MOLECULAR GENETIC ANALYSIS OF HEPATIC CYTOCHROME P-450s

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My heart aches and a drowsy numbness pains my sense,
as though of hemlock I had drunk,
or emptied some dull opiate to the drains
one minute past and "Leith-Walk" had sunk;

(Ode to a Grecian Urn - Keats)

DECLARATION

I declare

(a) that this thesis has been composed by myself, and

(b) that the work is either my own or the worker/ author involved is clearly stated

ABSTRACT

The genetic analysis and isolation of cDNA clones for a constitutively expressed hepatic cytochrome P-450 (P-450), PB-1 (P450-2C), which is involved in chemical detoxification is described for mice and humans. A rat PB-1 cDNA was used to isolate a full length human PB-1 cDNA clone from a liver cDNA library. The human cDNA clone, pHL5,5, has an insert of approximately 2 kilobases. Sequence analysis proved it to be full length encoding a protein of 490 amino acids 70% of which show identity with rat and rabbit PB-1 forms. The N-terminal sequence derived from pHL5,5 matches a human P-450 involved in the polymorphic oxidative metabolism of the drug mephenytoin (an anti-convulsant). Southern blot analysis indicated there are a minimum of 2 to 3 PB-1 genes in human; these were mapped to human chromosome 10q24.2 by a combination of somatic cell genetics and *in situ* hybridization. This locus was designated P450C2C.

A companion study was initiated in mice with the rat PB-1 cDNA clone, pTF-1. Southern blot analysis of rat and mouse genomic DNA detected 7-8 PB-1 genes in rodents which by a combination of mapping in recombinant inbred mouse strains, somatic cell hybrids and *in situ* hybridization with mouse PB-1 cDNA clones were shown to be clustered and mapped to the D1/D2 region of mouse chromosome 19. This locus was termed P450-2C. This region of mouse chromosome 19 contains a number of markers which are also on the long arm of human chromosome 10, indicating that these chromosomes contain conserved linkage groups. Three DNA haplotypes, termed P450-2C^{a}, P450-2C^{b} and P450-2C^{c}, were observed in recombinant inbred mouse strains. P450-2C showed tight linkage with constitutive aryl hydrocarbon

hydroxylase (AHH) activity. *In vitro* evidence suggests that this activity is also determined by human PB-1 P-450. Thus this locus could be involved in determining the metabolic fate of polycyclic aromatic hydrocarbons which are potential carcinogens or mutagens. The regulation of a number of P-450s, including PB-1, was investigated in rats and mice and a number of these were found to be regulated by pituitary factors in both species. A number of species differences in response to glucocorticoids and pituitary factors were also noted. The developmental regulation and tissue specific expression of P-450s and a number of other liver specific genes were also investigated. In general it was found that the foetal liver and yolk sac, two tissues of different embryonic lineage, shared a similar pattern of expression but are independently programmed for expression of the same set of serum protein genes.

Sequence comparison of rabbit, rat and human PB-1 cDNA clones was unable to identify orthologous forms between these species. The data suggests that the members of this family have become sufficiently diverged between these species that they no longer share common endogenous function in relation to the metabolism of steroids. This is discussed in relation to the rodent as a model for chemical detoxification of foreign compounds in humans.

ABBREVIATIONS

A	Adenine
AAF	2-acetylamino fluorene
AHH	Aryl Hydrocarbon Hydroxylase
ATP	Adenosine triphosphate
B[a]P	Benzo[a]pyrene
bp	base pairs
BNF	β-napthoflavone
C	Cytosine
CHO	Chinese hamster ovary cells
cM	Centimorgans
CsCl	Cesium chloride
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanidine triphosphate
dTTP	Deoxythymidine triphosphate
ddNTP	Dideoxynucleotide triphosphate
DBA	Di-benz[a,h]anthracene
DEAE	Diethylaminoethyl
DEPC	Diethylpyrocarbonate
DEX	Dexamethazone
DMBA	Dimethylbenzenthracene
DNA	Deoxynucleic acid
ds	Double stranded
EDTA	Ethylenediaminetetracetic acid
EtBr	Ethidium Bromide
G	Guanine
GH	Growth Hormone

GHCl	Guanidinium Hydrochloride
HEPES	N-2-Hydroyethylpiperazine-N-2-ethanesulphonic acid
Hx	Hypophysectomy
IP	Intraperitoneally
IPTG	Isopropyl-1-thio-β-D-galactoside
K	1000 revolutions per minute
kb	Kilobase pairs
kD	Kilodaltons
3MC	3-methylcholanthrene
mins	Minutes
3MMAB	3-methyl-4-methylaminobenzene
MOPs	3-[N-Morpholino]propanesulphonic acid
mRNA	Messenger ribonucleic acid
P-450	Cytochrome P-450
PB	Phenobarbital
PCN	Pregnenolone-16α-carbonitrile
O.D.	Optical density
RFLP	Restriction fragment length polymorphism
RFLV	Restriction fragment length variant
RI	Recombinant inbred
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDP	Strain distribution pattern
SDS	Sodium dodecyl sulphate
SM	Storage medium
ss	Single Stranded
T	Thymidine
TCA	Trichloroacetic acid

TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TE	10mM Tris pH7.5, 1mM EDTA
TEMED	N,N,N',N'-tetramethylene diamine
Tris	Tris (hydroxymethyl) aminomethan
UV	Ultraviolet
vol	Volume
VYS	Visceral yolk sac
XGal	5-bromo-4-chloro-3-indoyl-β-D-galactoside

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CHAPTER 1

INTRODUCTION

1.1. General background to drug detoxification

We live in a chemically challenging environment. The number of different products arising in nature and those from human synthetic capability via the agro-chemical industrial complex is in the order of hundreds of thousands. The diversity of human exposure can be illustrated by the content of one cigarette which contains over 1200 different compounds (Steadman, 1968). Western life style can be characterized by fifty years of good living before the incipient effects of chronic toxic exposure are felt and seen. Over a lifetime we absorb, imbibe, ingest and synthesize a wide range of potential toxins.

The introduction of new compounds into the environment has led to the establishment of a causal relationship between specific toxins and their pathological effects. The incidence of these effects can be estimated by a comparison of epidemiologic and pathological data from different countries. On this basis 70% of all cancers have been attributed to environmental factors (Doll and Peto, 1981). For example Japan has a higher incidence of stomach cancer and a lower incidence of intestinal cancer than in the United States (Haenszel and Kurihara, 1968). The cancer pattern of Japanese immigrants in the United States however reflects the normal U.S. incidence, which indicates that a non-genetic component is involved in cancer initiation. Such studies indicate that most of the major forms of cancer are avoidable. The use of tobacco is associated with 30% of all cancer deaths in the United States and Britain (Doll and Peto, 1981; Wynder and Gori, 1977) and probably responsible for more chronic effects such as emphysema and heart damage. Diet has been suggested to be another major cause of cancer (Ames, 1983; Ames et al 1987; Doll and Peto, 1981). These studies

suggest that a change from excess consumption of fat-rich products and alcohol to fibre-rich cereals, vegetables and fruits would have a beneficial effect on cancer incidence. The contribution of diet indicates that the problem of foreign compounds or xenobiotics in the environment is not a new one.

The type of compounds that are harmful to living organisms are those that cannot be incoporated into normal biological processes. These foreign compounds are termed xenobiotics. The exposure to xenobiotics during biological development has undoubtedly led to the evolution of a group of complex mechanisms which provide protection against chemical challenge. These include a number of anatomical, physiological and enzymatic systems that provide a means of resistance to harmful xenobiotics and have an obvious selective advantage. Skin, for example, acts as a major barrier to the outside environment. Most foreign compounds are hydrophobic and as such they would remain in the lipophilic environment of a multicellular organism indefinitely unless there was a mechanism for their excretion. In mammals the major routes for the elimination of waste products and other redundant molecules involves the aqueous solutions urine and bile. A number of enzymatic mechanisms have evolved to convert a plethora of structurally diverse foreign compounds to more polar derivatives and therefore make them suitable for rapid elimination (Jakoby, 1980). The liver is the major (but not the only) organ of detoxification where enzymes catalysing the oxidation, reduction, hydrolysis, rearrangement and conjugation of a broad range of foreign compounds are located.

Understanding the mechanisms of xenobiotic detoxification is a major goal of research into cancer, heart disease and aging. The major contribution to cell damage is the formation of chemically

reactive intermediates and oxygen radicals (Miller, 1970; Heidelberger, 1975) which can form covalent adducts with cell macromolecules (Miller, 1951). There are many non-enzymatic substances which act as anti-oxidants in the cell (Ames, 1983; Temple and Basu, 1987) which include small molecules in the diet including glutathione which acts as a nucleophilic acceptor for harmful electrophiles (Jakoby and Habig, 1980). Paradoxically as a result of the multiplicity and nature of biochemical reactions involved in foreign compound metabolism, certain compounds are formed which are pharmacologically and toxicologically more potent than the parent substrate. Many carcinogens are metabolized to chemically reactive ultimate carcinogens before they can exert a harmful effect (Rinkus and Legator, 1979; Singer and Kusmicrek, 1982).

The major enzyme system in the initial conversion of hydrophobic substances to hydrophilic products in mammalian cells is the cytochrome P-450 (P-450) monooxygenase system. Subsequent reactions further enhance the polarity of the xenobiotic which leads to its ultimate excretion. The initial oxidation of a foreign compound can be mediated by several different P-450s resulting in several different metabolic pathways. The ultimate balance between these pathways will determine the fate of a foreign compound. For example, there are competing pathways in the metabolism of benze (a)pyrene (B[a]P) (Nebert and Jenson, 1979) which will determine its potential as a carcinogen. Cytochrome P-450s are characterised by their broad substrate specificity. As a consequence this leads to low turnover rates and relatively poor substrate affinities. This can be compensated by high substrate concentrations, especially in the liver, the major site of this enzyme system (Astrom and DePierre, 1986; Waxman, 1986). The multiplicity of the P-450

system and the broad spectrum of enzymatic activities begs the question what is the function of these enzymes in the absence of xenobiotic challenge, i.e. do they have a natural substrate?

The epidemiological studies indicate that there is a non-genetic component in susceptibility to environmental cancers. There is strong evidence that genetically determined differences in drug metabolizing enzymes will also have a profound effect in determining a hosts response to xenobiotic challenge (Kalow et al, 1985). For example adverse drug reactions in humans can be associated with genetically determined differences in oxidative metabolism (Mahgoub et al, 1977). A fruitful line of research would be to identify the genetic factors which regulate the expression of drug metabolizing enzymes especially the cytochrome P-450 monooxygenase system whose central role in the initial conversion of xenobiotic compounds is undisputed.

1.2. Drug metabolism

The study of drug metabolizing enzymes has diverse roots, these being in pharmacology, cancer, chemical toxicity and mutation research. These research areas are all related to different aspects of the metabolism of foreign compounds. The consequence of this broad research is that the literature on drug metabolizing enzymes is eclectic to read and cover, with a certain inbuilt redundancy in many of the published papers. In addition, drug metabolising enzymes can be involved in many diverse physiological processes ranging from reproductive physiology (Gustafsson et al, 1983) to mechanisms of oxygen stress (Gonder et al, 1985). One way to illustrate the diversification of drug metabolising enzymes is in relation to chemical carcinogenesis.

1.2.1. Chemical carcinogenesis and toxins

Historically the area of chemical carcinogenesis can be divided into five periods. The hypotheses formulated during these periods formed the basis of current concepts in this area. The first four periods covering the last 250 years up to about 1950 were concerned with the nature of chemical toxicity and carcinogenesis, the last period deals with the characterization of the enzymes involved in these processes. The last period will be covered in its own sections.

The first crucial observation was the causal relationship between chemical exposure and cancer. This showed that environmental chemicals can be harmful to living organisms. There were early examples of etiological cancer agents, notably snuff. Hill (1761) published <u>Cautions against the immoderate use of Snuff</u> because of the fatal nasal polypusses (cancers) associated with its excessive use. The irritation associated with the growths led Hill to speculate as to whether snuff was a causative agent or accentuating an underlying trend (Boutwell, 1974). Apparently, the practice of tobacco smoking in the seventeenth century was as subversive as illegal drug use is today (Redmond, 1970). Another early report was by Percival Potts on the unusually high incidence of scrotal cancer in London chimney sweeps (Potts, 1775, quoted in Miller, 1970). This was subsequently attributed to their exposure to tars and soots. Chimney sweeps from Yorkshire and Europe had a lower incidence of this type of cancer (when compared to London chimney sweeps) because of better hygiene and use of protective clothing (Butlin, 1892, quoted in Miller, 1978). Subsequent to these studies the observations that industrial exposure to certain chemicals leads to an increase in specific types of cancer was the

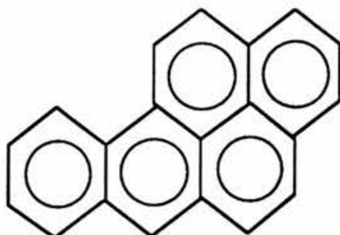
basis for establishing the causal relationship between the two. This led to the identification of specific groups of workers at risk from exposure to particular compounds (reviewed by Wynder and Gori, 1977). For example, an increased risk of bladder cancer was found in workers in the asphalt, coal and pitch, dye stuffs, paint and leather and shoe industries.

The second period of drug metabolism and cancer (1900- 1930s) can be associated with research into the nature of this relationship. Since human neoplasia cannot be experimentally induced or manipulated, a variety of animal tumour models were developed with rabbits, dogs, mice and rats. Initially, these model systems were usually based on topical administration of the suspected complex chemical mixtures and extracts with subsequent analysis of the extent of tumour formation. The first reports on the induction of skin cancer in rabbits and mice by coal tar was achieved by Yamagiwa and Ichikawa (1915 and 1918). Advancements in chemical techniques then allowed the fractionation and identification of the chemical components responsible. It was noted that tars and oils contained compounds with unusual fluorescence spectra which suggested the active agents were aromatic hydrocarbons (Hieger, 1930). The first synthetic carcinogen dibenz(a,h,)anthracene (DBA) (Fig. 1.1) was identified in 1930 (Kennaway and Hieger, 1930). It was tested because of its fluorescence spectrum which was similar to those observed in coal and tar products. B[a]P was first isolated from coal and shown to be carcinogenic in 1933 (Cook et al, 1933). New carcinogens are continuously being discovered from exposure of humans to various compounds. To date over 500 carcinogens have been identified (Rinkus and Legator, 1979) unequivocally. In addition the U.S.

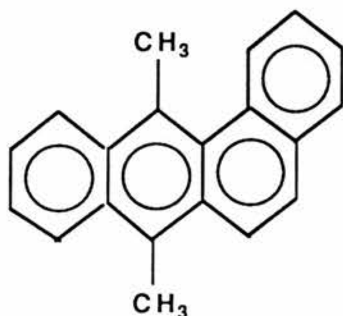
FIGURE 1.1.

Chemical structures of known carcinogens

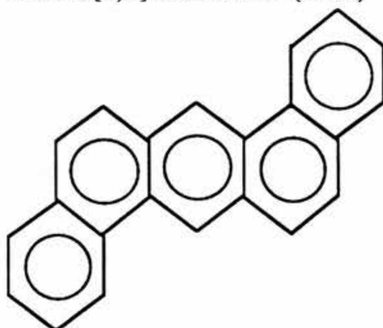
1. Benzo[a]pyrene (B[a]P)



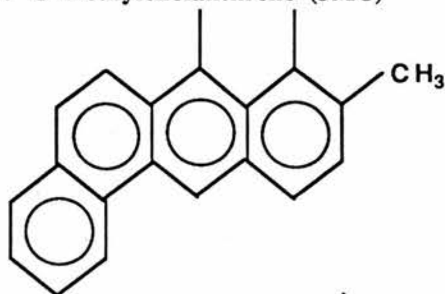
2. 7,12 Dimethylbenz[a]anthracene (DMBA)



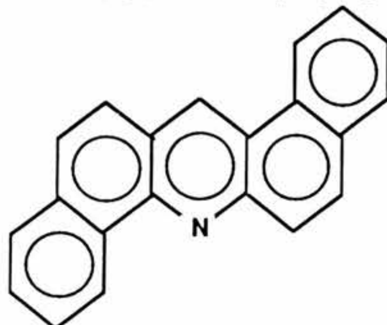
3. Dibenz[a,h]anthracene (DBA)



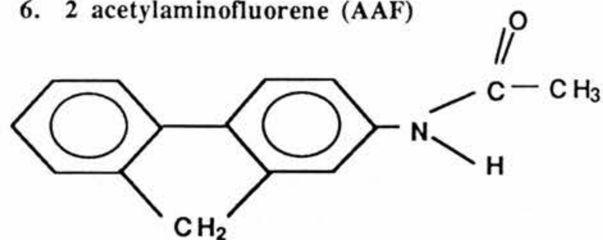
4. 3 methylcholanthrene (3MC)



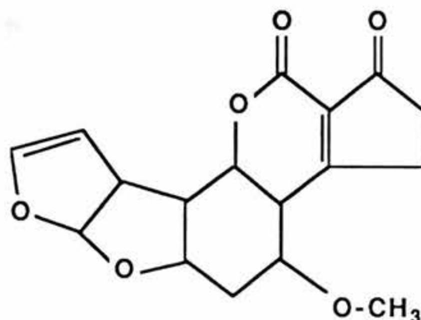
5. Dibenz[a,h]acridine (DB[a,h]AC)



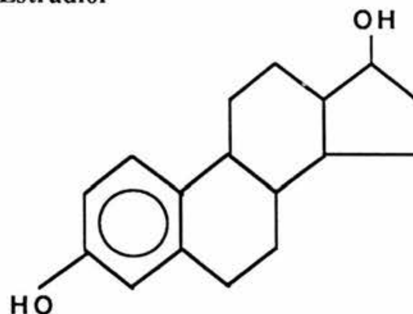
6. 2 acetylaminofluorene (AAF)



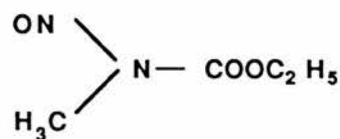
7. Aflatoxin B_1 (AFB_1)



8. Estradiol



9. N-Nitroso-N-methyl urea (NMU)



10. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)

National Cancer Institute lists nearly a 1000 suspected carcinogens (Technical Resources Inc, 1986).

The third period of research in the late 1930s was characterized by attempts to relate chemical structure to carcinogenic potency in order to devise models for chemical carcinogenesis. It was thought that fluorescence could be used as a general indicator of carcinogenic activity but this correlation did not hold (Hieger, 1930; Auerbach, 1976). Such attempts were unsuccessful as more and more diverse compounds were implicated as carcinogens and mutagens (Miller, 1978). One of the reasons for the diversity became apparent with the discovery of the multistage process of carcinogenesis (Berenblum, 1941). In these studies the development of mouse skin tumours could be delineated into three events; initiation, promotion and progression. Initiation could be affected by a single low dose of carcinogen such as an alkylating agent or polycyclic aromatic hydrocarbon (PAH) which may cause changes in cells which, by themselves, will not cause the development of a tumour. The second step, promotion, occurs over a period of weeks and its early stages are reversible by removal of the promoter (Boutwell, 1974) until commitment to a malignant tumour. The classic promoting agent is croton oil (Berenblum, 1941) whose active agent is a phorbol ester (Hecker, 1971). A promoter is an incomplete carcinogen and can only promote the carcinogenic process begun by an initiating agent which can be a chemical or a virus (Balmain and Pagnell1, 1983; Brown et al, 1986).

The fourth period (1940-1950s) was the realization that many chemical carcinogens and probably mutagens required metabolic activation before their harmful effects are manifested. For example it was shown that fluorescent derivatives of B[a]P were covalently

linked to mouse skin proteins after topical administration to live animals but this did not occur on dead skin (Miller, 1951). Thus the search for the reactive products of such a process and whether their formation had an enzymatic basis was initiated. The acceptance of the Watson-Crick model for the structure of the genetic material, DNA (Watson and Crick, 1953) had an obvious revolutionary effect on this research area because it provided a target for chemical carcinogens and mutagens. Previous concepts of the gene included an assortment of protein, protein-DNA complexes and nuclear versus plasma genes (Miller, 1951; Judson, 1979).

1.2.2. Mechanisms of chemical carcinogenesis and detection

The administration of many carcinogens to laboratory animals led to the identification of covalent DNA, RNA and protein adducts (Lawley and Brooks, 1963; Gelboin, 1968; Weinstein *et al*, 1976; Heidelberger, 1975; Ashurst *et al*, 1983; Liehr *et al*, 1987; Bradlow *et al*, 1986). In general, many carcinogens require metabolic activation to reactive intermediates before adducts are formed. Once these intermediates have been identified they can be synthesized *in vitro* and used as direct carcinogenic and mutagenic agents (Miller *et al*, 1961). In this case metabolism of xenobiotics can be regarded as a black box which identifies the ultimate carcinogens *in vivo*. Much of the work on detection of important intermediates in metabolic activation can be summarized as follows (Miller, 1970; Miller, 1978; Heidelberger, 1975):

1) Chemical carcinogens that are normally not chemically reactive must be converted to a chemically reactive form.
2) The activated metabolite is an electrophilic agent.

3) This activated metabolite reacts with nucleophilic groups in cellular macromolecules to initiate carcinogenesis.

The requirement for metabolic activation was used in an innovative step by Ames who incorporated rat liver fractions (which contain the activating enzymes) in a mutagenesis assay based on Salmonella Typhimurium (Ames et al, 1973a; Ames et al, 1973b; Ames et al, 1975). This assay system allowed the identification of potential carcinogens and the role of metabolic activation. In this assay revertants in strains of S. Typhimurium were selected that had defined mutations in the histidine operon. These strains were also deficient in DNA repair and lipopolysaccharide synthesis which increased the sensitivity of the assay by preventing the repair of DNA adducts and also by facilitating drug transport into the bacterium. Reversions of his^- to his^+ were used to detect mutagens in the presence of mammalian microsomes. Using this test it was shown that up to 95% of carcinogens are mutagens (McCann et al, 1975; Rinkus and Legator, 1979). However not all mutagens are carcinogen (Nagao and Sugimura, 1978) and some carcinogens are not genotoxic (Warren et al, 1980). These tests can also be used to measure inactivation of direct acting carcinogens and for studying genetic and tissue specific differences in carcinogenic activation by using samples from different sources (Hutton et al, 1979). The assay can also be used to assess the role of specific activating or deactivating enzymes. For example the mutagenic potential of B[a]P is altered when C57BL/6 mice are treated with 3 methylcholanthrene ((3MC) (Fig. 1.1.) prior to microsome preparation. The net number of revertants per plate increases from 57 to 182 in the presence of control and 3MC treated C57BL/6 microsomes respectively (Hutton et al, 1979).

The combination of carcinogen and mutagenicity tests allows some insight into the mechanism of actions of potentially harmful compounds to be observed. Cancer results from diverse types of damage to genetic material which can involve recessive and dominant mutations (Bishop, 1987), large scale rearrangements of DNA (Yunis, 1983) and point mutations (Barbacid, 1986). The idea that direct mutagenesis of DNA leads to neoplasia derives support from the effect of carcinogen/ DNA adducts on DNA replication, repair and transcription in vitro (Waring, 1981) and the activation of transforming genes in vivo by carcinogenic agents (Barbacid, 1986). A possible relationship between carcinogens and chromosomal defects and certain neoplasias involving deletions or trisomy (Mitelman et al, 1981) has been proposed. In a study of patients with acute non-lymphocytic leukemia (ANLL) 75% of patients with a history of exposure to mutagens and carcinogens had chromosomal abnormalities as compared to 32% of a non-exposed group with ANLL. A specific range of defects associated with chromosomes 5, 7 and 8 was found in the exposed group (Mitelman et al, 1981). This group included of people exposed to solvents (chemical/engineering/print workers), insecticides (farmers) and petro products (bus/truck drivers/petroleum workers). It was concluded that the karyotype of leukemia cells was influenced by the pattern of exposure. This type of pattern was also seen in patients with secondary ANLL resulting from therapies involving chemo- and radiation therapy for a previous malignancy (Rowley et al, 1981). Such changes may provide markers for ANLL due to chemical exposure.

Before considering direct acting or metabolically activated carcinogens in more detail it is worth noting a class of compounds which does not obey the rules for metabolic mutation. The

administration of clofibrate, an hypolidemic agent caused peroxisome proliferation in the parenchymal cells of the liver (Hess *et al*, 1965). The compound was demonstrated to cause hepatocarcinogenesis (Reddy *et al*, 1980). However it was negative on *Salmonella* mutagenesis assays and did not appear to have genotoxic effects (Warren *et al*, 1980). When tested *in vivo* and *in vitro* for DNA adduct formation by P^{32} labelling and chromatography no DNA adducts were found on exposure of rat liver to clofibrate. These assays were extremely sensitive and would detect 1 adduct/10^{10} nucleotides (Gupta *et al*, 1985). By these criteria cloribrate is not a chemical carcinogen according to the Millers (Miller, 1970; Miller, 1978). Clofibrate and other classes of compounds are thought to initiate hepatocarcinogenesis by virtue of their ability to cause peroxisome proliferation. Several functions are associated with peroxisomes which include respiration, glycerolipid and fatty acid metabolism, alcohol oxidation and peroxidation (Tolbert, 1981). Increased levels of H_2O_2 generated with peroxisomes are normally metabolised by catalase and other peroxidases. However, leakage of H_2O_2 from peroxisomes would have harmful effects on the rest of the cell. The subsequent formation of oxygen radicals can give rise to genotoxic effects and the peroxidation of lipid membranes (Goel *et al*, 1986). Clofibric acid is no longer used as a drug, however other classes of peroxisome proliferators are used extensively in the plastics industry and may thus pose a potential environmental risk (Rao and Reddy, 1987). The tissue specificity of the response has been postulated to be due to lipid receptor mediated mechanisms which induces a pleiotrophic response in liver (Rao and Reddy, 1987).

2-Acetylaminofluorene (AAF) is a potent genotoxic hepato-

carcinogen in rats and mice (Miller et al, 1961) which is eventually converted to a reactive intermediate N acetoxyaminofluorene (N-Aco-AF) and N-hydroxyacetylaminofluorene (N-OH-AF) (Bartsch et al, 1972). The major effect of these derivates is to mutate DNA. N-Aco-AF has been shown to bind DNA *in vitro* in a covalent manner (Drinkwater et al, 1978). Coliphage ØX174 DNA modified by acteyl-aminofluorene derivatives exhibit premature termination by DNA polymerases at the site of modification (Moore et al, 1981). Product analysis of modified DNA with N-Aco-AF shows that the majority of the interactive products are at guanine residues (Lutgerink et al, 1984). These adducts also affect transcription by a T7 RNA polymerase on a plasmid containing T7 promoter (Nath et al, 1987), the mechanism of which is postulated to be due to a slowing in elongation rate by T7 polymerase.

Protooncogenes have been identified as genetic targets that play a key role in carcinogenesis (Varmus, 1984). Transfection assays based on immortalized but non-transformed cells can detect activated protooncogenes, particularly members of the *ras* family from a variety of human tumours (Barbacid, 1986). The *ras* genes are known to acquire their transforming properties by point mutation at 12th and 61st codons. The *ras* protooncogenes code for proteins called p21 of 188 or 189 amino acid residues. Genetic damage appears to release the biochemical activities of certain proto-oncogenes from allosteric controls. The *ras* protein, p21, binds GDP and GTP and has GTPase activity. This enzymatic activity is reduced in mutant p21 proteins coded by activated oncogenes (Colby et al, 1986) which may result in sustained levels of otherwise normal bio-chemical activities. Mouse hepatomas induced by N-OH-AF have been shown to have activated c Ha-*ras* protooncogenes with mutations at

the 61st codon (Wiseman et al, 1986). Selective oligonucleotide hybridization demonstrated a CG to AT transition at the first position of the 61st codon which would cause a glutamine to lysine amino acid change in the polypeptide. This type of mutation would agree with the preference of AAF derivatives for deoxyguanosine (Lutgerink et al, 1984; Singer and Kusmierek, 1982).

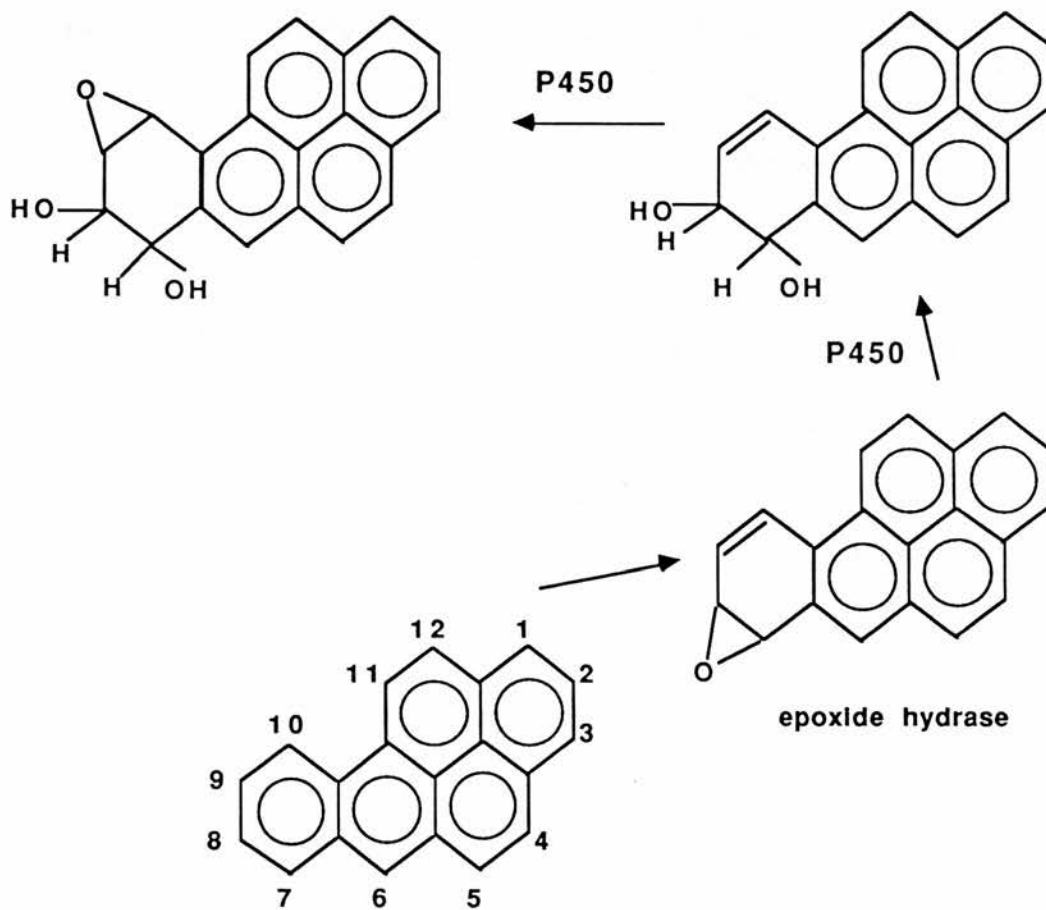
Direct mutation of c Ha-*ras* in rats treated with alkylating agents can produce mammary tumours, for example N-nitroso-N-methyl-urea (NMU) mutated the rat c Ha-*ras* at codon 12 causing CG to AT transition. Although NMU does not require metabolic activation it does show a restricted tissue specificity in the site of the tumours it induces (Heidelberger, 1975) which is indicative that mutagenesis by NMU is not sufficient for neoplasia to be initiated. Other factors such as requirement for target cells to be in a proliferative state, DNA repair and detoxification enzymes are also postulated to be involved (Barbacid, 1986).

Polycyclic hydrocarbons have also been shown to be directly involved in animal carcinogenesis models. The binding of B[a]P to DNA was completely dependant on the presence of liver microsomes (Gelboin, 1968). B[a]Ps can be metabolized to a variety of products with different carcinogenic potential (Nebert and Jensen, 1979). The non-K region dihydrodiols of 7 methylbenz(a)anthracene, 7, 12, dimethyl benze(a)-anthracene and B[a]P (Fig. 1.2) have been shown to transform a mouse fibroblast cell line at high efficiency, approximately 20 foci/10^3 surviving cells (Marquardt et al, 1976). These experiments also showed that the parent compounds had negligible transforming capabilities, 0.4-2 foci/10^3 surviving cells. Modified derivatives at other positions had insignificant transforming capabilities indicating that these metabolites are not

FIGURE 1.2.

Metabolic activation of Benzo[a]pyrenes. The 7,8 oxide is predominantly formed by cytochrome P-450s following diol formation by epoxide hydrase, the 7,8 and 9,10-epoxide is also formed by cytochrome P-450. The diol epoxides are more active, forming adducts and causing malignant transformation of mouse fibroblasts (Nebert and Jensen, 1979; Marquardt et al, 1976). Hydroxylation at 1,3,7 and 9 positions leads to non-active metabolites which are excreted following sulphate conjugation.

Metabolic activation of Benzo[a]pyrenes.



involved in cancer initiation. B[a]P epoxides have also been found to bind DNA and RNA covalently (Drinkwater et al, 1978; Weinstein et al, 1976) and cause premature termination by T7 RNA polymerase on BP-DNA adducts (Nath et al, 1987). For example, B[a]P diolepoxide DNA adducts have been found in peripheral blood lymphocytes of risk category coke workers (Harris et al, 1985). In in vitro experiments with a cloned chicken adult β-globin gene, B[a]P diolepoxide will form adducts in a non-random manner to a region 300bp immediately 5' to the RNA cap site (Boles and Hogan, 1984). This could conceivably cause a down regulation of RNA transcription. The major adduct formed with B[a]P diolepoxide is at the N2 position of guanine (Jeffrey et al, 1977; Ashurst et al, 1983). Mutation studies on shuttle vectors containing these adducts that are replicated in human cells show that of 61 base substitutions 45 were GC to TA transitions in the marker gene (Yang et al, 1987). In contrast, dimethylbenz(a)anthracene (DMBA) binding to DNA in mouse embryo cell cultures results in extensive binding to both adenine and guanine residues (Dipple et al, 1983. This could explain the reduced potency of B[a]P in comparison to DMBA in the two stage mouse skin carcinoma model (Bizub et al, 1986; Balmain and Pagnell, 1983). Mouse skin carcinomas initiated with DMBA, B[a]P and dibenz(c,h)acridine (DB(c,h)ACR) and promoted by phorbol-12 myristate 13-acetate shown differences in the ability to mutate the c Ha-ras oncogene and the ability of DNA from the resulting papillomas to transform NIH3T3 cells (Bizub et al, 1986). Both DMBA and DB(c,h)ACR induce an A to T transversion at codon 61 of c Ha-ras in papillomas which efficiently transform NIH3T3 cells. Although B[a]P induced skin carcinomas, these did not shown any mutation at codon 61 of c Ha-ras in the tumour nor was the tumour DNA able to

transform NIH3T3 cells. Thus the inability of B[a]P diolexpoide to mutate deoxyadenosine would decrease its carcinogenic potential in this experimental system. Similarly, when the alkylating agent N-methyl-N'-nitro-N-nitrosguanidine (MNNG) is used as an initiating agent in the mouse skin tumour model it shows different mutational specificity to DMBA (Quintilla et al, 1986). MNNG binds deoxyguanosine preferentially (Lawley and Brooks, 1963; Auerbach, 1974). Therefore the genotoxic specificity of a carcinogen can effect the type of cancer produced in combination with other factors such as cell proliferation and metabolism. In the majority of cases DNA/carcinogen adducts can be removed by alkyl-nucleotide transferases (Sekigucki and Nakabeppa, 1987) and excision repair enzymes (Walker, 1984). Mutations can be generated during the repair process or if unrepaired sites serve as templates for DNA replication (Drinkwater et al, 1978; Eadie et al, 1984; Loechler et al, 1984).

Carcinogen metabolism will also be a factor in the site of tumour formation. Cell specificity can be demonstrated in the activation of foreign compounds. Aflotoxin B_1 (AFB_1) and B[a]P are both metabolized by rat liver cells to water soluble products but only AFB_1 is positive on the V79 chinese hamster cell line mutation assay (Langenbock et al, 1978). When rat embryo fibroblasts are treated with these compounds only B[a]P is metabolized to a water soluble mutagen product on V79 assays. Other metabolic factors will inhibit skin tumourgenesis in mice by PAH. For example, prior treatment of mice with 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) will reduce the frequency of papilloma formation initiated by DMBA, B[a]P, 3MC and B[a]P diolepoxide. The mechanism of inhibition by TCDD is unknown but this compound induces many drug metabolizing

enzymes. For example a 21 fold increase in arylhydrocarbon hydroxylase (AHH) activity is seen in mouse epidermis on treatment with TCDD (DiGiovanni et al, 1980). This increases the formation of mutagenic products from PAHs but the additional biological affects of TCDD appear to protect the mouse skin from the carcinogenic potential of these compounds.

The formation of DNA adducts and resulting mutation is not a property unique to foreign compounds. It has been known for many years that estrogens have an etiological role in the development of breast cancer (Wynder and Gori, 1977; Doll and Peto, 1981). It has been noted by certain workers that a high proportion of women with breast cancer have an abnormal oxidative metabolism of estrogen which leads to the formation of high circulating levels of 16α-hydroxy metabolites (Schneider et al, 1982. This metabolite is capable of forming adducts with large macromolecules (Yu and Fishman, 1985; Bradlow et al, 1986). A model system for estrogen induced renal carcinoma in Syrian hamster identified DNA adducts in the kidney when natural 17β-estradiol and several synthetic steriod estrogens were used. Chromatographic analysis showed that these compounds induced the same modification of DNA (Liehr et al, 1986). These experiments led to the conclusion that the estrogens induced the binding of some unknown endogenous compound in target tissue. The site of the DNA adducts was subsequently localized to the renal cortex (Liehr et al, 1987). As this is also the site for cytochrome P-450 enzymes it was postulated that estrogen metabolism by cytochrome P-450 enzymes would lead to a reactive estrogen metabolite. Estrogen dependant human breast cancer has been correlated with the amplification of the HER-2/*neu* protooncogene (Slamon et al, 1987). This may be the target for the estrogen

derived metabolites which induces breat cancer. The amplification is postulated to lead to an increased tyrosine kinase activity encoded by the HER-2/neu protein (Bishop, 1987). In contrast the rat homologue to HER-2/*neu* was isolated from neuro- and glioblastomas induced in BDIX rats given a single dose of ethyl-nitrosoured (ENU). HER-2/*neu* was the transformating gene on NIH3T3 assay and was found to have an T to A transversion in the transmembrane region of the protein (Bargmann *et al*, 1986). In this case mutation rather than overexpression is associated with the transformed phenotype.

A multitude of natural carcinogens can be found in our diet including AFB_1 found in peanut butter, phosphorelins in celery and hydrazines in mushrooms (Ames, 1983). Derivatives of 4 hydroxy-methyl phenyl hydrazine produces lung and blood vessel tumour in mice (Toth *et al*, 1978). The metabolism of this compound to its reactive intermediate is thought to be via the mixed function oxidase system in liver and lung (Lawson and Chanhan, 1985; Lawson, 1987).

In addition to carcinogens, compounds which inhibit carcinogenesis have also been identified in our diet (Ames, 1983). Natural anticarcinogens in our diet include diallyl sulphide from garlic oil (Hayes *et al*, 1987) and β carotene from carrots (Temple and Basu, 1987). Diallyl sulphide inhibited the necrotic effects of an hepatocarcinogen, 1,2,dimethylhydrazine, by reducing its macro-molecular binding in cultured liver cells. Onion and garlic oil also have anti-promoter effects in mouse skin exposed to phorbol esters (Belman, 1985). It is thus becoming recognised that in addition to harmful compounds in the environment there are many with beneficial anti-carcinogenic and anti-promoting activities (Ames,

1983). The basis for the protective effects of diet are now being investigated with model systems originally developed to detect harmful compounds in the environment. Prevention is always better than cure!

1.3. The role of cytochrome P-450s in detoxification

In the fifties it was demonstrated that the liver was a major organ of detoxification and that there was a multiplicity of enzymes involved in this process. Rabbits and rats were shown to have a differential capacity for the liver N-demethylation of narcotics (Axelrod, 1956). This was measured by the rate of formation of formaldehyde from the narcotic and the appearance of the amine derivatives when incubated with liver homogenates. Rabbits were better at the N-demethylation of L-methadone (heroin substitute) and mperidine whereas rats had a higher activity towards morphine. These experiments implied that the enzymes of detoxification were different between species. Alexrod also noted sex differences in the enzymatic demethylation of narcotic drugs in rats. Female rat liver had a decreased metabolizing capacity which could be enhanced by treatment with testosterone. Support for the idea that the liver of a single species contained several different enzymes of detoxication was demonstrated by comparing the metabolizing capacity of rats before and after treatment with B[a]P (Cooney et al, 1959). B[a]P administration increased the rate of its own metabolism, as well as acetanilide, zoxazolamine and 3 methyl-4-methyl aminobenzene (3MMAB) yet decreased the metabolism of the narcotic, mperidine. B[a]P administration had no affect on chlorpromazine metabolism. An important conclusion from this work was that the metabolizing capacity of an organism could be modulated by exposure to a

xenobiotic.

1.3.1. **Cellular localization of xenobiotic metabolism.**

Subcellular fraction studies suggested that the drug metabolizing enzymatic activities resided in the endoplasmic reticulum of the liver (Axelrod, 1956; Cooney et al, 1959; Creaven and Williams, 1963). Studies on the hydroxylation of the corticosteroids also suggested a similar location for this activity in the bovine adrenal gland (Ryan and Engel, 1957). Of interest here was the fact that the 21 hydroxylation of corticosteroids could be inhibited by carbon monoxide but this inhibition was reversible by light. Subsequently bovine adrenal microsomes were found to have a carbon monoxide binding pigment. A corresponding liver carbon monoxide binding pigment was described by Omuro and Sato (1964) which was demonstrated to be a b type cytochrome. They called this protein cytochrome P-450 because the peak of the reduced carbon monoxide difference spectrum was at 450nm. The inhibitory effects of carbon monoxide on the oxidative demethylation of codeine and monomethyl-4-aminopyrine and the hydroxylation of acetanilide by rat liver microsomes was found to be reversed by illumination with light at 450nm (Cooper et al, 1965). The similarity between the photochemical action spectrum and the absorption spectrum suggested that the carbon monoxide binding pigment (P-450) was the terminal oxidase in drug metabolism in mammals. A similar effect was seen for the reversible inhibition of 17-hydroxyprogesterone metabolism by bovine adrenal microsomes (Cooper et al, 1965). When liver microsomes were treated with detergents with the aim of solubilizing cytochrome P-450s a new peak at 420nm appeared in the reduced carbon monoxide spectrum. This form was named P-420 to distinguish it from

the native catalytically active membrane bound form (Omuro and Sato, 1964). Both P-450 and P-420 could be shown to bind substrate by characteristic spectral changes (Imai and Sato, 1966).

1.3.2. Induction of cytochrome P-450s

The cytochrome P-450 system was found to be inducible by a vast variety of lipid soluble drugs (Remmer and Merker, 1965; Ernster and Ornehius, 1965; Sladek and Mannering, 1969). Phenobarbital(PB) and 3 methylcholanthrene (3MC) (now referred to as the classic P-450 inducers) could be shown to have differential effects on microsomal N-demethylating activities. PB and 3MC stimulated the N-demethylation of 3MMAB but only PB stimulated the N-demethylation of ethyl morphine (Sladek and Mannering, 1969). Both these compounds could act synergistically on the stimulation of 3MMAB metabolism. Previously it had been established that the 3MC induced co-spectrum was slightly different to that induced by PB (Alvares et al, 1967) in that the spectral peak was at 448nm as compared to 450nm for PB. This again supported the possibility that different forms of P-450 were present in rat liver. Another P-450 inducer was found to be pregnenolone-16α-carbonitrile (PCN). Following administration of the compound the spectral characteristics in microsomes were similar to PB and control but the substrate specificity was changed (Lu et al, 1972). Marker substrates for PB induction was found to be benzephetamine, for PCN, ethylmorphine and for 3MC benzo[a]pyrene.

1.3.3. Purification and reconstitution of cytochrome P-450 activity

The solubilization, resolution and reconstruction of a microsomal hydroxylase system was first achieved in rabbits (Lu and

Coon, 1968; Lu *et al*, 1969). This consisted of cytochrome P-450, NADPH cytochrome P-450 reductase and lipid factor (Table 1.1). When these fractions were recombined many hydroxylase activities could be regenerated. This was to prove a valuable tool in the analysis of the biotransformation of steroids (Einarsson *et al*, 1973) drugs, carcinogens and a variety of other foreign compounds (Lu and West, 1978). Genetic differences in mouse strains for inducibility of aryl hydrocarbon hydroxylase (AHH) expression was shown to reside in the microsomal fraction (Nebert *et al*, 1973). Additionally NADPH cytochrome c reductase could be substituted between the rat and mouse reconstituted systems indicating that differences between hydroxylation capacities for these two species also resided in the microsomal fraction.

1.4. Characterization of rat liver cytochrome P-450s

It soom became established that cytochrome P-450s play an important role in metabolism of endogenous substrates such as fatty acids and steroids, in the detoxification of many drugs and xeno-biotics, and in the activation of environmental agents to toxic, mutagenic and carcinogenic forms. In order to investigate the broad substrate specificity of the microsomal oxidase system many workers purified the P-450s responsible for specific P-450 mediated reactions. The ultimate objective of these studies was to determine the structural features of P-450s in relation to their function. For example, to identify common structural similarities which may relate to their monooxygenase function, heme and reductase binding sites. To establish the factors which determine substrate specificity, to

Table 1.1 Components of liver microsomal hydroxylation system and their functions

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Cytochrome P-450

Prosthetic group: Iron-Protoporphyrin IX

Function:
1. Oxygen and substrate binding site
2. Monooxygnation of lipophilic substances.
3. Determines substrate specificity of the system: multiple forms with overlapping substrate specificities

---

NADPH - cytochrome c reductase

Prosthetic groups: FMN and FAD

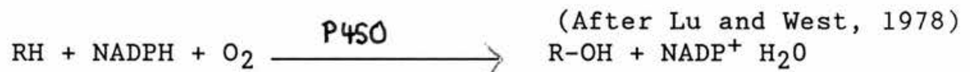
Function: Transfers electrons from NADPH to cytochrome P-450

Phospholipid

Active Component: phosphatidylcholine and possibly other lipids can be replaced by detergents

Function: Allows the formation of a complex between the reductase and the P-450 and therefore provides the environment for electron transfer from the reductase to the P-450.

(After Lu and West, 1978)



$$RH + NADPH + O_2 \xrightarrow{P450} R\text{-}OH + NADP^+ \; H_2O$$

evaluate how many P-450s exist and also whether differences in response to chemical challenge be associated with specific P-450 forms. These are central questions which have resulted in a multidisiplinary approach to investigate the role and nature of P-450s in metabolism.

The main areas of investigation that are currently being followed in this area are (i) the purification of cytochrome P-450 forms and reconstitution in in vitro assays to investigate substrate specificity; (ii) isolation of P-450 cDNAs to investigate the number of cytochrome P-450 forms and the extent of their structural similarities; (iii) the use of genetics to investigate role of P-450s in susceptibility to the chronic effects of chemical toxins and carcinogens.

Before considering these areas of research we have to discuss the complex problem of nomenclature.

1.4.1. Cytochrome P-450 nomenclature

The existence of multiple forms of mammalian cytochrome P-450s (Lu and West, 1980) has led to a variety of different nomenclatures (Astrom and De Pierre, 1986) for an increasing number of forms. It has been estimated that there is a minimum of 12 distinct P-450 forms in untreated rat liver (Waxman, 1986). Previous attempts at a unified nomenclature have depended on physical, biochemical, immunological properties, catalytic specificity and the inducibility of different forms (Lu and West, 1980). These nomenclatures were (are) confusing and outdated as the criteria for differentiating between different P-450 isozymes became well defined. For example the N-demethylation of ethylmorphine was originally associated with PB-

inducible P-450 forms but it was then found PCN induced this activity to a greater extent in female rats (Lu et al, 1972). To some extent the nomenclatures were adapted according to the activity of the P-450 form, for exampled those working on steroid hydroxylation would name the forms according to specific steroid hydroxylations e.g. P-$450_{15\beta}$ (MacGeoch et al, 1984). On the other hand, forms isolated in other laboratories were named in order of isolation e.g. P-450a-k (Ryan et al, 1982; 1984), form i being equivalent to P-$450_{15\beta}$. The inducibility of these isozymes and subsequent changes in enzyme activity can vary between species. For example 3MC which can significantly increase B[a]P activity in rat or mouse liver microsome preparations has no such effect on adult rabbit liver preparations (Atlas et al, 1977).

These deficiencies in nomenclature have been rectified to some extent with the accumulation of protein sequences and genetic data. Therefore, a nomenclature based primarily on sequence data but with reference to their chromosomal location has been proposed for different cytochrome P-450 families (Nebert et al, 1987). Each family is characterized as "P450" followed by an arabic number i.e. P450-1. Individual numbers can have a subscript number or be referred by their trivial name. Related subfamilies are designated by capital letters i.e. P450-2B and P450-2C. Within a given species any one protein is 40-60% similar to a protein in any of the other subfamilies. Subfamilies can have different chromosomal locations. Listed in Table 1.2. is the nomenclature of Nebert et al (1987) for forms presently isolated and the equivalent rat hepatic P-450s isolated by Wolf et al (1986). The rat hepatic forms isolated by different laboratories are also listed (Table 1.3.) and grouped according on the basis of biochemical and physiological

similarities.

Most of the work in this thesis is concerned with the P450-2C family of P-450s. In rat liver at least 5 different forms have been identified (Haniu et al, 1984; Ryan et al, 1982; Waxman, 1986; Wolf et al, 1986). Collectively these isozymes are related to PB-1 of (Wolf et al, 1986; Waxman and Walsh, 1983). The nomenclature used here will be that of Wolf (1986) with reference to Nebert et al (1987). Where no equivalent form exists the trivial and recommended nomenclature will be used. Certain P-450s have specific functions involved in steroid biosynthesis (Jefcoate, 1986). These forms are localized in a variety of steroidogenic tissues including the adrenal gland and are not noted for their drug metabolizing capacity. The nomenclature for these forms are included in Table 1.3.

1.4.2. Purification of cytochrome P-450 proteins

The definitive proof for the existence of multiple cytochrome P-450s came with their purification. Initial studies in this area were hampered by the instability and hydrophobic nature of P-450 proteins. This problem was overcome by the use of glycerol to stabilize the proteins and the use of non-ionic detergents (Imai and Sato, 1974) followed by hydrophobic chromatography and anion exchange chromatography (Van der Hoeven et al, 1974; Astrom and De Pierre, 1986). The use of rodents in toxicology assays has led to their use in the purification of P-450 isozymes. In initial purification schemes animals were treated with xenobiotics to increase hepatic P-450 content and to selectively increase the metabolism of specific substrates (Guengerich et al, 1982). The purification of catalytically active P-450 forms could then be

Table 1.2

Mammalian P450 nomenclature according to Nebert *et al* (1987) and equivalent rat hepatic forms isolated by Wolf, 1986. Names in brackets have not been isolated by Wolf *et al*, 1986.

	Nebert Gene Family	Wolf
hepatic	(P450-1	MC1a, MC1b
	(P450-2A	UT_1
	(P450-2B	PB_{3a}, PB_{3b}
	(P450-2C	PB_{1a}, PB_{1b}, PB_{2a}
	(P450-2D	(debrisoquine)
	(P450-2E	(ethanol)
	(P450-3	PB_{2b}, PB_{2c}
	(P450-4	(clofibrate)
adrenal	(P450-11	(11 β hydroxylation of steroids)
	(P450-17	(17 α hydroxylation of steriods)
	(P450-21	(21 hydroxylation of steriods)
	(P450-22	(cholesterol side chain cleavage)

Table 1.3

Cytochrome P-450 isozymes purified from rat liver micromes. Many forms of cytochrome P450 have been purified on the basis of their induction by foreign and endogenous compounds

Levin[a]	Wolf[b]	Waxman[c]	Guengerich[d]	Other groups	Nebert et al nomenclature	inducer[L]
a	UT_1	3	UT-F	-	P450-2A	3MC
b	PB_{3a}	PB4	PB-B	-	P450-2B	PB
c	$MC1_a$	-	IsF-G	-	P450-1	3MC/TCDD
d	$MC1_B$	-	BNF-B	-	P450-1	3MC/TCDD
e	PB_{3b}	PB-5	PB-D	-	P450-2B	PB
f	(PB-1)	(PB-C)	-	-	P450-2c	not induced
g	-	-	-	RLM_3[e]	P450-2C	not induced
h	PB_{2a},PB_{2d}	PB-2C	UTA	RLM5[e], P450M[f], $P450_{16\alpha}$[h], $P450vtD_325$-OH[g]	P450-2C	GH
i	-	PB-2d	UT_1	P450F[f], $P450_{15\beta}$[h]	P450-2C	GH
j	-	-	-	-	P450-2E	ethanol/isoazid
k	PB-1	PB-1	PB-C	-	P450-2C	PB
-	PB_{2b},PB_{2c}	PB_{2a}	PCN/E	$P450_p$[i]	P450-3	PCN/DEX
-	-	-	UT-H	$P450_{DB}$[j]	P450-2D	PB/3MC/DEX
-	-	-	-	CLO[K]	P450-4	clofibrate

Purified cytochromes are from the following Laboratories: a) Ryan et al, 1982; Ryan et al, 1984; Ryan et al, 1985; b) Wolf et al, 1986; Wolf, 1986; c) Waxman, 1984; Waxman and Walsh, 1982; Waxman and Walsh, 1983; Waxman et al, 1983; d) Guengerich et al, 1982; Larrey et al, 1984; e) Cheng and Schenkman, 1982; Cheng and Schenkman, 1983;; f) Matsumoto et al, 1986; g) Anderson and Jornvall, 1986; h) MacGeoch et al, 1984; Morgan et al, 1985a; i) Schuetz et al, 1984; j) Gouzalez et al, 1987; K) Tamburini et al, 1984; L) Also listed are inducers of some of these rat liver hepatic cytochrome P450s.

assessed for hydroxylation specificity in reconstitution systems. In addition to this approach sexual differences in P-450 mediated steroid hydroxylations were also used to identify and isolate novel enzymes (Einarsson *et al*, 1973). Female rats have a high 15 β hydroxylase activity towards steroid sulphates which is absent in male rats (Gustafsson and Ingelman-Sandberg, 1974; 1975). Whereas male rats have prominent 16 α hydroxylation activity towards testosterone (Einarsson *et al*, 1973).

The most comprehensive biochemical analysis of cytochrome P-450 structure and function has been determined with rat hepatic P-450 forms. This work provides the best basis with which to compare P-450 forms between species. A number of criteria have been used to differentiate between different forms. These are listed below.

a) Mobility on SDS gels: SDS polyacrylamide gel electrophoresis has proved useful in the determination of the purity of P-450 preparations and distinguishing between related forms. On the basis of electrophoretic mobility P-450s range in size from 48kd to 56kd (Black and Coon, 1986). Variations can be seen in the order of migration for purified forms between different laboratories due to differences in running conditions.

b) Peptide mapping: Peptide maps generated from electrophoretic separation of peptides obtained by proteolytic digestion have proved to be a rapid and useful means to examine similarities and differences of purified forms of P-450. Purified forms are digested with proteases and run in parallel to compare the patterns produced. For example, such analysis showed similarities between PB_{3a} and PB_{3b} (Waxman and Walsh, 1982), PB_{1a} and PB_{1b} (Wolf *et al*, 1986) and differences between PB_{3a} and PB_{1a} (Waxman and Walsh, 1983).

c) <u>Substrate specificity</u>: Various factors can influence the activaties of both purified P-450s and those measured in microsomal samples, so their activities can be influence by the phospholipid composition and perturbation of the membrane. Normal catalytic activity requires oxygen and the donation of two electrons mostly provided by NADPH. A complication is that one molecule of cytochrome P-450 reductase serves about twenty P-450 molecules (Peterson and Prough, 1986), thus leading to potential substrate competition between drugs occupying different P-450's. The order of affinity for the reductase as determined by Guengerich (1984) is UT-1 > PB_{3b} > PB_{2a} > MC_{1a} > PB_{3a} > PB_1 > MC_{1b}.

The various P-450s can have different but overlapping specificities. However, by comparing the activity obtained on substrate specificity in reconstitution systems with microsomes and inhibiting these activities with specific antibodies, the substrate specificity may be confirmed. A variety of substrates are currently being used as markers for specific types of P-450. For example, benzephetamine which has different turnover rates with purified P-450 enzymes with PB_{2a} and PB_{3a} having the highest (Table 1.4.) (Wolf <u>et al</u>, 1986) it is generally used as an indicator for induction of PB_{3a} by phenobarbital. Similarly the O-dealkylation of ethoxy-coumarin has significant turnover rates for many P-450s but MC_{1b} has over a 100-fold higher activity relative to the other forms listed in Table 1.4. Thus this activity can be used as a marker of 3MC induction. In control samples MC_{1b} is almost absent and this activity has been associated with the constitutively expressed PB-1 form (Waxman and Walsh, 1983). Other marker substrates are the phenoxazone analogues (Burke and Meyer, 1974). These compounds

Table 1.4

Metabolism of substrates by cytochrome P-450 isozymes*

	Enzyme	Rate (nmol product/min per nmol of P-450)				
		7 Ethoxy-Coumarin	Benz-Phetamine	Pentoxy-resorufin	Benzloxy resorufin	Ethoxy-resorufin
(P450-2C)	PB_{1a}	0.31	4.62	-	0.04	0.41
"	PB_{1b}	0.33	3.2	0.25	3.04	0.87
"	PB_{1c}	3.4	-	0.04	0.04	0.31
	PB_{2a}	0.69	15.9	0.04	0.3	0.46
(P450-3)	PB2b	0.17	3.2	0.02	0.08	0.02
"	PB2c	ND	ND	0.002	0.01	0.04
(P450-2B)	PB_{3a}	2.06	14.84	4.91	9.1	0.05
(P450-1)	MC_{1b}	56.7	3.82	0.33	0.68	28.0

ND = Not detectable

\- = Not determined

* = Wolf *et al*, 1986

Table 1.5

Marker activities for specific cytochrome P-450s

Cytochrome	Activity in microsomes
UT1 (P450-2A)	Testosterone 7 α hydroxylase
PB_{3a}/PB_{3b} (P450-2B)*	Dealkylation of Pentoxyresorufin Testosterone 16α hydroxylase
MC_{1a}/MC_{1b} (P450-1)*	7 ethoxycoumarin O deethylation B[a]P 3,9 hydroxylase
PB_{2a} (P450-2C)	constitutive testosterone 16α-hydroxylase
$P450_{15\beta}$ (P450-2C)	15 β hydroxylase
$P450_{2b}$ (P450-3)	6 β hydroxylation of steriods
UT-H (P450-2D)	Debrisoquine hydroxylation

* Only measurable following administration of an inducing agent.

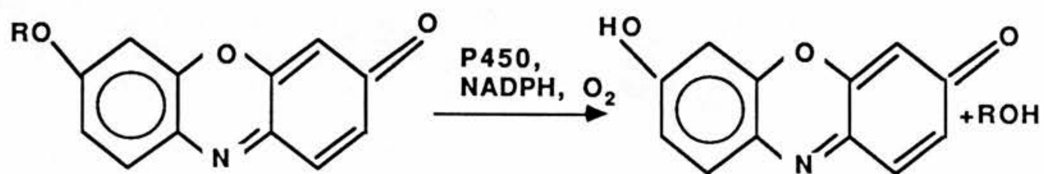
consist of a basic phenoxazone ring structure in which different alkoxy groupings are substituted at the C 7 position (Figure 1.3.). The substitution of different R groups alters the rate of metabolism by different purified P-450 forms. For example, pentoxyresorufin is fairly specific for PB_{3a} (Table 1.5) and ethoxyresorufin is specific for MC_{1b}. Benzyloxyresorufin has a more broad specificity, in the absence of PB_{3a} and MC_{1b} it is fairly specific for PB_{1a}. In addition to these substrates the metabolism of B[a]P has been used as a marker for MC_{1b} (23.4nmol/min per nmol cytochrome P-450) and PB_{2a} (1.8nm/min per nmole cytochrome P-450) (Ryan et al, 1982; 1984). In the metabolism of acetylaminoflourene by eight different isozymes of P-450s, MC_{1a} and MC_{1b} produced much higher levels of N-OH AAF the metabolite associated with the hepatocarcinogenic effects of this compound (Astrom and De Pierre, 1985). This metabolite was only observed with one other isozyme, PB_{2b}/PB_{2c} (P450-3), albeit at 100-fold reduced levels. Larrey et al (1984) reported the isolation of a cytochrome P-450 (UT-H, (P450-2D)) which metabolized the anti-convulsive drug debrisoquine at 20-fold greater rates when compared to eight other purified forms.

