). Beyond 1.0 pmol, the fold induction with phenobarbital begins to drop as the graph for phenobarbital-induced CAT activity begins to plateau. A possible explanation is that the number of constructs transfected per cell increases beyond that of which the cell can supply the appropriate factors to support maximal expression of each construct. Extrapolation of the graph below 0.25 pmol, passes though the x-axis y-axis intersection at zero zero, and therefore it may be assumed that transfecting even less than 0.25 pmol of DNA would have no effect on the fold of phenobarbital-induction. This result in a way is not unexpected, as transfection efficiency experiments performed earlier with ß-galactosidase (see section 4.2.B.4) revealed that at lower amounts of transfected construct DNA (up to 1.25 pmol) the number of tranformants increased proportionally with the amount of transfected DNA, but at higher amounts of transfected construct DNA (greater than 1.25 pmol) an increase was seen in the number of constructs transfected per cell.

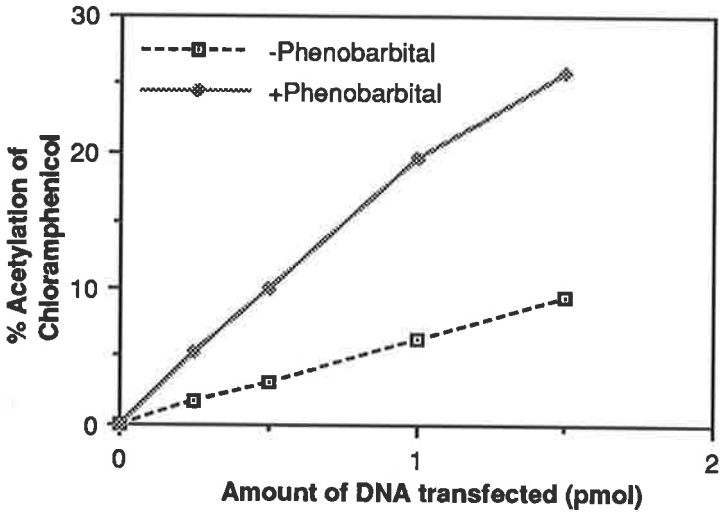
Using the same batch of primary hepatocytes as in the above experiment, 1.0 pmol of CYP2H1CAT-8.9 construct DNA was transfected, induced by 2 mM phenobarbital (rather than 500 μM) for 48 h and the fold increase was compared with that by 500 μM phenobarbital. Increasing the amount of added phenobarbital, led to a 7% increase in induction (results not shown), suggesting that 500 μM phenobarbital is near "saturation" for the phenobarbital "receptor" or the responsive mechanism operating in these cells under the conditions used. Future experiments were performed using 1 pmol of construct DNA (*i.e.* the upper limit of the linear range of CAT expression) and 500 μM phenobarbital for induction.

### 5.2.F. Effect of different media on phenobarbital-induction of CYP2H1CAT-8.9

Although William's E medium has been widely used as the culture medium of choice for chick embryo primary hepatocytes (Althaus *et al.*, 1979; Giger and Meyer, 1981; Lincoln

**Figure 5.4. Effect of the amount of CYP2H1CAT-8.9 construct transfected on expression of CAT activity in the presence and absence of phenobarbital**

Primary hepatocytes ($2 \times 10^7$) were transfected in the presence of 500 µg/ml salmon sperm DNA with 0, 0.25, 0.50, 1.00 or 1.50 pmol of CYP2H1CAT-8.9 by electroporation at 250 V and 960 µF. Each electroporated sample was halved and plated in William's E medium supplemented with 10% Nu-Serum for 20 h. The medium was then replaced with fresh medium and to one half was added phenobarbital to a final concentration of 500 µM and to the other was added solvent only. After a further 48 h incubation, the cells were harvested, CAT assays were performed on 5 µg of cellular protein extract and the results plotted.


30
20
10
0
0
1
2
-Phenobarbital
+Phenobarbital
% Acetylation of Chloramphenicol
Amount of DNA transfected (pmol)

*et al.*, 1988; Hamilton *et al.*, 1988), a report by Jauregui *et al* (1986) on long term culture of primary rat hepatocytes demonstrated that while William's E medium was able to maintain these cultures in the differentiated state (*i.e.* maintain expression of liver-specific genes) for up to 14 days, Chee's medium (Chee *et al.*, 1976), was able to maintain them for up to 28 days. This study also demonstrated that Chee's medium, unlike William's E medium, did not require the use of the expensive matrix "Matrigel" (a basement membrane matrix described earlier - see section 3.2.C), but a less expensive and more easily handled collagen matrix could be used. Chee's medium is a totally defined medium containing no serum supplements. The effect of Chee's medium on expression of CYP2H1CAT-8.9 in chick embryo primary hepatocytes was therefore investigated.

Work reported in section 3.2.C of this thesis, demonstrated that the level of induction of CYP2H1 mRNA with phenobarbital was similar whether chick embryo primary hepatocytes were plated directly onto plastic or onto Matrigel. However despite this earlier result, Matrigel was re-examined, this time to study its effect on the level of basal and phenobarbital-induced expression of CYP2H1CAT-8.9. Primary hepatocytes were transfected by electroporation with 1 pmol of CYP2H1CAT-8.9. Cell samples were split and cultured directly onto plastic or onto Matrigel in either William's E medium supplemented with 10% Nu-Serum or Chee's medium. The effect of 500 μM phenobarbital on expression of CYP2H1CAT-8.9 was determined by assaying for CAT activity. Results of this experiment are shown in Figure 5.5. Chee's medium allowed a 70% greater fold induction of CAT expression with phenobarbital than did William's E medium supplemented with 10% Nu-Serum. However, in Chee's medium the level of basal and phenobarbital-induced expression of CAT activity was only 10-20% of that in William's E medium supplemented with 10% Nu-Serum. The reason for this is unclear, especially since Waxman *et al* (1990) have recently published results showing that the presence of foetal bovine serum (10%) markedly reduces (>90%) phenobarbital-induction of CYP2B1 and CYP2B2 protein levels in rat liver. Similar effects of serum have also been reported previously, especially with respect to a loss of liver-specific functions in rat primary hepatocytes (Enat *et al.*, 1984; Jefferson *et al.*, 1984; Zaret *et al.*, 1988) and also in repressing the level of induction of CYPs (Schuetz *et al.*, 1988). Whether the 2.5% serum

**Figure 5.5. Effect of Matrigel and Chee's medium on the induction of CYP2H1CAT-8.9 with phenobarbital**

Primary hepatocytes ($2 \times 10^7$) were transfected in the presence of 500 µg/ml salmon sperm DNA with 2 pmol of CYP2H1CAT-8.9 by electroporation at 250 V and 960 µF. The cell sample was split, plated either directly onto plastic plates or onto plates that had been previously coated with Matrigel and cultured in William's E medium supplemented with 10% Nu-Serum or Chee's medium for 20 h. To one of each pair of plates was added phenobarbital to a final concentration of 500 µM and to the other was added PBS. The hepatocytes were incubated for a further 48 h, prior to harvesting and cell lysis. Protein extract (10 µg) was assayed for 1 h and the resultant autoradiographic exposure is shown.

**A.** William's E medium supplemented with 10% Nu-Serum

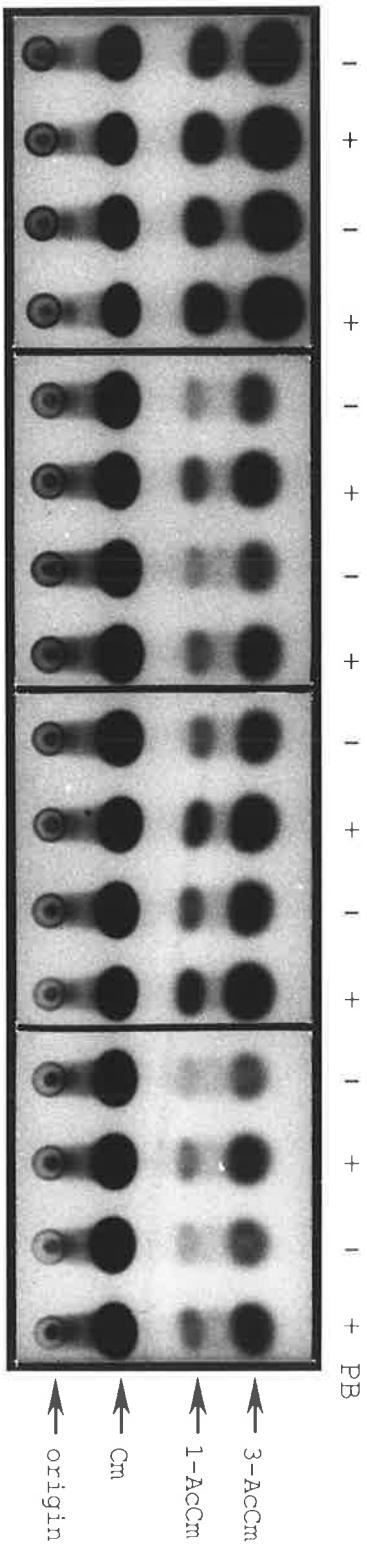
**B.** William's E medium supplemented with 10% Nu-Serum with Matrigel

**C.** Chee's medium

**D.** Chee's medium with Matrigel

The radioactivity in each spot was quantified by liquid scintillation counting and the percent of acetylated chloramphenicol was calculated. The results derived from four determinations are expressed as the mean fold induction with phenobarbital and the standard deviation values are shown in parentheses.

Fold induction with phenobarbital




1.5 (±0.2)

2.1 (±0.4)

2.6 (±0.2)

2.0 (±0.2)

present in the William's E medium supplemented with 10% Nu-Serum, which contains Newborn Calf Serum, is not enough to markedly influence phenobarbital induction of the *CYP2H1* promoter or whether adult rat and chick embryo primary hepatocytes differ in their response to serum is not known. It is also possible that the rat and chicken phenobarbital-inducible CYPs differ in their regulation by serum components. Despite a slightly greater fold induction of CAT activity with Chee's medium, William's E medium supplemented with 10% Nu-Serum was maintained for use in culture since the chick embryo primary hepatocytes had been characterised in this medium and the change would not offer significant advantages.

Matrigel did not significantly enhance the induction of expression of CYP2H1CAT-8.9 with phenobarbital. In fact, the presence of Matrigel reduced the recovery of cellular proteins from freeze/thawed cell lysates by 50-75% and reduced the total expression of basal and phenobarbital-induced CAT activity by 75-90%. One possible explanation is that during lysis of the cells, Matrigel binds cellular proteins of which the CAT enzyme is one. Alternatively, in the presence of Matrigel, the hepatocytes may be more refractory to lysis by freeze/thawing. The use of Matrigel was not further pursued. In all future experiments, chick embryo primary hepatocytes were cultured in William's E medium supplemented with 10% Nu-Serum and without the use of Matrigel.

### 5.2.G. Basal expression of CYP2H1CAT constructs and induction with phenobarbital

Attempts described to increase the fold induction by phenobarbital on expression of CAT activity from CYP2H1CAT-8.9 did not result in major increases, but in doing so, a method that enabled much greater precision of the fold induction with phenobarbital was developed. This method as mentioned earlier (see section 5.2.D), involved electroporation of a particular construct into primary hepatocytes, splitting the sample and treating one half of the sample with phenobarbital and the other without.

Using this more precise method, expression of the CYP2H1CAT constructs with and without phenobarbital was once again investigated (for preliminary experiments see section 5.2.B). Primary hepatocytes were transfected with CYP2H1CAT constructs (1 pmol) in the

presence of 500 μg/ml salmon sperm DNA by electroporation at 250 V and 960 μF. Each construct was transfected in triplicate. Following transfection, the sample was split and plated into each of two plates containing William's E medium supplemented with 10% Nu-Serum. The cells were incubated for 20 h and phenobarbital, to a concentration of 500 μM, was added to one plate and an equal volume of PBS added to the other. After incubation for a further 48 h, the cells were harvested, lysed and 5 μg of protein extract was assayed for CAT activity. Results for this experiment are shown in Figure 5.6. For comparison, this experiment was repeated, except that the transfected construct DNA was prepared by the traditional method of CsCl/ethidium bromide equilibrium density gradients instead of the QIAGEN column procedure (see section 2.3.A) which was used for all experiments described so far. In this repeat experiment, constructs were only transfected in duplicates rather than triplicates because of the difficulty in obtaining the required number of primary hepatocytes, and also to avoid possible problems arising from transfected hepatocytes remaining in electroporation buffer at 4°C (see section 2.7.A) for extended periods of time during the lengthy plating procedure required in such an experiment. These results are also shown in Figure 5.6. Basal expression of CAT activity, that is, expression in the absence of phenobarbital, did not vary significantly for all CYP2H1CAT constructs with the exception of CYP2H1CAT-4.7 which consistently expressed at levels 1.5 to 1.9-fold higher than the other constructs. Although basal expression of the shortest construct, CYP2H1CAT-0.5 is shown in experiment 1 to be the highest, in a repeat experiment (experiment 2 - see Fig. 5.6) this construct was demonstrated to express at a level similar to that of CYP2H1CAT-1.1 and CYP2H1CAT-1.7. This was confirmed in repeated experiments. The elevated expression in experiment 1, therefore appears to be an aberrant result, presumably due to this particular preparation of DNA. Interestingly, basal CAT expression of all of the constructs was comparable with that of pRSVCAT which contains the strong RSV long terminal repeat promoter/enhancer. Possible reasons for the strong promoter activity directed by the first 0.5 kb of the *CYP2H1* 5' flanking region will be discussed in detail in Chapter 7. As the basal level of expression for all CYP2H1CAT constructs was similar, except for the higher expression of CYP2H1CAT-4.7, the possibility existed of a positive regulatory region(s) between positions -4.7 and -1.7 kb, and a negative regulatory region(s) between positions

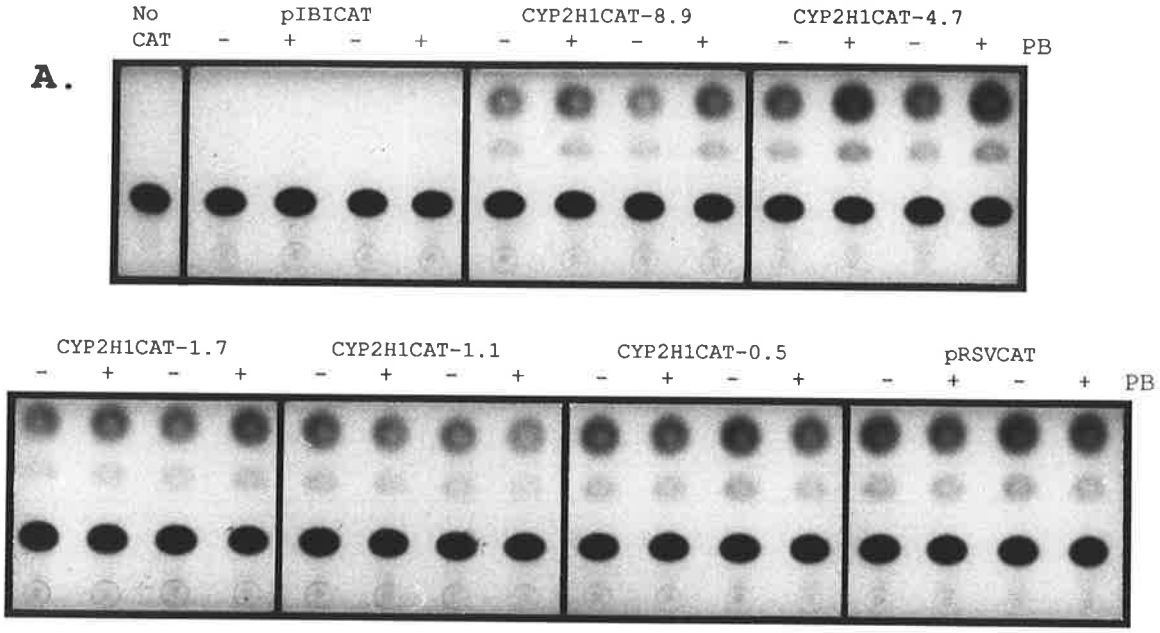
**Figure 5.6. Basal and phenobarbital-induced expression of CYP2H1CAT constructs**

**A.** Primary hepatocytes ($2 \times 10^7$) were transfected in the presence of 500 µg/ml salmon sperm DNA with 1.0 pmol of each construct by electroporation at 250 V and 960 µF. Each sample was split and cultured in William's E medium supplemented with 10% Nu-Serum for 20 h. To one of each pair of plates was added phenobarbital to a final concentration of 500 µM and to the other was added PBS. The hepatocytes were incubated for a further 48 h, prior to harvesting and cell lysis. CAT assays were performed on 5 µg of cellular protein extract for 1 h. The resultant autoradiographs of one such experiment (*i.e.* Experiment 2) are shown.

**B.** CAT activities were quantified by liquid scintillation counting and are expressed relative to the longest construct, CYP2H1CAT-8.9, in non-induced hepatocytes. A value of 100 has been arbitrarily given to this level of activity. The fold induction with phenobarbital was determined for each individually transfected construct, and the mean of these is given (NA - not applicable). Two different experiments were conducted. Experiment 1 was performed in triplicate using construct DNA prepared by the QIAGEN column procedure; the results are reported as the mean $\pm$ standard deviation. Experiment 2 was performed in duplicate using construct DNA prepared by two CsCl/ethidium bromide density gradient spins; the results represent the mean.

**C.** CAT assay results for the construct CYP2H1CAT-8.9Δ, which was made from CYP2H1CAT-8.9 by deletion of a 4.8 kb *Bam* HI fragment.

**A.**




**B.**

| Construct | CAT activity -PB | CAT activity +PB | Fold-induction with phenobarbital |
|---|---|---|---|
| CYP2H1CAT-8.9 | 100 | 238 | 2.38 (±0.09) |
| | 100 | 181 | 1.81 |
| CYP2H1CAT-4.7 | 177 | 302 | 1.72 (±0.09) |
| | 176 | 247 | 1.41 |
| CYP2H1CAT-1.7 | 122 | 167 | 1.37 (±0.08) |
| | 104 | 126 | 1.22 |
| CYP2H1CAT-1.1 | 134 | 127 | 0.95 (±0.04) |
| | 116 | 134 | 1.17 |
| CYP2H1CAT-0.5 | 192 | 187 | 0.98 (±0.12) |
| | 118 | 101 | 0.85 |
| pIBICAT | 0 | 0 | NA |
| | 0 | 0 | NA |
| pRSVCAT | 157 | 162 | 1.03 (±0.05) |
| No DNA | 0 | 0 | NA |
| | 0 | 0 | NA |

**C.**

| | | | |
|---|---|---|---|
| CYP2H1CAT-8.9Δ | 113 | 119 | 1.06 (±0.05) |

-8.9 and -4.7 kb that approximately negates the effect of the more proximal positive regulatory region(s).

Expression of CAT activity in hepatocytes transfected with constructs containing either 1.7, 4.7 or 8.9 kb of *CYP2H1* 5' flanking sequence was shown to increase when phenobarbital was added to the culture medium (see Fig. 5.6). The level of this induction consistently and significantly increased with increasing length of 5' flanking sequence beyond position -1.1 kb. Constructs containing either 0.5 or 1.1 kb of 5' flanking sequence showed no increase in expression after addition of phenobarbital. No drug-induction was observed with the control plasmid pRSVCAT, in which the CAT gene is under the control of the RSV long terminal repeat promoter/enhancer. Moreover, the promoterless plasmid pIBICAT, lacking *CYP2H1* 5' flanking sequence, did not express any detectable CAT activity even when large amounts of protein extract (50 μg) from transfected cells were assayed (results not shown) showing that expression of CAT activity was not being derived spuriously from the bacterial plasmid vector sequence.

This represents the first demonstration of phenobarbital-induction using a transient expression system in eucaryotes. These results suggest that multiple *cis* -acting elements involved in the overall response to phenobarbital reside between 1.1 and 8.9 kb upstream of the *CYP2H1* transcription start site. These elements must lie within at least three regions upstream of the gene; -8.9 to -4.7 kb , -4.7 to -1.7 kb and -1.7 to -1.1 kb.

In the preliminary experiments described in section 5.2.B, the response to phenobarbital of the constructs CYP2H1CAT-4.7 and -1.7 was variable. Highly reproducible results were, however, obtained using a modified transfection procedure. In this procedure, twice the number of hepatocytes (*i.e.* $2 \times 10^7$) were electroporated with the same amount of construct DNA, and the transfected cell sample was halved and plated separately. One plate was used for measurement of basal expression while the other plate was used for determination of phenobarbital-induced expression. This procedure enabled a consistent display of phenobarbital-induction of CYP2H1CAT-8.9, -4.7 and -1.7. These modifications were crucial for determining the regions within the *CYP2H1* gene 5' flanking region that were important in the phenobarbital response since the level of induction by phenobarbital was only of the order of 1.5 to 3-fold.

As mentioned earlier (see section 3.2.F), there are variations in basal expression of the *CYP2H1* gene and in the fold induction with phenobarbital between batches of chick embryo primary hepatocytes prepared on different days. This variation was also seen with the level of basal and phenobarbital-induced expression of CYP2H1CAT constructs, although the relative levels of basal expression and phenobarbital-induction between constructs remained quite constant. For instance, although the absolute fold induction with phenobarbital of CYP2H1CAT-8.9, -4.7 and -1.7 varied on different days with different batches of primary hepatocytes (coefficient of variation of 22%), the fold induction of CYP2H1CAT-8.9 was always greater than CYP2H1CAT-4.7, which in turn was always greater than CYP2H1CAT-1.7. For the above reasons, results from different batches of hepatocytes cannot be averaged. An analogous problem of variation in responsiveness of the *CYP17* gene to forskolin induced levels of cAMP between batches of primary bovine adrenocortical cells has been reported (Lund *et al.*, 1990).

### 5.2.H. Construction and expression of CYP2H1CAT-8.9Δ

From work in the previous section, elements involved in the response to phenobarbital appear to be located within a 7.8 kb region from position -8.9 to -1.1 kb of the 5' flanking region of the *CYP2H1* gene. An attempt to more precisely define the phenobarbital-responsive regulatory region was made in the following way. The longest and most highly phenobarbital-inducible construct CYP2H1CAT-8.9 was shown to contain only two *Bam* HI sites, one at position -5.9 kb and the other at position -1.1 kb (see Fig. 5.2). A third *Bam* HI site within the polylinker of pIBICAT was destroyed simultaneously with mutagenesis of the *CYP2H1* gene translation initiation ATG codon (see Fig. 5.1). CYP2H1CAT-8.9 was restricted with *Bam* HI, the 4.8 kb *Bam* HI fragment removed and the resultant vector recircularised to produce CYP2H1CAT-8.9Δ (see Fig. 5.2.C). This new construct was essentially the same as CYP2H1CAT-8.9, except that at least two of the regions shown to be involved in the phenobarbital response were removed. While the level of basal expression of CYP2H1CAT-8.9Δ in chick embryo primary hepatocytes was the same as that of CYP2H1CAT-8.9, expression of CAT activity from CYP2H1CAT-8.9Δ was not responsive to phenobarbital (see Fig. 5.6.C). By inference then, essential elements

for induction with phenobarbital must reside in the deleted 4.8 kb *Bam* HI fragment (position -5.9 to -1.1 kb). Expression studies designed to investigate the possible phenobarbital-inducing properties of the 4.8 kb *Bam* HI fragment are discussed in Chapter 6.

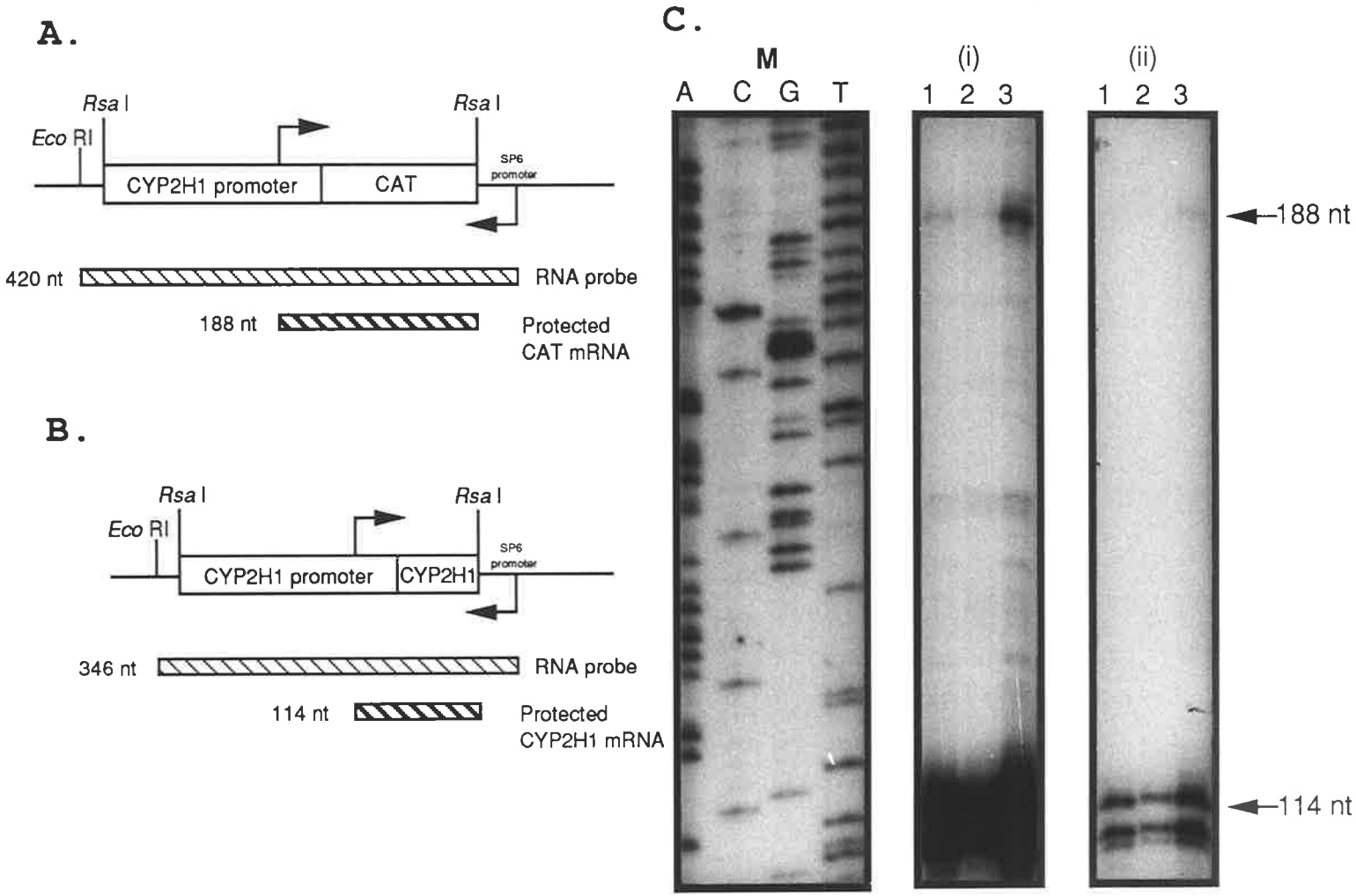
### 5.2.I. RNase protection analysis of RNA from primary hepatocytes transfected with CYP2H1CAT-8.9

RNase protection analysis was performed to investigate whether transcription of the CAT reporter gene driven by *CYP2H1* upstream sequences initiated at the correct *CYP2H1* transcription start site in both untreated and phenobarbital-treated hepatocytes transfected with the different CYP2H1CAT constructs. Templates for generating $^{32}$P-labelled RNA transcripts were made using the SP6 transcription vector, pGEM1. The transcription template for detection of CAT mRNA was constructed by excision and removal of a 366 bp *Rsa* I fragment, spanning the mRNA transcription start site, from CYP2H1CAT-0.5 and ligation into the *Hinc* II site of pGEM1 to create pGEM1-366 (see Fig. 5.7.A). Similarly, a 292 bp chicken genomic *Rsa* I fragment that spans the mRNA transcription start site of *CYP2H1* isolated from a pBR322 clone containing a 3.8 kb *Eco* RI/*Bam* HI chicken genomic fragment (Mattschoss *et al.*, 1986) was cloned into the *Hinc* II site of pGEM1 to create pGEM1-292 (see Fig. 5.7.B). This produced a template for detection of endogenous CYP2H1 mRNA. Both template plasmids were linearised with *Eco* RI. Synthesis of $^{32}$P-RNA from these templates produced the expected full length transcripts of 420 nt and 346 nt from pGEM1-366 and pGEM1-292, respectively. These RNA probes were hybridised to total RNA, isolated from untreated and phenobarbital-treated hepatocytes that had been transfected with CYP2H1CAT-8.9. Correct initiation from the *CYP2H1* gene transcription start site (Hobbs *et al.*, 1986) would be expected to result in protected mRNA species of size 188 nt and 114 nt for CAT mRNA and endogenous CYP2H1 mRNA, respectively. Major bands of these sizes were obtained from both uninduced and phenobarbital-induced cultures which is in keeping with initiation from the proposed *CYP2H1* start site. Bands of greater intensity were observed from phenobarbital-induced cells demonstrating elevated levels of endogenous CYP2H1 mRNA as well as CAT mRNA. In Figure 5.7.C, the drug-induced

**Figure 5.7. RNase protection analysis of total RNA from chick embryo primary hepatocytes transfected with CYP2H1CAT-8.9**

**A** and **B**. Diagrammatic representation of the constructs used as templates in the preparation of $^{32}$P-RNA transcripts for RNase protection. The expected size of protected mRNAs is shown for CAT mRNA (**A**) and the endogenous 3.5 kb mRNA derived from the *CYP2H1* gene (**B**).

**C**. Primary hepatocytes were transfected with 2 pmoles of CYP2H1CAT-8.9 by electroporation. Phenobarbital was added to a concentration of 500 µM and the hepatocytes were incubated for 48 hours. Control plates received an equal volume of PBS. Total RNA was then isolated and analysed in RNase protection assays. The $^{32}$P-RNA probes synthesized were hybridised to 20 µg (lane 1) and 8 µg (lane 2) of total RNA from non-induced hepatocytes and 8 µg (lane 3) of total RNA from phenobarbital-induced hepatocytes. Autoradiographs were allowed to expose for 120 hours (i) and 20 hours (ii) on Konica - Medical A x-ray film. The numbers displayed to the right of the autoradiographs correspond to the expected nucleotide lengths of the protected fragments. An M13mp18 DNA sequence ladder (M) was used to aid in size determination of the protected fragments.


A.
Rsa I
Rsa I
Eco RI
CYP2H1 promoter
CAT
SP6 promoter
420 nt
RNA probe
188 nt
Protected CAT mRNA
B.
Rsa I
Rsa I
Eco RI
CYP2H1 promoter
CYP2H1
SP6 promoter
346 nt
RNA probe
114 nt
Protected CYP2H1 mRNA
C.
M
A C G T
(i)
1 2 3
(ii)
1 2 3
188 nt
114 nt

increase in CAT mRNA amounts measured by RNase protection apparently exceeds the 2 to 3-fold increase seen in CAT activity from CYP2H1CAT-8.9 (see Fig. 5.6), but in repeated experiments, the fold induction of CAT mRNA more closely approximated that of CAT activity. Analysis of RNA from primary hepatocytes transfected with constructs CYP2H1CAT-4.7, 1.7 and 1.1 also revealed correct initiation of transcription in both untreated and phenobarbital-treated cultures (results not shown).

