Investigation of Genes Involved in an Iron and Chemically-Induced Malfunction of Haem Biosynthesis

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by

Ruth Ann Akhtar

Department of Pathology

University of Leicester

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346


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Abstract

Porphyria cutanea tarda (PCT) belongs to a group of diseases that result from malfunctions of the haem biosynthetic pathway. PCT is caused by a reduction in the activity of the enzyme uroporphyrinogen decarboxylase (UROD) which can be inherited as a mutation in the UROD gene or acquired through moderate liver damage. This sporadic type of PCT is commonly associated with exposure to alcohol, oestrogens, iron or polyhalogenated chemicals. Although the mechanism underlying the sporadic condition is still under speculation, a variation in the response to these agents has suggested a genetic predisposition, unrelated to a mutation in UROD, is required.

An experimental porphyria that develops in mice treated with hexachlorobenzene and iron has been studied because of its analogy with sporadic PCT. The severity of this porphyria is dependent on the strain of mouse used, again indicating a genetic susceptibility. To study the genetic element of the experimental condition, F_2 intercrosses were bred from the most susceptible and most resistant strains and a basic microsatellite linkage analysis was undertaken.

This analysis has revealed three chromosomes; 12, 14 and 17, that correlated with susceptibility to porphyria. One proposed candidate for the susceptibility locus chromosome 12 was the *Ahr* gene, which encodes the aromatic hydrocarbon receptor, was investigated further by restriction fragment length polymorphism analysis.

No correlation with the mouse *Urod* gene, either by linkage or expression analysis, was discovered, hence a mutation or polymorphism in this gene does not appear to explain the reduction in activity of this enzyme in the experimental condition. Two further genes *cyp1a1* and *cyp1a2* (encoding the cytochrome P450 isozymes 1A1 and 1A2) under regulational control of the *Ahr* gene were also examined extensively. Again no significant linkage was detected, more over, there appeared to be no significant correlation with expression or activity of either isozyme and susceptibility to the experimental condition.

1 - Introduction

Overview

The group of human diseases termed the porphyrias are caused by defects in the synthesis of haem. These defects are mostly the result of mutations in each of the enzymes in the pathway. The characteristic symptoms of these diseases are acute abdominal pain and photosensitivity of the skin, although both do not occur together in each type of porphyria.

One particular type, Porphyria Cutanea Tarda (PCT), is associated with decreased activity of the enzyme uroporphyrinogen decarboxylase (UROD) and unlike the other porphyrias it can be acquired as well as inherited. The acquired or sporadic form (S-PCT) is usually observed in patients with moderate hepatic damage associated with alcohol consumption or exposure to oestrogenic drugs, iron or toxic chemicals such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and hexachlorobenzene (HCB). Although no mutations in the UROD gene of sporadic patients have been found at present, there is thought to be an underlying genetic susceptibility present, as not everyone exposed to these toxins develops S-PCT.[1,2]

A similar porphyria can be caused in some inbred strains of mice, after exposure to polyhalogenated chemicals including TCDD, HCB, polyhalogenated biphenyls and also after iron overload. These experimental systems are thought to be of enough similarity to the human condition for them to be used to study the mechanism of induction of S-PCT. Currently, the mechanisms of both the human and experimental porphyrias are still speculative. From the experimental mouse models, there is strong evidence for an association with the hepatic microsomal cytochrome P450 (CYP) system, which is involved in the detoxification process of many xenobiotic chemicals. The main enzymes implicated being the CYP1A subfamily (CYP1A1 and CYP1A2) which are regulated by the ligand-activated transcription factor, the aromatic hydrocarbon (AH) receptor.[3,4]

Most of the polyhalogenated hydrocarbons that induce porphyria in experimental animals are poorly metabolised by these enzymes and it has been hypothesised that this could lead to an 'uncoupling' of the electron transfer between the cytochrome P450 and cytochrome P450 NADPH-dependant reductase system. According to this hypothesis, oxygen metabolites, such as superoxide anions, would be released which could lead to the inhibition of UROD by one of two ways; either by oxidising the substrate molecule uroporphyrinogen to the non-metabolisable form uroporphyrin, or by producing an inhibitor of UROD.[3]

However, this hypothesis does not take into account the influence that iron status has in both S-PCT and experimental porphyria. Hepatic iron is elevated in most S-PCT patients and experimentally iron potentiates the porphyrogenicity of TCDD and HCB in rodents. Theories as to the role of iron suggest that it might act as a catalyst in the production of hydroxyl or iron-oxygen radicals. These radicals could be formed from the oxygen metabolites produced from the uncoupled cytochrome P450 enzyme. The radicals could then go on to inhibit UROD by producing an hydroxylated analogue of the uroporphyrinogen substrate, which might act as a powerful inhibitor or cause inactivation of the enzyme at the active site.[3,5]

In terms of genetic susceptibility, it is possible that a polymorphism governing the expression of one of the cytochrome P450 isoforms, or in its controlling mechanism via the AH receptor, could account for the variation. However in mice, there is much evidence to suggest an incomplete correlation between this system and the development of porphyria. [6,7] In addition, as iron itself has been found to induce porphyria in some strains, this would imply a mechanism independant of induction of any of the cytochrome P450 isoforms investigated to date. [8]

It is possible that the genetic variation could occur at another stage of haem biosynthesis. For instance, the regulatory enzyme of the pathway aminolaevulinate synthase (ALAS) is reported to be stimulated by many porphyria-inducing compounds.[9] Another possiblity could be that an unknown

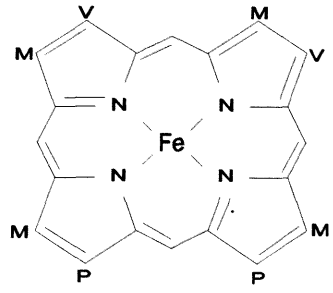
porphyria-inducing compounds.[9] Another possiblity could be that an unknown aspect of iron metabolism, causing a variation in free radical production and toxicity might be involved in the development of this porphyria.[8,10]
The subjects encompassed in this thesis cover a range of topics from toxicology, genetics and biochemistry, thus the background to this project is complex. The remainder of this chapter describes in more detail these aspects introduced above.