The catalytic properties of isolated cytochrome P-450 proteins have also been studied with endogenous substrates such as testosterone, androstenedione, progesterone, estrogen, cholesterol and vitamin D_3. Hydroxylations of these substrates in the liver can be both anabolic and catabolic.

Chloesterol and the C-27 steroids are involved in the biosynthesis of bile acids and vitamin D_3. The 25 hydroxylation of C-27 steroids and vitamin D_3 has been shown to be mediated by PB_{2a} (Anderson et al, 1983). The enzyme is expressed in male but not female rats (Anderson and Jornvall, 1986) which parallels the sex

FIGURE 1.3.

Metabolism of phenoxozone analogues by cytochrome P-450s. The R group determines the specificty of conversion by P-450s.


RO
O
O
N
P450,
NADPH, O2
HO
O
O
N
+ROH

difference in 25-hydroxylation activites.

Hepatic cytochrome P-450s are very active in the hydroxylation of steriod hormones. UT_1 is specific for the 7α-hydroxylation of testosterone (Ryan et al, 1984; Waxman, 1986). The 16α hydroxylation of progesterone and estradiol is carried out mainly by PB_{2a} (Cheng and Schenkman, 1984). This protein also metabolizes testosterone and androstenodion at this position (Waxman et al, 1983) and accounts for this male specific 16α-hydroxylation activity measured in adult rat microsomes (Waxman et al, 1985). However, PB_{3a} also has 16α-hydroxylase activity (Waxman, 1984) which can be induced in female rat microsomes by phenobarbital administration (Waxman et al, 1983). An equivalent form to PB_{2a}, which is female specific has estrogen 15β hydroxylase activity (MacGeoch et al, 1984; Cheng and Scharkman, 1984). The 6β hydroxylation of steroids is associated with PB_{2c} (Waxman et al, 1985). Antibodies to purified P-450s form can inhibit these activities in liver microsomes. For example 7α hydroxylase is abolished by anti UT_1 antibody (Waxman et al, Guengerich, 1985) and 6β oxidation hydroxylation by anti-PB_{2c} (Gonzalez et al, 1986b). Table 1.5. lists some marker activities for specific P450s in microsomal preparations

1.4.3. Induction of cytochrome P-450 isozymes

Numerous compounds have been shown to induce or suppress microsomal activities and associated cytochrome P-450 proteins. Classically, inducers of drug metabolism could be divided into three classes, those which respond to 3MC, PB or PCN. Clearly the 3MC type is associated with MC_{1a} and MC_{1b}, the PB type with PB_{3a} and PB_{3b} and the PCN type with PB_{2b}. It is now apparent that many

structurally diverse compounds can induce the same form of cytochrome P-450. Thus phenobarital and Aroclor 1254 (R) induce PB_{3a}, Aroclor 1254 (R) which is a complex mixture of polychorinates and biphenyls also induces MC_{1a} and MC_{1b} (Vlasuk et al, 1983). Table 1.6 lists a number P-450 isozymes and their relative level of expression in control and induced microsomes of male rats. An important observation is that in the absence of induction there are many P-450s with a high constitutive level of expression. Notably PB_{2a} (P450-2C) which in control rat microsomes is expressed at a level of 1.2nmol/mg of total microsomal protein. Other P-450 isozymes with significant levels of expression are PB_{2B}(P450-3), PB-1 (P450-2C) and UT-1 (P450-2A). The highly inducible forms of P-450, PB_{3a} (P450-2B) and MC_{1a} (P450-1) represent only 2.5% of P-450 content in untreated male rat liver. Therefore in the absence of induction it is the constitutively expressed forms of P-450 which will be responsible for detoxification of foreign compounds as well as the metabolism of steroids. A variety of P-450s are suppressed by the administration of inducing compounds. Phenobarbital treatment suppresses PB_{2a}, has a marginal effect on the levels of PB_{2c}, PB-1 and UT-1, while inducing the levels of PB_{3a} and PB_{3b} 50 and 10 fold respectively. β-napthaflavone (BNF) induces MC_{1b} whilst suppressing the levels of PB_{2a}. In general the levels of PB_{2a} are down regulated by many classical P-450 inducers. One conjecture is that this P-450 is too specialized in its function in steroid metabolism to be of use in xenobiotic metabolism.

1.4.4. N-terminal sequence analysis

A variety of methods for distinguishing between P-450 isozymes are described above, however, the most useful method for

Table 1.6

Microsomal levels of distinct liver P-450 isozymes in control and induced male rat liver microsomes

P-450 isozyme content (nmol/mg protein)

Isozyme		Treatment			
		Control	PB	BNF	PCN
(P450-2C)	PB_{2a}	1.2	0.49	0.33	0.33
(P450-3)	PB_{2b}	0.4	1.06	0.32	1.32
(P450-2C)	PB-1	0.36	0.69	0.26	0.31
(P450-2A)	UT-1	0.15	0.10	0.12	0.08
(P450-2B)	PB_{3a}	0.03	1.27	0.04	0.10
"	PB_{3b}	0.07	0.92	0.04	0.09
(P450-1)	MC_{1b}	0.04	0.04	1.41	0.06
"	MC_{1a}	0.03	0.03	0.57	0.03

The data is expressed as nanomole of each protein per milligram of total microsomal protein. PB = Phenobarbital; BNF - β naphtoflavone; PCN = Pregnenolene-5α carbonitrile. Data from Guengerich _et al_, 1982.

constructing identity between P-450 isozyme forms from different laboratories therefore facilitating the grouping together of different P-450 forms is N-terminal sequence analysis. This has formed the basis of the comparison given in Table 1.7 significant identity can be found between PB-1 and PB_{2c} described by Waxman, 1984; Waxman and Walsh, 1984 RLM5 and RLM3 described by Cheng and Schenkman, 1983, f-i (Haniu et al, 1984) and PB_{1a}, PB_{1b}, PB_{2a} and PB_{2d} (Wolf et al, 1986) (Table 1.7). These sequences are distinct from PB_{3a} and PB_{3b} (Wolf et al, 1986). A table of identities for the PB-1 P-450 forms shows that they are all related to each other (Table 1.8). A notable feature of all rat hepatic P-450s is the presence of a hydrophobic leader sequence. In a few cases notably cytochromes MC_{1a}, MC_{1b} and PB_{2d}, the N-terminal Met is removed by proteolysis (Haniu et al, 1984; Wolf et al, 1986). The differences in homology between P-450h and RLM5 are probably not due to strain variation as both proteins were isolated from uninduced Sprague-Dawley rats. P-450h is exactly the same as P450 PB_{2a} from N-terminal sequence analysis. One difference between the purification of Schenkman and Levin versus Waxman and Wolf is that the latter two groups used PB treated rats which marginally increase PB-1 levels whilst decreasing form PB_{2a} (Adesnik and Aitchison, 1986).

1.5. P-450 forms in other species

Multiple isozymes of P-450 have been purified from liver microsomes of many species notably rabbit, mouse and human (Table 1.9). In the rabbit at least 8 distinct forms have been identified (Johnson and Schwab, 1984). Similarly at least 6 distinct forms have been identified in humans (Wang et al, 1983). In spite of its use as a genetic model work on mouse purified P-450s has been

Table 1.7

Comparison of N-terminal sequences of PB-1 (P4502C) P-450s

	P-450 nomeclature	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		Rat Strain
Waxman	(PB-1	M	D	L	V	M	L	L	V	L	T	L	T	X	L	I	L					)	Sprague-Dawley
	(PB2c	M	D	P	V	L	V	L	V	L	T	L	X	(S)	L	L	L					)	
Schenkman	(RLM5	M	D	P	V	L	V	L	V	L	T	L	L	L	L	L						)	Sprague-Dawley
	(RLM3	M	D	P	V	V	V	L	L	L	M	L	F	F	L	L	L	L				)	
Levin	(f	M	D	L	V	T	F	L	V	L	T	L	S	S	L	I	L	L	S	L	W	)	
	(g	M	D	P	V	V	V	L	L	L	S	L	F	F	L	L						)	Sprague-Dawley
	(h	M	D	P	V	L	V	L	V	L	T	L	S	S	L	L	L	L	S	L	W	)	
	(i	M	D	P	F	V	V	L	V	L	S	L	S	F	L	L	L	L	Y	X	W	)	
	(PB1a	M	D	L	V	M	L	L	V	L	T	L	T	X	L	I	L	L	S	I		)	
Wolf	(PB2a	M	D	P	V	L	V	L	V	L	T	L	S	S	L	L	L	L	X	L		)	Wistar
	(PB2d	X	D	L	V	L	V	L	V	L	T	L	X	X	L	L	L	L	L			)	
	(PB3a	M	E	P	S	I	L	L	L	A	L	L	V	G	F	L	L	L	L	V		)	

'X' = not determinable 	'(S)' = most likely candidate

Data from:
Waxman: (Waxman, 1984; Waxman and Walsh, 1983)
Schenkman: (Cheng and Schenkman, 1983)
Levin: (Haniu *et al*, 1984)
Wolf: (Wolf *et al*, 1986)

Table 1.8.

Sequence homology of N-terminal sequences between PB-1 (P450-2C) proteins from different laboratories.

		PB-1	PB_{2c}	RLM_5	RLM_3	f	g	h	i	PB_{1a}	PB_{2a}	PB_{2d}	PB_{3a}
Waxman	(PB-1	100											
	(PB_{2c}	69	100										
Schenkman	(RLM_5	64	**93**	100									
	(RLM_3	73	73	67	100								
Levin	(f	80	73	47	53	100							
	(g	50	71	57	**93**	53	100						
	(h	70	**100**	**87**	71	27	37	100					
	(i	53	73	60	76	47	20	27	100				
Wolf	(PB_{1a}	**100**	71	70	69	**84**	67	59	56	100			
	(PB_{2a}	73	**100**	**87**	71	**84**	70	100	77	65	100		
	(PB_{2d}	75	92	82	84	71	**93**	33	**77**	**80**	**94**	100	
	$PB3_a$*	40	40	40	47	27	40	33	33	44	39	40	100

* Sequence of PB_{3a} (P450-2B) is included for comparison purposes.

Table 1.9

Comparison of P-450 izsozymes identified in 4 species

Rat	Mouse	Rabbit	Human
PB-1	$(C\text{-}P\text{-}450_{16\alpha})$	P450 1	P450mpI
		PB_{c1}	P450mpII
		PB_{c2}	
		PB_{c3}	
		LM3b	
MC_{1a}	P4503	LM4	
MC_{1b}	P4501	LM6	P4501
PB_{3a}	C2	LM_2	?
UT-H	?	?	$P450_{DB}$
PB_{2b}	?	?	P450NF
j	?	LM3a	P450j
UT_1	(Coh)	LM2	P4502A

( ) = tentative ? = unknown

Mouse forms: Harada and Negishi, 1984b; Negishi and Nebert, 1979; Huang e 1976; Kaipainen et al, 1984.
Rabbit forms: Johnson and Schwab, 1984; Leighton et al, 1984, Ozols et al, Tukey et al, 1985; Black and Coon, 1986.
Human forms: Adams et al, 1985; Disterlath et al, 1985; Gut et al, 1986; et al, 1986; Shimada et al, 1985; Wang et al, 1983; Watkins al, 1985; Phillips et al, 1985; Wrighton et al, 1986.

limited. Nonetheless, at least five distinct forms have been identified in this species (Table 1.9).

For the P-450s many substrate specificities are maintained across species. For example, the ethanol inducible forms from rat, rabbit and human. In contrast the rabbit P-450I is unique in having progesterone-21-hydroxylase activity. The metabolic pathway is usually associated with a highly specific P-450 located in the adrenal gland (Tukey et al, 1985). In addition to mammals P-450s have also been identified in Saccharomyces cerevisae, Bacillus megatherium, plants and Drosophilia Melanogaster (Black and Coon, 1986). Comparative analysis of P-450 isozymes from different species should provide evidence for conservation of functionally important activities and domains.

1.6. Molecular analysis of P450s

The number P-450 isozymes identified in rat liver is twelve (Waxman, 1986). The recombinant DNA approach has been used to elucidate the nature of this diversity at the genetic and molecular levels. A number of strategies have been used to clone P-450 cDNA and genomic clones. Initially as in the biochemical analysis the favoured animal models were rat, mice and rabbits. At the DNA level most of the analysis of P-450 isozymes has concentrated on the PB and MC inducible forms. They serve as useful models for the regulation of P-450s and their genomic organization.

1.6.1. Cloning strategies for P-450s

The induced forms of P-450 were cloned first because of the high frequency of recombinants in cDNA libraries synthesized from hepatic mRNA from induced animals. This class of mRNA was enriched

to use as a source for cDNA cloning by polysome precipitation (Hardwick et al, 1983; Negishi et al, 1981) or by size fractionation (Adesnik et al, 1981). Libraries were constructed in pBR322 and putative P-450 cDNA clones were identified by differential screening with ^{32}P labelled control and induced cDNA. The resulting plasmids were then used to hybrid select mRNA which could be translated in vitro to see if they correspond to purified P-450 forms. This strategy for cDNA cloning is now rarely used and the most commonly used method for isolating P-450 cDNA clones is a direct screen of expression libraries with affinity purified anti-P-450 antibodies (Freidberg et al, 1986; Gonzalez et al, 1986a).

1.6.2. Genomic Complexity of P-450s

In rats two distinct forms of 3MC inducible P-450 proteins have been identified MC_{1a} and MC_{1b} (Ryan et al, 1982, 1984). These can be distinguished by their N-terminal sequence (Haniu et al, 1984) and their catalytic activity towards B[a]P. MC_{1b} has greater activity than MC_{1a} (Guengerich et al, 1982. Only two 3MC inducible cDNA clones have been identified in rat liver each of which appears to correspond to the purified proteins by comparison with N-terminal sequence of the cDNAs (Kawajiri et al, 1984; Yakasaki et al, 1984). Orthologous forms have been identified in mouse liver (Negishi and Nebert, 1979; Gonzalez et al, 1985). Southern analysis indicate that this is the extent of MC inducible P450 gene family in rats, mice and humans. The structure of the mouse MC_{1a} and MC_{1b} genes is very similar, both being 6kb long containing 7 exons (Gonzalez et al, 1985).

cDNA clones corresponding to phenobarbital inducible PB_{3a} and PB_{3b} P-450 have also been isolated (Adesnik et al, 1981; Fuji-

Kuriyama et al, 1982). These two proteins have identical N-terminal sequences but PB3a has about 5-fold greater metabolising activity than PB_{3b} (Ryan et al, 1984). Southern blot analysis of genomic DNA demonstrated that there are many sequences similar to PB_{3a} in rat DNA (Atchison and Adesnik, 1986). Genomic cloning has identified eleven PB_{3a} related genes in rats (Mizukami et al, 1983; Atchison and Adesnik, 1983). These genes have adifferent genomic organization to MC_1 genes in that they are up to 20kb in length and are split into 9 exons.

The cloning of the PCN inducible P450s has identified two closely related cDNAs in the rat (Gonzalez et al, 1985b). Southern blot analysis indicates this is a complex gene family (Hardwick et al, 1983). However at present there is no data on the genomic organization of these genes.

Southern analysis and genomic cloning has illustrated that the potential number of P-450 genes in rats is greater than the proteins isolated to date. Whether these extra genes are pseudo genes or not is open to question, they may correspond to extrahepatic on developmentally expressed members of these families.

1.6.3. Functional Domains of P-450s

The number of complete cDNA, and therefore amino acid sequences of P-450s is rapidly increasing (Adesnik and Atchison, 1986; Nebert et al, 1987). Black and Conn (1986) list 10 complete primary structures, 4 of rat liver, 3 of rabbit liver, 2 of mouse liver and one of bovine adrenal gland. A summary of the molecular data shows that in rodents hepatic P-450s represent a superfamily composed of at least 7 distinct gene families, which are distinguishable from each other by Southern blot and sequence analysis. Direct

comparison of P-450 sequences provides an insight into the structural features required for common function. These can best be illustrated with three distinct inducible P-450 gene families P450-1 (MC), P450-2B (PB) and P450-3 (PCN). At the sequence level they show less than 35% amino acid identity to each other (Black and Coon, 1986; Gonzalez *et al*, 1986b). This indicates that these gene families diverged 200 million years ago (Gonzalez *et al*, 1985b). Comparison of hydropathy indices, which plots the relative hydrophobicity or hydrophilicity of the proteins, shows them to be generally very different, however two regions of similarity can be noted. All P-450s have a stretch of 15-25 hydrophobic amino acids after the initiator methionine although the N-terminal sequences of these P-450s are very different from each other. This stretch has been demonstrated *in vitro* to serve as a membrane insertion and stop signal during translation of the P-450 message (Sakaguchi *et al*, 1987). This feature has been noted in other mammalian P-450 forms (Heinemann and Ozols, 1983). A second similarity is a large segment of hydrophilic amino acids (residus 405-440).This region is present immediately before a highly conserved region containing the heme binding system which is present in P-450 proteins.

Overall comparison of P-450s reveals 3 regions which are conserved between distantly relative enzymes. These regions are confined to the N- or C-terminal regions of the proteins. The first conserved region was identified by comparing the sequence of P-450cam isolated from *Pseudomonces putida*, and the rabbit phenobarbital inducible P-450 LM2 (Heinmann and Ozols, 1983). This region is centered around Cys 152 of PB_{3a} in rats (Table 1.10) and region Cys 134 in P-450cam. These two forms show 10/18 matches. Two regions in MC_{1a} and MC_{1b} P450s show some similarity to this region,

although to a lesser extent. It is interesting that PB_{2a} shows essentially no similarities to the above proteins over this region. Indeed the cysteine in this region is missing from PB_{2a} as well as from P450-2A, P450-2D, P450-2E and P450-4 proteins of rat. A second cysteine containing region at Cys 436 in PB_{3a} is much more highly conserved between different P-450 forms (Table 1.10). All P-450s isolated to date have a cysteine at this region. It has been shown conclusively in P-450cam that the cysteine containing peptides provides the heme binding site (Poulos, 1986). A third region of similarity was described in two forms of rabbit P-450 (Ozols et al, 1981). This tridecapeptide region shows 50% homology between PB_{3a} and MC_{1a} and 45% overall identity between distantly related mammalian P-450s. The high conservation of these region suggests an important structural or functional role.

Regions of maximum dissimilarity between P-450s lie in the region between the N-terminal and C terminal conserved regions corresponding to regions 200 and 300 of the PB_{3a} protein sequence. Over this region MC_{1b} and PB_{3a} share 15% identity. MC_{1a} and MC_{1b} share over 70% amino acid similarity but only 35% idenity over region 200-300. These two forms have marked differences in their substrate specificity. Thus this region is thought to be involved in determining substrate specificity. Indeed, chimeric cytochrome MC P450s, where the central and carboxy portion of MC_{1b} is replaced by that of MC_{1a}, conferred the substrate specificity of MC_{1a} to this novel protein (Sakaki et al, 1987). Therefore an idealized cytochrome P-450 molecule consists of a hydrophobic protein of around 490 amino acids in length with a hydrophobic leader sequence for membrane binding, a central region involved with substrate binding and a carboxy terminal which contains the elements for

Table 1.10 Comparison of three conserved amino acid regions form a diverse cytochrome P-450s.

	P450	Sequence	aa
A)	PB3a rat	V E E R I Q E E A Q C - L V E E L R K	159
	MC1a "	L E E H V S K E A N H - L I S K F Q K	177
	MC1b "	L E E H V S K E A E Y - L V S K F Q K	179
	PB2b "	M F P I I E Q Y G D I - L V K Y L K Q	163
	MC1a "	I A S D P T S V S S C Y L E E H V S K	166
	MC1b "	I A S D P T L A S S C Y L E E H V S K	168
	P-450 cam	L E N R I Q E L A - C S L I E S L R P	142
B)	PB3a rat	F S T G K R I C L G E G I A R N E L F L F	449
	MC1a "	F G L G K R K C I G E I P A K W E V F L F	469
	MC1b "	F G L G K R K C I G E Y I G R L E V F L F	473
	PB2b "	F G N G P R N C I G M R F A L M N M K L A	456
	P-450 cam	F G H G S H L C L G Q S L A R R E I I V Y	368
C)	PB3a rat	M P Y T D A V I H E I Q R	358
	MC1a "	L P Y L E A P I L E I Y R	375
	MC1b "	L P Y L E A P I L E T P R	380
	PB2a "	R L Y P I G N R L F R V C	390
	LM2 rabbit	M P Y T D A V I H E I Q R	350

PB3a; Fuji-Kuriyama et al, 1982. MC1a: Yabusakai et al, 1984. MC1b: Kawjiri et al, 1984. P-450 cam; Haniu et al, 1982; LM2: Heineman and Ozols, 1983.

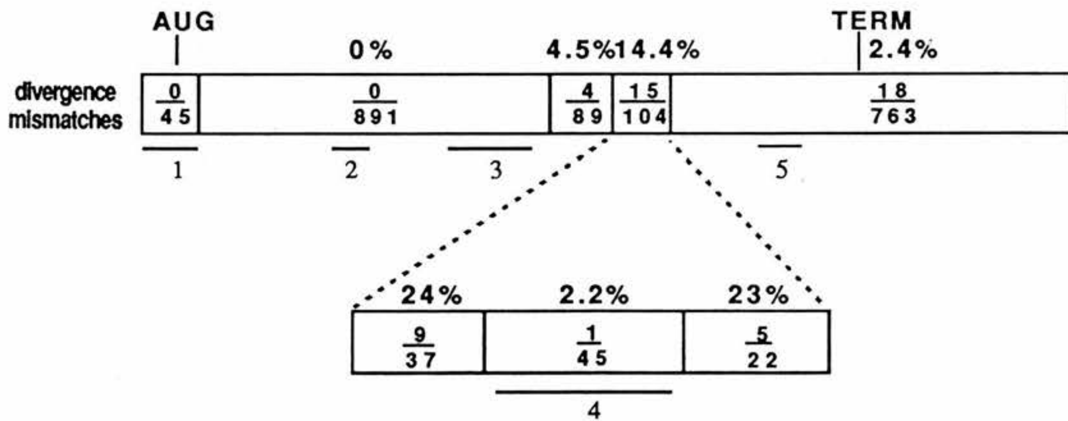
A) N-terminal cysteine fragment centered at Cys152 of PB3a
B) C-terminal cysteine fragment centered at Cys436 of PB3a.
C) The 'analogous tridecapeptide' region at Met346-Arg358 of PB3a.

reductase and heme binding to allow electron transfer to the substrate molecule (Fig. 1.4).

The above gives a rough idea of the structural requirements of the P-450 molecules. However, the factors which determine substrate specificity are even more subtle for example cDNA clones for the closely related phenobarbital inducible P-450s $PB3_a$ and $PB3_b$ have been identified (Fuji-kuriyama et al, 1982). $PB3_a$ has the same substrate specificity as $PB3_b$ but its specific activity is about 5 times greater than $PB3_b$ (Ryan et al, 1984). Sequence comparison of over 1900bp of these cDNAs indicate them to be 98% homologous overall but the distribution of homologous and non-homologous sequences is non-random (Adesnik and Atchison, 1986). The 5' 946bp are identical (Figure 1.4) whilst there are 36 divergent residues in the remaining sequence of the two mRNAs with 14 differences clustered to two divergent segments which flank the conserved 'tridecapetide' region. The gene encoding $PB3_a$ has been cloned and characterized in detail. The gene, which encodes a 2kb mRNA, is 14kb long and is split into 9 exons. The hypervariable region has been localized to exon 7 (Atchison and Adesnik, 1983; 1986). In an elegant study, Atchison and Adesnik sequenced a number of $PB3_a/PB3_b$ related genes which showed that at least one of the divergent segments appeared to result from a gene conversion event between an ancestral $PB3_a$ gene and a related donor gene (gene 3) of unknown function. A gene conversion event is a non-reciprocal recombination in which a segment of one gene replaces another. The sequence data also suggested extensive gene conversion within all the members of this family including exon 7 and 8 and the intron inbetween them. Generally, it has been found that the exons of related genes retain homology while intronic sequences diverge more

FIGURE 1.4.

Comparison of PB_{3a} and PB_{3b} cDNAs showing common P-450 features, 1-5, and regions of homology and non-homology within PB_{3a} and PB_{3b}. The position of the initiation codon is indicated by AUG and the termination codon by TERM.



1) Hydrophobic leader sequence
2) Cys 152 containing peptide
3) substrate specificity
4) analagous tridecapaptide
5) Cys 436 containing fragment

rapidly (Li et al, 1985b). Intron 7 of all the cloned PB_{3b} genes show 90% homology. Upstream sequences of exon 7 and downstream of exon 8 also show identity. Genomic blots of germline versus somatic tissue showed that the hypervariable region does not represent a somatic rearrangement of the PB_{3a} DNA configeration. Gene conversion has also been postulated to occur in the PB_{2b} (P450-3) gene cluster, the rabbit PBc1 (P450-2B) gene cluster and the MC_{1a} (P450-1) gene cluster (Adesnik and Atchison, 1986; Gonzalez et al, 1986b). It can be appreciated that the action of a gene conversion event can maintain homology between gene family members or introduce more diversity. New genes encoding proteins with new catalytic activities could be generated by one gene procuring a new DNA segment from a related gene or pseudogene. Whether this new gene becomes fixed could be due to random processes or due to selective pressure for new catalytic activity. Thus gene conversion could accelerate the evolution of new P-450 forms.

1.7. Genetics of Cytochrome P-450s in mice

The large number of different P-450 isozymes with overlapping substrate specificities suggests that genetic variation may not be a significant factor in our interaction with the chemical environment. Early demontrations of variation in the response of different mouse strains to barbituates suggested otherwise (Jay, 1955). The P-450 dependent monoxygenase system reduces the biological effects of drugs by providing their excretion. The rate at which a drug is metabolized will differ between individuals due to qualitative and quantitative differences in these enzymes. Altered rates of drug metabolism may result from modulation of P-450s by endogenous or

exogenous factors or by genetic polymorphism. In both cases the Km and Vmax for the substrates can be changed. In addition, if the xenobiotic is metabolized in a stereospecific manner then a change in the site of metabolism can have profound effects on drug disposition, dose response and toxicity. Genetic factors are known to play a central role in drug response thus it is important to be able to define the genetic background of an organism's drug metabolizing system under different chemical exposures. Thus rodent models for testing the toxicity of foreign compounds will be affected by the strains used. To understand the P-450 system it is critical to establish how well the drug metabolizing capacity of a particular species reflects that of another.

1.7.1. The Ah locus

The first evidence for the existence of polymorphic loci involved xenobiotic metabolism came from studies on inbred strains of mice. It was observed that the 3-hydroxylation of B[a]P [Arylhydrocarbon hydroxylase (AHH)], was strongly inducible in some mouse strains but not others (Nebert and Gielen, 1972). C57BL/6 mice were responsive to 3MC induction whereas DBA/2 mice were non-responsive. Appropriate crosses established that the inducibility was controlled by a single autosomal dominant gene (Nebert *et al*, 1972; Nebert and Gielen, 1972: Thomas *et al*, 1972). This locus was termed *Ah* for AHH responsiveness. Responsive (C57BL/6) strains carried the $\underline{Ah}^{b}$ allele whilst the non-responsive strain (DBA/2) carried the $\underline{Ah}^{d}$ allele. This locus was equivalent to a locus which also controlled the response to inflammation caused by skin exposure of DMBA (Taylor, 1971; Thomas *et al*, 1973). After topical application of DMBA, C57BL/6 mice show intense ulceration

and inflammation after a latency period of 10 days whereas DBA/2 mice show no such response. This experiment illustrated again that carcinogen metabolism was an important feature in a host's response to chemical challenge and the need to use the appropriate strain for carcinogen and toxicological testing. Fibrosarcomas initiated by subcutaneous injection of 3MC are also associated with the $\underline{Ah}^b$ allele in 14 strains of mice (Nebert and Jensen, 1979).

The Ah locus is thought to be responsible for induction of a number of different enzymes activities (Eisen et al, 1983) including aflotoxin B_1 hydroxylase (Gurtoo et al, 1978), glut athione transferase (Czosnek et al, 1984) and UDP glucuronyl transferase (Jakoby, 1980). Practically all mouse strains exhibit Ah locus associated responsiveness (Nebert et al, 1982; Taylor, 1984). The metabolism of many drugs and environmental pollutants by the P-450 dependent monooxygenase system represents an important rate limiting step in their excretion. Thus the pleiotropic effects of the Ah locus implies that it is a regulatory locus which affects the levels of many structural loci related to xenobiotic metabolism. The control of inducibility by the Ah locus thus represents a case where quantitative changes in the levels of drug metabolizing enzymes can have profound effects on an organism's drug metabolizing capacity. Another AHH inducer is 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD). This compound has about 30,000 times more potency than 3MC as an inducer (Poland and Glover, 1973). Following interaperitoneal administration of (^{14}C) labelled TCDD it was found to accumulate at high levels in the liver of C57BL/6, intermediate levels in (C567BL/6 X DBA/2)F1's and to a much lower degree in DBA/2 mice (Poland et al, 1976). This pattern mirrored the strain sensitivity to AHH induction by nanomolar amounts of TCDD. It was then

established that TCDD bound to a soluble hepatic cytosolic protein in C57BL/6 mice but not DBA/2 mice at the concentration tested (Poland et al, 1976). This was postulated to be due to a receptor involved in the induction mechanism. It was noteworthy that at higher doses (10-fold) TCDD is a potent inducer of AHH in Ah^a (DBA/2) mice (Poland et al, 1974; Niwa et al, 1975). The diminished sensitivity of non-responsive mice to AHH induction was interpreted as being due to a mutation in the TCDD binding protein/ receptor. Further alleles at the Ah locus were suggested by crossing DBA/2 and C3H/HJe (responsive) mice, an F_1 between these mice showed an additive mode of inheritance for AHH inducibility (Thomas and Hutton, 1973; Robinson et al, 1974).

A more complex result was obtained by Robinson et al (1974) using NIH substrains C57BL/6N (responsive) and AKR/N (non-responsive). At that time these sublines had been separated from their Jackson laboratory counterparts C57BL/6J and AKR/J for more than twenty years. In their cross of C57BL/6N with AKR/N they found non-responsiveness was a dominant trait. This reversal of dominance appeared to result from the specific interaction of these strains since both strains showed a normal dominance pattern in crosses to other strains. In a series of crosses of either C57BL/6J or N or AKR/N or J with F_1s of respectively (AKR/N X AKR/J) X (C57BL/N X C57BL/6 J) all the resulting progeny showed nearly a 100% responsiveness which was significantly different from the expected segregation based on the subline differences. This data was perplexing and may suggest that additional loci are involved in AHH induction. For example the TCDD receptor could be a multimeric protein where specific interactions are required by its subunits for it to function.

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The structural loci responsive to induction of AHH activity, by the _Ah_ locus have been associated with a P-450 in the mouse named cytochrome $P_1$450 (_P450-1_) (Robinson _et al_, 1974). Two forms of P-450 which were part of this family were isolated from induced C57BL/6 mice. One of which was $P_1$450 associated with AHH activity, (MC1b) and the other, $P_3$450 with acetanilide hydroxylase activity (MC1a) (Negishi and Nebert, 1979). Both forms of P-450 were inducible by TCDD and 3MC in C57BL/6 mice. Recombinant DNA studies have shown a stoichiometric relationship between translocation of a TCDD-receptor complex into the nucleus and the accumulation of $P_1$450 mRNA (Tukey _et al_, 1982). This suggested that the inducer-receptor complex interacts with chromatin to initiate transcription. Increases in the mRNAs for P_1 and P_3 450 are a direct result of increases in transcription rate as determined by nuclear run off assays (Gonzalez _et al_, 1984; Kimura _et al_, 1986). A _cis_ acting dioxin responsive element (DRE) has been located upstream of the $P_1$450 gene by splicing 5' $P_1$450genomic fragments to a reporter gene (Jones _et al_, 1986) and by mapping the sites _in situ_ with nuclei from induced hepatoma cell lines (Durrin and Whitlock, 1987).

The $P_1$450 and $P_3$450 genes have been assigned to chromosome 9 in mice using a panel of DNAs from Chinese hamster/mouse somatic cell hybrids (Tukey _et al_, 1984; Hildebrand _et al_, 1985a). The regional localization of the $P_1$450 gene was assigned by mapping in a set of recombinant inbred (RI) strains of mice. Briefly, RI lines result from the systematic inbreeding (>20 generations) of the selected F_2 pairs from a cross between two pre-existing progenitor strains. This essentially fixes within each resulting RI strain a specific pattern of recombination. The probability of recombination between any two loci is proportional to the distance between them. Strain

distribution patterns (SDP) can be generated for a set of allelic chromosomal markers. If differences exist in the progenitor strains for new markers then a SDP can be generated and compared to the previously mapped genes and thus determine relative chromosomal location (Taylor, 1978). By this method the P_1450 (P450-1) gene was assigned 7cM distal from the Thy-1 locus near Mpi-1 on chromosome 9 (Hildebrand et al, 1985a). The original SDP for the Ah locus did not allow regional assignment but excluded many parts of the mouse genome including chromosome 9 (Taylor, 1971; Wood and Taylor, 1979; Gurtoo et al, 1978) which would support the idea of Ah being a regulatory locus.

With the accumulation of new markers, the Ah locus has now been assigned to chromosome 12 in mice in the BxD RI series (Cobb et al, 1987). This places it about 10.5±3.7cM from the ApoB locus (Lusis et al, 1987).

1.7.2. Coh locus (P450-2B)

The metabolism of coumarin, a constituent of many plants, whose derivatives are used as anticoagulant drugs, is metabolized by the P-450 dependent monooxyg nases (Wood and Cooney, 1974). The basal and phenobarbital (PB) induced levels of this hepatic microsomal activity are genetically controlled in different strains of mice. In untreated DBA/2 mice this activity is three- to four-fold higher than AKR, C57BL/6 and C3H/He strains. After PB treatment this relative difference has increased to four- to six-fold. PB treatment induces coumarin hydroxylase activity two- to five-fold. 3MC has no effect on this activity which is therefore distinct from

the Ah locus. Analysis of the F_1s between DBA/2 and low activity strains shows that this trait is inherited additively. Comparison of pH optima, heat stability and Km values for coumarin hydroxylase activity show no significant differences between the DBA/2 and AKR strains or the F_1 hybrid (Wood, 1979). However, the heme ligands aniline and metyrapone show differential inhibition of this enzyme activity in DBA2/J and AKR mouse strains. Metyrapone preferentially inhibits in AKR microsomes whereas aniline preferentially inhibits DBA/2 microsomal coumarin hydroxylase activity. The classification of mice strains has been extended (Lush and Arnold, 1975; Lush and Andrews, 1978; Wood and Taylor, 1979) and an additional class was identified as containing medium metabolizers of coumarin or 7 ethoxycoumarin (Lush and Andrews, 1978). However the medium group was absorbed in to the high metabolism group when 4 methyl derivatives of coumarin or 7 ethoxycoumarin were used as substrates. These results were interpreted as evidence that there are two closely linked genes which determine cytochrome P-450 isozymes with different substrate specificities.

Additive inheritance of coumarin hydroxylase activity in the F_1 generation between DBA/2 and AKR suggested that a structurally different enzyme rather than an absence of regulatory process may account for the different levels of enzyme activity (Wood and Cooney, 1974). Backcross analysis of PB treated AKR, DBA/2 and (AKR X DBA/2)F_1 indicated that coumarin hydroxylase activity is inherited as a single autosomal trait (Wood and Taylor, 1979). The locus symbol was designated Coh with subscripts h or l for high and low activity alleles. Backcross analysis between (AKR X DBA/2)F_1 and AKR mice indicated that Coh was 21±6 cM from the albino locus on chromosome 7. Analysis of the SDP in the BXD RI series indicated

that Coh is closely linked to Gpi-I on the centromeric side of 7.

The analysis and characterization of PB induced P-450's in the mouse is very limited, the rat PB3a cDNA clone picks up multiple sequences in the mouse (Simmons and Kasper, 1983). On Southern blots of DNA from different inbred strains restriction fragment length variants can be detected. C57BL/6/DNA gives a different pattern to DBA/2 on EcoRI, BamHI and PvuII digestion. When these variants are mapped in the BXD series they show a 100% concordance with the Coh locus as determined by coumarin hydroxylase activity. However, in typing 15 mouse strains one discordant was found. CBA/Ca mice were typed as being Coh^l but on Southern analysis shows a DBA/2 (Coh^h) DNA pattern. This discordance tends to support the idea that there are two closely linked loci involved in the metabolism of 7-ethoxycoumarin (Lush and Andrews, 1978). This idea is further supported by semi-purification of PB induced P-450s from mouse (Huang et al, 1976; Kaipainen et al, 1984). Two PB inducible proteins termed A_2 and C_2 were isolated from liver microsomes of (C57BL/6 X DBA2)F_1 mice (Huang et al, 1976). These had molecular weights of 50 and 56kD respectively. In reconstitution experiments the fractions containing the A form had ten- to one hundred-fold greater coumarin hydroxylase activity than the C fractions. Interestingly, the A fractions also had significantly greater testosterone 7α-hydroxylase activity. This is a marker substrate for UT-1 P-450 in rats (Ryan et al, 1984; Waxman, 1984) UT-I protein from rats also has a molecular weight of 49kD. The C fractions had significantly greater N-demethylation activity (Huang et al, 1976) which is suggestive that they are more similar to rat PB_{3a} (Astrom and De Pierre, 1986). A P-450 Coh has been isolated from DBA/2 hepatic microsomes (Kaipainen et al, 1984) which has a

Vmax twice as high as, and a Km value tenfold less than a similar isolate from C57BL/6 in reconstitution systems. This protein also has a molecular weight of 49kd and antibodies raised to this protein will not cross react with the higher molecular weight major phenobarbital induced P-450. Antibodies to P-450 Coh will block coumarin 7 hydroxylase activity in DBA/2 and C57BL/6 microsomes. Thus indirectly the Coh locus appears to be distinct from but closely linked to the PB inducible P-450 locus on mouse chromosome 7.

1.7.3. PCN locus (P450-3)

There has been virtually no biochemical analysis done on the steroid inducible P-450s in mice. However, rat derived cDNA clones have been used to map this locus in mice. Analysis of the SDP of restriction fragment length variants in the BXD series showed that this locus was distinct from the Coh, Ah and P450-1 loci. A panel of DNAs from Chinese hamster/mouse hybrids was used to assign the locus to mouse chromosome 6 (Simmons et al, 1985).

1.8. Genetic analysis in humans

1.8.1. P-450s induced by Polycyclic Aromatic Hydrocarbons

The human cDNA homologues to the rat and mouse TCDD inducible P-450s have been isolated (Jaiswal et al, 1985; Quattrochi et al, 1986). This gene family has been assigned to human chromosome 15 (Hildebrand et al, 1985b) and regionally localized to 15q22-q24 (Amsbaugh et al, 1986) by in situ hybridization. This part of chromosome 15 in man appears syntenic with chromosome 9 in mice as

Mpi and Pkm2 also map to these regions (Cox and Gedde-Dahl, 1985).

1.8.2. The human Ah locus

The discovery of the Ah locus in mice stimulated the search for a similar pleiotropic locus in humans. In a study of 353 healthy subjects inducibility of cultured lymphocytes by 3MC varied widely from 1.3 to 4.5. times the basal level (Kellerman et al, 1973a). The population could be separated into low, high and intermediate inducibility. The inheritance of the human Ah locus was therefore thought to be autosomal co-dominance. High inducibility of AHH has been associated with susceptibility to bronchogenic carcinoma in one study (Kellerman et al, 1973b). However these findings were not confirmed by other laboratories who associated the increased AHH inducibility with the disease process itself (Paigen et al, 1977). Lymphocytes from tumour patients had reduced cell growth and reduced protein synthesis. Thus Paigen and colleagues used a progeny test because it estimates the AHH inducibility indirectly on healthy lymphocytes. The progeny test does not depend on the genetic mode of inheritance. Almost any type of inheritance should produce a shift in inducibility toward the high end of the range if patients with lung cancer are shifted towards this range. In this test no difference in induced AHH activity between lung cancer progeny and controls was observed (Paigen et al, 1977). Measurement of AHH in cultured lymphocytes of homozygotic and dizygotic twin pairs showed that basal and induced AHH activity and AHH inducibility are heritable traits (Paigen et al, 1978). Using mouse X human somatic cell hybrids segregating human chromosomes the Ah inducibility locus was assigned to human chromosome 2 (Weibel et al, 1982). In hybrids containing large fragments of chromosome 2 the human Ah locus has

been localized to 2q31-2pter (Ocraft et al, 1985). It is difficult to interpret segregation data from somatic cell hybrids for a regulatory phenomenon which is the end point. Therefore Ocraft and colleagues felt they had mapped the structural gene for AHH activity. The activity assays do not differentiate between the species origin of the structure gene. There is also a question mark over whether they are measuring a regulatory or a structural locus. However there are two compelling reasons for assignment of the Ah locus to chromosome 2 in humans. Firstly, the AHH gene has been assigned to chromosome 15. Secondly, human chromosome 2 has regions of synteny with mouse chromosome 12 (Povey et al, 1985: Lusis et al, 1987).

1.8.3. Human Pharmacogenetics

It is now well established that genetic variation can play a significant role in an individual's response to drug treatment. Genetic differences in the oxidative metabolism of a number of compounds have been observed (Eichelbaum, 1984; Mahgoub et al, 1977; Kleinbloesen et al, 1984; Kupfer and Preisig, 1984). Now these drugs serve as probes for variation in xenobiotic metabolism. The study of the host factors responsible for variations in drug metabolism, may give clues to the origins of individual susceptibility to environmental toxins and carcinogens.

1.8.4. Debrisoquine Hydroxylation

The levels of debrisoquine, an anti-hypersensitive agent, and its primary metabolite 4-hydroxydebrisoquine have been measured in the urine of healthy human volunteers after a single oral administration of debrisoquine (Mahgoub et al, 1977). The ratio

between excreted debrisoquine and its metabolite showed a bimodal distribution. Individuals could be characterized as extensive (EM) or poor metabolizers (PM) of the drug. Family studies demonstrated that the inability to metabolize debrisoquine was inherited as an autosomal recessive trait (Eichelbaum, 1984). In a population study, approximately 5 to 10% of the British Caucasian population are PM (Price-Evans et al, 1980). The debrisoquine polymorphism has been associated with susceptability to Parkinsons disease (Barbeau et al, 1985) and also susceptability to lung (Ayesh et al, 1985) and liver cancer (Idle and Ritchie, 1983). For example in a study of 245 bronchogenic carcinoma patients and matched smoking controls the frequency of PMs was reduced in the cancer population compared to the control population, 1.6% and 9.0% respectively (Ayesh et al, 1985). It was concluded that homozygous dominants have a high risk of developing lung cancer if they smoke. Similarly there is a low incidence of PMs in individuals with hepatocelluar carcinomas caused by exposure to AFB_1 (Peanuts) and hepatitis infection in Africans (Idle and Ritchie, 1985).

Parkinsons disease which is thought to be due to environmental factors has also been associated with a low incidence of PMs of debrisoquine (Barbeau et al, 1985). Other drugs which show variability in oxidative metabolism have also been correlated with debrisoquine metabolism (Idle and Ritchie, 1985).

1.8.5. Mephenytoin and Nifedipine metabolism

The oxidative metabolism of mephenytoin(an anti-convulsant) and nifedipine (a calcium channel blocker) have also been shown to be polymorphic in human populations (Kupfer and Preisig, 1984; Kleisbloesan et al, 1984). Mephenytoin is sold as a racemic

mixture, the S-enantiomer is quickly hydroxylated and excreted while the R-form is eliminated at a much slower rate (Kalow, 1986). In persons with mephenytoin hydroxylase deficiency both the enantiomers accumulate leading to overdose and toxicity. Mephenytoin hydroxylase deficiency occurs as a recessive trait (Inaba et al, 1986) in 5% of Caucasian populations (Kupfer and Presig, 1984) and in 23% of a Japanese population (Jurima et al, 1985). As yet the mephenytoin polymorphism has not been linked to a marker for altered susceptability to environmental toxins or carcinogens. There are currently no studies on this theme.

The metabolism of nifedipine is also bimodal in Dutch populations (Kleinbloesan et al, 1984). PMs of nifedipine represent 17% of the Dutch population. Again there are no reported environmental susceptibilities associated with the metabolism of this drug.

1.8.6. Human P-450s and Drug Metabolism

On the basis of oxidative metabolism of marker substrates the P-450s responsible for debrisoquine, mephenytoin and nifedipine metabolism have been isolated. The P-450, P450DB, responsible for debrisoquine hydroxylation has a molecular weight of 50kD (Gut et al, 1986) and has a high specific activity towards debrisoquine and buffuranol (Disterlath et al, 1985). A second related form P-450 DBII with higher Km for bufuranol and no determinable debrisoquine 4 hydroxylation activity (Gut et al, 1986) has also been isolated. In previous studies it had been shown that female DA rats were poor metabolizers of debrisoquine (Al-Dabbagh et al, 1985). When human anti-P-450_{DB} antibodies were used on Western blots a 20 fold lower amount of P-450DB was found in female relative to male DA rats (Gut

et al, 1986; Larrey et al, 1984). In humans lack of debrisoquine activity appears to be due to a structurally altered protein (Gut et al, 1985).

Debrisoquine is a P-450 which is constitutively expressed and can play a role in general xenobiotic metabolism. P-450_{DB} is distinct from the highly inducible forms on western blot analysis (Wang et al, 1982; Larrey et al, 1984).

By monitoring the oxidative metabolism of mephenytion two P-450s have been isolated from human liver which stereoselectively catalyse the 4-hydroxylation of S-mephenytoin (Shimada et al, 1986). These were termed P-450mpI and P-450mpII and had molecular weights of 48kD and 50kD respectively. These P-450s had no debrisoquine activity and appeared distinct from P-450DB. Western blot analysis could detect no differences between EM and PM hepatic microsomes. Neither P-450mpI or P450mpII was correlated with a deficiency in mephenytoin metabolism. Again this P-450 had high constitutive levels of expression in adult hepatic microsomes.

In the case of nefedipine it was found that male rats were most efficient at metabolizing this compound and that this activity could be induced with PB, PCN and dexamethasone (Guengerich et al, 1986). This suggested that the P-450 responsible for nifedipine metabolism was PB_{2b} (P450-3). In reconstitution assays with purified rat forms the P-450 had high activity towards this compound. The human P-450 responsible for nifedipine oxidation has been isolated and found to cross react with the rat anti-PB_{2b} antibodies. Monoclonal antibodies to human P-450_{NF} also reacted with the rat PB_{2b}. This human P-450 also had high catalytic activity towards steroids (Guengerich et al, 1986) and erythromycin (Watkins et al, 1985). P-450_{NF} had 17β-estradiol activity and at the 2-, 4- and 6β position

of testosterone which makes it similar to the rat form in that it metabolizes androgens (Astrom and De Pierre, 1986). Again this had high levels of expression in EM and PM human livers.

1. 9. Aims of this thesis

As described above, the cytochrome P-450s play a central role in the metabolism of drugs, toxins and steroids. Much of the biochemical analysis of these proteins has been carried out on purified forms from rats and the genetic analysis in mice has been limited and restricted to those forms which are only expressed after exposure to chemical inducing agents. Pharmacogenetic analysis has demonstrated that there is considerable genetic variation in P-450 expression in humans. In some cases these polymorphic differences can be related to susceptibility to exposure from environmental toxins and carcinogens. Initial biochemical analysis of the human P-450s involved in these effects suggest that they correspond to the constitutively expressed P-450 isozymes in rats and mice.

In the light of our current knowledge important questions need to be answered. Firstly, what role do these constitutive P-450s play in a host's response to chemical challenge? As rodents are used extensively for toxicological models, how well does the rodent drug metabolizing system reflect the human system. In an attempt to establish which genetic and regulatory factors govern P-450 expression I decided to use a molecular genetic approach to this problem. The organisms of choice were human and mice.

Mice were chosen as the animal model of choice because of their well characterized genetics, the availability of genetically homogenous inbred strains and subsequently identified genetic

mutants and variants. The cumulative data from this system represents an important contribution to our understanding of structure/function relationships, evolution and the derivation of mutant loci. Comparative biology with other systems can indicate the universality of conclusions reached.

When this investigation was begun I had access to a rat cDNA clone corresponding to a constitutively expressed P-450 of unknown function. It was decided to use this clone to characterize the chromosomal organization of any mouse genes detected by the cDNA and to identify any variants amongst different inbred strains. These variants would be analysed genetically with respect to candidate P-450 functions. A parallel study was begun in humans to isolate the human counterpart and characterise the human DNA with respect to known human pharmacogenetic responses. The regulation of this and other gene families were studied in rodents to establish potentially important regulatory mechanisms in humans.

CHAPTER 2

MATERIALS AND METHODS

2.1. Animals

2.1.1. Laboratory mice and DNAs

Inbred mice used for these studies were obtained from colonies maintained at the Western General Hospital Animal Unit (WG); The Jackson Laboratory, Bar Harbor, Maine (JL); AFRC Physiology and Genetics and Research Station (AFRC); Netherlands Cancer Institute (NCI); Charles River Laboratories (CR). The following is a list of different inbred mice used for DNA analysis: C57BL/6 (WG and JL), CBA/Ca (WG), C3H/HeJ (WG), DBA/2 (JL and CR) 129 (CR), C57/L (JL and AFRC), AKR (JL), AKR/FuRda (NCI), BALB/c By (WG and NCI), BALB/cHeA (NCI), STS/A (NCI), 020/A (NCI), C57BL/10 (a gift from P. Jones). RIII mice were represented by DNA from the C127 breast fibroblast cell line (Lowy et al, 1978). GRS/A DNA was a gift from Dr. J. Hilgers (Hilgers et al, 1975).

Spleen DNAs from the (C57BL/6J X DBA/2J), (C57/L X AKRJ) and (C57BL/6 X C3H/HeJ) RI strains were obtained from the Jackson Laboratory, Maine, U.S.A. (C57BL/6 X DBA/2) RI strain spleen DNAs were also a gift from Dr. G. Bul field. Spleen DNAs from the (020/A X AKR/FuRdA) and (C57BL/6 X BALB/c) RI strains were a gift of Dr. J. Hilgers.

2.1.2. Wild Mice and DNAs

Mus musculus musculus, *Mus musculus domesticus* and *Mus spretus* mice were a gift from Dr. G. Bul field. Spleen DNAs from a (C57BL/6 X *Mus spretus*)F_1 X C57BL/6) backcross were obtained from Dr. Louis Guenet, Pasteur Institute (Robert et al, 1985).

2.1.3. Rat Strains

Rats of the Wistar, Sprague-Dawley (SD) and Dark Agouti (DA) strains were obtained from the Western General Animal Unit.

2.2. Treatment of Animals

2.2.1. Xenobiotic induction

Male Wistar rats (200g) or male CBA/Ca mice (25g) were used. Animals were treated with either phenobarbital (80mg/Kg i.p. in 0.9% saline) or 3-methylcholanthrene (20mg/Kg i.p. in sunflower oil) (Wolf and Oesch, 1983).

2.2.2. Corticosteroid

Male Wistar rats (200g) or male CBA/Ca, BALB/c or C57BL/6 mice were treated with dexamethasone (200mg/Kg i.p. in corn oil) either three consecutive days before use (CBA/Ca) or 6 to 24 hours before use (BALB/c and C57BL/6).

2.2.3. Inflammation

Inflammation was induced in intact male CBA/Ca mice by intraperitoneal injection of <u>Escherichia coli</u> lipopolysaccharide (Serotype No. 0127:B8; Sigma). Three doses were administered in control animals or after administration of phenobarbital, 3 methylcholanthrene and dexamethazone on the three consecutive days prior to sacrifice (2 and 7.5μg lipopolysaccharide/animal) or 24 hr prior to sacrifice (25μg/animal).

2.2.4. Surgical treatment

Liver RNA from hypophysectomised CBA/Ca, C3H/He and BALB/c mice were a generous gift from Dr. R. E. Hill (Hill et al, 1985). Hypophysectomized Wistar rats were purchased from Charles River Laboratories.

2.3. Origins of Eukaryote DNAs

The origin of rodent DNAs has been specified, Human placental DNAs were a gift from Mrs. S. Christie, chimpanzee DNA (Pan troglodytes) was a gift of Mr. R. A. Buckland, sheep (Ovis orientalis) DNA was a gift of Dr. A. J. Clark and fruitfly (Drosophila melanogaster) DNA was a gift of Dr. D. Finnegan. Livers from pig (Sus scrofa domesticus) was a gift of Dr. A. Archibald, from chicken (Gallus gallus) was a gift of Dr. H. Sange and from the South African clawed frog (Xenopus laevis) was a gift of Dr. A. P. Bird. Chinese hamster (Cricetulus griseu) ovary cell pellets were a gift of Ms A. Hall. Liver from Rainbow Trout (Salmo gairdneri) was obtained from Dr. C. R. Wolf.

2.4. Somatic cell hybrids

Somatic cell genetics offers a unique method for understanding gene and chromosome structure in multicellular organisms. Genetic analysis of somatic cell hybrids bypasses the need for sex and lends itself to a molecular analysis of DNAs from such somatic cell lines. It complements mating studies in humans and mice (Puck and Kao, 1982). The fusion of somatic cells from different species can lead to the subsequent segregation and stabilization of chromosomes of

one particular species on the background of another e.g. human chromosomes on a mouse background (Harris, 1965). When rodent somatic cells are fused to human cells the resulting hybrids usually lose human chromosomes, so that often only one or a very few human chromosomes are retained in the hybrids (Nabholz et al, 1969). These human chromosomes can be identified by both cytogenetic and isozyme analysis (Kao et al, 1976). A panel of these hybrids can be used to assign recombinant DNA probes to a chromosomal location (Ruddle, 1981). Similarly somatic cell hybrids between Chinese hamster and mouse somatic cells can lead to the formation of a panel of hybrids which retain a set of mouse chromosomes (Ruddle, 1981). These mouse chromosomes are usually identified by isozyme analysis only (Lalley et al, 1976). In conjunction with other mapping techniques these Chinese hamster/mouse somatic cell hybrid panels can be used to assign recombinant DNA probes to their chromosomal location (Swan et al, 1979; Bennet et al, 1982).

2.4.1. Human/rodent somatic cell hybrids

A panel of human/rodent somatic cell hybrids is maintained at the M.R.C., C.A.P.C.U. by Mrs. S. Christie. The following somatic cell hybrid cell lines have been reported in Lund et al (1983), the numbers in brackets refer to their position on Table 3.1: SK81.Wg3H.L2.15.3.2. (1), C121 (22), CTP41.7 (14), CTP41.3 (15), H22.6.9 (16), Horp25.14 (17), ADP3.10.7 (18), PGME25.8 (20), THYB1.33 (24), THYB1.33.6 (25), SK81-RAG-G5 (12) and ALR.1B5.AG.58A (11). The following somatic cell hybrids were reported in Meehan et al (1988): SK81.Wg3H.L12.15.3 (2), SK81Wg3H:L2.38.2 (9), H22.6.13 (16), Horp25.4 (26), ADP3.10.8 (21), PGME25.9 (27) and X63.BTS4.F9.2 (3). The following hybrids have been described previously and were

a gift by Dr. N. Spurr: 1WILA4.9 (28) (Nabholz et al, 1969); DUR4.3 (4) and CTP41.A2 (20) (Heisterkamp et al, 1982); 3W4CIS (5), DTI.2R (6) and CTP34B4 (19) (Hobart et al, 1981); and Horp9.5 (7) (van Heyningen et al, 1975). The somatic cell hybrids SIF4A31 (13) and SIF-15 (8) were gifts of Dr. S. Povey.

The karyotype of these hybrids is included in Table 3.1. The characterization of the hybrids was by established methods (Lund et al, 1983).

2.4.2. Mouse/hamster somatic cell hybrids

The Chinese hamster/mouse somatic cell hybrids used in this study were a gift of Dr. J. Hilkens. The fusion procedure and characterization of the hybrids has been described (Hilkens et al, 1979; 1983). Briefly the presence of mouse chromosomes was established in each hybrid using variant chromosome markers between mouse and Chinese hamster. Chromosomal assignment of a recombinant DNA probe was made on the basis of its concordance with a variant mouse chromosomal marker (Hilkens et al, 1986).

2.5. Isolation of Eukaryotic DNA

In order to obtain high molecular weight DNA the procedure of Marshall and Burgoyne (1976) for preparing nuclei was used. To prepare DNA from spleens or cell lines polyamine stabilised nuclei were isolated as follows. Freshly excised or thawed material (~1g) was homogenized in 20mls of 0.3M sucrose - 3mM EDTA-'A' buffer.
1x 'A' Buffer is:

60mM	KCl	
0.5mM	spermine	
0.15mM	spermidine	
14mM	β-mercaptoethanol	
15mM	Tris/HCl	pH 7.4

Homogenization was carried out with a motor driver tight fitting teflon/glass homogenizer (Tri-R Stir-R) on ice. The resulting homogenate was layered over 15mls of 1.37M sucrose cushion in 1.5mM EDTA, 1.5mM EGTA-'A' buffer in a 50ml polypropylene tube and spun at 12k for 15 mins in a HB-4 (Sorvall) swing out rotor. The nuclear pellet was resuspended in 10mls 1.5mM EDTA-'A' buffer on ice and an equal volume of 2x pronase buffer (100mM Tris, 300mM NaCl, 200mM EDTA, pH10) was added.

The nuclei were lysed in 0.1% SDS and 100μg/ml of DNAse free RNAaseA (Sigma) was added to remove nuclear RNA. After digestion by RNAaseA for 1 hr at 37°C, protinase K (Sigma) or pronase (Calbiochem) at 100μg/ml in 1% SDS was added to digest protein. Incubation at 37°C was continued for a further 4hrs to overnight. Protein was further removed by phenol-chloroform extractions, the DNA remaining in the aequous phase. The DNA was spooled on a glass rod by the addition of 2 volumes of ice cold ethanol. Spooled DNA was washed in 70% ethanol air dried and unspooled in 1-4mls of T.E. (10mM Tris (pH8.0), 2mM EDTA) The DNA was left at 4°C overnight to go into solution and the OD determined at 260nm (DNA at an OD_{260} of 1 equals 50μg/ml). Typically one mouse spleen gave a yield of 1mg of DNA.

2.6. Preparation of prokaryotic DNA

2.6.1. Small scale preparation of plasmids

The LiCl boiling method was used for pUC and pEMBL based vectors (Wilimzig, 1985). 3ml of LB or 2x YT broth containing ampicillin (50µg/ml) was inoculated with a single bacterial colony and grown overnight at 37°C with shaking. The overnight culture was pelleted sequentially in a 1.5ml Eppendorf tube and the supernatant discarded. The pellet was resuspended in 400µl of TELT (50mM Tris-HCl (pH7.5), 62.5mM EDTA, 2.5M LiCl and 0.4% Triton X100). To this was added 40µl of 100mg/ml fresh lysozyme in TELT. The sample was boiled for 90 sec and cooled on ice for 10 minutes which precipitates rRNA and protein. The precipitate was pelleted by centrifugation for 8 mins and the supernatant removed to another tube containing 0.6 volume of isopropanol. This was spun again for 10 mins at 4°C, the resulting pellet was washed in 70% ethanol and dried and resuspended in 40µl of T.E. The plasmid was checked by diagnostic restriction enzyme digestion and electrophoresed on a 14 x 20cm agarose horizontal gel.

It was found that pBR322 and pKT218 plasmids did not respond well to TELT mini preps method so a scaled down version of the maxi plasmid preparation was used (Birnboim, 1983). 10ml overnight cultures of tetracycline resistant colonies were used and processed in the same way as the maxi-plasmid preparation except they were not loaded on CsCl gradients.

2.6.2. Large scale preparation of plasmid DNA

This method used a combination of differential precipitation to remove E.coli genomic DNA and CsCl equilibrium centrifugation

(Birnboim, 1983). Before CsCl centrifugation the plasmid DNA was usually clean enough for restriction enzyme digestion, isolation of inserts, radiolabelling and bacterial transformation. Plasmids were purified on CsCl gradients when used as cloning vectors and for use as in situ hybridisation probes to mitotic chromosome spreads.