It is interesting to note the much greater intensity of the protected fragment derived from the endogenous 3.5 kb CYP2H1 mRNA compared with that from CAT mRNA. This is most likely due to the combination of a low transfection efficiency (approximately 10-15% of cells with 1 pmol of construct DNA as seen in Fig. 4.7.C) and the known instability of CAT mRNA which together could account for this difference in mRNA levels.

## 5.3. DISCUSSION

The aim of the work in this chapter was to analyse the transcriptional regulation of the *CYP2H1* gene in chick embryo primary hepatocytes and to search for regions of the gene that are involved in the inductive response to phenobarbital. To do this, chimeric constructs containing the CAT reporter gene fused to various lengths of the *CYP2H1* gene 5' flanking region were made, transfected into chick embryo primary hepatocytes and expression investigated in the presence and absence of phenobarbital.

Strong expression of CAT activity was seen from all CYP2H1CAT constructs in chick embryo primary hepatocytes representing to the author's knowledge, the first report in eucaryotes of expression from the promoter of a phenobarbital-inducible *CYP* gene. Interestingly, basal expression of the shortest construct CYP2H1CAT-0.5 was shown to be of the same order of magnitude as that driven by the strong Rous Sarcoma Virus long terminal repeat promoter/enhancer. Therefore, the first 0.5 kb of *CYP2H1* 5' flanking region must contain positive regulatory elements capable of promoting high expression in these cells. This region contains sequences for a putative TATA box, an inverted CCAAT box and a CACCC box, and similarities to the binding sites for AP-1, the erythroid-specific NF-E2, and the liver-specific HNF-1 and HNF-3 *trans* -acting factors. The sequence of this region and putative *cis* -acting elements within it are shown and discussed in more detail in

Chapter 7. Increasing the length of 5' flanking region beyond position -0.5 kb resulted in relatively constant basal expression to position -1.7 kb (*i.e.* CYP2H1CAT-1.7). However, addition of further sequence between position -4.7 and -1.7 kb resulted in a consistent increase of 1.5 to 1.9-fold in basal expression providing evidence for the presence of positive regulatory elements. Additional sequence from position -8.9 to -4.7 kb "neutralised" the positive effect of the region -4.7 to -1.7 kb, resulting in CAT expression driven from 8.9 kb of 5' flanking region (as found in CYP2H1CAT-8.9) being similar to that from the early promoter (as found in CYP2H1CAT-0.5). Thus evidence for negative regulation of this gene also exists (for reviews on negative regulation refer to Robertson, 1988; Levine and Manley, 1989). The possibility was considered that the reduced expression of CYP2H1CAT-8.9 compared with that of CYP2H1CAT-4.7 was due to a lower transfection efficiency because of the size difference of these constructs, 14.7 kb and 10.5 kb, respectively. However, removal of a 4.8 kb *Bam* HI fragment from CYP2H1CAT-8.9 to create CYP2H1CAT-8.9Δ (9.9 kb) resulted in a construct that expressed at the same level as the parent construct despite its size. While this is not conclusive evidence for similar transfection efficiencies of the different size constructs, it seems most probable that this is so especially following a report that constructs ranging in size from 3 to 55 kb transfect by electroporation with equal efficiencies (Andreason and Evans, 1988). This result also implies that the 4.8 kb *Bam* HI fragment excised from CYP2H1CAT-8.9 contains both the putative positive and negative basal regulatory regions suggested above.

When expression of the CYP2H1CAT constructs was investigated in the presence of phenobarbital, an important finding was made. The expression of the three largest constructs CYP2H1CAT-8.9, -4.7 and -1.7 was enhanced with phenobarbital. CYP2H1CAT-8.9 consistently gave the highest induction and CYP2H1CAT-1.7 the least, although the fold induction was quite low ranging from 1.8 to 2.4-fold for CYP2H1CAT-8.9 down to 1.2 to 1.4-fold for CYP2H1CAT-1.7. At least three regulatory regions therefore appear to be important in the production of maximal induction with phenobarbital and within these regions, one or more phenobarbital-responsive elements as well as other basal or regulated elements that modulate this drug response may reside. There may, of

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Another possible explanation is that the *CYP2H1* gene promoter is functioning at a near maximal level in the absence of phenobarbital and that, upon drug induction, a further increase in expression is limited. The high levels of basal expression of the CYP2H1CAT constructs may be due to transfection of cells with multiple copies of each construct such that transcription factors become limiting. Alternatively, the process of electroporation itself, while apparently not affecting endogenous mRNA levels, may somehow raise the level of basal expression from the constructs and subsequently partially mask the induction by phenobarbital.

course, be other regulatory regions outside of this first 8.9 kb of 5' flanking region which in concert, modulate the total phenobarbital response. This is the first time in eucaryotes that transiently transfected constructs containing the promoter of a phenobarbital-inducible gene have been shown to respond to phenobarbital.

Experiments were performed to study the level of response to phenobarbital in the chick embryo primary hepatocytes using the most highly inducible construct, CYP2H1CAT-8.9, with the aim of increasing the fold induction with phenobarbital to a level similar to that found for the native *CYP2H1* gene in nuclear transcription run-on assays (see Chapter 3). Salmon sperm DNA as carrier was shown not to affect the level of phenobarbital-induced expression from CYP2H1CAT-8.9, although the absolute level of expression was drastically reduced in its absence. Likewise, increasing the amount of construct DNA transfected from 0.25 to 1.0 pmol resulted in no significant change in the level of phenobarbital response. Beyond 1 pmol, the level of induction appeared to decline. This may be due to transfection of multiple copies of this highly transcriptionally active construct, such that a level is reached where the supply of cellular transcription factors becomes limiting. A time course of induction of CAT activity revealed that the fold induction with phenobarbital increased with time. This raised the possibility that the actual fold increase in CAT activity with phenobarbital is greater than the measured 2 to 3-fold because of a relatively high background of CAT activity already formed at the time of administration of phenobarbital to the cultures. However, this would be unlikely to account for the difference in the magnitude of induction with phenobarbital between the endogenous *CYP2H1* gene and the expression of CAT activity. A probable explanation is that the first 8.9 kb of 5' flanking region of the *CYP2H1* gene does not contain all of the necessary regulatory regions for a maximal response. ★

Investigation into the use of both Chee's medium (Chee *et al.*, 1976) and Matrigel (Schuetz *et al.*, 1988) on expression of CYP2H1CAT-8.9, revealed a number of interesting findings. In Chee's medium, induction with phenobarbital was approximately 70% higher than in William's E medium supplemented with 10% Nu-Serum, but the absolute level of basal and induced expression in Chee's medium was 5 to 10-fold lower. Chee's medium may therefore lack some component necessary for efficient expression of this construct or

may contain a component that reduces expression. Matrigel in the presence of Chee's medium or William's E medium supplemented with 10% Nu-Serum, had no significant effect on the magnitude of phenobarbital-induction of expression of CYP2H1CAT-8.9. This is in keeping with results shown earlier (see section 3.2.C) demonstrating Matrigel to have no effect on the phenobarbital response of endogenous CYP2H1 mRNA levels.

As mentioned above, removal of a 4.8 kb *Bam* HI fragment from CYP2H1CAT-8.9 to create CYP2H1CAT-8.9Δ resulted in no change in basal expression. By inference then, there appears to be at least one positive and one negative regulatory region within the 4.8 kb *Bam* HI fragment. The phenobarbital-induced expression of these two constructs, however differs dramatically. Deletion of the 4.8 kb *Bam* HI fragment from CYP2H1CAT-8.9 removes the ability of the resulting construct CYP2H1CAT-8.9Δ to respond to phenobarbital. This region not only appears to contain positive and negative regions, but also the regulatory region(s) enabling responsiveness to phenobarbital. Two possibilities exist with respect to the elements involved in the phenobarbital response. The 4.8 kb *Bam* HI fragment may contain in total those regions necessary for the phenobarbital response. Alternatively, it may contain only some of the essential elements which act in conjunction to produce the phenobarbital response and when one or some of these are removed, non-responsiveness results. A direct analysis of the 4.8 kb *Bam* HI fragment and regions within it was undertaken and is discussed in Chapter 6.

In rat primary hepatocytes, the *CYP2B1* and *CYP2B2* genes have been shown to be transcriptionally regulated by phenobarbital provided Matrigel is employed (Schuetz *et al.*, 1990), but as yet, there have been no reports of expression of *CYP2B1* or *CYP2B2* gene promoter/reporter constructs. Rangarajan and Padmanaban (1989) have defined a 360 bp *Hae* III fragment (position -179 to +181 bp) of the rat *CYP2B2* (P450e) gene which when transcribed in rat liver nuclear lysates produces correctly initiated CYP2B2 mRNA mini-transcripts. The transcription rate of this fragment was shown to increase in liver nuclear lysates from phenobarbital-treated rats as compared to control rats and the binding of protein to the region -179 to +31 bp was greater in liver nuclear lysates from phenobarbital-treated rats. DNase I foot-printing analysis using nuclear extract from phenobarbital-treated rats revealed a protected region from -87 to -56 bp which contained a CCAAT box-like motif and

an imperfect palindrome, but as no foot-print from control rat liver nuclear extracts was performed, it is not known if this region is important for the phenobarbital response.

Other phenobarbital-inducible gene promoters that have been investigated in transient expression studies include those for the conjugating enzymes, UDP glucuronosyltransferase-2 ($UDPGT_r$-2) and the Ya sub-unit of glutathione S-transferase (GST Ya). To date, up to 0.35 kb of the rat $UDPGT_r$-2 gene (Mackenzie, 1990) and up to 1.6 kb of the mouse GST Ya gene (Daniel *et al.*, 1989) 5' flanking regions have been investigated in transient expression studies. These promoters have been shown to express basally in a number of hepatoma cell lines, but as yet no response to phenobarbital has been reported.

The first demonstration of a *cis* -acting region of DNA capable of conferring, to a heterologous gene, responsiveness to phenobarbital-like drugs (*i.e.* barbiturates) was performed in the procaryote *Bacillus megaterium*. Expression from a transfected construct, containing the first 1.6 kb of 5' flanking region of the barbiturate-inducible *CYP102* gene (previously $P450_{BM-3}$) from this bacterium fused to the CAT gene, increased upon addition of pentobarbital to the culture medium (Wen *et al.*, 1989). The response to barbiturates was shown to occur upon transfection of this construct into *Bacillus megaterium*, but not into *E.coli*, suggesting that *E.coli* does not contain the necessary factors for this response. Deletion studies of the first 1.6 kb of 5' flanking region of the *CYP102* gene have revealed two putative positive regulatory regions, but a barbiturate-responsive region or element has not yet been defined (Wen *et al.*, 1989). Whether the mechanism of phenobarbital-induction in procaryotes relates to that in eucaryotes remains to be seen.

Other *CYP* genes whose promoters have been investigated in expression studies include the well characterised TCDD-inducible rat, mouse and human *CYP1A1* (see reviews Whitlock, 1987; 1989; Whitlock *et al.*, 1989; Fujii-Kuriyama *et al.*, 1989) and the steroid hydroxylase bovine *CYP17* ($P450_{17\alpha}$) (Lund *et al.*, 1990), bovine *CYP11A1* ($P450_{scc}$) (Ahlgren *et al.*, 1990), murine *Cyp21a-1* ($P450_{C21}$) (Handler *et al.*, 1988) and murine *Cyp11b-1* ($P450_{11\beta}$) (Mouw *et al.*, 1989).

Transient expression studies reported in this chapter have demonstrated, for the first time in eucaryotes, basal expression of a phenobarbital-inducible *CYP* gene and increased

expression by phenobarbital of a phenobarbital-inducible gene. A 4.8 kb *Bam* HI fragment from the 5' flanking region of the *CYP2H1* gene (-5.9 to -1.1 kb) has been shown indirectly to contain the elements necessary for phenobarbital-induction. In the following chapter, the characteristics of this fragment are investigated in detail.

# CHAPTER 6

# ISOLATION AND ANALYSIS OF A PHENOBARBITAL-RESPONSIVE ENHANCER DOMAIN

## 6.1. INTRODUCTION

Experiments performed in Chapter 5 demonstrated, using transient expression studies, the presence of *cis* -acting regions responsive to phenobarbital in the 5' flanking region of the chicken *CYP2H1* gene. These phenobarbital-responsive regions appeared to be located within a 4.8 kb *Bam* HI fragment (position -5.9 to -1.1 kb). This phenomenon has been further studied by determining the effect of the 4.8 kb *Bam* HI fragment on expression of a heterologous promoter. To do this, the 4.8 kb *Bam* HI fragment was inserted upstream and downstream of the CAT reporter gene driven by an enhancerless SV40 promoter. These constructs were introduced into chick embryo primary hepatocytes by electroporation and the effect of phenobarbital investigated. In addition, a search for the functional regions of this 4.8 kb *Bam* HI fragment was initiated.

## 6.2. RESULTS

### 6.2.A. Synthesis and expression of SV40 promoter/CAT constructs containing the 4.8 kb *Bam* HI fragment

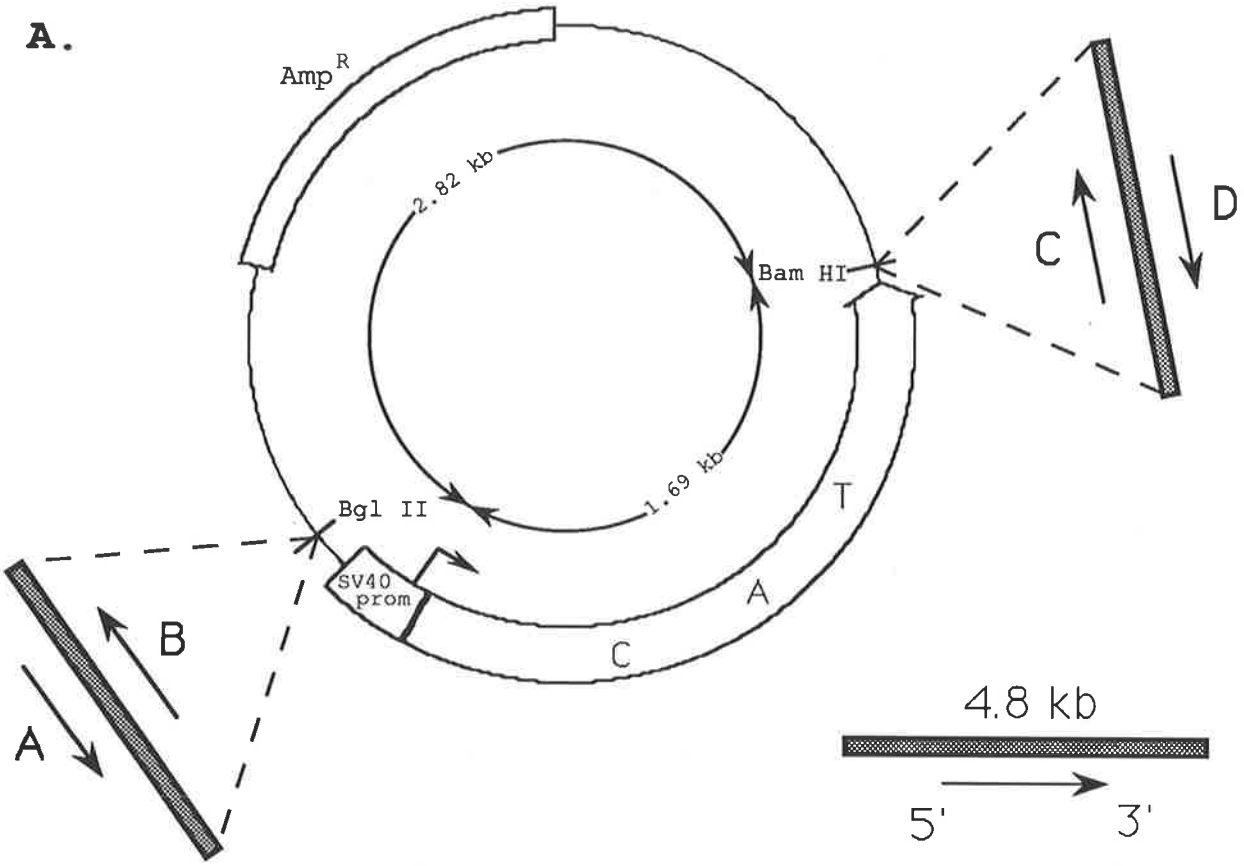
To directly demonstrate that the 4.8 kb *Bam* HI fragment from the 5' flanking region of the *CYP2H1* gene contains elements responsive to phenobarbital, the effect of this fragment on the expression driven from a heterologous promoter was investigated. The plasmid "pCAT™-promoter" (Promega) was chosen for this work. pCAT™-promoter is designed for detection of enhancers as it contains an enhancerless SV40 promoter upstream of the CAT reporter gene and polylinker cloning sites both immediately upstream of the SV40 promoter and downstream of the CAT gene (see Fig. 6.1.A). The 4.8 kb *Bam* HI fragment was cloned in both orientations into the *Bgl* II site and *Bam* HI site of pCAT™-promoter to produce the constructs pCATBg4.8, pCATBg4.8R, pCATBa4.8 and pCATBa4.8R (see Fig. 6.1.A). "Bg" and "Ba" refer to ligation of the 4.8 kb *Bam* HI fragment into the *Bgl* II and *Bam* HI sites respectively, while "R" refers to the reverse orientation with respect to the native promoter orientation.

**Figure 6.1. A 4.8 kb *Bam* HI fragment from the *CYP2H1* gene 5' flanking region confers phenobarbital-inducibility to the SV40 promoter**

**A.** A 4.8 kb *Bam* HI fragment (-5.9 to -1.1 kb) from the 5' flanking region of the *CYP2H1* gene was cloned in both orientations into the unique *Bgl* II and *Bam* HI sites of the pCAT™-promoter vector located about 150 bp and 2820 bp upstream of the SV40 promoter transcription start site, respectively (due to the circular nature of the plasmid, the *Bam* HI site can alternatively be considered about 1690 bp downstream of the SV40 transcription start site, immediately 3' of the CAT gene). The shaded box represents the 4.8 kb *Bam* HI fragment (not drawn to scale) while the arrow shows the 5' to 3' orientation of insertion. The resultant four constructs labelled A, B, C and D have been designated pCATBg4.8, pCATBg4.8R, pCATBa4.8 and pCATBa4.8R, respectively.

**B.** These constructs (2 pmol) were introduced into primary hepatocytes in triplicate by electroporation and the samples were halved and plated in William's E medium supplemented with 10% Nu-Serum for 20 h. Phenobarbital was then added to a concentration of 500 μM to one of each pair of plates and to the other was added PBS. The cells were incubated for a further 48 h prior to harvesting, lysis and assaying for CAT activity. CAT activity is expressed as the percent of acetylated chloramphenicol and the fold-induction with phenobarbital is given as the mean ± standard deviation. The results shown are indicative of a typical experiment using one batch of primary hepatocytes. Similar results were obtained with at least two other batches of primary hepatocytes prepared on different days.

**A.**




**B.**

| | Construct | CAT activity -PB | CAT activity +PB | Fold PB Induction Mean (± S.D.) |
|---|---|---|---|---|
| | pCAT™-promoter | 1.19 | 1.11 | 0.94 (±0.1) |
| A. | pCATBg4.8 | 1.00 | 14.03 | 14.0 (±0.9) |
| B. | pCATBg4.8R | 3.89 | 57.41 | 14.3 (±0.8) |
| C. | pCATBa4.8 | 2.24 | 18.00 | 8.1 (±0.4) |
| D. | pCATBa4.8R | 2.16 | 18.83 | 8.8 (±0.8) |

The vector pCAT™-promoter and the various constructs containing the 4.8 kb *Bam* HI fragment (2 pmol) were transfected into 2 x $10^7$ chick embryo primary hepatocytes by electroporation at 250 V and 960 μF in the presence of 500 μg/ml salmon sperm DNA. The samples were split and cultured overnight in William's E medium supplemented with 10% Nu-Serum. Phenobarbital or an equal volume of PBS (to control cultures) was then added and the cells incubated for a further 48 h. The results of assaying cell lysates for CAT activity are shown in Figure 6.1.B. Analysis of the basal level of expression from the enhancerless SV40 promoter revealed that the 4.8 kb *Bam* HI fragment conferred a slight positive effect of 1.5 to 3-fold, depending on the orientation of the construct and variation between batches of primary hepatocytes. In Figure 6.1.B, the basal level of expression of pCATBg4.8 is similar to that of pCAT™-promoter. However this was a unique situation, as on at least five other occasions, it was shown to express at a basal level of 1.5 to 2.0-fold higher than pCAT™-promoter (results not shown). The reason for this single variation is not known.

By far, the most striking observation was that the 4.8 kb *Bam* HI fragment conferred phenobarbital-responsiveness to the SV40 promoter (see Fig. 6.1.B). When the *Bam* HI fragment was placed immediately upstream of the SV40 promoter (*i.e.* in the *Bgl* II site), the addition of phenobarbital caused a 14.0-fold increase in expression of CAT activity with this fragment directed in the native orientation with respect to the promoter and a 14.3-fold increase when in the reverse orientation. Insertion of the 4.8 kb *Bam* HI fragment immediately downstream of the CAT reporter gene (*i.e.* in the *Bam* HI site), led to an 8.1-fold increase in expression of CAT activity by phenobarbital in the native orientation and a 8.8-fold increase when in the reverse orientation. Whether or not all of the elements necessary for the total phenobarbital response, as seen in the *CYP2H1* gene, are included in this fragment is not known, but it is clear that the elements essential to confer phenobarbital-inducibility to a heterologous promoter are present. From this data, it can be concluded that the 4.8 kb *Bam* HI fragment has the properties of an enhancer; it functions over large distances and is orientation independent. However, a distance-dependent mechanism is involved since insertion of the fragment immediately upstream of the enhancerless SV40 promoter resulted in a higher fold induction with phenobarbital (~14-fold) compared with

insertion of the fragment downstream of the CAT gene (~8-fold). Possible reasons for this result are proposed later (see section 6.3. Discussion).

Another aspect of these results is that the fold induction with phenobarbital is much higher from constructs containing the enhancerless SV40 promoter than it is from those with the native *CYP2H1* gene promoter (*i.e.* CYP2H1CAT constructs). This may be due to the strength of the respective promoters in chick embryo primary hepatocytes. The *CYP2H1* gene early promoter (up to -0.5 kb) can drive strong basal expression, equivalent to that of the RSV long terminal repeat promoter/enhancer, and it is possible that this level of expression approaches an upper limit in these cells such that upon addition of phenobarbital, a further increase in expression is limited. In contrast, the enhancerless SV40 promoter drives basal expression at a rate which is only about 1/100 of that from the *CYP2H1* gene promoter and, conceivably then, upon addition of phenobarbital an increase in expression from this SV40 promoter is not restricted.

It should also be pointed out that as with the CYP2H1CAT constructs (see Chapter 5), the absolute level of expression and fold induction of the pCAT™-promoter constructs with phenobarbital varied between batches of primary hepatocytes prepared on different days. For instance, with four separate batches of primary hepatocytes made on different days, phenobarbital induced the pCATBg4.8 construct 5, 14, 5 and 8-fold, respectively (results not shown). However, within a single batch, the level of expression of each construct and fold induction were remarkably consistent with coefficients of variation of less than 15%.

The 4.8 kb *Bam* HI fragment does confer phenobarbital-inducibility to the heterologous SV40 promoter in either orientation and at different distances and will subsequently be referred to as the "phenobarbital-responsive enhancer domain". The following section of experiments demonstrates the specificity of this domain in response to various drugs.

### 6.2.B. Effect of various drugs on expression of pCATBg4.8 and CYP2H1CAT-8.9

The chicken *CYP2H1* gene is known to be induced by the drugs phenobarbital, AIA and DDC, but not significantly by drugs such as TCDD (an aryl hydrocarbon) which induce members of the *CYP1* family or pregnenolone-16-α-carbonitrile (PCN) which induces members of the *CYP3* family. To investigate the specificity of drug-induction conferred by the 4.8 kb *Bam* HI fragment, chick embryo primary hepatocytes were transfected as described in the previous section, with pCAT™-promoter (2 pmol), pCATBg4.8 (2 pmol) or CYP2H1CAT-8.9 (1 pmol) and CAT activity measured following addition of AIA, 20-methylcholanthrene (an aryl hydrocarbon) or dexamethasone (a "PCN-like" drug). The results are shown in Table 6.1. Expression of pCATBg4.8 increased 4.6-fold with AIA while increases in expression of 1.5 and 1.1-fold occurred with 20-methylcholanthrene and dexamethasone, respectively. Upon treatment with these compounds, expression of the control plasmid pCAT™-promoter, did not vary significantly from the untreated state. Furthermore, expression of CYP2H1CAT-8.9 was induced 2.2-fold in the presence of AIA, while expression with 20-methylcholanthrene and dexamethasone only increased 1.3 and 1.1-fold, respectively (possible reasons for the lower fold induction by AIA of this construct when compared with pCATBg4.8 have been discussed in the previous section). These results demonstrate that the 4.8 kb *Bam* HI fragment responds substantially to AIA, marginally to 20-methylcholanthrene, but not significantly to dexamethasone.

To determine whether CYPs were expressed in chick embryo primary hepatocytes in response to 20-methylcholanthrene and dexamethasone as well as phenobarbital and AIA, total RNA was isolated from cultures that had not been transfected with construct DNA, but which had been treated for 48 h with each of the drugs of interest. Fractionated RNA was transferred by Northern blotting to Nytran filters and probed for specific CYP mRNAs. In rat liver, 20-methylcholanthrene and dexamethasone induce CYP1A and CYP3A mRNAs, respectively. To determine whether 20-methylcholanthrene and dexamethasone induce "CYP1A-like" and "CYP3A-like" mRNAs in chick embryo primary hepatocytes, Northern blots were probed using rat *CYP1A1* (P450c; Yabusaki *et al.*, 1984) and *CYP3A1* ($P450_{pcn1}$; Gonzalez *et al.*, 1985) cDNA probes. The results are shown in Figure 6.2.

**Table 6.1. Effect of various drugs on transient expression of pCAT™-promoter, pCATBg4.8 and CYP2H1CAT-8.9 in chick embryo primary hepatocytes.**

Primary hepatocytes ($2 \times 10^7$) were transfected in the presence of 500 µg/ml salmon sperm DNA with either pCAT™-promoter (2 pmol), pCATBg4.8 (2 pmol) or CYP2H1CAT-8.9 (1 pmol) in triplicate by electroporation at 250 V and 960 µF. Each sample was split and incubated in William's E medium supplemented with 10% Nu-Serum for 20 h. Drugs were then added to one of each pair to give final concentrations of 50 µg/ml of 2-allyl-2-isopropylacetamide (AIA), 1 µg/ml of 20-methylcholanthrene (20-MC) or 10 µM dexamethasone (Dex). The other of each pair (*i.e.* control) received an equal volume of solvent only. After 48 hours, the hepatocytes were harvested, lysed and the level of CAT activity determined. The results are given as the mean fold-increase in CAT activity, above that of the control, following addition of drug. Standard deviation values are given in parentheses. Similar results were obtained with at least 2 other different batches of primary hepatocytes.

**Table 6.2. Effect of growth hormone on the response of phenobarbital in transient expression of pCAT™-promoter, pCATBg4.8 and CYP2H1CAT-8.9 in chick embryo primary hepatocytes.**

Primary hepatocytes were transfected and cultured as described in Table 6.1. To the appropriate plates, phenobarbital (PB) was then added to a final concentration of 500 µM and porcine growth hormone (pGH) to 5 µg/ml. The cells were then further cultured, CAT assays performed on cell extracts and the results expressed as described for Table 6.1.

## Table 6.1

| Drug | pCAT™-promoter | | pCATBg4.8 | | CYP2H1CAT-8.9 | |
|---|---|---|---|---|---|---|
| **AIA** | 1.07 | (± 0.23) | 4.60 | (± 0.17) | 2.20 | (± 0.003) |
| **20-MC** | 1.24 | (± 0.15) | 1.47 | (± 0.35) | 1.31 | (± 0.16) |
| **Dex** | 0.84 | (± 0.06) | 1.11 | (± 0.18) | 1.14 | (± 0.04) |

## Table 6.2

| Drug | pCAT™-promoter | | pCATBg4.8 | | CYP2H1CAT-8.9 | |
|---|---|---|---|---|---|---|
| **PB** | 0.90 | (± 0.002) | 5.04 | (± 0.39) | 2.43 | (± 0.02) |
| **PB + pGH** | 0.77 | (± 0.01) | 5.41 | (± 1.36) | 2.49 | (± 0.15) |
| **pGH** | 1.06 | (± 0.03) | 0.99 | (± 0.03) | 1.08 | (± 0.08) |

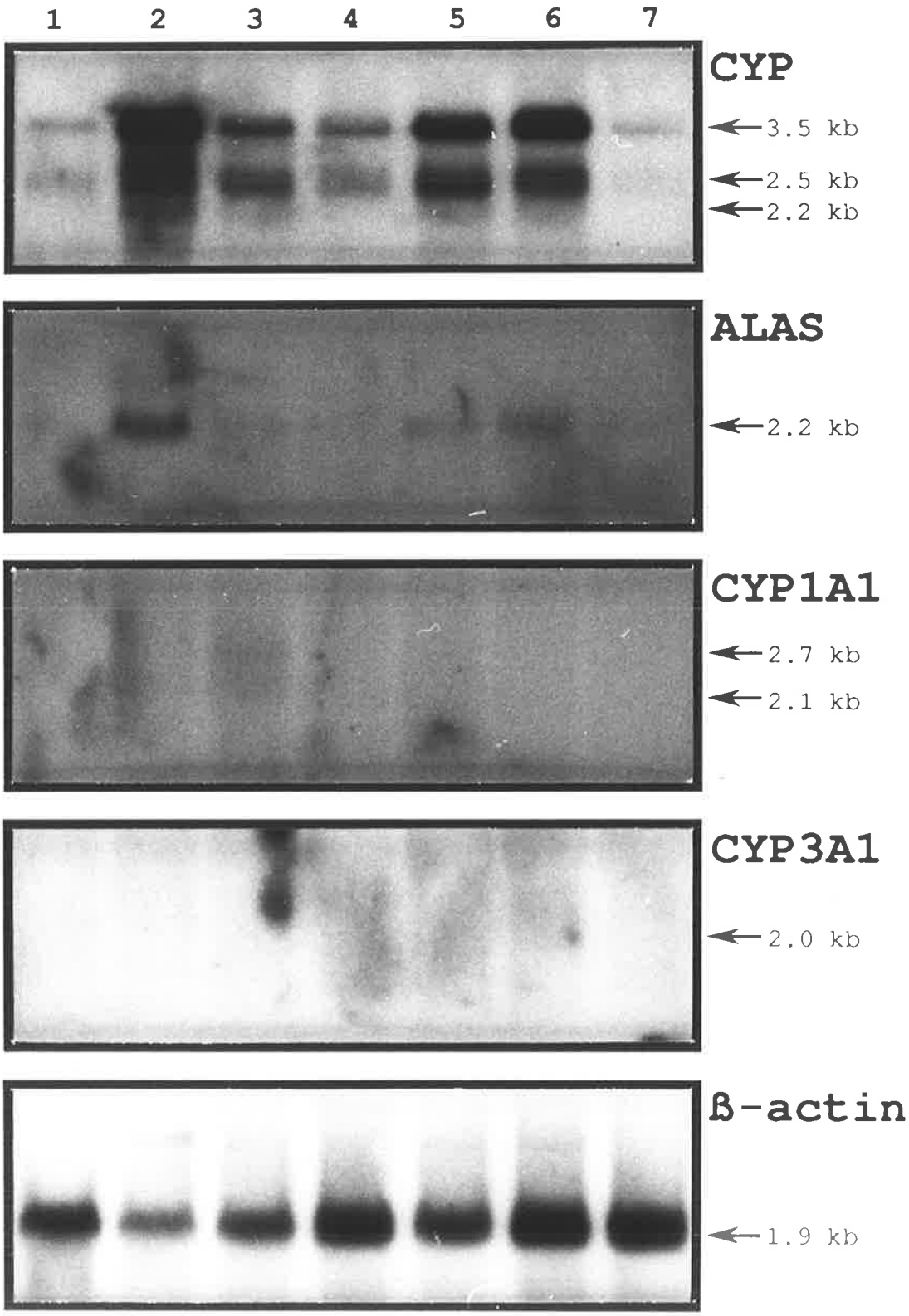
**Figure 6.2. Effect of various drugs and growth hormone on ALAS and several CYP mRNAs in chick embryo primary hepatocytes**

Primary hepatocytes ($1 \times 10^7$) were cultured in William's E medium supplemented with 10% Nu-Serum and incubated for 20 h. Drugs and porcine growth hormone were then added to give the following final concentrations: 50 µg/ml of 2-allyl-2-isopropylacetamide; 1 µg/ml of 20-methylcholanthrene; 10 µM dexamethasone; 500 µM phenobarbital; and 5 µg/ml of porcine growth hormone. The control cultures received PBS. The cells were further incubated for 48 h prior to harvesting and isolation of RNA. Total RNA (20 µg/lane) was run in a 1% agarose-formaldehyde gel and transferred to Nytran filters.