1.1 Haem, Haem Biosynthesis and Familial Porphyria

1.1.1 Haem

Haem is an essential molecule for many living organisms. It belongs to a class of biological molecules, known as the tetrapyrroles, which are ubiquitous to all living systems. Haem has a cyclic tetrapyrrole structure, (Figure 1.1) comprised of a porphyrin ring and contains a chelated ferrous (Fe^{2+}) iron atom.
Other tetrapyrroles include chlorophyll, which contains a magnesium ion and is critical for photosynthesis in plants and algae, and corrins such as vitamin B_{12} containing a chelated cobalt ion.
Haem acts as a prosthetic group for many proteins and is found in haemoglobin and myoglobin where it is critical for binding and transport of oxygen. Haem is also required for electron transport in cytochromes, for mixed function oxidation enzymes such as the cytochrome P450 enzymes, for decomposition of hydrogen peroxide by peroxidases and degradation of tryptophan in tryptophan pyrrolase. It also serves as a co-factor for several other enzymes including; prostaglandin endoperoxide synthase, indolamine 2,3-dioxygenase, nitric oxide synthase and in guanylate cyclase for production of cyclic GMP. [1]

Figure 1.1 Haem



Abbreviation used for side chains; V= vinyl, M=methyl, P= propionic acid

1.1.2 Haem Biosynthesis

The majority of haem in the body (80-90%) is required for haemoglobin and is produced by erythroid cells, although it is synthesised by all cells where it is required for the production of cytochromes. The parenchymal cells of the liver are the second largest producers of haem, where it is required mainly by cytochrome P450 enzymes.

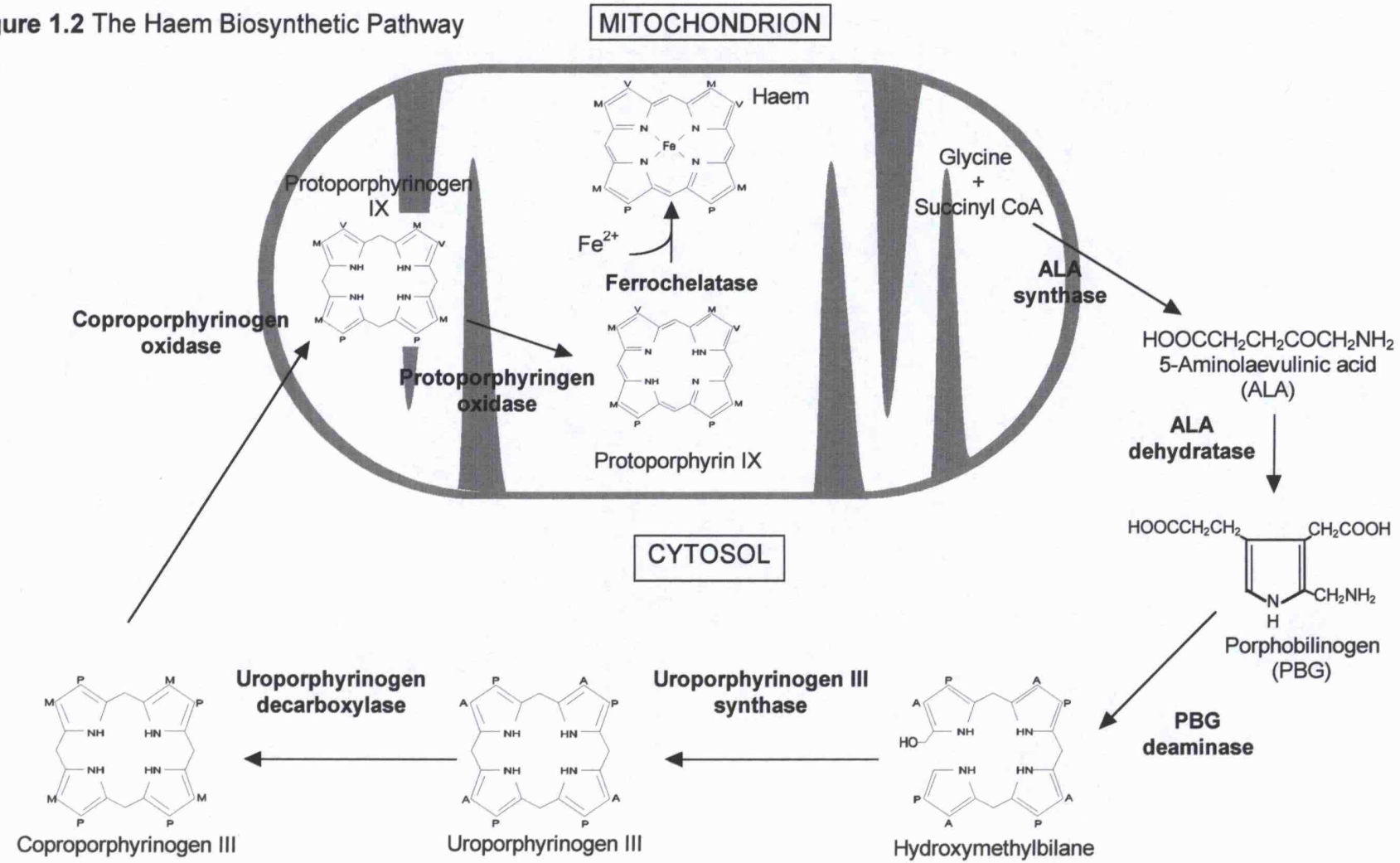
Haem synthesis occurs in the mitochondria and cytosol in eight enzyme catalysed steps. (Figure 1.2) The first of these occurs in the mitochondrion, glycine and succinyl-CoA undergo a condensation reaction catalysed by aminolaevulinic acid synthase (ALAS) to form 5-aminolaevulinic acid (5-ALA). This enzyme is also the main regulatory point for the pathway.

5-ALA is transported out to the cytosol, where the next four steps take place. Two molecules of 5-ALA are combined to form porphobilinogen (PBG), a monopyrrole, by ALA-dehydratase. Four molecules of PBG are polymerised to the first cyclic tetrapyrrole or porphyrinogen, by PBG-deaminase and uroporphyrinogen III cosynthase via the intermediate hydroxymethylbilane. The porphyrinogen formed is uroporphyrinogen III. Subsequently, this molecule undergoes decarboxylation of the acetic acid side chains catalysed by uroporphyrinogen decarboxylase (UROD) to form coproporphyrinogen III.