Single colonies were used to inoculate 5mls of LB (pBR322; pKT218) 2XYT (pUC or pEMBL) broth in the presence of the antibiotic tetracycline (20µg/ml) or ampicillin (50µg/ml) respectively. The resulting overnight cultures were used to inoculate 500mls of LB or 2XYT and grown overnight at 37°C with vigorous agitation.

The next day cells were harvested by centrifugation at 6K for 10 mins. The cell pellet was suspended in 2mls of GTE (50mM glucose, 2mM Tris-HCl pH8.0, 10mM EDTA) to which was added 8mls of lysozyme (10mg/ml) in GTE. This was left on ice for 30 mins. 20mls of fresh alkaline SDS (0.2M NaOH, 1% SDS) was added 10 mins later. 15ml of high salt solution (3M CH_3COOK, 2M CH_3COOH, pH5.0) was also added and mixed in. A white precipitate forms this is removed after 20 min incubation on ice by centrifugation at 8K for 10 mins. The supernatant was filtered through muslin and the DNA precipitated by addition of 2 vols of 100% ethanol and incubation at -20°C for 20 mins. The precipitate was collected by centrifugation at 10K for 20 min and redissolved in 6 mls of acetate MOPs (0.1M CH_3COONa, 0.05M MOPs pH8.0). The DNA is precipitated by the addition of 2 vols of 100% ethanol and incubation at -20°C for 20 min. The precipitate was collected by centrifugation at 10K for 10 min. The DNA is resuspended in 10mls of T.E. and the E.coli genomic DNA and rRNA is differentially precipitated by the addition of 0.5 vol. of 7.5M CH_3COONH_4 and incubation on ice for 20 min. This solution is then centrifuged at 12K for 20 min and the pellet is discarded. To the

supernatant 2 vols of 100% ethanol was added and the sample incubated at -70°C for 45 min (or -20°C overnight). The precipitate is collected by centrifugation and consists mainly of plasmid DNA, RNA and lipopoly saccharide. This was resuspended in 2mls of T.E. containing 50mM NaCl and SDS (0.1%). RNAaseA (DNAase free) was added at 100mg/µl and the solution incubated at 37°C for 15 min. The SDS concentration was increased to 1% and pronase or proteinase K added to 100µg/ml and incubated at 37°C for a further hour. An equal volume of phenol/chloroform (1:1) is added and the solution well shaken. The phases were separated by centrifugation at 400g for 10mins and the aqueous phase extracted sequentially with equal vols of chloroform and ether respectively. DNA was precipitated by addition of 1/10 vol of 2M CH_3COONa (pH5.5) to the aqueous phase and 2.5 vols of 100% ethanol and incubated at -70°C for ~1 hour. The DNA pellet is redissolved in 1ml of T.E. if purification is to be stopped here, it can be checked by restriction enzyme digestion for purity and concentration. For banding on CsCl density gradients the volume is increased to 3.21ml with T.E. and 3.55gm of CsCl is added (1gm/ml) and 340µl of 10mg/ml EtBr. The dark red solution is loaded into a 5ml polyallomer (Sorvall) tube and put in a TV865 vertical rotor (which allows the CsCl to reach equilibrium rapidly). Centrifugation was at 40K for a minimum of 18 hrs at 20°C. The plasmid band was visualised by exposure to U.V. (300nM) and collected with a syringe. The EtBr is removed by extraction with 2-butanol saturated with isoamyl alcohol. The DNA was precipitated with 2 vols of 70% ethanol following incubation at -70°C for 30 mins, the DNA was collected by centrifugation at 10K for 15 min and resuspended in 400µl of T.E. and reprecipitated with 0.2M CH_3COONa (pH5.5) and 2 vols of 100% ethanol. The DNA was checked by running

on agarose gels and by reading the O.D. at 260nm (1 OD_{260} = 50µg/ml DNA).

2.6.3. <u>Preparation of bacteriophage lambda DNA</u>

The method of Helms was used (1985). 1ml of overnight Q358 or LE 392 were infected at a high titre (10^5pfu) and plated out on large 20 x 20cm plates. After lysis the phage were collected by washing with 40mls of SM (50mM Tris-HCl (pH7.5), 0.01% gelatin, 10mM $MgSO_4$, 100mM NaCl). To this was added a few drops of chloroform and the phage suspension was spun at 3K for 10 min to remove debris. The phage were collected by centrifugation for 90 minutes in a 8x 50ml MSE fixed angle rotor at 35K using an MSE Prespin 65. The phage are resuspended in 3mls of phage lysate buffer (10mM Tris-HCl pH8.0) and the lysate is applied to a 1ml DEAE-cellulose (DE.52) column, the column run through is discarded. 5ml of chase buffer (10mM Tris-HCl [pH8.0]], 10mM $[CH_3COO]_2Mg$, 60mM CH_3COONa) is added and the run through discarded. Then 1ml of elution buffer (10mM Tris-HCl (pH8.0), 50mM $[Ch_3COO]_2Mg$) is added and the run through discarded. The next 0.6ml of elution buffer are collected and the phage lysed by the addition of 10µl of 100µg/ml proteinase K and 24µl of 10% SDS. After 5min 100µl of 3M CH_3COOK are added and the sample heated to 88°C for 20 min and then cooled on ice (a white precipitate forms). The white precipitate was removed by centrifugation (12K for 10 min) and to the transferred supernatant is added 20µl of 1mg/ml mussel glycogen to act as carrier. The DNA was precipitated by the addition of 700µl of isopropanol, collected by centrifugation, the pellet was washed with 70% ethanol, air dried and resuspended in 100µl of T.E. The DNA was checked for purity on agarose gels.

2.7. Analysis of DNA

2.7.1 Restriction endonuclease digestion

Restriction enzymes were purchased from Amersham International plc, BRL, Boehringer-Mannheim GmbH or New England Biolabs. Restriction endonuclease digestions were carried out according to the manufacturers' recommendations in either high, medium or low salt buffer (Maniatis et al, 1982). A 4-5 fold of excess of enzyme was used over a 5-12 hr incubation period. The reaction was stopped by the addition of 0.1 vol of 10x stop buffer (20% Ficoll, 100mM EDTA).

High salt buffer:	100mM NaCl, 10mM $MgCl_2$
	500M Tris-HCl (pH7.5),
	10mM β-mercaptoethanol
Medium salt buffer:	50mM NaCl, 10mM $MgCl_2$
	10mM Tris-HCl (pH7.5)
	10mM β-mercaptoethanol
Low salt buffer:	10mM $MgCl_2$
	10mM Tris-HCl (pH7.5)
	10mM β-mercaptoethanol
SmaI/KpnI buffer:	20mM KCL, 10mM $MgCl_2$
	10mM Tris-HCl (pH8)
	10mM β-mercaptoethanol

Usually 5-10μg of genomic DNA and 0.5μg to 1μg of plasmid DNA were digested for subsequent analyses.

2.7.2. Gel electrophoresis of DNA

DNA was analysed on horizontal agarose gels (Sigma Type II) of 0.8 to 1.5% in TAE buffer (0.04M Tris acetate, 0.002M EDTA (pH8.0).

Gels were run at a voltage of 0.3 to 5v/cm. After each run gels were stained by addition of a drop of EtBr (10mg/ml) in buffer for 10 min and destained in buffer for 20 min before visualization of DNA under UV illumination. Gels were photographed with Kodak Plus-X professional film and developed through a Fugi film X-ray auto processor.

2.7.3. Southern Blotting

Gels were transferred to nitrocellulose or Hybond-N nylon sheets (Amersham) according to Southern (1975). Gels were irradiated with U.V. for 5 min to break up large DNA fragments (this improves their transfer) and then soaked in denaturing solution (1.5M NaCl, 0.5M NaOH) for 45 min with shaking. Gels were then transferred to neutralizing solution (3M NaCl, 0.5M Tris-HCl, pH7.5) and left for 45 min to 3 hrs. After neutralization DNA was transferred from the gels to nitrocellulose or Hybond in 20x SSC (3M NaCl/0.3M sodium citrate) through a wick of 3mm paper. DNA was immobilized on nitrocellulose by baking at 80°C *in vacuo* or on Hybond by exposure to a U.V. light box for 5 minutes.

2.7.4. Probe Stripping of filters

Filters were stripped of radiolabelled probes by immersion in 500mls of 1% SDS at 95°C and allowed to cool to room temperature.

2.8. Isolation of insert DNA

The cDNA inserts were isolated from their parental plasmid DNA by preparative agarose gel electrophoresis according to Dretzen *et al* (1982). Plasmid DNA (~50μg) was digested to completion with the appropriate enzyme and electrophoresed on 1.2% low melting point agarose (FMC). The relevant band was localized by EtBr staining and UV visualization and a slit inserted in front of it with a fine point scalpel. A piece of DEAE nitrocellulose (Schlier and Schull) which had been equilibrated in running buffer was inserted into the slit and electrophoresis continued until the DNA fragment was run "in" to the DEAE paper. The paper containing bound DNA was washed in DEPC treated water and the DNA was eluted off in 400μl of 2M NaCl in T.E. at 68°C for 20 mins. After elution the paper was removed and the DNA precipitated with two vols of 100% ethanol. After recovery of DNA by centrifugation it was resuspended in 50μl of T.E. and 1/10 vol. run on gel to determine the DNA concentration.

2.9. DNA Size markers

Three sets of DNA size markers were used. *Hind*III digested cI 857 Sam 7 lambda phage DNA, *Eco*RI digested lambda phage DNA and *Hae*III cut OX174 coliphage DNA. Generally 0.5-1.0μg of marker DNA was run per track.

Molecular Weight markers	OX174 (*Hae*III)	lambda (*Hind*III)	lambda (*Eco*RI)
Sizes of fragments in kb	1353	23130 (sticky)	21226 (sticky)
	1078	9416	7421
	872	6682	5804
	603	4361 (sticky)	5643
	310	2322	4878
	281	2027	3530 (sticky)
	271	564	
	234	125	
	194		
	118		
	72		

2.10. Autoradiography

Radioactive ^{32}P-labelled nucleic acids hybridized to nitrocellulose or Hybond filters were visualized by exposure to Kodak X-AR5 film. Filters were placed in X-ray cassettes with an intensifying screen and preflashed film laid on them. Cassettes were then stored at -70°C for the relevant exposure time. (Typically 3 days to 2 weeks).

2.11. Bacterial culture

2.11.1. Media used for culture of E. Coli

L Broth:	10g tryptone (Difco)	
	5g yeast "	
	10g NaCl	/litre pH7.2

For the growth of plasmids glucose (BDH Chemicals) was added to 2g/1.

For growth of bacteriophage lambda maltose (Sigma) was added to 2g/1 and $MgSO_4$ to 0.5g/1 (2mM) was added before autoclaving.

L-Agar contains in addition 15g agar/1 and 6.5g/1 of agarose (Sigma, Type II) for Top agarose.

CY Agar:	10g caseamino acids (Difco)	
	5g yeast	
	3g NaCl	
	1g KCl	
	10g Agar	/litre pH7.2

for CY Agarose 6.5g of Agarose were added instead of agar.

H-agar:	10g tryptone
	4g NaCl
	12g Agar

H agarose contained only 8g of agarose instead of agar.

2xYT:	16g Tryptone	
	10g Yeast extract	
	5g NaCl	/litre pH7.0

Minimal Agar plates:

15g Bacto agar		
10.5g K_2HPO_4		
4.5g KH_2PO_4		
1g $(NH_4)_2SO_4$		
0.5g sodium-	)	
citrate $2H_2O$	)	make as 100x concen-
0.2g $MgSO_4.7H_2O$	)	trate and filter
5µg thiamine HCL	)	sterilize, add
2g glucose	)	separately to above.

2.11.2. Antibiotics

Ampicillin (Sigma) was made up as a 1000x stock in sterile ddH_2O at 50mg/ml.

Chloramphenicol (Sigma) stock solution was made up at 36mg/ml in 50% ethanol and used on plates at a concentration of 20µg/ml.

Tetracycline (Sigma) was made up as 500x stock at 12.5mg/ml in 50% methanol.

2.12. Bacterial strains

2.12.1. pBR322 vector strain

E.coli K-12 HB101 (hrs^-, hrm^-, $recA^-$, gol^-, str^-, $B1^-$). This strain is commonly used as a recipient in transformation of pBR322 based vectors and is a good host for large scale growth and purification of plasmids (Bolivar et al, 1977).

2.12.2. pUC and pEMBL vector strains

E.coli K12 JM83 (ara, Lacpro, strA, thi, o80d, lac ZW 15) (Veira and Messing, 1982). This strain can be a host for the high copy pUC (Veira and Messing, 1982; Norrander et al, 1983) and pEMBL (Dente et al, 1983) vectors. Both these plasmids contain the β-galactosidase genes. In the presence of XGal and ampicillin the JM83 strain carrying these vectors appear blue, if the β-galactosidase gene has been disrupted by insertion of DNA into the cloning sites of these vectors the colonies will appear white but will still be ampicillin resistant.

2.12.3. M13 coliphage strain

E.coli K12 JM101 (W lac pro, thi, supE, F'traD36, pro AB, lac q^2 M15). This strain is used for the propagation of M13 coliphage. The mutation $lac^{q}2$ mutation leads to overproduction of the lac repressor. To compete out the repressor IPTG is added so that XGal can be metabolized to its blue substrate by the β-galalactosidase carried on the M13 mp8/9 coliphages. Again when this gene is disrupted by the insertion of DNA the M13 plaques will appear clear.

2.12.4. Bacteriophage lambda strains

LE392 ($hsdR^-$, SupE, SupF) (Kaiser and Murray, 1985). This strain was used for the propagation of the lambda gt 11 human liver cDNA library, it is deficient in restricton ($hsdR^-$) to protect introduced DNA.

E.coli Q358 ($hsdR^-$, SupE)

E.coli Q359 ($hsdR^-$, SupE, p2).

This strain carries a P2 lysogen so that spi^- recombinants of

phage will more readily grow on this host (Karn _et al_, 1983; Frischauf _et al_, 1983).

2.13. Recombinant libraries

2.13.1. Liver cDNA libraries

a) Humans: A human liver cDNA library of 230,000 members (Woods _et al_, 1982) was used to screen for human PB-1 P450 cDNAs. This library was made from RNA from a single white male individual and was constructed in the pBR322 deletion derivative vector pKT218 (Talmadge _et al_, 1980). This plasmid is deleted with respect to most of the β-pencillinase gene and so is only tetracycline resistant. A second human cDNA library in lambda gt11 of 300,000 members was obtained from Clontech (California).

b) Mouse: A DBA/2 male liver cDNA library was obtained from Clontech, California, USA. This library of 100,000 members was constructed in the pBR322. vector.

2.13.2. DBA/2 mouse genomic library

This library was a gift of Dr. R. E. Hill and was constructed in the bacteriophage EMBL3 vector (Frischauf _et al_, 1983; Karn _et al_, 1983) by size selecting a Sau3A partial digest of DBA/2 mouse spleen DNA and cloning into the _Bam_HI sites of this vector.

2.14. Ligation of DNA fragments

Ligations were performed by the following procedure: 200ng of linearized vector DNA was incubated with a 3-5 fold molar excess of

insert DNA in ligation buffer (50mM Tris-HCl pH7.5, 10mM $MgCl_2$) plus 1mM DTT, 1mM rATP and 10 units of T4 DNA ligase in as small a volume as possible. Incubations were carried out at 12-16°C overnight and then stored at -20°C before use.

2.15. Vectors

2.15.1. pBR322

This plasmid (Fig.2.1) has proved a popular cloning vector for cDNA libraries, because it is small (4.3kb), carries five unique restriction sites for insertion of DNA (Bolivar et al, 1977). It contains two selective markers which confer resistance to the antibiotics tetracycline and ampicillin. Insertion into the PstI site inactivates the β-pencillinase gene which codes for ampicillin resistance. This site has been utilized for cloning by the oligo (dG) oligo (dC) trailing method (e.g. Barth et al, 1982).

2.15.2. pKT218

This plasmid is a deletion derivative of pBR322 (Talmadge et al, 1980). It has been deleted for most of the β-pencillinase gene from the PstI site toward the pencillinase signal sequence at position 218 from the EcoRI site (Fig. 2.1). The PstI was recreated by ligation of PstI linkers. It was hoped that cDNA libraries constructed in this vector would more readily secrete recombinant products.

2.15.3. pUC 8/9

These are a series of highly popular high copy number plasmids with multiple cloning sites for insertion of DNA fragments (Vieira

and Messing, 1982). These plasmids carry the ampicillin resistance gene and the β-glactosidase gene (Fig. 2.1) The multiple cloning sites are positioned in the amino terminus of the β-galactosidase gene. Insertion of DNA fragments into these sites disrupts gene function which can be tested by a histochemical reaction on agar plates. The functional β-galactosidase gene can convert X-Gal to a blue indolyl derivative, in recombinant pUCs the colonies whilst still ampicillin resistant remain colourless in the presence of X-Gal.

2.15.4. pEMBL 8/9

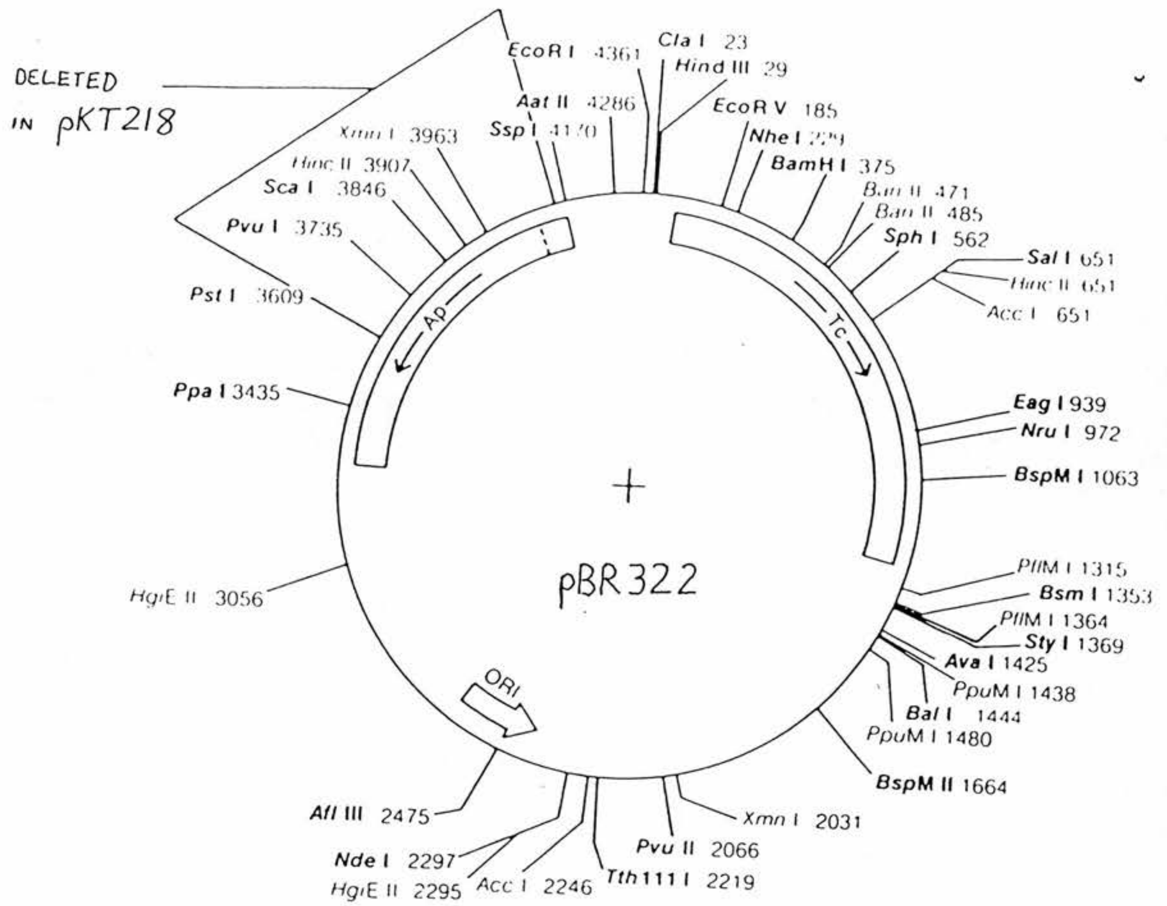
These plasmids (Fig.2.1) are derivatives of pUC 8/9 which contain the region of the Fl genome carrying all the elements required for DNA replication and morphogenesis (Dente et al, 1983). Thus upon super infection with Fl phage, virus capsids contain Fl ss DNA or pEMBL ss DNA at about the same frequency. In the same manner as pUC plasmids the pEMBL variety contain multiple cloning sites in the amino terminal of the β-galactosidase gene and the ampicillin resistance gene. The ss DNA from pEMBL recombinants can be used as templates for sequencing using the universal primer. However, it was found that large fragments inserted into pEMBL tended to rearrange when going through the Fl replication step (R. E. Hill and R. Meehan, unpublished observations). Therefore these vectors were only used to maintain recombinant DNA fragments and not as sources of sequencing templates.

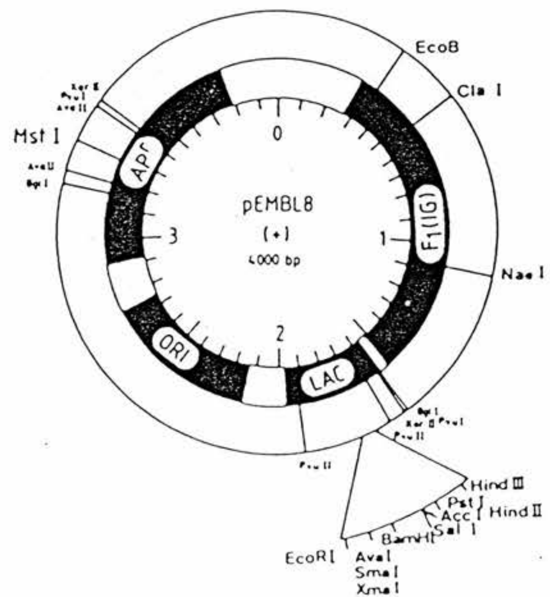
2.15.5. M13 mp8/9

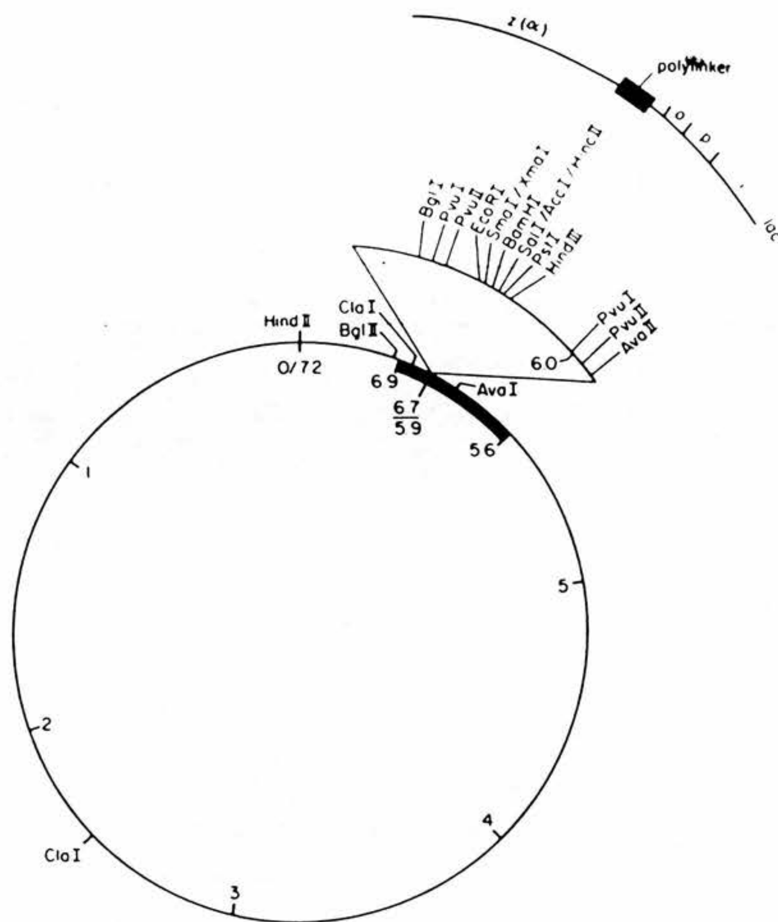
The bacteriophage vector M13 mp8/9, M13 18/19 were developed as cloning vectors for DNA sequencing (Messing, 1983; Norrander et al,

FIGURE 2.1.

Restriction maps of vectors used in this study. pBR322 was first described by Bolivar et al, 1977. pKT218 is a deletion derivative of this (Talmadge et al, 1980). pEMBL is from Dente et al, 1983, pUC from Veira and Messing, 1982 and the bacteriophage vector M13 mp8/9 from Messing, 1983. Also shown is the polylinker region for pUC8/9, pEMBL 8/9 and m13 mp8/9. The insertion of the polylinker region into the β-galactosidase gene is also shown.


DELETED IN pKT218
EcoR I 4361
Cla I 23
Hind III 29
Aat II 4286
EcoR V 185
Ssp I 4170
Nhe I 229
Xmn I 3963
BamH I 375
Hinc II 3907
Ban II 471
Sca I 3846
Ban II 485
Pvu I 3735
Sph I 562
Sal I 651
Hinc II 651
Pst I 3609
Acc I 651
Ap
Tc
Ppa I 3435
Eag I 939
Nru I 972
BspM I 1063
PflM I 1315
pBR322
Bsm I 1353
HgiE II 3056
PflM I 1364
Sty I 1369
Ava I 1425
PpuM I 1438
ORI
Bal I 1444
PpuM I 1480
BspM II 1664
Afl III 2475
Xmn I 2031
Pvu II 2066
Nde I 2297
HgiE II 2295
Acc I 2246
Tth111 I 2219


EcoB
Cla I
Mst I
0
APr
pEMBL8
(+)
4000 bp
3
1
F1(IG)
Nae I
2
ORI
LAC
Pvu II
Hind III
Pst I
Acc I
Hind II
Sal I
BamHI
EcoRI
Ava I
Sma I
Xma I



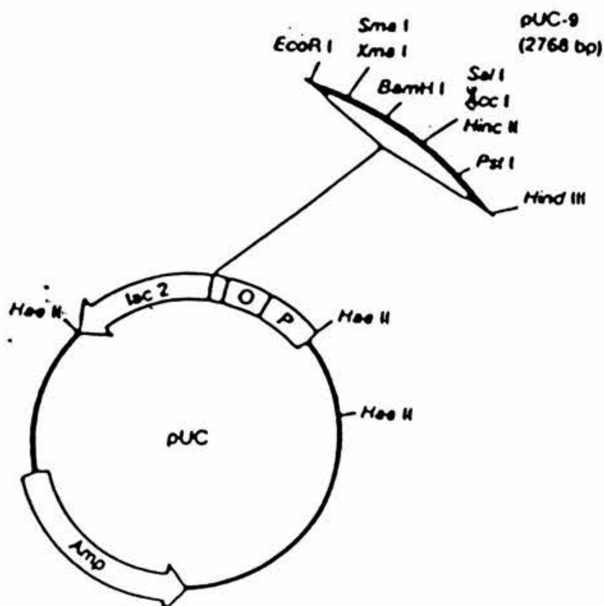
M13 mp8

A T G A C C A T G A T T A C G A A T T C C C G G G G A T C C

EcoRI — SmaI — XmaI — BamHI

G T C G A C C T G C A G C C A A G C T T G G C A

SalI / AccI / HincII — PstI — HindIII



1983). During the life cycle of the M13 bacteriophage its genome can exist in two forms: as double stranded DNA during the replicative phase inside the host bacterium or as a single stranded DNA filamentous phage which is secreted by the host into the growth medium. As in the pUC plasmids foreign DNA is cloned into the poly linker region of the aminoterminal of the lac Z gene in the M13 replicative form (RF) (Fig. 2.1). Recombinant M13 phage can be identified as clear "plaques" when plated on JM101 host E.coli in the presence of IPTG and X-Gal. Annealing of the universal oligonucleotide primer next to the multiple cloning site of the ss form allows inserts to be used as templates for copying by the Klenow fragment of E.coli DNA polymerase I. Thus inserts can be copied from the multiple cloning site and used as templates for the dideoxy sequencing reaction (Sanger et al, 1977).

2.15.6. EMBL 3 bacteriophage lambda

This is a replacement vector for cloning fragments of 9-20kb (Karn et al, 1983; Frischauf et al, 1983). The selection scheme for recombinants depends on plating these phage on P2 lysogen containing strains which will select against phage carrying a functional gam gene. This recombinant phage (Spi^-) in which the gam gene has been replaced will only grow on such a host e.g. Q359.

2.15.7. cDNA clones

A large number of liver cDNA clones were used in this study and these are listed in Table 2.1. A clone for muscle actin, pAM (Minty et al, 1981) was also used.

TABLE 2.1

Liver cDNA probes used in this study

Plasmid	Species	Product	References
plv54	mouse	contrapsin	Barth *et al*, 1982
plv56	"	MUP	"
plv1806	"	apolipoprotein A1	"
plv1706	"	albumin	"
plv1796	"	α1-Antitrypsin	"
LVA329	mouse	transferrin	Clissold and Bishop, 1981
pR17	rat	cytochrome P450-2B	Adesnik *et al*, 1981
p5'2	"	cytochrome P450-2B	Adesnik *et al*, unpublished
pTF-1	"	cytochrome P450-2C(f)	Freidberg *et al*, 1986
pTF-2	"	cytochrome P450-2C(PB-1)	"
pCL46	mouse	cytochrome P450-1	Negishi *et al*, 1981
pPB3-15	mouse	cytochrome P450-2C	This study
pM8-1	"	"	"
pPB5-21	"	"	"
pPB4-17	"	"	"
pm_1EH4	mouse	metallothionein	Durnam *et al*, 1980
pHL5,5	human	cytochrome P450C2C	This study
pHL1,5	human	"	"
pPB2-29	human	unknown	Wolf *et al*, unpublished
pR17-13	"	cytochrome P450C2B	Miles *et al*, unpublished
pPCN	"	cytochrome P450C3	Stevenson *et al*, unpublished
pMC_{1b}	"	cytochrome P450C1	Stevenson *et al*, unpublished
plivS-5	mouse	prealbumin	Derman *et al*, 1981
plivS-7	"	unknown	"
plivS-8	"	"	"
plivS-9	"	"	"
plivS-10	"	"	"
plivS-11	"	"	"

2.16. Bacterial transformation by plasmid DNA

2.16.1. pBR322, pUC, pEMBL and pKT218

Competent E.coli cells were prepared by the $CaCl_2$ method according to Maniatis et al (1982). The strain of choice was grown from an overnight culture to an OD_{550} of 0.6. The culture was chilled on ice and spun down (3K for 5 min) and resuspended in 0.5 volume of 50mM $CaCl_2$ and kept on ice for a further 20 mins then the cells were spun down again and resuspended in 1/10 of the original volume of 50mM $CaCl_2$. The cells were then stored at 4^oC and used for up to 5 days after their preparation for transformation by DNA.

For transformation DNA was added to 200µl of cells and stored on ice for 20 min and then heat shocked at 42^oC for 90 sec. The cells were returned to the ice for a further 20 min and then 1ml of appropriate broth was added and the mix incubated at 37^oC to allow for expression of the selectable marker. 20µl of the incubation mix were plated directly on the plates of choice and incubated at 37^oC overnight.

2.16.2. M13

The procedure of Messing (1983) was used here. The E.coli strain JM101 was used for M13 transformations which requires the production of a pilus by the host cell to be infective. The pilus is encoded by an F plasmid. A colony of JM101 is picked from minimal (+glucose) plates and grown up in 20ml of 2XYT to an OD_{550} of 0.3 and competent cell prepared as in the previous section. The cells are aliquoted into 200µl portions, the ligation mix added and the mix kept on ice for 40 min In the meantime a fresh culture of JM101 is prepared for the lawn by adding 2ml of 2XYT to the dregs of

the culture left over from making the competent cells. After incubation on ice the ligation/competent cells mix is heat shocked at 42°C for 90 sec while cells are being heat shocked, 30µl of BClG (20mg/ml in DMF); 20µl of IPTG (24mg/ml in water) and 0.2ml of exponential JM101 are added to 4ml of molten top H-agarose. The agarose is added to the transformed cells and plated out on minimal (glucose) plates or H plates and incubated at 37°C overnight.

2.17. RNA extraction

2.17.1. Guanidinium hydrochloride method

For the vast majority of RNA extractions the method of Cox (1968) was used. 1g of fresh tissue was placed in 20mls of 8M guanidinium hydrochloride (GHCL) solution (8M GHCL, 0.01M EDTA, 0.05M Tris, pH7.5) and homogenized in an omnimix MSE at maximum speed for 45 secs. Cellular debris is removed by a clearing spin at 8K for 10 mins. The supernatant is removed to a fresh tube and the nucleic acids precipitated by the addition of 0.5 vol of 100% ethanol and incubation at -20°C for 45 mins (maximum incubation time). The RNA is collected by centrifugation at 10K for 10 mins in fixed angle or swing out rotor. Depending on the pellet size, it is resuspended in 5-10mls of 6M GHCL with the help of a tight fitting glass to glass dounce homogenizer. The RNA is again precipitated by addition of 0.5 vol of absolute alcohol and incubation at -20°C for greater than 45 min. The RNA is collected by centrifugation and resuspended in 6M GHCL as before, precipitated again and resuspended in DEPC treated H_2O (0.1% diethylpyrocarbonate in ddH_2O which has been autoclaved to inactivate this methylating agent). One tenth vol of 2M NaOAc pH5.5

is added and 2 vols of absolute alcohol and the mix incubated over night at -20°C. The RNA is again collected by centrifugation and the precipitation step repeated so as to remove traces of GHCL. Then the RNA is resupended in 1-2mls of DEPC H_2O and the RNA concentration measured by measuring its OD_{260} (for RNA 1 OD_{260} = 40μg/ml) and stored at -70°C.

2.17.2. Lithium chloride/urea method

This method was used when RNA was to be extracted from frozen tissues and follows the procedure described by Jackson et al (1985). RNA was prepared by homogenizing 1g of frozen tissue in 20mls of 3M LiCl/6M urea. The RNA was allowed to precipitate overnight at 4°C and collected by centrifugation at 10K for 30 mins at 0°C. The pellet is resuspended in 8ml of 3M LiCl/6M Urea and spun again at 10K for 30 mins at 0°C. At this point the RNA is resuspended in 6 mls of T.E. plus 0.5% SDS to which is added proteinase K to 50μg/ml and incubated at 37°C for 30 mins. The RNA solution was extracted sequentially with phenol, chloroform and ether. Ribonucleic acid is precipitated by the addition of 1/10 vol of 2M CH_3OONa and 2.5 vols of absolute ethanol and incubation at -20°C for greater than three hours. The RNA is collected by centrifugation at 10K for 10 mins and resuspended in 1-3mls of DEPC H_2O and its concentration determined spectrophotometrically at 260nm and then stored at -70°C.

2.17.3 Isolation of poly(A)± RNA.

The method of Aviv and Leder (1972) was followed. Before loading, the RNA was denatured at 80°C for 10 mins and then an equal volume of 2x loading buffer (20mM Tris pH7.6, 0.5M NaCl; 1mM EDTA, 0.1% SDS) was added. This was loaded to a 1ml oligo (dT)-cellulose

column which had been equilibriated with loading buffer. The flow through was collected and reapplied to the column. The column was then washed in loading buffer minus SDS and then eluted with 3 volumes of elution buffer (10mM Tris pH7.5, 1mM EDTA), 0.5ml fractions were collected and assayed spectrophotometrically for RNA. The most concentrated fractions were pooled and the RNA precipitated by the addition of CH_3COONa and ethanol as before.

2.17.4. Gel electrophoresis and analysis of RNA

RNA was size fractionated through formaldehyde denaturing gels by a modification of Derman *et al* (1981) according to Meehan *et al* (1984). RNA was suspended in 50% formamide, 6% formaldehyde and 10mM sodium phosphate, pH6.5. Samples were heated to 50°C for 15-30 min prior to loading and then bromophenol blue/orange G dye added in the presence of Ficoll (10%). The electrophoresis buffer was 10mM sodium phosphate pH6.5 and samples were run at 30V for 10-16 hours. The gels were soaked in 10x SSC prior to transfer to nitrocellulose or Hybond-N (Amersham) in 10x SSC according to Southern (1975). Filters were then baked *in vacuo* at 80°C if nitrocellulose or if Hybond exposed to UV for 5 minutes to bind the RNA to the filter prior to hybridization.

2.18. Radiolabelling of DNA

2.18.1. Nick translation

Double stranded DNA (dsDNA) was labelled by the nick translation (Rigby *et al*, 1977) reaction. *E.coli* DNA polymerase repairs nicks in dsDNA by adding a residue to the 3'-OH terminus of

the nick. It also has 5' exonuclease activity with a net result that nucleotides are removed in a 5' direction and replaced by addition at the 3' end. Typical reactions contained 0.1-0.2μg of DNA in 20μl. The reaction contained 2μl of 10x nick translation buffer (500mM Tris pH7.5, 50mM $MgSo_4$, 0.1M β-mercaptoethanol, 10mg/ml BSA and 60mM each of dATP, dCTP and dGTP), 30-40μCi of α-^{32}P dTTP (>800Ci/mmol [Amersham]), 1μl of DNAase 1 (BRL) (0.1μg/ml freshly diluted from a 1mg/ml stock) and 1-5 units of E.coli DNA polymerase I. This reaction was incubated at 16°C for 1-2 hours and the percentage incorporation of the labelled mix determined by trichloroacetic acid precipitation (TCA), (Furlong, 1967). GF/4B filters (Whatman) were spotted with the radioactive mix and counted in a Packard Tri-carb scintillation counter. The filter is then washed extensively with 5% TCA and counted again. The percentage of counts remaining on the filter gives an estimate of incorporation. Normally specific activities were greater than 10^8dpm/μg of DNA.

The reaction was terminated by the addition of EDTA to 20mM. Labelled DNA was isolated by SP-50 chromatography. Briefly, the mix is applied to a 5ml SP-50 column in 1x column buffer (0.3mM NaCl, 10mM NaOAc pH5.0). Large molecules are excluded from the column and pass through more rapidly than small unincorporated nucleotides. Separation is monitored by a Geiger counter and the labelled fraction collected in 0.3ml. Before use as probes the labelled DNA is denatured in a boiling water bath for 10 mins in a 4ml loose capped Sarstedt tube. The DNA is then quenched on ice for 10 mins before addition to the hybridization mix.

2.18.2. Random priming

This method was a modification of that reported by Feinberg and

Vogelstein (1983; 1984) using Klenow fragment to extend random hexamers (Pharmacia) with the DNA of interest as template. Double stranded linear DNA is denatured by boiling for 2 minutes in the presence of 0.2μg of hexamers, cooled on ice made up to 20μl by the addition of 2μl of 10x nick translation buffer (previous section) 30-40μCi α-^{32}P TTP, DEPC H_2O and 1-3 units of Klenow. Klenow retains only the 5'-3' polymerase activity and the 3'-5' exonuclease activity but lacks the 5'-3' exonuclease activity. Because of this the labelling reaction can be carried out overnight at 37°C without degradation of the product. Percentage incorporation was measured by TCA precipitation and the reaction terminated by diluting it in 400μl of T.E. The mix is not cleaned up any further but heat denatured before use as a hybridization probe.

2.18.3. End-labelling reaction

This labelling reaction is mediated by T4 polynucleotide kinase which catalyses the transfer of the α-phosphate of ATP to a 5'-OH terminus in DNA or RNA (Richardson, 1971). Typically 5-10 pmoles of 5' ends are labelled in a reaction. The reaction mix consists of 1μl of 10x kinase buffer (0.5M Tris, pH7.6, 0.1M $MgCl_2$, 50mM DTT, 1mM spermidine, 1mM EDTA), 5-10pM of 5' ends, 80mCi -^{32}P ATP (3000Ci/mmol) and 10-20 units of T4 polynucleotide kinase (Biolabs). The reaction mix is incubated at 37°C for no more than 30 mins and stopped by addition of 2μl of 0.2M EDTA. The reaction can be checked by running the sample (typically 20 mers on a denaturing acrylamide gel or by hybridizing to a reference plasmid. The reaction is used without further manipulation as a probe.

2.19. Hybridization conditions of radiolabelled DNA to DNA or RNA immobilized on membranes

2.19.1. Random primed or nick translated DNA.

Filters were prehybridized in sealed plastic bags in a 68°C water bath for a minimum of 30 mins. Prehybridization mix is:

5x SSC
2x Denhardts
0.1% PPi
0.5% SDS
150-200µg/ml denatured salmon sperm DNA
10% Dextran sulphate.

20x Denhardts is : 0.2% BSA 'Sigma'
0.2% PVP 360 (Sigma)
0.2% Ficoll 400 (Sigma)

After prehybridization the radiolabelled DNA probe is added and hybridized overnight at 68°C. Standard wash conditions were a total of 2 litres of 2x SSC, 0.1% SDS and 0.1% PPi at 68°C per filter over a period of 1 hour or more. Stringent wash conditions were 0.1-0.05x SSC, 0.1% SDS, 0.1% PPi at 68°C . Low stringency hybridization and wash conditions were done at 55°C in the absence of dextran sulphate.

2.19.2. End labelled oligonucleotides

These hybridizations used the same hybridization mix as for random primed or nick translated conditions except that dextran sulphate was omitted. The temperature used varied from 37°C to 50°C and hybridization was carried out overnight at the required temperature. Filters were washed in a large volume of 4x SSC, 0.1%

SDS, 0.1% PPi at two or three degrees higher than the hybridization temperature.

2.20. Screening of recombinant libraries

2.20.1. Liver cDNA libraries

A method derived from that of Grunstein and Hogness (1975) was used to bind DNA from plasmid containing colonies onto nitrocellulose. Bacterial colonies were plated out on sterile nitrocellulose sheets (Schleicher and Schuell) on top of agar plates. Replica filters were made by placing a fresh sheet of "damp" nitrocellulose on top of the colonies and pressing them together to transfer colonies; filters were then marked for orientation. The filters were peeled apart and if necessary placed on fresh agar plates. One replica was retained on a master plate and stored at 4°C. Filters to be probed were placed colony side up on a sheet of Whatman 3MM chromatography paper saturated with 10% SDS for 2 min. Filters were then denatured on paper soaked in denaturing solution (0.5M NaCl, 1.5M NaCl) for 5 minutes. Filters were then neutralized on paper saturated in neutralizing solution (0.5M Tris, pH7.5, 1.5M NaCl) for a further 5 mins before rinsing in 2x SSC for 5 mins. The filters were then air dried at room temperature and baked at 80°C under vacuum for a minimum of 2 hours to bind DNA to the filters. Subsequently filters were hybridized with radiolabelled probes of interest and colonies identified to be of interest picked from the master plate and rescreened until plasmid pure and suitable for DNA minipreparation.

2.20.2. Lambdaphage genomic and cDNA libraries

The method of Benton and Davis (1977) was used. For screening bacteriophage lawns agarose was used in place of agar in the top layer of the plates and the plates incubated for at least 3 hours at $4^{o}C$ before "lifts" were taken. This prevents a peeling away of the top layer. Nitrocellulose filters fresh from the box were carefully lowered onto a lawn of plaques for a minimum of 1 minute. For subsequent lifts absorption times were doubled, i.e. 2, 4 min etc. The nitrocellulose filters were orientated on the plate by marking both filter and plate with india ink. Then, as in the Grunstein Hogness procedure, the filters were peeled off and floated on denaturing solution (30 sec), neutralizing solution (5 mins) and 2x SSC (5 mins). Filters were then dried and baked. These filters were then screened with radiolabelled probes and as necessary rescreened until plaque pure.

2.21. Sequencing in M13

As mentioned in the text most of the sequencing of the human PB-1 P450 insert cDNA clone pHL515 was done by Janice Sweeney. However it was my responsibility to subclone all the relevant PstI fragments of pHL5,5 into M13 mp9, grow up the recombinants and get a partial sequence on each template in order to identify what part of the PB-1 mRNA transcript that pHL5.5 cDNA corresponded to. After this was done Janice continued the sequence with an Amersham ^{35}S sequencing kit according to their protocol. After a sequence of 250-300 nucleotides had been determined sequencing proceeded using a synthetic 18mer primer which was made on an Applied Biosystems DNA synthesizer by the I.C.R.F. Synthesis service or by John Inglis.

Overlapping and complementary sequences were read for verification and tested by restriction mapping of pHL5,5. Each sequence was determined a minimum of 5 times.

2.21.1. Subcloning of PstI fragments of pHL5,5 into M13 and DNA preparation

The three *PstI* insert fragments from pHL5,5 were first subcloned into pEMBL9 and re-isolated to prevent contamination and resubcloned into double stranded M13 for preparation of the single stranded template for sequencing by the dideoxy method (Sanger *et al*, 1977). JM101 were transformed with the relevant ligation mix and recombinant phage identified by the blue/white colour test on X-gal/IPTG plates. Recombinant phage were picked with a tooth pick into 1ml of a log culture of JM101 in 2XTY medium and grown for a further 5 hrs at 37°C. The culture was transferred to Eppendorf tubes and the supernatant cleared of cells by centrifugation for 5 min. The supernatant (containing single stranded phage) was transferred to a fresh Eppendorf tube and 200µl of PEG/NaCl (20% PEG 6000/2.5M NaCl). The tube was shaken and allowed to stand for 15 min at room temperature. The phage were precipitated by centrifugation for 5 min and the supernatant discarded. The tubes were inverted on tissues for 5 min and then 100µl of T.E. was added to the pellet. After resuspension the solution was extracted 1 to 3 times with an equal volume of ultra-pure phenol (BRL). The DNA in the aqueous phase was precipitated by the addition of 1/10 volume of 2M CH_3COONa (pH5.5) and 2.5 volumes of ethanol. This was incubated overnight at -20°C. After 10 min centrifugation the resulting pellet was washed in 70% ethanol and resuspended in 50µl T.E. In some cases 5µl of the miniprep was run on agarose gels to check for

the presence of DNA.

2.21.2. Sequencing reaction and polyacrylamide gel electrophoresis

5 to 8µl of single stranded template was added to 2.5ng of universal primer DNA (BRL) and 1µl of annealing/sequencing buffer (10x is 100mM Tris pH8.0; 50mM $MgCl_2$) and made up to 10 with sterile ddH_2O. The annealing reaction mix was placed in a 60°C oven for 1-2 hours after which it was quenched on ice for 2 mins.

Four different sequencing reactions are performed, each supplied with all four dNTPs but each reaction containing a different ddNTP. Incorporation of a ddNTP terminates the primer extension reaction carried out by the Klenow fragment of E.coli DNA polymerase. This is because there is no 3'-OH group available to form the next phosphodiester bond. The ratio of dNTP:ddNTP determines the frequency and size of DNA fragments generated by the Klenow fragment but all fragments originate at the primer and terminate at the site of ddNTP incorporation. By the use of labelled NTP (^{35}S or ^{32}P) these fragments can be visualized by autoradiography on high resolution acrylamide gels. The smallest fragment is the first band on the gel and represents the first base of the sequence. The next band up is the next smallest etc., etc. Gels are read upwards towards the comb and in a 5' to 3' direction from the primer.

The following solutions of dNTPS and ddNTPs were made up:

	A^o	C^o	G^o	T^o
0.5mM dATP	1	20	20	20µl
0.5mM dCTP	20	1	20	20µl
0.5mM dGTP	20	20	1	20µl
T.E.	20	20	20	20

A' mix: equals vols A^o plus 0.1mM ddATP

C' mix: equals vols C^o plus 0.1mM ddCTP

G' mix: equals vols G^o plus 0.1mM ddGTP

T' mix: equals vols T^o plus 0.1mM ddTTP

After quenching on ice, 2 units of Klenow (~0.5µl) and 10µCi of α-^{32}P dTTP (>800Ci/mmol) were added to the annealing mix and 2.5µl of this aliquoted to four tubes marked A, T, G and C. Then 2µl of the relevant dNTP/ddNTP mix were added and the reaction allowed to proceed for 15 min when 2µl of chase solution (0.5mM TTP) were added and the reaction continued for a further 15 min. The reaction was terminated by the addition of 4µl formamide dye mix (100ml formamide, 0.1g xylene cyanol FF, 0.1g brophenol blue, 4ml of 0.5M EDTA). The samples were placed in a boiling water bath for 3 minutes directly before loading 3-5µl aliquots onto a sequencing gel.

Sequencing gels were prepared as follows: two glass 20cm x 40cm plates were cleaned with ethanol. One plate, with notched rabbit ears, was coated with siliconizing solution (2% dimethyl-dichlorosilane in 1,1,1-trichloroethane [BDH]) whilst the other unnotched plate was treated with -methacryloxypropyl-

trimethoxysilane (Sigma). Buffer gradients were used to get more even spacing of bands over the length of the gel which was made up in TBE (10.8g Tris base, 5.5g boric acid, 0.93g EDTA, pH8.4/litre). A 40% stock of acrylamide (19:1) acrylamide:NN'-methylenebis acrylamide) was made up and de-ionized by mixing with amberlite MB1 resin for 30 mins and subsequently filtered. 80ml of light solution (36.8g urea, 8ml 10x TBE, 12ml 40% acrylamide) and 20ml of heavy solution (9.2g urea, 5ml 10x TBE, 3ml 40% acrylamide, 1g sucrose, 1mg bromophenol blue) were prepared. Before pouring 150µl and 40µl of 10% ammonium persulphate (fresh) and 80µl and 40µl of TEMED was added to the light and heavy solutions respectively. 12.5ml of light solution followed by 12.5ml of heavy solution was drawn up into a 25ml pipette and poured at a steady flow rate between the two glass plates and topped up with light solution. Then the comb was added and gels allowed to set for 45 min. After setting the gels were clamped to a Bio-Rad sequencing apparatus. The buffer tanks were filled with TBE buffer and the comb removed. 3-5µl of denatured DNA were loaded per slot and the gel connected to an LKB 2197 power supply and run at approximately 1500v at 0.46mA until the bromophenol blue marked dye had run off the bottom. The gels were then removed from the gel tank and prized apart with the gel sticking to the non-notched plate. Gels were fixed in 10% acetic acid, 10% methanol for 10 minutes and washed extensively under tap water for 5-10 min. The gel was dried to the glass plate by baking at 80°C for greater than 1 hour. The gels were placed in direct contact with X-AR film and exposed at room temperature overnight. Sequences were read directly into the computer for data storage and manipulation.

2.22. Oligonucleotides used in this study

A variety of oligonucleotide probes were used in this study to identify selected cDNA clones as cytochrome P-450s and to distinguish between members of the same cytochrome P-450 family. These probes were synthesized on the basis of sequence comparisons between rabbit PB-1 cDNA clones (Leighton et al, 1984) and rat PB-3 cDNA clones (Fuji-Kuriyama et al, 1982). Oligonucleotides were synthesized by ICI (a personal gift of Dr. M. Rose), I.C.R.F. and by J. Inglis (a personal gift). All oligonucleotides, except for sequencing oligonucleotides, were made anti-sense so they could be used in northern blot analysis.

b and e oligomers. Rat cytochromes P-450b and P-450e are extremely homologous forms of the rat P-450 family P450-2B. They are over 97% homologous over their primary cDNA structure (Fuji-kuriyama et al, 1982; Adesnik and Atchison, 1986; Atchison and Adesnik, 1986) but there is a region of hypervariability centered around amino acids Ser 334 to Thr 339 for P-450b and Ser 334 to Ser 339 for P-450e. Over this region 4 out of 18 nucleotides are mismatched and thus oligonucleotides directed to this region can be used to distinguish these forms at the RNA (Omiecinski et al, 1985) and DNA level. Interestingly a sequence for mouse P-450b has been published which differs from the rat in this region (Stupans et al, 1984) so an oligo was made from this sequence as well.

A/s 5'

Rat P450b (PB3a)

GGT TGG TAG CCG GTG TG

Rat P-450e (Pb3b)

GGA TGG TGG CCT GTG AG

Mouse P-450b (PB3a)

GGT GGG TAG CCT GTG AG

Mouse P-450b has 3/17 mismatches with rat P-450b and 3/17 mismatches with rat P-450e.

oligonucleotide: C128

Comment:

This oligonucleotide was made to a conserved region between a rabbit P-450, P-450 PBc2 (P450-2C) (Leighton et al, 1984) and a rat P-450, P-450b (P450-2B) (Fuji-Kuriyama et al, 1982). At the time it was thought these forms were orthologous but subsequently (through this work and others) this was found not to be the case. However, this oligo turned out to be a very useful probe for identifying P-450 cDNA probes as discussed in the text. The sequence of the oligonucleotide was as follows:

A/S 5' 3'

T C C G C A G T T C C T C C A C C A A

A G G C G T C A A G G A G G T G G T T

3' 5'

Amino Acid Arg Leu Glu Glu Val Leu

The Leu corresponds to residue 153 in P-450b (P450-2B) (Fuji-kuriyama et al, 1982).

oligonucleotide: C129

mer C129

This oligonucleotide was derived from rat P-450b (P450-2B) (Fuji-kuriyama et al, 1982) from a conserved region around cysteine thought to be part of the heme binding region of P-450's (Black and Coon, 1986). This turned out to be fairly rat specific.

```
A/S  5'                                    3'

          GGC  AAT  GCC  TTC  GCC  AAG  ACA

          CCG  TTA  CGG  AAG  CGG  TTC  TGT

Amino Acid:  Alu  Ile  Gly  Glu  Gly  Leu  Cys
```

oligonucleotides: RWE296 and RWE297

It was soon realised how oligonucleotides directed to amino acids around region 150 of the Phenobarbital inducible P-450s would be a useful method for identifying potential P-450 cDNA clones so a set of mixed antisense oligos were made to Glu_{153} to Arg_{158}. These were made in two sets.

RWE296

```
  5'                                        3'

   G T T T T A  C G  T A A  T T C  T T C
             C    T C   G C     C
             G
             T
```

RWE297

```
  5'                                        3'

   G T T T T A  C G  A A A  T T C  T T C
             C    T G   G C     C
             G
             T
```

Both of these are 17 mers of a 144 mix.

2.23. In situ hybridization to metaphase chromosomes

This method of mapping is a useful addition of the genetic approach where genes can be localized physically on a chromosome or chromosomes and compared to the genetic map. This method was a collaboration with J.R. Gosden, D. Rout and R.M. Speed. I identified and prepared probes deemed useful as in situ hybridization probes.

2.23.1. Human metaphase spreads

Chromosomes were prepared from peripheral blood lymphocytes stimulated with PHA and cultured in RPM1 1640 with 15% fetal calf serum in the usual way. After 72h the cells were synchronised by the addition of 200µg/ml BrdU, cultured overnight, and released the following morning by replacing the BrdU with 2 x 10^{-5}M thymidine. Culture was continued for 5h 15 min, colcemid added for 40 min, harvesting and fixation was carried out in the usual way. Chromosome preparations were air-dried on acid alcohol cleaned slides.

0.5µg of pHL5,5 was labelled with ^{3}H-dATP, dCTP and dTTP (Amersham) by random oligonucleotide primed-synthesis (Feinberg and Vogelstein, 1983; 1984) to a specific activity of ~10^{7} dpm/µg. In order to eliminate hybridisation of the repeat sequence, the labelled probe denatured and was annealed with 200µg of total human DNA in 5x SSC in a volume of 80µl, for 30 min at 70°C. The remainder of the hybridisation mix (tRNA, formamide, dextran sulphate) was added as described previously (Gosden et al, 1986) to make final concentrations of 50% formamide; 10% dextran sulphate 2x SSC; 400µg/ml tRNA. The chromosomes were treated with RNAse A and the chromosome DNA was denatured in 70% formamide; 0.6 x SSC at

70°C, while the probe was denatured at 70°C for 5 min and applied to the slides as described previously (Gosden _et al_, 1986). The slides were incubated at 37°C overnight and washed in 50% formamide, 1x SSC at 40°C (4 x 5 min) and 1x SSC at 40°C (4 x 4 min) before dehydrating through an alcohol series and dipping in Ilford L4 liquid nuclear emulsion. Slides were exposed at 4°C for 7 days, developed and fixed as described previously and thoroughly dried. They were then stained in Hoescht 33258 (50μg/ml in 2 x SSC) for 20 min, the slides were flooded with 2x SSC in a Petri dish and exposed to UV for 17 min, before rinsing thoroughly in 2x SSC and staining in Wright's stain for 8 min. This produced clear G-bands, enabling simultaneous analysis of karyotype and grain distribution to be performed.

2.23.2. Mouse metaphase spreads

Chromosomes were prepared from CD female bone marrow cells by standard methods (Ford and Hamerton, 1956) and the chromosomes karyotype identified on a subset by trypsin banding. Hybridization conditions were the same as for human except that slides were not post-banded just developed and fixed as in Gosden _et al_ (1986). The probes used were pHL5,5, pPB3-15 and lambda 35.1. G-banded karyotypes were constructed following the trypsin banding method of Gallimore and Richardson (1973).

CHAPTER 3

ISOLATION, CHARACTERIZATION AND MAPPING OF A HUMAN PB-1 P-450 cDNA CLONE

3.1. Detection of sequences homologous to rodent P-450s in human genomic DNA

In order to initiate a study into human P-450 genes it is important to determine whether cDNA probes corresponding to rodent P-450s could detect human homologues. Three distinct classes of cDNA probes were available. Firstly, two rat cDNA probes pR17 and p5'2 which correspond to the highly PB inducible P-450, PB_{3a} (P450-2B). These probes together include over 85% of the coding region for this P-450 (Kumar et al, 1983). Secondly, a mouse cDNA probe pCL46, corresponding to the 3MC inducible P-450, P_1450 (P450-1). This clone contains a 1.1kb insert which corresponds primarily to the 3' untranslated region of P_1450 mRNA (Negishi et al, 1981; Adesnik and Atchison, 1986). Thirdly, two clones pTF-1 and pTF-2 which were isolated from a rat cDNA library with antibodies raised to a constitutive form of P-450, PB-1 (P450-2C). These clones contained inserts of 1200 and 400 base pairs respectively (Freidberg et al, 1986) and were subsequently shown to correspond to forms f and PB-1 respectively by comparing their sequence with almost full length clones for these P-450s (Freidberg et al, 1986; Gonzalez et al, 1986a).

Southern blots of rat, mouse, CHO, chimp and human DNA digested to completion with restriction enzymes MspI and EcoRI were hybridized overnight under low stringency conditions with the respective probes. Low stringency conditions in this instance are hybridization at 55°C with standard hybridization mix but without the addition of dextran sulphate. Blots were washed in 2 x SSC at 55°C. As shown in Fig. 3.1a, pR17/p5'2 detects multiple bands in rodent (CHO, mouse, rat) and to a lesser extent in human DNA samples. The same observation can be made for Southern blots

FIGURE 3.1.

A) Hybridization of pR17 (P450-2B) to various DNAs at 55°C. 10µg DNA from various species was digested with EcoRI (R) and MspI (M). Electrophoresed on agarose gels, Southern blotted and hybridized with pR17 plasmid under 'low' stringency conditions. DNAs are as follows: 129, male mouse DNA; BWJ, mouse hepatoma cell line DNA; Wistar, male rat DNA; FAZA rat hepatoma cell line DNA; CHO, Chinese hamster ovary cell line DNA; F_2, female human DNA; Mo, male human DNA. Hybridisation was at 55°C in the absence of dextran sulphate and washes were 2 x SSC at 55°C.

B) Hybridization of pTF-1 (P450-2C) to various DNAs at 55°C. DNA digestion, samples and hybridization conditions as in Fig. 3.1.A.

A

Hybridization of pR17 to various DNAs at 55° c.

B

Hybridization of pTF – 1 to various DNAs at 55° C.