The RNA samples were loaded as follows:

lane 1, PBS
lane 2, 2-allyl-2-isopropylacetamide
lane 3, 20-methylcholanthrene
lane 4, dexamethasone
lane 5, phenobarbital
lane 6, phenobarbital and porcine growth hormone
lane 7, porcine growth hormone

The filters were probed individually for phenobarbital-inducible CYP mRNAs with pCHP3, for ALAS mRNA with p105B1 and for ß-actin mRNA with a chicken ß-actin cDNA clone. These filters were washed with a solution containing 0.1x SSPE, 0.1% $NaDodSO_4$ and 0.05% sodium pyrophosphate at 65°C for 1 h. In addition, cDNAs for the rat CYP1A1 and CYP3A1 mRNAs were used to probe for the chicken homologues. These filters were washed with a solution containing 2x SSPE, 0.1% $NaDodSO_4$ and 0.05% sodium pyrophosphate at 49°C for 1 h. To obtain suitable autoradiographs, the filters probed with pCHP3, ß-actin and CYP3A1 were exposed to normal sensitivity film for 1, 3 and 3 days, respectively while those probed with p105B1 and CYP1A1 were exposed to sensitive film for 10 and 7 days, respectively.


1
2
3
4
5
6
7
CYP
3.5 kb
2.5 kb
2.2 kb
ALAS
2.2 kb
CYP1A1
2.7 kb
2.1 kb
CYP3A1
2.0 kb
ß-actin
1.9 kb

Using the rat *CYP1A1* cDNA probe, two low abundance mRNAs were detected following treatment of primary hepatocytes with 20-methylcholanthrene. In aryl hydrocarbon-induced rat liver, *CYP1A1* cDNA detects a 2.7 kb CYP1A1 mRNA and a 2.1 kb CYP1A2 mRNA (Kawajiri *et al.*, 1984; Yabusaki *et al.*, 1984). Hence, the two faint but detectable bands seen in this experiment probably represent the chicken CYP1A1 and CYP1A2 mRNAs (see Fig. 6.2, lane 3), since the genes for these two mRNAs are highly conserved across species (Nebert *et al.*, 1991). Despite several attempts, no mRNA species could be detected using the rat *CYP3A1* cDNA probe, even following dexamethasone treatment. It is possible that the sequence of the chicken *CYP3A* (*i.e.* dexamethasone-inducible *CYP* ) mRNA differs significantly from the rat *CYP3A1* mRNA sequence and hence is not be detected under the hybridisation and washing stringencies used. However, PCN-P450 protein (presumably belonging to the *CYP3A* sub-family) can be detected immunologically using a monoclonal antibody against rat PCN-inducible CYP (*i.e.* CYP3A1), in chick embryo liver and in adult chicken liver (Lorr *et al.*, 1989). This protein is induced poorly by phenobarbital in the chick embryo and more strongly by phenobarbital and dexamethasone in adult chicken (Lorr *et al.*, 1989). It is not known whether the rat *CYP3A1* cDNA probe would detect the mRNA corresponding to this protein in chick embryo liver. Therefore, it is not possible, from the experiments performed here, to determine whether dexamethasone can function to induce CYP3A-like mRNAs in these primary hepatocytes.

Filters were probed for the chicken phenobarbital-inducible CYPs and as well for ALAS and ß-actin. Three phenobarbital-inducible CYP mRNAs of size 3.5, 2.5 and 2.2 kb were, as expected from earlier work, induced strongly by AIA and phenobarbital. 20-Methylcholanthrene also significantly induced these three CYP mRNAs, although to a lesser extent than AIA and phenobarbital while dexamethasone had little, if any, effect (see Fig. 6.2). Interestingly, from transient expression studies, 20-methylcholanthrene marginally induced the constructs containing the 4.8 kb *Bam* HI fragment and dexamethasone had no significant inductive effect. Taken together, these findings suggest one of two possibilities. Either 20-methylcholanthrene is acting as a "phenobarbital-like" drug in this system or it is acting via the classic Ah receptor transduction pathway through dioxin-responsive elements (DREs) within the 4.8 kb *Bam* HI fragment. Isolation of the

phenobarbital-inducible elements will be necesary to conclusively determine if aryl hydrocarbons such as 20-methylcholanthrene can exert an effect through the same induction pathway as phenobarbital. Such minor levels of induction of a specific CYP by drugs that strongly induce another class of CYPs is relatively common amongst the members of the CYP superfamily (Gonzalez and Nebert, 1990). ALAS mRNA amounts were induced by AIA, phenobarbital and 20-methylcholanthrene to levels that corresponded with those of the phenobarbital-inducible CYP mRNAs, once again demonstrating a link between ALAS and CYP induction by drugs. The levels of ß-actin mRNA remained relatively consistent, although AIA caused a significant decrease and this may reflect prolonged exposure of the hepatocytes to this drug since no effect is seen at earlier times (results not shown).

### 6.2.C. Effect of growth hormone on expression of pCATBg4.8 and CYP2H1CAT-8.9

Yamazoe *et al* (1987) initially reported that growth hormone was able to suppress phenobarbital-induction of the rat CYP2B1/2 (previously P450b/e) proteins in the liver. Subsequently, human growth hormone added to rat primary hepatocyte cultures, has been shown to completely block transcriptional induction of *CYP2B1/2* genes by phenobarbital (Schuetz *et al.*, 1990) and to suppress the phenobarbital-response of androstenedione 16ß-hydroxylase (*i.e.* one of the enzyme activities of the CYP2B1 protein) by greater than 85% (Waxman *et al.*, 1990). It was of interest then to investigate the effect of growth hormone on phenobarbital-induced expression of constructs containing phenobarbital-responsive regions following introduction into chick embryo primary hepatocytes.

To investigate this, chick embryo primary hepatocytes were transfected as in section 6.2.B. Following overnight incubation, porcine growth hormone to a concentration of 5 µg/ml and phenobarbital to a concentration of 500 µM were added to the culture medium and an equal volume of phosphate buffered saline was added to control plates. Obviously the use of chicken growth hormone would have been desirable, but this was not available and therefore the readily available porcine growth hormone was used. The concentration of porcine growth hormone used was approximately five times greater than the amount of human growth hormone shown to completely repress phenobarbital-induced transcription of

*CYP2B1/2* genes in rat primary hepatocytes (Schuetz *et al.*, 1990). Table 6.2 shows that the hormone elicited no effect on basal or phenobarbital-induced expression of pCATBg4.8 and CYP2H1CAT-8.9 in chick embryo primary hepatocyte cultures. A similar result was also obtained for the level of the endogenous 3.5 kb CYP2H1 mRNA upon treatment of control and phenobarbital-induced cultures with growth hormone (see Fig. 6.2). Possible reasons for the different responses of chick embryo primary hepatocytes and rat primary hepatocytes to growth hormone are suggested later in the discussion. No further attempt was made to study the effect of growth hormone on phenobarbital-induction of the *CYP2H1* gene. Instead, efforts were focused on narrowing down the region(s) within the 4.8 kb *Bam* HI fragment responsible for induction by phenobarbital.

### 6.2.D. Synthesis and expression of SV40 promoter/CAT constructs containing smaller fragments of the phenobarbital-responsive enhancer domain.

To further define the elements within the 4.8 kb *Bam* HI fragment (phenobarbital-responsive enhancer domain) necessary for the phenobarbital-response, the following strategy was employed. The 4.8 kb *Bam* HI fragment was cut into smaller fragments and each of these was individually ligated into the pCAT™-promoter. The basal and phenobarbital-induced expression of the resultant constructs in chick embryo primary hepatocytes was examined. Initially the 4.8 kb *Bam* HI fragment was completely digested with *Bgl* II resulting in six fragments of approximate size 220, 290, 780, 900, 920, 1740 bp. Unfortunately, after several attempts to ligate these fragments into the unique *Bgl* II site of pCAT™-promoter, only clones containing the 220, 290, and 780 bp fragments were obtained and these were designated pCATBg-220, -290 and -780, respectively. The reason for this inability to clone the 900, 920 and 1740 bp fragments is not clear.

As it was important to clone subfragments representing the entire 4.8 kb *Bam* HI fragment, different restriction enzymes were used to cut the 4.8 kb fragment, and the generated pieces were cloned into an alternative vector pBCSVp-1 (*i.e.* a pBluescriptKS$^+$/CAT/SV40 promoter chimeric plasmid; Clark *et al.*, 1989). pBCSVp-1 contains the enhancerless SV40 promoter fused immediately upstream of the CAT gene

which has been ligated into the unique *Bam* HI site of pBluescriptKS$^+$, and is essentially the same as pCAT™-promoter except that contains more suitable polylinker sites for cloning. Prior to dissection of the 4.8 kb *Bam* HI fragment and cloning of the resultant pieces, the entire 4.8 kb fragment was end-filled and ligated into an *Eco* RV linearised and dephosphorylated unique site of the pBCSVp1 vector in a 5' to 3' orientation immediately upstream of the SV40 promoter. This clone was designated pBCSVp1-4840 and although it is essentially the same as pCATBg4.8, pBCSVp1-4840 was transfected into chick embryo primary hepatocytes to check for basal expression and response to phenobarbital. Expression of the pCATBg4.8 construct was also examined for comparison. Both constructs, as expected, expressed similarly and were induced by similar amounts with phenobarbital (results not shown). Following this result, the 4.8 kb *Bam* HI fragment was dissected and the fragments cloned into pBCSVp1. The cloning strategy is shown in Figure 6.3. All restriction fragments were blunted and cloned into a dephosphorylated *Eco* RV cut pBCSVp-1 vector. Where possible the orientation of the fragments were determined by restriction mapping. The arrows represent the restriction fragments and the orientation (5' to 3') in which they were inserted.

To analyse expression of the various constructs, the following experiment was performed. Each construct (2 pmol) was transfected by electroporation into chick embryo primary hepatocytes. The samples were then split, cultured overnight in William's E Medium supplemented with 10% Nu-Serum and phenobarbital or an equal volume of PBS (to control cultures) was added to the culture medium. Following a further 48 h incubation, the cells were harvested, lysed and cell extracts were assayed for CAT activity. These results are shown in Figure 6.3. The basal expression of CAT activity of both SV40 promoter/CAT constructs, pBCSVp1 and pCAT™-promoter were the same and were given an arbitrary value of 1.0. Neither of these constructs induced with phenobarbital. In this figure, the results of all pBCSVp1-derived constructs and all pCAT™-promoter-derived constructs have been related to the level of expression of pBCSVp1 and pCAT™-promoter, respectively. As shown in Figure 6.3, various regions of the large 4.8 kb *Bam* HI fragment contain elements capable of modulating expression of the enhancerless SV40 promoter. Two distinct regions were shown to positively regulate basal expression of CAT activity.

**Figure 6.3. Expression of SV40 promoter/CAT constructs containing fragments of the phenobarbital-responsive enhancer domain**

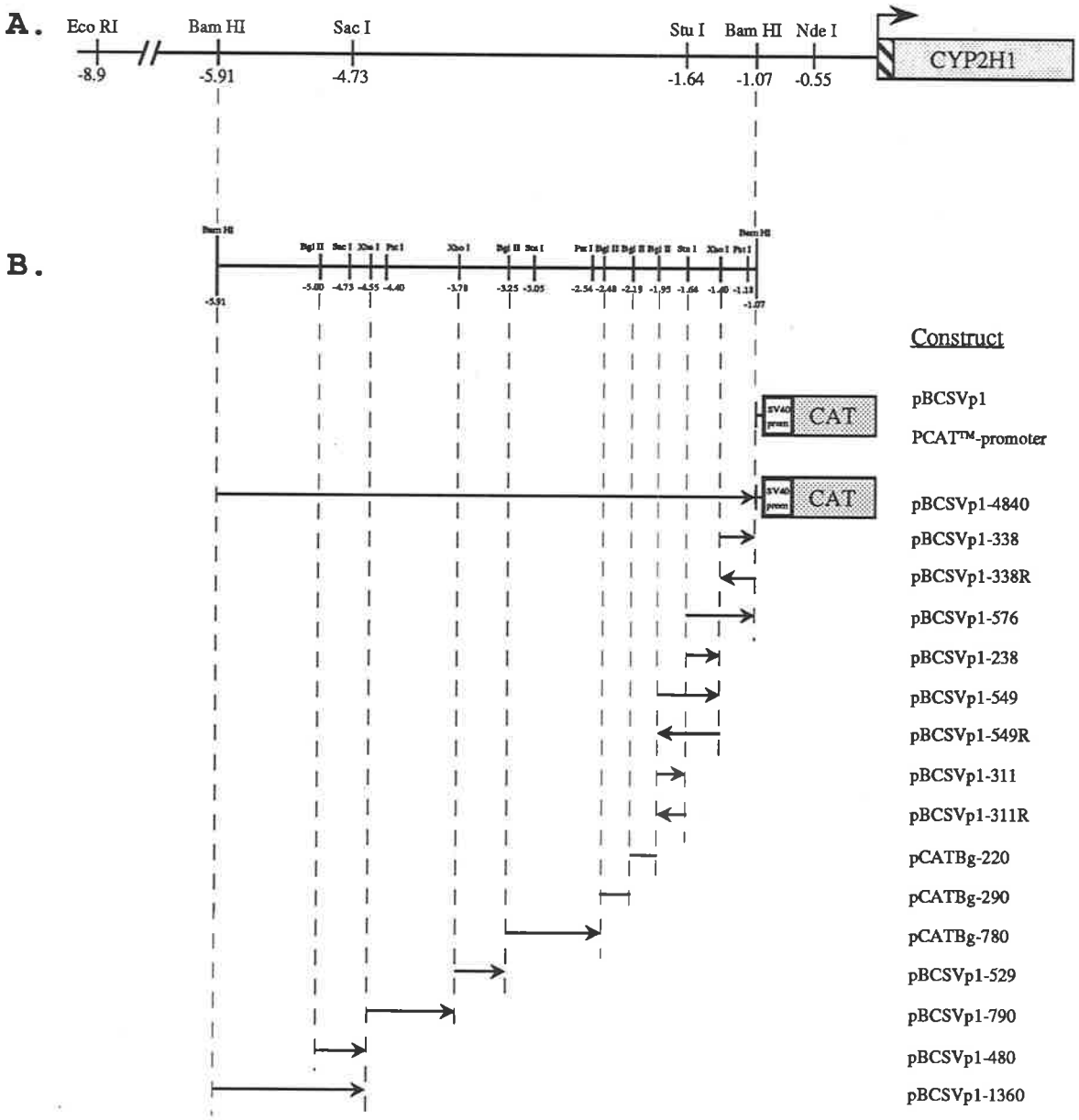
**A.** Diagrammatic representation of the 5' portion of the *CYP2H1* gene.

**B.** Diagrammatic representation the 4.8 kb phenobarbital-responsive enhancer domain and SV40 promoter/CAT constructs and the results of expression of these 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, except for those subfragments in which the orientation was not determined, where a line without an arrowhead has been used. The resultant constructs have been designated pBCSVp1-X and pBCSVp1-XR where "X" refers to the size (bp) of the inserted subfragment and "R" refers to the reverse orientation (*i.e.* 3' to 5') with respect to the orientation in the native gene. Several of the subfragments were cloned into the *Bgl* II site in pCAT™-promoter (see Fig. 6.1) and are designated pCATBg-X where "X" refers to the size (bp) of the inserted subfragment.

Primary hepatocytes ($2 \times 10^7$) were transfected with 2 pmol of each construct, the sample was split and the cells were incubated in William's E medium supplemented with 10% Nu-Serum for 20 h. To one of each pair was added phenobarbital to a final concentration of 500 µM and to the other was added PBS. Following a further 48 h incubation, the hepatocytes were harvested and the CAT activity in 50 µg of protein extract was determined. The results of these assays are represented in relative terms in two ways.

**"Basal"** refers to the expression of CAT activity from each construct, in the absence of phenobarbital, relative to the expression of the parent construct, pBCSVp1 or pCAT™-promoter. This result shows the ability of each subfragment to enhance the strength of the SV40 promoter.

**"PB-induced"** refers to the fold increase in CAT expression from each construct upon the addition of phenobarbital. This result shows those subfragments which are able to confer phenobarbital inducibility to the SV40 promoter.




| Construct | Relative Expression | |
|---|---|---|
| | Basal | PB-induced |
| pBCSVp1 | 1.0 | 0.9 |
| PCAT™-promoter | 1.0 | 0.9 |
| pBCSVp1-4840 | 2.0 | 4.8 |
| pBCSVp1-338 | 0.6 | 1.0 |
| pBCSVp1-338R | 0.6 | 1.0 |
| pBCSVp1-576 | 0.7 | 0.9 |
| pBCSVp1-238 | 1.6 | 1.6 |
| pBCSVp1-549 | 3.4 | 3.8 |
| pBCSVp1-549R | 2.0 | 4.0 |
| pBCSVp1-311 | 4.0 | 1.0 |
| pBCSVp1-311R | 2.0 | 1.0 |
| pCATBg-220 | 1.0 | 0.9 |
| pCATBg-290 | 1.0 | 1.0 |
| pCATBg-780 | 0.9 | 0.9 |
| pBCSVp1-529 | 15.3 | 1.0 |
| pBCSVp1-790 | 1.0 | 1.0 |
| pBCSVp1-480 | 0.7 | 1.3 |
| pBCSVp1-1360 | 1.0 | 2.0 |

The strongest of these was a 529 bp *Xho* I/*Bgl* II fragment (position -3.78 to -3.25 kb in pBCSVp1-529) which increased basal expression driven from the SV40 promoter by over 15-fold, but showed no response to phenobarbital. The other region was a 549 bp *Bgll* I/*Xho* I (position -1.95 to -1.40 kb in pBCSVp1-549) which possesses basal enhancer activity, increasing CAT expression by 3.4-fold in the native orientation (pBCSVp1-549) and by 2.0-fold in the opposite orientation (pBCSVp1-549R). Importantly however, this region was able to confer phenobarbital-inducibility in either orientation to the SV40 promoter leading to a 3.8 to 4.0-fold increase in CAT activity in the presence of phenobarbital. To further define the functional regions, this 549 bp fragment was cut with *Stu* I to produce a 311 bp *Bgl* II/*Stu* I fragment (position -1.95 to -1.64 kb) and a 238 bp *Stu* I/*Xho* I fragment (position -1.64 to -1.40 kb) which were then analysed in a similar way to determine their effect on basal and phenobarbital-induced expression of CAT activity. In isolation, the 311 bp fragment (pBCSVp1-311 and pBCSVp1-311R) exhibited basal enhancer activity, increasing basal expression of CAT activity by 4.0-fold in the native orientation and 2.0-fold in the reverse orientation. These results were similar to those obtained when the 311 bp fragment was tested in conjunction with the 238 bp fragment as found in the pBCSVp1-549 and pBCSVp1-549R constructs. In contrast to the pBCSVp1-549 and -549R constructs, the pBCSVp1-311 and -311R constructs were not responsive to phenobarbital, implying that the phenobarbital-responsive element resided in the 238 bp fragment. Confirmation of this was seen with analysis of expression of pBCSVp1-238 which contains this 238 bp fragment. Basal expression was shown to increase slightly by 1.6-fold and in the presence of phenobarbital this expression was increased by a further 1.6-fold. Therefore, there seemed to be co-operation between elements within the 311 bp basal enhancer fragment, with those of the 238 bp phenobarbital-inducible fragment, to increase the level of response to phenobarbital. One other region of the 4.8 kb *Bam* HI fragment was shown to respond to phenobarbital in this assay system. A 1360 bp *Bam* HI/*Xba* I fragment (position -5.91 to -4.55 kb in pBCSVp1-1360) was able to confer phenobarbital-inducibility to the SV40 promoter and in response to phenobarbital, expression of CAT activity consistently increased by 2.0-fold.

Analysis of enhancer activity, has identified three regions that increase basal expression of the heterologous enhancerless SV40 promoter (*i.e.* -3.78 to -3.25 kb, -1.95 to -1.64 kb and -1.64 to -1.40 kb) and two regions that are able to confer phenobarbital-responsiveness (*i.e.* -5.91 to -4.55 kb and -1.64 to -1.40 kb).

In Chapter 5 (section 5.2.G and 5.2.H) evidence was provided that the 4.8 kb *Bam* HI fragment should contain positive and negative regulatory regions as well as the elements necessary for phenobarbital-inducibility. The experimental data above confirms the presence of regions responsive to phenobarbital and also positive regulatory regions. However, detection of negative regulatory regions in this system because the enhancerless SV40 promoter expresses weakly in chick embryo primary hepatocytes, is difficult. Determination of a negative effect on such a promoter goes beyond the limits of accuracy in this system and hence, no mention of possible negative regulatory regions within the 4.8 kb *Bam* HI fragment has been made.

## 6.3. DISCUSSION

As described in the previous chapter, when a 4.8 kb *Bam* HI fragment (position -5.9 to -1.1 kb) was removed from a construct containing the first 8.9 kb of the chicken *CYP2H1* gene 5' flanking region fused to the CAT reporter gene (*i.e.* CYP2H1CAT-8.9) to produce CYP2H1CAT-8.9Δ, phenobarbital-inducibility no longer occurred. This provided indirect evidence that the fragment contained elements necessary for the phenobarbital response. To directly test this, the 4.8 kb *Bam* HI fragment was analysed for its ability to modulate expression of CAT activity driven by an enhancerless SV40 promoter in chick embryo primary hepatocytes both in the presence and absence of phenobarbital. The results have demonstrated that the 4.8 kb *Bam* HI fragment acts as a "phenobarbital-responsive enhancer domain", which is able to confer phenobarbital-responsiveness to the heterologous enhancerless SV40 promoter when tested in either orientation and at various distances from this promoter. The level of induction was, however, distance dependent. When this fragment was placed immediately upstream of the enhancerless SV40 promoter (pCATBg4.8 and pCATBg4.8R) an approximate 14-fold induction was seen with phenobarbital, whereas when placed a further 2.7 kb upstream of the enhancerless SV40 promoter (pCATBa4.8 and

pCATBa4.8R) an approximate 8-fold induction resulted (see Fig. 6.1). Due to the circular nature of the plasmid, the insertion site in these latter constructs can also be considered to be about 1.69 kb downstream of the enhancerless SV40 promoter transcription start site, immediately 3' of the CAT reporter gene. Such distance dependence may be a real phenomenon or it may be due to the constraints of being in a circular supercoiled plasmid. In any case, this phenobarbital-responsive enhancer domain is able to function over a distance of at least 1.69 kb which strongly indicates that it would be functional when located in its true position in the *CYP2H1* gene, 1.07 kb upstream of the transcription start site.

To investigate the specificity of drug-induction conferred by the 4.8 kb *Bam* HI fragment, CYP2H1CAT-8.9 which contains the 4.8 kb *Bam* HI fragment with its own promoter and pCATBg4.8 which contains the 4.8 kb *Bam* HI fragment fused to the heterologous enhancerless SV40 promoter were introduced into chick embryo primary hepatocytes, and the response to AIA, 20-methylcholanthrene and dexamethasone observed. The "phenobarbital-like" drug AIA, that is known to induce members of the *CYP2* gene family (Elliott *et al.*, 1989), produced a substantial response, increasing the levels of expression of CAT activity from these constructs. 20-Methylcholanthrene, an aryl hydrocarbon that induces members of the *CYP1* gene family, marginally induced expression from these constructs while dexamethasone, a "PCN-like" drug that induces members of the *CYP3* gene family, had little if any effect on expression from these constructs. Northern blot analysis of total RNA from chick embryo primary hepatocytes showed that the CYP2H1 mRNA level was induced markedly by AIA, marginally by 20-methylcholanthrene, but not significantly by dexamethasone. A correlation therefore exists between the specificity of induction by drugs of endogenous CYP2H1 mRNA levels and of constructs containing the 4.8 kb *Bam* HI fragment, marked induction only occurring with phenobarbital and "phenobarbital-like" drugs.

Following a report by Schuetz *et al* (1990) that human growth hormone blocked phenobarbital-induced transcription of *CYP2B1* and *CYP2B2* genes in adult rat primary hepatocytes, the effect of porcine growth hormone was investigated in chick embryo primary hepatocytes using the phenobarbital-inducible constructs CYP2H1CAT-8.9 and pCATBg4.8. No effect of porcine growth hormone on phenobarbital-induction of CAT

activity from these constructs was seen and, in keeping with these results, the levels of endogenous CYP2H1 mRNA from untreated and phenobarbital-treated chick embryo primary hepatocytes did not alter significantly with the addition of growth hormone for 48 h. Whether porcine growth hormone is active to any degree in avian species is not known. Alternatively, the chicken *CYP2H1* gene in chick embryo liver or primary hepatocytes may not be regulated by growth hormone while it is also possible that chick embryo liver at this stage of development may have no or only a few growth hormone receptors. Perhaps, the response to growth hormone may be a chronic one, since both Schuetz *et al* (1990) and Waxman *et al* (1990) incubated their rat primary hepatocyte cultures for 96 h, compared with the 48 h incubation used here. The molecular basis for the effect of growth hormone on CYP expression in rat liver will be of interest.

To delimit the functional control regions within the phenobarbital-responsive enhancer domain, the 4.8 kb *Bam* HI fragment was cut into smaller fragments and each of these tested individually for the ability to modulate expression of CAT activity driven from an enhancerless SV40 promoter. Three regions at position -3.78 to -3.25 kb (529 bp), -1.95 to -1.64 kb (311 bp) and -1.64 to -1.40 kb (238 bp) of the *CYP2H1* gene were shown to enhance basal expression by 15.3, 2.0 to 4.0 and 1.6-fold, respectively. Of particular interest is the 15-fold increase in basal expression mediated by the 529 bp fragment. Since the 4.8 kb *Bam* HI fragment only gives a 2-fold increase in basal expression, the substantial enhancing effect of the isolated 529 bp fragment must be silenced within the context of the 4.8 kb *Bam* HI fragment either by negative regulatory regions present or because the 529 bp fragment is now positioned further upstream (~2.2 kb) from the enhancerless SV40 promoter.

In addition to the three regions shown to enhance basal expression of CAT activity, two regions at position -5.91 to -4.55 kb (1360 bp) and -1.64 to -1.40 kb (238 bp) were shown to confer to the enhancerless SV40 promoter responsiveness to phenobarbital by 2.0 and 1.6-fold, respectively. The more distal of these phenobarbital-responsive regions (the 1360 bp region) has not been further investigated. The more proximal of these phenobarbital-responsive regions (the 238 bp region) was however, studied in some detail and it was demonstrated that the response to phenobarbital, mediated by this fragment,

★ A third, although unlikely, possibility is that a phenobarbital-responsive element resides at approximately position -1640 bp and upon restriction with *Stu* I is destroyed.

increased from 1.6-fold to approximately 4.0-fold when the adjacent 311 bp basal enhancer fragment was also included in the constructs. Two possible explanations for this result are depicted diagrammatically in Figure 6.4 and are as follows. In the first possibility (see Fig. 6.4.A), the assumption is made that a single phenobarbital-responsive element is able to confer responsiveness to phenobarbital. Therefore, the 238 bp phenobarbital-responsive region must contain one or more phenobarbital-responsive elements while the 311 bp basal enhancer region contains only elements that do not respond to phenobarbital, but which are able to increase basal expression. An increase in the magnitude of response to phenobarbital when both of these fragments are juxtaposed, may then be due to a synergistic effect whereby basal element(s) act in co-operation with the phenobarbital-responsive element(s). A precedence for this idea is seen in the well characterised TCDD-inducible *CYP1A1* gene. In the mouse gene (*Cyp1a-1* ), a dioxin-responsive enhancer (position -1302 to -820 bp) consists of four copies of the aryl hydrocarbon (Ah) receptor recognition motif, each of which has been shown by mutation to contribute to the overall response of TCDD on this gene (Fisher *et al.*, 1990). Furthermore, in this enhancer region, a GC box known to bind a ubiquitous transcription factor Sp1 was shown to act synergistically with the recognition motif for the liganded Ah receptor to cause an increase in drug-induced expression. However, the GC box motif in isolation from the Ah receptor motif, did not exhibit any intrinsic response to TCDD. In the alternative possibility (see Fig. 6.4.B), the assumption is made that two or more phenobarbital-responsive elements are required to co-operate to bring about responsiveness to phenobarbital and that the magnitude of response to phenobarbital increases with the number of elements involved (note that, basal elements cannot increase the magnitude of the phenobarbital response; *i.e.* fold induction). Hence, the 238 bp phenobarbital-responsive region must contain at least two phenobarbital-responsive elements, while the 311 bp basal enhancer region must contain one such element. Other elements that do not respond to phenobarbital, but which increase basal expression may reside in both the 238 and 311 bp regions. Further discussion of the two proposed mechanisms shown in Figure 6.4 is given in Chapter 7 where the sequences of these two regions are analysed in detail.