Coproporphyrinogen is then transported back in to the mitochondrion where it is converted to protoporphyrinogen IX, by coproporphyrinogen oxidase. The mechanism by which coproporphyrinogen is transported back into the

mitochondrion is unkown but this could possibly be a protein-dependent active transport process. Protoporphyrinogen IX undergoes six electron oxidation to become the penultimate precursor of haem, protoporphyrin IX, catalysed by protoporphyrinogen oxidase. The final step of haem synthesis is the insertion of ferrous iron by ferrochelatase.[1,2,11]

Figure 1.2 The Haem Biosynthetic Pathway



Schematic diagram showing the eight steps of haem biosynthesis in relation to their cellular location. Enzymes are indicted in bold type. Abbreviations; CoA-coenzyme A, A- acetic acid, P-propionic acid, M-methyl, V-vinyl.

1.1.3 Regulation of Haem Biosynthesis

Haem has the potential to be toxic to the cell. It can interact with hydrogen peroxide, which is produced by normal oxidative metabolism, to produce reactive oxygen species that might damage cell membranes, proteins and DNA. Thus haem biosynthesis is under fine genetic control.

The regulatory point for biosynthesis occurs at the first step, catalysed by ALAS. There are two closely related isoforms of this enzyme, ALAS-1 and ALAS-2, encoded by separate genes on two different chromosomes in higher vertebrates.[11-13]

ALAS-1 is expressed in all tissues except erythroid (bone marrow and spleen) and has been identified in humans, rats and chicken, the hepatic enzyme being the most studied.

Evidence for the regulatory role of ALAS-1 comes from many observations. It has a much lower catalytic activity and half-life, compared with the other enzymes of the pathway, although this is sufficient to produce the necessary amount of haem required for the cell under normal circumstances.[1,3,11]

ALAS-1 activity is thought to be regulated by haem through a negative-feedback mechanism and several hypotheses have been suggested to explain the inhibitory action of haem. These include the direct inhibition of ALAS-1 activity, repression of ALAS-1 mRNA synthesis, repression of ALAS-1 mRNA translation or inhibition of transport of the ALAS-1 protein into the mitochondrion. The regulatory haem is thought to exist briefly as a 'free-haem pool', when not associated with a haemoprotein.[3] At least two of these pools are thought to exist; one in the cell nucleus where it may regulate the transcription of the ALAS-1 mRNA and the other in the cytosol, where it may act by reducing the stability of ALAS-1 mRNA and inhibit the transport of ALAS-1 into the mitochondrion.[11]

The activity of ALAS-1, in the liver is known to be stimulated by chemicals such as phenobarbital and the porphyria inducing chemical TCDD.[9] This is believed to be coupled to a demand for haem, from an increase in synthesized P450 apoproteins also induced to metabolise chemicals.[1,3,11]

Regulation of haem synthesis in erythroid cells differs from that in the liver. In erythroid cells it is linked to cell proliferation, differentiation and haemoglobin synthesis and is regulated by iron via the erythroid form of ALAS (ALAS-2). ALAS-2 cannot be affected by drugs which induce hepatic porphyrias and there is no apparent feedback control mechanism.

ALAS-2 is thought to be regulated post-transcriptionally by iron via an iron regulatory element (IRE) and an IRE-binding protein (IRE-BP). The ALAS-2 mRNA has a sequence that forms a stem-loop structure upstream of the start codon which is bound by IRE-BP. IRE-BP acts as a metal 'sensor', when iron concentration in the cell is low the IRE-BP binds strongly to the IRE. If iron levels are high the protein is modified and releases the IRE.[11,12,14,15]

A similar control mechanism exists for ferritin and the transferrin receptor. Transferrin binds ferric iron (Fe^{3+}) absorbed in the gut and transports it to other cells. Ferritin forms a protein shell for storage of reduced ferrous iron, preventing it from damaging the cell by reacting with oxygen radicals. When iron concentrations are low, binding of the IRE-BP prevents ferritin translation. The IRE/IRE-BP complex is located at the 5' end of the transcript, where it may inhibit the ribosomal subunits from translating the mRNA. The reverse situation is found with the transferrin receptor when the iron concentration is low. The IRE is located at the 3' end of this transcript, where binding of the IRE-BP is thought to stabilise the mRNA, thus increases leading to an increase in the the amount of transferrin receptor and resulting in increased iron uptake.[11,14]

1.1.4 Abnormalities of Haem Biosynthesis - The Porphyrias

Mutations in enzymes involved with metabolic processes are responsible for many inherited disorders. Investigations into conditions such as the porphyrias and the mutations giving rise to them, has aided the characterisation of the enzymes involved and the biochemical processes that they mediate.

Mutations in all of the enzymes of the haem biosynthetic pathway, except for the first, ALAS, are responsible for causing most of the porphyrias. Each porphyria is characterised by a build up of porphryins or their precursor

molecules, 5-ALA and PBG. Sporadic or type I porphyria cutanea tarda (caused by defective UROD) is the only porphyria that can be acquired as a result of liver damage by alcohol consumption, oestrogen containing drugs or other toxins.[1-4]

Under normal cicumstances, loss of haem precursors from the body is less then 2.5% of the total daily amount of 5-ALA used for haem synthesis. No pathways exist to metabolise any excess precursors so they or their oxidised derivatives, are excreted in the urine, faeces and bile, depending on their solubility. Occasionally, the urine of patients with certain porphyrias is coloured red and fluoresces under ultra-violet light. [1,2] The excretion pattern of these porphyrins is characteristic and along with the clinical symptoms, can be used for diagnosis of each disease. These patterns can be determined by high peformance liquid chromatography or thin-layer chromatography. [17,18]

Porphyrias are classified as hepatic or erythropoeitic, depending on the primary site of accumulation. Hepatoerythropoietic porphyria a homozygous abnormality of UROD is an exception as it results from porphyrin accumulation at both sites.

There are two main clinical features of the porphyrias which cause an acute or cutaneous condition. These symptoms can occur together or alone, depending on the type of porphyria.