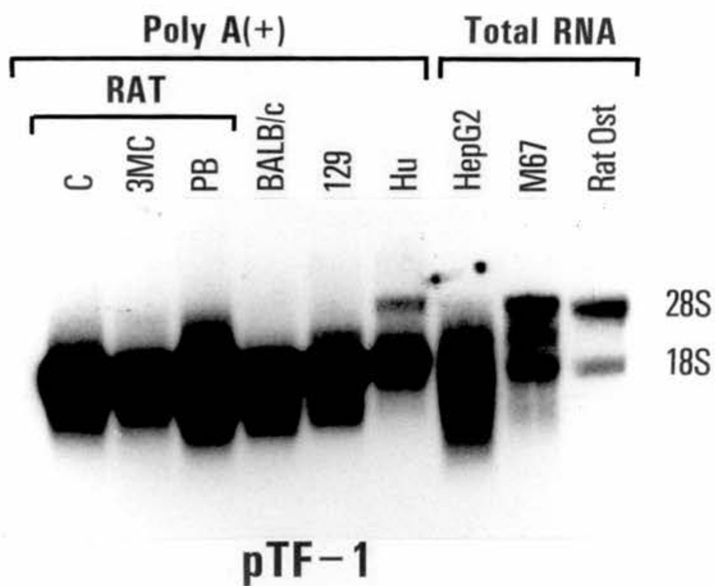
analysed with the pTF-1 probe (Fig. 3.1B), however the pattern appears to be distinct between the two sets of probes. The probes are comparable in that each encodes about 85% of the coding region of their respective cDNAs. Thus both probes detect multiple homologous sequences in human DNA digests. In contrast under these conditions pCL46 gave only a background smear in both rodent and primate DNA digests (data not shown). To obtain a distinguishable pattern of hybridization with this probe, higher stringency conditions had to be used. Southerns were hybridized with pCL46 at 68°C under standard hybridizations including dextran sulphate and washed in 2 x SSC at 68°C. In this case two distinct bands could be detected in digest of rodent DNA and a faint single band at 4.4kb for EcoRI digests human and chimp DNA (data not shown).

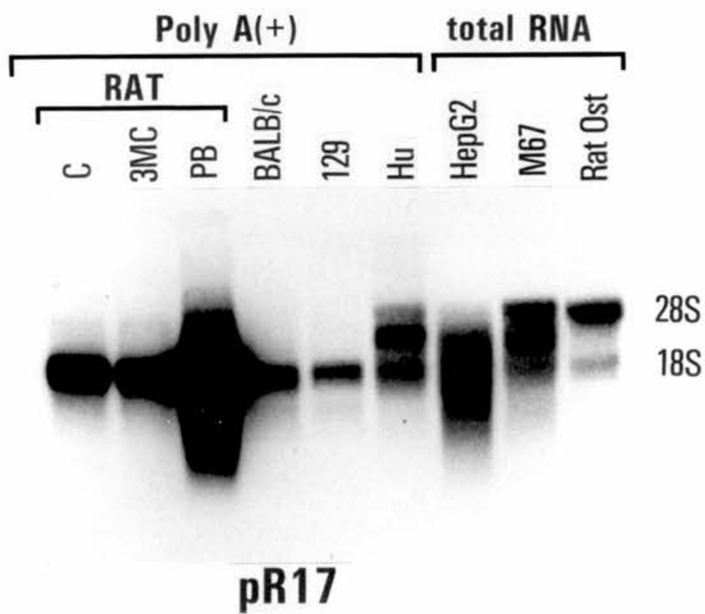
3.2. Detection of homologous sequences to rodent P-450s in human hepatic mRNA.

Identical Northern blots with control and induced rat hepatic mRNA preparations and mouse and human hepatic RNAs were probed under low stringency conditions with pR17/p5'2, pTF-1 and pCL46. pCL46 only detected a 2.3kb mRNA in the 3MC induced rat liver preparation and none in untreated rat, mouse and human hepatic RNA samples even after long exposure (data not shown). However, both pTF-1 and pR17/p5'2 detect transcripts in human polyA$^+$ RNA preparations and to a lesser extent in total human preparations (Fig.3.2). The human 2.1kb mRNA detected by pTF-1 appeared to be slightly larger than the rodent equivalents but it also has a high constitutive level of expression. The 3kb mRNA detected in human samples by pR17/p5'2 was also larger than its rodent counterpart and as in untreated rodents had a low level of expression. For pTF-1 rats and mice appear to

FIGURE 3.2.

Northern blot analysis of hepatic RNA from rodent and human. 2ug of polyA$^+$ and 15ug of total RNA were fractionated on denaturing agarose gels. Northern blotted and hybridized with the indicated probes at 55°C. Wistar rat male liver RNA were (c) control; (3MC) 3 methylcholanthrene induced; (PB) phenobarbital induced; Mouse RNAs were from untreated BALB/c and 129 males; Human RNAs were from adult males Hu and M67 respectively and from a human hepatoma cell line HepG 2 (Knowles *et al*, 1980); Rat ost is RNA from a rat osteosarcoma cell line which is negative for P-450 expression. Size markers were the 18S and 28S ribosomal bands.





Northerns hybridized at 55° C.

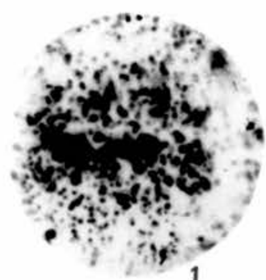
have comparable levels of expression in adult liver and this is marginally induced by PB administration of rats. However the highly PB inducible P-450 mRNA has a higher basal level of expression in rats when compared to untreated mice. Other controls on these blots were RNA from HepG2 a human hepatoma cell which expresses many liver specific functions (Knowles <u>et al</u>, 1980) and RNA from a rat osteosarcoma cell line which is negative for P-450 expression. The HepG2 RNA sample appeared slightly degraded but even still no significant transcripts were observed. The two bands seen in the rat osteosarcoma RNA correspond to the non-specific binding of the probes to ribosomal RNA. These experiments deomonstrated that the rodent probes identify human P-450 counterparts and that these probes can be used to isolate the homologous human genes.

3.3. Isolation of a human P-450 cDNA

To isolate a human P-450 cDNA clone I screened a human liver cDNA library constructed in a pBR322 derived vector (Woods <u>et al</u>, 1982). The only probes which offered any chance of success were pTF-1 and pR17/p5'2. Inserts were derived from these probes and used to screen 10-20,000 colonies by the method of Grunstein and Hogness (1975) (Fig.3.3). Most of the hybridization on these filters appeared non-specific as both pR17/p5'2 and pTF-1 gave similar background (data not shown). Two colonies were identified which gave strong signals with pTF-1 insert on filters (Fig.3.3). These were picked along with colonies which gave slightly increased hybridization with pTF-1 or pR17/p5'2 for secondary screening. Only the two strongly hybridizing colonies came through the screen with pTF-1 (Fig.3.4). No colonies were identified with pR17/p5'2. The failure to identify human homologues for pR17/p5'2 may be due to

FIGURE 3.3.

Primary screen of a human cDNA library with pTF-1 insert. Colonies (2000 per filter) were screened *in situ* by the method of Grunstein and Hogness (1975) under low stringency conditions . The arrows indicate the resulting positive colonies.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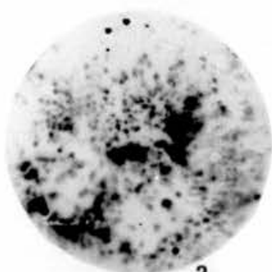
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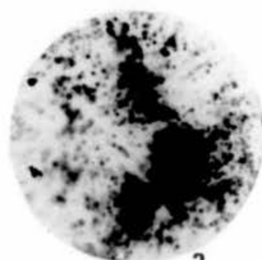
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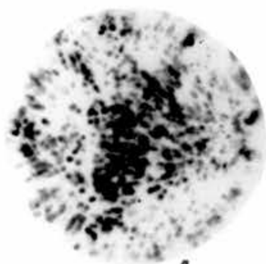
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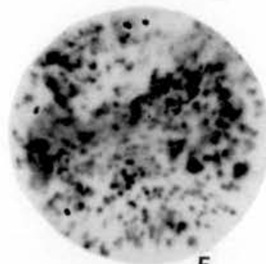
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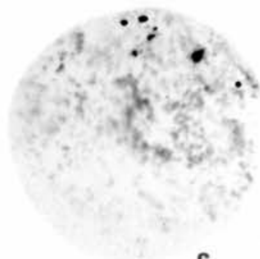
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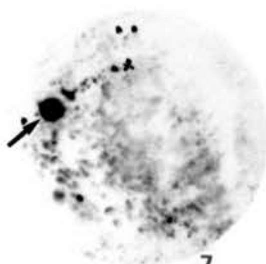
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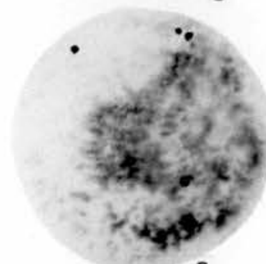
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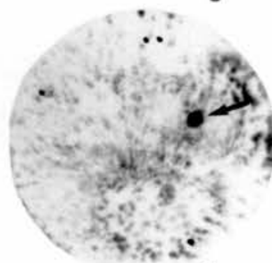
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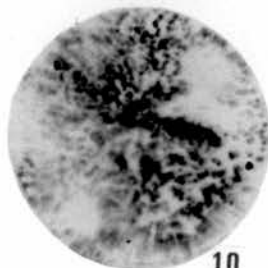
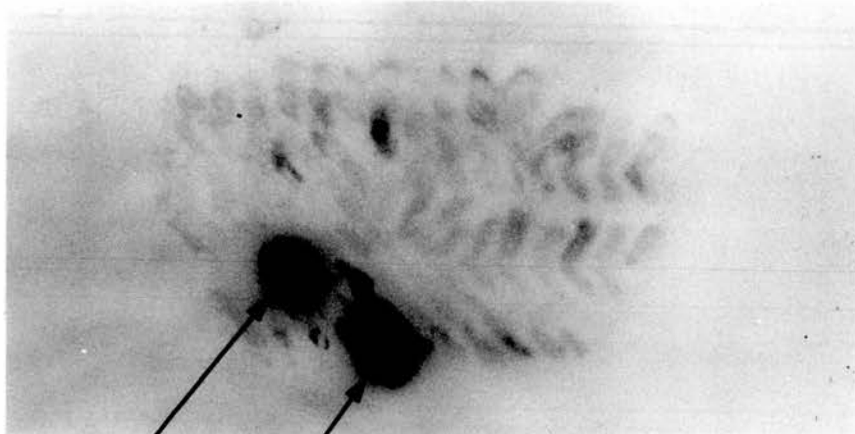

10

FIGURE 3.4.

Secondary screen of human liver cDNA library with pTF-1 insert.
Approximately 50 picks were streaked onto nitrocellulose filters and screened as before under low stringency conditions. The indicated colonies were picked and grown up in bulk for plasmid DNA preparation.



Secondary screen of hman liver cDNA library with pTF – 1 insert (hybridized at 55^{0}C).

failure to identify human homologues for pR17/p5'2 may be due to their low representation in human liver mRNA and subsequently constructed cDNA libraries. This library was originally amplified on plates which might further reduce the chance of identifying a low abundance message.

3.4. Characterization of human cDNA clones

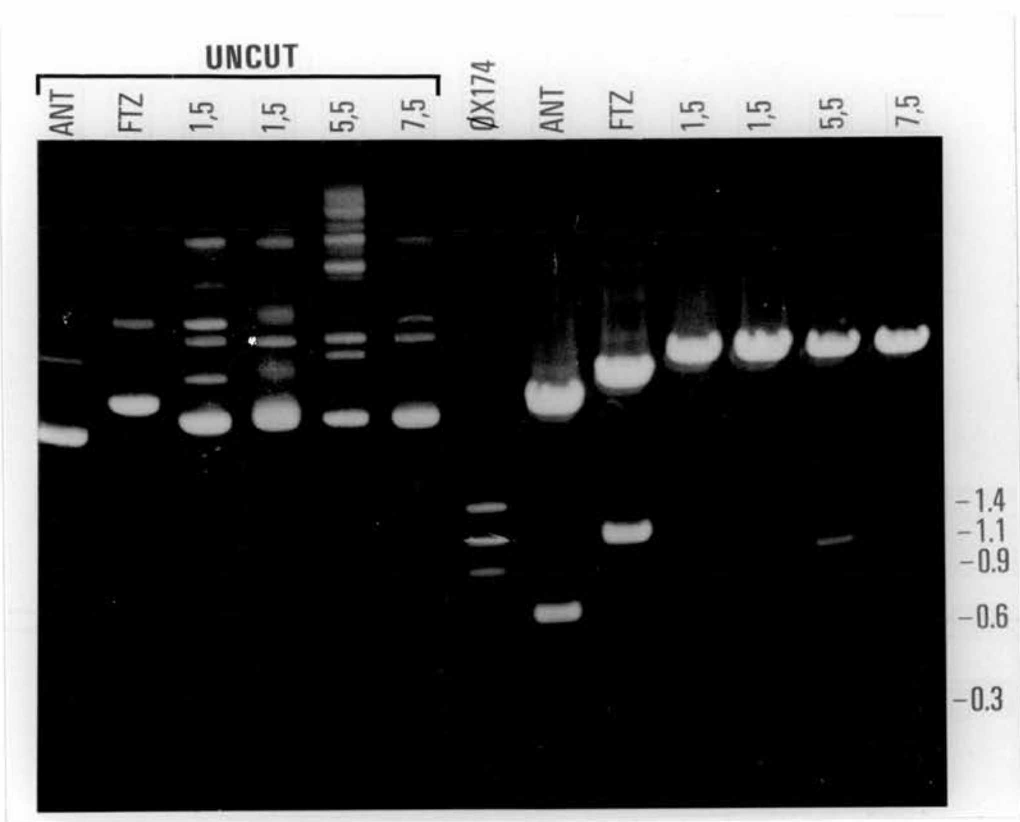
The colonies identified with the pTF-1 insert were grown up in bulk and their plasmids isolated on CsCl gradients. Digestion with _Pst_-I releases their inserts from the parental pKT218 plasmid. Analysis by agarose gel electrophoresis revealed that pHL1,5 and pHL5,5 had inserts of 700 and 2,000 base pairs (bp) respectively. The large plasmid contained two internal _PstI_ sites (Fig.3.5). This gel was transferred to nitrocellulose and then probed with the pTF-1 insert under high stringency conditions 68°C (+ dextran) and washed in 2 x SSC at 68°C (Fig.3.5B). Only the two larger inserts of pHL5,5 and the 700bp pHL1,5 insert hybridised to the pTF-1 insert. Negative controls on these blots were the drosophilia homeo box containing probes _Ant_ and _Ftz_ (kindly provided by B. McGinnis) which failed to light up. If the experiment was repeated substituting the pTF-2 insert as a probe only the larger 900bp _PstI_ fragment from pHL5,5 lit up and the 700bp fragment from pHL1,5 (data not shown). The insert from pTF-2 corresponds to amino acid residues 132-268 of the PB-1 protein (Freidberg _et al_, 1986; Gonzalez _et al_, 1986) whereas pTF-1 corresponds to amino acid residues 87 to 490 of the f protein (Freidberg _et al_, 1986; Gonzalez _et al_, 1986). One of the assumptions that could be made is that the insert in pHL1,5 represents a truncated form of a human cDNA. Additionally it seemed that the 900 and 700bp _PstI_ fragments of pHL5,5 contained the main

FIGURE 3.5.

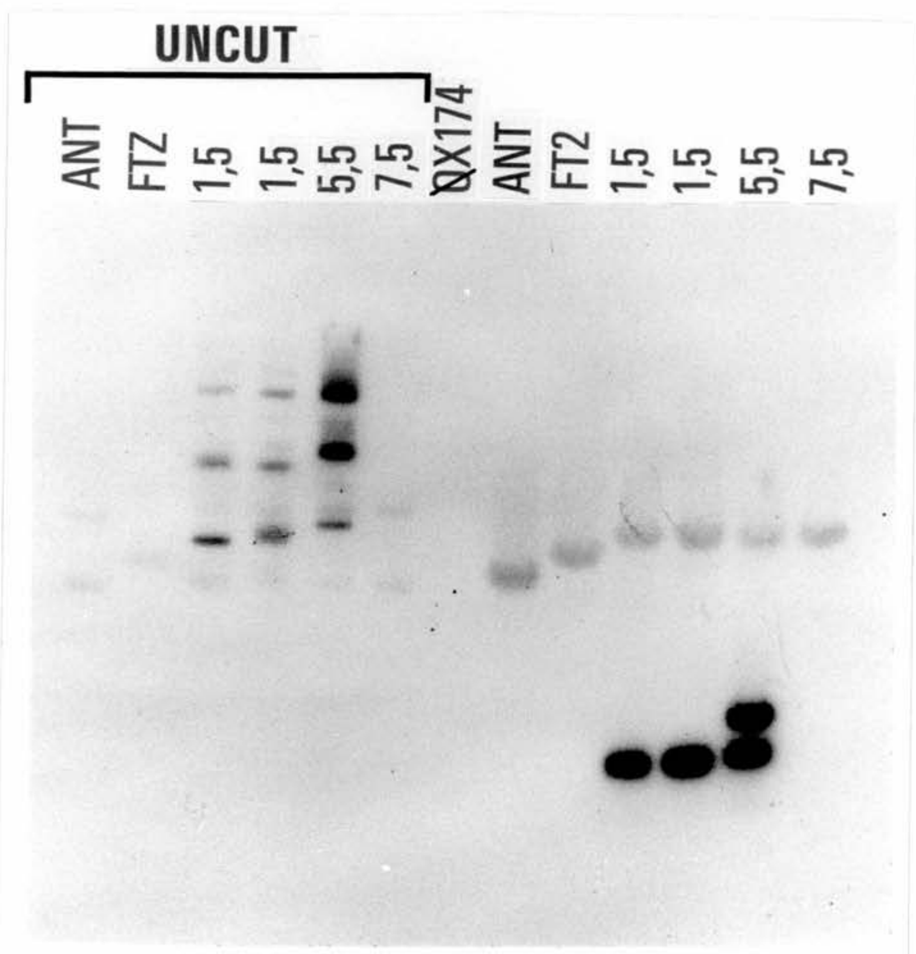
A) Size estimation of inserts from putative human P450 containing plasmids. Plasmids were run digested and undigested. pHL1,5 (1,5), pHL5,5 (5,5) and pHL7,5 were digested with PstI. pHL7,5 represents the parental plasmid vector without an insert. The plasmids ANT and FT2 contain Drosophilia homeo box fragments and were digested with BamHI/PvuII and PvuII respectively. The size marker is HaeIII digested OX174.

B) Detection of human containing P-450 plasmids. The gel in (A) was Southern blotted and probed with pTF-1 insert under normal stringency conditions, i.e. 68°C. The autoradiographic bands in 1,5, 1,5 and 5,5 correspond to human cDNA homologues of rat PB-1 hepatic P-450s.

A



B



coding region corresponding to the rat PB-1 P-450. The small 400bp Pst-I fragment of pHL5,5 represents either an extreme 5' or 3' part of human cDNA with no significant homology to the rodent PB-1 cDNA probes.

3.5. Oligonucleotide probing of P-450 cDNAs

The plasmid pHL5,5 was further characterized by probing with oligomers which corresponded to two conserved cysteine containing regions of cytochrome P-450. C128 is an antisense 19mer to the Cys152 region of rat PB_{3a} which was designed on the basis of homology between rat PB_{3a} (Fuji-kuriyama et al, 1982) and a rabbit P-450 (Leighton et al, 1984). Overall these P-450s share 50% homology at the DNA level. Over the Cys 152 region they have an 8 amino acid region of identity and a 16/19 nucleotide identity (Fig. 3.6). A second oligomer, C129, was made to the Cys435 region of the rat PB_{3a} cDNA sequence. Over this region the two P-450 forms shared only 50% identity. It was reasoned that if divergent P-450s maintain nucleotide sequence similarity over conserved regions then oligomers to these regions would identify potential P-450 cDNA clones. That this was the case was proved by using the C128 oligo which hybridized to plasmid p5'2 and pTF-2 (Fig. 3.7 A and B) which share only 50% sequence homology overall (Freidberg et al, 1986). This oligonucleotide also hybridized to the 900bp and 700bp fragment of pHL5,5 and pHL1,5 respectively. The pTF-2 clone has a 16/19 match-up with CL28 with a maximum stretch of 12 nucleotide homology. pTF-1 has 15/19 match but only a 5 nucleotide run of maximum homology which does not allow for stable hybrid formation. Although the hybridization to the human clones is much reduced compared to

FIGURE 3.6.

Oligomers derived from conserved cytochrome P-450 regions. Two peptides are shown for 4 P-450s together with the nucleotide sequence. Rabbit Pbc2 (P450-2C) (Leighton et al, 1984); rat P-450 PB_{3a} (P450-2B) (Fuji-kuriyama et al, 1982); PB-1 and f (P450-2C) (Freidberg et al, 1986). '*' denotes the template for the antisense oligomer. ' - ' denotes mismatches between the oligomer and P-450 cDNA sequence for the respective forms.

	Cys152 (C128)	Cys436 (C129)
	Amino Acid	Amino Acid
Rab PBc2	C L V E E L R K	C L G E A L
Rat PB2a	C L V E E L R K	C L G E G I
Rat pTF-2(PB-1)	C L V E E L R K	- - - - - -
Rat pTF-1 (f)	C L V E E L R K	C V G E G L
	C128	C129

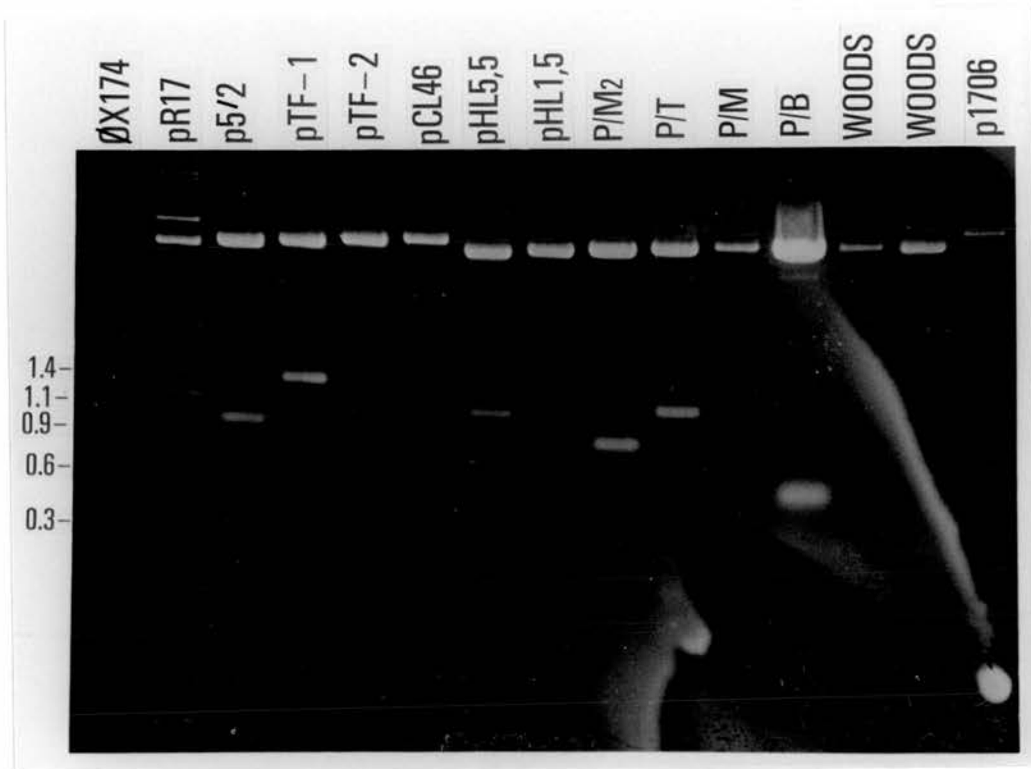
	Nucleotide	Nucleotide
Rab PBc2	CTGTGGAGGAAACTGAGAA	TGTGTGGGAGAGGTCCTGGCC
*Rat PB3A	TTGGTGGAGGAACTGCGGA	TGTCTTGGCGAAGGCATTGCC
Rat pTF2(PB-1)	CTTGTGGAGGAACTGAGGA	---------------------
Rat pTF1(f)	CTAGTGGAAGAACTGAGGA	TGTGTTGGAGAGGGCCTGGCC

FIGURE 3.7.

A) Gel photograph of PstI digested P-450 containing plasmids. OX174, size marker; pR17 and p5'2, rat PB_{3a} cDNAs (P450-2B); pTF-1, pTF-2, rat PB-1 cDNAs (P450-2C); pC146, mouse P-450 (P450-1), pHL5,5 human PB-1 P450 (P450C2C); p/M2, p/T, p/M and p/B are subclones of the PstI inserts from pHL5,5; Woods - PstI digested cDNA library; p1706 mouse albumin.

B) The above plasmids probed with oligomers direct against conserved regions of rat PB_{3a} cDNA. C129 is towards Cys 436 of PB_{3a} and C128 is towards Cys 152 of PB_{3a} cDNA.

A



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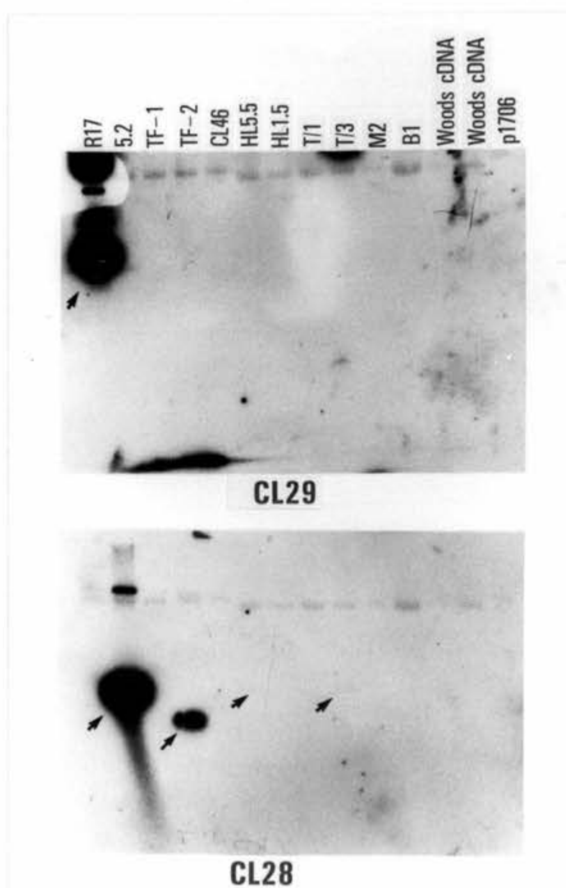


the rodent clones it is significantly above background. This was further evidence these were P-450 containing plasmids are almost full length. As suspected from the sequence comparison C129 only lit up pR17 in this panel of plasmids. Subsequently a mixed series of oligonucleotides to region Cys 152 was made which hybridizes to all P-450 containing plasmids which encoded the conserved sequence - Glu Glu Leu Arg Lys -. This oligomer mix gave a pattern of hybridization similar to C128 but the relative signal intensity was similar for each plasmid (see Chapter 4).

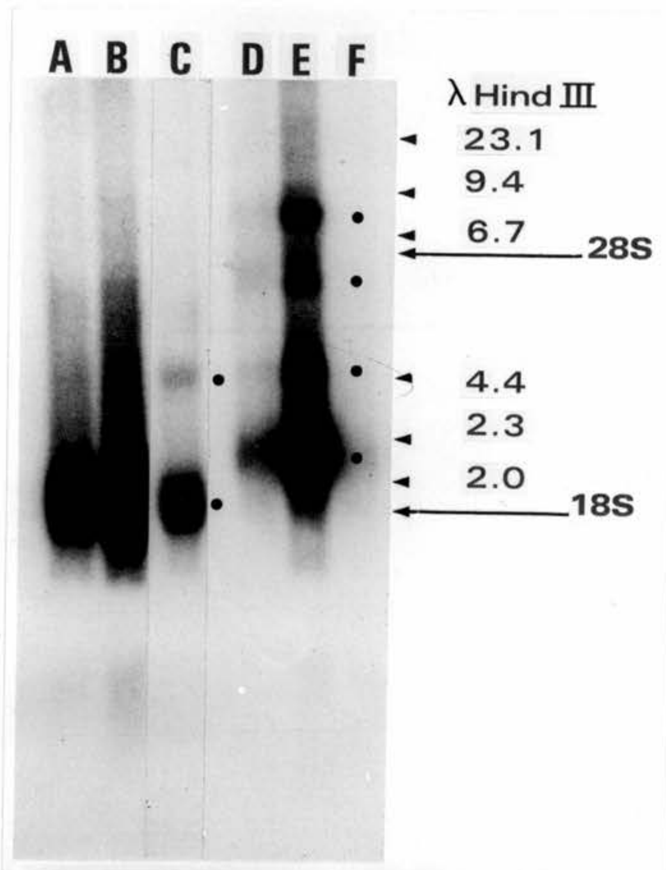
3.6. Northern Blot analysis

The human clone pHL5,5 was further characterised by using Northern blots of treated and untreated rodent liver and human liver RNA samples. Phenobarbital treated rats show a marginal increase in PB-1 related hepatic mRNA on hybridization with pTF-1. This was also the case when pHL5,5 was used, i.e. the major transcript at approximately 18S (Fig. 3.8) was increased two-fold by phenobarbital treatment (Fig. 5B). In human liver an RNA transcript was observed with a high level of expression which was larger than its rodent equivalent (Fig. 8 D and E). The pattern of hybridisation with the human clone was the same as when the rat pTF-1 is used as a probe to rodent and human liver RNA samples (Fig. 3.2). Interestingly minor transcripts could also be seen in the rodent and human RNA samples which are larger than the major forms. These may correspond to processing intermediates or to alternative RNA species.

The level of expression of PB-1 related mRNAs is around 0.25%. This calculation is based on the number of PB-1 containing plasmids found on antibody screening of an 8,000 member pBR322 cDNA library (Freidberg et al, 1986). Three clones were identified, if we accept

FIGURE 3.8.

<u>Northern blot analysis of human and rodent liver RNA samples with the PB-1 cDNA clone pHL5,5.</u> Lane A, rat liver control polyA$^+$, RNA, (1μg); Lane B, PB induced rat liver polyA$^+$ RNA (1μg); Lane C, C57BL/6 mouse liver control total RNA (10μg); Lane D, normal human male liver total RNA; Lane E, normal human male liver polyA$^+$ RNA (2.5μg); Lane F, '●' indicates multiple transcripts in human tracks. Size markers were denatured <u>Hind</u>III fragments and 28S and 18S ribosomal RNA markers. All these samples were run on the same gel but superfluous tracks were removed.


A B C D E F
λ Hind III
23.1
9.4
6.7
28S
4.4
2.3
2.0
18S

that a cDNA clone has a 1/6 chance of being in the right orientation for expression then this corresponds to 18 PB-1 clones/8000 or approximately 0.25% of the total mRNA content. This corresponds to 1000 mRNA copies/hepatocyte (Hastie and Bishop, 1976). A comparable level of expression is seen with pHL5,5 in human adult male liver.

3.7. Southern blots to genomic DNA

pHL5,5 was used as a probe against Southern blots of human and rodent DNA to determine the genomic complexity detected by this cDNA. This experiment was hampered by the presence of a low level repeat which was subsequently localized to the 400bp *PstI* fragment of pHL5,5 (see below). However washing at high stringency (0.01 x SSC at 68°C) removed this background and revealed a unique pattern of hybridization of some complexity (Fig. 3.9). In humans the amount of hybridizable DNA detected by pHL5,5 ranged from 70-150 kilobases (kb) depending on the restriction enzyme used, whereas in rodents the pattern was more complex (even at this high stringency wash). This implies that pHL5,5 is a member of a conserved small multigene family or corresponds to a very large gene.

3.8. Crude restriction map of pHL5,5

A restriction map of pHL5,5 was determined by standard methods and later confirmed by sequence analysis (Fig. 3.10). The three *PstI* fragments were subcloned into the multicloning site vector pEMBL9 (Dente *et al*, 1983). Hereafter these clones are referred to as p/T (900bp *PstI* fragment), p/M (600bp *PstI* fragment) and p/B (400bp *PstI* fragment)

FIGURE 3.9.

Southern blot analysis of primate and rodent DNAs with pHL5.5 This blot was washed at high stringency (0.01 x SSC at 68°C). The restriction enzymes used were BamHI (Lanes A and B) and EcoRI (Lanes C-H). Lane A and C human liver DNA; lane B and D, AKLO a human cell line DNA; Lane E, male chimp DNA; Lane F, Chinese hamster WG8H cell line DNA; lane G, Wistar male rat DNA; Lane H, C57L mouse male DNA.

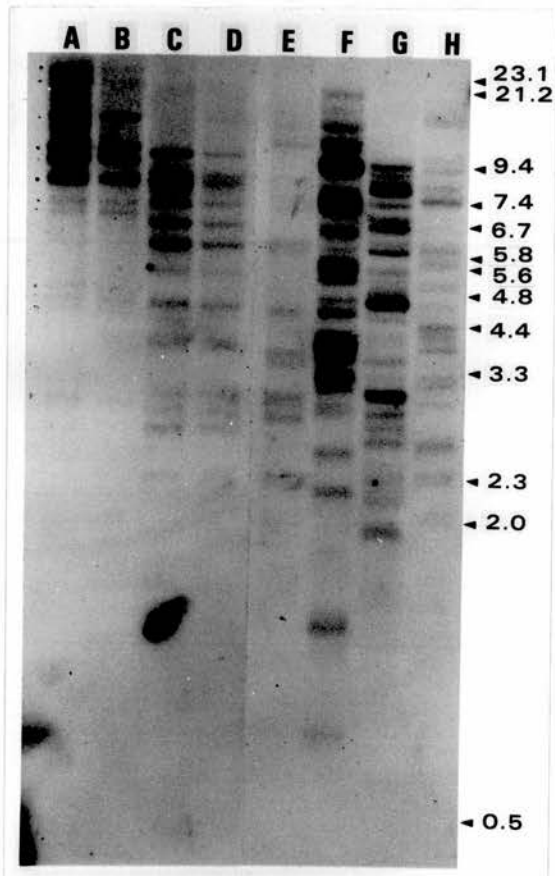
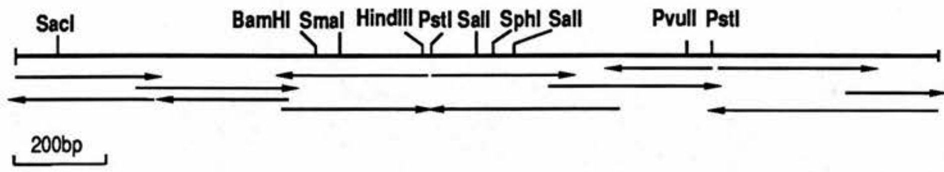

A B C D E F G H
23.1
21.2
9.4
7.4
6.7
5.8
5.6
4.8
4.4
3.3
2.3
2.0
0.5

FIGURE 3.10.

Restriction map of the cDNA insert of pHL5.5. The restriction map was derived as described in the text. Also indicated is the sequencing strategy; the PstI fragments were subcloned into M13 mp8 and sequenced by the dideoxy chain termination method. The arrows indicate the segments and direction of sequences (underlined) read from synthetic oligonucleotide primers (see Materials and Methods). The orientation of the cDNA in the parental pKT218 plasmid is such that the EcoRI site in the vector is 3' to the second indicated PstI site.


SacI
BamHI
SmaI
HindIII
PstI
SalI
SphI
SalI
PvuII
PstI
200bp

3.9. Sequence of pHL5,5

The pEMBL vector also contains sequences of the F_1 coliphage required for DNA replication and morphogenesis (Dente et al, 1983). On superinfection with F_1 phage pEMBL containing bacteria also produce pEMBL ss DNA in virion capsids. This can be isolated and used as a template for DNA sequencing by the dideoxy chain termination method (Sanger et al, 1977). However it was found that large fragments cloned in pEMBL tended to rearrange when going through the F_1 replication step (R. Hill and R. Meehan, unpublished observations). Therefore the three PstI fragments were reisolated from pHL5,5 and subcloned into double stranded M13 mp8 at the PstI site for the single stranded DNA template. The initial sequencing was carried out by myself which identified polyGC tails in p/T and p/B subfragments. This is in accordance with the method of library cDNA construction and identified these fragments as representing the 5' end and 3' ends of the cDNA. Sequencing of the p/M subfragment identified a sequence with significant homology to the pTF-1 cDNA sequence. This confirmed that pHL5,5 was a human homologue, of the rat PB-1 P-450. At this point the sequence of pHL5,5 became a collaborative effort with Ms Janice Sweeney. After a sequence of 250-300 nucleotides had been determined, sequencing continued using a synthetic 18 mer primer which was made by an Applied Biosystems DNA synthesiser (synthesis carried out by J. Inglis and the I.C.R.F. synthesis laboratory). Overlapping and complementary sequences were read for verification. Each sequence was determined a minimum of 5 times. The sequence strategy is also illustrated in Fig. 3.10.

3.10. pHL5,5 encodes a P-450 which is a member of the P450C2C family of proteins and is related to a human P-450 which metabolizes mephenytoin.

The complete sequence of pHL5,5 is presented in Fig. 3.11. the accurate size of the PstI fragments was determined to be 894, 640 and 430bp respectively. The sequence surrounding the first ATG is included in the consensus sequence (A/G) NNATG (A/G) characteristic of an active start codon (Kozak, 1984). An open reading frame of 1470 nucleotides was found which terminates 37 nucleotides before the second internal PstI site. There is a 3' untranslated region of nearly 400 nucleotides mostly encoded by the small PstI fragment. The most obvious polyA$^+$ addition site is AATTAAAA which is in the expected position 25-30bp from the polyA$^+$ tail. It is possible that there is a longer 5' leader sequence which would account for the larger size of the mRNA transcript alternatively the size difference could be due to polyA$^+$ tail length which is normally 200-300 nucleotides long (Hastie and Bishop, 1976).

The primary coding sequence of pHL5,5 predicts a protein of 490 amino acids long with a calculated molecular weight of 55.6 kd. The first 29 amino acids from the putative N-terminal sequence is identical to that reported for a human P-450 which metabolizes mephenytoin (Shimada et al, 1986). The molecular weight of the mephenytoin P-450 is 50kd on SDS gels. This size difference between predicted and estimated molecular masses has been noted for many P-450s (Black and Coon, 1986). The primary coding sequence of pHL5,5 can be aligned with rat P-450 forms, PB-1, f and M (Freidberg et al, 1986; Gonzalez et al, 1986a; Yoshioka et al, 1984) without the need for insertions or deletions. At the amino acid level these P-450s show greater than 70% homology (Table 3.1). Thus this human

FIGURE 3.11.

The nucleotide sequence of pHL5,5. This corresponds to the human PB-1 cDNA (P450C2C) sequence and its predicted amino acid sequence. The amino acid sequence underlined corresponds to the N-terminal sequence determined for P450mp (Shimada *et al*, 1986). The boxed sequence corresponds to the putative heme binding site around cys435 (Black and Coon, 1986). The two internal PstI sites are also illustrated.

```
1
GGGGGGGGGGCGGCAATGGATTCTCTTGTGGTCCTTGTGCTCTGTCTCTCATGTTTGCTT
              M  D  S  L  V  V  L  V  L  C  L  S  C  L  L   15
61
CTCCTTTCACTCTGGAGACAGAGCTCTGGGAGAGGAAAACTCCCTCCTGGCCCCACTCCT
 L  L  S  L  W  R  Q  S  S  G  R  G  K  L  P  P  G  P  T  P   35
121
CTCCCAGTGATTGGAAATATCCTACAGATAGGTATTAAGGACATCAGCAAATCCTTAACC
 L  P  V  I  G  N  I  L  Q  I  G  I  K  D  I  S  K  S  L  T   55
181
AATCTCTCAAAGGTCTATGGCCCTGTGTTCACTCTGTATTTTGGCCTGAAACCCATAGTG
 N  L  S  K  V  Y  G  P  V  F  T  L  Y  F  G  L  K  P  I  V   75
241
GTGCTGCATGGATATGAAGCAGTGAAGGAAGCCCTGATTGATCTTGGAGAGGAGTTTTCT
 V  L  H  G  Y  E  A  V  K  E  A  L  I  D  L  G  E  E  F  S   95
301
GGAAGAGGCATTTTCCCACTGGCTGAAAGAGCTAACAGAGGATTTGGAATTGTTTTCAGC
 G  R  G  I  F  P  L  A  E  R  A  N  R  G  F  G  I  V  F  S  115
361
AATGGAAAGAAATGGAAGGAGATCCGGCGTTTCTCCCTCATGACGCTGCGGAATTTTGGG
 N  G  K  K  W  K  E  I  R  R  F  S  L  M  T  L  R  N  F  G  135
421
ATGGGGAAGAGGAGCATTGAGGACTGTGTTCAAGAGGAAGCCCGCTGCCTTGTGGAGGAG
 M  G  K  R  S  I  E  D  C  V  Q  E  E  A  R  C  L  V  E  E  155
481
TTGAGAAAAACCAAGGCCTCACCCTGTGATCCCACTTTCATCCTGGGCTGTGCTCCCTAC
 L  R  K  T  K  A  S  P  C  D  P  T  F  I  L  G  C  A  P  Y  175
541
AATGTGATCTGCTCCATTATTTTCCATAAACGTTTTGATTATAAAGATCAGCAATTTCTT
 N  V  I  C  S  I  I  F  H  K  R  F  D  Y  K  D  Q  Q  F  L  195
601
AACTTAATGGAAAAGTTGAATGAAAACATCAAGATTTTGAGCAGCCCCTGGATCCAGATC
 N  L  M  E  K  L  N  E  N  I  K  I  L  S  S  P  W  I  Q  I  215
661
TGCAATAATTTTTCTCCTATCATTGATTACTTCCCGGGAACTCACAACAAATTACTTAAA
 C  N  N  F  S  P  I  I  D  Y  F  P  G  T  H  N  K  L  L  K  235
721
AACGTTGCTCTTATGAAAAGTTATATTTTGGAAAAAGTAAAAGAACACCAAGAATCAATG
 N  V  A  L  M  K  S  Y  I  L  E  K  V  K  E  H  Q  E  S  M  255
781
GACATGAACAACCCTCAGGACTTTATTGATTGCTTCCTGATGAAAATGGAGAAGGAAAAG
 D  M  N  N  P  Q  D  F  I  D  C  F  L  M  K  M  E  K  E  K  275
841                                           Pst I
CACAACCAACCATCTGAATTTACTATTGAAAGCTTGGAAAACACTGCAGTTGACTTGTTT
 H  N  Q  P  S  E  F  T  I  E  S  L  E  N  T  A  V  D  L  F  295
901
GGAGCTGGGACAGAGACGACAAGCACAACCCTGAGATATGCTCTCCTTCTCCTGCTGAAG
 G  A  G  T  E  T  T  S  T  T  L  R  Y  A  L  L  L  L  L  K  315
961
CACCCAGAGGTCACAGCTAAAGTCCAGGAAGAGATTGAACGTGTGATTGGCAGAAACCGG
 H  P  E  V  T  A  K  V  Q  E  E  I  E  R  V  I  G  R  N  R  335
1021
AGCCCCTGCATGCAAGACAGGAGCCACATGCCCTACACAGATGCTGTGGTGCACGAGGTC
 S  P  C  M  Q  D  R  S  H  M  P  Y  T  D  A  V  V  H  E  V  355
1081
CAGAGATACATTGACCTTCTCCCCACCAGCCTGCCCCATGCAGTGACCTGTGACATTAAA
 Q  R  Y  I  D  L  L  P  T  S  L  P  H  A  V  T  C  D  I  K  375
1141
TTCAGAAACTATCTCATTCCCAAGGGCACAACCATATTAATTTCCCTGACTTCTGTGCTA
 F  R  N  Y  L  I  P  K  G  T  T  I  L  I  S  L  T  S  V  L  395
1201
CATGACAACAAAGAATTTCCCAACCCAGAGATGTTTGACCCTCATCACTTTCTGGATGAA
 H  D  N  K  E  F  P  N  P  E  M  F  D  P  H  H  F  L  D  E  415
1261
GGTGGCAATTTTAAGAAAAGTAAATACTTCATGCCTTTCTCAGCAGGAAAACGGATTTGT
 G  G  N  F  K  K  S  K  Y  F  M  P [F  S  A  G  K  R  I  C  435
1321
GTGGGAGAAGCCCTGGCCGGCATGGAGCTGTTTTTATTCCTGACCTCCATTTTACAGAAC
 V  G  E  A  L  A  G] M  E  L  F  L  F  L  T  S  I  L  Q  N  455
1381
TTTAACCTGAAATCTCTGGTTGACCCAAAGAACCTTGACACCACTCCAGTTGTCAATGGA
 F  N  L  K  S  L  V  D  P  K  N  L  D  T  T  P  V  V  N  G  475
1441
TTTGCCTCTGTGCCGCCCTTCTACCAGCTGTGCTTCATTCCTGTCTGAAGAAGAGCAGAT
 F  A  S  V  P  P  F  Y  Q  L  C  F  I  P  V  !              490
1501                   Pst I
GGCCTGGCTGCTGCTGTCAGTCCCTGCAGCTCTCTTTCCTCTGGGGCATTATCCATCTTC
1561
ACTATCTGTAATGCCTTTTCTCACCTGTCATCTGACATTTTCCCTTCCCTGAAGATCTAG
1621
TGAACATTCGACCTCCATTACGGAGAGTTTCCTATGTTTCACTGTGCAAATATATCTGCT
1681
ATTCTCCATACTCTGTAACAGTTGCATTGACTGTCACATAATGCTCATACTTATCTAATG
1741
TTGAGTTATTAATATGTTATTATTAAATAGAGAAATATGATTTGTGTATTATAATTCAA
1801
AGGCATTTCTTTTCTGCATGTTCTAAATTAAAAAGCATTATTATTTGCTG(A)42(GA)10
(G)14(GC)10
```

TABLE 3.1.

Percent similarity of amino acid sequences among the P450C2C gene family in three species. (A) overall similarity (B) over region 200-300 of the protein.

(A)

	Hu PB-1	Rat PB-1	f	m	RabI	Pbc1	Pbc2	Pbc3	PB_{3a}
	1	2	3	4	5	6	7	8	9
1. Hu PB-1	-								
2. Rat PB-1	74.8	-							
3. Rat f	72.2	75.8	-						
4. Rat M((h)	75.5	72.6	69.8	-					
5. Rabbit I	76.8	74.5	69.2	74.3	-				
6. Rabbit PBc1	71.8	67.6	64.3	31.0	72.8	-			
7. Rabbit PBc1	73.9	70.2	65.8	71.6	74.2	84.0	-		
8. Rabbit PBc3	68.7	65.3	62.3	66.7	66.0	64.5	65.5	-	
9. PB_{3a}	49.6	50.1	48.9	53.3	51.3	50.7	51.4	49.1	-

(B)

	1	2	3	4	5	6	7	8	9
1. Hu PB-1	-								
2. Rat PB-1	59	-							
3. Rat f	54	64	-						
4. Rat M((h)	67	64	54	-					
5. Rabbit I	66	65	55	72.7	-				
6. Rabbit PBc1	67	61	55	67.0	65.9	-			
7. Rabbit PBc2	67	65	56	69	65.9	78	-		
8. Rabbit PBc3	53.6	47	43	43.4	51.1	58	54.5	-	
9. PB_{3a}	40	40	36	43	43.3	41	41	39.4	-

Rat PB-1 and f : Gonzalez et al, 1986
Rat M : Yoshioka et al, 1987
Rabbit form I : Tukey et al 1985
Pbc1, PBc2, Pbc3 : Leigton et al, 1984
Rat PB_{3a} (P450C2B): Fuji-kuriyama et al, 1982
Human PB-1 : This study

P-450 is now referred to as human PB-1 and is a member of the P450C2C family of P-450s (Nebert et al, 1987). Human PB-1 also has 50% homology with the highly phenobarbital inducible rat form PB_{3a} (P450-2B) (Fuji-Kuriyama et al, 1983) and other members of the P450-2- subfamily of P-450s in rats (Nebert et al, 1987). This family includes 5 distinct members showing 50% amino acid similarity to each other and includes P450-2A, P450-2B, P450-2C, P450-2D and P450-2E. With the other members of the superfamily of P-450s human PB-1 shows less than 35% homology.

Human PB-1 also has 45% amino acid identity with the partial sequence of human UT_1 (P450C2A) (Phillips et al, 1985) but only 35% homology with members of the P450C1 and P450C3 gene families in humans (Quattrochi et al, 1986; Beaune et al, 1986). The nomenclature of P-450 gene families inserts a C into the human forms. Thus P450C2C in humans is the same as P450-2C in rats, mice and rabbits. The C corresponds to cDNA as proteins corresponding to all the human P-450 cDNAs have not yet been identified (Nebert et al, 1987).

Human PB-1 also contains the conserved regions found in all cytochrome P-450s (Table 3.2). It is interesting that the N-terminal conserved fragment has a cysteine at amino acid 144 whereas the equivalent rat forms have an arginine at this position, over this region pHL5,5 is most similar to PB-1a of rat. This cysteine at 144 has not been described in any other P-450 forms. In the conserved C-terminal fragment there is a glycine at position 444 which has not been found in other P-450 forms. Whether these changes are functionally significant is unknown.

The DNA sequence detected by the C128 oligomer shows 6/19 mismatch with a continuous stretch of 8 nucleotides showing maximum

TABLE 3.2.

Comparison of three conserved amino acid regions form diverse cytochrome P-450s with human PB1 P-450.
A) N-terminal cysteine fragment centred at Cys 152 of PB3a
B) C-terminal cysteine fragment centred at Cys 436 of PB3a
C) The 'analogous tridecapeptide' region at Met346-Arg358 of PB3a

	P-450	sequence																			aa
A)	PB-1 hu	I	E	D	C	V	Q	E	E	A	R	C	–	L	V	E	E	L	R	K	158
	PB3a rat	V	E	E	R	I	Q	E	E	A	Q	C	–	L	V	E	E	L	R	K	159
	PB1a "	I	E	D	R	V	Q	E	E	A	R	C	–	L	V	E	E	L	R	K	158
	f "	I	E	D	R	V	Q	E	E	A	Q	C	–	L	V	E	E	L	R	Q	158
	PB2A(M) rat	I	E	D	R	I	G	E	E	A	Q	C	–	L	V	E	E	L	R	K	158
	UT-1 rat	V	E	E	R	I	I	E	E	A	G	Y	–	L	J	K	M	L	Q	G	161
	Db-1 "	T	L	R	T	F	G	M	G	K	K	S	–	L	E	E	W	V	T	K	158
	Db-1 "	T	L	R	D	F	G	V	G	K	K	S	–	L	E	Q	W	V	T	K	158
	j "	N	E	A	R	I	Q	R	E	A	Q	F	–	L	V	E	E	L	K	K	170
	CLO "	L	D	K	Y	E	Q	L	A	G	Q	D	–	S	S	I	E	J	F	Q	186
	PB2b "	M	F	P	I	I	E	Q	Y	G	D	T	–	L	V	K	Y	L	K	Q	163
	MC1a "	L	E	E	H	V	S	K	E	A	N	H	–	L	J	S	K	F	Q	K	177
	MC1b "	L	E	E	H	V	S	K	E	A	E	Y	–	L	V	S	K	F	Q	K	179
	MC1a "	J	A	S	D	P	T	S	V	S	S	C	Y	L	E	E	H	V	S	K	166
	MC1b "	I	A	S	D	P	T	L	A	S	S	C	Y	L	E	E	H	V	S	K	168
	P-450 cam	L	E	N	R	I	Q	E	L	A	–	C	S	L	J	E	S	L	R	P	142

	P-450																						aa
B)	PB-1 hu	F	S	A	G	K	R	I	C	V	G	E	A	L	A	G	M	E	L	F	L	F	448
	PB3a rat	F	S	T	G	K	R	I	C	L	G	E	G	I	A	R	N	E	L	F	L	F	449
	PB1a "	F	S	A	G	K	R	M	C	A	G	E	G	L	A	R	M	E	L	F	L	F	448
	f "	F	S	T	A	K	R	I	C	V	G	E	G	J	A	R	Q	E	L	F	L	F	448
	PB2A(M) rat	F	S	A	G	K	R	I	C	A	G	E	A	L	A	R	T	E	L	F	L	F	446
	UT-1 rat	F	S	T	G	K	R	F	C	L	G	D	E	L	A	K	M	E	L	F	L	L	450
	Db-1 "	F	S	A	G	R	R	A	C	L	G	E	P	L	A	R	M	E	L	F	L	F	459
	Db-2 "	F	S	A	G	R	R	A	C	L	G	E	P	L	A	R	M	E	L	F	L	F	459
	j "	F	S	A	G	K	R	V	C	A	G	E	G	L	A	R	M	E	L	F	L	L	450
	CLO "	F	S	G	G	A	R	N	C	I	G	K	Q	F	A	M	S	E	M	K	V	I	468
	PB2b "	F	G	N	G	P	R	N	C	I	G	M	R	F	A	L	M	N	M	K	L	A	456
	MC1a "	F	G	L	G	K	R	K	C	J	G	F	J	P	A	K	W	E	V	F	L	F	469
	MC1b "	F	G	L	K	G	R	K	C	I	G	E	Y	I	G	R	L	E	V	F	L	F	473
	P-450 cam	F	G	H	G	S	H	L	C	L	G	Q	S	L	A	R	R	F	J	J	V	J	368

	P-450														aa
C)	PB-1 hu	M	P	Y	T	D	A	V	V	H	E	V	Q	R	357
	PB3a rat	M	P	Y	T	D	A	V	J	H	F	I	Q	R	358
	PB-1	M	P	Y	T	D	A	H	D	H	E	V	Q	R	357
	f	M	P	Y	T	D	A	V	I	H	E	J	G	R	357
	PB2a (M) rat	M	P	Y	T	D	A	V	V	H	E	I	Q	R	357
	UT-1 rat	M	P	Y	T	Q	A	V	I	N	E	L	Q	R	369
	Db-2 "	M	P	L	T	N	A	V	J	H	E	V	Q	R	356
	j "	M	P	Y	M	D	A	V	V	H	E	I	Q	R	359
	CLO "	J	P	Y	T	T	M	C	J	K	E	A	L	R	379
	PB2b "	R	L	Y	P	I	G	N	R	L	F	R	V	C	390
	MC1a "	L	P	Y	L	E	A	P	I	L	E	I	Y	R	375
	MC1b "	L	P	Y	L	E	A	P	I	L	E	I	P	R	380
	LM2 rabbit	M	P	Y	T	D	A	V	I	H	E	J	Q	R	350

alignment. This could explain the slight hybridization observed with this oligomer. The sequence is:

C128 T T G G T G G A G G A A C T G C G G A

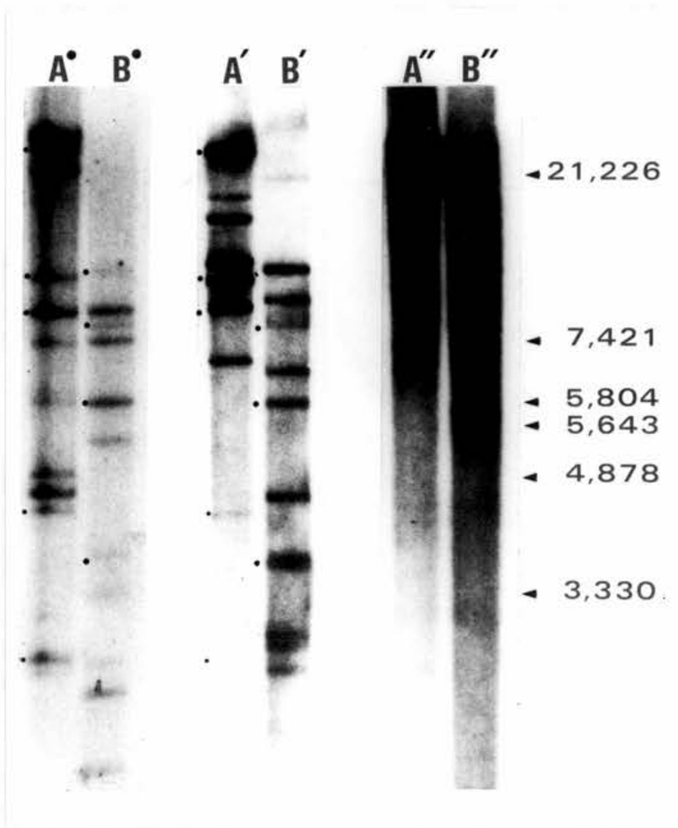
PB-1 C T T G T G G A G G A G T T G A G A A

3.11. Genomic complexity of human P450C2C

The high stringency Southern blots of pHL5,5 to human and rodent DNAs (Fig. 3.9) revealed an unique pattern of hybridization of some complexity. The result implied that human PB-1 is a member of a small multigene family or corresponds to a very large gene. The former interpretation seems reasonable because of the multiple PB-1 forms identified in many species including humans (Adams et al, 1985; Shimada et al, 1986). In order to investigate this hypothesis, the DNA of one individual was digested with EcoRI and BamHI and probed with the separate PstI fragments from pHL5,5. The 894bp fragment represents the 5' coding half of the PB-1 transcript, the 640bp fragment represents the 3' coding half of the PB-1 transcript and the 430bp represents the 3' end of the transcript. In order to obtain a minimum gene count for the P450C2C locus identical Southern blots were probed with these PstI fragments. Any DNA fragments which hybridize to both 5' and 3' probes must represent separate and distinct genes or pseudogenes. Therefore the fragments in common give a value for the minimum number of genes. As can be seen for Fig. 3.12, there are a number of DNA fragments in common with the 894bp fragment and 640bp fragment PstI fragments. The 430bp fragment gives a persistent smear at this stringency wash (2 x SSC wash) and at lower stringency (0.1 x SSC). However 2 major fragments do appear to be in common to all three probes. The use of the 430bp untranslated region probe may provide an

FIGURE 3.12.

Complexity analysis of the P450C2C DNA pattern. Three identical Southern transfer of *Bam*HI (A) and *Eco*RI (B) digested human DNA were probed with the three individual *Pst*I fragments from p/T, p/M and p/B respectively. The p/T fragment corresponds to 5' half of the cDNA. (Lanes A^{o} and B^{o}). The p/M fragment corresponds 3' coding half of the cDNA (Lanes A' and B'). The p/B fragment corresponds to the 3' untranslated region of the cDNA (Lanes A" and B"). '●' indicates fragments in common.


A•
B•
A′
B′
A″
B″
21,226
7,421
5,804
5,643
4,878
3,330

underestimate of the gene number because this region can show great diversity between members of the same multigene family (Li et al, 1985a). If we consider the coding region probes then a minimum count would be 3 genes for PB-1 in human. Also apparent in the original Southern blot with pHL5,5 plasmid was the greater complexity of rodent PB-1 sequences relative to primate. Gene counts for rodent were obtained from Southern blots of EcoRI, BamHI and KpnI digests of rat and mouse DNA with the two coding region probes (Fig. 3.13 A and B). In mouse the minimum estimate for the number of PB-1 genes was 5-7 and for rat 4-6 genes. In the case of rat this is in rough agreement with the number of different PB-1 forms purified to date, which presently stands at 6.

3.12. Chromosomal location of human PB-1 P-450.

It was of interest to know the relationship of the different members of the PB-1 gene family to each other i.e. whether they mapped to a similar or distinct chromosomal location. Somatic cell hybrid DNAs which contained selected human chromosomes on a Chinese hamster ovary (CHO) or mouse cell background were used.

To establish that human PB-1 genes could be distinguished on these backgrounds a Southern blot were carried out in which CHO DNA, DBA/2 mouse DNA and human male DNA (MO) were digested with a number of restriction enzymes and probed with a mixture of p/M and p/T inserts. Although the resulting pattern was complex BamHI digests of human and rodent DNA suggested that a triplet of human fragments at 4kb could be distinguished on a rodent background (data not shown). Additionally Southern blots of BamHI digests of human placental DNAs probed with p/T and p/M did not detect any restriction fragment length polymorphism (RFLP) (data not shown).

FIGURE 3.13.