★

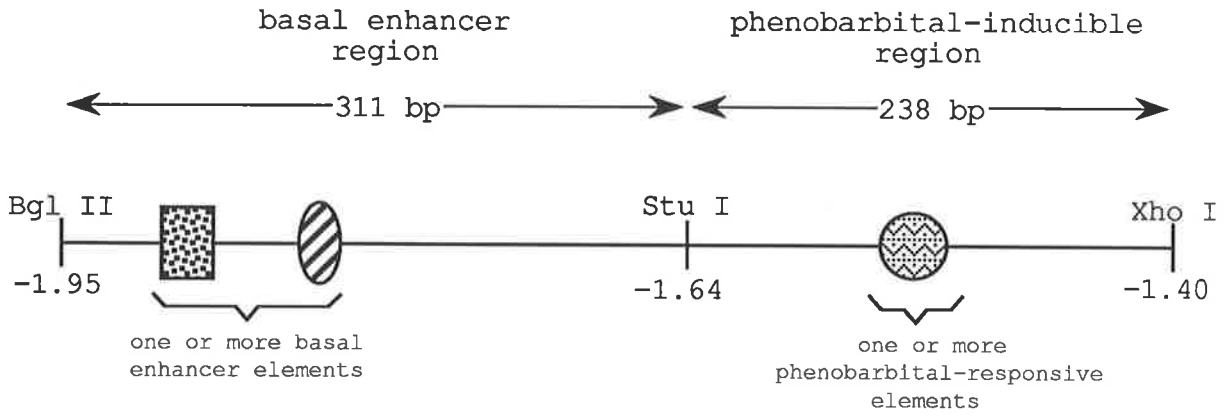
**Figure 6.4. Possible models for induction by phenobarbital at the gene level**

**A.** A model in which one phenobarbital-responsive element is able to confer phenobarbital-responsiveness to a promoter. Basal elements (*i.e.* elements that do not confer responsiveness to phenobarbital), in conjunction with phenobarbital-responsive elements, synergistically increase the magnitude of the response to phenobarbital.

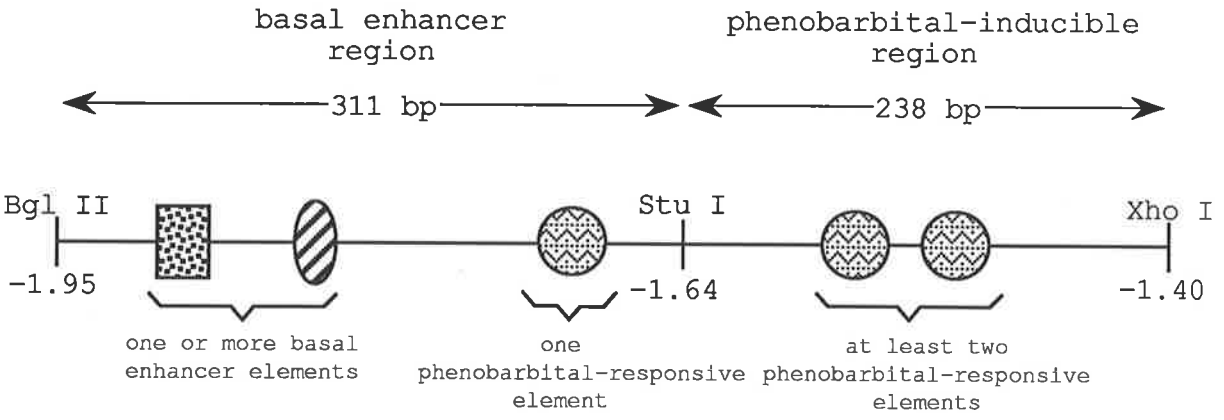
**B.** A model in which at least two phenobarbital-responsive elements are required to interact to bring about responsiveness to phenobarbital.

In both models, the presence of multiple phenobarbital-responsive elements results in a greater magnitude of response to phenobarbital.

**A.**


basal enhancer region
phenobarbital-inducible region
311 bp
238 bp
Bgl II
-1.95
Stu I
-1.64
Xho I
-1.40
one or more basal enhancer elements
one or more phenobarbital-responsive elements

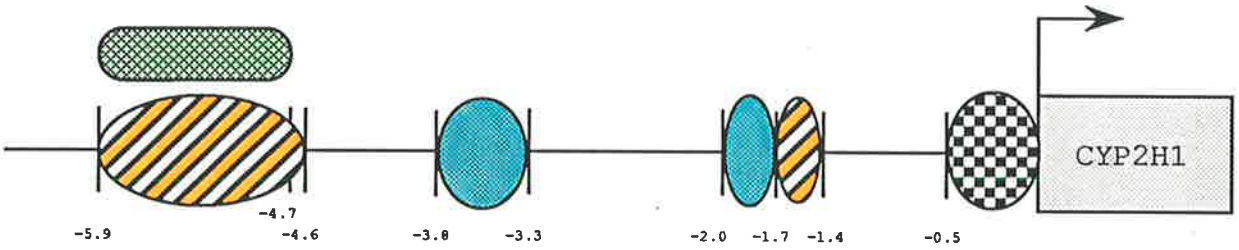
**B.**


basal enhancer region
phenobarbital-inducible region
311 bp
238 bp
Bgl II
-1.95
Stu I
-1.64
Xho I
-1.40
one or more basal enhancer elements
one phenobarbital-responsive element
at least two phenobarbital-responsive elements

The identification in this chapter of specific regulatory regions within the 4.8 kb *Bam* HI fragment, in conjunction with the results obtained from transient expression studies of *CYP2H1* gene promoter/CAT reporter constructs in Chapter 5 (see section 5.2.G), has enabled the proposal of a model to explain the expression patterns seen in these transient studies. Figure 6.5 depicts the determined regulatory regions of the 5' flanking region of the *CYP2H1* gene. In discussing this model, the results of transient expression studies in Chapter 5 will be briefly summarised. Expression of CYP2H1CAT constructs in chick embryo primary hepatocytes revealed that the first 0.5 kb of promoter was able to drive strong basal expression. Increasing the promoter length to 1.7 kb resulted in no alteration in basal expression. Further increasing the promoter length to 4.7 kb resulted in an increase in basal expression which may be due to the inclusion of the two basal enhancer regions. Basal expression of a construct containing the entire 8.9 kb of 5' flanking region (CYP2H1CAT-8.9) decreased to a level similar to that found with only 0.5 kb of 5' flanking region, suggesting a distant negative regulatory region beyond the *Sac* I site at position -4.73 kb. Induction by phenobarbital was not seen for constructs containing 0.5 or 1.1 kb, but was seen with 1.7, 4.7 and 8.9 kb of *CYP2H1* gene 5' flanking region and the fold induction increased with the length of this region. These results can be explained in the following way. The shortest construct to induce with phenobarbital, CYP2H1CAT-1.7 contains the most proximal phenobarbital-responsive region. CYP2H1CAT-4.7, while containing only the proximal phenobarbital-responsive region, does contain basal enhancer regions which may serve to increase the response to phenobarbital. Expression from the longest construct CYP2H1CAT-8.9, resulted in the greatest fold induction by phenobarbital. As well as containing the proximal phenobarbital-responsive region and the two basal enhancer regions, the distal phenobarbital-responsive region was also present in this construct, suggesting that this latter region does play a role in the overall response of the *CYP2H1* gene promoter to phenobarbital. The model as shown in Figure 6.5 explains why removal of the 4.8 kb *Bam* HI fragment from CYP2H1CAT-8.9, produced a construct CYP2H1CAT-8.9Δ that did not respond to phenobarbital, since both phenobarbital-responsive regions were contained within this fragment.

**Figure 6.5. Diagrammatic representation of the functionally important regions the *CYP2H1* gene 5' flanking region**

Diagram of the *CYP2H1* gene 5' flanking region showing those regions which have been demonstrated to be functionally important in transient expression studies (see Fig. 5.6 and Fig. 6.3). "Basal enhancer region" refers to a region of DNA which acts as an enhancer in the absence of phenobarbital and the enhancement of which is unaltered in the presence of drug. "Phenobarbital-inducible region" refers to a portion of DNA which may not necessarily enhance transcription in the absence of phenobarbital, but which does result in activation of transcription in the presence of drug.




strong promoter region

phenobarbital-inducible region

basal enhancer region

negative regulatory region

As mentioned earlier in section 6.2.A, the 4.8 kb *Bam* HI fragment behaves as a phenobarbital-responsive enhancer domain that is able to confer phenobarbital-responsiveness to an enhancerless SV40 promoter in either orientation and at different distances from the promoter. There is however, a distance-dependent mechanism operating such that the fold induction by phenobarbital decreased from approximately 14-fold to approximately 8-fold when the fragment was moved from immediately upstream of the SV40 promoter to a site 2.7 kb further upstream. The reason for this distance effect is not known. However, since there is a distance-dependent effect, the fact that a large fragment can be reversed without altering expression requires explanation. One possibility is that the phenobarbital-responsive elements are positioned equidistant from the ends of the 4.8 kb fragment and this would result in an enhancer domain which, although large, would confer a similar magnitude of response to phenobarbital in either orientation.

The 5' flanking region of the *CYP2H1* gene contains a number of regions that appear to be important in the basal expression of the gene and in response to phenobarbital. The sequence of a number of these regions has been determined and analysed in Chapter 7 in an attempt to identify putative elements and possibly a phenobarbital-responsive element involved in the control of expression of this gene.

# CHAPTER 7

# SEQUENCE EXAMINATION OF FUNCTIONALLY IMPORTANT REGULATORY REGIONS OF THE CHICKEN HOUSE-KEEPING ALAS GENE AND *CYP2H1* GENE

## 7.1. INTRODUCTION

In the study of gene regulation, valuable information may be gained by examination of the sequence of the promoter and enhancer regions of a gene. The sequences of many *cis* -acting elements have been reported (Locker and Buzard, 1990) and identification of known elements within a gene control region may help to explain the types of regulation that govern expression of the gene.

Simple gene sequence analysis in conjunction with gene expression studies may also provide an important initial step in the identification of new *cis* -acting elements. As *cis* -acting elements may be palindromic, especially if bound by a *trans* -acting factor which functions as a dimer, this provides one way to search for unknown elements. This approach is limited because the majority of known *cis* -acting elements are not palindromic. Often, however, *cis* -acting elements important in the control of a specific gene, are repeated either in the same or reverse orientation. Such repeats also tend to occur within a similar region of the gene such as the immediate promoter region and/or an enhancer region. Multiple copies of a particular element within a gene control region may enable a greater response of the gene to a specific *trans* -acting factor (Schatt *et al.*, 1990). A good example of this is found in the dioxin-responsive enhancer of the TCDD-inducible *Cyp1a-1* gene, where four dioxin-responsive elements are located within approximately 400 bp (*i.e.* position -1302 to -820 bp) and each of these elements has been shown to contribute to the magnitude of response of gene expression to TCDD (Fisher *et al.*, 1990).

In this chapter, the 5' flanking sequence including the 5' untranslated region of the chicken house-keeping ALAS gene is examined for known *cis* -acting elements. In addition, the sequence of the functionally important regions of the *CYP2H1* gene 5' flanking region is determined and, along with the previously determined sequence of the chicken *CYP2H1* gene (*i.e.* -1065 to +2698 bp; Mattschoss, 1987), is analysed for possible *cis* -acting elements.

## 7.2. RESULTS

### 7.2.A. Analysis of the sequence of the chicken house-keeping ALAS gene 5' flanking and 5' untranslated region

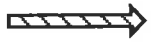
The entire sequence of the chicken house-keeping ALAS gene including 994 bp of 5' flanking sequence and approximately 1 kb of 3' flanking region has been published (Maguire *et al.*, 1986) and further 5' flanking sequence to -1556 bp (*i.e.* a *Hind* III site) has been determined by A. Day (a former Ph. D. student in this laboratory). A number of putative *cis* -acting elements including a TATA box, CCAAT boxes and GC boxes have been reported (Maguire *et al.*, 1986). As described in section 4.3, mutagenesis studies, using expression in *Xenopus Laevis* oocytes, demonstrated that the TATA box was essential for expression driven from the ALAS promoter and that, of the other putative elements, only the GC box at position -79 to -74 bp contributed significantly to expression at least in this heterologous system (Loveridge *et al.*, 1988). In the homologous system, other control elements may of course be important. In an attempt to gain a better understanding of the regulatory mechanisms of the ALAS gene, a more comprehensive analysis of this promoter region was undertaken.

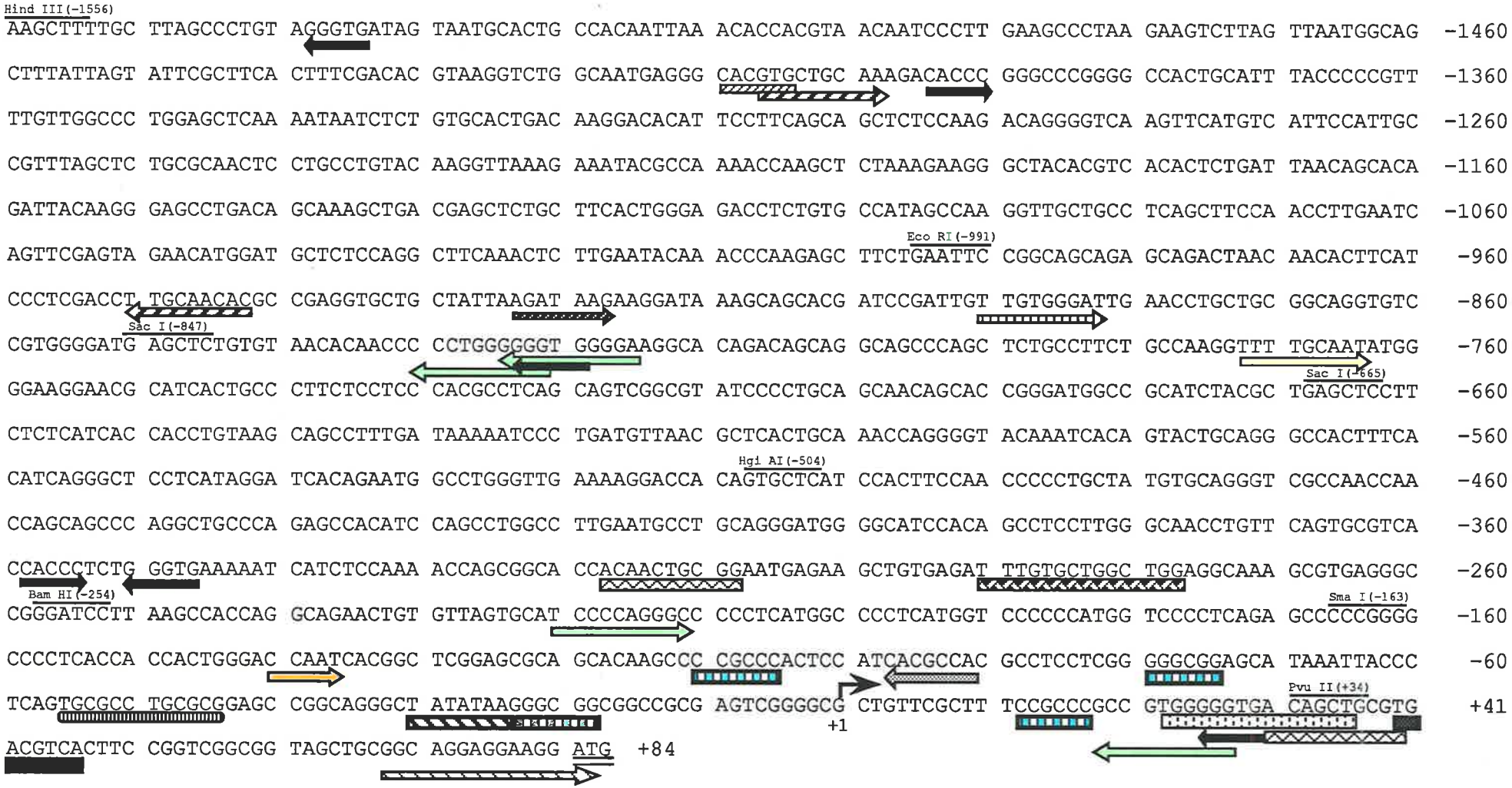
Analysis of the ALAS promoter was performed by searching for known *cis* -acting elements. To do this, a computer programme called "Find" was used, which is part of a sequence analysis software package written by the Genetics Computer Group from the University of Wisconsin (for details, see section 2.10). This allows the user to make a list of consensus sequences of *cis* -acting elements and then to search for these *cis* -acting elements within a known sequence. A list of *cis*- acting elements, collected by the present author, was used to search the promoter of the chicken house-keeping ALAS gene. The sequence of the chicken ALAS promoter is given in Figure 7.1 and the positions of putative *cis* -acting elements are shown.

A TATA box at position -30 to -24 bp of the chicken ALAS gene is probably important in the positioning of the transcription start site at the "G" residue (position +1 bp). As already stated above, mutagenesis studies have shown that this TATA box is essential for expression from the chicken ALAS gene promoter in *Xenopus laevis* oocytes (Loveridge *et*

**Figure 7.1. Known sequence of the chicken house-keeping gene 5' flanking region and 5' untranslated region and the position of putative *cis*-acting elements**

The following is a list of *cis*-acting elements, their consensus sequences and their diagrammatic representation

| Diagrammatic Motif | Cis-acting element | Consensus Sequence | Reference |
|---|---|---|---|
|  | TATA box | TATA | Breathnach and Chambon, 1981 |
|  | GC box | GGGCGG | Briggs et al., 1986 |
|  | CCAAT | CCAAT | Santoro et al., 1988 |
|  | Ig/EBP C/EBP | TTNNGCAAT | Roman et al., 1990 |
|  | NF-E1 | G/AA/TGATA/TA/GG/T | Mignotte et al., 1989a |
|  | AP-2 | TCCCCANG/CG/CG/C | Imagawa et al., 1987 |
|  | AP-4-like sequence | T/CCAGCTGC/TGG | Mermod et al., 1988 |
|  | CRE | TGACGTCA | Roesler et al., 1988 |
|  | MLTF/USF/c-myc | CACGTG | Blackwell et al., 1990 |
|  | CACCC | CACCC | Mantovani et al., 1988 |
|  | Acute phase/IL-6 RE | T/ATC/GTGGGAA/T | Hattori et al., 1989 |
|  | NRF-1 | T/CGCGCAT/CGCGCA/G | Evans and Scarpulla, 1990 |
|  | TRE | GGAG/CGTGACA/CGG/CA | Izumo and Mahdavi, 1988 |
|  | DRE | TNGCGTG | Fisher et al., 1990 |
|  | Hemopexin A sequence | GTGNNGC/TAA | Poli et al., 1989 |
|  | BTE | AGC/AAGGAGGNG/AT/GGG/AC/A | Yanagida et al., 1990 |
|  | sequence identical to a region of the *CYP2H1* gene | TTTGTGCTGGCTGG | |


Hind III(-1556)
AAGCTTTTGC TTAGCCCTGT AGGGTGATAG TAATGCACTG CCACAATTAA ACACCACGTA ACAATCCCTT GAAGCCCTAA GAAGTCTTAG TTAATGGCAG -1460
CTTTATTAGT ATTCGCTTCA CTTTCGACAC GTAAGGTCTG GCAATGAGGG CACGTGCTGC AAAGACACCC GGGCCCGGGG CCACTGCATT TACCCCCGTT -1360
TTGTTGGCCC TGGAGCTCAA AATAATCTCT GTGCACTGAC AAGGACACAT TCCTTCAGCA GCTCTCCAAG ACAGGGGTCA AGTTCATGTC ATTCCATTGC -1260
CGTTTAGCTC TGCGCAACTC CTGCCTGTAC AAGGTTAAAG AAATACGCCA AAACCAAGCT CTAAAGAAGG GCTACACGTC ACACTCTGAT TAACAGCACA -1160
GATTACAAGG GAGCCTGACA GCAAAGCTGA CGAGCTCTGC TTCACTGGGA GACCTCTGTG CCATAGCCAA GGTTGCTGCC TCAGCTTCCA ACCTTGAATC -1060
Eco RI(-991)
AGTTCGAGTA GAACATGGAT GCTCTCCAGG CTTCAAACTC TTGAATACAA ACCCAAGAGC TTCTGAATTC CGGCAGCAGA GCAGACTAAC AACACTTCAT -960
CCCTCGACCT TGCAACACGC CGAGGTGCTG CTATTAAGAT AAGAAGGATA AAGCAGCACG ATCCGATTGT TGTGGGATTG AACCTGCTGC GGCAGGTGTC -860
Sac I(-847)
CGTGGGGATG AGCTCTGTGT AACACAACCC CCTGGGGGGT GGGGAAGGCA CAGACAGCAG GCAGCCCAGC TCTGCCTTCT GCCAAGGTTT TGCAATATGG -760
Sac I(-665)
GGAAGGAACG CATCACTGCC CTTCTCCTCC CACGCCTCAG CAGTCGGCGT ATCCCCTGCA GCAACAGCAC CGGGATGGCC GCATCTACGC TGAGCTCCTT -660
CTCTCATCAC CACCTGTAAG CAGCCTTTGA TAAAAATCCC TGATGTTAAC GCTCACTGCA AACCAGGGGT ACAAATCACA GTACTGCAGG GCCACTTTCA -560
Hgi AI(-504)
CATCAGGGCT CCTCATAGGA TCACAGAATG GCCTGGGTTG AAAAGGACCA CAGTGCTCAT CCACTTCCAA CCCCCTGCTA TGTGCAGGGT CGCCAACCAA -460
CCAGCAGCCC AGGCTGCCCA GAGCCACATC CAGCCTGGCC TTGAATGCCT GCAGGGATGG GGCATCCACA GCCTCCTTGG GCAACCTGTT CAGTGCGTCA -360
CCACCCTCTG GGTGAAAAAT CATCTCCAAA ACCAGCGGCA CCACAACTGC GGAATGAGAA GCTGTGAGAT TTGTGCTGGC TGGAGGCAAA GCGTGAGGGC -260
Bam HI(-254)
Sma I(-163)
CGGGATCCTT AAGCCACCAG GCAGAACTGT GTTAGTGCAT CCCCAGGGCC CCCTCATGGC CCCTCATGGT CCCCCCATGG TCCCCTCAGA GCCCCCGGGG -160
CCCCTCACCA CCACTGGGAC CAATCACGGC TCGGAGCGCA GCACAAGCCC CGCCCACTCC ATCACGCCAC GCCTCCTCGG GGGCGGAGCA TAAATTACCC -60
Pvu II(+34)
TCAGTGCGCC TGCGCGGAGC CGGCAGGGCT ATATAAGGGC GGCGGCCGCG AGTCGGGGCG CTGTTCGCTT TCCGCCCGCC GTGGGGGTGA CAGCTGCGTG +41
+1
ACGTCACTTC CGGTCGGCGG TAGCTGCGGC AGGAGGAAGG ATG +84

*al.*, 1988) and presumably this element is necessary for correct initiation and expression of the ALAS gene in chicken cells.

ALAS is a house-keeping gene expressed in all cells (see section 1.3.A). Often the promoters of house-keeping genes do not contain a TATA box to precisely position the transcription initiation start site, but rather consist of a GC-rich promoter containing several GC boxes which in some way loosely position RNA polymerase II within a confined region from which transcription may initiate (Dush *et al.*, 1988). Unlike TATA box-containing genes which usually have a major transcription start site, genes that do not contain a TATA box often initiate transcription from multiple sites and some such GC-rich promoters support bidirectional transcription (Dush *et al.*, 1988). Interestingly, chicken ALAS gene promoter contains four putative GC boxes in various orientations within a region of only 130 bp from position -110 to +18 bp. It is possible that these GC boxes are used in conjunction with, or in place of, the TATA box in particular tissues, under specifically induced conditions or during development.

A putative CCAAT box is located at position -141 to -137 bp in the ALAS gene 5' flanking region. This core element is bound by a family of *trans* -acting factors, each of which require slightly different flanking sequence for optimal binding (Santoro *et al.*, 1988; Short, 1988). According to the flanking sequence of the CCAAT box in the ALAS gene promoter, the CCAAT-binding proteins CP1 and CP2 are most likely to bind to this putative element (Chodosh *et al.*, 1988). In relation to the CCAAT box is the CCAAT/enhancer binding protein (C/EBP) which is abundant in the liver and adipose tissue, but limited or absent in other tissues (Birkenmeier *et al.*, 1989; Xanthopoulos *et al.*, 1989). C/EBP has been shown to bind to two completely different sequences including a CCAAT-like sequence and the core enhancer element (or AP-3 consensus sequence; Johnson *et al.*, 1987). It now appears that C/EBP belongs to a family of proteins that bind to similar sequences, some of which appear to be expressed in most cell types such as AGP/EBP (Chang *et al.*, 1990) and Ig/EBP (Roman *et al.*, 1990) while others are tissue-specific such as C/EBP (Birkenmeier *et al.*, 1989; Xanthopoulos *et al.*, 1989), LAP which is highly enriched in the liver (Descombes *et al.*, 1990) and the inducible NF-IL6 which is expressed in adult liver only following induction with interleukin-1, interleukin-6 or lipopolysaccharide (Chang *et al.*,

1990). To add to the complexity of gene regulation by this family of proteins is the ability of each member to form dimers via leucine zippers. The possible production of heterodimers as well as homodimers could greatly broaden the range of responses of a gene by this one element, especially if the different members elicit different degrees of gene activation. A recognition motif for the C/EBP family of proteins, of sequence TTNNGCAAT, is found at position -772 to -764 bp in the 5' flanking region of the ALAS gene. It is possible that this element is important in the control of the ALAS gene in all tissues, with the degree of its influence on expression, dependent on the presence and abundance of the various members of the C/EBP family of proteins in each tissue. It is also possible that this element plays a role in the strong expression of the ALAS gene in the liver in response to drugs, especially since several members of the C/EBP family of *trans* -acting proteins are enriched in the liver.

As ALAS is expressed in all tissues, it might be expected that in certain tissues where larger amounts of ALAS are required, there may be tissue-specific mechanisms by which this increase is executed. As mentioned above, the enrichment of C/EBP in the liver is one way in which genes containing C/EBP binding sites may be expressed at higher levels in the liver than in other tissues. The ALAS gene promoter contains two other putative elements for factors which are either liver-specific or enriched in the liver. One is termed the Hpx A consensus which is found in the haemopexin gene, C-reactive protein $\alpha$ gene and haptoglobin gene (Poli *et al.*, 1989). The other is called the interleukin-6-responsive element (IL-6 RE) (Hattori *et al.*, 1989). IL-6 has emerged as a major systemic alarm signal and it is produced by virtually all injured tissues in response to damaging influences such as bacterial products, viral infection, and inflammation-associated cytokines. A major role of IL-6 is to elicit the "acute phase" plasma protein response (reviewed by Baumann and Gauldie, 1990; Sehgal, 1990). In general, the aim of the response is to seal the site of tissue injury and to reduce the systemic effects of tissue injury. Many of the promoters for acute phase protein genes contain either IL-6 REs or elements for *trans* -acting factors that respond to IL-6. One might speculate that ALAS is needed for the supply of haem during the acute phase of tissue injury or during the healing process which follows. In this way, although not a plasma protein, ALAS may belong to the battery of acute phase proteins.

As discussed in Chapter 4 (see section 4.3), a consensus cAMP-responsive element (CRE) exists in the 5' untranslated region of the ALAS gene (position +40 to +47 bp). Although a functional CRE has not been reported in the 5' untranslated region of any gene reported so far (Montminy *et al.*, 1986; Roesler *et al.*, 1988), the ALAS gene might be expected to respond to cAMP, especially since cAMP induces steroid hydroxylase CYPs in steroidogenic tissues and a concommitant rise in ALAS activity is also seen (for details see section 1.2.C.1.f). However, this increase in ALAS activity has not been proven to be a direct effect of cAMP. Other evidence to suggest that cAMP might have a role in ALAS expression is that freshly isolated rat and chick embryo primary hepatocytes require added cAMP for induction of ALAS levels by drugs, although cAMP alone has no inducing effect (Edwards and Elliott, 1972; Srivastava *et al.*, 1979). Once again, the effect of cAMP on ALAS could be an indirect one, in the absence of evidence to the contrary. Other *trans* -acting factors known to mediate cAMP-responsiveness are AP-1 and AP-2 (Bohmann *et al.*, 1987; Imagawa *et al.*, 1987). Although no AP-1 elements were found, two overlapping consensus AP-2 elements (position -830 to -821 bp and -824 to -815 bp) are also present in this promoter. Waterman *et al* (1990b) have evidence that there may be a number of other *trans* -acting factors which mediate the response of cAMP, but as the precise consensus binding sequences for these factors have not been defined, it is not known if any of these are present in the ALAS 5' flanking region.

ALAS is essential for the production of haem for respiratory cytochromes. Co-ordinate expression of several proteins of the electron transport chain has been observed in response to various metabolic and physiological signals (Williams *et al.*, 1987). A protein designated nuclear respiratory factor-1 (NRF-1) has been shown to bind to sequences found within the promoters of genes involved in respiration. Examples are the human cytochrome $c_1$ gene, the rat cytochrome c oxidase subunit VIc gene and the mouse mitochondrial RNA processing RNA gene which encodes the RNA moiety of a ribonucleoprotein endonuclease involved in generating primer RNAs for mitochondrial DNA replication (Evans and Scarpulla, 1989). A sequence very similar to the reported NRF-1 consensus sequence (Evans and Scarpulla, 1990) is found at position -55 to -44 bp of the chicken ALAS gene. Similar sequences are also present in the human and rat house-keeping ALAS genes (May,

B.K., personal communication). It is possible that this putative element links expression of the ALAS gene with the co-ordinate expression of other genes important in the respiratory process.

Another element of interest is the putative binding site for the erythroid-specific factor NF-E1 which has also been called Eryf-1, EF-1, GF-1, EFγa (Evans *et al.*, 1988; Plumb *et al.*, 1989; Tsai *et al.*, 1989). NF-E1 elements appear predominantly in the promoter and enhancer regions of the erythroid-specific genes including the α-globin and ß-globin family of genes in many species and also in porphobilinogen deaminase, the third enzyme of the haem biosynthetic pathway (Mignotte *et al.*, 1989a; 1989b). It is possible that this element is required for expression of the house-keeping form of ALAS in erythroid cells in their development, prior to expression of the erythroid-specific form of ALAS.

Another region of interest in the ALAS gene promoter is that of a putative basic transcription element (BTE), which is reported to exist approximately between position -80 and -40 bp in a number of *CYP* genes including rat *CYP1A1, CYP2B1, CYP2B2* and *CYP2E1*, and human *CYP11A1* and *CYP21A1* as well as other genes such as mouse apolipoprotein A, mouse α-globin, chicken X gene and *Xenopus Laevis* $\alpha_1$-globin (Yanagida *et al.*, 1990). The BTE of the rat *CYP1A1* gene is required for maximal inducible expression of this gene in response to aromatic hydrocarbon inducers (Yanagida *et al.*, 1990). In the chicken ALAS gene, the BTE strongly resembles the BTE of the human *CYP11A1* gene promoter, except that it is found downstream of the transcription start site within the 5' untranslated region.