The main symptoms of the acute porphyrias are sudden attack of abdominal pain, in around 90% of cases and neurological abnormalities which develop in 60% of patients. Both of these symptoms are thought to stem from axonal degeneration and secondary demyelination. Biochemically, acute porphyrias are associated with an increase in the concentration of the haem precursors 5-ALA and PBG. How abnormal levels of these precursors elicit a neurotoxic effect is unknown. Most evidence points to a neurotoxic effect of 5-ALA, which is able to cross the blood-brain barrier. Acute porphyrias are aggrevated by drugs, steroid hormones, diet, alcohol, infectious disease and a reduced diet.[1,2]

The symptom of cutaneous porphyria is skin photosensitivity caused by dermal phototoxicity of excess porphyrins. Exposure of porphyrins to light at a

wavelength of approximately 400nm in the presence of oxygen, leads to the formation of singlet oxygen or other oxygen radicals. These reactive species are capable of causing local lipid peroxidation and release lysozymes, damaging tissue leading to skin lesions. There are no acute attacks and cutaneous porphyrias are not affected by drugs. [2]

For most of the inherited porphyrias the enzymes loose 50% of normal activity as a result of a heterozygotic mutation. In these cases, one allele expresses the functional enzyme and one allele carries a mutation leading to a partial loss of activity. The nature of these mutations are highly heterogeneous, with mutations discovered at different sites in each gene. The very rare homozygous cases of porphyria can be caused by compound heterozygous mutations. Enzyme activity may fall as low as 10% of normal but retain enough activity to produce some haem, otherwise a total lack of activity would be lethal.[1,2]

It is not pertinent to describe in depth the individual inherited porphyrias here, but a brief overview is given in the following section and Table 1.1. The spontaneous form of PCT is discussed in more detail in section 1.2.

Table 1.1 The Familial Human Porphyrias

Enzyme	Porphyria	Initial site of Porphyrin Accumulation
ALA Dehydratase	ALA Dehydratase Porphyria	Liver
PBG Deaminase	Acute Intermittent Porphyria	Liver
Uroporphyrinogen III Cosynthase	Congenital Erythropoietic Porphyria	Erythroid Cells
Uroporphyrinogen Decarboxylase	Porphyria Cutanea Tarda (Types II & III)	Liver
	Hepatoerythropoietic Porphyria	Liver
Coproporphyrinogen Oxidase	Hereditary Coproporphyria	Liver
Protoporphyrinogen Oxidase	Variegate Porphyria	Liver
Ferrochelatase	Erythropoietic Protoporphyria	Erythroid Cells

Summary of the inherited human porphyrias associated with each of the enzymes from the haem biosynthetic pathway. The initial site of accumulation of porphyrins or their precursors, 5-ALA and PBG in the liver or erythroid cells is used to classify the disease as hepatic or erythropoietic.

ALA Dehydratase Porphyria (ADP)

ADP is a hepatic porphyria and a very rare autosomal recessive disorder caused by mutations in ALA dehydratase. It is seen only in homozygotes and only six cases are known to date. Depressed activity of ALA dehydratase leads to a rise in the levels of the precursor 5-ALA. The symptoms of ADP are neurological with no cutaneous photosensitivity and are aggravated by diet, stress and alcohol.[1]

Acute Intermittent Porphyria (AIP)

AIP is autosomal dominant and the most common of the inherited hepatic porphyrias. A reduction in PBG deaminase activity (usually 50% of normal but reduced to 15% in rare homozygous cases) leads to a build up of 5-ALA and PBG. The symptoms of AIP are acute attacks rather than cutaneous, although 90% of individuals with a mutation in PBG deaminase may never develop any symptoms. AIP is more common in females than males and could be triggered by hormonal changes. It is often induced by drugs including the contraceptive pill and reduction in diet, which are thought to aggravate AIP by stimulating ALAS activity. Mutations in the PBG deaminase gene are highly heterogeneous, with over 50 mutations so far recorded.[1] AIP has been also been recorded as sporadic cases, where the disease cannot be traced in members of the patients family. This sporadic form is thougt to be caused by a *de novo* mutation in the PBG deaminase gene.[19]

Congenital Erythropoietic Porphyria (CEP)

CEP is a rare autosomal recessive disease, seen only in homozygote cases and is characterised by a decrease in the activity of uroporphyrinogen III cosynthase. This causes a build up of uroporphyrinogen I and coproporphyrin I isomers. CEP is an erythropoietic porphyria with symptoms including photosensitivity and haemolysis. Several mutations in uroporphyrinogen III cosynthase are known, the most common mutation is a cysteine to arginine change. Patients homozygotic for this mutation have the most severe cases of CEP.[1]

Porphyria Cutanea Tarda (PCT)

PCT is caused by defective UROD is the most prevalent of all the porphyrias and thousands of cases have been reported world-wide. There are two main types of PCT. Type I is the acquired or sporadic form and seems to be more common. Type II, which is the familial form, makes up about 20-30% of all cases of PCT.

Type II is inherited as an autosomal dominant disorder. Several mutations have been discovered, including point mutations and a splice site mutation leading to an exon deletion in the UROD gene. The familial form of PCT is characterised by decreased UROD activity (50% of normal) in all tissues. It was distinct from type I, in which UROD activity is only reduced in the liver, until a second familal PCT was described. [1,2] Studies on patients with PCT have found siblings with decreased heptic UROD activity but without any abnormality in the erythrocytic enzyme. This class of PCT has been refered to as type III.[20,21]

There is some suggestion that type I or sporadic PCT could in fact have a genetic predisposition factor. Whether these patients were an example of this or a true third class of PCT is unclear.

The biochemical pathology of PCT leads to an accumulation of uroporphyrin and heptacarboxylic porphyrin in the skin and liver, causing photosensitivity and in long term cases may be associated with liver cancer.[22-24] S-PCT is decribed in more detail in section 1.2.