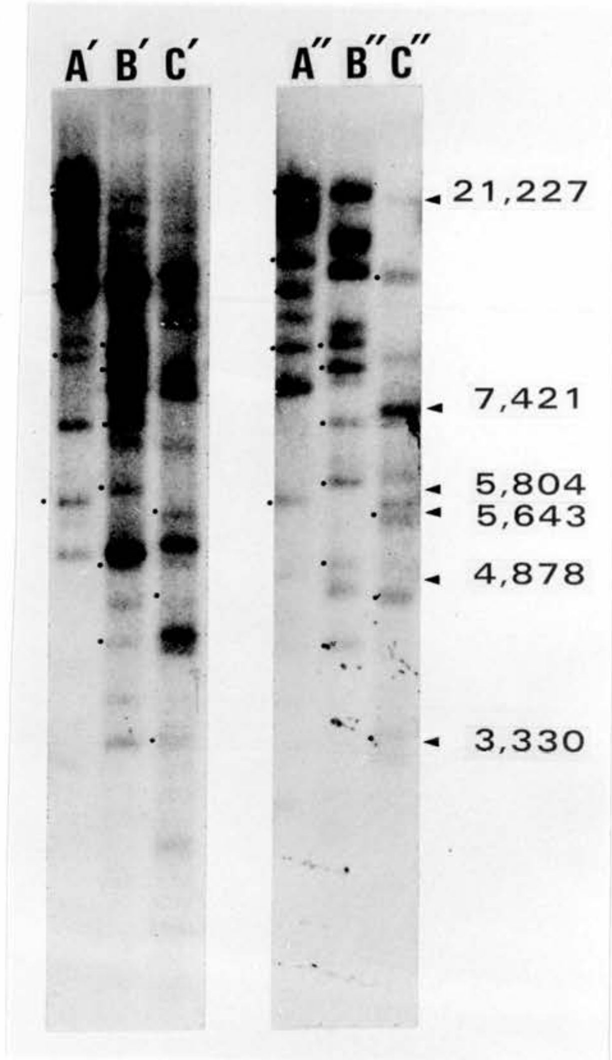
Gene counts in rat and mouse

A) Male wistar rat DNA was digested with *Eco*RI (A), *Bam*HI (B) and *Kpn*I (C). Identical blots were run and probed with p/T (A', B' and C') and p/M (A", B" and C").

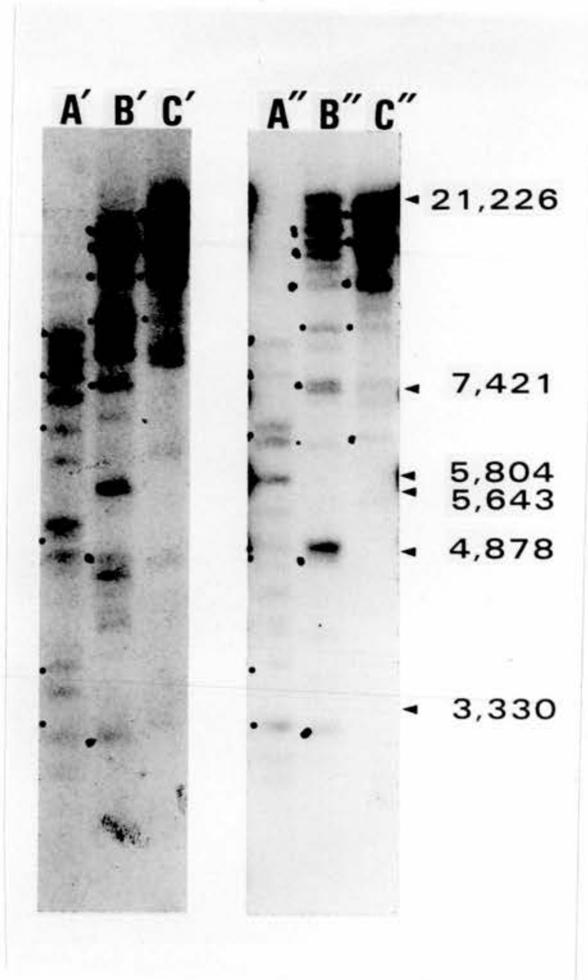
B) Male C57L DNA was digested with *Eco*RI (A), *Bam*HI (B) and *Kpn*I (C). Identical blots were run and probed with p/T (A', B' and C') and plan (A", B" and C").

'●' fragments in common.

A



B

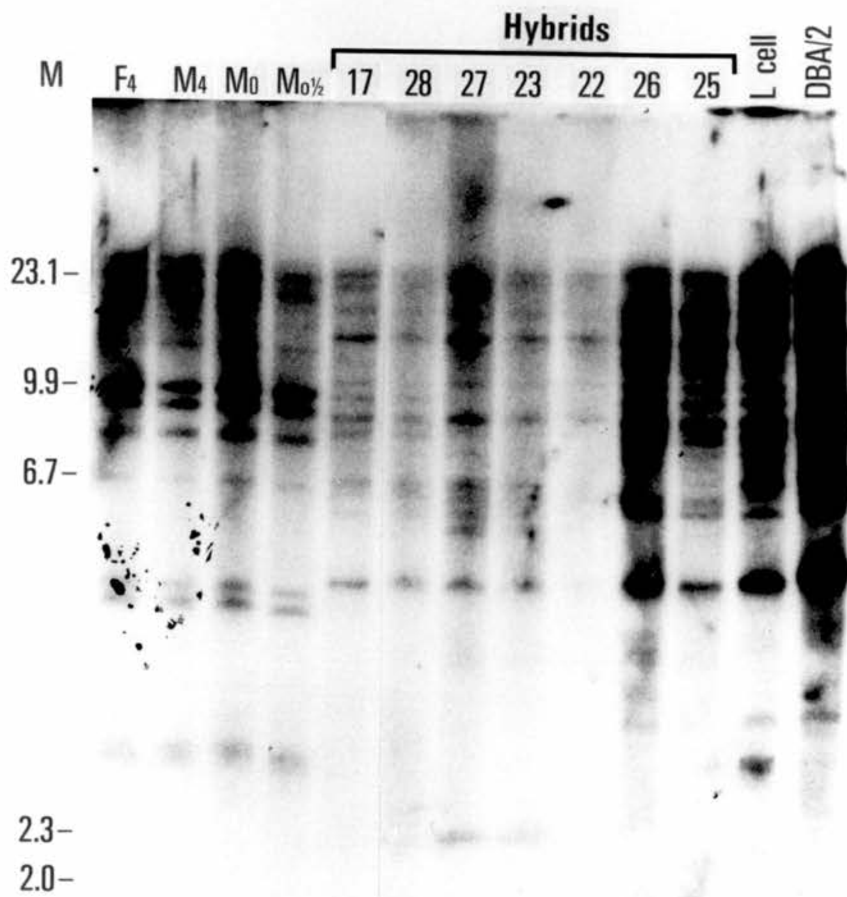


Therefore the different sources of the hybrids would not be compromised by RFLPs.

Initially a small panel of mouse/human somatic cell hybrid DNAs was analysed. Most of the hybrids used contained single human chromosomes (Fig. 3.14). As controls human male and female DNA was run as well as the parental rodent DNAs. This would indicate whether PB-1 mapped to either the X or Y chromosomes. In this initial analysis none of the hybrids appeared to contain human sequences homologous to the p/T and p/M inserts used as probes. The human chromosomal content of these hybrids had previously been characterized in our laboratory and thus human chromosomes 2, 4, 7, 11, 13, 15, 21, 22, X and Y could be excluded for the location of human PB-1 genes. A screen of a larger representative panel of rodent/human somatic cell hybrids identified one hybrid which contained human DNA fragments which hybridized with p/T and p/M. A major triplet of human PB-1 fragments can be observed at around 4kb and a minor fragment at ~2.3kb (Fig. 15 A and I). Analysis of these hybrids showed human chromosome 10 was concordant with this signal. In order to confirm this assignment a range of other chromosome 10 containing hybrids from rat, mouse and Chinese hamster fusions were studied. These hybrids gave 100% concordance between the human signal and chromosome 10 (Table 3.3). Three hybrids deserve special mention; numbers 2 and 9 (Table 3.3) are sister subclones with an almost identical human chromosome content except that hybrid 2 contains a chromosome 10 and hybrid 9 contains a chromosome 9. In this case only hybrid 2 was positive for human fragments (Fig. 3.16, 1 and 2). The third hybrid (mouse) of note is hybrid 3 which has a diploid number of human chromosome 10 and gave an increased signal intensity relative to the mouse background when probed for human

FIGURE 3.14.

Southern blot analysis of human/rodent somatic cell hybrids probed with p/T and p/M. 10μg of DNA were digested with *Bam*HI, fractionated on agarose gels, and blotted. The human chromosome content of the hybrids is indicated on Table 3.1. L cell and DBA/2 represent the parental rodent cell line DNA. F4 is human female DNA, M4 and Mo human male DNA, $Mo_{1/2}$ is 5μg of DNA instead of 10μg.



Hybridization of P/T and P/M to human/mouse somatic cell hybrids.

FIGURE 3.15.

Southern blot analysis of a representative panel of human/rodent somatic cell hybrid DNA with p/T and p/M. Lane A, hybrid 1, Lane B, hybrid 24; lane F, hybrid 21; Lane G, hybrid 17; Lane H, hybrid 10; Lane I, hybrid 1; Lane J, hybrid 27; Lane K, hybrid 20; Lane L, hybrid 14; Lane M, hybrid 12; Lane N, WG3H (control hamster DNA); lane O, C57L (mouse control DNA; Lane P, human female placental DNA only 1/4 of the amount loaded compared to the hybrid DNA). '◄' on the right hand side indicates fragments there mapped to chromosome 10. Only one hybrid (Lanes A and I) were positive for human bands. See Table 3.1 for karyotype of hybrids and Materials and Methods for their origin. Size markers were λ HindIII.

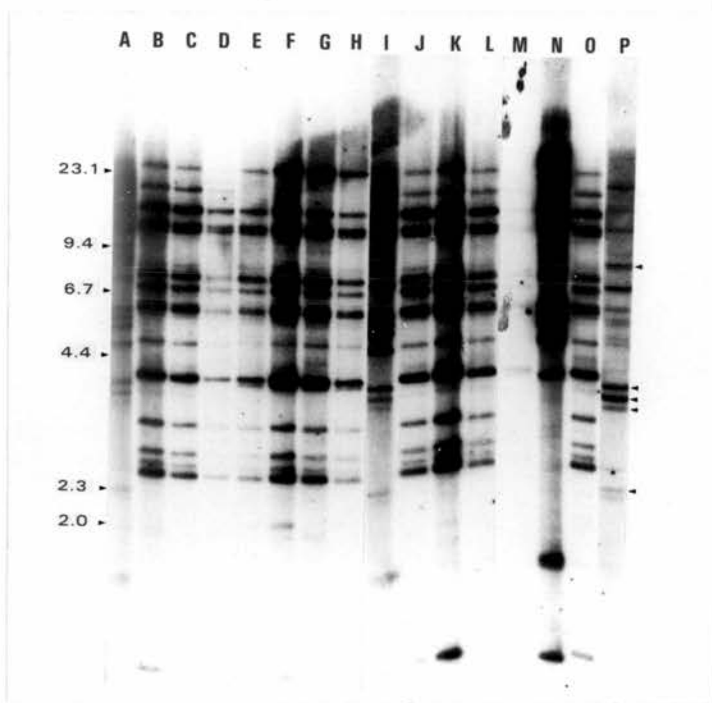

A B C D E F G H I J K L M N O P
23.1
9.4
6.7
4.4
2.3
2.0

TABLE 3.3
Segregation of PB-1 P-450 with Human Chromosomes in Cell Hybrids

Hybrid cell	Human Chromosomes ‡																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
^1	-	+	-	(+)	(+)	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	+	-	-	-
^2	-	+	+	-	+	-	-	+	-	+	-	+	-	+	-	-	-	+	-	-	+	-	-	-
^3	+	+	+	-	-	+	+	+	-	+	-	+	-	+	-	+	+	+	-	-	+	+	+	-
^4	-	-	+	-	+	-	-	-	-	+	+	+	+	+	+	-	+	+	-	+	+	+	+	-
^5	-	-	-	-	-	-	+	-	-	+	+	-	-	+	+	-	+	-	-	-	+	-	+	-
^6	-	-	+	-	-	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	+	-	-
^7	-	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-	-	-	-	-	+	+	-
^8	-	+	-	-	-	+	+	-	-	+	-	-	-	+	+	-	-	-	-	-	-	-	+	-
9	-	+	+	-	+	-	-	+	+	-	-	+	-	+	-	-	-	+	-	-	+	-	-	-
10	+	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	+	+	+	-	+	-	-
*11	+	-	+	-	+	+	-	-	+	-	+	+	-	+	-	+	-	-	-	-	+	-	-	-
*12	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
13	-	-	+	+	+	+	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	+	-
14	-	-	-	-	-	+	-	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-	+	-
15	-	-	-	-	-	-	+	+	-	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-
*16	+	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+	+	+	+	-	+	-	-
*17	-	+	-	+	-	-	+	-	-	-	+	-	-	-	+	-	-	-	-	-	+	-	+	-
*18	+	-	+	-	-	+	-	-	-	-	+	-	+	-	-	-	-	-	+	-	+	+	-	+
19	+	+	+	-	+	+	+	+	-	-	-	+	-	+	-	+	+	+	-	-	-	-	+	-
20	-	+	+	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-
21	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+
22	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
27	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Percent Concordant	53	71	64	64	64	57	61	67	89	100	71	71	69	69	69	46	75	71	61	71	71	69	64	64

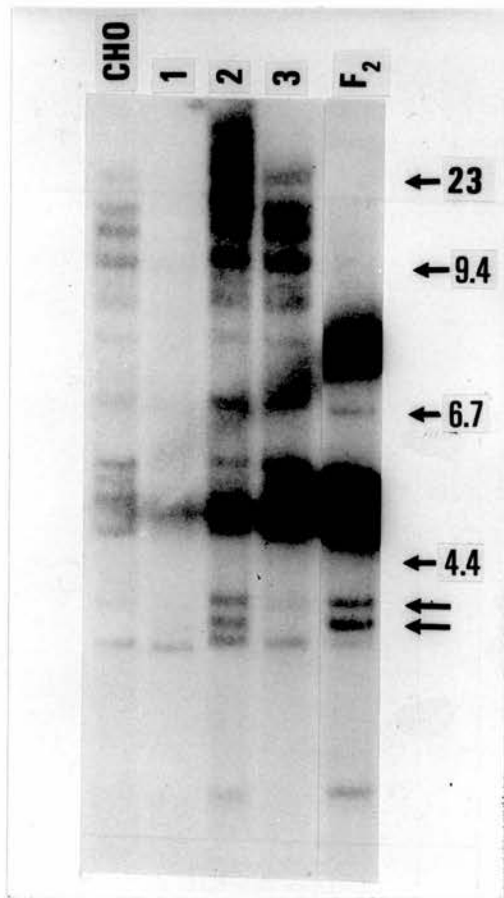
* These hybrids also contain unidentified human marker chromosomes and some identified translocations, see Lund et al (1983), but these abnormal human chromosomes did not give rise to any aberrant segregation patterns for the PB-1 P- 450 cDNA clone.

‡ "+" indicates the presence of the human chromosomes >20% of the metaphases analysed, (+) indicates the presence in 10% of metaphases analysed. "-" indicates the absence of the human chromosome in the metaphases analysed.

^ Hybrids 1 to 8 are chromosome 10 containing hybrids.

FIGURE 3.16.

Southern blot analysis of a mini panel of human/rodent somatic cell hybrid DNA probed with p/T and p/M. CHO, Chinese hamster; Lane 1, hybrid 1; lane 2, hybrid 2; lane 3, hybrid 9; F_2, human female DNA, 1/4 loaded in comparison to hybrid DNAs.



CHO
1
2
3
F2
23
9.4
6.7
4.4

PB-1 sequences (data not shown). To reduce the complexity of hybridization and to map more of the human fragments duplicate blots of hybrid DNAs were probed with p/T and p/M respectively. Nearly all the PB-1 fragments appeared to be located on chromosome 10 (data not shown).

In order to sublocalise the PB-1 P-450 genes and to ascertain whether they were clustered on human chromosome 10 pHL5,5 was used by Dr. J. R. Gosden as a probe to metaphase spreads from human blood lymphocytes. The cDNA probe was annealed with total human DNA to eliminate hybridization of the repeat sequence (Sealey et al, 1985; Porteous et al, 1986). Twenty three cells were analysed with a total of 111 grains. Of these 21 grains (23%) were localized on chromosome 10 and of these 13 (62%) were in the region 10q24.1-10q24.3 with a peak at 10q24.2 (Fig. 3.17). From this it was concluded that the PB-1 genes do appear to be clustered on human chromosome 10. In accordance with the nomenclature this locus was designated P450C2C.

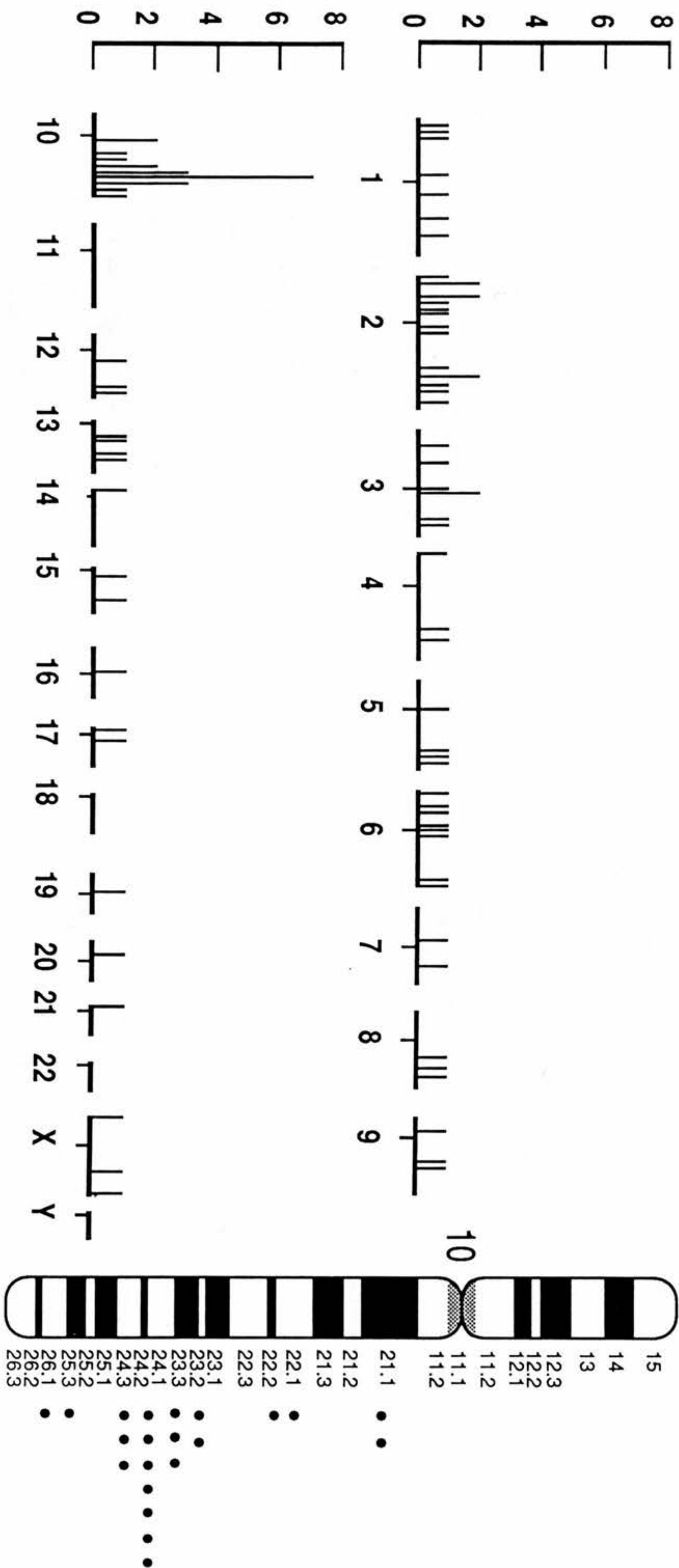
3.13. RFLP search for P450C2C

It is important to note that all adverse drug side effects so far identified with P-450s have been associated with constitutively expressed forms and not the highly inducible forms beloning to the P450C1 and P450C2B gene families (Wolf, 1986). RFLPs for P450C2C would be useful for doing linkage analysis in relation to oxidative metabolism in phenotyped families.

A panel of nine individual placental DNAs was digested with eight restriction enzymes, fractionated on agarose gels, Southern blotted and probed with inserts derived from p/T and p/M subclones

FIGURE 3.17

Autoradiographic grain distribution following hybridization in situ of pHL5.5 to human chromosomes. The horizontal axis shows the chromosomes, with the centromeres indicated by a vertical bar. The vertical axis shows the total grains found at each site in the 23 cells analysed. Note the peak of grains in the long arm of chromosome 10, insert shows a detailed grain distribution on that chromosome.

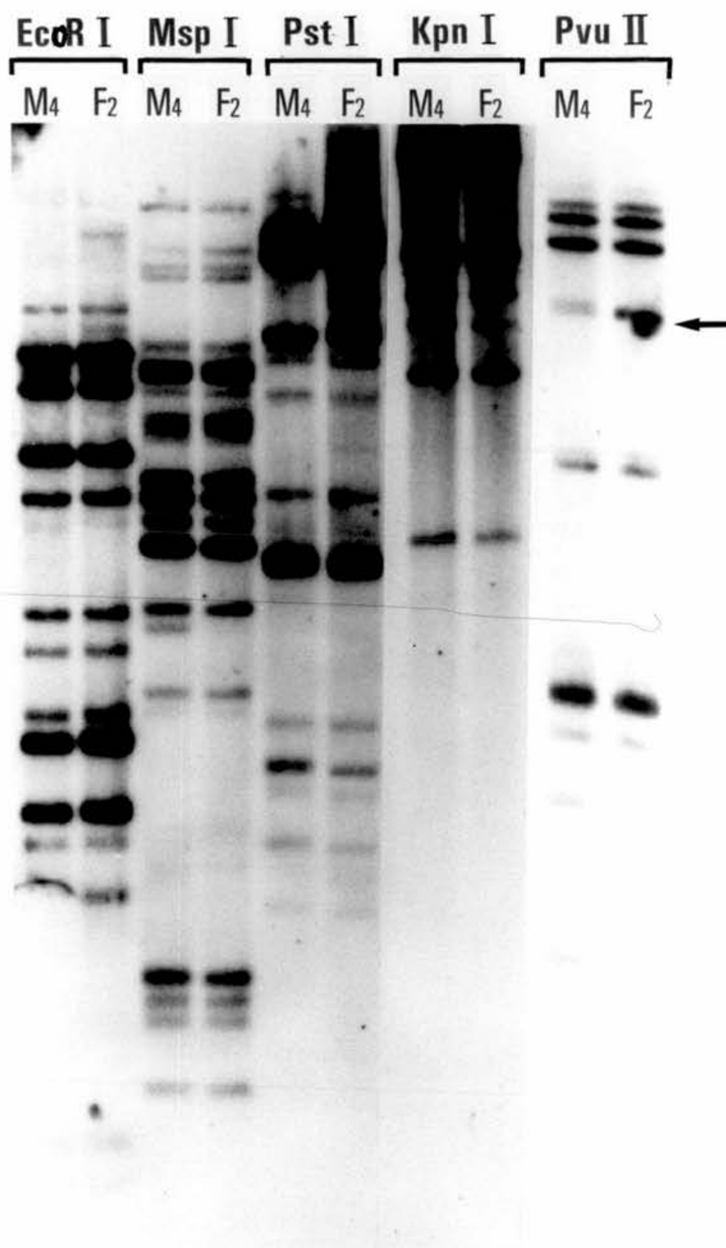


Total grain counts
0 2 4 6 8
0 2 4 6 8
1 2 3 4 5 6 7 8 9
10 11 12 13 14 15 16 17 18 19 20 21 22 X Y
10
15
14
13
12.3
12.2
12.1
11.2
11.1
11.1
11.2
21.1
21.2
21.3
22.1
22.2
22.3
23.1
23.2
23.3
24.1
24.2
24.3
25.1
25.2
25.3
26.1
26.2
26.3

of pHL5,5. No variant patterns of hybridization were seen with EcoRI, BamHI, HindIII, PstI or KpnII digests of human DNA. On PvuII digestion two individuals (Table 3.3) showed an apparent shift in hybridization of a 10kb fragment. The same two individuals showed a loss of a 3kb band on MspI digestion (Table 3.4). This was rechecked by taking a representative from each different group M_4 and F2 and digesting again with EcoRI, MspI, PstI, KpnI and PvuII (Fig. 3.18). Again on PvuII digestion F_2 showed a change on the migration of a 10kb band, F_2 also showed the loss of a 3kb MspI fragment. Due to the complexity of hybridization in all these experiments it is difficult to see the appearance of new bands of hybridization, concomitant with the loss of the MspI fragment and shift in the PvuII fragment. However M_4 digested with PvuII does look like it contains two fragments, one slightly larger than the other at 10kb (Fig. 3.18). However the proximity of the two bands at 10kb does not make it useful for distinguishing between the different variants for this particular enzyme. The MspI variants did not look useful either as again the pattern was too complicated to determine between homo- or heterozygotes. TaqI digestion initially looked very promising as 5/9 individuals had a variant fragment at approximately 1kb (Figure 3.19) which could be observed even after digestion overnight with TaqI at 65°C. Again no concomitant change could be seen in the individuals without this extra 1kb band. This fragment could only be detected with the p/T subfragment which corresponds to 5' coding region of pHL5,5. Even with this reduced complexity of hybridization no additional variants could be identified either with p/T or p/M subclones. Partial digestion of the DNA could be ruled out because when duplicate blots of TaqI digested DNA were probed separately with p/T or p/M no

FIGURE 3.18.

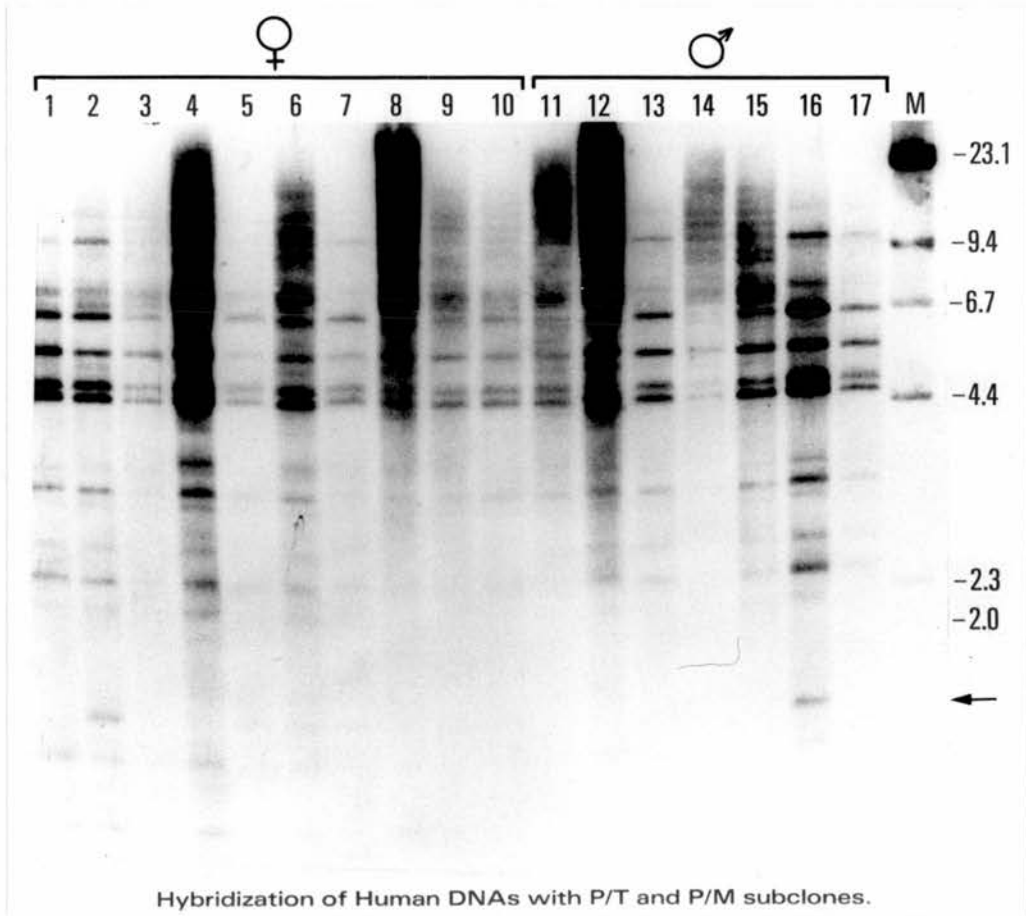
Concordance of MspI and PvuII RFLP. Male (M_1) and Female (F_2) DNA were cut with the indicated restriction enzymes and analysed as in Fig. 3.15. Arrows indicate PvuII polymorphism in F_2 and possible doublet in M.



Hybridization of P/T and P/M to human DNA digests.

FIGURE 3.19

<u>TaqI polymorphism detected by p/T and p/M.</u> DNA from 17 individual lymphoblastoid cell lines DNAs was digested with <u>TaqI</u> and analysed as in Fig. 3.18. Size markers, λ <u>Hind</u>III and the arrow indicates the polymorphic band.



Hybridization of Human DNAs with P/T and P/M subclones.

TABLE 3.4

Restriction enzymes used in polymorphism search and number of variants detected

	[1]Placental DNA	[2]Lymphoblastoid DNA	[3]UTAH DNA	Total
BamHI	0/9	-	-	0/9
EcoRI	0/9	-	-	0/9
HindIII	0/9	-	-	0/9
[4] PvuII	2/9	-	-	2/9
[4] MspI	2/9	-	-	2/9
KpnI	0/9	-	-	0/9
PstI	0/9	-	-	0/9
TaqI	5/9	6/17	6/14	17/40

[1] Panel of 9 random DNA samples

[2] 17 random samples provided by Dr. P. Middleton

[3] UTAH family DNA, generation II provided by Dr. N. Spurr.

[4] Same two individuals gave a variant pattern of hybridization on digestion with PvuII and MspI and probed with p/T and p/M.

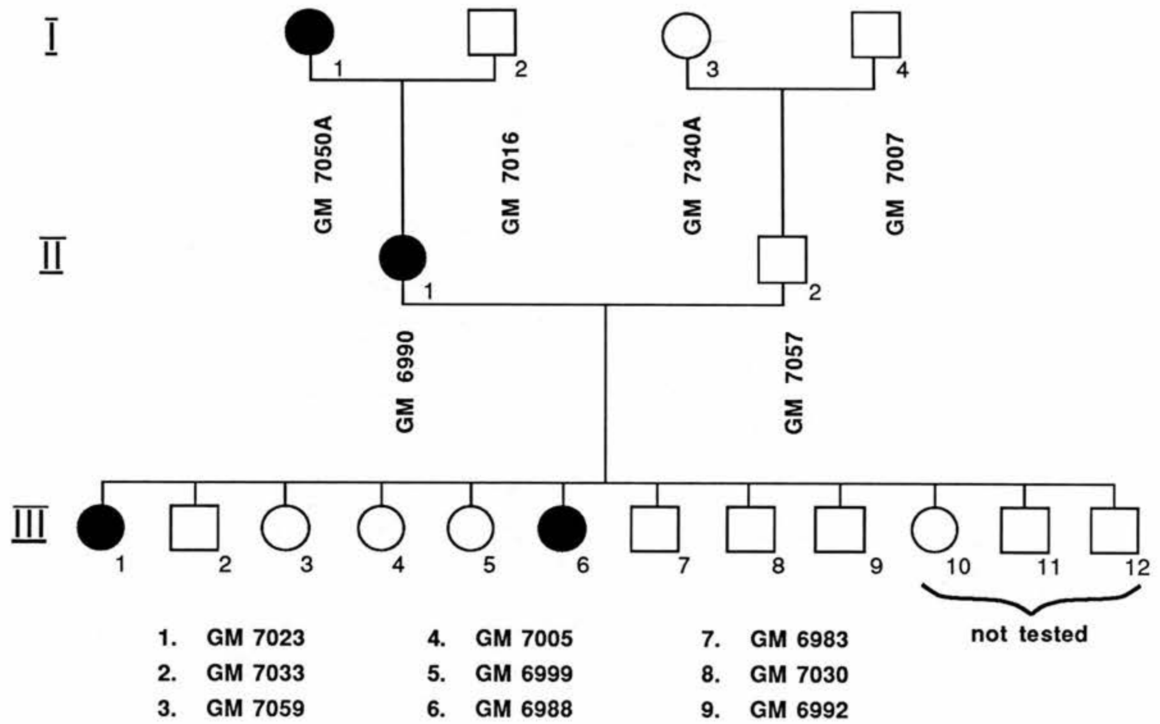
partials were seen with the individuals who had the 1kb band when probed with the p/M subclone (data not shown). In a UTAH family pedigree the 1kb Taq fragment showed a consistent inheritance through three generations (Fig. 3.20). This pedigree was originally chosen as GM6990 showed an intensely hybridizing *TaqI* 1kb band. It was hoped this might have been a homozygote but only one parent had this band on *TaqI* digestion and 2 out of 9 progeny which suggests it was heterozygous for this fragment. Single allele polymorphisms are of no use in trying to look for segregation of genotype and linked phenotype in families. This has been remedied for *P450C2C* by Dr. N. Spurr who has identified a simple 2 allele polymorphism with *XmnI* digestion of human DNA and probing with the p/T subclone (Dr. N. Spurr, pers. comm.). This can now be used in family studies to see whether there is tight linkage between *P450C2C* and mephenytoin metabolism.

3.14. Discussion

One of the aims of this study was to isolate and characterize a human cytochrome P-450 cDNA, preferentially a constitutive form as these had been implicated as being polymorphic with respect to the oxidative metabolism of many marker substrates (Kalow *et al*, 1986). By a number of criteria one of the two clones isolated was shown to correspond to the rat P-450s PB-1, f and M. This included a characteristic induction pattern on northern blots of hepatic mRNA, Southern blot analysis and sequence comparison. Additionally the human PB-1 appeared to correspond to a human P-450 involved in the metabolism of mephenytoin, P-450mp, which is polymorphic in humans. Recently antibodies to P-450mp were used to screen a human liver

FIGURE 3.20.

Pedigree analysis of TaqI polymorphism. Filled circles represent members of this pedigree which have the 1kb TaqI band. Each individual DNA was digested with TaqI and analysed as in Fig. 3.29. "□" = male. "O" = female.


UTAH PEDIGREE K-1331
I
1
2
3
4
GM 7050A
GM 7016
GM 7340A
GM 7007
II
1
2
GM 6990
GM 7057
III
1
2
3
4
5
6
7
8
9
10
11
12
1. GM 7023
2. GM 7033
3. GM 7059
4. GM 7005
5. GM 6999
6. GM 6988
7. GM 6983
8. GM 7030
9. GM 6992
not tested

bacteriophage λ gt11 library (Umbenhauer et al, 1987). The amino acid sequence of this cDNA shows 5/485 amino acid differences with that encoded by pHL5,5 which shows these two clones are closely related if not the same. When the overall amino acid composition was compared between P-450mp and that derived from the mp cDNA and pHL5,5 there was a large difference in the determination of several amino acids (Table 3.5). This was particularly the case for the alanine, aspartic acid/asparagine and glycine. These differences could reflect the limits of accuracy in the determinations of amino acid composition. Alternatively the mp cDNA and pHL5,5 code for a protein related to but not one identical with that of P-450mp.

3.14.1. How many forms of human PB-1 are there?

A number of criteria suggest there is more than one form of human PB-1 P-450 that are not allelic variants of each other. Firstly, more than two forms of P-450mp are detected in a single liver sample (Shimada et al, 1986) of 48kd and 50kd respectively. Western blot analysis of human liver microsomes with anti-rat PB-1 detects two bands (Adams et al, 1985; L. Forrester, pers. comm.) of 50kd and 54kd respectively. This difference in size estimates could be due to differences in gel electrophoresis conditions. The larger size detected by the rat antibodies, 54kd, is in closer agreement with the predicted molecular weight of human PB-1 P-450 of 55.6kd.

Northern blot analysis of human liver mRNA samples detected multiple bands which may correspond to different PB-1 transcripts. In the initial screen of the Woods cDNA library two cDNA clones were isolated which hybridized with rat pTF-1 insert. This same probe was used to screen another human liver cDNA library constructed in λ gt11. Four positive plaques were isolated each of these had the

TABLE 3.5

Amino Acid compositions of purified P-450mp protein, derived from mp cDNA and pHL5,5 cDNA sequences.

	*P-450mp	*mpcDNA	pHL5.5. cDNA
Ala	31 ± 7	18	18
Arg	22 ± 5	21	20
Asx	33 ± 5	47	47
Glx	41 ± 6	46	46
Gly	39 ± 1	27	28
His	12 ± 2	12	12
Ile	26 ± 4	34	34
Leu	51 ± 5	57	59
Lys	46 ± 12	35	35
Met	12 ± 1	13	14
Phe	26 ± 4	32	31
Pro	31 ± 2	31	31
Ser	31 ± 4	31	32
Thr	23 ± 4	24	24
Trp	3 ± 1	3	3
Tyr	14 ± 3	11	13
Val	30 ± 3	29	30

* Data from Umbenhauer <u>et al</u>, 1987

same restriction map as pHL5,5 on PstI and EcoRI digestion (data not shown). This suggested that this is the major form of PB-1 which is expressed and corresponds to the pHL5,5 cDNA. In addition the Woods and λ gt11 liver libraries were grown up and DNA prepared from them. These were digested with restriction enzymes to release their inserts and Southern blotted. When pTF-1 was used as a probe most of the bands detected appear to correspond in size to those seen with pHL5,5 (data not shown).

The complex pattern of hybridization seen on Southern blots of human DNA also suggest the presence of multiple genes. A minimum gene count would suggest 2-3 genes. In human breast tissue a human PB-1 protein is detected on western blots with anti-rat PB-1 antibodies which is intermediate in size between the 2 liver forms (L. Forrester, pers. comm.) which would support this estimate. An extensive tissue search has not been made for expression of PB-1 forms but PB-1 proteins have also been detected in human kidney (Beaune et al, 1987) which might suggest the presence of additional members of this gene family.

3.14.2. Function of cytochrome P-450 PB-1 and oxidative polymorphism

In view of the structural homology between human PB-1 and the human enzymes involved in mephenytoin metabolism it would appear that the human PB-1 cDNA is part of this gene family, P450C2C. In the case of mephenytoin, 2 phenotypes have been identified, trivially named extensive (EM) and poor metabolizers (PM) (Eichelbaum, 1984; Kupfer and Preisig, 1984). Population and family studies have established that these differences are genetic in character and that the PM phenotype is inherited as an autosomal

recessive (Inaba et al, 1986; Jurima et al, 1985). Comparative intersubjective studies have shown discordance between the mephenytoin and a polymorphism in the oxidation of the drug debrisoquine (Jurima et al, 1985). Poor metabolizers of mephenytoin exist at a frequency of 5% in Caucasian populations which suggests that carriers of the recessive allele should exist at frequency of 0.35 (Hardy-Weinberg) in the population. There is no correlation between the amount of immunoreactive P-450mp in human livers and mephenytoin metabolism (Shimada et al, 1986). This suggests that the difference in the metabolism is due to a structurally altered form.

Some drugs have been found to induce immunoallergic hepatitis in humans with the appearance of circulating antibodies in human sera directed against cell organelles (Homberg et al, 1985; Mackay, 1985). The major epitope of these antibodies was found to be kidney and liver endoplasmic reticulum in rat (Homberg et al, 1984). Subsequently it was found that 12 out of 20 sera recognised a human P-450 which was identified as that which metabolizes mephenytoin (Beaune et al, 1986b). A great number of these sera were from patients who were given the anaesthetic, tienilic acid. This P-450 (P-450mp) was specific for the metabolism of tienilic acid at the 5 position and can lead to the formation of reactive intermediates which form adducts to microsomal proteins. However administration of tienilic acid did not itself induce the immuno allergic hepatitis response which is only observed in 1/10,000 patients. Some sort of secondary event must be required. It is suggested that this cytochrome could be alkylated by a reactive intermediate and migrate to the hepatocyte membrane surface which would then be recognised by the immune system leading to the auto-

immune hepatitis response (Beaune et al, 1986b). This observation is seen with several types of hepatitis induced by drugs with concomitant production of liver and kidney auto-antibodies which are distinct from each other and are thought to be directed against many hepatic cytochrome P-450 forms (Mackay, 1985).

In vitro studies have demonstrated that steroids such as cortisone, 21-deoxycortisone and progesterone are potent inhibitors of S-mephenytoin hydroxylase in human liver (Inaba, 1986). None of the purified forms of P450mp have been reported to have significant steroid hydroxylation activity towards the 21 position of progesterone or the 2 position of 17 β-estradiol (Umbenhauer et al, 1987). 2- and 4-hydroxylation of 17 β-estradiol have been attributed to P-450 NF (P450-3) in human liver (Beaune et al, 1986a) which is not structurally related to P-450mp. Other steroid hydroxylation reactions have yet to be reported for P-450mp. Purified forms of P-450mp also had significant AHH activity but this activity could not be inhibited with anti-P-450mp antibodies (Shimada et al, 1986).

3.14.3. Comparison of human P450C2C cDNA with rabbit and rat P450-2C cDNAs: identification of possible orthologous genes?

The identity of functional and historical counterparts between related gene families is complicated because it is difficult to know whether the comparison has been made between orthologous or paralogous forms. Orthologous forms are those genes which are identical by descent and have been separated by speciation events. Paralogous forms share their origin by gene duplication. This distinction is

best illustrated with the P450C1 gene family. In rat, rabbits, mouse and human this gene family consists of two closely related members which share 70% homology (Nebert et al, 1987). The duplication event which created this family appears to have happened prior to the split that led to the human rodent and rabbit lineages. $P_1450(MC_{1b})$ in human shows significantly higher homology to P_1450 in mouse, rat and rabbit than to $P_3450(MC_{1a})$ forms in these species (Table 3.6). Indeed the orthologous form of this gene family appear to share greater functional properties between species than do the paralogous forms in relation to substrate specificity and induction characteristics (Jaiswal et al, 1985; Negishi et al, 1979).

In Table 3.1 is listed the overall amino acid percent similarity of members of the P450-2C gene family in three different species. This comparison indicates that rat forms are as similar to each other as they are to the human form. The same observation can be made when comparing human and rabbit forms. Only differences of greater than 5% are significant in these comparisons (Nebert et al, 1987). Thus rabbit forms Pbc1 and Pbc2 are clearly more similar to each other than to the other forms listed. Rabbit form Pbc3 is border line but appears to be distinct from all the other forms listed. However this sequence represents only two thirds of a putative P-450 form (Leighton et al, 1984).

The substrate specificity of P-450s appears to lie within the 200-300 amino acid region of the protein. This region of P450C2C protein was compared to identify any similarities for forms between different species. In all comparisons the percentage similarity for this region was significantly less than that seen for the overall comparisons. Over this region human PB-1 showed less homology to rat forms f and PB-1 in comparison to rat M and rabbit forms I, Pbc1

TABLE 3.6.

Percent similarity of amino acid sequence among members of P450C1 gene family in four species (adapted from Nebert et al, 1987).

		1	2	3	4	5	6	7	8	9
1.	Human P_3450	-								
2.	Human P_1450	72	-							
3.	Mouse P_3450	73	68	-						
4.	Mouse P_1450	69	80	73	-					
5.	Rat MClb(d)	75	67	93	71	-				
6.	Rat MCla(c)	68	79	69	93	70	-			
7.	Rabbit form 4	77	68	73	67	73	67	-		
8.	Rabbit form 6	65	71	60	72	59	72	68	-	
*9.	Rat PB_{3b}(c)	36	35	35	36	35	35	36	34	-

* Included as a control, member of P450-2B gene family.

Human P_1450 = mouse P_1450, rat MCla(c) and rabbit form 6.

Human P_3450 = mouse P_3450, rat MClb(d) and rabbit form 4.

and Pbc2. This suggested that human PB-1 is functionally more related to these forms. Rat M is associated with the male specific 16α hydroxylation of testosterone and 2 hydroxylation of estradiol (Yoshioka et al, 1987). Rabbit form I also metabolizes estradiol at the 2 position and the 21 hydroxylation of progesterone (Tukey et al, 1987). No function has been assigned to rabbit forms Pbc1 or Pbc2.

Another method for comparing rates of change is to use nucleotide sequence comparisons which distinguish between those nucleotide changes which cause amino acid replacements (non-synonymous changes) and those that do not (synonymous changes) in coding regions.

Because synonomous changes are subject to fewer functional constraints it is a more reliable estimate of the rate of change between two genes. To estimate the number of substitutions of both types of sites in the P450-2C family of proteins I have used the method of Li et al (1985a). This method gives a K_A (number of substitutions per asynonomous site) and K_S (number of substitutions per synonomous site) value for the genes compared. The K_A values for all functional genes that have been analysed are lower than the K_S values, but show a range of values depending on the functional constraints placed on the protein (Li et al, 1985b). In this comparison the program identified rabbit form Pbc3 as being already too divergent to meanifully estimate substitution rates.

In Table 3.7 the K_A and K_S values for all the comparisons of rabbit, rat and human forms is presented. In all the pairwise combinations of human PB-1 with rat and rabbit forms the K_A's are very similar and significantly less than the K_S values. The K_S comparison discriminates between the various forms. Rat M is more

TABLE 3.7.

Number of substitutions per synonomous (K_S) and non-synonomous (K_A) sites between P-450 genes.

*Synonomous rates (K_S)

	Hu PB-1	Rat PB-1	f	M	Rab I	PBc1	PBc2
	1	2	3	4	5	6	7
1. Hu PB-1	-						
2. Rat PB-1	.66±·08	-					
3. Rat f	.62±.06	.56±.06	-				
4. Rat M	.69±.07	1.0±.1	.91±.09	-			
5. Rabbit I	.54±.06	.81±.08	.78±.08	.74±.02	-		
6. Rab PBc1	.49±.05	.80±.08	.77±.08	.83±.09	.59±.06	-	
7. Rab PBc2	.46±.05	.80±888	.73±.07	.77±.08	.55±.06	.3±.04	-

* Asynonomous rates (K_A)

	Hu PB-1	Rat PB-1	f	M	Rab I	PBc1	PBc2
	1	2	3	4	5	6	7
1. Hu PB-1	-						
2. Rat PB-1	.14±	-					
3. Rat f	.17±.02	.16±.02	-				
4. Rat m	.13±.02	.17±.02	.19±.02	-			
5. Rabbit I	.16±.02	.19±.02	.21±.02	.19±.02	-		
6. Rab Pbc1	.16±.02	.19±.02	.22±.02	.17±.02	.02±.02	-	
7. Rab Pbc2	.14±.02	.18±.02	.20±.02	.16±.02	.18±.02	.085±.009	-

* = calculated with program of Li _et al_, 1985b

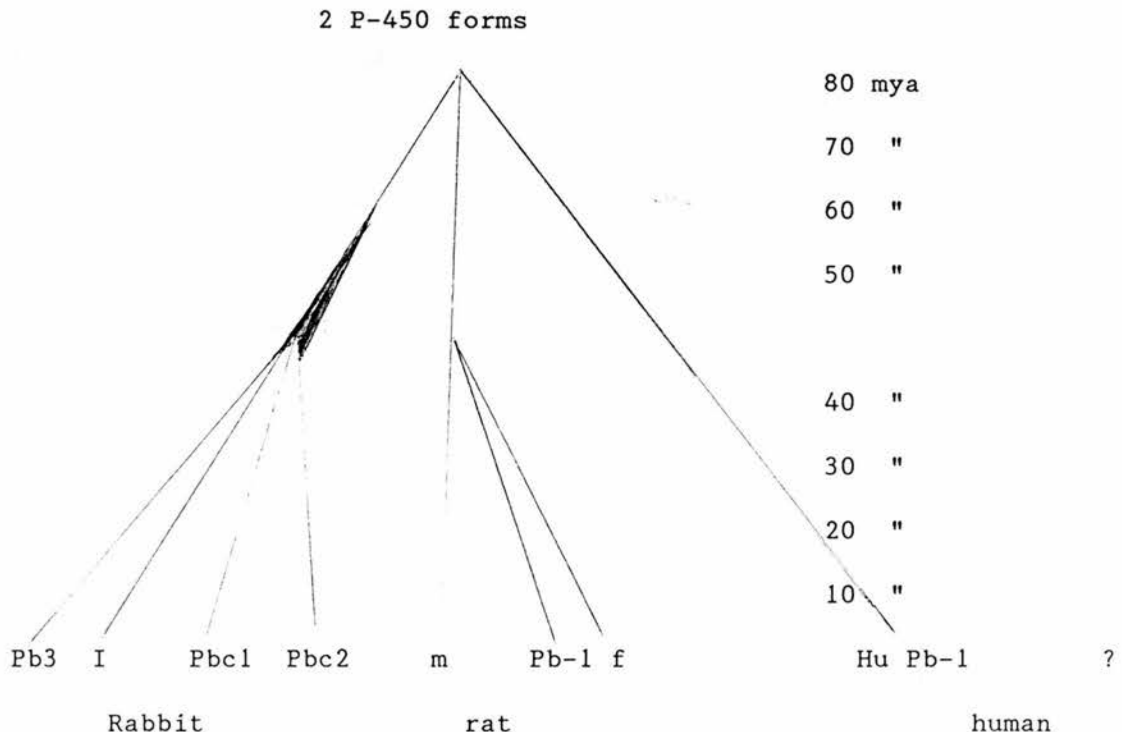
similar to human PB-1 than to the other rat forms whereas rabbit form I is as similar to human PB-1 as it is to rabbit forms Pbc1 and Pbc2. The comparison also distinguishes two groups of P-450s within the P450-2C family in rat and rabbit. This is paradoxical as the two groups are similarly close to human PB-1. It would suggest that the two groups duplicated at or before the rat, rabbit and human radiation which is thought to have occurred 80 million years ago (mya). A prediction from this analysis is that a second unidentified member of this gene family exists in humans which may show greater identity with the PB-1/f or Pbc21/Pbc2 group than M or form I in rats and rabbits respectively. The existence of two members in the second group which show greater identity with each other also suggests that a second duplication event occurred in the rabbit and rat lineages. These may have been independent events.

Another observation is that in general the rabbit forms show a lower rate of divergence in relation to human PB-1 than the rat forms. The average human/rabbit K_S is 0.5 whereas the average human/rat K_S is 0.66. The K_S between rats and rabbits is significantly higher than that between humans and rats or humans and rabbits. However these calculations are based on a number of assumptions. Firstly, the rate of nucleotide substitution is the same in each species, secondly, no gene conversion has taken place and thirdly, that the comparison is between equivalent forms.

Wu and Li (1985) have estimated the rate of synonomous substitution has been on average about two times higher in the rodent lineages than in the human and artiodactyl lineages. This increase is attributed in part to the generation effect, that is rodents have shorter generation times and thus higher mutation rates. As yet there is no comprehensive data on human/rabbit

comparisons but the lower K_S seen for rabbits and humans could be due to a reduced nucleotide substitution rate in rabbits when compared to rats. Gene conversion events between PB-1 genes in rats and rabbits also could lead to higher rates of change between related forms. There are probably greater than 9 genes for the P450-2C family in rats and rabbits which would be ample material for gene conversion to occur. The environmental niche of rabbits and rats is distinct from humans thus the selective pressures of the chemical environment will be different. After gene duplication in rabbits and rats had occurred a comparable functional equivalent to the human PB-1 form might be no longer maintained. In such cases there are no strictly orthologous forms between rabbits, rats and humans for members of the P450C2C family. The difference in substitution rates (K_S rat > K_S rabbit > K_S human) would give a false picture of orthology between rabbit and human forms.

A possible divergence time could be represented as follows.



At some point in time the rate of nucleotide substitution would

begin to differ between the three species when this occured would have a bearing on the timing of the gene duplication events. The above scheme is based on a divergence time of 3.3 x 10^6 my for a 1% difference in amino acid change to be observed for rabbits and approximately half that time for rats. One prediction of this scheme is that orthologous forms to M, PB-1 and f could exist in mice as the duplication event for PB-1 and f is prior to the mouse/rat divergence time estimated to be 25 mya (Sarich, 1985).

3.14.4. Map location of human P-450s

Each cytochrome P-450 involved in xenobiotic metabolism appears to map to a distinct chromosomal position. The majority map to different chromosomes (Table 3.8). The exception are the P450C2B and P450C2A which both map to chromosome 19. P450C2A has been localized to 19q13.1-qter with P450C2B mapping to the same chromosome. Members of these two P-450 families share 50% sequence homology (J. Miles, pers. comm.). The P-450 families with the prefix P450C2 all share 50% sequence homology to each other (Nebert et al, 1987). The localization of P450C2E is to chromosome 10 (F.J. Gonzalez, per. comm.). The assignment of P450C2C and P450C2E to human chromosome 10 provides much needed molecular markers for this chromosome. HMG8 (Grzeschik and Kazazian, 1985) shows only 28 mapped loci for chromosome 10 (including fragile sites) and of these only 8 were molecular probes.

3.15. Summary

In this chapter the isolation of a cDNA corresponding to a constitutively expressed cytochrome P-450 is described.

TABLE 3.8

Chromosomal locations of hepatic P-450s in humans

P450 family	Chromosomal locations
P450C1	15q22-qter
P450C2A	19q13.1-13.3
P450C2B	__ 19
P450C2C	10q23-q24
P450C2D	22q11.1-qter
P450C2E	10
P450C3	7
P450C4	?

P450C1: Nebert et al, 1987

P450C2A: Nebert et al, 1987

P4502B: Dr. C. R. Wolf, pers. comm.

P450C2C: This study

P4502D: Nebert et al, 1987

P3402E: Dr. F. J. Gonzalez, pers. comm.

P450C3: Dr. C. R. Wolf, pers. comm.

Characterization of this clone shows that it corresponds to a P-450 responsible for the metabolism of mephenytoin (an anti-convulsant in humans) and tienilic acid (an anaesthetic). This P-450 also corresponds to a rat and rabbit P-450s involved in steroid oxidations, the human P-450mp has the potential to bind steroids but no specific metabolic transformation has been reported for it. Southern blot analysis indicates that this cDNA encodes a member of a small multigene family, P450C2C, which maps to chromosome 10q23-10q24. This cytochrome P-450 does not correspond to any of the hepatic cytochrome P-450 gene families previously mapped in humans. Sequence comparisons indicate that related forms of this P-450 are rapidly evolving in different species which may imply a consequent change in substrate specificity. Therefore these may not serve as useful models for human oxidative metabolism by members of P450C2C gene family. However it does illustrate the flexibility of this gene family in relation to environmental chemical stress. On average cytochrome P-450 gene families are more complex in rodent and rabbits than in humans. This may reflect a selective pressure for increased P-450 function in response to an increase in chemical diversity faced by these species.

CHAPTER 4

THE PB-1 (*P450-2C*) GENES IN THE MOUSE

4.1. INTRODUCTION

A number of mouse models have been developed for chemically induced metabolism and carcinogenesis (Gurtoo et al, 1978; Stout and Becker, 1986; Drinkwater and Ginsler, 1986; Reynolds et al, 1987). It is of interest to assess the role of P-450s in these model systems and to see how applicable they are to human experience. Therefore I decided to use the powerful mapping tool of recombinant inbred strains of mice (Taylor, 1978) to determine the chromosomal organization and assess some candidate function for the PB-1 (P450-2C) gene family in mice. The approach depends on the identification of restriction fragment length variants (RFLVs) between progenitor strains which can be used as linkage markers in sets of RI lines.

I had two rat PB-1 cDNA clones available, pTF-1 and pTF-2 which contained inserts of 1227 and 410 base pairs respectively and were shown to have 80% nucleic acid sequence identity (Freidberg et al, 1986). pTF-1 was chosen for the identification of PB-1 related sequences in the mouse for the following reasons. Firstly, when identical Southern blots of rodent and human DNA were probed with pTF-1 and pTF-2 respectively it was found that pTF-2 did not detect any additional DNA fragments (data not shown). Secondly, on a screen of a mouse DBA/2 genomic library every positive plaque detected by pTF-2 was also detected by pTF-1. This indicated that pTF-2 although distinct from pTF-1 did not detect additional genes.

4.2. Genomic complexity PB-1 P-450 related sequences and identification of RFLVs.

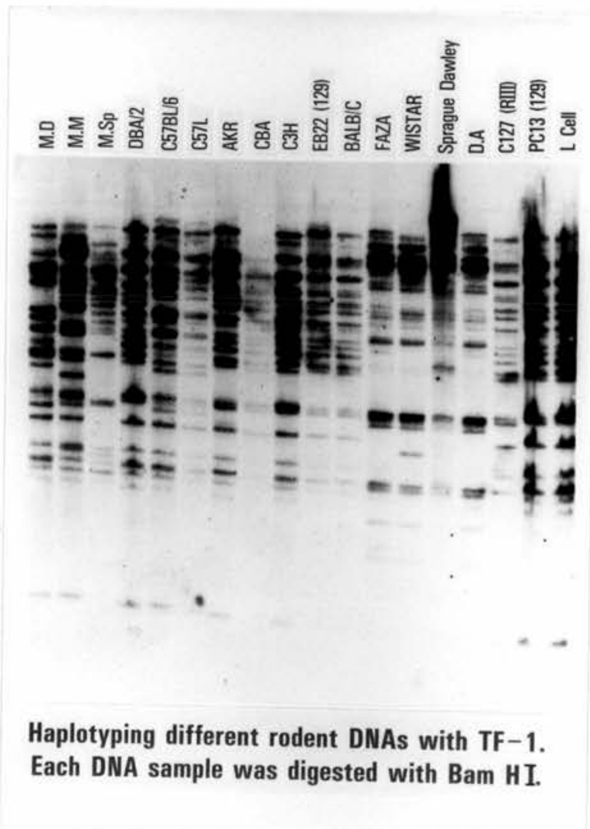
Firstly to demonstrate that the rat PB-1 cDNA can detect mouse

sequences Southern blots of BamHI digested rat and mouse DNA were probed with pTF-1. This revealed a complex pattern of hybridization with up to 25-30 bands representing 150-200 kb of DNA (Fig. 4.1). The pattern of hybridization in rat and mouse DNA was very similar indicating the presence of several related genes in both species. In the previous Chapter gene counts with 5' and 3' human PB-1 cDNA clones indentified a minimum of 7-8 genes for the P450-2C gene family in rodents.

In the inbred strains examined two basic patterns could be observed, a DBA/2 pattern seen in DBA/2, AKR, CBA/Ca, C3H/He, 129 and BALB/c, and a C57BL/6 pattern seen in C57BL/6 and C57L. When longer gels were run with BamHI and HindIII digests of inbred strain DNA the DBA/2 pattern could be further subdivided between DBA/2 and AKR (Fig. 4.2). DBA/2 has RFLV at 12kb and 4.8kb in BamHI digests not seen in AKR which has an additional band at ~15kb. Similar observations can be made on HindIII digests. The C57BL/6 and C57L patterns appear quite distinct from the DBA/2 and AKR pattern with a minimum of 6 polymorphic fragments on HindIII digestion. To further extend the RFLV search I used C57BL/6 and DBA/2 as representative strains and cut these DNAs with a number of different enzymes (Fig. 4.3). On this analysis SstI appear to generate the largest number of variant fragments. SstI was subsequently used to examine a number of other mouse strains with pTF-1. Again 3 patterns of hybridization could be observed (Fig. 4.4). This suggested that the DNA pattern was segregating as a haplotype i.e. the members of the PB-1 (P450-2C) gene family are linked. Most of the inbred strains examined could be classified as possessing one of three DNA haplotypes designated $P450\text{-}2C^{a}$, $P450\text{-}2C^{b}$ and $P4502C^{c}$ (Table 4.1). On SstI digest the four strains designated $P450\text{-}2C^{b}$ differed from P450-

FIGURE 4.1.

Southern blot analysis of BamHI digests of genomic DNA (5ug) from rodents. The mouse DNA samples were M.D., *Mus musculus domesticus*; M.M.. *Mus musculus musculus*; M.sp, *Mus spretus*; C56BL/6, C57L, AKR, CBA/Ca, C3H/He, BALB/C and four DNA preparations from mouse cell lines (strain in parentheses: EB22 (129), C127 (RIII), PC13 (129) and L cell (unknown). The blot was probed with ^{32}P-labelled pTF-1 *PstI* insert.



Haplotyping different rodent DNAs with TF−1. Each DNA sample was digested with Bam HI.

FIGURE 4.2.

Southern blots of HindIII and BamHI digests of genomic DNA (5μg) from inbred strains probed with pTF-1: The indicated mouse strains were probed as in Fig. 4.1. M, λ HindIII size markers.