In brief, other putative elements within the ALAS gene 5' flanking region and 5' untranslated region include a DRE or XRE core sequence at position -91 bp which may be important for the response of ALAS to aryl hydrocarbons, several CACCC boxes at positions -1534, -1390, -819, -354, -346 and +30 bp, a binding motif for MLTF/USF/c-myc at position -1404 bp, two AP-4-like motifs at positions -308 and +40 bp and a thyroid hormone-responsive element (TRE) at position +36 bp.

Transient expression studies of ALAS promoter/CAT constructs in chick embryo primary hepatocytes reported in Chapter 4, demonstrated that the construct, ALASCAT-163, containing 197 bp of promoter (-163 to +34 bp) was able to express at a low level. The

presence of additional 5' flanking sequence to -1700 bp made no difference to this level of expression, indicating one of several possibilities. The putative elements beyond position -163 bp may be non-functional in this tissue. Alternatively, negative regulatory elements may be scattered throughout this 5' flanking region, effectively neutralising the effects of the positive elements. This is however, unlikely to be the case in that all six ALASCAT constructs containing 1700, 847, 665, 504, 254 and 163 bp of 5' flanking region respectively, express similarly. It is more likely that many of these elements may be tissue-specific or stage-specific during development or utilised only under certain circumstances such as upon drug induction or during an acute phase response.

Expression of the ALAS gene is required in all cells for the production of haem. ALAS is known to be the rate-limiting enzyme of the haem biosynthetic pathway in the liver and adrenal cortex (see section 1.3) and this might also be expected in other tissues. If so, one can imagine that the ALAS gene would need to be regulated differently in different tissues depending on the haem requirement of a particular tissue. This may help to explain the complex array of putative *cis*- acting elements shown in Figure 7.1 and, no doubt, while some of the elements shown may not be functional, there will be others that remain to be discovered.

In summary, mutagenesis studies of the chicken ALAS gene in *Xenopus laevis* oocytes revealed that the TATA box at position -30 to -24 bp and a GC box at position -79 to -74 bp were important for expression from this promoter. However, this work needs to be repeated in a homologous system such as in chick embryo primary hepatocyte cultures to gain a clearer picture of what elements are important *in vivo*. It will also be necessary in these future expression studies to include in the constructs the putative CRE and TRE which were not present in the ALASCAT constructs tested to date. Of interest will be the expression of ALASCAT constructs in extrahepatic tissues of the chick embryo such as heart, erythroid and fibroblasts. The role of promoter elements important in these tissues may be investigated by foot-printing techniques and gel retardation using nuclear protein extracts from the various chick embryo tissues and by site directed mutagenesis studies.

## 7.2.B. Sequence determination of functionally important regions of the *CYP2H1* gene promoter

Transient expression studies of *CYP2H1* gene promoter/CAT constructs in chick embryo primary hepatocytes revealed that the first 0.5 kb of the *CYP2H1* gene promoter was able to drive strong expression and that a number of regions within the *CYP2H1* gene 5' flanking region were able to modulate the strength of the SV40 promoter (for details see section 6.3 and Fig. 6.5). In brief, three regions at position -3.78 to -3.25 kb (529 bp), -1.95 to -1.64 kb (311 bp) and -1.64 to -1.40 kb (238 bp) were able to enhance basal expression of the SV40 promoter, while two regions at position -5.91 to -4.55 kb (1360 bp) and -1.64 to -1.40 kb (238 bp) were shown to confer responsiveness to phenobarbital. These regions were therefore considered to play a role in expression of the *CYP2H1* gene *in vivo*. It was decided to sequence each of these regions except for the 1360 bp region, which could not be entirely sequenced easily, and to search for known *cis* -acting elements. The appropriate regions were also searched for repeated sequences, inverted repeats and palindromes which may aid in defining elements involved in the response to phenobarbital. Prior to commencement of this expression work, sequence of the 5' flanking region of the *CYP2H1* gene had been determined up to a *Bam* HI site (-1065 bp) by L. Mattschoss of this laboratory. Therefore the sequence of important regions beyond this point needed to be determined.

### *7.2.B.1. Sequencing of putative functionally important regions of the* CYP2H1 *gene*

The constructs used in transient expression studies described in Chapter 6 were made using the pBCSVp-1 plasmid which is derived from pBluescriptKS$^+$ (Stratagene). pBluescriptKS$^+$ contains primer binding sites enabling DNA which is cloned into the polylinker site to be sequenced on both strands using either single stranded or double stranded DNA as a template in sequencing reactions. Despite this, the way in which the pBCSVp-1 constructs containing *CYP2H1* gene 5' flanking region were made, enabled sequence of the *CYP2H1* gene promoter regions to be determined using available primers by a double stranded DNA sequencing technique only and also only in one orientation. However, upon screening, all pBCSVp-1 constructs containing inserts of *CYP2H1* gene

promoter regions were obtained in both orientations and these were used to determine and confirm the sequence of both strands of the relevant regions.

Sequencing was performed as described in the Materials and Methods (see section 2.3.E) using either the reverse sequencing primer or the KS primer (Stratagene). The reverse sequencing primer enabled accurate determination of the sequence of the insert which was nearest the primer while the KS primer enabled a greater number of base pairs to be read. The regions that were sequenced included -1.95 to -1.64 kb (in pBCSVp1-311 and pBCSVp1-311R), -1.64 kb to -1.40 kb (in pBCSVp1-238 and pBCSVp1-238R), -1.40 to -1.07 kb (in pBCSVp1-338 and pBCSVp1-338R) and -3.78 to -3.25 kb (in pBCSVp1-529 and pBCSVp1-529R). It should be noted that the orientation of all the clones were determined by restriction mapping prior to sequencing, except for the clones pBCSVp1-529 and pBCSVp1-529R which could not be determined in this way. Clones of opposite orientation were therefore chosen by complementarity-testing (for details see section 2.3.F) prior to sequencing and the actual orientation of each insert was determined from the sequence of the cloning restriction sites. This section of work was performed in collaboration such that all of the clones were made by the author while the actual sequence determination was performed by Satish Dogra of this laboratory.

When added to the pre-existing *CYP2H1* gene 5' flanking sequence of -1065 bp (a *Bam* HI site), the newly determined sequence extended the known sequence to -1952 bp (a *Bgl* II site) with an extra segment of 529 bp of sequence between -3.78 kb (an *Xho* I site) and -3.25 kb (a *Bgl* II site). It should be mentioned that the only region able to modulate expression from the SV40 promoter that was not sequenced, was the much larger 1360 bp region (position -5.91 to -4.35 kb) shown to confer responsiveness to phenobarbital. The phenobarbital-responsive element(s) within this region remain to be more precisely defined before sequencing.

### 7.2.B.2. *Analysis of the known sequence of the chicken* CYP2H1 *gene for* cis-*acting elements*

As described for the chicken house-keeping ALAS gene promoter in section 7.2.A, the known sequence of the chicken *CYP2H1* gene was analysed by searching for known

consensus *cis* -acting sequences, using a programme called "Find" (see section 2.10). Many putative *cis* -acting elements were found and these are shown in Figure 7.2.

An overall view of the position of putative *cis* -acting elements reveals three regions in which the majority of these elements are clustered. One region from postion -504 to +1 bp incorporates the immediate 5' flanking region or early promoter region of the gene and correlates with the region shown to promote strong basal expression of CAT activity. A second region from position -1915 to -1380 bp (536 bp) relates to the 549 bp *Bgl* II/*Xho* I fragment which, in pBCSVp1-549 or pBCSVp1-549R (see Fig. 6.3), enhances basal expression from the SV40 promoter and confers to the SV40 promoter, the ability to respond to transcriptional induction by phenobarbital. The third region from position -3726 to -3285 bp (442 bp) is found within the 529 bp *Xho* I/*Bgl* II fragment, which increases basal expression driven from the SV40 promoter by over 15-fold (see Fig. 6.3). Thus, it appears that the majority of identified putative *cis* -acting elements of the known *CYP2H1* gene sequence, cluster into regions which seem to be functionally important as determined by transient expression studies.

The early promoter region of the *CYP2H1* gene contains a prominent TATA box at position -32 to -25 bp which is probably important in directing the transcription initiation site of the *CYP2H1* gene. A core consensus CCAAT box (-70 to -66 bp), which may be bind any one of a number of CCAAT-binding proteins (see section 7.2.A) is present in this region. Of interest also is the presence, at position -101 to -85 bp, of an almost perfect palindrome spanning 17 bp which contains within it, two inverted sequences matching 8 out of 9 bp to the consensus NF-E2/AP-1 element. This element has been shown to bind a factor termed NF-E2, as well as AP-1 (Mignotte *et al.*, 1989a). NF-E2 and another factor termed NF-E1 were originally thought to be erythroid-specific as the sequence motifs to which they bind, were found in the promoters and enhancers of many of the globin genes as well as the erythroid-specific promoter of porphobilinogen deaminase, the third enzyme of the haem biosynthetic pathway (Mignotte *et al.*, 1989a; 1989b; Plumb *et al.*, 1989). It is now known that, while NF-E1 and NF-E2 are important in expression of erythroid genes, both *trans* -acting factors are also present in leucocytes in mammals (Martin *et al.*, 1990;

**Figure 7.2. Known sequence of the chicken *CYP2H1* gene and 5' flanking region and the position of putative *cis*-acting elements**

The 5' flanking sequence and exonic sequence is given in uppercase type while the intronic sequence is shown in lowercase type. The following is a list of *cis*-acting elements, their consensus sequences and their diagrammatic representation.

| Diagrammatic Motif | Cis-acting element | Consensus Sequence | Reference |
|---|---|---|---|
|  | TATA box | TATA | Breathnach and Chambon, 1981 |
|  | eH-TF | TGTTTGC | Zaret et al., 1990 |
|  | LF-A1 | TGACCT | Ochoa et al., 1989 |
|  | NF-E1 | G/AA/TGATA/TA/GG/T | Mignotte et al., 1989a |
|  | NF-E2/AP-1 | TGACTCAGC | Mignotte et al., 1989a |
|  | CCAAT | CCAAT | Santoro et al., 1988 |
|  | Ig/EBP | TTNNGCAAT | Roman et al., 1990 |
|  | AP-1 | TGACTCA | Nakabeppu et al., 1988 |
|  | AP-2 | TCCCCANG/CG/CG/C | Imagawa et al., 1987 |
|  | AP-3 | TGTGGA/TA/TA/TG | Weiher et al., 1983 |
|  | MLTF/USF/c-myc | CACGTG | Blackwell et al., 1990 |
|  | CACCC | CACCC | Mantovani et al., 1988 |
|  | Acute phase/IL-6 RE | T/ATC/GTGGGAA/T | Hattori et al., 1989 |
|  | sequence identical to a region of the chicken ALAS gene | TTTGTGCTGGCTGG | |
|  | 10 bp repeat | | |
|  | 11 bp repeat | | |
|  | similarity to 11 bp repeat | | |
|  | 12 bp repeat/LF-A1 | | |
|  | Palindrome | | |


Xho I(-3778)
CTCGAGACCT CTCCCAGTCT GTCTATAAAG TGTCTTGTGC TCACCTCTTC ATCTTGGGTG GCCTGTAGCA GACTCCCACG ATAACGTCAG TCTTATTGGC -3682
CTTCACTTTT ATCCTGACTC AACCCTATCA TCACCAACAT TAATTTCCAT ACATACACAC TCTTTCTTAA CACAGAGGGC TACTACGCCA CCACCCTTCC -3582
TGCCTTGCCT ATCTATCTCT TTTGAAAAGT AAGTAGCCAT CCGTCACAGC ACTCCAGCTG TGCGATTCAT TCCACCAACG TTTCTGTGAT AGTGACCATA -3482
TCATAGCTTT CCGACCCCAC GATGGCCTCC AGCTCCTCCT GTTTAGTATC CATGATGCGT TCATTAGTGT AAAGGCACCT GAGCCGGGCC TCCTTTCGAG -3382
TGCAGTAGCC CTCAGACCCT CGTGACCATA CTCACGTGTT TCCACAGCCA CCAACCTCCC ACCAACCCTC GTGTTCTTCC TACAAAAGGG TGAGTCATCC -3282
Bgl II(-3250)
TCCTCGTTCA CCAAGACACA AGACCGTGAG ATCT.....(~1300 base pairs).....
Bgl II(-1952)
AGATCTGAAA GTGTAGCCAA AGGTTTTGCT ACTCATCAAC GATTGCTGAA CTTTTCTGAG ATTTAATATT GTCAGTTGGT GAATTTGCCC ATTGACAACT -1856
GTCTTTGTGA TGTGCGCTGT TTGCAAAGCA CATGCACTTG TCAACTTACT GTCCCAATTC CTGACTTTTG CTGCCTGGCT TGGGGAAGGG CCTCTTCTCA -1756
TTTAATCCCG ACTATCATTT AAAACTAAGC TCTCTTCAAA GATGATCAGC AACAGGAACA ACAGAGTGCT TTACTGCTGA GGTCACCGTA CAGATCACAC -1656
Stu I(-1641)
AACAGGTGAT AAGGCCTGAA CTTCCTTGCC CTTTCAGAGA CCGAGCCAAT ACATAGCAAT CTGTCGTACG CCCTAAATGA CTGGAGAGCA GTTATGTCAG -1556
TGGCCTGGTC CTGAGTCAAC TGAGTTGTGT TTTGGGTCCT GGGAGTTCAG ACACAAATAT TTAACCAAAC CTTTTGTGCT GGCTGGTTAA ATGGCATTTC -1456
Xho I(-1403)
TGCAATCACC TGAATCACCT GAAAAATAAT GACAAGGTTT GTTACTTATC TCGAGTCCTT CCATTGCAGC AGGTCACAAG TAGGCATGTT TTATAGTTAT -1356
TGAATTATGA AGTTAACATT TAACCTTAAT ACCGCTGGTG ATGGCAAAGC TCTGTCTTGT AAGGCAGGAG TTGACATCGT GATTTTCTGC AAGGGGCTAA -1256
Pst I(-1178)
AAAGAATCCT ACCTGAAATT GATAAGATTT TAGCAGCAGA GATGTAATTC GCAGCAGAAT AGACGTGATG CTTCCTGCAG GCTAATCTGT GAAATAAGAA -1156
Bam HI(-1065)
CATTCAGTGA AGTAATACAT CTTAATGTAA GGACACAGGA GCCTAAGGAT GGATGCACAA CATCACAACA ACAGTCAAGT CAGATAGGGA TCCCGTCTAA -1056
CACTGGCTGA AAACGAGGTC CCAAGAATAG CAGCAATGCA ACCACGGAGG AATTGTTTTT TGATTGCTCT CCAGCGTCCA GAGAGTCCTG GGCATTTTTG -956
Pst I(-862)
AATACCCTGA ATGGATTTTT CTGCCATGAC TTTGCTTGAT TACTTTTGAA CGCATATAAA CTTAACAGCT ACATCCTGGC TAATGGTGTT CTGCAGCTCA -856
CGATCTGTGA AGAACTGTGT CCCTTTCCCT GTATAAACGG TGCCAGTTGC TGTTTTAGCA TTTTCTGACA CCATGCAGAT GGCCATAAAG CTGTGGGAAG -756
TTGTCCAGGG GGCAGCCGCT GCTTAGCCAG TGCTGGCTGT TGGTTGGCAG TGAAACATGG CATCAAACTA TAAGCACAGA ATAGTCATAA AGCAGGTGTT -656
TTTACTCAGT GCTGAGCACT CATGCTGGAT GAAGGGGGCG ATATTCCCTC CAAACCTGTA TACACGATGG CAAGAAATGT ACATACTTAA AGAACAAGCT -556
Nde I(-547)
GCTTACATAT GCATTAGATG TTTAAGAAAA GGTTGGGCCG AGTCCCCAGA ATGACTAACG TTTTGCCCCA CCCCGTGACT TAGTTCCTTT ACACACGCGT -456
GGTATCTCTC GGTGGTCGTC CGGTGGTCTT CTCTGATGAA GGCTGGATGT CTTTCTCGCA GTTTACTCTC TGACCTGGGT CCCTTTTCGC ATCCACAGCT -356
CCCTCCACTG CGCGCACCTG TTGGAGGGGC TGGCAGGAGT CATCCTACGC ATAAGGACAA GAGTTGAAGC ATACACTCAG CAGCTAGGCT CTAGTATTTC -256
Rsa I(-178)
TGCATTGTGA GATCATTTGG GAGCATTAGA TCATTATTAG TACCACATGA ATGATTACCC AAAGTCACAC AAGCTGTACA TGTAAAAAAG TCAGAGTAAA -156
CAATGAGTTC ATCCCTAGTT TGTTCATTCT AATCTTGAGC AGATTAATAA GTAACCTGCT GCCTCAGCAG GAACAGGGAG CTGATATTGG CTGATTTAAT -56
Nco I(+40)
CCACGTGCTT TTGTTCTACA GCTTATAAAT ACCTCAGGTT TGCAGGTCCC AGTTCAGACT CTTCTGACAC TTGACATCTC TTCCTCTGCC CACCATGGAC +45
+1
Rsa I(+114)
Exon 1 TTCCTGGGAT TGCCCACAAT CCTCTTGCTG GTCTGTATCT CATGCCTTCT CATTGCTGCA TGGAGGAGTA CATCGCAAAG AGGGAAGGAG CCTCCTGGTC +145


CCACGCCAAT CCCCATCATT GGAAATGTTT TTCAGCTGAA CCCGTGGGAC TTGATGGGAA GCTTTAAGGA Ggtaaggctg cctctttcat ttctgtgttg +245
tctccctggc ctgtagaaca aagcccagtc cagacagtct cagcttaatt cacagcttct gtgtaaatgt ttgcttttga tgtattacgc tgcctactgg +345
aattaccata cctatgttga gacctgcact ggcctcacga cagtactgca gtatacaaca gatttctaga caaggtattg atgcttttgc catattcacc +445
acatcccttc tgtacccatc ccctcagtgc tctggcaaca acgccgagca ctgttttagg gaggaccttg ctatgctttg ttgtctctgc tccctgccag +545
gtaggtataa tctccaggga ggggttgtca cgagtataag ggatgaatca aaggagttga agatgaatgg tgctgtctgt tccctccccc ggatactgtc +645
atgcatctgt tcaacagaac ctgtattgca gtgcctggga gagaataggt ggagtaagtt tggctttgga gccggttcct gaggggactg tgatggaatg +745
gtggcagatg gatgggaaag agctaagaaa tgaaatggtt cccagaaaat ttgcgtaggg gtcttattca ccaggcgaag acctgcaaac ttaatttttc +845
catgattaac atccagatgt gttgttcctt gtgttcttcc ttcctgcagC TCAGCAAAAA GTACGGTCCT ATCTTCACAA TACATTTAGG CCCCAAAAAG +945
Exon 2 ATTGTGGTGC TGTATGGCTA CGACATTGTG AAAGAAGCCC TAATTGATAA CGGAGAAGCC TTCAGTGGGA GAGGAATACT GCCTCTGATT GAAAAGCTCT +1045
TCAAGGGCAC AGgtgcgtcc caggtaacac tgccatacag aactcgatcg tgtcattgga acaggaatcc acctgtgagg gcagtggagg agtgactgga +1145
gggtaataca ctacatttct acataaaaat tatggaataa agcattaaaa gcccaaccca ttacttgggc ctagcagtag gttgaagccc tggagaactg +1245
tttaaggtcc taggttgcct tcagtgagtt cctttcaatt ttcagcacca ttcatattct ttctacagtt tcgttcccaa aaggtgcccc taagatctta +1345
ctgttattct tccatttctg aaaatactgg caaagtccct acagctcagg agccatcaac tgaacacaac actgacaagc aaatacaggc tgtgctagtt +1445
aagggtccta ctcaagatta ggccctgggc tcattcacca tgacagtccc cagcagagca tttattcaag taaatgcttg tcccgtggta ttttcagtaa +1545
tgatacgatg tgtgaaggtg catcactgca catctgggaa tttgtcttag gcacctgctt gtggccaagt tgactttagc accagtggtc cctttctatt +1645
gacagcccat tgtcacttct ctttttctgg aaataaacgg tggtgccttg atcagattga tctatttgtc acctccataa ctgcaggaag attagggttt +1745
ggggcatatc ccaattgtgg tagatatcta ttccagaacg aggttggggg gcagaaaatt tacttactca tggaatcttt gttgttcctt cttgtgacag +1845
Exon 3 GCATTGTGAC CAGCAATGGG GAGACCTGGA GGCAACTGCG ACGATTTGCC CTCACCACCC TGCGTGATTT TGGAATGGGG AAGAAGGGCA TTGAGGAGCG +1945
AATCCAGGAG GAAGCTCATT TTCTGGTGGA GAGGATCAGG AAGACACACG gtaggacact gacgtgtgtc tgctgggtaa cactgcgtat ctgcaatgag +2045
actgtaagaa aattcagtct ggattcacaa ataacaatcc ctgctaccaa tctgagaaaa tgtgctcatc tctactgctc ttttcatttc actgattctg +2145
aattgtatat ggcctcactg taagcttcca agtctcggtc gctttgttac gctgtatact acattctatg ctttactatg tactaaaagg cttcacaagt +2245
aactgaaaag tccagaagcg tttttatact taatgctaac aataaataga taggactact aacctactag acttacttac gctgagcatt actatgctta +2345
ccacttctga caagatatcc gatgaggttt tgctggcgag gagtcgcgag caaggcagag aagggcaggc tccagtgctg cacagaagag gctgatcctt +2445
aacgttacca ttttctcttc catctttttt cctttagaat tttataactt tccatcttga tgattctcat cttccatact tgagatatac cttaccgcgc +2545
tctccaggtt caactttcgt gtctttcatg gatctaatct catttttttct cacgcttcca tagttattta cttagaaaat tctgtagccg tgctgtcaaa +2645
Eco RI (+2695)
actctatttt tgatcacggg tatctgaagt ttcattgggt atggtggaa ttc

Romeo *et al.*, 1990). It seems unlikely, however, that the *CYP2H1* gene would be expressed in either erythroid cells or leucocytes.

Two putative AP-1 binding sites are found at positions -504 to -498 bp and -320 to -314 bp. While these sequences differ from the consensus TGACTCA motif, sequences identical to those in the *CYP2H1* gene promoter have been shown to bind AP-1 and to function in an AP-1-dependent manner in response to phorbol ester stimulation of protein kinase C (Angel *et al.*, 1987: Lee *et al.*, 1987). Interestingly, three other consensus AP-1 sites are found within the other two more distal regions of clustered putative *cis* -acting elements (position -3667 to -3661 bp, -3291 to -3285 bp and -1544 to -1538 bp). This strongly suggests that the family of c-jun and c-fos proteins which dimerise to produce functional AP-1, are important in the regulation of expression of the *CYP2H1* gene.

A putative binding site for the liver-specific factor, LF-A1 is located in the promoter region at position -385 to -380 bp and as well, further upstream at positions -1385 to -1380 bp and -1676 to -1671 bp. LF-A1 binding sites were first demonstrated in the promoters of $\alpha_1$-antitrypsin, apolipoprotein AI, antithrombin III and transferrin to be important in liver-specific expression (Ochoa *et al.*, 1989) and may also be involved in the control and level of expression of the *CYP2H1* gene in the liver.

Located only 16 bp upstream of the TATA box at position -54 to -49 bp is the sequence CACGTG which is found again further upstream within a putative enhancer region at position -3348 to -3343 bp. This is a putative binding site for the c-myc protein and a factor called Major Late Transcription Factor (MLTF) or Upstream Sequence Factor (USF) that binds to the Adenovirus major late promoter (Blackwell *et al.*, 1990). c-*myc* is an oncogene which functions in cell proliferation and differentiation, although the molecular mechanism by which this occurs is unknown. These factors belong to a family of proteins which also includes a myogenic determination protein MyoD and the constitutively expressed E2A protein (Blackwell *et al.*, 1990). Each of these proteins contain a basic-helix-loop-helix (bHLH) motif, which is important for homo- and heterooligomerisation and specific DNA binding (Murre *et al.*, 1989; Lassar *et al.*, 1989; Davis *et al.*, 1990; Voronova and Baltimore, 1990). Members of this family have been shown to bind to the core sequence CA--TG with different flanking sequence being important for the specific binding

of different members (Murre *et al.*, 1989; Lassar *et al.*, 1989; Cai and Davis, 1990; Blackwell and Weintraub, 1990). Recently, the bHLH domain of c-myc has been shown to specifically bind the sequence CACGTG, which is also specifically bound by MLTF/USF (Blackwell *et al.*, 1990). The role of c-myc and/or other members of this family of proteins in the control of *CYP2H1* gene expression remains to be seen, bearing in mind that the two putative c-myc motifs do occur within regions of the 5' flanking region which appear to be functionally important in expression.

As discussed above, a number or putative binding sites for known *trans* -acting factors are found within the early promoter region of the *CYP2H1* gene. It remains to be seen which of these elements as well as others not yet defined, are involved in driving transient expression from this promoter at a level similar to that of the strong Rous Sarcoma Virus long terminal repeat when tested in chick embryo hepatocytes (expression results are shown in section 5.2.G).

The second region of clustered putative *cis* -acting elements, as mentioned earlier, corresponds to a region which is able to enhance basal expression driven from the SV40 promoter and to confer to this promoter responsiveness to phenobarbital. Elements within this region that are common to the *CYP2H1* gene early promoter include two CCAAT boxes, one with similarity to the CP2 consensus sequence (Codosh *et al.*, 1988), an AP-1 binding site and two motifs for the liver-specific factor, LF-A1. cAMP may mediate a response through the numerous putative AP-1 binding sites in this promoter and, in addition, through a putative AP-2 motif (-1779 to -1770 bp), although there is no evidence that cAMP controls this promoter.

Two putative NF-E1 recognition motifs (-1411 to -1404 bp and -1650 to -1643 bp) are located in this region. As mentioned earlier, NF-E1 is an erythroid and leucocyte-specific factor and its possible function in expression of the *CYP2H1* gene is not clear.

As well as for LF-A1, this region of the *CYP2H1* gene 5' flanking region contains the consensus binding sequence, at position -1838 to -1832 bp, for another liver-specific factor designated eH-TF. eH-TF motifs have been shown to exist in the promoters of $\alpha_1$-antitrypsin, albumin, $\alpha$-fetoprotein and UDP glucuronosyl transferase-2, and in the Hepatitis B virus at position +1190 bp (Shaul and Ben-Levy, 1987; Mackenzie, 1990; Zaret

*et al.*, 1990). Also, in relation to liver-specific expression, is the presence of putative binding sites for Ig/EBP at positions -1458 to -1450 bp and -1914 to -1906 bp. While Ig/EBP is ubiquitously expressed, other members of the family to which Ig/EBP belongs and which bind to similar sequences, such as C/EBP and LAP, are enriched in the liver (Chang *et al.*, 1990). Hence these motifs may play an important role in the control and/or level of *CYP2H1* gene expression in the liver in particular as well as in extrahepatic tissues.

This second cluster of putative motifs in the region, from position -1915 to -1380 bp (536 bp), is of interest as the sequence between the *Stu* I site (-1641 bp) and the *Xho* I site (-1403 bp) is able to confer responsiveness to phenobarbital. This region contains a putative CCAAT box, an AP-1 site, an Ig/EBP motif and a NF-E1 binding site. Although responsiveness to phenobarbital may be mediated indirectly through one of these factors, it is also possible, for reasons of specificity, that the phenobarbital-responsive element is unique. A search for a putative phenobarbital-responsive element is described in the next section.

The third region of clustered putative motifs occurs within an *Xho* I/*Bgl* II fragment (-3778 to -3250 bp) which was able to enhance basal expression of the enhancerless SV40 promoter by over 15-fold when tested alone (see Fig. 6.3). This region contains the following putative elements; two AP-1 sites, an AP-3 motif (or core enhancer element), three CACCC boxes, a CCAAT box and a MLTF/USF or c-myc binding site. These elements, bound by their respective *trans* -acting factors may function as a strong basal enhancer in chick embryo primary hepatocytes.

Outside of the three regions of clustered putative *cis* -acting elements, there are very few other elements, and they tend to be scattered throughout the rest of the gene. Of particular note is the eH-TF binding site in intron 1, an IL-6-responsive element in intron 1 and another in intron 2 and six putative CCAAT boxes, all of which are spatially isolated except for the two inverted CCAAT boxes in exon 1.

Schaffner *et al* (1988) have performed extensive expression studies with the aim of studying enhancer function. They found that while extremely strong promoters can be accommodated within a DNA sequence of less than 50 bp, strong enhancers are usually greater than 200 bp in length. The shortest enhancer detected by their screening method was

84 bp long. From these results, it was reasoned that enhancers are composed of multiple elements or modules (*i.e.* clusters of elements) consisting of a combination of different DNA *cis* -acting elements or multiple copies of the same element. Furthermore, in general, the greater the number of such elements or modules, the greater the activation of transcription. From these results it is possible that expression of the *CYP2H1* gene is driven by the early promoter (*i.e.* region 1) with the aid, presumably via a "looping" mechanism, of two enhancer modules (*i.e.* region 2 and region 3). According to this model of gene activation, isolated elements play little or no role in gene regulation.

It should be stressed that the discussion of these putative *cis* -acting elements, in relation to functional regions as determined by transient expression studies, does not take into account negative regulation of this gene, which has not been focused on in this thesis. Negative regulation is vital to the homeostatic control of genes and must not be overlooked. However, negative regulation may take on an number of forms in which positive *trans* -acting factors are displaced or their effect neutralised, and to date, there are few examples of *cis* -acting sequences to which bind "true" silencer-type *trans* -acting factors (Locker and Buzard, 1990).

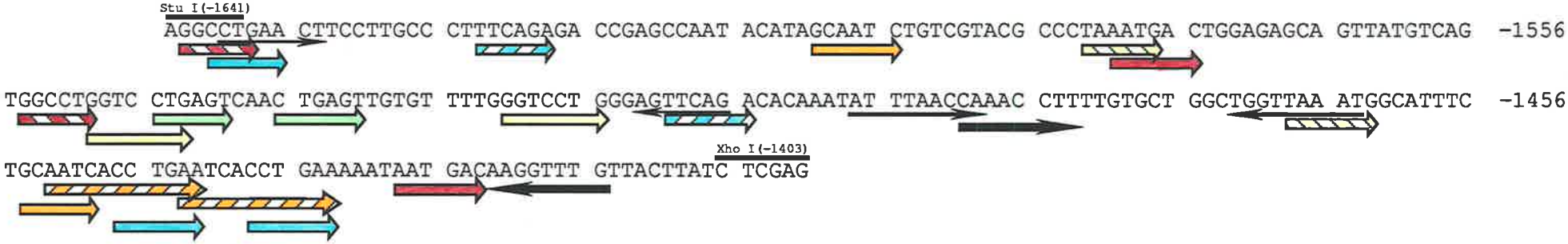
### *7.2.B.3. Search for a phenobarbital-responsive element*

An attempt was made to search for a phenobarbital-responsive element by sequence analysis. In this process, the following assumption was made based on known *cis* -acting element consensus sequences; that responsiveness to phenobarbital is either conferred by a repeated motif of size 6 bp or greater (*i.e.* either a direct repeat or an inverted repeat) or that the phenobarbital-responsive element is palindromic.