Hepatoerythropoeitic Porphyria (HEP)

Homozygous mutations in UROD cause the very rare hepatoerythropoietic porphyria, with only 20 cases reported to date in the world. It is clinically similar to CEP and excess uroporphyrin accumulates in the liver and bone marrow. Patients have less than 10% of normal UROD activity which is explained by a very unstable enzyme with a half-life of about 7 hours, the normal being over 80 hours. Again mutations are heterogeneous, with several point mutations and a large deletion reported.[25] One of these was reported in a patient heterozygous for the mutation G281E, with familial PCT, who came from a

family with a history of HEP. In this instance HEP may be the homozygous form of type II PCT .[26]

Hereditary Coproporphyria (HCP)

HCP is an autosomal dominant, heterozygous disorder caused by a deficiency of coproporphyrinogen oxidase which leads to a build up of coproporphyrin. The homozygous form is very rare and a more severe disorder. The symptoms it produces can be both neurological and cutaneous, although only 30% of cases suffer skin photosensitivity. HCP is more common in females than in males and is aggravated by steroid hormones and barbiturates, probably by stimulating haem synthesis to worsen the condition.[1]

Variegate Porphyria (VP)

Variegate porphyria is a low penetrance autosomal dominant disorder of protoporphyrinogen oxidase. The heterozygous disorder is more prevalent than the homozygous condition. Protoporphryin builds up causes the main neurological and cutaneous symptoms. VP can also occur with porphyria cutanea tarda where it is known as 'dual porphyria'. [1]

VP is reportedly more common in South Africa than anywhere else in the world and is thought to have come from two Dutch settlers in the 17th century. The main mutation found in South African VP patients, R59W, is thought to be the ancestral mutation as one of the families used in the study was known to be descended from the Dutch settlers. VP has also been claimed to be the cause of the 'Royal Malady' of King George III.[27,28]

Erythropoietic Protoporphyria (EPP)

Decreased activity of ferrochelatase, the final enzyme of haem biosynthesis, induces the final and most common erythropoeitic porphyria. EPP is autosomal dominant and characterised by severe burning of the skin on exposure to sunlight. A subset of patients (5-10%) also suffer liver failure. Protoporphyrin accumulates in erythrocytes, where it produces free radicals when excited by light. This leads to damage to cells by lipid peroxidation and activation of the

complement cascade. Heterozygotes have less than 50% of normal ferrochelatase activity. Apparent homozygotes are usually compound heterozygotes. A dominant homozygous form would be lethal, as no haem would be formed. [1]

1.2 Sporadic Porphyria Cutanea Tarda

1.2.1 Clinical Features of Sporadic Porphyria Cutanea Tarda

As stated previously, PCT arises from a defect in the activity of the fifth enzyme of haem biosynthesis, UROD, and seems to be the only human porphyria which can be acquired as well as inherited.

PCT is the most common of all the porphyrias and the sporadic type (S-PCT) is apparently most prevalent in Europe, South Africa and South America. S-PCT presents mainly in men at middle age but it is becoming increasingly frequent in women.

The symptoms are cutaneous photosensitivity with no acute attacks caused by adverse effects from drugs. Patients typically present with lesions on sun-exposed area of the skin such as the back of the hands and on the face, which can be followed by ulceration and scarring. Severe cases may have a scleroderma-like appearance of the hands, tapering fingers, abnormalites of the nails and occasionally a partial resorbtion of the terminal phalanges. More mild cases may have a fine hair growth on the face. Hepatic iron levels are often elevated or in the upper range of normal such that around 80% of S-PCT patients have some degree of hemosiderosis.[29] In long term cases, there appears to be an increased chance of developing hepatocellular cancers, than with some other liver diseases.[1,2,4,22-24]

To date, no mutations in the UROD gene or promoter sequence of S-PCT patients have been discovered and there is no drop in enzyme concentration associated with lack of activity. These finding have led to the suggestion that an inhibitor of UROD is produced in the liver, or that some other gene(s) can influence enzyme activity. [31,32]

1.2.2 Sporadic Porphyria Cutanea Tarda Precipitating Factors

The most common exacerbating factor of S-PCT appears to be alcohol consumption. Oestrogens, used for hormone therapy, contraception and prostate cancer treatment have also been reported to be associated with the onset of S-PCT. [1-4] There are also cases of S-PCT being triggered by pregnancy. [33]

The association with iron overload has led to the most effective treatments for S-PCT to include depletion therapy. The symptoms of PCT are usually alleviated by phlebotomy, to lower hepatic iron levels, often this treatment is often supplemented with low doses of chloroquine, which acts by complexing with uroporphyrin, promoting its excretion and possibly depressing ALAS activity.[34] Exposure to some polychlorinated aromatic chemicals can also cause S-PCT and this is discussed in more below.

Recently, cases of S-PCT have been reported in patients with HIV and hepatitis B and C infection. The relationship between these viral infections and development of the disease is unclear. Hypotheses suggest that it could be triggered by an interference with the cytochrome P450 system, ineffective hematopoeisis leading to an increase in iron concentration or alteration in the metabolism of hormones such as oestrogen. [35-39]

The one common factor that all of these agents have is that they cause some form of liver damage, from cirrhosis induced by alcohol to inflammation and necrotic foci caused by oestrogen.[3]

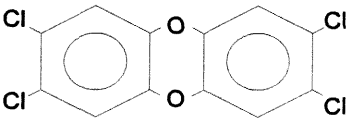
1.2.3 Chemicals Responsible for Sporadic Porphyria Cutanea Tarda

A number of polychlorinated cyclic hydrocarbons have been reported to cause a toxic porphyria with the same symptoms as S-PCT.[1,3,40] The structures of some of these compounds are shown in Figure 1.3. Exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has been linked to the development of S-PCT in chemical incidents.[41] Cases of S-PCT have also been reported after exposure to polychlorinated (PCB) and polybrominatedbiphenyls (PBB) and triazine herbicides.[1,3,40,42] The most notorious incident of toxic porphyria

happened in Turkey in the 1950's. In this incident, seed wheat treated with hexachlorobenzene (HCB) was widely consumed by a population in Eastern Turkey, instead of it being planted, causing several thousand cases of porphyria with PCT-like symptoms.[43] The action of HCB in causing S-PCT and the experimental porphyria in animals is explored further in section 1.3.