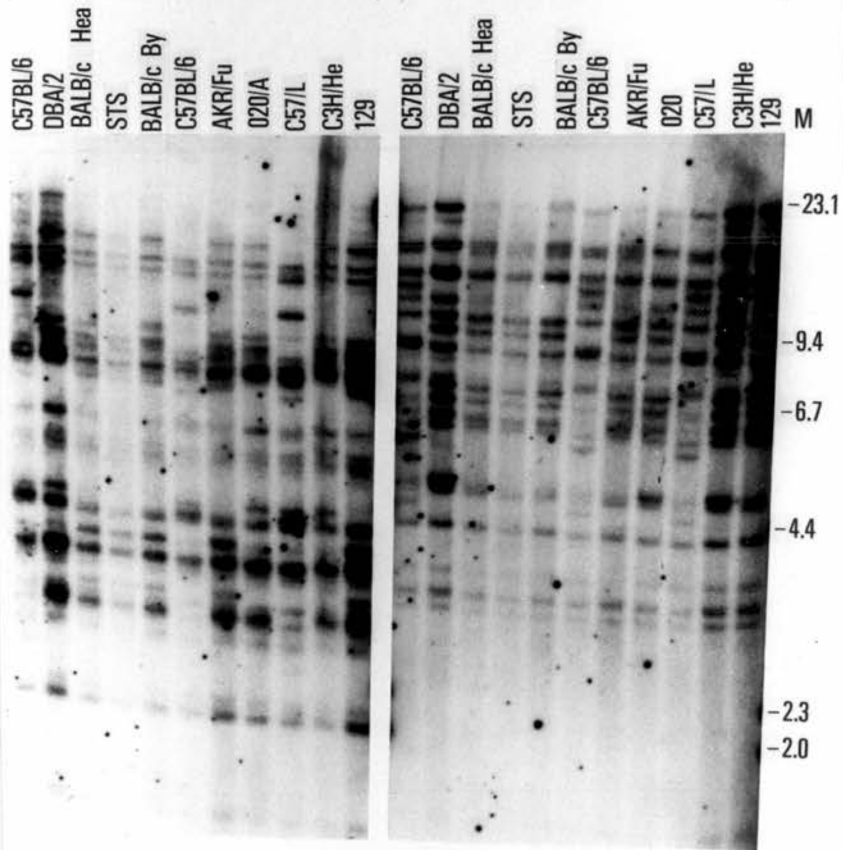
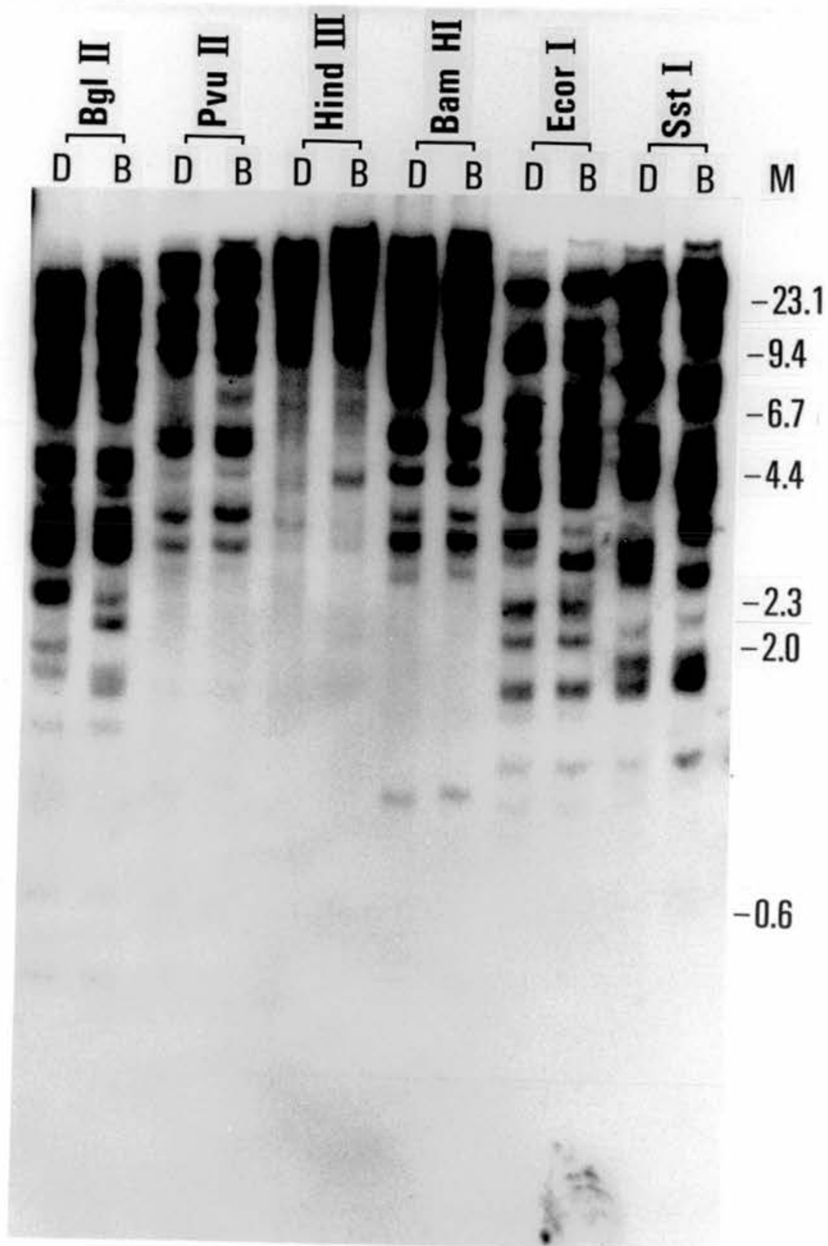

pTF-1
Hind III
Bam HI
C57BL/6
DBA/2
BALB/c Hea
STS
BALB/c By
C57BL/6
AKR/Fu
020/A
C57/L
C3H/He
129
C57BL/6
DBA/2
BALB/c Hea
STS
BALB/c By
C57BL/6
AKR/Fu
020
C57/L
C3H/He
129
M
-23.1
-9.4
-6.7
-4.4
-2.3
-2.0

FIGURE 4.3.

RFLV search between C57BL/6 and DBA/2. D, DBA/2; B, C57BL/6; M, *Hind*III. C57BL/6 and DBA/2 genomic DNA were cut with the indicated restriction enzymes and analysed as in Fig. 4.1.



Southern Blot of DBA/2 and C57BL/6 DNAs cut with different restriction enzymes and probed with pTF-1.

FIGURE 4.4.

Haplotype analysis of rodent DNAs digested with SstI and probed with pTF-1. M, λ *Hind*III; A, DA rat; B, wistar rat; C, 129 mouse; D, C3H/He, mouse; E, AKR, mouse; F, C57L mouse; G, DBA/2 mouse. The blot was analysed as in Fig. 4.1.

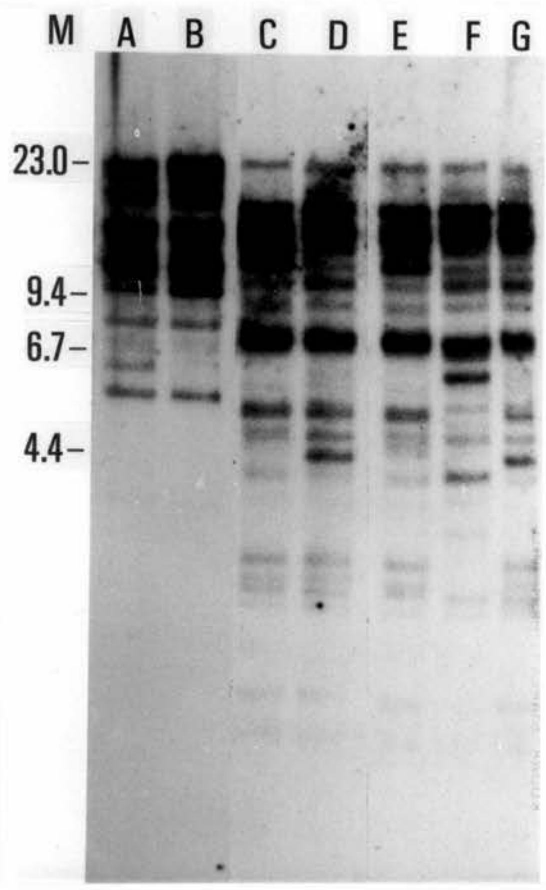

M A B C D E F G
23.0–
9.4–
6.7–
4.4–

Table 4.1.

Distribution of P450-2C Haplotypes Among Inbred Mouse Strains

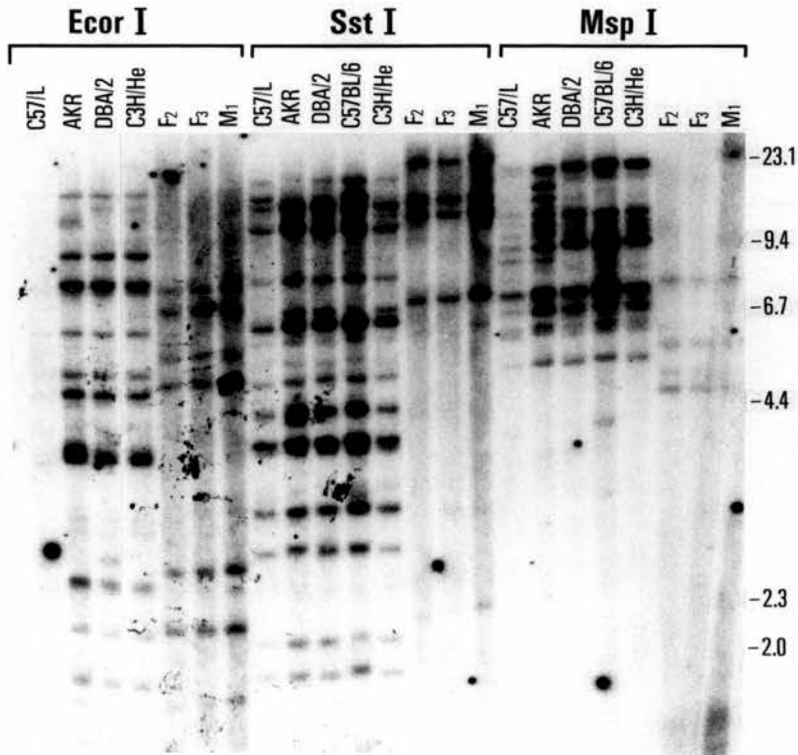
Haplotype	Inbred Strains
a	C57BL/6, C57BL/10, C57L
b	DBA/2. C3H/He, 020, CBA/Ca
c	AKR, AKR/Fu, BALB/c, 129, STS, GRS

2C[c] in two variant fragments (Fig. 4.4 D vs C). P450-2C[a] differed from P450-2C[b] and P450-2C[c] with respect to a minimum of 9 different fragments (Fig.4.4 F vs G and E). When pTF-2 was used as a probe the pattern of hybridization was less complex and fewer variant fragments were seen. For example on SstI digestion DBA/2 (P450-2C[b]) and AKR (P450-2C[c]) cannot be distinguished from each other (Fig. 4.9). The difference between C57BL/6 (P450-2C[a]) and DBA/2 (P450-2C[b]) is one additional fragment in the C57BL/6 track at ~16kb and loss of a band at ~7kb (Fig. 4.5). This suggests that most of the variants detected by pTF-1 lie at the 3' end of the gene. However pTF-2 was able to detect differences between C57L and C57BL/6 on MspI digestion (Fig. 4.5). Where C57L has an additional band at 9.4kb this is seen in AKR mice as well but not in DBA/2, C57BL/6 or C3H/He mice (Fig. 4.5). AKR can be distinguished from C57L by two additional bands at 15-18kb. These do not appear to represent a partial digest because when these blots are probed with a 3' P-450 probe C57L gives the same pattern as C57BL/6 (see later).

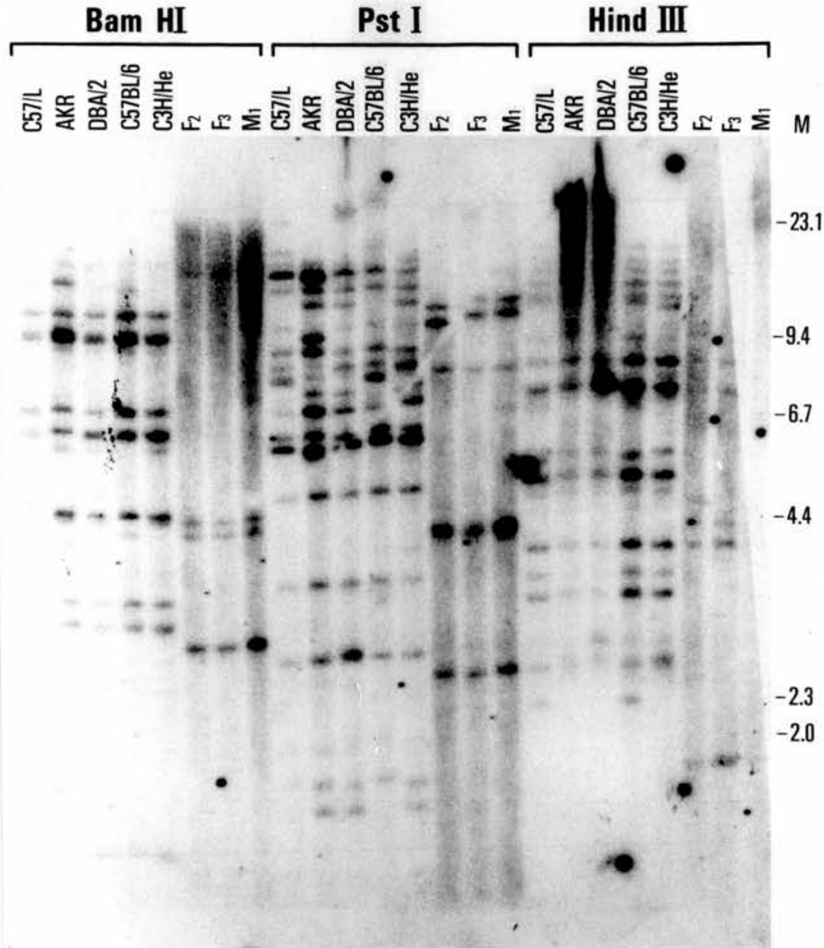
The distribution pattern has been established among inbred strains for DNA variants detected by a rat PB_{3a} (P450-2B) cDNA probe (Simmons and Kasper, 1983). These authors detected two DNA haplotypes, a DBA/2 pattern and C57BL/6 pattern and typed a number of mouse strains with respect to this pattern. The distribution of these haplotypes was distinct from that seen for P450-2C haplotypes which implied these two loci were not closely linked. I repeated their analysis using a probe for P450-2B with the additional strains used in the pTF-1 analysis and found an extra variant haplotype. 129 mice had a distinct pattern from DBA/2 and C57BL/6 (Fig. 4.6). This was also detected in independent 129 samples and extraembryonic cell line DNA derived from these mice. Thus three haplotypes can

FIGURE 4.5.

Southern blot analysis of human and rodent DNA with pTF-2. Blots were hybridized with ^{32}P-labelled *PstI* insert from pTF-2. The mouse strains and restriction enzymes used are indicated. The human DNA preparations were female, F_2 and F_3, and Male M_1. M λ *HindIII* size markers.



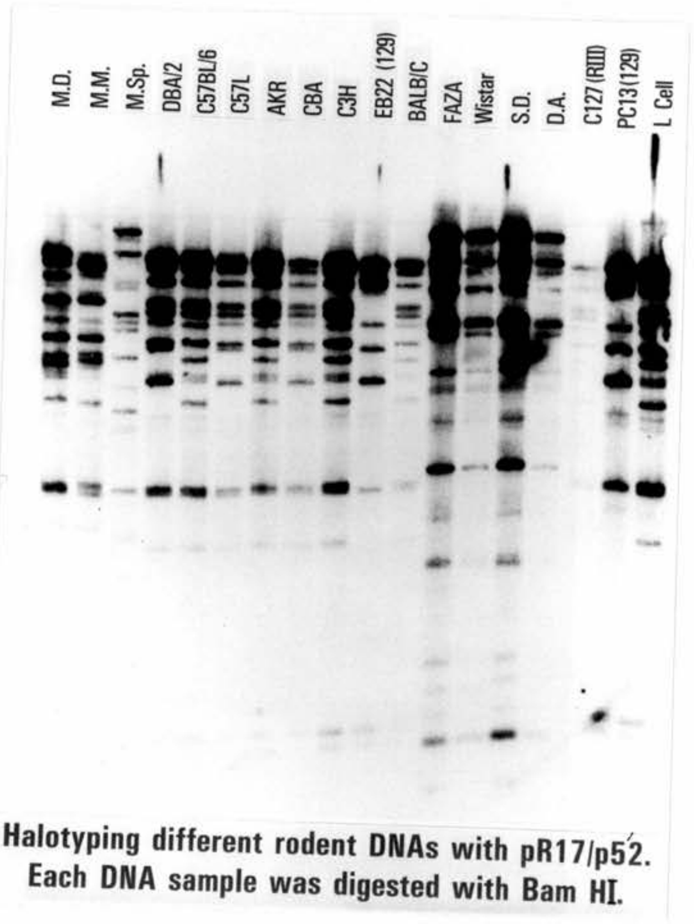
pTF-2
Ecor I
Sst I
Msp I
C57/L
AKR
DBA/2
C3H/He
F2
F3
M1
C57/L
AKR
DBA/2
C57BL/6
C3H/He
F2
F3
M1
C57/L
AKR
DBA/2
C57BL/6
C3H/He
F2
F3
M1
-23.1
-9.4
-6.7
-4.4
-2.3
-2.0



pTF-2
Bam HI
Pst I
Hind III
C57/L
AKR
DBA/2
C57BL/6
C3H/He
F2
F3
M1
M
-23.1
-9.4
-6.7
-4.4
-2.3
-2.0

FIGURE 4.6.

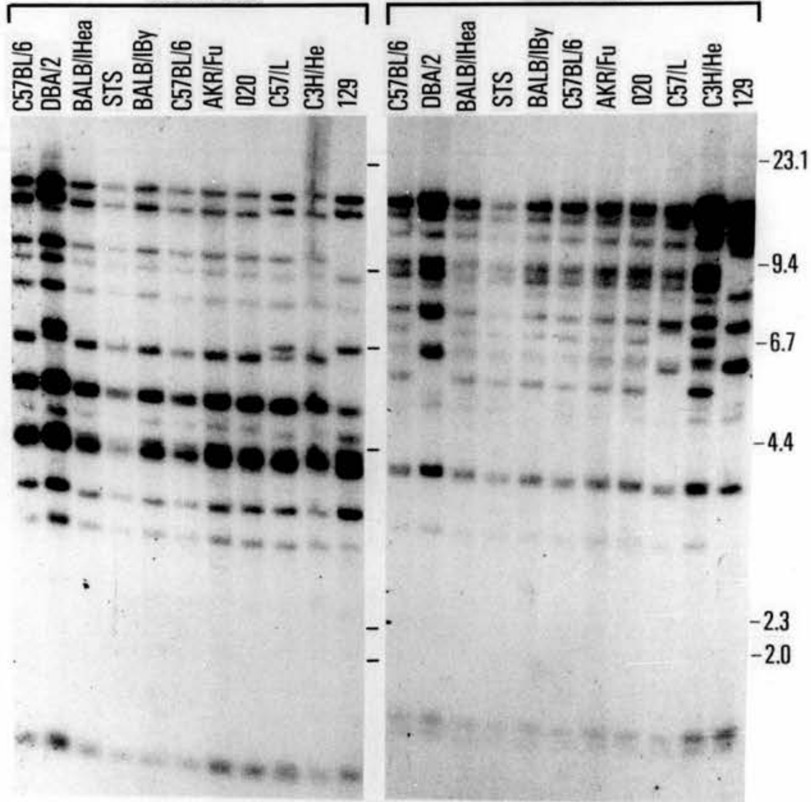
Haplotyping with pR17/p5'2 (P450-2B). See Legend in Figure 4.1. for source of DNA samples. Blot was hybridized with ^{32}P labelled *PstI* inserts from pR17 and p5'2.



Halotyping different rodent DNAs with pR17/p5′2. Each DNA sample was digested with Bam HI.

FIGURE 4.7.

Further haplotyping with pR17/p5'2 (P450-2B). The same blot as in Fig. 4.2. was stripped and rehybridized with pR17 and p5'2 ^{32}P-labelled *PstI* inserts.



pR17/p5'2
Hind III
Bam HI
C57BL/6
DBA/2
BALB/IHea
STS
BALB/IBy
C57BL/6
AKR/Fu
020
C57/L
C3H/He
129
C57BL/6
DBA/2
BALB/IHea
STS
BALB/IBy
C57BL/6
AKR/Fu
020
C57/L
C3H/He
129
-23.1
-9.4
-6.7
-4.4
-2.3
-2.0

Table 4.2.

Distribution of P450-2B haplotypes among inbred mouse strains.

Haplotype	Inbred Strains
a	C57BL/6, AKR, C3H/He, A/He, A, BUB/Bn, PL, RF, SJL, BALB/c Hea, BALB/c By, STS, 020, AKR/Fu
b	DBA/2, C57L, C57BR/cd, DBA/1, CBA/Ca
c	129/Sv

Data from Simmons and Casper (1983) and this study.

be seen for the P450-2B locus as well (Table 4.2) which I have designated P450-2B^{a}, P450-2B^{b} and P450-2B^{c} these do not show concordance with the P450-2C haplotypes for the strains examined in common. One point of note is that both CBA/Ca and 129 mice show discordance between the Coh phenotype (Wood and Taylor, 1979) and P450-2B. In the case of CBA/Ca these mice have low coumarin hydroxylase (Coh^{L}) activity which is seen in C57BL/6 (P450-2B^{a}) but has a DBA/2 (P450-2B^{b}) DNA haplotype pattern. Similarly 129 mice have high coumarin hydroxylase (Coh^{h}) activity but a distinct DNA haplotype (P450-2B^{c}) that is different to the DBA/2 and C57BL/6 patterns. Interestingly the CBA/Ca and 129 strains have been classified as medium metabolizers of coumarin and 7-ethoxycoumarin by Lush and Andrews (1978) which supports the idea that these are two closely linked loci involved in coumarin metabolisms.

4.3. Genomic organization of P450-2C

Recombinant inbred mouse strains were used to determine the relative chromosomal location and organization of the members of the PB-1 gene family. The BXD series of mouse strains was initially used as it has been extensively characterized with chromosomal markers and contains the largest number of independent lines. Figure 4.8 shows the hybridization patterns observed in a number of BXD RI DNA samples when digested with SstI and probed with pTF-1. For 26 lines analysed each RI line showed the inheritance of either the C57BL/6 (P450-2C^{a}) or the DBA/2 (P450-2C^{b}) progenitor haplotype. No intermediate patterns were observed (Table 4.3) which demonstrates the PB-1 genes map to a single chromosome and are present as a large cluster within a maximum of 1cM of DNA. This locus was designated P450-2C in accordance with the new cytochrome

FIGURE 4.8.

Southern blot analysis of SstI digests of genomic DNA from BXD mouse strains and their progenitors. Blot was probed as in Figure 4.1.

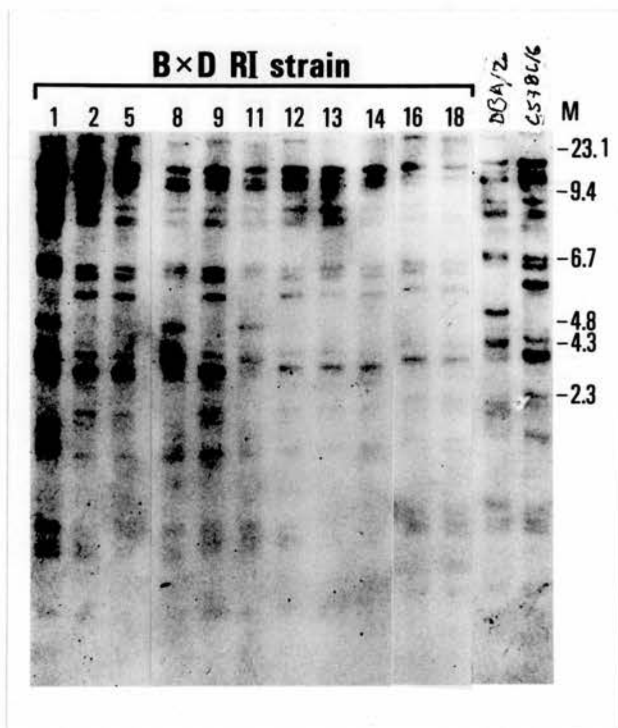

B×D RI strain
1
2
5
8
9
11
12
13
14
16
18
DBA/2
C57BL/6
M
-23.1
-9.4
-6.7
-4.8
-4.3
-2.3

TABLE 4.3.

Inheritance of P450-2C Restriction Fragment Variants in RI Strains

	RI strain	Progenitor DNA pattern
AKXL	5,9,12,21,24,29,37,17	A
	6,7,8.13,14,16,38,19,25,28	L
BXD	2,5,9,12,13,14,15,16,18,22	B
	24,25,27,28,29,30,31	
	1,6.8.11.19.20.21.23.32	D
BXH	3,4,6,9,12,14	B
	2,7,8,10,11,19	H
*CXB	D,J,K,N,O,P	C
	E,G,I,L,Q,R	B
OXA	A,B,F,G,I,N,M,N,**H**	O
	C,D,E,J,K,L	A

* Determined with pM8-1 only. DNAs from 5 sets of RI strains were digested with restricton enzymes, Southern blotted and probed with pTF-1 and pM8-1. The progenitor of the various sets are as follows. AKXL (AKR X C57L), BXD (C57BL/6 X DBA/2), BXH (C57BL/6 X C3H/He), CXB (BALB/c X C57BL/6) and OXA (020 XAKR/Fu). Bold letters, A, B. C, D, H, L and O denote the inherited progenitor DNA pattern from AKR, C57BL/6, BALB/c, DBA/2J, C3H/HeJ, C57L and 020 respectively. The restriction enzymes used to type the various RI strains were as follows: SstI, (AKXL, BXD, BXH, CXB, OXA), HindIII (AKXL, BXD, BXH, CXB, OXA), BamHI (BXH), MspI (BXH) and TaqI (BXD).

P-450 nomenclature (Nebert et al, 1987). Comparison of the strains distribution pattern (SDP) obtained with those markers previously mapped demonstrated that P450-2C was not tightly linked to any known genomic loci including the PCN (P450-3) (Simmons et al, 1985) and the P450-2B (Simmons and Kasper, 1983) loci. Loose linkage (20/26) was observed to the Env-25 locus on chromosome 12 (Blatt et al, 1983).

Possible linkage for the P450-2C locus was further investigated using the AKXL (Fig. 4.9), BXH (Fig. 4.10), CXB and OXA RI series. No intermediate haplotype patterns were observed. This provides further evidence that P450-2C exists as a clustered array. Again no tight linkage was found with any known genomic markers including the P450-1 locus on chromosome 9 (Hildebrand et al, 1985a). pTF-2 was tested in the BXD and AKXL RI lines and gave 100% concordance with pTF-1 (data not shown).

4.4. Linkage between P450-2C and constitutive AHH activity.

It has been established several years ago that mice of the AKR progenitor strain have a high uninduced liver AHH activity (Hutton et al, 1979). Mice of the C57L and C57BL/6 strain have a significantly lower basal liver AHH activity associated with it (Hutton et al, 1979; J. J. Hutton, pers. comm.). This difference in AHH activity was shown to be heritable and a SDP pattern was obtained for the AKXL RI series (B.A. Taylor and J. J. Hutton, pers. comm.). This segregation showed tight linkage (17/18) with the P450-2C locus, the only discordance being in the AKXL 17 line (Table 4.4). In spite of the use of a number of restriction enzymes as well as different PB-1 cDNA clones no recombination within the P450-2C locus in the AKXL 17 line was observed. The genetic distance

FIGURE 4.9.

Southern blot analysis of SstI digests of genomic DNA from AKXL mouse strains and their progenitors. Blot was probed as in Figure 4.1.

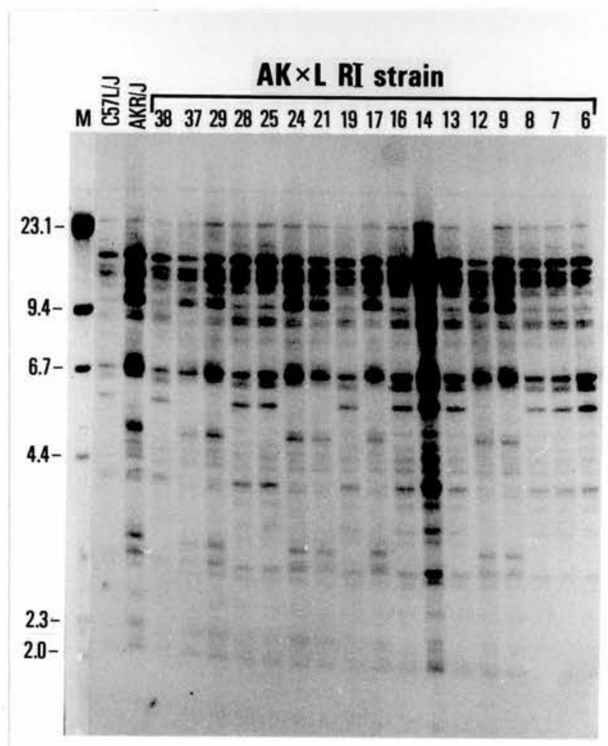
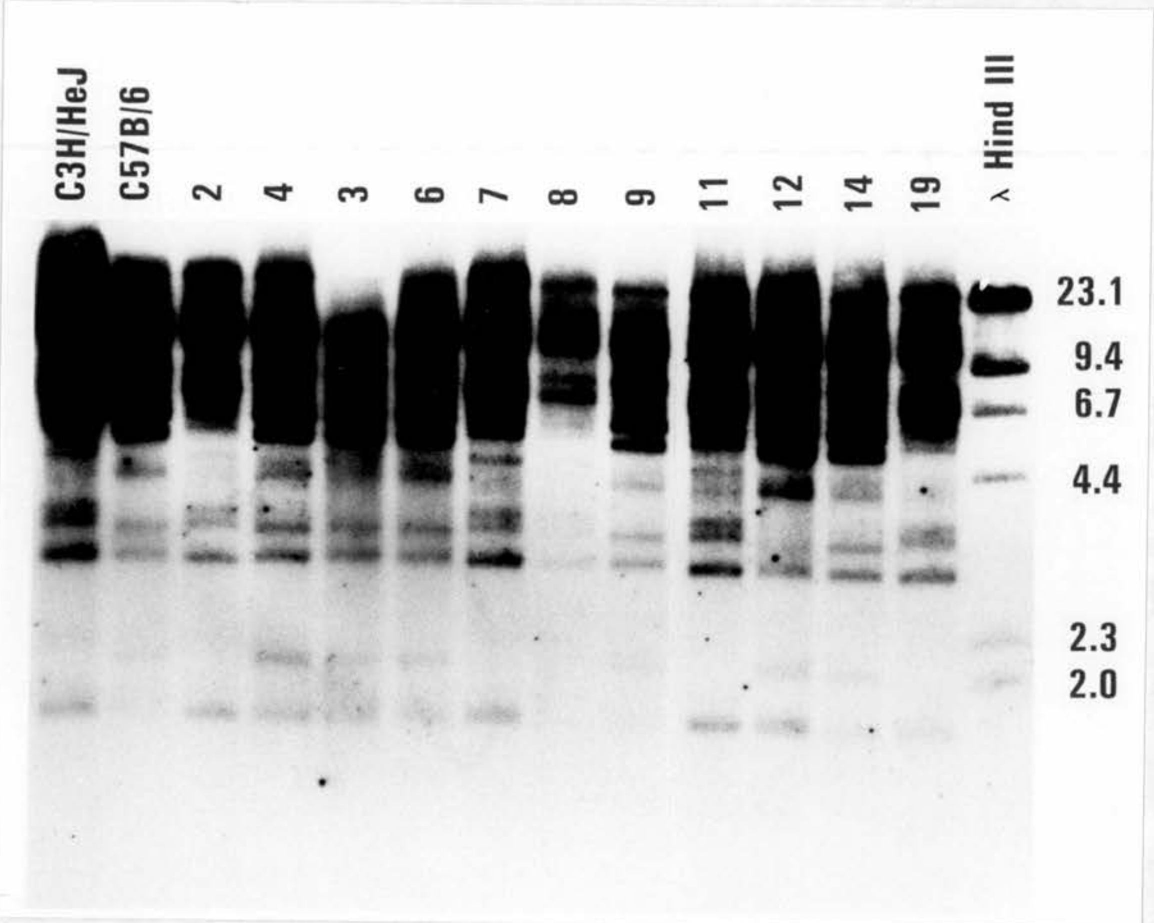

AK×L RI strain
M
C57L/J
AKR/J
38 37 29 28 25 24 21 19 17 16 14 13 12 9 8 7 6
23.1–
9.4–
6.7–
4.4–
2.3–
2.0–

FIGURE 4.10.

Southern blot analysis of MspI digests of BXH mouse strains and their progenitors. Blot was analysed as in Figure 4.1.



C3H/HeJ
C57B/6
2
4
3
6
7
8
9
11
12
14
19
λ Hind III
23.1
9.4
6.7
4.4
2.3
2.0

TABLE 4.4.

Distribution of Constitutive Liver AHH activities (nmole/g tissue/min) and P450-2C in the AKXL Recombinant Inbred Strains

Strain	AHH Activity (mean ± SE)	Genotypes* (con. AHH)	Progenitor P450-2C DNA pattern
5	42.5±3.9	A	A
6	21.1±1.8	L	L
7	24.4±1.6	L	L
8	21.8±2.0	L	L
9	30.4±1.9	A	A
12	32.0±2.9	A	A
13	24.4±1.5	L	L
14	22.7±0.9	L	L
16	19.0±1.1	L	L
17	23.0±1.3	A X	L
19	21.6±1.0	L	L
21	28.0±2.6	A	A
24	34.8±4.4	A	A
25	22.2±1.7	L	L
28	19.9±1.6	L	L
29	29.8±2.4	A	A
37	34.4±2.1	A	A
38	30.4±1.4	L	L

* "A" and "L" refers to the pattern observed for the parental AKR/J and C57L/J strains respectively. The "X" represents an apparent recombination event that occurred between the two loci.

between P450-2C and constitutive AHH activity is 1.52cM and the 95% confidence limits are 0.04 to 11.55cM (Silver, 1985).

4.5. Non-linkage between P450-2C and candidate functions

The oxidative metabolism of estradiol is mediated by cytochrome P-450 reactions (Waxman, 1984; Cheng and Schemkman, 1983). A correlation between high circulating 16α-hydroxy estradiol levels *in vivo* and the incidence of breast tumours has been reported in humans (Schneider *et al*, 1982) and mice (Bradlow *et al*, 1985; 1986). In C3H/He mice the extent of estrogen 16α-hydroxylation is increased relative to C57BL/6 mice. This difference was shown to be heritable and a SDP established for the BXH series of RI strains (Bradlow *et al*, 1985; 1986). No linkage was observed between the *P450-2C* locus and the reported SDP for this activity (Table 4.5). Also it was established that the *P450-2C* locus is not linked with either the e poxidation of the hepatocarcinogen aflatoxin B_1 (Gurtoo *et al*, 1978) or the induction of liver tumours by *N*-ethyl-*N*-nitrosourea (Drinkwater and Ginsler, 1986).

4.6. Isolation of mouse PB-1 cDNA clones.

I decided to isolate mouse PB-1 cDNA clones for a number of reasons. Firstly they might improve the analysis of various mouse haplotypes and detection of possible recombination events in RI lines. Secondly, the use of mouse cDNAs would facilitate the analysis of Chinese hamster/mouse somatic cell hybrids for those hybrids which retained mouse chromosomes containing the *P450-2C* locus.

A male DBA/2 liver cDNA library (Clontech) constructed in pBR322 was plated out to a density of 20,000 colonies per 20 x 20cm

TABLE 4.5.

Distribution of P450-2C and estrogen 16α-hydroxylase activity in the BXH RI strains. The estrogen 16α-hydroxylase activity is from Bradlow et al, 1985; 1986.

Strain	[1] P450-2C progenitor DNA pattern	[2] 16α-hydroxylase phenotype
2	H	B
3	B	H
4	B	B/H
5	B	H
7	H	B
8	H	B
9	B	B
10	H	H
11	H	B
12	B	H
14	B	H
19	H	B

1) "B" and "H" refers to the pattern observed for the parental C57BL/6 and C3H/He strains respectively.

2) In Bradlow et al, 1985 strain BXH4 was reported to have a H phenotype but in Bradlow et al, 1986 this was switched to a B phenotype.

nitrocellulose filter and screened with pTF-1 insert by the method of Grunstein and Hogness (1975). Approximately 0.5% of the clones were identified as being homologous to pTF-1 insert. Four clones came through the secondary and tertiary screening. These were termed pPB3-15, pM8-1, pPB5-21 and pPB4-1. On Pst-I digestion pPB3-15 and pM8-1 had inserts of 1350 and 650bp respectively. pPB4-1 and pPB5-21 had lost a PstI site because PstI digestion only linearized these plasmids. These plasmids were then cut with MspI whose sites are deficient in eukaryotic genomes (Bird, 1986), this gives a fragment of approximately 2kb of which 110 base pairs can be attributed to pBR322 if the plasmid is unrearranged (Fig. 4.11).

4.7. Characterization of mouse PB-1 cDNA clones.

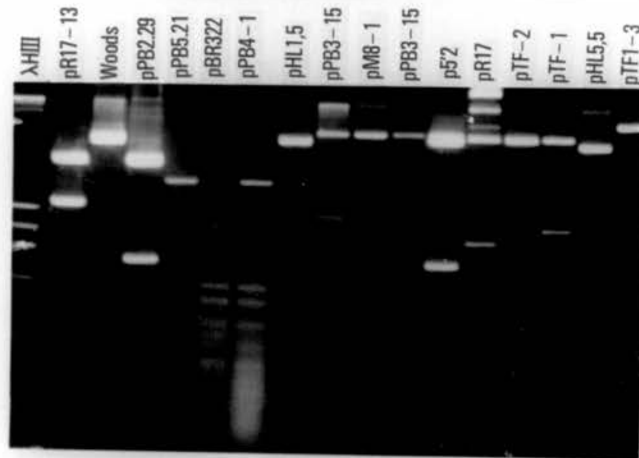
Firstly restriction digestions of purified plasmids were Southern blotted and shown to hybridize with pTF-1 and not with pR17/5'2 (P450-2B) (data not shown). Secondly, a mixed oligomer to the conserved Cys152 region of rat PB_{3a} was used to probe the mouse cDNA clones. pPB3-15, pPB4-1 and pPB5-21 all lit up with this oligomer (Fig. 4.11). This suggested that these clones were at least 3/4 full length. pM8-1 could be a short 3' prime which did not contain this region. Northern blots with these probes showed they detected a 2kb mRNA with a high level of expression which was slightly phenobarbital inducible (data not shown). Preliminary sequence analysis of pPB3-15 and pM8-1 sequests that pM8-1 is a truncated version of pPB3-15 and that coding portions of these cDNAs are 88% homologous to the rat PB-1 cDNA (J. Sweeney, pers. comm.).

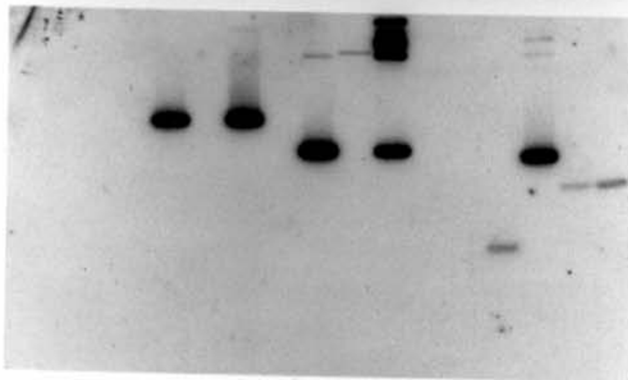
4.8. Haplotyping with mouse PB-1 cDNA clones

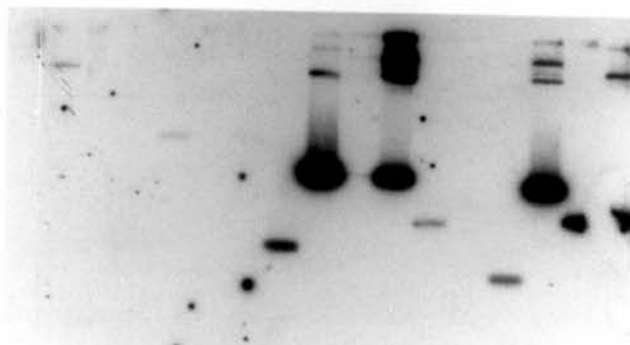
A number of different restriction digests of mouse inbred

FIGURE 4.11.

Sizing and identification of mouse PB-1 (P450-2C) cDNA probes. All plasmids except pPB5-21, pPB4-1 and pBR322 were digested with *Pst*I. The aforementioned three plasmids were digested with *Msp*I. Plasmids were fractionated on a 1.2% agarose gel, Southern blotted and hybridized to mixed oligomers directed towards the conserved Cys152 fragment of rat PB_{3a} (P450-2B) protein. Size markers were λ *Hind*III and OX174 *Hae*III. PB-1 cDNA plasmids were pM8-1, pPB3-15, pPB5-21 and pPB4-1 (mouse); pTF1-3 and pHL5,5 (Human); pTF-2 and pTF1 (rat). Also included were rat PB_{3a} cDNAs pR17 and p5'2, a human P450C2B cDNA pR17-13 and the Woods cDNA library.







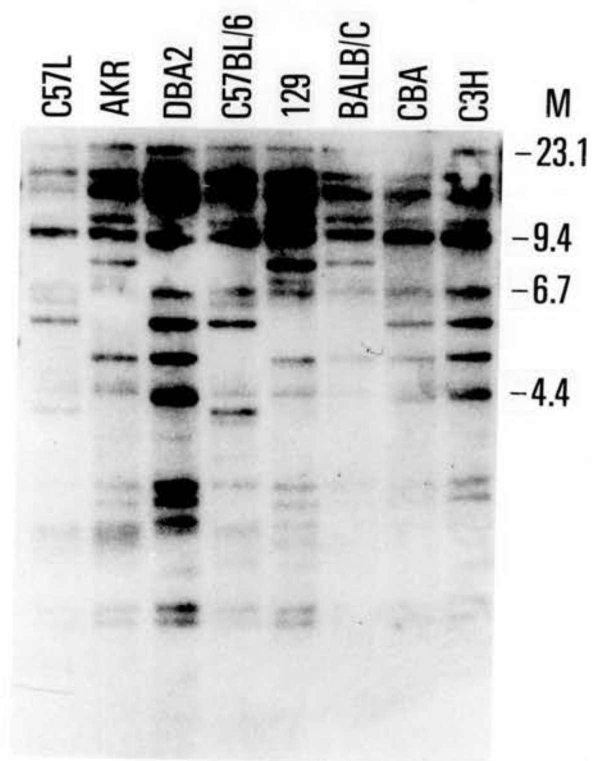
P450-2C and P450-2B cDNA plasmids probed with conserved mixed oligomers to Cys152 region of PB3a protein.

strain DNAs were probed with pM8-1, pPB3-15 and pPB5-21. All showed the same haplotype distribution detected by pTF-1. A number of differences were noted. For instance, pM8-1 was a highly polymorphic probe and detected many variant fragments on SstI digestion (Fig. 4.12). pPB3-15 had a low level repeat associated with it but even against this background it gave a similar hybridization pattern and the same strain distribution pattern in the progenitor strains as pTF-1. pPB5-21 did not detect as many variant fragments as the pM8-1 and pPB5-15 probes (Fig. 4.12 and 4.13). However it did give a similar pattern to pTF-2 which suggests that this is a 5' PB-1 cDNA clone lacking the 3' region which detects the variant fragments in mouse RI lines.

The pM8-1 cDNA clone was investigated further because of its polymorphic nature. Mouse strain DNAs were probed with pM8-1 to see if the DNA haplotypes detected by pTF-1 and pTF-2 could be further subdivided. Examples of such analysis are shown in Figures 4.14 and 4.15. As shown in Figure 4.14, BALB/c DNA digested with HindIII appeared to give a new variant fragment at 9.4kb when probed with pM8-1, but this variant band could not be detected in subsequent experiments. With every digest looked at additional variants not uncovered by the pTF-1 probe could be detected by pM8-1 but no additional subdivisions could be detected i.e. the only haplotypes detected by pM8-1 corresponded to $P450\text{-}2C^{a}$, $P450\text{-}2C^{b}$ and $P450\text{-}2C^{c}$. Of note is that pM8-1 could detect no differences between C57BL/6 and C57L even on probing MspI digests of their strains (Fig. 4.15). This is the same blot in which pTF-2 detected a difference between these strains.

FIGURE 4.12.

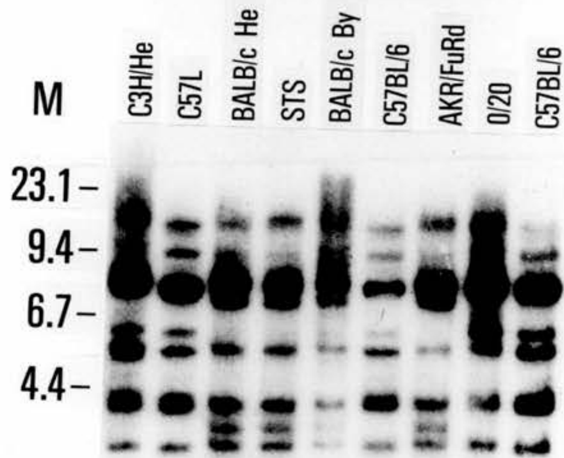
Southern blots of SstI digests of mouse strain genomic DNA probed with pM8-1. The indicated mouse strain DNAs were used and the blot probed with ^{32}P-labelled PstI insert from pM8-1. M, λ HindIII size markers.



pM8 – 1
(SstI)

FIGURE 4.13.

Haplotyping with pPB5-21. *Hind*III digests of the indicated mouse strain DNAs were Southern blotted and probed with ^{32}P-labelled *Msp*I insert from pPB5-21 (p5-21).



p5−21, Hind III
M
C3H/He
C57L
BALB/c He
STS
BALB/c By
C57BL/6
AKR/FuRd
0/20
C57BL/6
23.1–
9.4–
6.7–
4.4–

FIGURE 4.14.

Haplotyping with pM8-1. The same blot as in Figure 4.2 was stripped and reprobed with ^{32}P-labelled *Pst*I insert from pM8-1.

pM8-1

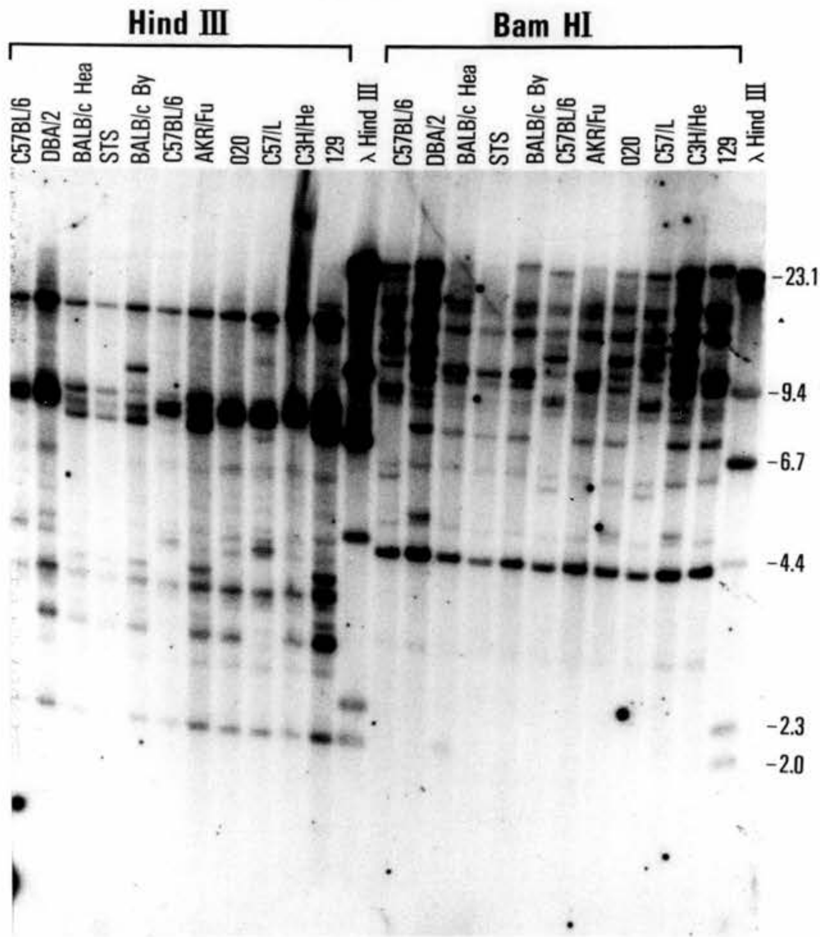
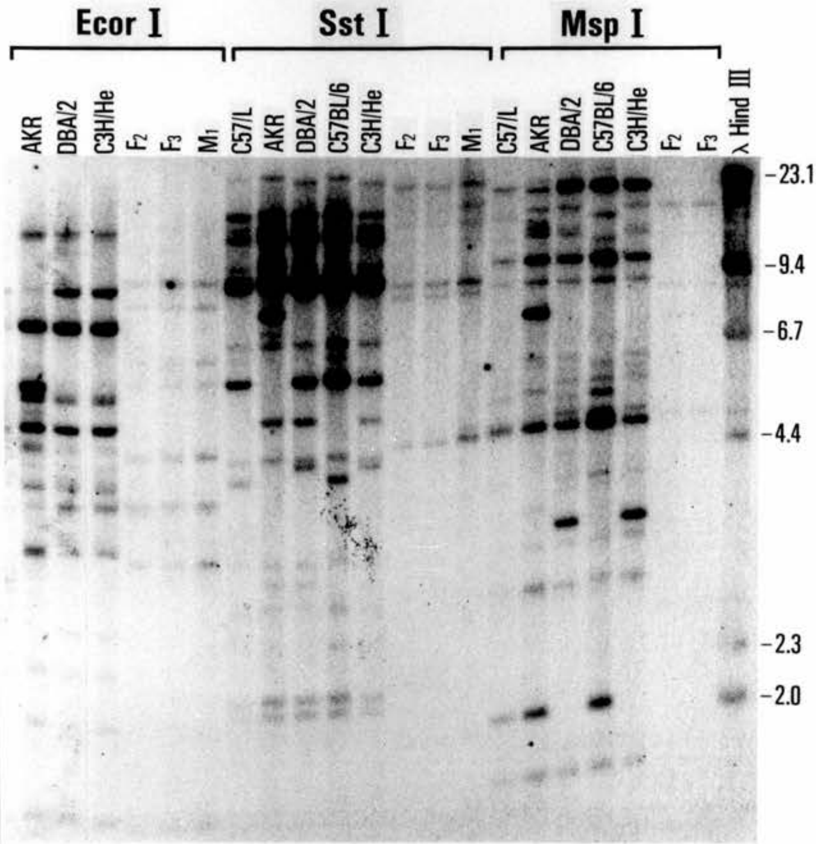
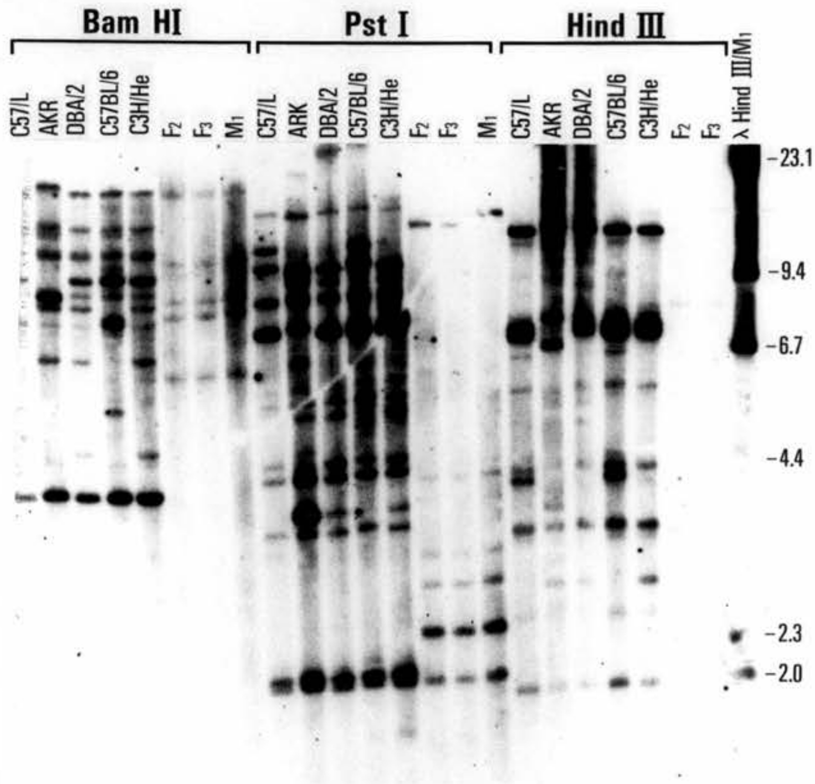


FIGURE 4.15.

Mouse haplotyping with pM8-1. The same blots as in Figure 4.5. were stripped and reprobed with ^{32}P-labelled *PstI* insert from pM8-1.


pM8-1
Ecor I
Sst I
Msp I
AKR
DBA/2
C3H/He
F2
F3
M1
C57/L
AKR
DBA/2
C57BL/6
C3H/He
F2
F3
M1
C57/L
AKR
DBA/2
C57BL/6
C3H/He
F2
F3
λ Hind III
-23.1
-9.4
-6.7
-4.4
-2.3
-2.0



pM8-1
Bam HI
Pst I
Hind III
C57/L
AKR
DBA/2
C57BL/6
C3H/He
F2
F3
M1
C57/L
ARK
DBA/2
C57BL/6
C3H/He
F2
F3
M1
C57/L
AKR
DBA/2
C57BL/6
C3H/He
F2
F3
λ Hind III/M1
-23.1
-9.4
-6.7
-4.4
-2.3
-2.0

4.9. Mapping with pM8-1 in RI lines

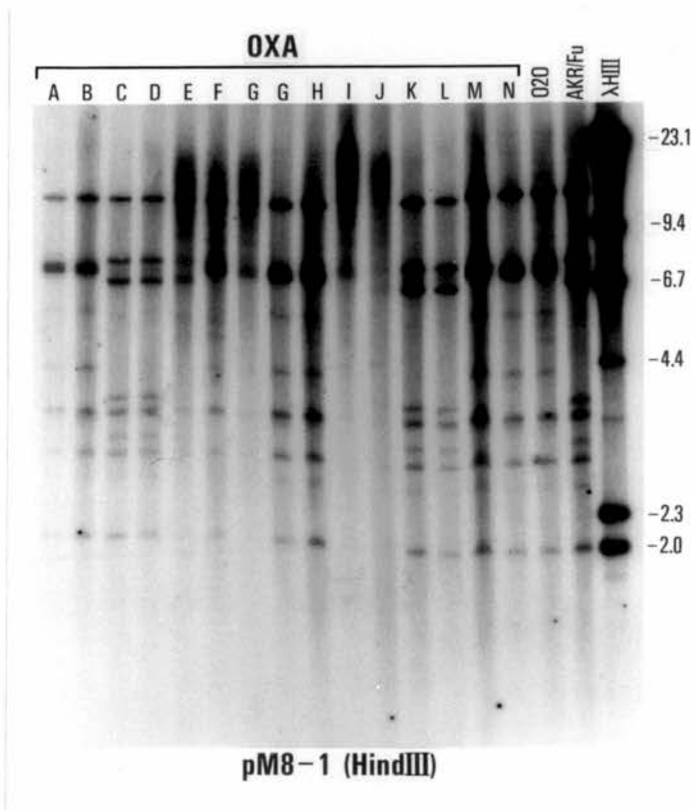
pM8-1 was used to map in the BXD, AKXL, BXH, CXB and OXA RI strains. An example is shown for the OXA, BXH and BXD RI lines (Fig. 4.16) in all these analysis pM8-1 gave a 100% concordance with pTF-1. When used in the AKXL RI lines pM8-1 could not detect any possible recombination events within *P450-2C* in the AKXL 38 line (data not shown).

4.10. Chromosomal location of P450-2C locus

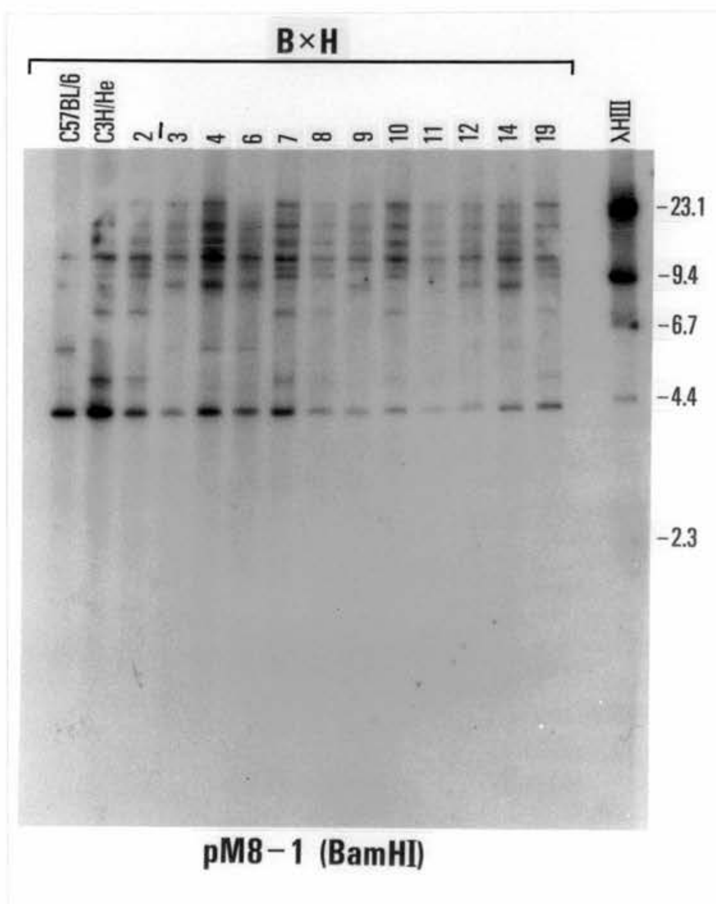
From the genetic and molecular analysis of the mouse PB-1 cDNA clones it was certain these clones represented the same locus mapped with the rat pTF-1 cDNA. A panel of Chinese hamster/mouse hybrid DNA (kindly provided by J. Hilkens) was used to determine the chromosomal assignment of *P450-2C*. The parental cell lines were digested with a number of restriction enzymes, Southern blotted, hybridized and washed at high stringency to find the conditions which best suited the detection of mouse PB-1 sequences on a Chinese hamster background. For pM8-1 this turned out to be *Hind*III digest and for pPB5-2 *Bgl*II digestion. Fig. 4.17 shows a series of *Hind*III digested mouse, Chinese hamster and hybrid DNAs Southern blotted and probed with pM8-1. Of note is that the fragments mapped in this experiment correspond to those mapped in the OXA RI lines on *Hind*III digestion (Fig. 4.16). Hybrids were also analysed by *Bam*HI and *Bgl*II digestion and pM8-1 probing and a segregation pattern obtained. As pPB5-21 detected additional fragments not observed with pM8-1 it was also used to probe *Bgl*II digests of Chinese hamster/mouse somatic cell DNA (Fig. 4.18). This gave 100% concordance with pM8-1 for hybrids analysed in common which suggested that these sequences were linked on the same chromosome.

FIGURE 4.16.