The sequence of the 238 bp *Stu* I/*Xho* I fragment, which was shown using transient expression studies to confer phenobarbital-responsiveness to the enhancerless SV40 promoter (see Fig. 6.3), was analysed for direct repeats, inverted repeats and palindromic sequences. This was performed using computer sequence analysis programmes called "Repeat" and "Inverted Repeat" (see section 2.10) while palindromes were searched for manually. The results are diagrammatically represented in Figure 7.3. No palindromic sequences of 6 bp or more were found. Of interest are the many repeated sequences that

**Figure 7.3. Position of repeats and inverted repeats in the 238 bp fragment that is able to confer phenobarbital-responsiveness to the SV40 promoter**

Repeated sequences are shown by coloured arrows and inverted repeated sequences are indicated by black arrows.


Stu I(-1641)
AGGCCTGAA CTTCCTTGCC CTTTCAGAGA CCGAGCCAAT ACATAGCAAT CTGTCGTACG CCCTAAATGA CTGGAGAGCA GTTATGTCAG -1556
TGGCCTGGTC CTGAGTCAAC TGAGTTGTGT TTTGGGTCCT GGGAGTTCAG ACACAAATAT TTAACCAAAC CTTTTGTGCT GGCTGGTTAA ATGGCATTTC -1456
Xho I(-1403)
TGCAATCACC TGAATCACCT GAAAAATAAT GACAAGGTTT GTTACTTATC TCGAG

occur within this 238 bp fragment. One of the direct repeats is 11 bp in length and is partially overlapping (position -1442 and -1433 bp). The sequence of this repeat is AATCACCTGAA which contains within it the minidyad sequence CA--TG; as mentioned in section 7.2.B.2, this belongs to the family of *trans* -acting factors including c-myc, MyoD and MLTF/USF. Whether this region is important in the response to phenobarbital or in the control of expression of the *CYP2H1* gene remains to be determined. Other sequences which are found more than twice within this region include CCTGAA (position -1636, -1442 and -1433 bp) and the truncated version of this sequence CTGAA which, in addition, is also found inverted at postion -1506 bp. Interestingly, an extended version of this sequence CTGAACT is found twice in the 238 bp *Stu* I/*Xho* I fragment (position -1636 and -1506 bp) and is also present in the adjacent 311 bp *Bgl* II/*Stu* I fragment (not shown) which in conjunction with the 238 bp fragment confers to the SV40 promoter a greater response to phenobarbital (see Fig. 6.3)

Although any of the repeated sequences shown in Figure 7.3 are possible candidates for a phenobarbital-responsive element, it is conceivable that such an element may not be detected in this way. For instance, recognition of the phenobarbital-responsive element would be missed if the element is not palindromic and not repeated (at least not repeated in the 238 bp fragment) or if the consensus sequence is less than 6 bp, or if the consensus sequence is greater than 6 bp, but contains gaps of non-essential base pairs within it.

Due to the large number of repeated sequences within this fragment, a putative phenobarbital-responsive element could not be identified. The sequence of the 238 bp *Stu* I/*Xho* I fragment was then compared using a "Bestfit" programme (see section 2.10) to the first 1540 bp of 5' flanking sequence of the *B.megaterium CYP102* gene, which is the only sequence other than the 238 bp fragment that has been shown to respond to phenobarbital in transient expression studies. The best match between the two sequences was an 8 out of 9 bp similarity; *CYP2H1* contained the sequence TGTTACTTA at position -1408 bp while *CYP102* contained the sequence TGTTAATTA at position -748 bp. It will be of particular interest to determine the possible role of this element.

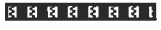
In an attempt to isolate regions or elements important in expression of the *CYP2H1* gene and especially in the response to phenobarbital, comparisons were made with the

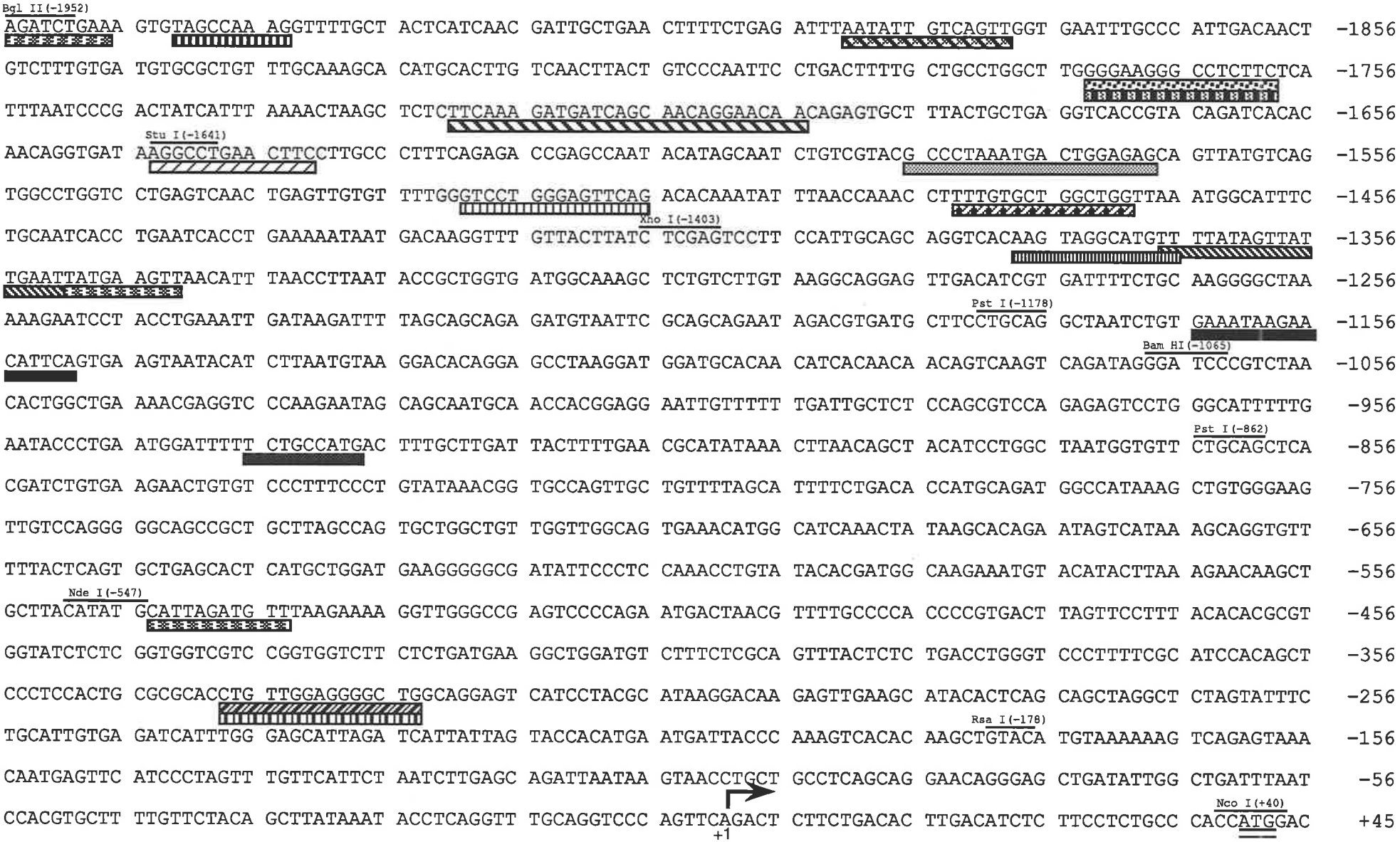
published promoter sequence of other phenobarbital-inducible genes, which includes rat *CYP2B2* (Jaiswal *et al.*, 1987), rabbit *CYP2C1* and *CYP2C4* (Zhao *et al.*, 1990), bacterial *CYP102* (Ruettinger *et al.*, 1989) and *CYP106* (He *et al.*, 1989), rat phenobarbital-inducible aldehyde dehydrogenase (Dunn *et al.*, 1989), rat and mouse glutathione S-transferase Ya subunit (Telakowski-Hopkins *et al.*, 1986; Daniel *et al.*, 1989) and UDP glucuronosyl transferase-2 (Mackenzie, 1990). As a programme able to identify sequences common to three or more sequences was not available, the following strategy for comparison was employed. The entire published 5' flanking sequence and 5' untranslated region for each gene were used in the comparisons. Initially, a programme called "Bestfit" which finds the region of highest similarity between two sequences (see section 2.10) was used to compare the promoter of the *CYP2H1* gene with that of each of the other phenobarbital-inducible genes. This gave a region of highest similarity (or the "Bestfit") with the *CYP2H1* gene promoter and the position of these sequences with respect to the *CYP2H1* gene promoter are demonstrated in Fig. 7.4. These Bestfit sequences were then searched for in all of the other phenobarbital-inducible gene promoters. In this way it was hoped that sequences such as the phenobarbital-inducible element that are common to some or all of the phenobarbital-inducible promoters would be identified. A sequence with the consensus AAGTAGGNATGTT, was found in the promoters of the genes for *CYP2H1* (-1366 bp), *CYP2C1* (-331 bp ) and twice in *CYP102* (-1298 and -97 bp). Interestingly, the two sequences in the *CYP102* gene promoter reside within regions designated R1 and R2 that have been reported to be important in maximal barbiturate-induced expression of this gene (Wen *et al.*, 1989). This is probably not the phenobarbital-inducible element, as it is located ouside of the phenobarbital-responsive region in the *CYP2H1* gene promoter, but may be important in gene expression. No other strikingly obvious sequences common to more than two of the phenobarbital-inducible gene promoters were found.

Unfortunately, there are problems associated with this strategy. Firstly, the Bestfit programme only identifies the best fit between two sequences and even if the phenobarbital-responsive element is present in both sequences, but is not the most extensive match, then it will be missed. Secondly, the phenobarbital-inducible element(s) may be located outside of the reported sequence of the gene promoters analysed. The only other promoter sequence

**Figure 7.4. Sequence similarity of the *CYP2H1* gene promoter with other phenobarbital-inducible gene promoters**

The region of greatest similarity between the *CYP2H1* gene promoter and other phenobarbital-inducible gene promoters was determined using a programme called "Bestfit". The position of these sequences in the *CYP2H1* gene promoter are shown. *a.* The number in brackets represents the position of the 5' most base pair of the Bestfit sequence in the gene from which it comes.

| Diagrammatic Motif | Gene | Bestfit Sequence[a] | Reference |
|---|---|---|---|
|  | Chicken house-keeping ALAS | TTTGTGCTGGCTGG (-277) | Maguire et al., 1986 |
|  | Chicken house-keeping ALAS | GCCTTGAATGCCTGCAGGG (-404) | Maguire et al., 1986 |
|  | *B.megaterium CYP102* | TTAAAAGATGA-CAGAGACAGGAAGAA (-1695) | Ruettinger et al., 1989 |
|  | *B.megaterium CYP106* | TTTTATACTAATTGTATAAAAATGT (-119) | He et al., 1989 |
|  | Rat *CYP2B2* | AATAATATCAGTT (-1369) | Jaiswal et al., 1987 |
|  | Rat *CYP2B2* | AGGCGTGAACATC (-63) | Rangarajan and Padmanaban, 1989 |
|  | Rat *CYP2B2* (CCAAT box) | TAGCCAAAG (-80) | Rangarajan and Padmanaban, 1989 |
|  | Rat *CYP2B2* (13 bp palindrome) | AACATCTGAAGTT (-56) | Rangarajan and Padmanaban, 1989 |
|  | Rabbit *CYP2C1* | AAATAGGAATGTT (-331) | Zhao et al., 1990 |
|  | Rabbit *CYP2C4* | GTCGTGGTAGTTCAG (-474) | Zhao et al., 1990 |
|  | Rat ALDH-PB | TCTGCCATG (-219) | Dunn et al., 1989 |
|  | Rat GST Ya subunit | GGGAAGGG-CTGTTC (-70) | Telakowski-Hopkins et al., 1986 |
|  | Rat GST Ya subunit | CTGTGGGAAGGGCTG (-73) | Telakowski-Hopkins et al., 1986 |
|  | Mouse GST Ya subunit | GGGAGGGG-CTGTTC (-70) | Daniel et al., 1989 |
|  | Mouse GST Ya subunit | CTGTGGGAGGGGCTG (-73) | Daniel et al., 1989 |
|  | $UDPGT_r$-2 | GAAATAAGAGCTTTCA (-253) | Mackenzie, 1990 |


Bgl II(-1952)
AGATCTGAAA GTGTAGCCAA AGGTTTTGCT ACTCATCAAC GATTGCTGAA CTTTTCTGAG ATTTAATATT GTCAGTTGGT GAATTTGCCC ATTGACAACT -1856
GTCTTTGTGA TGTGCGCTGT TTGCAAAGCA CATGCACTTG TCAACTTACT GTCCCAATTC CTGACTTTTG CTGCCTGGCT TGGGGAAGGG CCTCTTCTCA -1756
TTTAATCCCG ACTATCATTT AAAACTAAGC TCTCTTCAAA GATGATCAGC AACAGGAACA ACAGAGTGCT TTACTGCTGA GGTCACCGTA CAGATCACAC -1656
Stu I(-1641)
AACAGGTGAT AAGGCCTGAA CTTCCTTGCC CTTTCAGAGA CCGAGCCAAT ACATAGCAAT CTGTCGTACG CCCTAAATGA CTGGAGAGCA GTTATGTCAG -1556
TGGCCTGGTC CTGAGTCAAC TGAGTTGTGT TTTGGGTCCT GGGAGTTCAG ACACAAATAT TTAACCAAAC CTTTTGTGCT GGCTGGTTAA ATGGCATTTC -1456
Xho I(-1403)
TGCAATCACC TGAATCACCT GAAAAATAAT GACAAGGTTT GTTACTTATC TCGAGTCCTT CCATTGCAGC AGGTCACAAG TAGGCATGTT TTATAGTTAT -1356
TGAATTATGA AGTTAACATT TAACCTTAAT ACCGCTGGTG ATGGCAAAGC TCTGTCTTGT AAGGCAGGAG TTGACATCGT GATTTTCTGC AAGGGGCTAA -1256
Pst I(-1178)
AAAGAATCCT ACCTGAAATT GATAAGATTT TAGCAGCAGA GATGTAATTC GCAGCAGAAT AGACGTGATG CTTCCTGCAG GCTAATCTGT GAAATAAGAA -1156
Bam HI(-1065)
CATTCAGTGA AGTAATACAT CTTAATGTAA GGACACAGGA GCCTAAGGAT GGATGCACAA CATCACAACA ACAGTCAAGT CAGATAGGGA TCCCGTCTAA -1056
CACTGGCTGA AAACGAGGTC CCAAGAATAG CAGCAATGCA ACCACGGAGG AATTGTTTTT TGATTGCTCT CCAGCGTCCA GAGAGTCCTG GGCATTTTTG -956
Pst I(-862)
AATACCCTGA ATGGATTTTT CTGCCATGAC TTTGCTTGAT TACTTTTGAA CGCATATAAA CTTAACAGCT ACATCCTGGC TAATGGTGTT CTGCAGCTCA -856
CGATCTGTGA AGAACTGTGT CCCTTTCCCT GTATAAACGG TGCCAGTTGC TGTTTTAGCA TTTTCTGACA CCATGCAGAT GGCCATAAAG CTGTGGGAAG -756
TTGTCCAGGG GGCAGCCGCT GCTTAGCCAG TGCTGGCTGT TGGTTGGCAG TGAAACATGG CATCAAACTA TAAGCACAGA ATAGTCATAA AGCAGGTGTT -656
TTTACTCAGT GCTGAGCACT CATGCTGGAT GAAGGGGGCG ATATTCCCTC CAAACCTGTA TACACGATGG CAAGAAATGT ACATACTTAA AGAACAAGCT -556
Nde I(-547)
GCTTACATAT GCATTAGATG TTTAAGAAAA GGTTGGGCCG AGTCCCCAGA ATGACTAACG TTTTGCCCCA CCCCGTGACT TAGTTCCTTT ACACACGCGT -456
GGTATCTCTC GGTGGTCGTC CGGTGGTCTT CTCTGATGAA GGCTGGATGT CTTTCTCGCA GTTTACTCTC TGACCTGGGT CCCTTTTCGC ATCCACAGCT -356
CCCTCCACTG CGCGCACCTG TTGGAGGGGC TGGCAGGAGT CATCCTACGC ATAAGGACAA GAGTTGAAGC ATACACTCAG CAGCTAGGCT CTAGTATTTC -256
Rsa I(-178)
TGCATTGTGA GATCATTTGG GAGCATTAGA TCATTATTAG TACCACATGA ATGATTACCC AAAGTCACAC AAGCTGTACA TGTAAAAAAG TCAGAGTAAA -156
CAATGAGTTC ATCCCTAGTT TGTTCATTCT AATCTTGAGC AGATTAATAA GTAACCTGCT GCCTCAGCAG GAACAGGGAG CTGATATTGG CTGATTTAAT -56
Nco I(+40)
CCACGTGCTT TTGTTCTACA GCTTATAAAT ACCTCAGGTT TGCAGGTCCC AGTTCAGACT CTTCTGACAC TTGACATCTC TTCCTCTGCC CACCATGGAC +45
+1

known, without doubt, to contain a barbiturate-responsive element(s), as determined by transient expression studies, is *CYP102* (Wen *et al.*, 1989). The *CYP2B2* gene promoter (-179 to +31 bp) may also contain elements important in the phenobarbital-response, but as yet this has not been conclusively shown (Ragarajan and Padmanaban, 1989). Finally, such comparison techniques rely on the mechanism of phenobarbital-induction and hence the sequence of the phenobarbital-responsive elements to be conserved between species. This however, may not necessarily be the case.

## 7.3. CONCLUSION

An extensive analysis of the sequence of the promoter regions of the chicken house-keeping ALAS gene and the *CYP2H1* gene revealed the presence of many putative *cis* -acting elements. At least for the *CYP2H1* gene, these putative elements were found clustered, and significantly, within the regions of the promoter that appear to be functionally important as determined by transient expression studies. The importance of these putative elements in the regulation of the ALAS and *CYP2H1* genes remains to be determined with the aid of mutagenesis and transient expression studies.

In an attempt to identify a putative phenobarbital-inducible element, a 238 bp fragment which conferred to the SV40 promoter responsiveness to phenobarbital, was analysed for direct repeats, inverted repeats and palindromes. As many repeats were identified, this exercise was not extremely fruitful in the recognition of a possible sequence that responds to phenobarbital.

Comparisons were also performed between the *CYP2H1* gene promoter and the published promoter sequences of other phenobarbital-inducible genes. Once again, little valuable information was gained due to the unavailability of computer programmes able to compare more than two sequences and due to restricted sequence data that is known to contain regions responsive to phenobarbital.

The phenobarbital-responsive element awaits identification. Further deletions of the 238 bp fragment that confers responsiveness to phenobarbital followed by transient expression studies of such deletion constructs may be required for this. Alternatively, foot-printing techniques and gel retardation studies may be used to identify regions bound by

proteins. The ultimate test however, for delineation of this element will be the ablation of responsiveness to phenobarbital using mutagenesis and the demonstration of conferral of phenobarbital-responsiveness by short multimerised oligomers of the specific phenobarbital-responsive sequence to a heterologous promoter.

# CHAPTER 8

# SUMMARY AND CONCLUDING DISCUSSION

## 8.1 SUMMARY AND CONCLUDING DISCUSSION

The work in this thesis has focused on understanding, at the molecular level, how foreign compounds induce the phenobarbital-inducible class of CYPs and also the house-keeping form of ALAS, and the role that haem plays in modulating the induction of ALAS. Chick embryos (17-18 day) and the primary hepatocytes derived from these embryos have been used as model systems in which to study the regulation of these genes.

Prior to commencement of the work described in this thesis, it was known that the drugs, phenobarbital and AIA, when administered to adult rats, induced the hepatic ALAS gene at the transcriptional level, and that haemin administration repressed the induction of this gene (Srivastava *et al.*, 1988; 1990). These findings in rat liver, supported the "haem repression model" for ALAS regulation as originally proposed by Granick (1966) and then modified by others (DeMatteis, 1978; May *et al.*, 1986) in which it was proposed that haem, through a repressor protein, inhibits transcription of the ALAS gene while drugs, via CYP apoprotein induction and consequent haem removal, derepress the synthesis of ALAS. However, it should be pointed out that no satisfactory model has been proposed to account for the induction of the phenobarbital-inducible CYPs by a wide range of structurally dissimilar compounds. In chick embryo liver, phenobarbital and AIA were known to induce the rate of transcription of the ALAS gene and of two *CYP* genes, designated *CYP2H1* and *CYP2H2* (Hansen *et al.*, 1989). This activation of transcription accounted predominantly for the increase in the ALAS mRNA levels and partly for the increase in the CYP mRNA levels. A substantial post-transcriptional mechanism by drugs also seemed to be required to account for the observed induced levels of CYP mRNAs, but whether this involved the modulation of mRNA stability was not known (Hansen *et al.*, 1989). The effect of haem on CYP regulation had not been reported. It had been known since the early work of Granick (1966) that haemin addition resulted in a dramatic reduction in the level of ALAS activity in chick embryo primary hepatocytes, although it was not known whether this reduction in ALAS was due to a transcriptional or post-transcriptional mechanism. Subsequently, it was shown that haemin administration to chick embryos specifically blocked the transport of the ALAS precursor protein into the mitochondria (Hayashi *et al.*, 1972; Srivastava *et al.*,

1983). Whether haem also acted in a transcriptional fashion, as in the rat, or at other post-transcriptional levels to inhibit ALAS synthesis in chick embryo liver was not clear.

It was therefore of interest to investigate the mechanisms of control involved in the response of CYPs and ALAS to phenobarbital and haem in chick embryo liver and in primary hepatocyte cultures derived from this source. Inherent in this investigation was the important question of whether drug-induction of ALAS occurs indirectly via derepression of haem-inhibited ALAS synthesis as a result of induced CYP protein synthesis, as proposed in the "haem repression model".

The transcription rates of the ALAS and *CYP* genes and the corresponding mRNA levels were therefore measured in chick embryo liver and primary hepatocytes in response to phenobarbital and haemin treatment. The most important findings from this work were as follows. Drugs increased the transcription rates for the ALAS and *CYP* genes in both systems. Haemin or ALA administration however, had no significant effect on these drug-induced transcription rates, but substantially decreased the levels of ALAS mRNA while CYP mRNA levels remained unaltered. The finding that haem did not repress ALAS gene transcription was unexpected and contrary to what has been established for expression of the rat hepatic ALAS gene (Srivastava *et al.*, 1988; 1990). This strongly implied that, in chick embryo liver, haem acts post-transcriptionally, possibly by specifically destabilising the ALAS mRNA in addition to the known effect it has on blocking transport of the ALAS precursor protein into mitochondria (Hayashi *et al.*, 1972; Srivastava *et al.*, 1983).

From the studies described above, a revised model for the regulation of ALAS and CYPs in chick embryo liver is proposed. It is suggested that drugs increase the transcription rates of the ALAS and *CYP* genes, by a common and as yet undetermined mechanism as discussed further below, and that the increased ALAS gene transcription rate is not dependent on the cellular haem pool being lowered. In addition, an unknown post-transcriptional mechanism, seen only in chick embryo liver but not in primary hepatocytes, also contributes substantially to the drug-induced increase in CYP mRNA amounts (Hansen *et al.*, 1989).

Under conditions where the level of regulatory haem is in excess, that is, when the amount of haem synthesized or entering the cell exceeds that required for CYPs and other

haemoproteins, it is postulated that haem both prevents transport of ALAS precursor and destabilises the ALAS mRNA. Both mechanisms would result in a rapid decline of mitochondrial ALAS activity in view of the short half-life of the mature protein (Ades *et al.*, 1983) and the resulting short half-life of the mRNA (Drew and Ades, 1989a). The control of haem biosynthesis can therefore occur rapidly, and by employing two levels of negative regulation, the cell can avoid a potentially toxic build up of haem and its precursors. At the same time, activation of the ALAS gene by a wide range of drugs and other foreign and endogenous compounds, ensures that ALAS mRNA is rapidly synthesised for the immediate supply of ALAS and consequently haem when needed.

To briefly summarise, in the chick embryo liver, the regulation of ALAS and CYP levels differ from that seen in the adult rat liver. In chick embryo liver, administered drugs activate transcription of the ALAS and *CYP* genes. Haem reduces ALAS mRNA levels, but does not affect ALAS gene transcription. However, in the rat liver, there is convincing evidence that administration of haemin or ALA represses transcription of the ALAS gene (Yamamoto *et al.*, 1988; Srivastava *et al.*, 1988; 1990), but is not involved in destabilisation of the ALAS mRNA (Yamamoto *et al.*, 1988). It is intriguing that these species use two completely different mechanisms to bring about the same response; that is, repression of ALAS synthesis. An interesting question is why, in chick embryo liver, haem represses ALAS via a transport mechanism and possibly mRNA destabilisation, while in rat liver the repressive effect of haem is at the transport and transcriptional level. In relation to this, it is interesting that the half-lives of the ALAS mRNAs in rat liver and in chick embryo primary hepatocytes are reported to be 20 min and 220 min in the absence of added haemin (Yamamoto *et al.*, 1988; Drew and Ades, 1989a), respectively. It seems likely that because the rat hepatic ALAS mRNA is so unstable, a rapid decrease in its level can be obtained solely by repressing ALAS gene transcription. In contrast, a rapid decrease in the level of the more stable chick embryo liver ALAS mRNA, could be achieved by haem destabilising the mRNA. An alternative explanation for the differences in the mechanisms by which haem acts to repress ALAS in adult rat and chick embryo liver is that they may reflect a variation between embryonic and adult stages of development. Experiments involving the administration of haemin to adult chickens and determination of ALAS mRNA half-lives in

the presence and absence of haemin should answer this. A difficulty with the rat system is understanding why haem also represses transcription of *CYPs* and the genes for other drug metabolising enzymes (Srivastava *et al.*, 1990). This question remains to be answered.

As discussed above, the ALAS and *CYP2H1* genes were rapidly induced by phenobarbital and "phenobarbital-like" drugs in chick embryo liver and primary hepatocytes. Nothing is known about the mechanism by which these structurally diverse drugs activate gene transcription. A receptor has not been found and it is not known if, or how many, intermediate factors are required for signal transduction. The approach taken to tackle this problem was to analyse the 5' flanking region of the ALAS and *CYP2H1* genes for regions that responded to phenobarbital using transient transfection studies in chick embryo primary hepatocytes, with the subsequent aim of isolating the regulatory protein(s) involved in gene activation. No such region, responsive to drug, was found in the first 1700 bp of 5' flanking region of the ALAS gene under conditions in which drugs were known to activate endogenous ALAS gene transcription. This implied that the *cis* -acting elements necessary for the drug response were located either within the gene, downstream of the gene or further upstream than -1700 bp and were not present in the constructs examined. An exciting result however, was the identification of a 4.8 kb *Bam* HI fragment in the 5' flanking region of the *CYP2H1* gene, spanning position -5.9 to -1.1 kb, that was able to confer phenobarbital-responsiveness to a heterologous enhancerless SV40 promoter. This fragment was termed a "phenobarbital-responsive enhancer domain" as it could function in either orientation and at various distances from the enhancerless SV40 promoter. Experiments with various drugs that induce several classes of CYPs, demonstrated that this domain responded strongly to phenobarbital and AIA. Dissection of the 4.8 kb *Bam* HI fragment revealed multiple regions that were able to function as basal enhancer regions or as phenobarbital-responsive regions. Basal enhancer regions increased expression from the enhancerless SV40 promoter to a similar degree both in the presence and absence of phenobarbital, while phenobarbital-responsive regions were able to increase expression from this promoter to a greater extent in the presence of drug. Interestingly, the combination of both a basal enhancer region and a phenobarbital-responsive region, synergistically increased the magnitude of the response to phenobarbital. A precedent for this is seen in the mouse TCDD-inducible *Cyp1a-1* gene,

where a GC box known to bind the *trans* -acting factor Sp1, does not confer a TCDD-response to a heterologous promoter, but in conjunction with a dioxin-responsive element (DRE), synergistically increases the effect of the DRE on a heterologous promoter (Fisher *et al.*, 1990).

Two phenobarbital-responsive regions of size 1360 and 238 bp were shown to exist in the 5' flanking region of the *CYP2H1* gene. Sequence analysis of the smaller region and a comparison with known promoter sequences of other phenobarbital-inducible genes, failed to identify a common sequence that may represent the phenobarbital-responsive element or motif. It is possible that the phenobarbital-responsive element may not be readily detectable or may be located elsewhere in these genes or their flanking regions. Further analysis of the 1360 bp region is necessary to localise the phenobarbital-responsive element(s) to a smaller region, and a subsequent sequence comparison of such a region with that of the 238 bp region will be important.

Of interest is the phenomenon of how structurally different compounds can induce many different phenobarbital-responsive genes. Further deletion experiments and/or foot-printing and gel retardation experiments, in conjunction with mutagenesis studies, will be necessary to define the sequence(s) responsible for conferral of the response to phenobarbital in the chicken *CYP2H1* gene. Identification of a phenobarbital-responsive element should enable isolation of the protein factor through which phenobarbital exerts its stimulatory effect. This would be the first step in unravelling the mechanism of the signal transduction process used by phenobarbital. It remains to be seen whether a broad specificity receptor or multiple receptors exist to which drugs bind or whether there is one or more *trans* -acting factors activated by these drugs, which in turn act on the appropriate genes. In the latter situation, one would still need to explain how the many different drugs activate the specific *trans* -acting factors. The basic work described here on the regulation of the *CYP2H1* gene in chick embryo primary hepatocytes should permit the elucidation in the future of the mechanism by which structurally diverse compounds are able to elicit a common activational response upon a gene.

# PUBLICATIONS

## ABSTRACTS

Drug-induced CAT activity using cytochrome P450 promoter-CAT constructs transfected into primary chicken hepatocytes.

Hansen, A.J., Hahn, C.N., and May, B.K. (1990) 12th Annual Conference on the Organisation and Expression of the Genome

Identification of an enhancer that is responsive to phenobarbital in the 5' flanking region of a chicken cytochrome P450 gene.

Hahn, C.N., Hansen, A.J., Dogra, S., and May, B.K. (1991) 13th Annual Conference on the Organisation and Expression of the Genome

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Transcriptional Regulation of the Chicken *CYP2H1* Gene: Localisation of a phenobarbital-responsive enhancer domain.