Figure 1.3 Structures of two important porphyria inducing chemicals

Hexachlorobenzene



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

1.2.4 Genetic Predisposing Factors

It has been hypothesised that S-PCT requires a genetic predisposition because patients with liver damage associated with the above factors do not, on the whole, develop porphyria. As an illustration, cirrhosis of the liver occurs in 30-40% of alcoholics, but the frequency of S-PCT amongst alcoholics with cirrhosis is only 2%.[2,3] In addition, exposure to chemical agents does not always induce S-PCT either. In the Turkish incident, although thousands of people were exposed to HCB not all developed S-PCT, even in families who prepared food and ate together.[44]

A mild hepatic siderosis is found in most S-PCT patients, thus the inheritance of an iron overload disorder, hemochromatosis, has been suggested as a possible genetic predisposing factor.[45]

Hemochromatosis is a common hereditary disorder, inherited as an autosomal recessive trait, with only homozygotes suffering from the disease. However mild iron overloading is possible in heterozygotes and it is estimated that 10-13% of Caucasians carry one haemochromatosis allele.[45] In fact cases of S-PCT in haemochromatosis patients have been reported.[45-47]

Investigation into the inheritance of haemochromatosis in S-PCT patients has been hampered as the gene had not been identified. The gene for haemochromatosis had been located on chromosome 6 and was linked to the major histocompatibility complex (MHC). S-PCT patients were typed for haemochromatosis by HLA typing, with the presence of the HLA-A3 antigen as a marker of the disease. In one study of 21 S-PCT patients and their relatives, 62% sufferers were found to have the HLA-A3 antigen, all had some degree of siderosis and 26% of relatives had elevated transferrin saturation and serum ferritin levels, which is common to the disorder.[48] However, these results have been disputed and whatsmore the HLA-A3 anitgen occurs in only 70% of haemochromatosis homozygotes. [49-51]

Recently the human gene thought to be responsible for haemochromatosis has been cloned. The gene was located three meagbases telomeric to the MHC and has been identified as a novel MHC class I antigen, HLA-H. Its function and relation to iron metabolism is not clear. Two mutations in this gene have been discovered so far. These mutations occur in 83% of haemochromatosis homozygotes.[52] A link between S-PCT cases and haemochromatosis would possibly be shown by screening the patients for these mutations.

Iron levels are not always elevated and are rarely found at the levels seen in haemochromatosis. This may be due to the penetrance of the haemochromatosis mutations or be the result of more than one predisposing gene.

1.3 Experimental Porphyria

1.3.1 Hexachlorobenzene as a Porphyrogenic Agent

Hexachlorobenzene was first reported as a porphyria-inducing agent in 1960.[43] After an incident in Turkey, Between 1955 and 1959, nearly 4,000 cases of a cutaneous porphyria, with symptoms similar to PCT, were estimated to have occurred. The cause of this epidemic was traced to seed wheat which had been treated with fungicide and insecticide. This contained 10%

hexachlorobenzene (HCB) as an agent against the fungus *Tilletia tritici.* The wheat had been used for human consumption instead of being planted, which explained the widespread epidemic. This incident was the first evidence for a toxic agent causing an acquired porphyria in man. [43]

A great deal of work was undertaken to determine whether HCB was the cause of this outbreak. Experiments using rats, mice, guinea pigs and rabbits showed a varied response to HCB which depended on species, strain and sex, although HCB was toxic to all.

Rats fed 0.2% HCB in their diet eventually became porphyric with excretions of porphryins and porphyrin precursors in the urine but no porphyria was induced after a large, single dose. Rabbits showed the closest response to the human condition and guinea pigs and mice were more susceptible to the neurotoxic action of HCB and died before any porphyria developed. [40,53,54]

The use of inbred mouse strains which are genetically defined for many biochemical processes, was regarded as unsuitable although this would be a useful approach to elucidate the mechanism of UROD inhibition. It was discovered that mice given HCB in the diet, at a concentration of 0.02%, overcame the neurotoxic effect and they survived long enough to develop porphyria. [6]

These experiments confirmed that the incident in Turkey was due to exposure to HCB and eventually provided the evidence that PCT could be acquired as well as inherited. The Turkish incident affected different ethnic groups of Turks, Kurds and people who had been repatriated from the Balkans, where their families had lived for several centuries. Therefore the genetic heterogeneity of these people would imply that a racial characteristic was an unlikely cause of the porphyria. However, in the light of modern molecular genetics it would seem unwise to conclude that a genetic predisposition was not involved.

1.3.2 Similarities between Hexachlorobenzene-Induced Experimental Porphyria and Human Sporadic Porphyria Cutanea Tarda

There are several similarities between human S-PCT and the experimentally induced porphyria. Both can be brought about by exposure to HCB. They are characterised by increased excretion of porphryins, especially uroporphryin and heptacarboxylic porphyrin in the urine. Porphyrin levels in the liver are raised massively and there is an associated marked depression of UROD activity. Increased impairment of liver function is observed and there are pathological changes in liver morphology including; degeneration, necrosis and fibrosis. There is also an increased chance of the development of hepatocellular cancer in both the human condition and in experimental condition in rodents. [3,55]

The porphyrogenic activity of HCB and other related chemicals appears to be enhanced by oestrogen and iron in rats and mice as well as humans. Female rats are more susceptible than males to induction of porphyria implying an association with oestrogen. Dosing males with oestradiol will increase the rate of development whereas ovariectomy in females will reduce porphyria. [4,40]

Since siderosis is common in patients with S-PCT a great deal of work on the effect of iron in experimental porphyria has been carried out. For instance, large doses of iron given to rats and mice before exposing them to HCB or TCDD increases the severity of the porphyria.[6,7,56,57]

Strain variation of animals to susceptibility suggests that there might be a genetic factor in the process of porphyria development. Similarly, in humans most patients with chronic liver damage from alcohol or oestrogen do not develop PCT and many of those people exposed to HCB in Turkey, did not develop the disease either. These similarities mean that as an animal model, experimental porphyria induced by HCB is closely related to the human condition of S-PCT and may be used as a useful model in elucidating the mechanism of human toxicity.

1.3.3 Toxicology of Hexachlorobenzene

Like many other organochlorine pesticides, HCB is a wide spread environmental pollutant, with high bioaccumulation potential. HCB was manufactured and used as a pesticide to prevent fungal growth on grain but this practice was discontinued in many countries by 1965. It was also used in the production of fireworks, ammunition and synthetic rubber. HCB can also be produced as a by-product of chemical manufacture; such as solvents and chlorinated compounds, from combustion of waste and from wood preserving plants.