Mapping in RI strains with pM8-1. The indicated RI strains and their progenitors were analysed as in Figure 4.12.



pM8−1 (HindIII)



pM8−1 (BamHI)

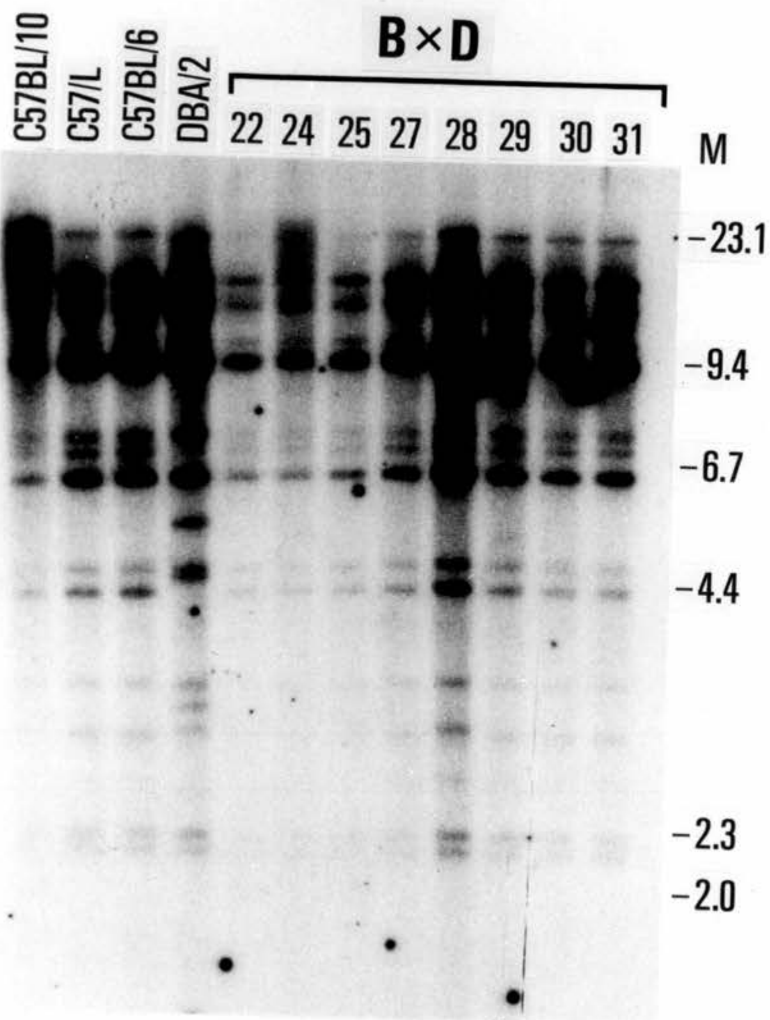
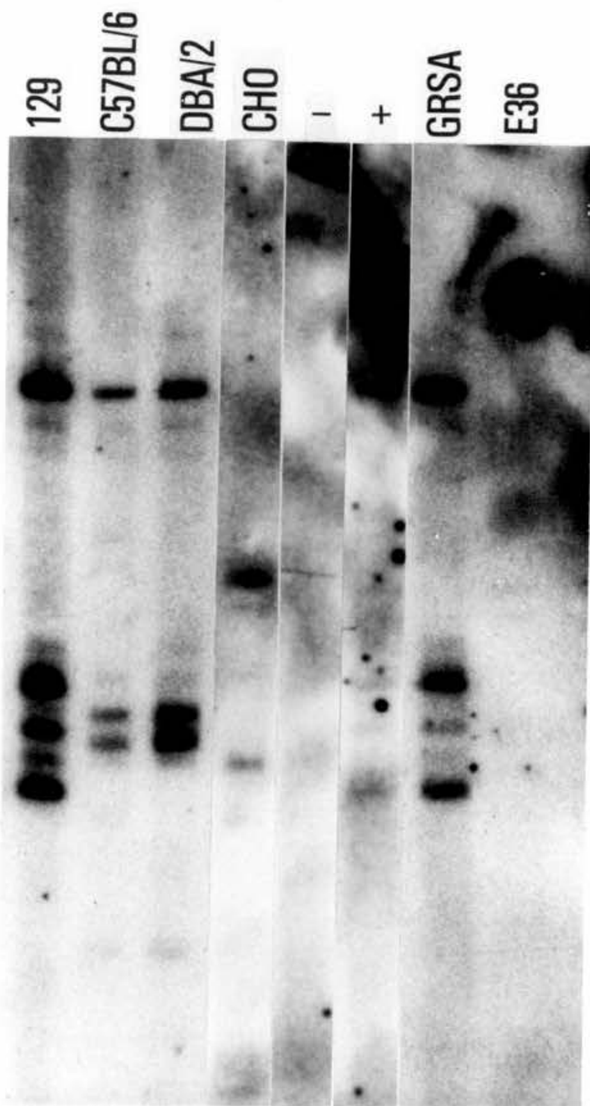

pM8－1
C57BL/10
C57/L
C57BL/6
DBA/2
B×D
22 24 25 27 28 29 30 31
M
－23.1
－9.4
－6.7
－4.4
－2.3
－2.0

FIGURE 4.17.

Southern blot analysis of HindIII digests of Chinese Hamster/mouse somatic cell hybrid DNAs and their parental cell lines. The blot was probed as in Figure 4.12. parental cell lines were E36 (Chinese hamster) and GRSA (mouse). Also included are mouse strain DNAs showing the *Hind*III polymorphism detected by pM8-1 and another Chinese hamster cell line, CHO. Two Chinese hamster/mouse somatic cell hybrids were analysed in this analysis, '+' is a hybrid positive for mouse DNA and '-' is a hybrid negative for mouse DNA.


pM8-1
129
C57BL/6
DBA/2
CHO
-
+
GRSA
E36

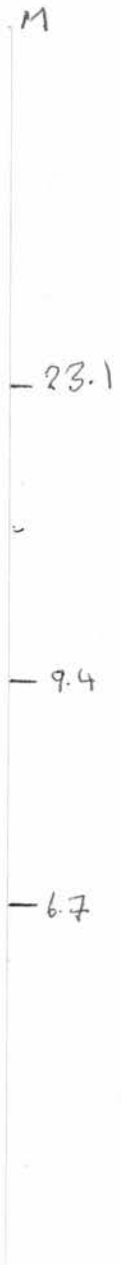
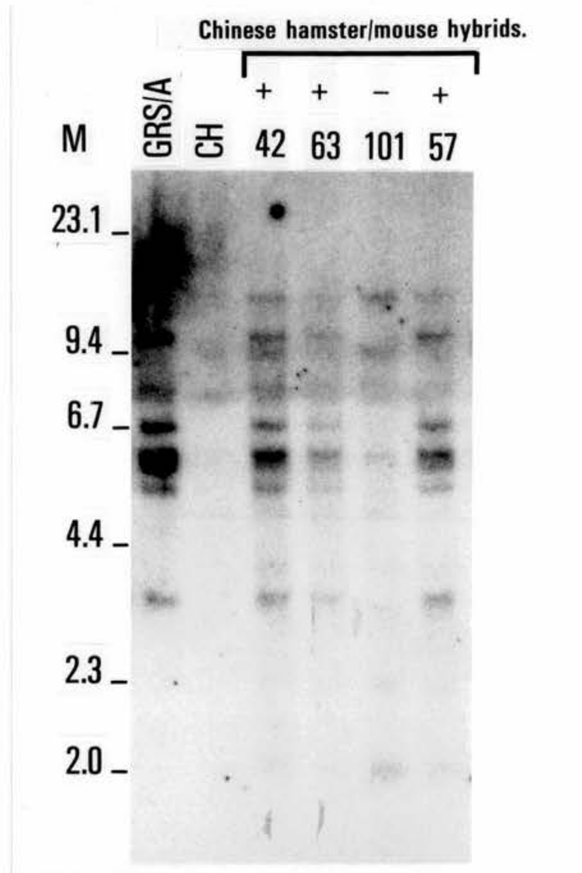
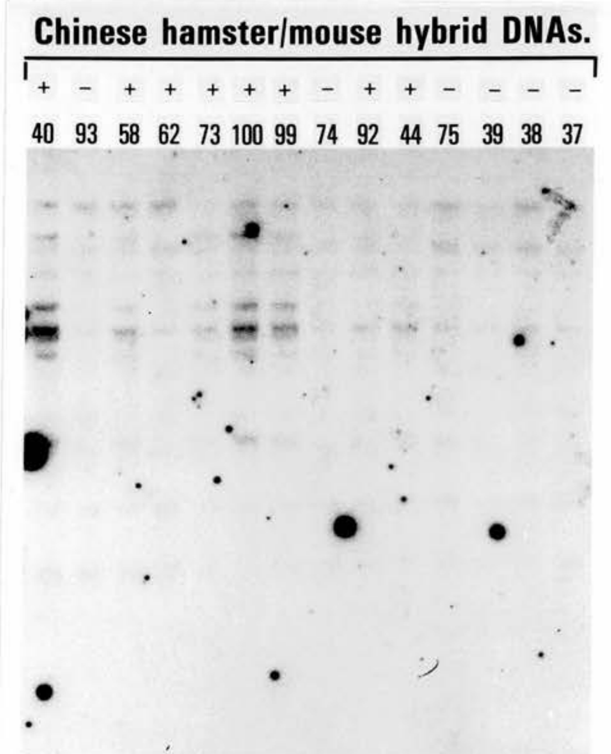

M
23.1
9.4
6.7

FIGURE 4.18.

Chinese hamster/mouse somatic cell hybrid DNAs digested with BglII and analysed with pPB5-21. Blots were analysed as in Figure 4.13.





"+"=Positive for mouse PB-1 fragments.
"−"=Negative for mouse PB-1 fragments.

These experiments were done blind and the resulting segregation was analysed by J. Hilkens to see whether P450-2C segregated with any chromosomal markers in these hybrids. Table 4.6 shows that P450-2C has100% concordance with Got-1 on chromosome 19.

In situ hybridization was used as an additional approach to map the P450-2C locus. Unlike human chromosomes most mouse chromosomes are telocentric and do not vary much in size (Miller and Miller, 1981). However specific chromosome rearrangements can be used to unequivocally identify specific mouse chromosomes. In collaboration with R. M. Speed and J. R. Gosden this approach was used to localize the P450-2C locus. A wild strain of mouse (Mus musculus), CD, has all its chromosomes apart from the 19, X and Y fused as metacentrics (Capanna et al, 1975). The 19, X and Y are readily distinguishable as acrocentrics. Firstly a G-banded karyotype from trypsin banded somatic chromosome preparations from the bone marrow of CD mice was made. In female CD mice the 19 is readily distinguishable from the X on account of its much smaller size (Fig. 4.19). The karyotype showed that the CD mice were of the right chromosome constitution.

The pPB3-15 cDNA probe was labelled with tritium and then annealed with total mouse DNA to eliminate the hybridization of the repeat sequence (Sealey et al, 1985; Porteous et al, 1987). The annealed probe was used against metaphase spreads of CD mouse bone marrow cells. A representative metaphase spread is shown in Fig. 4.20. In this figure three out of the four chromosome 19s have grains associated with them. Ten cells were analysed with a total of 88 grains, of these 21 (23.9%) were located on mouse chromosome 19 and of these, 19 (91.5% of total) were localized to within the 19 D1/D2 region at the tip (Fig. 4.21).

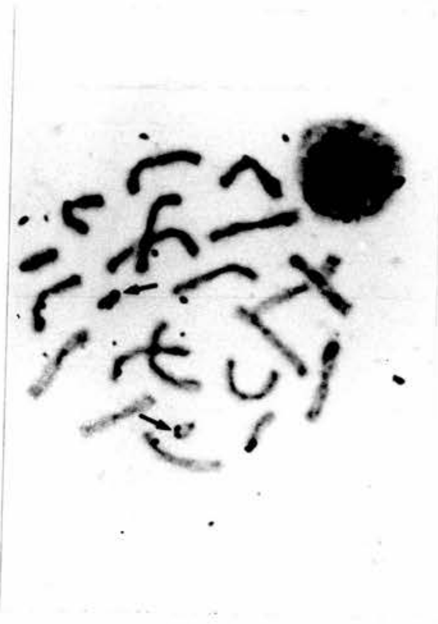
FIGURE 4.19.

The trypsin-giemsa-banding patterns of the karyotype of a female CD mouse. Figures underneath represent the fusions between the respective acentrics. Note the small acrocentric chromosome 19. (Kindly provided by R. M. Speed.)

Karyotype of CD mouse (♀).

FIGURE 4.20.

Metaphase spread of mouse chromosomes hybridized with tritiated pPB3-15. Hybridization was done *in situ* with pPB3-15 to mouse chromosomes from CD female mice. Note 3/4 chromosome 19s are labelled (arrows) (provided by D. Rout).



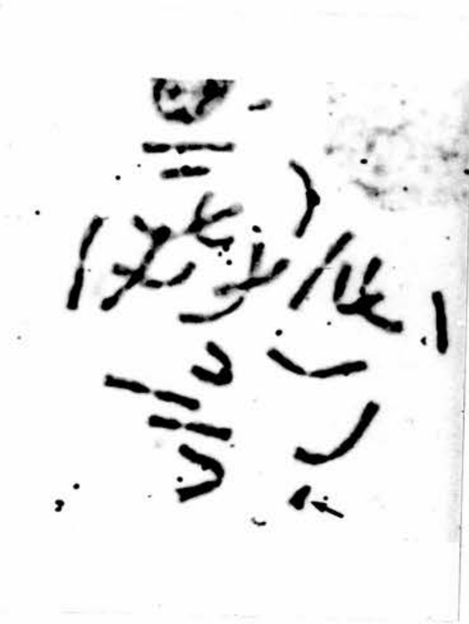
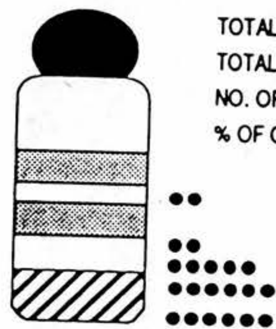


FIGURE 4.21.

Autoradiographic grain distribution of pPB3-15 on mouse chromosome 19.

Localisation of P450-2C on mouse chromosome19



TOTAL NO. OF GRAINS IN 10 CELLS = 88
TOTAL NO. OF GRAINS ON 19 = 21 (24%)
NO. OF GRAINS WITHIN D1/2 REGION = 19
% OF GRAINS WITHIN D1/2 REGION = 91.5%

TABLE 4.6.

Segregation of P450-2C in mouse-hamster hybrids*

Chromosome	Marker	Concordanct	Discordant	Concordancy % (N=TOT)
1	PEP3	15	9	62.5 (N=24)
2	SDH1	11	12	47.8 (N=23)
	AK1	12	12	50.0 (N=24)
3	RASN	5	7	41.6 (N=12)
4	PGD	12	12	50.0 (N=24)
5	PEP7	13	11	54.1 (N=24)
6	TPI1	10	14	41.6 (N=24)
	LOC2	5	8	38.4 (N=13)
7	GPI1	12	12	50.0 (N=24)
	LDH1	12	12	50.0 (N=24)
	PEP4	8	10	44.4 (N=18)
8	GR1	18	6	75.0 (N=24)
	APRT	15	5	75.0 (N=20)
	DIA	15	8	65.2 (N=23)
9	MOD1	14	10	58.3(N=24)
	MPI1	15	9	62.5 (N=24)
	PK3	15	9	62.5 (N=24)
10	PEP2	14	10	58.3 (N=24)
11	GALK	8	6	57.1 (N=14)
	TCN2	6	6	50.0 (N=12)
	SHU	9	7	56.2 (N=16)
12	ACP1	14	9	60.9 (N=23)
13	ARSB	21	2	91.3 (N=23)
14	NP1	11	13	45.8 (N=24)
	ES10	12	12	50.0 (N=24)
15	ARSA	12	12	50.0 (N=24)
	MYC	9	6	60.0 (N=15)
	INT1	6	2	75.0 (N= 8)
16	SOD1	14	10	58.3 (n=24)
	LOC3	8	5	61.5 (N=15)
17	GLO1	10	6	62.5 (N=16)
	SOD2	16	8	66.8 (N=24)
	C3	12	6	66.6 (N=18)
	PIM-2	15	7	68.1 (N=22)
18	PEP1	12	8	60.0 (N=20)
19	GOT1	24	0	100.0 (N=24)

* In total 24 hybrid DNAs were analysed but markers were available for only a proportion of these hybrids. Analysis performed by J. Hilkens. (See (Hilkens et al, 1986) for explanation of markers.)

4.11. Discussion

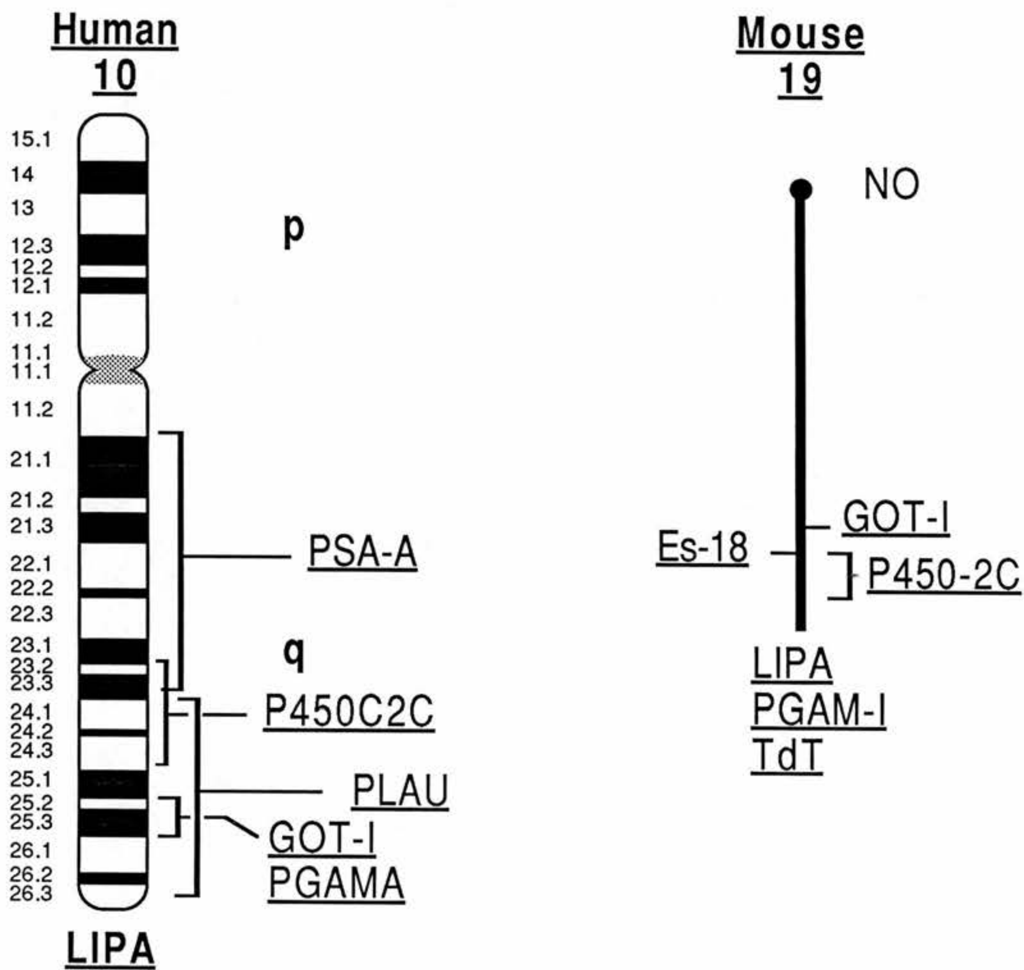
The power of mouse genetics for analysis of a multigene family as complex as cytochrome P-450 is evidenced by its dual ability to determine chromosomal organization and gene function. Genetics can provide the best evidence for in vivo function of specific P-450 families (Simmons and Kasper, 1985; Nebert et al, 1981; Taylor, 1984). In this Chapter it has been shown that P450-2C is a clustered multigene family, it is not linked to any other known P-450 loci and is associated with constitutive AHH metabolism.

4.11.1. P450-2C forms part of a syntenic linkage group in human and mouse

P450-2C was mapped to the tip of chromosome 19 by mapping in somatic cell hybrids and by in situ hybridization to mouse metaphase chromosomes. This mapping data suggests that P450-2C in mice and its equivalent P450C2C in human map to regions of homology Got-1, PGAM, LIPA, and TdT all map to chromosome 19 in mice and chromosome 10 in human (Greschik and Kazazian, 1985; Yeng Fang et al, 1986; Davidson and Roderick, 1986). P450C2C , Got-1, PGAM and TdT have been localized to 10q23-10q25 with P450C2C being centered at 10q24.2. In mice the in situ hybridization experiment cannot distinguish the relative order of markers, but in human Got-1 is more distal than P450C2C (Figure 4.22). When the human PB-1 cDNA plasmid pHL5,5 is used as a probe against mouse metaphase spreads it also preferentially hybridizes to the D1/D2 region of chromosome 19 (J. Gosden, pers. comm.). The extent of the chromosomal homology could be estimated by utilizing more flanking markers from human chromosome 10 and mouse chromosome 19. For instance if PSA-A could be mapped in the mouse this could detect how far up this region of

FIGURE 4.22.

Homologous loci containing PB-1 genes in mice (P450-2C) and humans (P450C2C). Shown are mouse human chromosomes 19 and 10 respectively with the localization of the P450-2C genes and their flanking markers. Loci at the end of the chromosomes have not been localized. The human chromosomal and regional mapping was performed using somatic cell hybrids, *in situ* hybridization and family studies (Grzeschik and Kazazian, 1985; Bruns *et al*, 1987; Yang-Feng *et al*, 1986). The mouse chromosomal locations were determined by recombination analysis and in somatic cell hybrids (Yang-Feng *et al*, 1986; Davidson and Roderick, 1986).



Homologous loci containing PB-I genes in mice (P450-2C) and humans (P450C2C).

synteny goes on the respective chromosomes.

4.11.2. Size of P450-2C locus

No recombination between any of the P450-2C haplotypes could be observed in 81 RI strains. This represents a maximal genetic distance of 0.96cM (95% confidence limits) Taylor, 1978). A centimorgan of DNA in the mouse is approximately 2 x 10^6bp. 0.96cM is equivalent to approximately 1920kb of mouse DNA. pTF-1 detects up to 200kb of genomic DNA on Southern blot analysis. pTF-2 and pM8-1 represent 5' and 3' coding regions for the rodent PB-1 mRNA. When the same blot is probed and stripped and reprobed with pM8-1 and pTF-2 respectively (Fig. 4.5 and 4.15) it can be estimated that there are 5-7 bands in common representing a minimum of 5-7 genes in the mouse. These probes also hybridize very well to human DNA (Figs. 4.5 and 4.15) and give a minimum estimate of 2-3 genes in humans. This estimate agrees with the minimum gene count estimated with the human 5' and 3' cDNA probes (Chapter 3). The number of $PB3_a$ (P450-2B) genes in rat has now been found to be 11 (Adesnik and Atchison, 1986) and has a similar genomic complexity on Southern blots to PB-1 (P450-2C) genes (Fig. 4.6 vs Fig. 4.1). The minimum size of a PB-1 (P450-2C) gene is 20kb in DBA/2 mice (data not shown) which is equivalent to that found for PB3a of 15-25kb (Atchison and Adesnik, 1983; Mizukari et al, 1983; Suwa et al, 1985). Thus a maximum estimate of the size of PB-1 (P450-2C) gene family is 11-14 genes in mice and 4-6 in humans.

4.11.3. P450-2C and function

The apparent recombination between P450-2C and constitutive AHH might suggest that these are different but closely linked loci.

However it is likely that constitutive AHH activity is encoded by P450-2C as purified PB-1 (P450-2C) proteins from rat, rabbit and human have this activity (Waxman and Walsh, 1983; Runcy and Johnson, 1985; Shimada et al, 1986). So this appears to be a highly conserved function. This constitutive AHH activity was determined several years ago, given the modest difference between AKR/J and C57L/J progenitors a single discordant RI strain might be due to statistical variation. Antibodies to PB-1 will inhibit this function in certain microsomal preparations (Adams et al, 1985). A third possibility is that P450-2C has a closely linked regulator of constitutive AHH metabolism. Proteins within the PB-1 (P450-2C) family therefore have the potential to play a role in the deactivation or activation of polycyclic hydrocarbons. Their role in carcinogenesis remains to be established. It has been proposed that the diminished potency of 3MC with respect to TCDD as an AHH inducer in whole animals as compared to tissue culture results from the metabolic inactivation of 3MC in whole animals (Poland and Glover, 1973; Niwa et al, 1975). In studies of expression pf P-450s in hepatoma cells (tissue culture) I found that PB-1 (P450-2C) mRNA levels are not maintained whereas MC1a (P450-1) levels are (data not shown). Thus loss of PB-1 expression may be concommitant with the inability to inactivate 3MC as an AHH inducer.

PB-1 proteins in rats also have activity towards steroids at the 2, 15β and 16α positions and the 25 hydroxylation of vitamin D (Astrom and De Pierre, 1986). A male specific form of PB-1 has estrogen 16α-hydroxylation activity in rats and probably in mice (Harada and Negishi, 1984). This sexual dimorphism is not seen *in vitro* (Bradlow et al, 1985; 1986); taken together with our BXH RI data this would suggest that circulating levels of 16α-

hydroxyestrogen are not determined by P450-2C.

In rat six distinct forms of PB-1 isozymes have been isolated (Haniu et al, 1984; Wolf et al, 1986b) termed P-450 - PB_{1a}, PB_{1b}, f, g, h and i. Sequence analysis of PB_{1a}, f and h (M) cDNA clones show them to have greater than 75% amino acid identity (Freidberg et al, 1986; Gonzalez et al, 1986a; Yoshioka et al, 1987). The estimate of divergence time between these forms is 50-80 mya (Chapter 3) which is prior to the mouse/rat divergence time of 25 mya (Sarich, 1985). The locus defined as P450-2C has the potential to code for all these PB-1 isoenzyme forms so orthologous forms might be expected in both species. Indeed preliminary sequence analysis of pPB3-15 shows it to have 88% identity with PB_{1a} of rat at the nucleotide level over the sequences compared (J. Sweeney, per. comm.). This makes pPB3-15 more similar to PB_{1a} of rat than either f or h(M). Thus pPB3-15 could encode the mouse orthologue of rat PB_{1a}. PB_{1a} in rat is active in the hydroxylation of steroids at the 16α- and 16β-positions (Astrom and De Pierre, 1986) and is induced 2-4 fold on phenobarbital administration in rats (Freidberg et al, 1986).

4.11.4. Haplotyping of P450-2C and P450-2B in mice

The finding that members of P450-2C map close together supports the idea that these particular genes arose by a process of duplication from an ancestral gene after the rodent/human split. This process seems to have been happening over a longer time scale. Members of the P450-2C and P450-2B multigene families have 50% amino acid identity in rats (Gonzalez et al, 1986a) the same will probably be true in mice. This suggests that they arose by a duplication of an ancestral gene 200 million to 300 mya (Nebert et al, 1987).

After these genes began to diversify, two distinct gene families would have resulted and become separated genetically at (or post) the initial amplification event. This initial amplification event seems to have led to an explosion of P-450 gene families. To date there are 5 P450-2-like distinct multigene sub-families showing 50% amino acid identity in rat and humans (Nebert et al, 1987), four of which map to distinct locations in humans. P450C2B and P450C2A both map to chromosome 19 in human (N. J. Spurr, pers. comm.). Linkage of related gene families has also been noted for multigene families encoding the protease inhibitors α anti-trypsin and contrapsin in mice (Hill et al, 1985). Both of these proteins share 50% amino acid identity and map to within 1cM on mouse chromosome 12. This linkage is also seen for these gene families in humans (R. Hill, pers. comm.). Therefore it would not be surprising if P450-2B and P450-2A were found to be in the same proximity in the mouse genome. The haplotyping of P450-2B in mice tentatively suggests this. It shows discordance with coumarin hydroxylase activity in two strains of mice which suggests a closely linked locus is responsible for this activity. Purification schemes for $P450_{Coh}$ in mice suggest it has testosterone 7α-hydroxylase activity (Huang et al, 1976; Kaipainen et al, 1984), this activity is normally associated with proteins encoded by members of the P450-2A gene family (Nagata et al, 1987). Therefore P450-2B and P450-2A could map to within 1cm in mice.

The $P450\text{-}2B^c$ haplotype in 129 mice is very unusual in that it is seen only in this strain and looks like loss of a gene in comparison to other inbred mice strains. 129 mice are also unusual in that females express 10-fold less protein in comparison to mice of the $P450\text{-}2B^a$ and $P450\text{-}2B^b$ haplotypes (Noshiro et al, 1986). The

PB3a protein in mice has 16α-testosterone activity (Devore et al, 1985). The lack of this activity does not appear to have adverse effects on these mice as they maintain a high 16α-estrogen hydroxylase activity in vivo (Bradlow et al, 1985) which would appear to rule out the contribution of PB_{3a} form to this activity. Conceivably it could be performed by another member of this gene family which is extrahepatic in its expression.

Two of the three P450-2C haplotypes detected by pTF-1 and pM8-1 are very similar and are examplified by AKR ($P450\text{-}2C^c$) and DBA/2 ($P450\text{-}2C^b$). These two strains are distinct from C57BL/6 ($P450\text{-}2C^a$). If pTF-2 is used as a probe the haplotypes are very similar which suggests most of the variation that is being detected is due to differences in the 3' part of P450-2C gene members. The cDNA encoded by pTF-2 corresponds to the region of the protein that is putatively involved in substrate specificity and the first conserved cysteine containing fragment whose function is unknown. Thus we might expect members of the P450-2C family to have similar substrate specificity between different mouse strains but correspondingly different specific activities due to differences in heme and reductase binding sites as detected by pM8-1. This could explain why AKR ($P450\text{-}2C^c$) mice have a substantially greater constitutive AHH activity as compared to C567BL/6 ($P450\text{-}2c^a$) and DBA/2 (($P450\text{-}2C^b$) mice (Hutton et al, 1979). In this context it would be useful to develop a set of specific substrates which detect these differences in different mice strains for proteins encoded by the P450-2C locus.

CHAPTER 5

PATTERN OF LIVER GENE EXPRESSION IN MOUSE VISCERAL YOLK SAC AND FOETAL LIVER

5.1. INTRODUCTION

In the developing embryo many complex programs of activation of cellular and gene processes are programmed resulting in the differentiated adult organism. For mammals this will mean that at critical periods the fetus may have different sensitivities to toxicological agents which may or may not cross the placental barrier. For example in mice and rats it has been shown that the MC1b (P450-1) is not inducible by 3MC in liver until a couple of days prior to birth (Chen et al, 1982; Negishi and Nebert, 1979). Similarly the PB_{3a} (P450-2B) genes do not become responsive to phenobarbital in liver until the early neonatal period in rats (Giachelli and Omiecinsk, 1986).

A pertinent question would be when, or if, does the foetal liver resemble the adult liver in its pattern of expression? The visceral yolk sac (VYS) one of the extra-embryonic membranes surrounding the mouse embryos expresses a number of liver functions including alpha-fetoprotein (AFP) and transferrin (Dziadek and Adamson, 1978; Janzen et al, 1982; Adamson, 1982; Dziadek and Andrews, 1983). However the extent of the overlap in the programme of gene expression between the VYS and the foetal and adult liver is not known. The VYS consists of visceral endoderm and mesoderm and it is the visceral endoderm which is thought to express the liver functions (Dziadek and Adamson, 1978; Adamson, 1983). The visceral endoderm has very different embryological origins to the liver, it is purely an extra-embryonic tissue derived from a population of primitive endoderm cells which arise on the fifth day of development and subsequently gives rise only to cells in the visceral and parietal yolk sacs (summarized in Hogan et al, 1986). Descendants

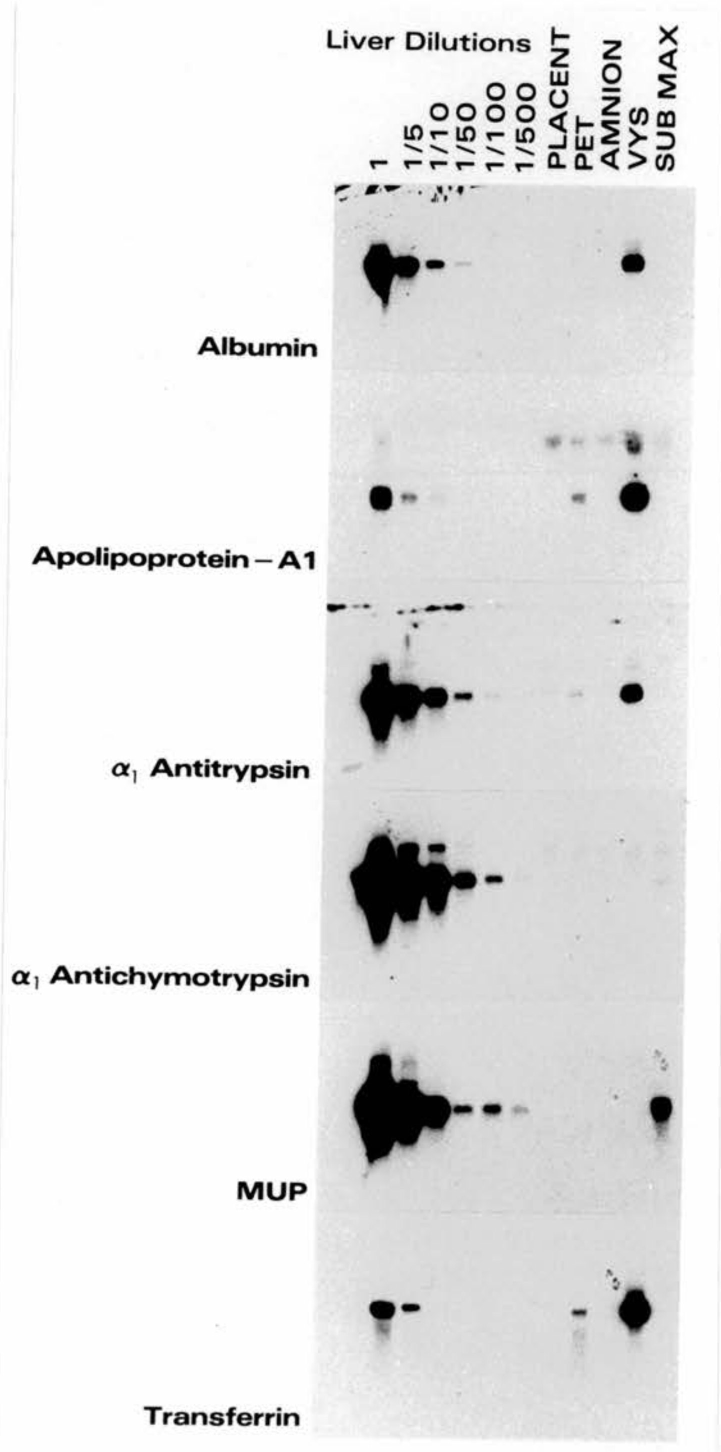
of the primitive endoderm do not colonize any tissues in the foetus proper (Gardner and Rossant, 1979). In contrast, the liver develops from an outgrowth of the gut endoderm at around 9 days of development. This diverticulum is derived from the definitive endoderm which traces back to the embryonic ectoderm (Hogan et al, 1986). When this work was begun I had available a number of liver cDNA clones for mouse liver serum proteins (Barth et al, 1982) which have subsequently been identified as coding for albumin, apolipoprotein AI (ApoAI) (Millar et al, 1983; alpha1antitrypsin (α_1-Pl), contrapsin a member of the antichymotrypsin family (Hill et al, 1986) and the major urinary proteins (MUPs) (Bennet et al, 1982; Shaw et al, 1983). These were used to characterise the pattern of serum protein gene expression in mouse VYS and foetal liver. Subsequently I used the cytochrome P-450 genes and a set of liver specific genes of unknown function (Derman et al, 1981) to see whether the observations for the serum protein genes could be extended to these other hepatic genes.

5.2. Expression of serum protein genes in VYS

Radioactive probes corresponding to albumin, ApoAl, α_1-PI, contrapsin and MUPs were hybridized to total RNA extracted from several extra-embryonic tissues of 13.5 day post-coitus (p.c.) embryos (kindly provided by B.L.M. Hogan) and several adult tissues (Fig. 5.1). The mRNAs coding for albumin, ApoAI and α_1-PI were all detected at relatively high levels in the VYS RNA population. By comparison with the signal obtained using dilutions of adult liver RNA it can be concluded that albumin and α_1-PI are present at ~10% of the level found in adult liver. Surprisingly the concentration of ApoAI RNA in the 13.5 day p.c. VYS is several fold higher than

FIGURE 5.1.

RNA blot analysis to quantify the expression of serum protein genes in extra-embryonic tissues relative to adult liver. 2μg of total RNA from adult mouse liver (and dilutions) and submaxillary gland (submax), placenta, parietal endoderm and trophoblast (PET), amnion and VYS were size fractionated on a series of 1.5% agarose formaldehyde gels and transferred to a set of nitrocellulose filters. Each filter was probed separately but under identical conditions with (^{32}P) labelled cDNA corresponding to the proteins indicated.


Liver Dilutions
1
1/5
1/10
1/50
1/100
1/500
PLACENT
PET
AMNION
VYS
SUB MAX
Albumin
Apolipoprotein – A1
α1 Antitrypsin
α1 Antichymotrypsin
MUP
Transferrin

that in the adult liver. It has been inferred from immuno-precipitation of radioactively labelled protein synthesis by isolated tissue that transferrin mRNA level is expressed at high levels in the VYS (Adamson, 1982; Janzen et al, 1982). This was confirmed by hybridizing a labelled transferrin cDNA probe (Clissold and Bishop, 1981) to the same series of mRNAs. The transferrin mRNA in 13.5 p.c. VYS is also several fold higher than in adult liver. None of these four mRNAs could be detected within RNA from 13.5 p.c. amnion, placenta or adult submaxillary gland. In each case a weak positive signal, corresponding to ~10% of the intensity observed with the VYS preparation, was obtained with parietal yolk sac RNA. It is not clear whether this represents true expression of these genes by parietal endoderm cells which are thought to arise from primitive endoderm during development via differentiating visceral endoderm (Hogan and Tilly, 1981). The other possibility is that the parietal yolk sac preparations are contaminated with some VYS material (Dziadek and Andrews, 1983).

In contrast to these results no expression was seen for MUPs or contrapsin in 13.5 day VYS or any of the other extra-embryonic tissues at this time. Based on a comparison with a series of liver dilutions it can be calculated that there are <30 copies per cell of MUP mRNA and <10 copies of contrapsin mRNA in these extra-embryonic tissues.

5.3. Developmental regulation of serum protein mRNA expression in the VYS and liver

By analysis of RNA-cDNA hybridization kinetics it has been estimated that each of these serum proteins has a characteristic time of appearance in the liver during foetal development (Barth et al, 1982). It was of interest to know whether this pattern of gene expression is mimicked in the VYS which would imply that the genes are under the same controls in the two tissues.

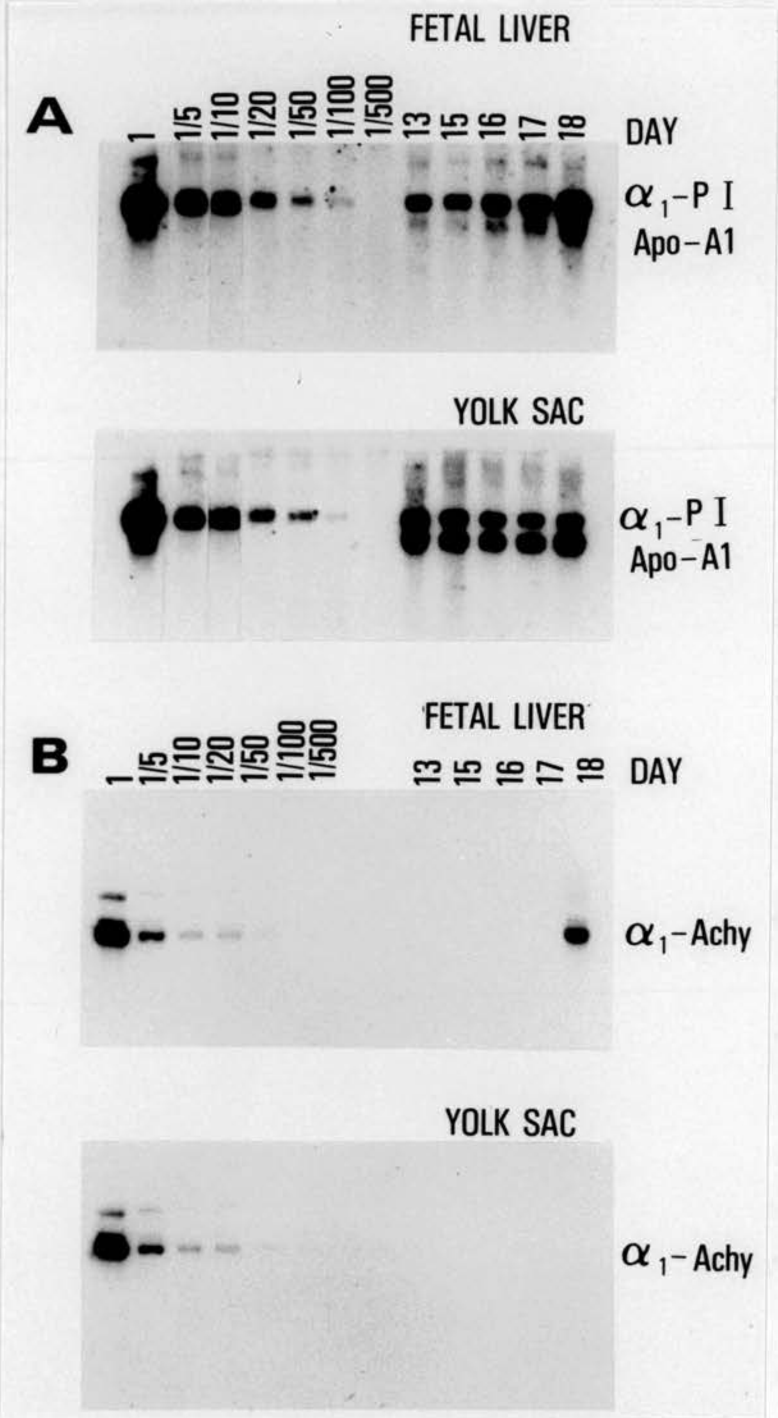
Figure 5.2 shows the result of an experiment in which α_1-PI, ApoAI and contrapsin mRNA levels were determined by Northern blot analysis of total RNA preparations from VYS and livers dissected from foetuses at the 13th and 15-18th days of pregnancy. As reported previously there is a 10- to 20-fold increase in the levels of α_1-PI and ApoAI mRNA in the liver between 13 and 18 days (Barth et al, 1982). However in the VYS there is little change in these mRNAs during this period of development. At the earliest times studied here the levels of these mRNAs are considerably higher in the VYS than in the foetal liver. The mRNA for contrapsin is first detectable in the liver on the 18th day of development. However as shown in Fig. 5.2B, there is no detectable level of contrapsin related mRNAs in the VYS at this stage of development.

5.4. Serum protein gene expression in visceral endoderm and mesoderm

The VYS is made up of two layers, an outer epithilium of endoderm cells, highly specialised for both the secretion and the uptake of nutrients (Franke et al, 1983) and a mesodermal layer in which blood islands and vessels first differentiate (Hogan et al, 1986). Synthesis of AFP and transferrin is restricted to the

FIGURE 5.2.

RNA blot analysis to determine the developmental pattern of expression of serum protein mRNA in the VYS and foetal liver.
2μg of total RNA from adult mouse liver (and dilutions), foetal liver and yolk sac were treated as in Figure 5.1. The numbers 13-18 refer to the days of pregnancy when the tissues were dissected at around 12 noon e.g. on the 13th day the embryos were 13.5 days old. Panel A was probed with two (^{32}P) labelled cDNAs corresponding alpha$_1$-PI and ApoAI. Panel B was probed with a cDNA corresponding to contrapsin a member of the alpha$_1$-antichymotrypsin (α_1-achy) family.



A
FETAL LIVER
1
1/5
1/10
1/20
1/50
1/100
1/500
13
15
16
17
18
DAY
α_1-P I
Apo-A1
YOLK SAC
α_1-P I
Apo-A1
B
FETAL LIVER
1
1/5
1/10
1/20
1/50
1/100
1/500
13
15
16
17
18
DAY
α_1-Achy
YOLK SAC
α_1-Achy

endoderm layer (Dziadek and Adamson, 1978; Adamson, 1982). To see if this is true for other serum proteins total RNA from the separated layers (kindly provided by B.L.M. Hogan) was used in northern blots for analysis with α_1-PI and ApoAI. As shown in Fig. 5.3, α_1-PI and ApoAI mRNA are present at much higher levels in the endoderm than mesoderm. Longer autoradiographic exposure of the filters showed some specific RNAs in the mesodermal RNA but this may be due to incomplete separation.

5.5. Cytochrome P-450s are expressed in extrahepatic sites

A number of cDNAs corresponding to members of the P450-1, P450-2C, P450-2B and P450-3 were used to probe northern blots of RNA from various adult mouse tissues, all showed expression in the small intestine (Table 5.1; Figs. 5.4 and 5.5) and except for pR17 (P450-2B) also showed expression in the kidney. Both these organs are associated with absorption and excretion of compounds from and to the outside environment. What was unusual was that pTF-1 (P450-2C) detected multiple sized transcripts in the small intestine. These were also evident in 16 day foetal liver and 9.5 day embryo (Fig. 5.4). pR17 (P450-2B) was unusual in that mRNA transcripts were also detected in the submaxillary gland when the level of the detection in this experiment was unable to detect any signal in uninduced mouse liver (Table 5.1). The two members of the inducible family could also be shown to have different expression characteristics. The mRNA corresponding to MC1b (P450-1) had a high constitutive level of expression in the kidney and the gut when absent elsewhere. The MC1a form had high constitutive levels in the liver and appeared absent elsewhere in uninduced animals. Thus these also correspond to liver specific genes which may show expression in the VYS.

FIGURE 5.3.

RNA blot analysis to quantify the levels of expression of serum protein genes in the VYS endoderm and mesoderm. 2µg of total RNA from adult mouse liver (and dilutions), 13.5 day p.c. VYS endoderm (and dilutions) and VYS mesoderm (and dilutions) were treated as in Figure 5.1. The filter was probed with a mixture of(^{32}P) labelled cDNAs for $alpha_1$-PI and ApoAI.

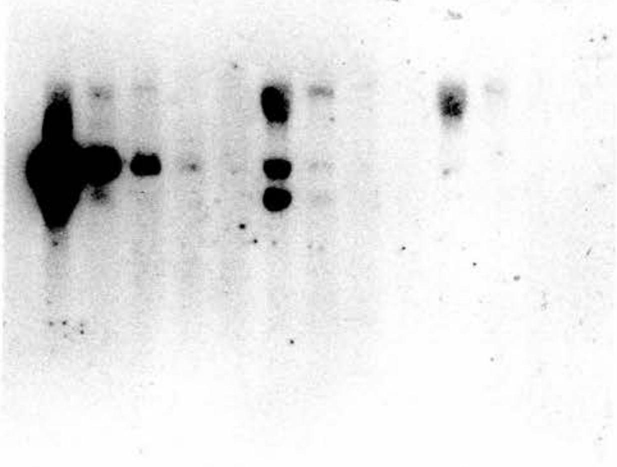

LIVER
ENDODERM
MESODERM
1
1/10
1/50
1/100
1/500
1
1/10
1/50
1/100
1
1/10
1/50
1/100
α1-P1
Apo-A1

FIGURE 5.4.

RNA blot analysis with liver derived cdNA probes to determine the pattern of expression in mouse adult and foetal tissues. (1) 1μg of polyA$^+$ RNA and 10μg of total RNA from mouse and human tissues were treated as in Figure 5.1 and hybridized with (^{32}P) labelled probes as indicated. Abbreviations are Lv, liver; Kd; kidney; Tes, testes; Br, brain; Sm, submaxillary gland; SI, small intestine; M67 male human liver RNA; Y/S, yolk sac; Am, amnion; Meso, visceral mesoderm. The probes were pLivS-8, (mouse cDNA); pLivS-9 (mouse cDNA); pPB2-29 (human cDNA); pTF-1 (rat P450f cDNA); pMC1b (human MC1b cDNA).

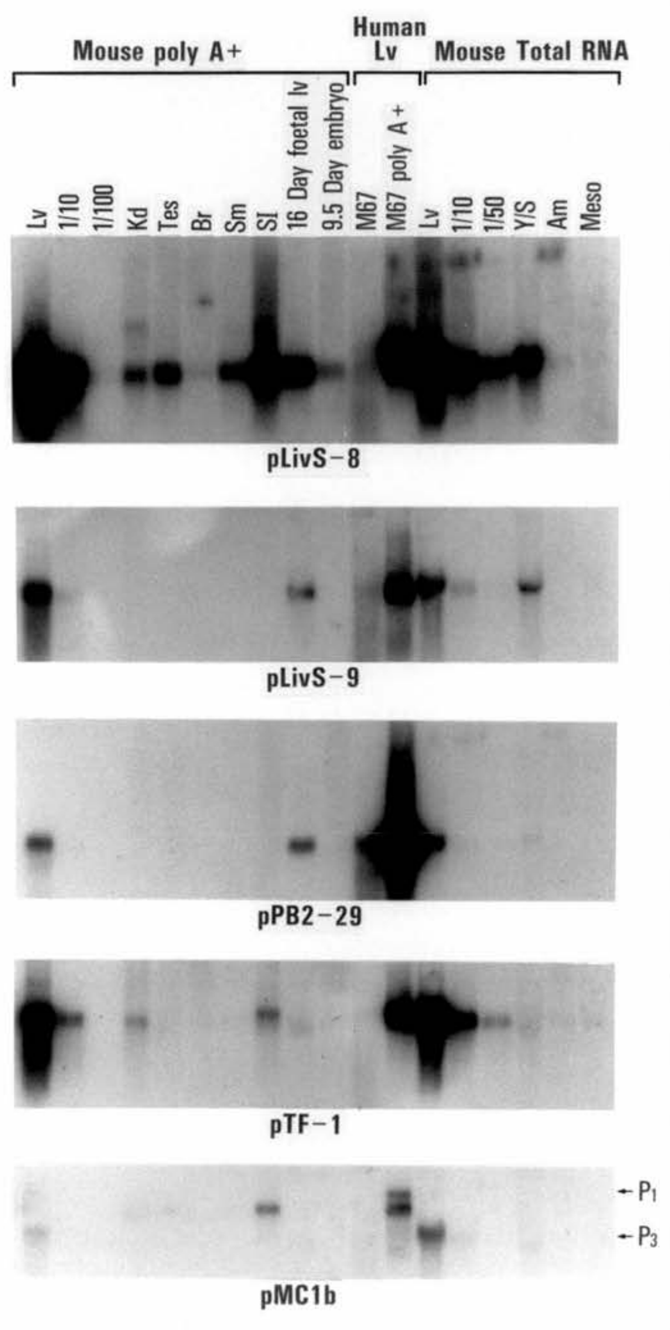
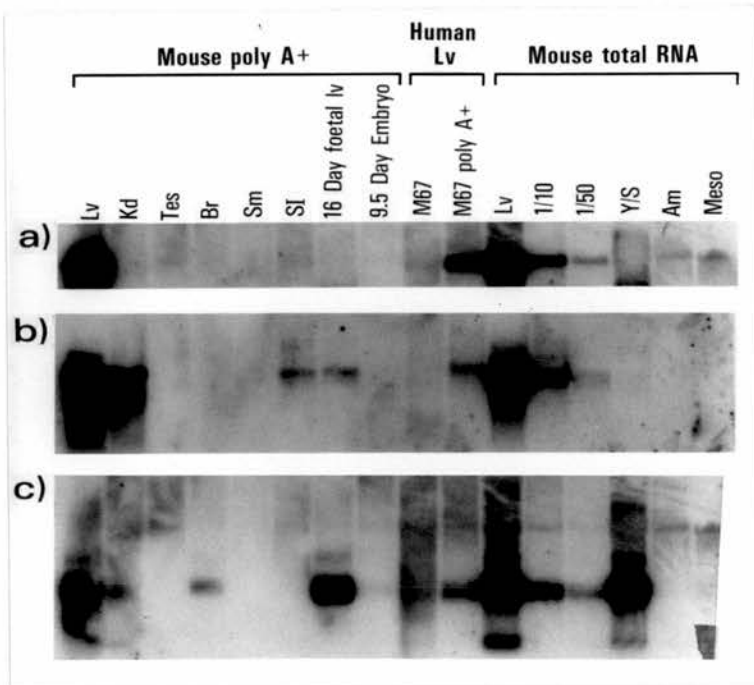

Mouse poly A+
Human Lv
Mouse Total RNA
Lv
1/10
1/100
Kd
Tes
Br
Sm
SI
16 Day foetal lv
9.5 Day embryo
M67
M67 poly A+
Lv
1/10
1/50
Y/S
Am
Meso
pLivS-8
pLivS-9
pPB2-29
pTF-1
P1
P3
pMC1b

FIGURE 5.5.

RNA blot analysis with liver derived cDNA probes to determine the pattern of expression in mouse adult and foetal tissues (2). Blots were treated as in Figure 5.4. and abbreviations are the same as in Figure 5.4. The probes used were (A) pLivS-11 (mouse cDNA), (B) pLivS-7 (mouse cDNA) and (C) pLivS-5 (mouse cDNA).



Mouse poly A+
Human Lv
Mouse total RNA
Lv
Kd
Tes
Br
Sm
SI
16 Day foetal lv
9.5 Day Embryo
M67
M67 poly A+
Lv
1/10
1/50
Y/S
Am
Meso
a)
b)
c)

TABLE 5.1.

(1) **Relative mRNA levels of liver derived cDNA clones**

Clone	(2) percent steady state mRNA (male liver)	Liver	Kidney	Testes	Brain	Sm	S.I.	16d f liv	13d VYS	Am	VM	9.5d E
pLivS-5	0.1	1	0.01	-	0.01	-	-	1	1	-	-	0.01
pLivS-7	0.07	1	0.1	-	-	-	0.05	0.05	0.02	-	-	-
pLivS-8	0.07	1	0.05	0.1	0.001	0.5	0.2	0.1	0.2	-	0.01	0.05
pLivS-9	0.05	1	-	-	-	-	-	0.1	0.2	-	-	-
pLivS-10	0.04	1	0.1	-	-	-	0.1	0.1	0.1	-	-	N.D.
pLivS-11	0.015	1	-	0.1	-	-	0.01	-	-	0.01	0.01	-
pPB2-29	?	1	-	-	-	-	-	1	0.5	-	-	
pTF-1(P450-2C)	0.2	1	0.03	-	-	-	0.05	0.01	.001		-	0.001
pPCN (P450-3)	~0.1	1	0.1	-	-	-	1	0.02	-	-	-	-
pR17 (P4502B)	~0.02	-	-	-	-	1	1	-	-	-	-	-
pMC1b(P4501) MC1a	~0.01	1	-	-	-	-	-	-	-	-	-	-
MCb	~0.001	-	1	-	-	-	2	-	-	-	-	-

(1) Relative to highest expressing adult tissue

(2) pLivS 5-11 taken from Powell et al, 1984; the rest are guesstimates based on levels relative to un-induced P-450s.

' - ' = not detectable; N.D. = not done.

SM, submaxillary gland; S.I., small intestine; 16d f liv, 16 day foetal liver; 13 d VYS, 13 day visceral yolk sac; Am, amnion; VM, visceral mesoderm; 9.5d E, 9.5 day whole embryo

5.6. Pattern of expression of cytrochrome P-450 and unknown mouse liver derived cDNA clones in foetal liver and yolk sac

In this section the developmental regulation of the cytochrome P-450s and a number of liver specific genes were investigated by northern blot analysis in uninduced animals. Figures 5.4 and 5.5. show a series of northern blots containing RNA from extra-embryonic tissues, foetal liver, various adult mouse tissues, whole 9.5 day mouse embryo and human liver preparations. In general genes expressed at high levels in 16 day foetal liver are also expressed in 13 day yolk sac e.g. pPB2-29, pLivS-5, pLivS-8 and pLivS-9. Also included in this category is pLivS-10 (Table 5.1). Other mRNA species which show late developmental gene expression have low to undetectable levels of expression in foetal liver and yolk sac e.g. pTF-1, pLivS-7, pLivS-11, pPCN12, pMC1b and pR17. Some cDNAs which show low levels of expression in foetal lever also show low levels of expression in the amnion and visceral mesoderm, but clearly these genes are not liver specific in their pattern of expression (Table 5.1). For example pLivS-8 is very promiscuous in its expression pattern. pLivS-11 is not detectable in foetal liver or VYS but can have low levels of expression in the small intestine, testes, amnion, visceral mesoderm and 9.5 day whole embryo. Thus these experiments seem to confirm the pattern of expression observed for the mouse serum proteins, i.e. those liver genes that expressed early in development are also expressed in the developing yolk sac.

5.7. Discussion

In this Chapter a number of liver specific genes have been shown to have high levels of expression in the VYS as well as the liver. It is important to consider whether the corresponding

proteins are serving useful functions during early embryonic developments. The presence of high levels of ApoAI mRNA in the VYS suggests this tissue is actively synthesising and secreting other components of high density lipoprotein (HDL) and low density lipoprotein (LDL) particles. When yolk sacs are labelled with (^{35}S) methionine and proteins immunoprecipitated from the culture medium with antisera to HDL and LDL many components corresponding to apolipoproteins ApoAI, ApoIV, ApoB and ApoE can been seen on SDS polyacrylamide gels (Meehan et al, 1984; Shi and Heath, 1984). Functional studies with embryonal carcinoma cell lines suggest that HDLS can act as growth factors, presumably supplying lipids necessary for embryonic growth (Shi and Heath, 1984; Heath and Deller, 1983). The fate of the other serum proteins is unknown. Also the relative contribution of the liver and VYS to the total pool of these proteins at different stages of embryonic development is not known. However the northern blot analysis suggests that the VYS of the 13 day foetus has a considerably higher concentration of some of these sequences than the liver at this stage (Fig. 5.2A). Thus it seems reasonable to assume that these proteins together with AFP are involved in nurturing and protecting the growing embryo before the liver takes over these functions. The identity of many of the liver derived cDNA clones are unknown we can only speculate as to their function but they may correspond to other components of HDL and LDL particles especially those showing expression in the gut as well. The only P-450 that was detected at significant levels in the developing embryo corresponded to members of the P450-2C gene family. This may reflect its endogenous role in the metabolism of steroids and other effector molecules.

One of the unknowns is whether the genes expressed in the VYS

and foetal liver are the same. There is only one gene for albumin, ApoAI and transferrin in the mouse so the same gene is being expressed in both tissues. Southern blot analysis with pLivsS-5, 8, 9 and pPB2-29 show them to have a simple pattern of hybridization (data not shown) which suggests they are single genes, therefore the same gene is being expressed in different tissues. pLivS-7 and 10 have a more complex pattern of hybridization which is suggestive of more than one gene. There are multiple α_1-PI (Hill et al, 1985) and P450-2C genes so it is open to question whether the same genes are being expressed. It is obvious that multiple sized transcripts could result from different genes, alternative splicing sites or attenuation signals.

Studies on cell lineages in mouse embryos using chimaeras have shown that the visceral endoderm and liver have very different lineages. This suggests that the two tissues are independently programmed to express the same set of genes. It is interesting that the haematopoietic system is also located in the VYS in the early embryo prior to migration to the foetal liver (Moore and Metcalf, 1970). The VYS in the human is a much less prominent organ than in the mouse but immunological studies suggest that the human yolk sac is capable of synthesizing AFP, albumin, α_1-PI and transferrin (Gitlin and Perricelli, 1970). The possibility thus exists that patients with teratomas and yolk sac carcinomas may have raised levels of serum proteins such as α_1-PI and apolipoproteins in addition to AFP (Newlands and Reynolds, 1983). In future it will be of interest to determine the cis and trans-acting factors which control the expression of these commonly expressed genes in the foetal liver and VYS.

The lack of expression of a number of cytochrome P-450 genes is

intriguing. It could be assumed that the placenta provides enough of a barrier to prevent foreign compounds harming the developing embryo. However this is patently not the case for a number of foreign compounds which cause a birth defect such as thalidomide. In the next chapter it will be demonstrated that the regulation of many cytochrome P-450s is dependent on a number of trophic factors which are themselves expressed late in development.