Hahn, C.N., Hansen, A.J., and May, B.K. (1991) *J. Biol. Chem.* **266**, in press

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# Transcriptional Regulation of the Chicken *CYP2H1* Gene

## LOCALIZATION OF A PHENOBARBITAL-RESPONSIVE ENHANCER DOMAIN*

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**The mechanism by which the drugs phenobarbital and 2-allyl-2-isopropylacetamide induce levels of chicken cytochrome P-450 (*CYP*) mRNAs has been investigated in primary hepatocyte cultures from 17-day-old chick embryos. It has been demonstrated that three *CYP* mRNAs of 3.5, 2.5, and 2.2 kilobases (kb) are strongly induced by phenobarbital in primary hepatocytes, as found previously in chick embryo liver *in ovo* (Hansen, A. J., Elferink, L. A., and May, B. K. (1989) *DNA (NY)* 8, 179–191), and that, at least for the 3.5-kb mRNA, this is predominantly a result of enhanced transcription of the corresponding gene, *CYP2H1*. Transient transfection assays were carried out in primary cultures using constructs containing different lengths of *CYP2H1* gene 5′-flanking sequence fused to the reporter chloramphenicol acetyltransferase (CAT) gene. These experiments established that *cis*-acting elements located in the first 0.5 kb of the *CYP2H1* gene 5′-flanking region direct high basal expression of the CAT gene, but do not mediate phenobarbital inducibility. When constructs containing more than 1.1 kb of *CYP2H1* gene 5′-flanking sequence were examined, phenobarbital induction of CAT expression was observed, and a drug-responsive domain between positions −5.9 and −1.1 kb was identified. This domain has the properties of an enhancer, since it is able to confer phenobarbital responsiveness to the enhancerless SV40 promoter when tested in either orientation or at different distances from the promoter. The enhancer domain also responds to 2-allyl-2-isopropylacetamide, but whether the action of the two drugs is mediated by a single nuclear receptor interacting with common DNA elements in the domain remains to be established.**

The cytochrome P-450 (CYP)[1] gene superfamily currently consists of 27 gene families classified according to amino acid sequence similarity, and these are found in a total of 23 eukaryote and 6 prokaryote species (Nebert *et al.*, 1991). This complex system of genes encodes enzymes that catalyze oxidation reactions on a myriad of substrates. Ten families of CYPs exist in mammals, six coding for CYPs involved in steroid metabolism, while the remaining four families are involved primarily in catabolism of endogenous steroids and fatty acids, in addition to exogenous drugs, plant metabolites, food additives, carcinogens, and many environmental pollutants. The reactions catalyzed by the latter four families of enzymes occur primarily in the liver and, following the action of conjugating enzymes, ultimately lead to increased water solubility of the substrates and hence more rapid excretion from the body.

Of great interest are the mechanisms by which a vast range of foreign chemicals are able to induce specific CYPs and conjugating enzymes in the liver. In rat and mouse, administration of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and other structurally similar aryl hydrocarbons induce members of the *CYP1* family, namely *CYP1A1* and *CYP1A2* (Nebert *et al.*, 1991). The mechanism by which this occurs has been well characterized and involves the binding of TCDD to the cytosolic aryl hydrocarbon (Ah) receptor, translocation of this complex into the cell nucleus, and specific interaction with the DNA at multiple responsive elements located in the 5′-flanking region of the inducible genes (Fujisawa-Sehara *et al.*, 1988; Denison *et al.*, 1988, 1990). There is strong evidence that these elements act synergistically to form drug-responsive enhancers that mediate the increased rate of TCDD-induced gene transcription (Fujisawa-Sehara *et al.*, 1987; Fisher *et al.*, 1990).

When rats are administered phenobarbital, there is an increase in the synthesis of CYPs that are members of the *CYP2* gene family. Increased amounts of two rat hepatic *CYP* mRNA species following phenobarbital treatment have been shown by nuclear transcription run-on assays to occur as a result of an increase in the transcription of the *CYP2B1* and *CYP2B2* genes (previously designated P-450b and P-450e), respectively (Hardwick *et al.*, 1983). In the rabbit, three *CYP* genes (*CYP2C1*, *CYP2C2*, and *CYP2C4*) have been shown in a similar way to be induced transcriptionally by phenobarbital (Zhao *et al.*, 1990). The prokaryote, *Bacillus megaterium* ATCC 14581, contains three reported *CYP* genes, one of which, *CYP102* (previously P-450$_{BM-3}$), is strongly induced transcriptionally by pentobarbital and other barbiturates (Wen *et al.*, 1989). Unlike the TCDD-inducible CYPs, little is known about the mechanism(s) by which phenobarbital exerts its effect. No evidence has been found for a phenobarbital receptor (Poland *et al.*, 1980, 1981; Tierney and Bresnick, 1981) and Fonne and Meyer (1987) have shown, in rat, that the CYP enzyme itself appears not to be the receptor. Recent work on the *CYP2B2* gene promoter, however, has revealed evidence that phenobarbital increases the amount of a protein binding to a 223-bp fragment located immediately upstream of the translation initiation ATG codon (Rangarajan and

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[1] The abbreviations used are: CYP, cytochrome P-450; AIA, 2-allyl-2-isopropylacetamide; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; CAT, chloramphenicol acetyltransferase; bp, base pair(s); nt, nucleotide(s); kb, kilobase(s); RSV, Rous sarcoma virus; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Padmanaban, 1989). Whether this binding protein is directly involved in the drug induction mechanism is still not clear. Therefore. the intriguing inherent problem of how the same CYPs of the *CYP2* gene family are able to be induced by a wide range of apparently structurally dissimilar compounds such as phenobarbital, 2-allyl-2-isopropylacetamide (AIA), and 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) remains to be elucidated (Elliott *et al.*, 1989). In an attempt to investigate this problem, we are studying the regulation of phenobarbital-inducible *CYP* genes in the chicken. In previous work, we have shown the existence of at least two genes encoding phenobarbital-inducible CYPs (Mattschoss *et al.*, 1986). One of these genes, *CYP2H1* (previously designated gene A), encodes a 3.5-kb mRNA while the other gene, *CYP2H2* (previously designated gene B) gives rise to a 2.2-kb mRNA. Both of these mRNAs in liver are highly induced following phenobarbital administration to chick embryos, and we have shown that these increases are a result of increased gene transcription and a post-transcriptional mechanism (Hansen *et al.*, 1989). A phenobarbital-inducible mRNA of size 2.5 kb is also present, probably due to an alternate splicing event of the RNA transcribed from either *CYP2H1* or *CYP2H2* (Hansen *et al.*, 1989).

One approach to further investigate and more precisely define the mechanism by which phenobarbital activates transcription of the chicken *CYP* genes is to localize any *cis*-acting elements in the promoter that are able to mediate phenobarbital induction. Studies have shown that addition of phenobarbital or "phenobarbital-like" drugs such as AIA and glutethimide to cultured chick embryo primary hepatocytes results in increased CYP protein levels (Althaus *et al.*, 1979; Oron and Bar-Nun, 1984; Sinclair *et al.*, 1989). In the present study, we have focused on the *CYP2H1* 3.5-kb mRNA and have established that there is an increase in levels of this mRNA following addition of phenobarbital to cultured chick embryo primary hepatocytes and that this is predominantly a result of transcriptional activation of the *CYP2H1* gene. This system has therefore been employed for transient expression analysis of fusion gene constructs. Our results demonstrate that the *CYP2H1* gene early promoter is able to direct high expression of the CAT gene and that, with longer constructs extending beyond −1.1 kb, this expression is enhanced following phenobarbital addition. A phenobarbital-responsive enhancer domain has been identified which confers marked inducibility to a heterologous SV40 promoter.

## MATERIALS AND METHODS[2]

## RESULTS

*Phenobarbital Induction of CYP mRNAs in Chick Embryo Primary Hepatocytes*—Previous studies in our laboratory have established that when 17–18-day-old chick embryos are injected with phenobarbital, there is a substantial rise in the hepatic levels of at least three *CYP* mRNAs of 3.5, 2.5, and 2.2 kb (Hansen *et al.*, 1989). Two hepatic *CYP* cDNA clones designated pCHP3 and pCHP7, and of lengths of 2712 bp and 2167 bp, respectively, have been isolated. The pCHP3 clone is derived from the 3.5-kb mRNA and the pCHP7 clone from the 2.2-kb mRNA (Hobbs *et al.*, 1986; Hansen *et al.*, 1989). Although no cDNA has as yet been found for the 2.5-kb mRNA, it is clear that all three mRNAs are highly related. There is 92% sequence similarity between the coding regions of pCHP3 and pCHP7, and pCHP3, when used as a probe in Northern blot analysis, hybridizes strongly to all three *CYP* mRNAs (Hansen *et al.*, 1989).

It has been known for many years that when cultures of chick embryo primary hepatocytes are treated with phenobarbital, levels of CYP protein are induced as measured by enzyme activity or immunological techniques (Althaus *et al.*, 1979; Giger and Meyer, 1981). However, it was not known whether this reflects an increase in *CYP* mRNA amounts following drug addition. Initial experiments were therefore performed to investigate whether the three drug-inducible *CYP* mRNAs detected in chick embryo livers (Hansen *et al.*, 1989) are expressed in primary hepatocytes and whether these mRNAs are induced following phenobarbital treatment. Primary hepatocytes were prepared from the livers of 17–18-day-old chick embryos using the previously described collagenase method of Giger and Meyer (1981). Hepatocytes were cultured under various conditions as described below, and the effect of phenobarbital on mRNA levels was analyzed. Hepatocytes were cultured in the following media: William's E medium alone; William's E medium supplemented with 10 μg/ml insulin and 300 ng/ml dexamethasone; William's E medium supplemented with 10% Nu-Serum; Dulbecco's modified Eagle's medium supplemented with 10% Nu-Serum; Dulbecco's modified Eagle's medium/Ham's F12 (1:1) supplemented with 1% ITS$^+$; and Opti-MEM supplemented with 1% ITS$^+$. The cells were plated on standard plastic Petri dishes. Cells in William's E medium supplemented with insulin and dexamethasone were also plated on Petri dishes precoated with Matri-gel, a connective tissue-like matrix (Schuetz *et al.*, 1988). All plates were incubated for 16 h to allow for the attachment of hepatocytes, and, at this time, phenobarbital was added to a final concentration of 500 μM. The cells were incubated for 4 h more prior to harvesting, and total RNA was isolated. Total RNA (20 μg) was fractionated on a 1% agarose-formaldehyde gel and transferred to nitrocellulose, and the amounts of three CYP mRNAs were examined using the probe, pCHP3. In preliminary experiments, it was found that *CYP* mRNAs of the expected sizes 3.5, 2.5, and 2.2 kb were present in primary hepatocytes under all culture conditions tested, and, while the amounts of these mRNAs were low in the absence of phenobarbital, they were increased substantially following addition of this drug (results not shown). William's E medium supplemented with 10% Nu-Serum was chosen for all further studies because levels of the drug-induced *CYP* mRNAs were consistently higher than in the other media. In addition, cell attachment to the dishes was found to be more rapid and efficient in this medium and, importantly, a greater number of hepatocytes survived in this medium following electroporation (as described later).

Fig. 1 shows the results for a Northern blot analysis using pCHP3 as probe; total RNA was isolated from hepatocytes cultured in William's E medium supplemented with 10% Nu-Serum in the presence and absence of phenobarbital. For comparison, total RNA from the livers of 17-day-old chick embryos, treated with or without phenobarbital, was also examined. It can be seen that, in hepatocytes, low basal levels of three *CYP* mRNAs of 3.5, 2.5, and 2.2 kb are visible, and the amounts of these mRNAs are greatly elevated by phenobarbital treatment for 4 h. In liver, the basal levels of the three mRNAs are very low (not visible in Fig. 1), but can be seen readily after phenobarbital induction. Furthermore, the levels of mRNAs detected by the pCHP3 probe are highest for the 3.5-kb mRNA and lowest for the 2.2-kb mRNA in both the drug-induced hepatocytes and chick embryo liver.

To investigate whether the *CYP* mRNAs induced by phe-

[2] "Materials and Methods" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

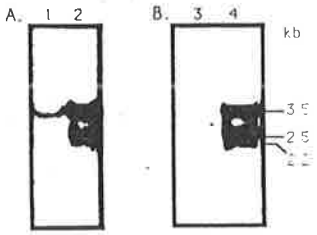



FIG. 1. **Comparison of phenobarbital-inducible *CYP* mRNAs in chick embryo primary hepatocytes and chick embryo liver.** *A*, primary hepatocytes were prepared as described under "Materials and Methods" and allowed to attach to the Petri dishes for 16 h in William's E medium supplemented with 10% Nu-Serum. Phenobarbital was then added to a final concentration of 500 $\mu$M while control plates received an equal volume of PBS. After 4 h, total RNA was isolated, and 20 $\mu$g were run on a denaturing formaldehyde-agarose gel. The RNA was transferred to nitrocellulose and probed with nick-translated pCHP3 (*lane 1*, control; *lane 2*, phenobarbital-treated). *B*, three 17-day-old chick embryos were each treated with 4 mg of phenobarbital in PBS by injection through the allantoic membrane and into the fluid surrounding the embryo. Three control embryos received an equal volume of PBS. After 4 h, the embryos were decapitated, the livers were removed and appropriate ones were pooled, and total RNA was isolated. RNA was treated and probed as in *A* (*lane 3*, control; *lane 4*, phenobarbital-treated). Molecular size markers were generated by digestion of pBR322 with *Acc*I and *Hinc*II (not shown).

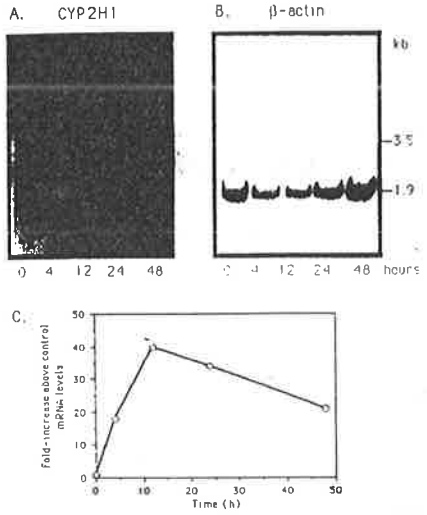



FIG. 2. **Time course of the 3.5-kb *CYP2H1* mRNA levels following phenobarbital treatment of chick embryo primary hepatocytes.** Primary hepatocytes were cultured as described in Fig. 1*A* in the presence of 500 $\mu$M phenobarbital for 0, 4, 12, 24, and 48 h. Total RNA was isolated, and 20 $\mu$g were run on a denaturing formaldehyde-agarose gel. RNA was transferred to nitrocellulose, probed with nick-translated pCHPB15 (*A*) or chicken $\beta$-actin cDNA (*B*), and autoradiographed. Molecular size markers were generated by digestion of pBR322 with *Acc*I and *Hinc*II (not shown). Autoradiographs were quantified using a computing densitometer. The level of the 3.5-kb mRNA was standardized to the $\beta$-actin mRNA level at the corresponding time, and the -fold increase in 3.5-kb mRNA compared to the zero time point was plotted (*C*). The level of the 3.5-kb mRNA did not vary significantly in the control cultures, without phenobarbital, throughout the 48-h period (results not shown).

nobarbital in primary hepatocytes are identical with those in liver, total RNA from drug-induced hepatocytes was analyzed by Northern blots using as probes, pCHPB15 and pCHP7-AB1. These probes are specific for the hepatic *CYP* 3.5- and 2.2-kb mRNAs, respectively, and hybridized to the unique 3′-untranslated region of these mRNAs (Hobbs *et al.*, 1986; Hansen *et al.*, 1989). Using these probes, the mRNAs of expected size were detected providing strong evidence that the 3.5- and 2.2-kb mRNAs induced in hepatocytes are the same as those induced in liver (results not shown). It seems also likely that the *CYP* mRNA of 2.5 kb which is detected by pCHP3, but not the 3′-specific probes (Hansen *et al.*, 1989), is also identical in the hepatocytes and liver.

We chose to examine in detail the drug induction in primary hepatocytes of the highly expressed *CYP* mRNA of 3.5 kb using the 3′-specific probe, pCHPB15. In order to determine the kinetics of induction of the 3.5-kb mRNA in primary hepatocytes, total RNA was isolated at 0, 4, 12, 24, and 48 h after the addition of phenobarbital to the medium. Following gel electrophoresis and Northern blotting onto nitrocellulose, duplicate filters were probed separately with either $^{32}$P-labeled pCHPB15 or with a $^{32}$P-labeled chicken $\beta$-actin cDNA clone as control. It can be seen in Fig. 2 that, following phenobarbital addition, the level of the 3.5-kb mRNA was elevated 18-fold at 4 h, reached a maximum of 40-fold at 12 h, and then declined gradually to 21-fold at 48 h. By contrast, in this and other experiments, the level of $\beta$-actin mRNA remained largely unaffected in the presence of phenobarbital throughout this time period.

*Nuclear Run-on Analysis of CYP2H1 Gene Transcription*—It was important to determine whether the increase in the level of the 3.5-kb mRNA following the addition of phenobarbital to primary hepatocytes was a result of increased transcription of the corresponding *CYP2H1* gene. To investigate this, nuclei were isolated from untreated and phenobarbital-treated primary hepatocytes at 0, 4, 12, 24, and 48 h following the addition of phenobarbital, and transcription was allowed to run-on in the presence of [$\alpha$-$^{32}$P]UTP. Total RNA was isolated and hybridized to filter-bound cDNA clones; pCHPB15 was used to determine the transcription rate of the *CYP2H1* gene transcription while a chicken $\beta$-actin cDNA clone monitored transcripts from the $\beta$-actin gene as control. The degree of nonspecific hybridization was determined using immobilized pBR322 plasmid DNA, the vector into which the above two cDNA clones had been inserted. A low level of transcription of the *CYP2H1* gene was observed in nuclei isolated from untreated primary hepatocytes (Fig. 3). When hepatocytes were treated with phenobarbital for 4 h, there was an increase of approximately 10-fold in the rate of *CYP2H1* gene transcription. This rate increased to a maximum of 36-fold measured 12 h after phenobarbital addition, but gradually declined to 9-fold at 48 h (the latter result indicated that the *CYP2H1* gene remains transcriptionally active 48 h after the initial addition of phenobarbital). By contrast, the transcription rate of the $\beta$-actin gene remained relatively unchanged over the 48 h of drug treatment, demonstrating that the effect of the drug on *CYP2H1* gene transcription was not a general response. From these studies, it can be seen that at 12 h after phenobarbital addition to primary cultures there is a maximal 36-fold increase in the steady state level of the 3.5-kb mRNA, and this corresponds with a 40-fold increase in the *CYP2H1* gene transcription rate measured at this time (see Fig. 2). Hence, these results indicate that the increase in the amount of the 3.5-kb mRNA following drug induction of hepatocytes can be accounted for predominantly by enhanced *CYP2H1* gene transcription. We have therefore employed this system to investigate the mechanism of drug action on *CYP2H1* gene transcription using transient transfection assays.

*Transient Expression of CYP2H1CAT Chimeric Gene Con-*

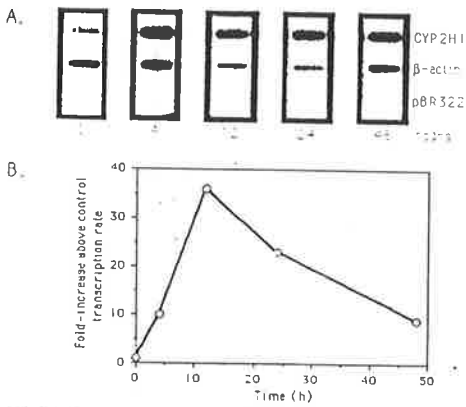



FIG. 3. **Nuclear transcription run-on time course of the *CYP2H1* gene following phenobarbital treatment of chick embryo primary hepatocytes.** Primary hepatocytes were cultured as described in Fig. 1*A* in the presence of 500 μM phenobarbital for 0, 4, 12, 24, and 48 h. Nuclei were isolated and allowed to run-on in the presence of [α-$^{32}$P]UTP, and the nascent [$^{32}$P]RNA transcripts were hybridized to duplicate slots of filter-bound pCHP3, chicken β-actin cDNA, and pBR322. Following autoradiography (*A*), transcription rates were quantified from slot blots using a computing densitometer and averaged. The rate of transcription from the *CYP2H1* gene was standardized to that of the control β-actin gene, and the fold increase in the transcription rate compared to the zero time point was plotted (*B*). Nonspecific hybridization to pBR322 was undetectable.

*structs*—In previous work, pCHP3 was used to screen a chicken genomic Charon 4A λ library and a number of overlapping clones for the *CYP2H1* gene were isolated (Mattschoss *et al.*, 1986). One of the genomic clones, designated λ3, has been shown by restriction mapping and Southern blot hybridization analyses using pCHP3 to contain approximately 9 kb of the 5′-flanking sequence (Mattschoss *et al.*, 1986). To locate *cis*-acting regulatory regions that may be involved in both the basal and phenobarbital-induced expression of the *CYP2H1* gene, increasing the lengths of the 5′-flanking region excised from λ3 were inserted upstream of the bacterial CAT reporter gene. The resulting chimeric reporter gene constructs contained between 0.5 and 8.9 kb of the *CYP2H1* gene upstream sequence. These constructs also contained the entire 5′-untranslated region of 39 bp as well as the translation initiation ATG codon of the *CYP2H1* gene (Hobbs *et al.*, 1986). Initial attempts to express these fusion constructs in chick embryo primary hepatocytes following electroporation were unsuccessful as no CAT activity could be detected in cell lysates. It was reasoned that if initiation of CAT protein synthesis was occurring at the initiation ATG codon of the *CYP2H1* gene, a CAT protein with additional N-terminal amino acids would be generated which may be inactive. The initiation ATG codon of the *CYP2H1* gene contained in these constructs was therefore altered by site-directed mutagenesis, thus ensuring that translation of CAT mRNA is initiated from the correct ATG codon. The resulting constructs were designated CYP2H1CAT-8.9, 4.7, 1.7, 1.1 and 0.5, where the numerals indicate the length in kilobases of the 5′-flanking region in each construct (see Fig. 4). These constructs were introduced into chick embryo primary hepatocytes using electroporation. The results from a transient transfection experiment are shown in Fig. 4. The basal expression of CAT activity remained fairly constant for all CYP2H1CAT constructs with the exception of CYP2H1CAT-4.7 which consistently expressed at a higher level. Although basal expression of the shortest construct, CYP2H1CAT-0.5, is shown in this experiment to be the highest, repeated experiments demonstrated that this construct expressed at a level similar to that of CYP2H1CAT-1.1 and CYP2H1CAT-1.7. Interestingly, basal CAT expression of all of the constructs was comparable with that of pRSVCAT which contains the strong RSV long terminal repeat promoter, and possible reasons for the strong promoter activity directed by the first 0.5 kb of the *CYP 2H1* 5′-flanking region will be discussed later. The most striking aspect of these results is the induction of CAT activity following addition of phenobarbital to cells transfected with constructs containing either 1.7, 4.7, or 8.9 kb of *CYP2H1* 5′-flanking sequence. The level of induction consistently and significantly increased with increasing length of the 5′-flanking sequence beyond 1.1 kb. The constructs containing either 0.5 or 1.1 kb of the 5′-flanking sequence showed no increase in expression after the addition of phenobarbital. No drug induction was observed with the control plasmid, pRSVCAT, in which the CAT gene is under the control of the Rous sarcoma virus long terminal repeat. Moreover, the control plasmid pIBICAT, lacking *CYP2H1* 5′-flanking sequence, does not express any detectable CAT activity even when large amounts of protein extract (50 μg) from transfected cells are assayed. The results suggest that *cis*-acting elements responsive to phenobarbital reside between 1.1 and 8.9 kb upstream of the *CYP2H1* transcription start site.

To more precisely locate the region required for drug induction, a construct designated CYP2H1CAT-8.9Δ was synthesized from CYP2H1CAT-8.9 by deletion of an internal 4.8-kb *Bam*HI fragment from the 5′-flanking region of the gene (see Fig. 4). This construct upon transfection into primary hepatocytes, expressed in a manner similar to CYP2H1CAT-1.1, but unlike CYP2H1CAT-8.9, was no longer responsive to phenobarbital (Fig. 4*C*). This indicated that the *cis*-acting element(s) responsible for phenobarbital activation are located between positions −5.0 and −1.1 kb of the *CYP2H1* 5′-flanking region.

*RNase Protection Analysis of RNA from Transfected Primary Hepatocytes*—RNase protection analysis was performed to investigate whether transcription of the CAT reporter gene driven by *CYP2H1* upstream sequences was initiated at the correct *CYP2H1* transcription start site in both untreated and phenobarbital-treated hepatocytes. A transcription template was synthesized by cloning into the *Hinc*II site of the SP6 transcription vector pGEM1, a 366-bp *Rsa*I fragment which spanned the *CYP2H1* gene transcription start site and encompassed 146 bp of the CAT gene from CYP2H1CAT-0.5 (Fig. 5*A*). Linearization of the resultant plasmid, pGEM1-366, with *Eco*RI produced a template for SP6 polymerase synthesis of a 420-nt transcript. Initiation from the *CYP2H1* gene transcription start site (Hobbs *et al.*, 1986) in the various CYP2H1CAT constructs would yield an expected RNase-protected *CYP2H1*/CAT hybrid mRNA of 188 nt (Fig. 5*A*). The SP6 transcript of 420 nt was hybridized with total RNA isolated from primary hepatocytes transfected with CYP2H1CAT-8.9, but not treated with phenobarbital. Following subsequent RNase treatment, a major band migrating at 188 nt was observed (Fig. 5*C*, *lane 1*). Similarly, total RNA was also isolated from hepatocytes treated with phenobarbital following transfection with the CYP2H1CAT-8.9 construct. When this RNA was subject to protection analysis, a major band of 188 nt was also observed, but showed a greater intensity relative to the corresponding band in untreated RNA (Fig. 5*C*, compare *lanes 2* and *3*). These results showed that transcription of the mRNA was initiated from the correct *CYP2H1* start site of the CYP2H1CAT-8.9 construct in both

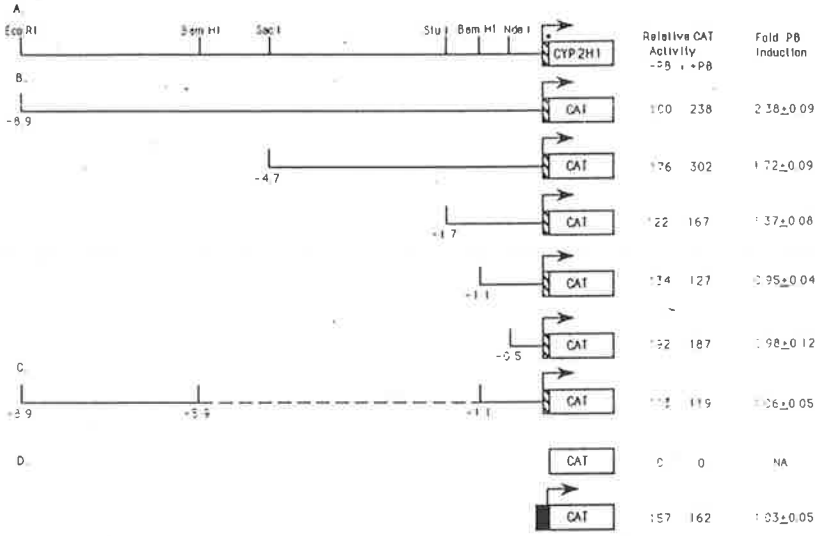



FIG. 4. **Transient expression of CYP2H1CAT chimeric constructs in chick embryo primary hepatocytes.** *A*, diagrammatic representation of the 5′ portion of the *CYP2H1* gene. The *line* represents the 5′-flanking region, while the *striped box* represents the complete 5′-untranslated region of the gene. An *asterisk* marks the translation initiation ATG codon. *B*, diagram of constructs containing various lengths of the 5′-flanking region of the *CYP2H1* gene inserted upstream of the CAT reporter gene. The *CYP2H1* translation initiation ATG codon has been mutated in these constructs. *C*, diagram of a construct from which an internal 4.8-kb *Bam*HI fragment has been deleted. *D*, diagram of the control promoterless plasmid pIBICAT and positive control pRSVCAT. The RSV long terminal repeat promoter is shown as a *black box*. 1 pmol of each construct was transfected by electroporation into primary hepatocytes and incubated in the presence or absence of phenobarbital for 48 h as described under "Materials and Methods." Hepatocytes were harvested, cell lysates were prepared, and CAT assays were performed on 5 μg of soluble lysate protein for 30 min by the method of Gorman *et al.* (1982). CAT activities are expressed relative to that of the longest construct CYP2H1CAT-8.9, in noninduced hepatocytes. A value of 100 is arbitrarily given to this level of activity. The results shown are indicative of a typical experiment using one batch of primary hepatocytes in which each construct was transfected in triplicate and the resultant CAT assay values were averaged. The -fold induction with phenobarbital was determined for each individually transfected construct and is given as the mean ± S.D. (*NA*, not applicable). Similar results were obtained with at least three batches of primary hepatocytes prepared on different days.

untreated and phenobarbital-treated hepatocytes and that the amount of CAT mRNA was increased following drug treatment. Analysis of RNA from primary hepatocytes transfected with constructs CYP2H1CAT-4.7, -1.7, and -1.1 also revealed correct initiation of transcription in both untreated and phenobarbital-treated cultures (results not shown). In Fig. 5*C*, the drug-induced increase in CAT mRNA amounts measured by RNase protection apparently exceeds the 2- to 3-fold increase seen in CAT activity from CYP2H1CAT-8.9 (see Fig. 4), but, in repeated experiments, the fold induction of CAT mRNA more closely approximated that of CAT activity. An internal control was carried out to determine whether endogenous levels of the *CYP* 3.5-kb mRNA in primary hepatocytes also increased in response to phenobarbital following transfection of the cells with CYP2H1CAT-8.9. A construct containing a 292-bp chicken genomic *Rsa*I fragment spanning the transcription start site of the native *CYP2H1* gene was cloned into the *Hinc*II site of pGEM1 to create pGEM1-292 (Fig. 5*B*). The 346-nt transcript generated by SP6 polymerase protected an expected 114-nt fragment, and the amount of this transcript was greater in RNA isolated from the phenobarbital-treated hepatocytes (Fig. 5*C*, compare *lanes 2* and *3*) showing that the level of endogenous 3.5-kb mRNA is elevated upon addition of the drug. Two other major bands of 111 nt and 112 nt were also protected and probably correspond to alternate transcription start sites. However, the reason that these alternate start sites are not seen for initiation of the CAT mRNA is not clear. A much greater intensity of the RNase-protected fragment derived from the endogenous 3.5-kb mRNA was seen compared with that derived from protection of CAT mRNA (Fig. 5*C*) and may reflect a low efficiency of transfection and CAT mRNA instability.