HCB is a white crystalline solid, insoluble in water but soluble in organic solvents, fats or vegetable oils. It is resistant to metabolism and persistent in the body fat of a variety of animals including invertebrates, fish, reptiles, birds and mammals as well as in humans. The chemical is distributed throughout the world, but is particularly concentrated near waste dumps. It has a half-life of three to six years in soil and up to one year in water.

Most exposure to HCB comes from contaminated diet. It is absorbed in the gut and distributed throughout the body. As it is fat soluble HCB is mainly deposited in adipose tissue where its concentration may be ten times higher than in the liver. HCB is poorly metabolised and excreted slowly.[58,59]

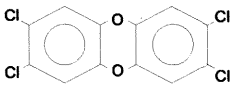
Enlargement of the liver in rodents following exposure to HCB is thought to result from an increase in the smooth endoplasmic reticulum. This happens in conjunction with the stimulation of drug-metabolising enzymes, the hepatic microsomal cytochrome P450 system. HCB is thought to induce some of these enzymes by acting as a weak ligand for the aromatic hydrocarbon receptor (AH receptor).[60] There is little or no evidence to support the view that HCB is a human carcinogen but since it can cause PCT and increased incidences of liver cancer have been reported in porphyria patients, the possibility exist that HCB does have carcinogenic potential in humans. [58]

1.4 Association of the Aromatic Hydrocarbon Receptor and Cytochrome P450 System with Experimental Porphyria

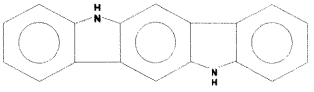
1.4.1 The Aromatic Hydrocarbon Receptor

The aromatic hydrocarbon (AH) receptor or 'dioxin' receptor was originally identified from studies on the interaction of TCDD in C57BL and DBA/2 mice.[61] Since then much of the work on the interaction and effects of TCDD (and other related halogenated aromatic hydrocarbons, Figure 1.4) and the AH receptor, has concentrated on rodents. AH receptor mRNA is widely distributed in mammalian tissues, including lymphocytes, brain, myocardium, skeletal muscle, bladder, breast tumour cells as well as the liver. The receptor has also been discovered in chick embryos and trout, so it appears to be ubiquitously distributed throughout vertebrates. Despite a huge amount of work on the AH receptor, there is no endogenous ligand known.

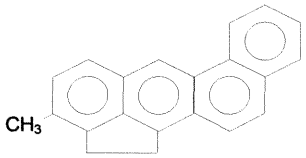
Figure 1.4 Aromatic Hydrocarbon Receptor Ligands



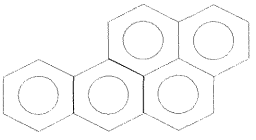
2,3,7,8-TCDD



Indolo[3,2-*b*]carbazole



3-Methylcholanthrene



Benzo[a]pyrene

Cloning and sequencing of the AH receptor gene (*Ahr*) and its partner protein the AH nuclear translocator (ARNT) has revealed its biological function as a ligand-activated transcription factor.[67] At first, its mechanism of action was

thought to be similar to steroid receptors. However, the structures of the AH receptor and ARNT were found to contain a DNA binding helix-loop-helix motif, similar to Per and Sim that form a heterodimeric transcription factor, instead of the zinc-finger structure steroid receptors possess.

The AH receptor has been studied for its role in cell toxicity after binding foreign compounds, of which TCDD is considered the prototype molecule. On binding these chemicals, the receptor undergoes a conformational change and is translocated to the cell nucleus, where it regulates transcription of a number of genes. The genes known of so far are mainly drug-metabolising enzymes but there are also a number of cell differentiation and growth proteins which apparently are regulated by the AH receptor. Genes regulated by the AH receptor encode cytochrome P450 1A1, 1A2 and 1B1, glutathione-S-transferase Ya, UDP-glucuronosyltransferase, NAD(P)H:quinone oxido-reductase (NQO_1) and aldehyde dehydrogenase (ALDH3c), and are all enzymes associated with drug-metabolism. The epidermal growth factor receptor, oestrogen receptor, plasminogen activator inhibitor 2, interleukin 1β, transforming growth factors α and β2, are also thought to be regulated by the AH receptor and are all cell growth genes. Thus the effect on the regulation of these genes by the AH receptor may be critical to the carcinogenic effect of TCDD and other similar compounds.[62-64]

1.4.2 The Mouse Aromatic Hydrocarbon Receptor

The AH receptor is known to exist as four allelic variants of the gene *Ahr*, in the mouse. [65,66] Two of these alleles are responsible for the pharmacogenetic differences between some inbred mouse strains. The most studied alleles are the Ahr^{b-1} and Ahr^{d} , which were identified in C57BL mice and DBA/2J mice, respectively. Mouse strains with the Ahr^{b-1} allele are known as 'responsive' strains, whereas mice with Ahr^{d} allele are termed 'nonresponsive'. These terms refer to the affinity with which the AH receptor binds to TCDD; there is a ten-fold difference in binding between the two with the nonresponsive strains having a much weaker affinity. This difference in affinity leads to a difference between Ahr^{b-1} and Ahr^{d} strains to induction of cytochrome P450 1A1. [61,62]

When the receptor gene was cloned the difference between the two alleles was characterised, although it has not yet explained the variation in affinity for TCDD. [67] The sequences are highly conserved except that the *Ahr*^{d} allele encodes a larger protein of 104 kDa, as opposed to the *Ahr*$^{b\text{-}1}$ receptor which is 95kDa. The difference in size is due to an alteration in the *Ahr*^{d} sequence which changes the stop codon to an arginine, producing a longer transcript. The *Ahr*^{d} gene is the same size as that of another allele, *Ahr*$^{b\text{-}2}$, which is found in the strains BALB-c, A/J, C3H/HeJ and CBA/J. Therefore the size of the receptor itself, is not likely to be the cause of the lowered affinity as the *Ahr*$^{b\text{-}2}$ allele encodes a receptor with a much higher binding affinity. Another polymorphism between *Ahr*$^{b\text{-}1}$ and *Ahr*^{d} alleles replaces a leucine with a proline residue, which may alter the secondary structure of the AH^{d} receptor and possibly cause the lowering of its ligand binding ability. The lower affinity of the AH^{d} receptor may be caused by alterations at the binding sites of the ARNT or heatshock protein subunits which complex with the receptor, this could affect ligand binding or the efficiency of the complex as a transcription factor.[68,69]