CHAPTER 6

REGULATION OF CYTOCHROME P-450 EXPRESSION BY ENDOGENOUS COMPOUNDS IN RODENTS

6.1. Introduction

An important property of P-450s is that their levels of expression can be modulated by foreign compounds (see 1.4.3). This can be achieved by a diverse number of mechanisms including transcriptional activation, protein and mRNA stabilisation. Initially inducers of drug metabolism could be divided into the phenobarbital or 3MC type. The 3MC induction of P-450s is associated with the MC1a and MC1b forms in rodents (Guengerich et al, 1982; Negishi and Nebert, 1979). Numerous studies suggest that this process is due to transcriptional activation of the respective genes (Kimura et al, 1986; Gonzalez et al, 1984; Adesnik and Atchison, 1986) which is mediated by the binding of a receptor/ inducer complex to regulatory elements located in the 5' region of the MC1a and MC1b genes (Tukey et al, 1982; Durrin and Whitlock, 1987). The mechanism of action of phenobarbital in the induction response has not been elucidated, this is primarily due to the many structurally diverse compounds which induce in a similar manner to phenobarbital. For example phenobarbital and trans non-achlor treatment of rats leads to induction of PB_{3a} and PB_{3b} (P450-2C) proteins as well as PB_{2c} (P450-3) (Schuetz et al, 1986). Macrolide antibiotics will also induce PB_{2c} (Wrighton et al, 1985). A 23-fold increase in transcription of PB_{3a} and PB3b (P450-2B) genes is seen one hour after treatment of rats with phenobarbital (Hardwick et al, 1983). In a similar manner PCN and dexamethazone treatment of male Sprague-Dawley rats leads to 2.7- and 6-fold increase in the transcription of PB_{2c} (P450-3) genes after 6 hours and 1 hour respectively (Simmons et al, 1987). Attempts to isolate a PB-receptor complex in the proteins of rat serum, rat liver cytosol or rat liver nuclei (Tierney and Bresnick, 1981) have proved

unsuccessful. This suggests the mode of action is not by a receptor mediated mechanism in the liver.

In the case of family P450-3 the dose of dexamethazone required to induce these enzymes is 50 times greater than the dose required to activate the glucorticoid receptor (Schuetz and Guzelian, 1984) indicating that if a receptor mechanism applies, it is different. Unlike dexamethazone, organochlorine pesticides increase the accumulation of PB_{2c} (P450-3) protein by decreasing its rate of degradation when added to dexamethazone-induced primary cultures of hepatocytes (Schuetz et al, 1986).

Within certain P-450 gene families many of the proteins are constitutively expressed at high levels. Some of these are still inducible by foreign compounds, others are not. Freidberg and colleagues (1986) demonstrated that the mRNA levels of PB-1 increased four fold in male and female rats on phenobarbital administration, whereas another member of their family, form f, was not affected. Form f has four fold higher levels in female than male rats. These mRNAs are switched on at puberty in male rats with this increase appearing to be due to transcriptional activation (Gonzalez et al, 1986a). Similar observations can be made for the regulation of members of the P450-2A (Nagata et al, 1987), P450-3 (Gonzalez et al, 1986b), P450-2D (Gonzalez et al, 1987), P450-2E (Song et al, 1986) and P450-4 (Hardwick et al, 1987) gene families. Age related alterations in drug metabolizing activities can also be observed in cytochrome P-450 content in male and female rats (Kamataki et al, 1985). For example constitutive AHH decreases progressively in male adult rats between the ages of 3 to 25 months. From these types of studies it can be appreciated that there are a number of factors which are involved in the endogenous regulation of

cytochrome P-450 isozymes. As mentioned earlier (Chapter 5) the inducible P-450s only become responsive to the inducing compound at specific times during development. In the case of PB_{3a}, Pb_{3b} and MC1a this appears to be postnatal in rodents. Therefore these isozymes are subject to some form of endogenous modulation as well.

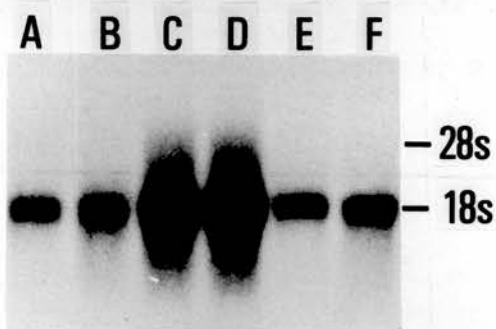
The genetic characterization of inbred mouse strains make them a powerful model for the study of multigene families such as the P-450 system. Nebert and colleagues have used this property extensively for a molecular genetic characterization of the structural loci for MC1a and MC1b in the mouse (Nebert and Jansen, 1979; Adesnik and Atchison, 1986; Eisen, 1986). Apart from this and recent work by Negishi and colleagues (Noshiro et al, 1986; Devore et al, 1986) extremely little work has been done on cytochrome P-450s in mouse liver. This includes studies on the regulation of the P-450 isozymes by endogenous and exogenous inducing agents. In this Chapter factors which affect the expression of P-450s in mice have been studied to try to ascertain whether differences in the levels of expression exist between different inbred mouse strains and to make interspecies comparisons in the regulation of cytochrome P-450s in mice and rats.

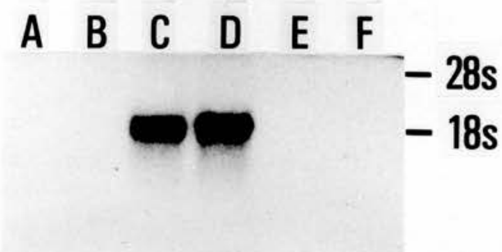
6.2. Effect of phenobarbital and 3MC on mouse hepatic P-450s mRNA

The expression of PB_{3a} (P450-2B) and PB-1 (P450-2C) gene families by the classical P-450 inducers as well as a number of endogenous type compounds was investigated. Figure 6.1 shows a northern blot analysis of hepatic total RNA from CBA/Ca mice treated with various compounds and probed with pR17 and pTF-1. It can be seen that as in rats phenobarbital treatment induces mouse PB-1 mRNA (pTF-1) and PB_{3a} mRNA (pR17) (Fig.6.1C) whereas 3MC has no effect

FIGURE 6.1.

Induction of PB_{3a} (P450-2B) and PB-1 (P450-2C) mRNAs in mouse liver by phenobarbital. 10μg of total RNA from the livers of CBA/Ca mice were fractionated on formaldehyde gels, blotted and hybridized with the indicated [^{32}P] labelled probes. pTF-2 rat P450 PB-1 cDNA; pR17 rat P-450 PB_{3a} cDNA. A, control; B, injected with crude interleukin extract; C, phenobarbital treated; D, phenobarbital plus interleukin; E, 3MC treated; F, 3MC plus interleukin.


A
B
C
D
E
F
28s
18s
pTF-2


A
B
C
D
E
F
28s
18s
pR17

on the mRNA levels of these P-450s (Fig. 6.1.E). Crude extracts of interleukin I from WEHI-TG cell culture medium did not affect the levels of these P-450s alone or in conjunction with phenobarbital or 3MC.

6.3. Dexamethazone induces PB_{3a} (P450-2B) mRNA in mice

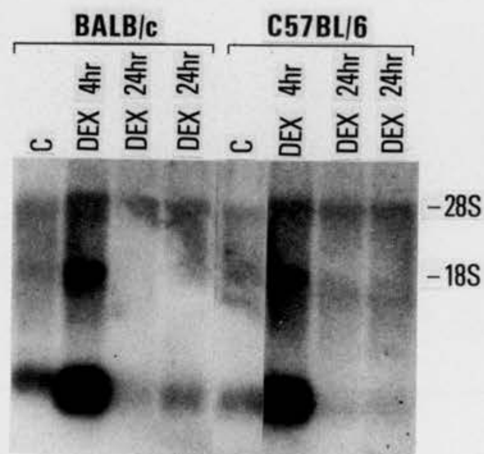
The mRNA levels of PB-1 and PB_{3a} were examined in mouse hepatic mRNA after treatment with dexamethazone. In both BALB/c and C57BL/6 mice no effect was seen on PB-1 levels after 4 hour and 24 hour dexamethazone treatment (Fig. 6.2). In contrast, PB_{3a} mRNA was induced by dexamethazone and required 24 hours for maximal effect. The induction rate was much slower than the glucocorticoid receptor mediated induction of metallothionein by dexamethazone which suggests that the mechanism of induction of PB_{3a} may be different to that of metallothioneine (Fig. 6.2).

6.4. Hypophysectomy induces PB-1 (P450-2C) and PB_{3a}(P450-2B) mRNAs in mice

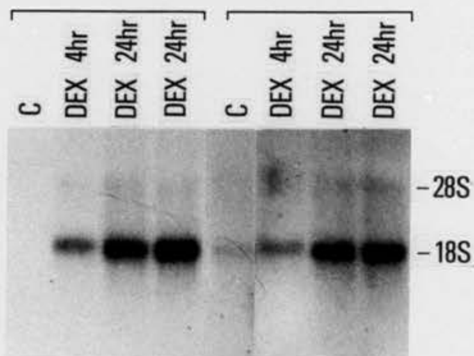
The pituitary and hypothalamus are responsible for producing many of the trophic and stimulating hormones, notably adreno-corticotrophin hormone (ACTH) and growth hormone (GH) which regulate corticosterol synthesis (Jefcoate, 1986) and are themselves regulated by steroids (Oostrom et al, 1983; Diamond and Goodman, 1985). The hypothalmic pituitary axis has been shown to be involved in the regulation of male and female specific P-450s in rats (Adesnik and Atchison, 1986). It was therefore of interest to what effect removal of the organs would have on the levels of P-450s in the mouse. Figure 6.3 shows that in hypophysectomized C3H/He and CBA/Ca the hepatic levels of PB-1 and PB_{3a} mRNAs are increased.

FIGURE 6.2.

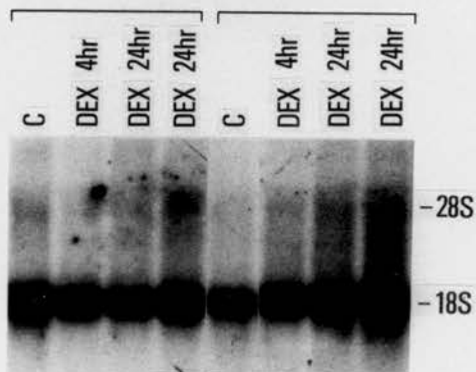
Dexamethazone induces PB_{3a} (P450-2B) mRNA in the mouse. 10μg of hepatic RNA from the indicated mouse strains were treated as in Fig. 6.1. and probed with the indicated [^{32}P] labelled probes. M_1pEH_4, mouse metallothionein cDNA probe; pR17, rat PB_{3a} cDNA; pTF-1, rat f cDNA; C, control mice vehicle only; DEX 4 hr, treated with dexamethazone for 4 hours; DEX 24 hr, treated with dexamethazone for 24 hours.



m1pEH4



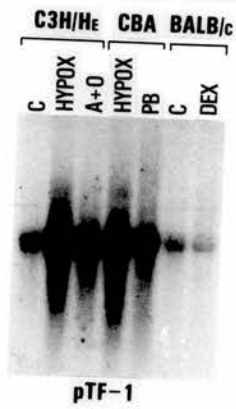
pR17



pTF-1

FIGURE 6.3.

Hypophysectomy induces PB-1 (P450-2C) and PB_{3a} (P450-2B) mRNA in mouse liver. 10μg of hepatic mRNa from the indicated mice strains (CBA = CBA/Ca) were treated as in Figure 6.1. pTF-1, rat f cDNA probe; pR17, rat PB_{3a} cDNA probe; C, control mice; Hypox, Hypophysectomized mice; A + O, adrenalectomized and ovarectomized; PB, phenobarbital treated; DEX, dexamethazone treated for 24 hours. Surgically treated mice RNAs kindly provided by Dr. R. E. Hill.


C3H/He
CBA
BALB/c
C
HYPOX
A+O
HYPOX
PB
C
DEX
pTF-1

C3H/He
CBA
BALB/c
C
HYPOX
A+O
HYPOX
PB
C
DEX
pR17

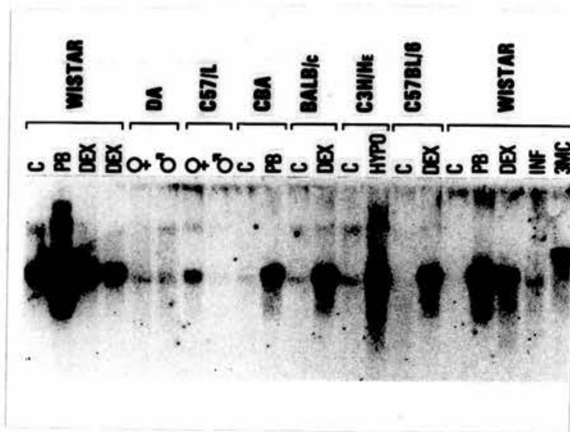
Additionally this increase is similar to that observed on treating mice with phenobarbital for both PB-1 and $PB3_a$, and dexamethazone for $PB3_a$ (Figure 6.3). This induction response was also seen in hepatic RNA from hypophysectomized DBA/2, C57BL/6, BALB/c, Swiss and [BALB/c x C57BL/6]F_1 mice (data not shown). Also shown on this blot (Fig. 6.3) is that female mice who have undergone adrenalectomy and ovarectomy also show increased levels of $PB3_a$ and PB-1 hepatic mRNA levels. Again this effect was also seen in C3H/He, DBA/2 and C57BL/6 female mice (data not shown). As controls for these experiments the mouse contrapsin cDNA p54 was used in the same northern blots. The expression of this mRNA has been shown to be abolished in hypophysectomized mice (Hill et al, 1985) but to be unaffected by adrenalectomy or ovarectomy (R. Hill, pers. comm.). The expression of contrapsin was only decreased in the hypophysectomized animals (data not shown). Dexamethazone plus hypophysectomy and dexamethazone plus phenobarbital could not superinduce PB-1 or $PB3_a$ mRNA further (data not shown).

6.5. Effect of Dexamethazone treatment of rats on P-450 mRNA levels

The $PB3_a$ gene is part of a multigene family of 8-10 genes in rats and mice. In adult rats 2 major enzymes have been identified which share 97% sequence homology (see Introduction) but these can be distinguished by synthetic oligonucleotide probes (Omiecinski et al, 1985). An oligomer directed towards the major phenobarbital inducible form $PB3_a$ was synthesized (kindly provided by J. Inglis) and used to probe northerns of rodent hepatic RNAs. Figure 6.4 summarizes a number of observations. Firstly in rats the basal level of $PB3_a$ mRNA varies, when the basal level is high it can be

FIGURE 6.4.

Use of a synthetic oligonucleotide probe to detect rat and mouse hepatic PB_{3a} mRNA. 15μg of total RNA or 2μg of poly A^+ from the livers of rat and mouse strains were treated as in Fig. 6.1. The blot was hybridized with the mouse MC1b 3' cDNA probe, pCL46, which only detected a 23S mRNA in 3MC treated rats. The blot was subsequently hybridized with an oligomer that distinguishes between the PB_{3a} and PB_{3b} mRNAs in rats. This oligomer is directed towards the PB_{3a} form. The second set of Wistar RNA samples on the right of the Figure are the polyA^+ samples. Rat strains, Wistar and Dark agout; Mouse strains, C57L (C57/L), CBA/Ca (CBA), BALB/c, C3H/He, C57BL/6. Treatments, C, control; PB, phenobarbital; DEX, dexamethazone; Hypo, hypophysectomy; INF, inflammed; 3MC, 3 methylcholanthrene; '♀' = female; '♂' = male.



← 23S MC1b

induced further with phenobarbital but not with dexamethazone. If the basal level is low then dexamethazone can lead to the accumulation of PB_{3a} mRNA. Secondly this oligomer is efficient in detecting an mRNA in mice that gives the same pattern of expression as the pR17 rat PB_{3a} cDNA probe when it is used in northern analyses. The RNA detected by this oligomer increases in mice on dexamethazone, phenobarbital and hypophysectomy treatment. Thirdly, mice appear to have a consistently lower basal level of PB_{3a} mRNA when compared to rats and there is a sex difference in the pattern of expression of PB_{3a} in mice but not rats. Female mice have higher basal levels of PB_{3a} mRNA than male mice. The same series of results can be seen when pR17 is used as a probe (data not shown). The same northern was previously probed with the 3' end of the mouse MC1b cDNA and shown only to be induced on 3MC treatment (the high molecular weight band in 3MC treated Wistar rat, Fig. 6.4) and not affected by hypophysectomy or dexamethazone treatment. If the same blot is reprobed with a human MC1b cDNA clone which detects both MC1a and MC1b then these mRNAs are only affected by 3MC treatment (data not shown). In a similar manner to mice dexamethazone had no significant effects on the levels of PB-1 mRNA levels in rats (data not shown).

6.6. Expression of P-450 mRNAs in little mice

Little mice arose from a spontaneous mutation in C57BL/6 mice (Eicher and Beamer, 1976). The defect due to the _lit_ mutation causes reduced growth rate in mice. The mutation is recessive, adult _lit/lit_ mice although normally proportioned are reduced in body size because of their failure to maintain normal neonatal and pubertal growth rates. This condition could be reversed by addition

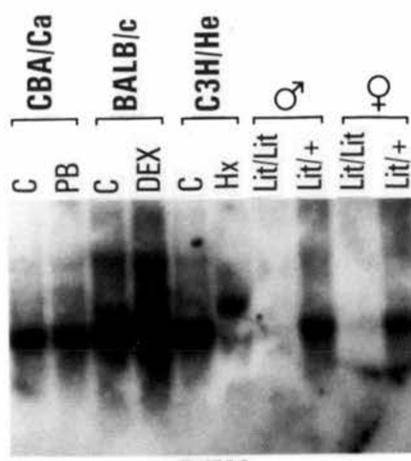
of growth hormone (GH) (Beamer and Eicher, 1976). Unlike other growth-defected mouse strains such as Snell and Ames or hypophysectomized animals, little mice have a defect only in GH with normal levels of other pituitary hormones such as luteinizing hormone and follicle-stimulating hormone (Phillips et al, 1982).

Hepatic RNAs from male and female lit/+ and lit/lit mice were kindly provided by Dr. R. Al-Shawi and used in conjunction with hypophysectomized, dexamethazone and pheobarbital treated hepatic RNAs for northern analysis. In Figure 6.5 it can be seen that the levels of PB-1 mRNA are relatively unaffected in the little mice as compared to phenobarbital and hypophysectomy treatment. In contrast male lit/lit mice have induced levels of PB_{3a} mRNA but the effect is not as great as that seen with phenobarbital, dexamethazone or hypophysectomy treatment. Female mice have higher levels of PB_{3a} mRNA and removal of growth hormone from male mice appears to restore PB_{3a} mRNA levels to that of female levels. The same result could be achieved with PB_{3a} oligomer (data not shown). Interestingly in a control experiment with pDEX (a cDNA for rat contrapsin) the levels of contrapsin mRNA were depressed in lit/lit mice as in hypophysectomised animals. This suggests that the levels of this mRNA are maintained by the presence of growth hormone.

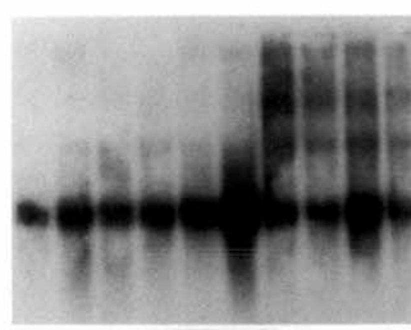
To extend the analysis a human PB_{2a} (P450-3) cDNA, $pPCN_{12}$, was used in similar northern blots. The mRNA level of P-450s within this family, P450-3, also increased in mice on phenobarbital, dexamethazone and hypophysectomy treatment (Fig 6.6). In lit/lit male mice a slight induction was observed relative to lit/+ males which brought it up to the levels seen in female mice (data not shown) but the effect was not as great as that observed in mice which had been induced with phenobarbital, dexamethazone or by

FIGURE 6.5.

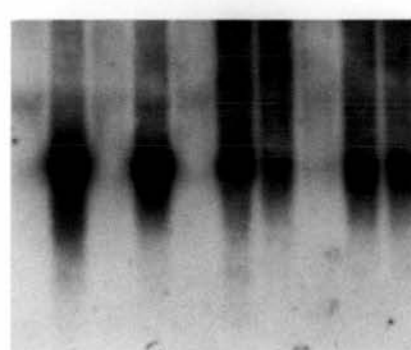
Expression of hepatic PB_{3a} (P450-2B) and PB-1 (P450-2C) mRNA in little mice. 15μg of total mRNA from the indicated mice strains were treated as in Figure 6.1 and probed with [^{32}P] labelled pDEX (rat contrapsin), pR17 (rat PB_{3a} cDNA) and pTF-1 (rat f cDNA). C, PB (from CBA/Ca mice); C, DEX (from BALB/c mice); C, Hx (from C3H/He mice). The little mice are on a C57BL/6 background. lit/lit homozygous growth deficient, lit/+ heterozygous normal growth. '♀' = female; '♂' = male.



pDEX



pTF-1



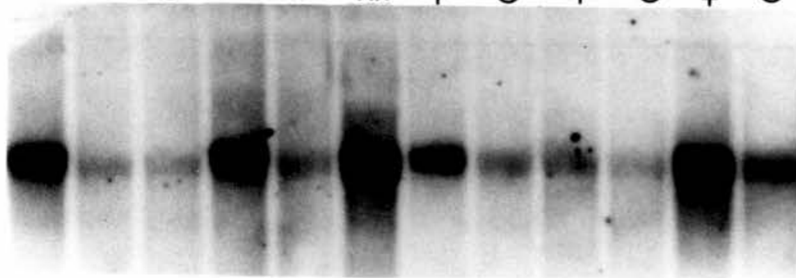
pR17

FIGURE 6.6.

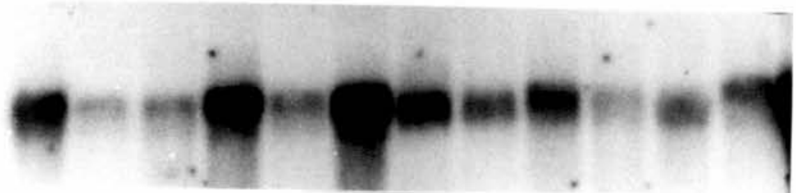
Induction of PB_{2a} (P450-3) in mice by phenobarbital, dexamethazone and hypophysectomy in mice. 15μg of total RNA from the indicated mouse and rat strains were probed with $pPCN_{12}$ (a human PB_{2c} cDNA probe) and pR17 (rat PB_{3a} cDNA). PB,C from CBA/Ca mice; C, DEX from BALB/c, C , Hx from C3H/He; '♀', '♂' from C57L, '♀', '♂' from 129, '♀', '♂' from SD rats.

CBA/Ca **BALB/c** **C3H/He** **C57L** **129** **SD**

PB C C DEX C Hx ♀ ♂ ♀ ♂ ♀ ♂



pR17



pPCN12

hypophysectomy.

6.7. Effect of hypophysectomy on P-450 levels in rats

To ensure that efficient hypophysectomy had taken place in the rats hepatic RNAs from operated and sham operated rats were probed with pDEX (Fig. 6.7). It can be seen that in all hypophysectomized animals that contrapsin mRNA levels have decreased due to loss of pituitary function. In male rats hypophysectomy induces $PB3_a$ mRNA levels slightly but has no effect on female $PB3_a$ mRNA levels. This induction is not as pronounced as with phenobarbital treatment. The overall levels of PB-1 mRNA were unaffected by hypophysectomy in male rats, in contrast female hypophysectomized rats show a decrease in PB-1 mRNA levels (Fig. 6.7). Neither PB_{2a} or MC1a and MC1b mRNA levels appeared to be affected by hypophysectomy in male or female rats (data not shown). An anonymous human liver cDNA probe, pPB2-29, which had been previously shown not to be induced by foreign compounds or affected by hypophysectomy was also used to check the relative mRNA loadings of the different preparations. The variation in mRNA levels seen with this probe cannot account for the slight induction or repressive effects seen for P-450 and contrapsin related mRNAs in the hypophysectomized rat liver.

6.8. Suppression of rat and mouse PB-1 P450 during acute phase response induced by bacterial endotoxin

Mammalian liver responds to systemic injury such as acute inflammation by changes in the activity of a number of genes. Adult mouse hepatocytes respond in vivo to inflammation by an increased synthesis and secretion of α_1-acid glycoprotein, haptoglobin,

FIGURE 6.7.

Effect of hypophysectomy on hepatic P-450 levels in rats. 15μg of total RNA from Wistar rat liver were treated as in Figure 6.1 and hybridized to [^{32}P] labelled pDEX (rat contrapsin), pR17 (rat PB_{3a}), pTF-1 (rat f), pPB2-29 (an anonymous human liver cDNA probe). Hx = hypophysectomy, C = sham operated, C, control; PB, phenobarbital; 3MC, 3 methylcholanthracene. '♂' = male, '♀' = female.

♂ ♀ ♂

Hx Hx Hx Hx C Hx Hx Hx Hx C C PB 3MC

-28S
-18S

pDEX

-28S
-18S

pR17

-28S
-18S

pTF-1

-28S
-18S

pPB2-29

RATS

hemopexin and serum amyloid A. In addition the synthesis of albumin, ApoAI and MUPs is reduced (Baumann et al, 1983; 1984). In rats traumatic injury decreased the metabolism of a number of P-450 mediated activities (Griffith et al, 1984). Many of these responses are thought to be mediated by lymphokines (Baumann et al, 1986; Balkwill et al, 1984). For instance tumour necrosis factor leads to an overall reduction in P-450 content in mice (Ghezzi et al, 1986). This would therefore represent another form of regulation of constitutive P-450 levels.

Male CBA/Ca mice were treated with phenobarbital, 3MC alone or concomitant with bacterial endotoxin to assess the effect the acute phase response had on hepatic mRNA levels of specific P-450s. Northern blot analysis showed that the levels of PB-1 mRNA (Fig. 6.8) decreased with increasing doses of endotoxin in control animals. The same affect was observed with phenobarbital and 3MC treated mice who also had increasing doses of endotoxin. The reason why the control mice in this experiment had low basal levels of PB-1 mRNA is unclear. The mRNA levels of PB_{3a} and MC1a and b were only detected in phenobarbital and 3MC induced animals respectively and did not appear to be affected by endotoxin treatment (data not shown). Metallothionein was used as a marker for acute phase response which was significantly induced by endotoxin treatment (Fig. 6.8). Methallothionein induction by endotoxin was potentiated when dosed with phenobarbital and 3MC. It was interesting that metallothionein mRNA level was induced by 3MC alone. This observation was also observed for other acute phase markers such as serum amyloid A, fibrinogen and hemopexin (W. Liao, pers. comm.).

PB-1 mRNA was also suppressed on turpentine induced inflammation of rats (Fig. 6.9), (RNA kindly provided by R.E. Hill)

FIGURE 6.8.

Acute phase represses PB-1 (P450-2C) mRNA levels in mice. 10μg of total liver RNA from CBA/Ca mice were treated as in Figure 6.1. and hybridized to [^{32}P] labelled pTF-1 (rat f cDNA), m_1pEH-4 (mouse metallothionein cDNA). C, control mice, vehicle only, L = 2μg dose of endotoxin; M = 7.5μg dose of endotoxin; H = 25μg dose of endotoxin. PB = phenobarbital; 3MC, 3 methylcholanthrene.

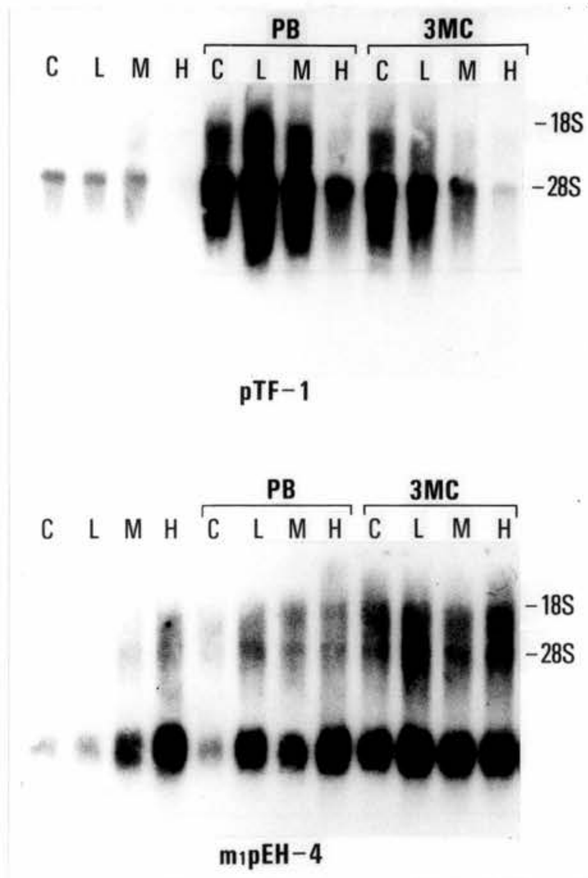
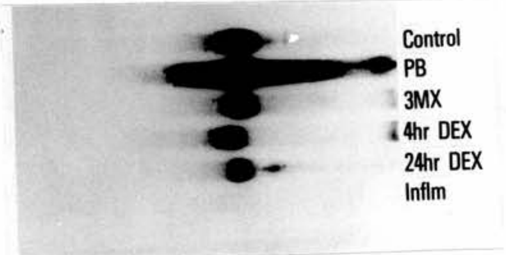

PB
3MC
C L M H C L M H C L M H
-18S
-28S
pTF-1
PB
3MC
C L M H C L M H C L M H
-18S
-28S
m1pEH-4

FIGURE 6.9.

Inflammation represses PB-1 (P450-2C) expression in rats. 2μg of polyA$^+$ from rat liver RNA was treated as in Figure 6.1. and hybridized to [^{32}P] labelled pTF-1 (rat f cDNA); PB, phenobarbital; 3MC, 3 methylcholanthrene; DEX, dexamethazone; Inflm, acute phase induced rat mRNA (kindly provided by Dr. R. E. Hill).


Control
PB
3MX
4hr DEX
24hr DEX
Inflm

but had no visible effects on $PB3_a$ on MC1b levels (data not shown).

6.9. Discussion

One of the most surprising findings in the regulation experiments was the inductive effect of dexamethazone on $PB3_a$ levels in mice but not rats. This result led to a more comprehensive biochemical analysis of this phenomenon with Dr. L. M. Forrester which demonstrated that the change in mRNA levels was reflected in changes in protein levels and enzymatic activities. Firstly increases in the metabolism of the monooxygenase substrates benzphetamine, benzyloxyresorufin and pentoxyresorufin (marker substrates for $PB3_a$ in rats [Wolf et al, 1986; Ryan et al, 1982]) were significantly elevated in mice but not rats following dexamethazone treatment. Secondly polyclonal antibodies to rat $PB3_a$ were used to inhibit the microsomal metabolism of benzyloxyresorufin in rat and mouse to see if the reactions were mediated by equivalent P-450s in both species. At the highest concentration this antibody inhibited 95% of the metabolism of this substrate in mouse and rat samples. Antibody to rat $PB2_c$ had no inhibitory effect on this activity. Thirdly, Western blot analysis with $PB3_a$, PB-1 and $PB2_c$ antibodies agreed closley with the observations made on RNA and activity analysis. Together these results suggest that increased levels of $PB3_a$ mRNA observed in the mouse leads to increased levels of $PB3_a$ protein and subsequent activities mediated by these P-450s.

A difference between rats and mice P-450 regulation has been reported for the compound 1,4,bis[2-3,5-dichlorophyridyloxy]-benzene[TCPOBOP] (Poland et al, 1981). TCPOBOP is a highly potent inducer of benzphetamine metabolism in mice, a marker substrate for

'phenobarbital-like' induction but has no effect in rats even at high doses. Whether TCPOBOP has other 'dexamethazone-like' properties has not been established.

6.9.1. Potential mechanism of species difference

The species differences in the regulation of certain members of the PB-1 (P450-2C), PB_{3a} (P450-2B) and PB_{2c} (P450-3) gene families allows some speculation about the site and mode of action of the compounds which regulate these proteins.

First of all growth hormone does play a role in the regulation of these P-450 families in mice and PB-1 and PB_{3a} in rats. Certain members of the PB-1 family are regulated in a sex-specific manner in mice and rats (Morgan et al, 1985; Noshiro and Negishi, 1986). Testosterone 16α-hydroxylation is higher in male rodents than in female rodents. Hypophysectomy abolishes this sex difference. The male predominant expression of this activity can be restored by pulsatile but not continuous secretion of growth hormone in lit/lit mice (Noshiro and Negishi, 1986) and hypophysectomized rats (Morgan et al, 1985). This observation is in agreement with previous findings by Norstedt and Palmiter (1984) concerning the male predominant expression of MUPs. A similar conclusion has been drawn to explain the female specific expression of $P\text{-}450_{15\beta}$ (P450-2C) in rats (MacGeoch et al, 1985). This P-450 has 16 fold higher levels in female than in male rats. Hypophysectomy reduces the sex difference which can be restored by continuous but not pulsative infusion of growth hormone. The northern blots using pTF-1 as a probe will not distinguish between these two members of the P450-2C gene family in rats or mice, thus the overall changes in the expression of members of this gene family is being measured on

northern blots of phenobarbital treated, hypophysectomized, adrenalectomized and ovarectomized and in lit/lit mice. However, it is obvious that phenobarbital administration and surgical treatment of normal mice has more profound effects than just the absence of growth hormone in lit/lit mice. This implies that some other factor besides growth hormone has a suppressing effect on the levels of members of this gene family in mice.

The level of PB_3 related mRNA is sexually dimorphic in mice but not in rats (this study and Noshiro et al, 1986). Growth hormone also appears to play a role in this dimorphism in mice (Noshiro and Negishi, 1986) but again hypophysectomy has greater inducing effects than the absence of growth hormone. The species difference in response to dexamethazone may be related to this i.e. dexamethazone affects the level of pituitary factors in mice but not in rats. Adrenalectomy coupled with ovarectomy had the same effect as hypophysectomy in mice on the levels of PB_{3a} mRNA. The expression of PB_{2a} suggests a similar mode of regulation to that for PB_{3a} in mice and rats.

Although these experiments are by no means conclusive it does suggest the exciting prospect that there exists a single mechanism for the regulation of many P-450s in mice. Hypophysectomized mice offer an assay with which to examine this process, i.e. different pituitary factors such as ACTH can be added back to mice to see if they can suppress the levels of the PB_{3a} related P-450s. Does phenobarbital act by effecting the secretion of tropic factors from the pituitary? What effect does adrenalectomy have on P-450 levels in the rat? Is the increase in expression in P-450s in hypophysectomized and dexamethazone treated mice due to transcriptional activation?

6.9.2. The phenobarbital-inducible gene family in the mouse

No differences in phenobarbital or dexamethazone inducible PB_{3a} mRNA levels were observed in any of the mouse strains studied. This is interesting because the metabolism of coumarin, which has been ascribed to the phenobarbital-inducible P-450s within the PB_3 family, has been shown to be strain dependent (Wood and Cooney, 1974; Lush and Andrews, 1978; Wood and Taylor, 1979). In addition no sex differences have been reported in the metabolism of coumarin whereas the PB_{3a} mediated metabolism of benzyloxyresorufin and pentoxyresorufin is higher in female mice than in male mice (L. Forrester, pers. comm.). This again argues that the Coh locus is distinct from the major forms of phenobarbital inducible P-450 in mice. It would be of interest to know whether dexamethazone or hypophysectomy could affect coumarin hydroxylase activity in mice.

6.9.3. Extrapolation between species

There are many similarities in cytochrome P-450 isozyme composition between species and orthologous forms between rat, mouse, rabbit and human have been identified (Nebert et al, 1987). However, it is clear that this gene superfamily has evolved very rapidly and to different extents in different species. Thus the human P-450 superfamily appears less complex than that in small mammals. As a consequence it is difficult to establish which model may best reflect the mechanism of isozyme regulation in humans. Do PB_3 related mRNAs in humans respond to dexamethazone and pituitary regulation?

6.9.4. Inflammation and P-450s

The acute phase response also appear to play a role in the

regulation of P-450 levels. The northern blot experiments have again signposted a number of ways in which this may be investigated, i.e. whether these probable lymphokine factors act directly or indirectly on the P-450 genes to suppress their level of expression.

CHAPTER 7

DISCUSSION AND PROSPECTUS

7.1. Comparison of rodent and human P-450s

A number of factors prevent an easy extrapolation of experimental data on P-450 function obtained in rodent models to human experience. These are a consequence of differences in gene number, organization, substrate specificity and regulation.

Rodents appear to have at least twice the number of PB-1 (P450-2C), PB_{3a} (P450-2B) and PB_{2b} (P450-3) genes (this study; Simmons et al, 1985; Beaune et al, 1986; J. Miles, pers. comm.). This will undoubtedly lead to differences in detox ification (and activation capacity) in the different species. The metabolism by P-450s within specific gene families is often conserved although there is increasing evidence that this is not always the case. For example, mephenytoin is metabolized principally by PB-1 P-450 (P450C2C) in humans (Shimada et al, 1986) but is metabolized principally by PB_{2b} (P450-3) in rats (Shimada and Guengerich, 1985). The steroidal activities attributed to rat PB-1 (P450-2C) forms have not been noted in humans (Umbenhauer et al, 1986), although steroids are inhibitors of S-mephenytoin activity in human microsome preparations (Inaba, 1986). In contrast constitutive AHH activity seems to involve members of the mouse (P450-2C) multigene family (this study) and at a functional level rat, rabbit and human purified forms of PB-1 P-450 (Waxman and Walsh, 1983; Raucy and Johnson, 1985; Shimada et al, 1986). This therefore seems to be a highly conserved activity and suggests that this gene family has the potential to play a role in the deactivation or activation of polycyclic hydrocarbons. It is not at present clear whether orthologous forms within the different species are performing this activity. Analysis of the sequence relationship of the various rat, rabbit and human PB-1 forms (Chapter 3) has not been able to satisfactorily identify

any orthologous forms between the different species examined. Preliminary sequence analysis suggests that the more closely related species, rat and mouse, do have orthologous P-450 forms. It is possible to speculate that the chemical environment experienced by rodents is different to that of humans. House mice are best known for their consumption of human foods but they do show a preference for cereal grains and their derivatives (Sage, 1981). Aboreal species of rodents also show a preference for cereals but can indulge in insects, fungi and moss sporangia (Sage, 1981). Thus diet, a major source of foreign chemicals to a host, is perhaps not sufficiently different between house mice and humans to explain the differences in gene multip licity. Whether this is also applicable to inbred strains of mice is not known but the genomic complexity of the P450-2C and P450-2B loci of the inbred and wild mouse strains is very similar (Chapter 4).

Differences in gene number have been noted between human and rodents for a number of other gene families. For example the protease inhibitor genes $alpha_1$-anti-trypsin and $alpha_1$-antichymotrypsin exist as single copy genes on human chromosome 14 (Rabin et al, 1986). In mice they are both members of two small multigene families on chromosome 12 (Hill et al, 1985). The mouse major urinary proteins are encoded by a cluster of approximately 35 genes on chromosome 4 (Bennet et al, 1982; Bishop et al, 1982). As yet no human homologues to the MUPs have been conclusively identified. The multiple protease inhibitor genes has been speculated to be an important defence against parasites in mice (Hill and Hastie, 1987) whereas the major urinary proteins are possibly important as carriers for behavioural cues (Shaw et al, 1983).

What are the functions of the rodent P-450s? There are three

major possibilities:

1) They evolved as a need for detoxification.

2) They evolved as a need to metabolise hormones.

3) A mixture of 1 and 2.

In an English population of Mus musculus domesticus the warfarin resistance gene (War) was found to reside on chromosome 7 (MacSwiney and Wallace, 1978). In a similar manner to the P-450s, warfarin resistance exhibits age and sex dependent variability. In the light of the physiological regulation of P-450s in mice by hormones (Chapter 6) it is possible to speculate that the Coh loci which maps to chromosome 7 (P450-2B and P450-2A) could be responsible for warfarin resistance. Interestingly total hepatic testosterone hydroxylase activity shows circannual variation in inbred strains of mice (Ford et al, 1979). The highest level of activity occurred in December and was nearly four times lower by April. This variation in testosterone metabolism existed in both sexes and showed a reciprocal relationship to the number of births per female per month. Whether this relationship is causal or casual is not known. This raises the possibility that the increased number of P-450 genes in rodent has less to do with chemical detoxification and more to do with behavioural response to hormonal cues. This would explain why many P-450s are responsive to changes in hormonal status. Particularly the families which have diverged most. Transcripts corresponding to members of the PB_3 (P450-2B) gene family have been found in the adrenal and preputial glands in uninduced rats (Adesnik and Atchison, 1986) and in mouse submaxillary gland (Chapter 5). PB-1 (P450-2c) genes are expressed in human (L. Forrester, pers. comm.) and mouse mammary tissue (data not shown). Thus it is possible that the role of these P-450 forms is in either

the synthesis or catabolism of hormonal factors.

Not all P-450 gene families are amplified in rodents with respect to humans. There are only two members of P450-1 family in all species (Nebert et al, 1987) and one member in P450-2E for both humans and rats (Song et al, 1986). Gene number for the steroid-iogenic P-450s is also very similar between species (Nebert et al, 1987). In spite of this, it is still feasible that even these P-450s have not preserved common functions. The existence of multiple members in other P-450 families in rodents could lead to transfer of substrate specificity between P-450 families.

A powerful strategy for the elucidation of human P-450 substrate specificity (particularly for forms which are difficult, if not impossible to purify) is, the cloning out of individual full-length cDNAs and putting these cDNA clones in an expression system such as yeast and testing in vitro for enzyme activity towards marker substrates. This however should not be at the expense of rodent research which offers a source of multiple P-450s which can be utilized to determine those regions of the protein which are important for their regulation, function and for the evolution of new substrate specificities.

How substrate specificity relates to in vivo effects is further complicated by the differences in P-450 regulation between species as exemplified by the study between rats and mice, two closely related species (Chapter 6). Dexamethazone and hypophysectomy induces PB_{3a} (P450-2B) in mice but not rats. Hypophysectomy also effects the levels of other P-450s in both species. It is not known which model best reflects the situation in humans. Regulation of PB_3 (P450C2B) is of potential pharmacological and toxicological importance in view of the wide use of glucocorticoids in combination

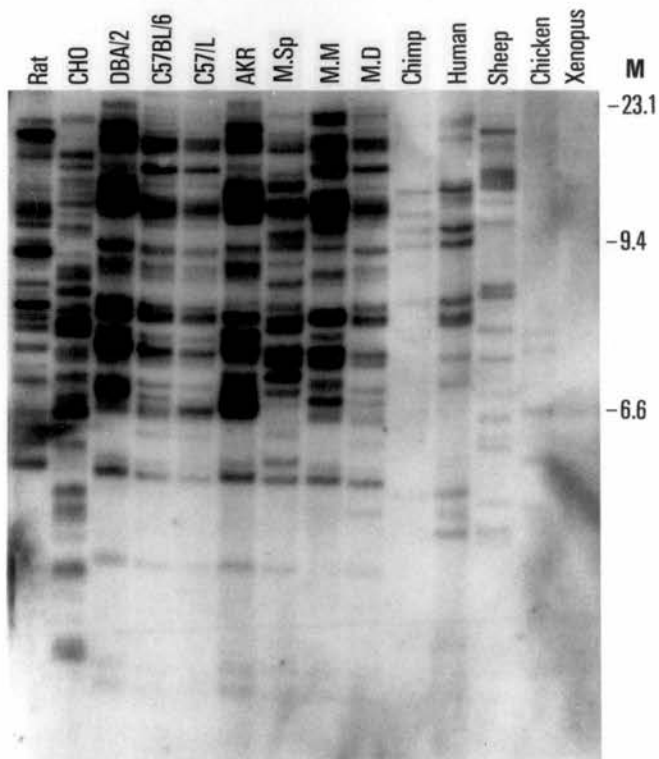
with other drugs in therapy and the high activity of certain proteins within the PB_3 family to a variety of exogenous and endogenous substrates. PB_{2C}(P450C3) has been shown to be inducible by dexamethazone in human liver and in a human hepatoma cell line (Molowa et al, 1986) as well as in rats and mice (Chapter 6). There are however no reports on the effect of this compound on PB_3 expression in humans. The coordinate regulation of a number of P-450 isozymes by hypophysectomy found here indicated that foreign compounds may exert their inductive effects by perturbing the hypothalmus and pituitary rather than by direct effects on the liver. This also raises other intriguing questions. For example at what level of control is induction by hypophysectomy regulated, i.e. is it by a transcriptional or post-transcriptional mechanism? Another experimental approach to study the regulation of human P-450s is to isolate the genomic clones for these genes and make transgenic mice which carry these genes (Palmiter and Brinster, 1986). The factors which regulate these genes in mice could provide clues to their endogenous regulation in humans, i.e. are they sex specific and do they respond in the same way as their mouse counterparts?

7.2. P-450s in other species

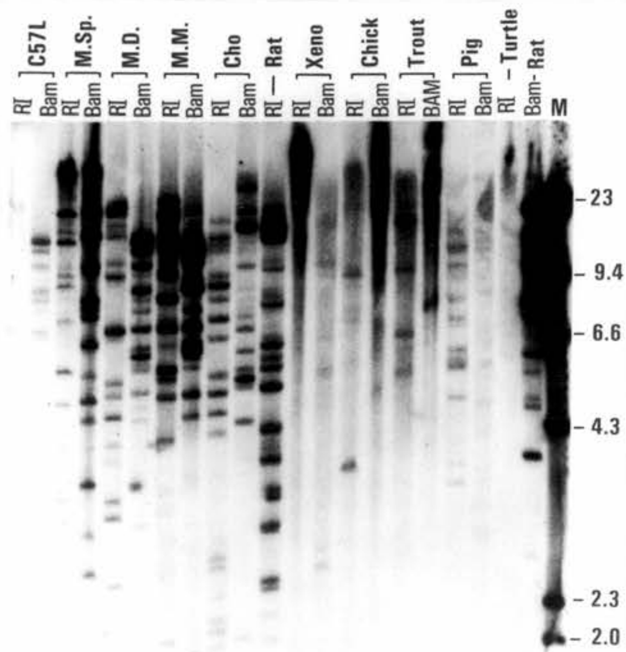
Sequence comparisons, indicate that the mammalian cytochrome P-450 gene families arose from a primordial gene and through a series of gene duplications and gave rise to a series of distinct gene families. The hybridization of rat pTF-1 (P450-2C) and pR17 (P450-2B) to Southern blots of DNA from diverse species is shown in Figure 7.1. The signal intensity is intense in mammals which are closely

FIGURE 7.1.

Southern blot analysis of a variety of genomic DNA samples probed with pTF-1 (P450-2C) and pR17/p5'2 (P450-2B). In the case of pTF-1 5μg of the indicated DNAs were digested with BamHI and used on Southern analysis. M.Sp, *Mus* spretus; M.M., *Mus mus musculus*; M.D. *Mus mus domesticus*. In the pR17/p5'2 Southern 5μg of the indicated DNAs were digested and Southern blotted.



pTF-1 vs Species

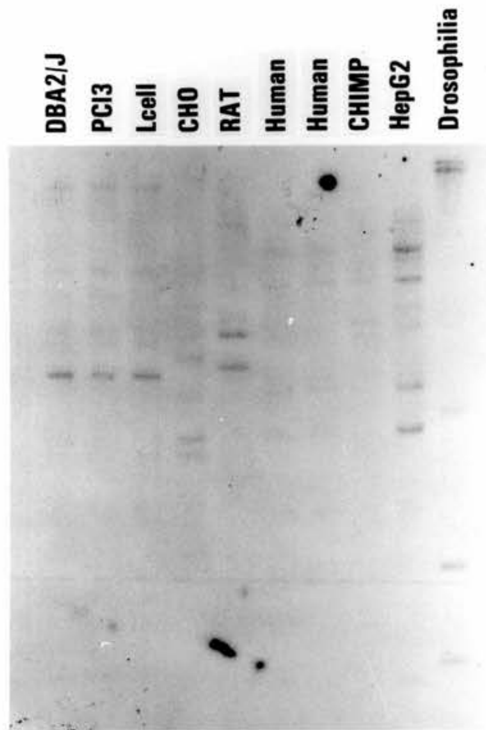


Conservation of PR17/p52 across different species DNAs.

related to rat such as mouse and Chinese hamster. Significant hybridization can also be seen for more distantly related species including chicken, rainbow trout and the African clawed toad (Xenopus). On other blots signal could also be detected with Drosophila melanogaster and Saccharomyces cerevisae DNA samples. In the latter case the same signal was detected in S.cerevisae with both pTF-1 and pR17 cDNA probes (data not shown) which may indicate a common ancestral region. Oligomers to conserved regions of cytochrome P-450 also detect specific bands in Southern blots of DNA from diverse species (Fig. 7.2). Both C128 and C129 which are derived from the rat $PB3_a$ cDNA (see Materials and Methods) detect DNA fragments in rat, mouse Chinese hamster, human and Drosophila melanogaster (Figure 7.2). These data suggest that there is a high degree of conservation of these genes and it is clear that in many species several genes must exist in each family. In Chapter 3 we saw that the C128 oligomer detected cDNAs from distinct P-450 families. If this is used in northerns to rodent RNA samples (Figure 7.3) several distinct transcripts can be observed in rat liver, one of which is highly phenobarbital inducible and probably corresponds to $PB3_a$ (P450-2B) mRNA. A lower molecular weight mRNA probably corresponds to a PB-1 (P450-2C) transcript whilst the identity of an even lower molecular weight transcript is unknown. Multiple transcripts can also be observed in mouse liver and other tissues notably 15 day foetal liver and possibly the yolk sac. This suggests there may be foetal forms of expressed P-450s. If oligomers to the $PB3_a$ (P450-2C) hypervariable region of rat and mouse are used in Southern blots they also detect a large number of fragments in rat, mouse and human genomic DNA (Figure 7.4). The rat $PB3_a$ oligomer detected the highly phenobarbital inducible mRNA in

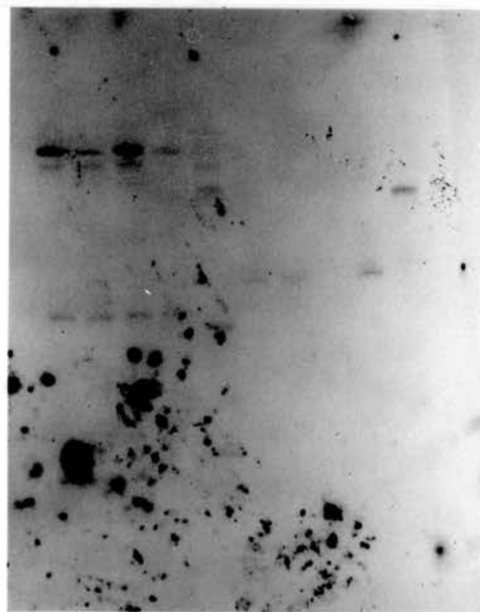
FIGURE 7.2.

Southern blot analysis of a variety of DNAs probed with oligonucleotides derived from rat PB_{3a} (P450-2B). 10µg of the indicated DNA samples were digested with *PstI* and Southern blotted. Mouse DNAs, DBA/2, L cell, PC13; Chinese hamster, CHO; Rat; human; chimp, chimpanzee; HepG2 human liver hepatoma cell line; Drosophilia, *Drosophila melanogaster*.



Detection of sequences homologous to an oligonucleotide directed against a conserved region of the rat PB−3 gene.

CL 28

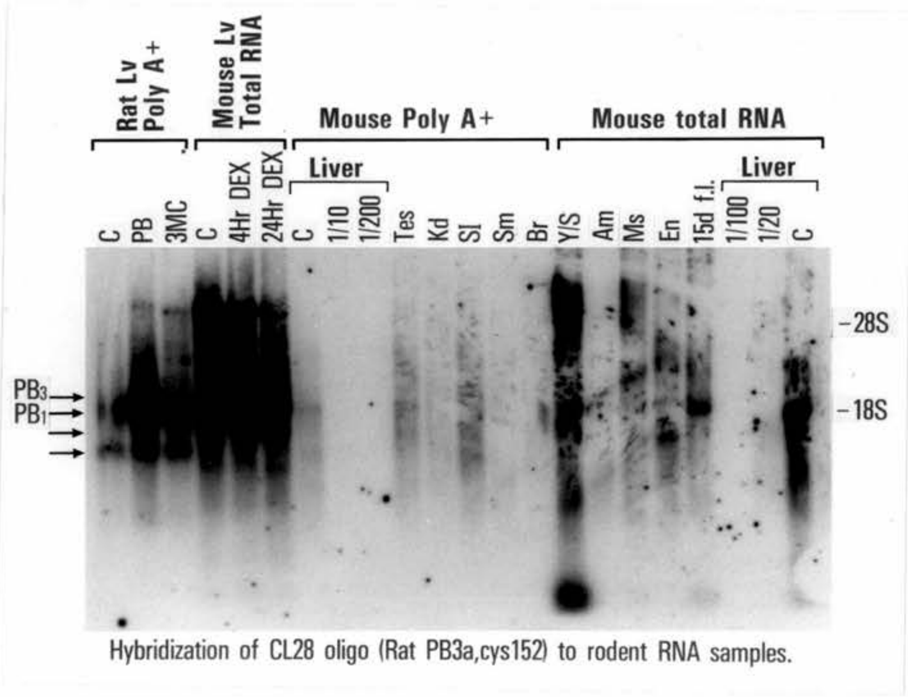


Southern analysis of DNA's from different species using an oligonucleotide directed against the heme binding region of a rat P.450 gene − PB−1, PB−3.

CL 29

FIGURE 7.3.

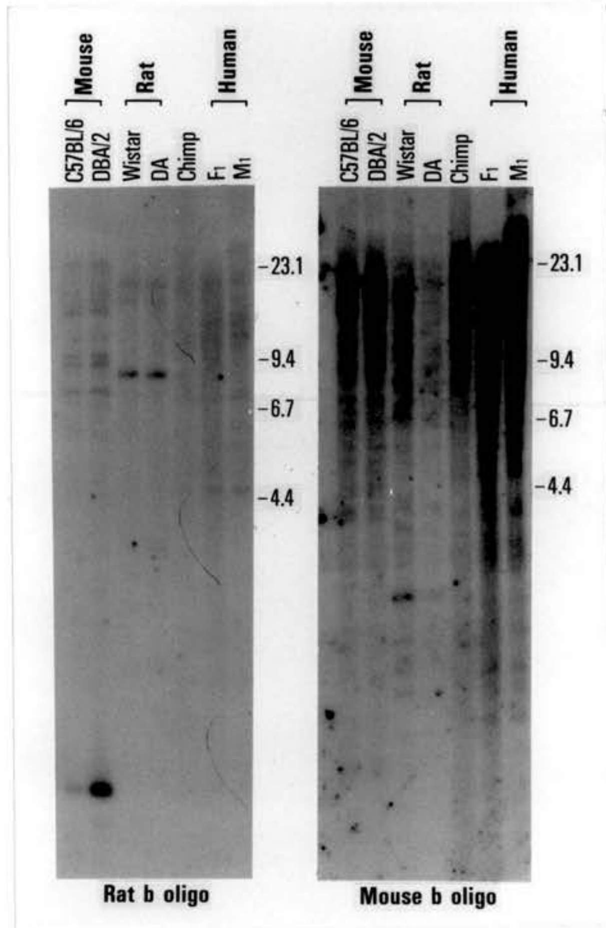
Northern blot analysis of rodent tissue RNA samples with C128 oligomer. 10μg of total RNA or 2μg of polyA^{+} RNA were fractionated on formaldehyde gels and northern blotted. Lv, liver; Tes, testes; kd, kidney; S.I., small intestine; Sm, submaxillary gland; Br, brain; Y/s visceral yolk sac; Am, amnion; Ms, visceral mesoderm; En, visceral endoderm; 15d f.l., 15 day foetal liver. C = control; PB = phenobarbital treated; 3MC = 3 methylcholanthrene treated; 4 hr DEX, 4 hour dexamethazone treated; 24 hr DEX; 24 dexamethazone treated. Also indicated are dilutions of control mouse liver RNA.



Hybridization of CL28 oligo (Rat PB3a,cys152) to rodent RNA samples.

FIGURE 7.4.

Southern blot analyses of rodent and primate DNAs with rat and mouse *PB*$_{3a}$ (P450-2B "hypervariable" oligomers. 5μg of the indicated DNA samples were digested with *Bam*HI and probed with the indicated oligomers.



C57BL/6
DBA/2
Mouse
Wistar
DA
Rat
Chimp
F1
M1
Human
-23.1
-9.4
-6.7
-4.4
Rat b oligo
Mouse b oligo

rats and mice (Chapter 6) whilst the mouse oligomer detected multiple transcripts on Northern blots none of which is phenobarbital inducible (data not shown). It now appears that the original sequence analysis of mouse PB_{3a} was wrong (D.W. Nebert, pers. comm.) which would explain the unusual results obtained with this oligonucleotide. From this brief analysis it can be appreciated that many organisms contain cytochrome P-450s which have become adapted to specific functional requirements of the organism. As such by our present analysis they represent a highly maleable system for the metabolism polycyclic aromatic hydrocarbons.

7.3. Conclusion

The study of the rodent liver cytochrome P-450s offers an opportunity for a fuller understanding of the cellular, hormonal and genetic factors which determine tissue and species specific patterns of P-450 gene expression. An investigation of closely related species such as the genus *Mus* may provide comparisons with which to devise mechanisms for the evolution of substrate specificity and function for the P-450 gene families. A comparison of rodent and human PB-1 (*P450-2C*) gene families has identified similarities in forms of linkage groups and selected metabolic activities but significant differences in relation to gene number and by inference the potential metabolizing activities of this locus in the two different species.

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APPENDIX I

Publications arising out of this Thesis

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ADDENDUM

1) The typing errors have been corrected.

2) The strain of bacteriophage lambda used was c1857 Sam7 from Boehringer Mannheim.

3)It is apparent from biochemical analysis that there is a multiplicity of P450 forms corresponding to P450IIC in rodent and human liver microsomal preparations(Shimada et al,1986 Gut;at al,1986;Wolf et al,1986). These obsevations have been reinforced by the isolation of distinct cDNAs coding for these forms in rat(Friedberg etal,!987) and human(Meehan et al,1988;Umbenhauer et al,1987;Okino et al,1987,J.B.C.,262,16072 -16079). Southern blots with human and rodent P450IIC cDNAs to both human and rodent genomic DNA detects numerous hybridizing fragments. Sequence analysis shows that a high degree of identity exists between different forms of P450IIC in and between rodent and human(Meehan et al,1988;Okino et al,1987). Therefore gene fragments identified on Southern blots with a distinct P450IIC probe,e.g. pHL5,5 in figure 3.9, also correspond to related P450IIC genes. To get a minimum estimate of gene number,identical Southern blots of human and rodent genomic DNA were probed with 5' and 3' human(figures3.12;3.13) and rodent(figures4.5;4.15) cDNA probes. Firstly it is apparent that the complexity of hybridization observed in rodent is greater, three times, than human irrespective of wheather a human or rodent probe is used. Secondly the distinct halves of the cDNA probes pick up a number of fragments in common. These fragments must represent pieces of DNA where the two probes are contigious and minimally could represent a distinct gene. When a number of fragments are seen in common this must represent a minimum gene count for the restriction enzyme used.

The estimate for the minimum number of P450IIC genes in humans was two to three,and three times that number in rodents,and agrees with estimates made on molecular cloning and biochemical analysis.There is no conflict between the estimates as stated in the abstract about gene number for P450IIc gene family in human and rodent,and the experimental evidence presented in chapters 3 and 4.

4) The frequency of recessive poor metabolizera of mephenytoin in the British caucasian population has been recalculated using the Hardy-Weinberg equilibrium equation.

5) All the additional CXB RI strains were developed by Dr.J.HILGERS (J.Hilgers,pers. comm.)

6)The hybrids used un this study were not charaacterized cytologicaly but only by the use of biochemical and molecular markers. Therefore the table of hybrids on page 154 cannot be presented in a more conventional manner(see Hikens et al,1986, for details of somatic cell hybrid construction and marker analysis). The assignment of P4502C to chromosome 19 in mouse was supported by <u>in situ</u> hybridization analysis of mouse metaphase chromosomes and by apparent synteny with the human locus. Linkage analysis of a set of interspecific mouse backcross is being investigated using mouse chromosome 19 markers and P4502C.

7) The text description onpage 152 referring to figure4.16 has been ammended.

8) Size markers have been included for figure 4.17.

9) A number of criteria were used to ascertain the integrity of RNA samples; by O.D. absorbance at 320,280,260 and 240nm, by visualization of RNA on ethidium bromide stained denaturing gels and comparing to a set of standards, and by blotting with a battery of liver derived cDNA probes. Treatments know to effect particular RNA species were checked with the corresponding cDNA.

Only effects of greater than five fold were taken to be significant in the experiments assesing the regulation of P450 mRNA levels.