*Transient Transfection of a CYP2H1 5′-Flanking Fragment from −5.9 to −1.1 Fused to a Heterologous Promoter*—As described, removal of the 4.8-kb *Bam*HI fragment from the 5′-flanking region of the *CYP2H1* gene resulted in CAT expression no longer being responsive to phenobarbital (Fig. 4*C*). To directly test for the presence of phenobarbital-responsive elements in the 4.8-kb *Bam*HI fragment, this fragment was inserted into the pCAT™-promoter, a pUC19 plasmid vector containing the CAT gene under the control of the enhancerless SV40 promoter (Fig. 6). The fragment was inserted in both orientations at an upstream *Bgl*II site and at a downstream *Bam*HI site relative to the CAT gene. These constructs were designated pCATBg4.8, pCATBg4.8R, pCATBa4.8, and pCATBa4.8R where *Bg* and *Ba* refer to the *Bgl*II and *Bam*HI cloning sites, respectively, and where the *R* refers to the reverse (*i.e.* 3′ to 5′) orientation. These constructs were transfected into primary hepatocytes, the cells were treated with and without phenobarbital for 48 h, and CAT assays were performed on the cell extracts (Fig. 6). The control pCAT™-promoter, expressed at very low but detectable levels that were not altered by the presence of phenobarbital. However, upon insertion of the 4.8-kb *Bam*HI fragment, expression of the CAT gene from the heterologous SV40 promoter was markedly induced following addition of pheno-

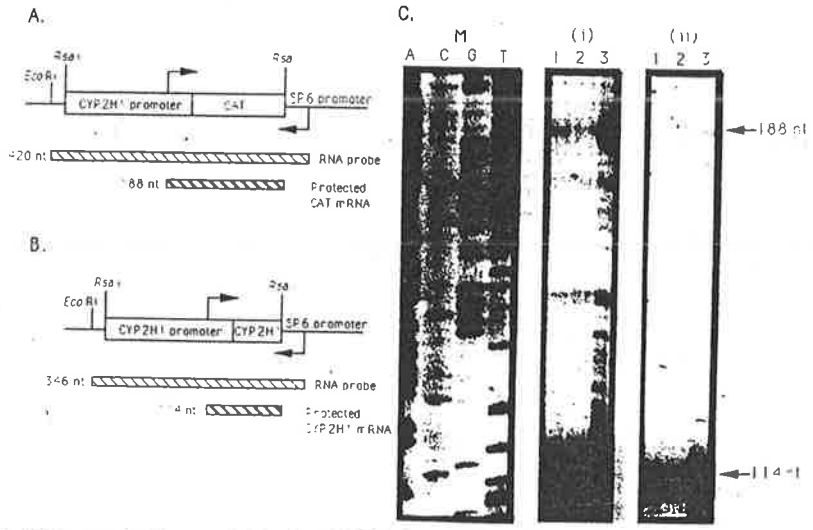



FIG. 5. **RNase protection analysis of total RNA from chick embryo primary hepatocytes transfected with CYP2H1CAT-8.9.** *A* and *B*, diagrammatic representation of the constructs used as templates in the preparation of [$^{32}$P]RNA transcripts for RNase protection. The expected size of protected mRNAs is shown for CAT mRNA (*A*), and the endogenous 3.5-kb mRNA derived from the *CYP2H1* gene (*B*). *C*, chick embryo primary hepatocytes were prepared and transfected with 2 pmol of CYP2H1CAT-8.9 by electroporation. Phenobarbital was added to a concentration of 500 $\mu$M, and the hepatocytes were incubated for 48 h. Control plates received an equal volume of PBS. Total RNA was then isolated and analyzed in RNase protection assays. The [$^{32}$P]RNA probes synthesized were hybridized to 20 $\mu$g (*lane 1*) and 8 $\mu$g (*lane 2*) of total RNA from noninduced hepatocytes and 8 $\mu$g (*lane 3*) of total RNA from phenobarbital-induced hepatocytes. Autoradiographs were allowed to expose for 120 h (*i*) and 20 h (*ii*) on Konica-Medical A x-ray film. The *numbers* displayed to the *right* of the autoradiographs correspond to the expected nucleotide lengths of the protected fragments. An M13mp18 DNA sequence ladder (*M*) was used to aid in size determination of the protected fragments.

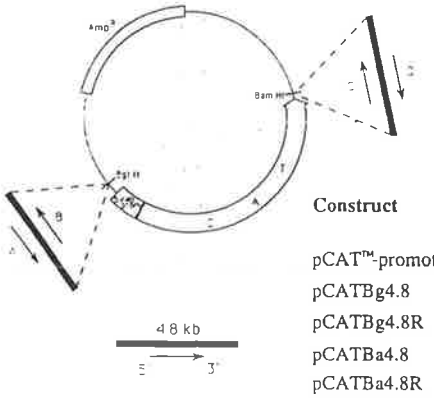
barbital to the culture medium. Expression of CAT activity from pCATBg4.8 or pCATBg4.8R was increased approximately 14-fold, while expression of pCATBa4.8 or pCATBa4.8R increased approximately 8-fold. The absolute fold of phenobarbital induction varied somewhat between experiments, but within an experiment using the same batch of primary hepatocytes, relative results were most consistent and this can be seen from the standard deviation values given. The lower level of induction of pCATBa4.8 and pCATBa4.8R by phenobarbital may be due to a distance-dependent effect relative to the SV40 promoter. These results therefore show that the 4.8-kb *Bam*HI fragment can functionally interact with and confer phenobarbital inducibility to a heterologous promoter, in either orientation and at different positions. Thus, this fragment contains sequence which displays the properties expected of a phenobarbital-responsive enhancer.

*Effect of Various Drugs on Expression of pCATBg4.8 and CYP2H1CAT-8.9*—Of interest is the mechanism by which a broad range of structurally different drugs can cause induction of the same phenobarbital-inducible *CYPs* (Elliott *et al.*, 1989). To investigate this, primary hepatocytes were transfected with pCATBg4.8 or CYP2H1CAT-8.9, and CAT expression was measured following addition of AIA, 20-methylcholanthrene, or dexamethasone. The results are shown in Table I. Expression of pCATBg4.8 increased 4.6-fold with AIA while increases in expression of 1.5- and 1.1-fold occurred with 20-methylcholanthrene and dexamethasone, respectively. In the presence of either AIA, 20-methylcholanthrene, or dexamethasone, expression of the control plasmid pCAT$^{TM}$-promoter, did not vary significantly from the untreated state. Furthermore, expression of CYP2H1CAT-8.9 was induced 2.2-fold in the presence of AIA while expression with 20-methylcholanthrene and dexamethasone only increased 1.3- and 1.1-fold, respectively. These results demonstrate that the 4.8-kb *Bam*HI fragment with phenobarbital-responsive enhancer properties responds significantly to AIA. However, 20-methylcholanthrene and dexamethasone do not have a marked effect of induction on either of these constructs. These results are in keeping with experiments performed in this laboratory where we have shown that the three *CYP* mRNAs are strongly induced in chick embryo liver by the apparently unrelated drugs phenobarbital and AIA (Hansen *et al.*, 1989), and by DDC, glutethimide, and mephenytoin, but are only poorly induced by $\beta$-naphthoflavone (an aryl hydrocarbon) or dexamethasone [3]

## DISCUSSION

Three closely related *CYP* mRNAs of 3.5, 2.5, and 2.2 kb are induced in the livers of phenobarbital-treated chick embryos (Hansen *et al.*, 1989) while studies with adult hens have shown that induction occurs in a tissue-specific fashion with a marked increase of these mRNAs occurring only in the liver, small intestine, and kidney (Hansen *et al.*, 1989). Other compounds with no apparent structural similarity such as AIA (Hansen *et al.*, 1989), DDC, glutethimide, and mephenytoin also induce the levels of these mRNAs in chick embryo livers.[3] In the present work, we have demonstrated for the first time that phenobarbital increases the levels of three *CYP* mRNAs in chick embryo primary hepatocytes. We have focused on the regulation of the 3.5-kb mRNA and have established that the increase in this mRNA in primary hepatocytes is predominantly a result of enhanced transcription of the correspond-

[3] C. N. Hahn, A. J. Hansen, and B. K. May, unpublished data.




| Construct | Fold PB Induction |
|---|---|
| pCAT™-promoter | 0.94±0.1 |
| pCATBg4.8 | 14.0±0.9 |
| pCATBg4.8R | 14.3±0.8 |
| pCATBa4.8 | 8.1±0.4 |
| pCATBa4.8R | 8.8±0.8 |

FIG. 6. **A 4.8-kb *BAM*HI fragment from the *CYP2H1* gene 5′-flanking region confers phenobarbital inducibility to the SV40 promoter.** A 4.8-kb *Bam*HI fragment (-5.9 to -1.1 kb) from the 5′-flanking region of the *CYP2H1* gene was cloned in both orientations into the unique *Bgl*II and *Bam*HI sites of pCAT™-promoter vector located about 150 bp and 2820 bp upstream of the SV40 promoter transcription start site, respectively. (Due to the circular nature of the plasmid, the *Bam*HI site can alternatively be considered about 1690 bp downstream of the SV40 transcription start site, immediately 3′ of the CAT gene.) The *shaded box* represents the 4.8-kb *Bam*HI fragment (not drawn to scale), while the *arrow* shows the 5′ to 3′ orientation of insertion. The resultant four constructs labeled *A*, *B*, *C*, and *D* have been designated pCATBg4.8, pCATBg4.8R, pCATBa4.8, and pCATBa4.8R, respectively. These constructs were introduced into primary hepatocytes by electroporation, and the effect of the presence and absence of 500 μM phenobarbital for 48 h was analyzed by assaying cell extracts for CAT activity. The results shown are indicative of a typical experiment using one batch of primary hepatocytes in which each construct was transfected in triplicate and assayed for CAT activity, and the -fold induction with phenobarbital was given as the mean ± S.D. Similar results were obtained with at least three batches of primary hepatocytes prepared on different days.

TABLE I

*Effect of various drugs on transient expression of pCAT™-promoter, pCATBg4.8, and CYP2H1CAT-8.9 in chick embryo primary hepatocytes*

2 pmol of pCAT™-promoter and pCATBg4.8 and 1 pmol of CYP2H1CAT-8.9 were introduced into primary hepatocytes in triplicate by electroporation. Drugs were added to give final concentrations of 50 μg/ml AIA, 1 μg/ml 20-methylcholanthrene, or 10 μM dexamethasone. Controls received an equal volume of solvent only. After 48 h, the hepatocytes were harvested and lysed, and the level of CAT activity was determined. The data shown are representative of a typical experiment performed in triplicate using one batch of primary hepatocytes. Results are given as the average fold increase in CAT activity following addition of drug, and standard deviation values are given in parentheses. Similar results were obtained with at least two other different batches of primary hepatocytes.

| Drug | pCAT™-promoter | pCATBg4.8 | CYP2H1CAT-8.9 |
|---|---|---|---|
| AIA | 1.07 (±0.23) | 4.60 (±0.17) | 2.20 (±0.003) |
| 20-Methylcholanthrene | 1.24 (±0.15) | 1.47 (±0.35) | 1.31 (±0.16) |
| Dexamethasone | 0.84 (±0.06) | 1.11 (±0.18) | 1.14 (±0.04) |

ing gene, *CYP2H1*. Previously, it was shown in livers of phenobarbital-treated chick embryos that the level of the 3.5-kb mRNA was increased as a result of both transcriptional and one or more post-transcriptional mechanisms (Hansen *et al.*, 1989). The reason for a post-transcriptional mechanism not being detected in primary hepatocytes in the present study is not known.

To investigate the mechanism of phenobarbital induction of the *CYP2H1* gene, transient transfection assays have been undertaken in the primary hepatocyte cultures. Reporter gene fusion constructs were synthesized with various lengths of the *CYP2H1* gene 5′-flanking region fused to the CAT gene. When 0.5 kb of flanking region was tested, strong basal expression was observed, with the level of measured CAT activity in cell lysates being comparable to that obtained with the positive control construct, pRSVCAT. The expression of CAT activity by this construct was, however, unaffected by phenobarbital addition indicating that elements in this region can direct high basal expression, but not drug inducibility. Several putative *cis*-acting DNA regulatory elements are located in the first 0.5 kb of the 5′-flanking sequence including a consensus TATA box, an inverted CCAAT box, a CACCC box, and five sequences similar to the binding site for liver-specific factors (Frain *et al.*, 1989; Zaret *et al.*, 1990). The contribution of these putative elements to basal and tissue-specific expression of the *CYP2H1* gene is not known but is being examined. The high level of basal expression directed by the first 0.5 kb of the 5′-flanking sequence was reduced by different amounts when increasing lengths of the *CYP2H1* gene 5′-flanking region were tested. The results imply that basal expression is subject to stimulatory elements in the first 0.5 kb of the 5′-flanking region and a combination of positive and negative regulatory elements further upstream.

Of most interest in the present study is the demonstration of a phenobarbital-responsive enhancer domain. Constructs containing 1.7, 4.7, and 8.9 kb of the 5′-flanking region all demonstrated increased CAT expression upon the addition of phenobarbital to the culture medium, and the level of this induction increased with lengths of the 5′-flanking region from position -1.1 to -8.9 kb suggesting the involvement of multiple *cis*-acting elements in the mechanism of induction. A 4.8-kb *Bam*HI fragment spanning positions -5.9 to -1.1 kb was deleted from the most highly inducible construct, CYP2H1CAT-8.9, to produce CYP2H1CAT-8.9Δ which was then shown to be no longer responsive to phenobarbital. Subsequently, when this 4.8-kb *Bam*HI fragment was inserted into the expression vector pCAT™-promoter, a substantial increase in the level of CAT activity was achieved with phenobarbital. Induction was totally dependent on the presence of the 4.8-kb *Bam*HI fragment in the vector and was observed when this fragment was placed in either orientation and at different distances from the promoter indicating that this phenobarbital-responsive domain has the properties of an enhancer. However, a distance-dependent effect was observed since induction of CAT activity with phenobarbital decreases from 14-fold to 8-fold when the enhancer domain is shifted from a position immediately 5′ of the SV40 promoter to a position about 2.7 kb further upstream. The reason for this phenomenon should become apparent when the phenobarbital-responsive element(s) within the enhancer domain have been localized. It was apparent from transient expression studies that the level of phenobarbital-induced CAT activity mediated by the enhancer domain located in the expression vector pCAT™-promoter was considerably greater than that seen in constructs containing the *CYP2H1* gene 5′-flanking sequences (compare Fig. 4 and Fig. 6). This finding may indicate that the *CYP2H1* promoter, unlike the enhancerless SV40 promoter, is functioning near the maximal level in the absence of phenobarbital and that, upon drug addition, further increases in expression are limited.

An important question is whether an intracellular phenobarbital receptor exists which recognizes different inducers. In this context, an Ah receptor has been shown to exist for polycyclic aryl hydrocarbons such as TCDD (reviewed by

Whitlock, 1989). Extensive studies into the control of the *CYP1A1* gene have shown that TCDD (and other structurally related aromatic hydrocarbons) bind to an intracellular Ah receptor which then interacts with several dioxin-responsive elements in the 5′-flanking region of the mouse *CYP1a1* gene to increase gene transcription (Fisher *et al.*, 1990). Recently, Issemann and Green (1990) isolated a cDNA clone for a mouse peroxisome proliferator activated receptor (mPPAR). Interestingly, this receptor was shown to bind a broad range of structurally distinct foreign compounds including clofibric acid, Wy-14,643, and monoethylhexylphthalate, but not phenobarbital. Whether there is a "phenobarbital receptor" with a broad specificity for foreign inducing compounds such as phenobarbital, AIA, DDC, and mephenytoin remains to be elucidated. In the present work, the upstream enhancer domain responds to both phenobarbital and AIA, but it is not known whether the same or different *cis*-acting elements in the enhancer are responsible for this.

Our studies to date imply that a combination of positive and negative *cis*-acting DNA elements in the *CYP2H1* gene 5′-flanking region control basal expression and that response to phenobarbital is mediated by elements in an upstream enhancer domain. To our knowledge, this is the first demonstration of a phenobarbital-responsive enhancer domain in a eukaryotic gene, although studies in the bacterium *B. megaterium* have defined upstream regions involved in the induction of transcription by barbiturates of the *CYP102* ($P\text{-}450_{BM\text{-}3}$) gene (Wen *et al.*, 1989). Whether the mechanism of phenobarbital induction is conserved between prokaryotes and eukaryotes remains to be determined. We are now examining in detail the DNA elements within the strong early promoter and also in the enhancer domain of the *CYP2H1* gene 5′-flanking region in an attempt to gain an insight into the intriguing mechanism by which foreign compounds are able to transcriptionally activate this gene.

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Supplemental material to Transcriptional Regulation of the Chicken *CYP2H1* Gene: Localization of a phenobarbital-responsive enhancer domain by Christopher N. Hahn, Antony J. Hansen and Brian K. May

## MATERIALS AND METHODS

White Leghorn fertilised eggs were obtained from Parafield Poultry Research Centre, Parafield Gardens, South Australia. Sodium phenobarbital was obtained from May and Baker, Ltd, and AIA was a generous gift from Roche, Australia. Restriction endonucleases, DNA polymerase I (Klenow fragment), T4 DNA ligase, [α-$^{32}$P]dCTP, [α-$^{32}$P]dATP, [α-$^{32}$P]dCTP, nick translation kits, sequencing kits and SP6 RNA transcription kits were obtained from Bresatec, Ltd., South Australia. RNasin, pGEM1 and pCAT$^{TM}$-promoter were purchased from Promega, USA and pBH76 from Integrated Sciences Pty. Ltd., Sydney, Australia. pRSVCAT was kindly provided by G. R. MacGregor, Baylor College of Medicine, Houston. Chicken *CYP2H1* cDNA clones, pCHP3 and pCHPR15 have been described previously (Hobbs *et al.*, 1986). The chicken β-actin cDNA clone was a generous gift from S. Dalton. Williams E medium, Supherum and other tissue culture media were obtained from Flow Laboratories Australasia Pty. Ltd., Sydney, Australia. Collagenase Type IV, RNase A and RNase T1 were purchased from Sigma. Nitrocellulose filters were purchased from Schleicher and Schuell. OpuScint IIiSafe scintillation fluid and restriction endonuclease *Sna* I were purchased from Pharmacia. [$^{14}$C]Chloramphenicol was obtained from Amersham Corp. and thin layer chromatography silica gel 60 $F_{254}$ sheets were purchased from E. Merck, Darmstadt, FRG. All enzymatic manipulations were performed according to standard procedures (Maniatis *et al.*, 1982) unless stated otherwise. Densitometry measurements were performed using a Molecular Dynamics computing densitometer Model 300A and ImageQuant software version 2.0.

*Plasmid Constructions* - A suitable reporter system was prepared by removal of the CAT gene from pSV2CAT (Gorman *et al.*, 1982) as a *Hind* III/*Bam* HI fragment, end-filling using DNA polymerase I (Klenow) fragment) and ligation into the *Hin*cII site of pIB176 such that the 5' end of the CAT gene was nearest the unique *Sma* I site of the pentalinker. This CAT vector was called pIBICAT. A series of constructs were then made in which various lengths of *CYP2H1* gene 5' flanking region including the complete 5' untranslated region were cloned into this *Sma* I site, 5' of the CAT gene in pIBICAT. This was done in the following way. A 0.5, 1.1 and 8.9 kb fragment representing different lengths of 5' flanking region were obtained by restricting the previously reported clone λ3 (Matsuoka *et al.*, 1988) with *Sde* I/*Nco* I, *Bam* HI/*Nco* I and *Eco* RI/*Nco* I, respectively. Thus, each fragment contained a common 5' *Nco* I end corresponding to the ATG translation initiation site of the *CYP2H1* coding sequence. Each fragment was then end-filled and ligated into the unique *Sma* I site. To ensure that translation could not initiate at the ATG initiation codon preserved by end-filling the *Nco* I site of the *CYP2H1* gene, this ATG sequence was altered by site directed mutagenesis to produce a *Sal* I site. A proximal *Bam* HI site, adjacent to this ATG was simultaneously created. This resulted in constructs which were designated CYP2H1CAT-0.5, CYP2H1CAT-1.1 and CYP2H1CAT-8.9. Constructs CYP2H1CAT-1.1 and CYP2H1CAT-8.9 were subsequently made by digesting CYP2H1CAT-8.9 with *Sna* I and *Sal* I, respectively, removing the excised 5' flanking region and recircularising the resultant vector. CYP2H1CAT-3.9 was produced by digesting CYP2H1CAT-8.9 with *Bam* HI, removing the 4.5 kb *Bam* HI fragment and recircularising the resultant vector. To investigate enhancer properties on a heterologous promoter, the *CYP2H1* 4.5 kb *Bam* HI fragment was cloned in both orientations into the *Bgl* II site and *Bam* HI site of pCAT$^{TM}$-promoter to produce the constructs pCATBgl-R, pCATBgl-NR, pCATBal-F and pCATBal-NR, respectively. (Bgl = *Bgl* II, Bal = *Bal* I, F = forward orientation of the 4.5 kb *Bam* HI fragment into the *Bgl* II and *Bam* HI sites, respectively while R refers to the reverse orientation with respect to the native promoter orientation.

*Preparation of chick embryo primary hepatocytes* - Hepatocytes were prepared from 17-18 day old chick embryos by the method of Greer and Meyer (1981), but with minor variations. 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 2 mM EDTA to remove blood cells followed by 4 ml of 0.05% collagenase in Hank's balanced salts solution (HBSS) to initiate digestion of intercellular 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 into fresh 0.05% collagenase - HBSS, cut into small pieces (1 - 2 mm) with fine tipped scissors and incubated at 37°C for 30 min with gentle shaking every 5 min and gentle pipetting, up and down, using a 10 ml glass pipette every 10 min to aid in separation of the cells. Collagenase was removed by washing with HBSS (2 ml/ liver). Contaminating erythrocytes were lysed in an ammonium chloride solution as described by Sassa and Kappas (1977). Following two washes with William's E medium (2 ml/ liver) to remove cell debris and hemoglobin from lysed erythrocytes, the hepatocytes were resuspended in William's E medium (1 ml/ liver) and the viscid determined. Typically, 2 x 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.

*Preparation of DNA* - The *rec* A$^+$ host, *E. coli* XL1-blue was used for cloning of all plasmids. This was done to prevent the production of multimeric forms of plasmid seen upon cloning in *rec* A$^-$ strains, which may lead to complications in interpretation of CAT assay data. Plasmid DNA was prepared using the QIAGEN column procedure as outlined by the manufacturer (QIAGEN, Dusseldorf, FRG). All construct DNA preparations were quantified by spectrophotometry at $A_{260}$ followed by analysis of known amounts on a standard 1% agarose gel to ensure consistency between preparations with regard to quantity and degree of supercoiling. For comparison, DNA was also prepared using the alkaline lysis procedure followed by two CsCl/ethidium bromide equilibrium density gradients, dialysis and ethanol precipitation (Maniatis *et al.*, 1982). Both procedures consistently produced a majority of supercoiled species that expressed comparably in transient transfection studies. However, in this paper the reported results for transient transfection assays were performed only with QIAGEN column prepared plasmid DNA.

*Transfection of hepatocytes and cultivation conditions* - Transfection of DNA into chick embryo primary hepatocytes was performed by electroporation. Hepatocytes were resuspended on ice cold 20 mM HEPES pH7.05 containing 137 mM NaCl, 5 mM KCl, 0.7 mM $Na_2HPO_4$ and 6 mM dextrose (Graham *et al.*, 1973) at a cell density of 2.5 x 10$^7$ cells/ ml and sheared salmon sperm DNA added as carrier in a final of 500 μg/ ml. Hepatocytes (2 x 10$^6$) and 2-10 μg/ ml of construct DNA for 'apoptosis' 4 μg of a 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 (two) 60 mm diameter petri dishes containing 5 ml of William's E medium supplemented with Supherum to a final concentration of 10% and containing 50 μg/ ml gentamicin. Hepatocytes were allowed to adhere to the petri dish and to recover at 37°C, under a CO-/air mix (5:95) for 16 hours. The media was changed to remove dead and non-adherent cells and the remaining healthy cells were treated as follows. To one of the two plates was added a 100 mM phenobarbital solution in phosphate buffered saline (PBS) to a final concentration of 500 μM (phenobarbital-treated) and to the other an equivalent volume of PBS (control). The cultures were further incubated for 48 hours prior to harvesting. For determination of the effects of $AlA_6$, 20-methylcholanthrene or dexamethasone, these drugs were added instead of phenobarbital. To harvest chick embryo primary hepatocytes, culture media was decanted and replaced with 1 ml of 40 mM Tris-HCl pH7.5 containing 1mM EDTA and 150 mM NaCl. The cells were dislodged by scraping with a rubber policeman, transferred into an eppendorf tube and pelleted in an Eppendorf Microfuge for 10 seconds. The supernatant was then removed and the pelleted cells were stored at -80°C until required.

*CAT assay* - Hepatocytes were lysed by three freeze/thaw cycles and centrifuged for 10 min in an Eppendorf Microfuge to pellet cell debris. Protein estimation was performed on the supernatant fraction and then all cell lysates were heated at 65°C for 10 min to remove an inhibitor (Fromm *et al.*, 1985; Sleigh, 1986). It was noted that lysates not heated revealed markedly reduced CAT activity. CAT assays were performed by the method of Gorman *et al.* (1982). Protein extract (5 μg) was assayed for 30 min for the CYP2H1CAT and pRSVCAT constructs, while 50 μg of protein extract was assayed for 120 min for the pCAT$^{TM}$-promoter chimeric constructs. Following autoradiography, CAT assays were quantified by cutting out the spots from TLC plates, placing them into scintillation vials containing 1 ml of OpuScint IIiSafe scintillation fluid and counting acetylated and unacetylated [$^{14}$C]chloramphenicol fractions. Results were expressed as a percentage of chloramphenicol acetylated. In this system CAT assays were linear to 70% conversion of chloramphenicol to the acetylated forms and all assays were performed within this range. It should be noted that within an experiment, duplicate or triplicate assays of CAT activity did not vary by more than 15% of the mean. However, between experiments there was some variation, probably due to preparation of different batches of primary hepatocytes. A similar problem has been reported for the preparation of primary bovine adrenocortical cells (Lund *et al.*, 1990). Thus, results of CAT assays for a given construct represent triplicates, individually transfected and assayed, on the same day using the same batch of primary hepatocytes. All comparisons of constructs were also performed using the same batch of primary hepatocytes.

*RNase protection analysis* - Templates for generating $^{32}$P-labelled RNA transcripts were made using the SP6 transcription vector, pGEM1. The transcription template for detection of CAT mRNA was constructed by excision and removal of a 366 bp *Rsa* I fragment, spanning the mRNA transcription start site, from CYP2H1CAT-0.5 and ligation into the *Hin*cII site of pGEM1 to create pGEM1-366. Similarly, a 292 bp chicken genomic *Rsa* I fragment that spans the mRNA transcription start site of *CYP2H1* was cloned into the *Hin*c II site of pGEM1 to create pGEM1-292, a template for detection of endogenous *CYP2H1* mRNA. Both template plasmids were linearised with *Eco* RI. Synthesis of RNA transcripts labelled with [α-$^{32}$P]UTP was performed using a kit and protocol supplied by Bresatec, Ltd. RNasin at a concentration of 1 U/μl was added to prevent RNA degradation. Full length transcripts of 420 nt and 326 nt from pGEM1-366 and pGEM1-292, respectively were obtained. These were purified by separation on a 6% polyacrylamide, 7 M urea, Tris-borate standard sequencing gel, excision of the full length major band, and elution in 10 mM Tris-HCl pH8.0 containing 1 mM EDTA and 0.1% $NaDodSO_4$ at room temperature overnight. RNase protection assays were performed on total RNA isolated from primary hepatocytes using the method of Krtee and Melton (1987). To ensure that protected RNA fragments were a quantitative measure of the amount of mRNA species present, controls in which double the amount of radioactive probe was added to the assay were performed. These revealed that the probe was in excess. To further check the linearity of this system, increasing amounts of RNA were assayed. An appropriate increase in intensity of protected fragment was observed (results not shown). In order to size the length of the RNA protected fragments, sequence derived from M13mp18 using the universal sequencing primer was run in adjacent tracks. In addition, because of the difference in mobility of DNA and RNA fragments, a control transcript of known size (189 nt) provided with the SP6 RNA transcription kit was simultaneously run and the mobility of the M13mp18 sequence markers adjusted appropriately to enable accurate size determination of RNA fragments.

*Transcription run-on analysis* - For the isolation of nuclei all steps were performed at 4°C, cells and all solutions were kept on ice, and equipment was precooled. Chick embryo primary hepatocyte cultures were harvested at various times after the addition of phenobarbital by decanting the culture media, replacing it with 1 ml of 150 mM NaCl, 40 mM Tris-HCl pH7.5, 1 mM EDTA and scraping the cells from the plates with a rubber policeman. Nuclei were isolated by a modification of the method of Clayton and Darnell (1983) as outlined by Schuetz *et al.* (1990). The nuclei were finally resuspended in storage buffer (20 mM Tris-HCl pH7.9, 75 mM NaCl, 0.5 mM EDTA, 50% glycerol) at a density of 1.5 x 10$^7$ nuclei/ 50 μl, frozen on dry ice and stored at -80°C until required. In our laboratory, nuclei over 2 years old have been shown to be transcriptionally active with a loss of less than 10% total activity upon storage.

*In vitro* transcription reactions were performed using 1.5 x 10$^7$ nuclei in 150 μl of 100 mM Tris-HCl pH7.9 containing 50 mM NaCl, 4 mM $MgCl_2$, 0.5 mM $MnCl_2$, 4 mM EDTA, 1 mM DTT, 50 μM phenylmethylsulfonyl fluoride, 40% glycerol, 1 U/μl RNasin, 1 mM each of ATP, CTP and GTP, 2 μM UTP, and 100 μCi [α-$^{32}$P]UTP (3000 Ci/ mmol) at 26°C for 10 min. The reaction was stopped by adding 750 μl of 0.5% $NaDodSO_4$, 10 μg RNA transcripts were isolated and purified by the method of Wagner *et al.* (1983). Extension of the incorporation of [$^{32}$P]UTP into total RNA was performed by the TCA precipitation method of Marzluff (1978). The rate of incorporation of [$^{32}$P]UTP into RNA by nuclei isolated from hepatocyte cultures untreated and phenobarbital-treated has been shown to be linear for 15 min. The procedures for binding specific recombinant plasmid cDNA clones to nitrocellulose filters, for hybridisation of $^{32}$P-RNA to the filter-bound target cDNA clones, and washing filters following hybridisation have been described previously by Hansen *et al.* (1989). In these present studies, 2 μg/slot of plasmid cDNA clones were employed and were shown to be saturating for each of the $^{32}$P-RNA transcripts examined under basal and phenobarbital-induced conditions. Exposure times were adjusted so that signals were in the linear range of the film used (Kodak X-OMAT AR). Autoradiographs were quantified by densitometry.

*RNA isolation and Northern analysis* - Total RNA was isolated from 2 x 10$^7$ chick embryo primary hepatocytes by the method of Chomczynski and Sacchi (1987). Yields were typically 80-100 μg. Northern blot analysis using 20 μg of total RNA per track was carried out exactly as previously described by Hansen *et al.* (1989). Chick cDNA clones being radiolabelled by nick-translation using [α-$^{32}$P]dATP and [α-$^{32}$P]dCTP. Exposure times were adjusted so that the signals were within the linear range of the film used (Konica - Medical A). Autoradiographs were quantified by densitometry.