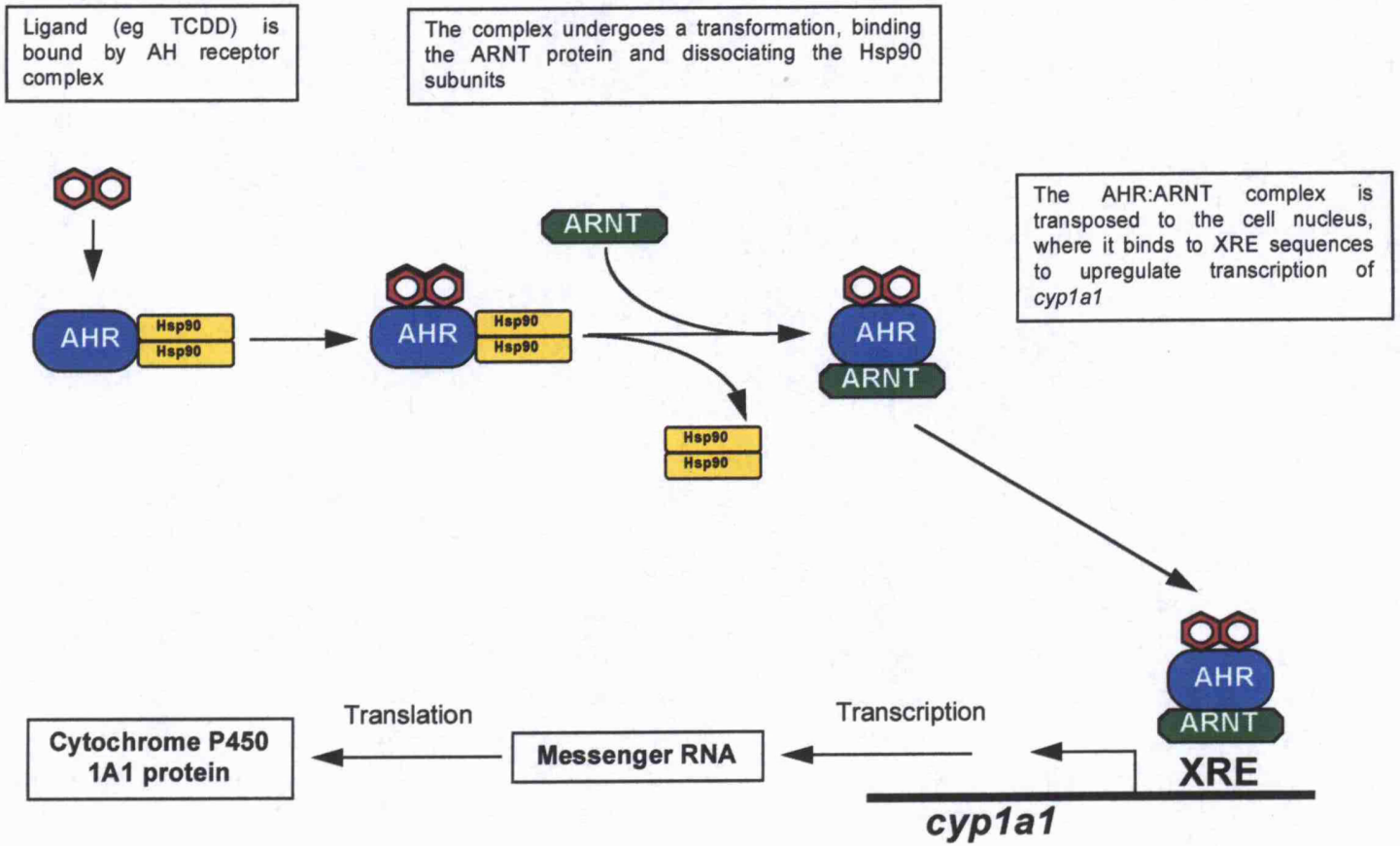
Although this section is dedicated to the mouse AH receptor biology, it is worth noting here some of the similarities and differences between the mouse and human receptors. The human AH receptor gene is similar in size to the mouse *Ahr*^{d} gene and it has a much lower affinity for chlorinated chemicals than that of mice or rats with 'high responding' phenotypes. A higher affinity type of AH receptor has been reported in human cell lines which results in greater induction of CYP1A1. It is thought that individuals with the high affinity AH receptor and who smoke are apparently at greater risk of developing lung, larynx and oral cavity cancers.[68,69]

1.4.3 The Mechanism of Induction of Genes via the Aromatic Hydrocarbon Receptor

One of the most studied mechanisms mediated by the AH receptor is the induction of cytochrome P450 1A1 by TCDD (Figure 1.5). The AH receptor resides in the cell cytoplasm complexed to two heat shock proteins (Hsp90) that act as molecular chaperones, which are thought to keep the AH receptor in a

ligand-binding conformation and repress its DNA binding activity. Once the AH receptor binds to a ligand, the complex then binds to a second protein, the nuclear translocator ARNT. This complex is transferred to the nucleus and the Hsp90 subunits dissociate. (It is not certain if this occurs before or after the translocation to the nucleus.) The DNA sequences that the AH receptor/ARNT complex binds are known as dioxin or xenobiotic responsive elements (XREs). Once the complex binds to the XRE, of which there are several upstream of the transcription start-site, it can upregulate transcription of *cyp1a1* or other target genes. [62-64]

Figure 1.5 Induction of Cytochrome P450 1A1 by the Aromatic Hydrocarbon Receptor



Outline of the induction of cytochrome P4501A1 via the aromatic hydrocarbon receptor (AHR) and ligand-activated transcription factor. Abbreviations; Hsp90, Heat shock protein 90, ARNT; AHR nuclear translocator protein, XRE; xenobiotic responsive element.

1.4.4 The Cytochrome P450 Mono-Oxygenase Enzymes

Among the many genes regulated by the AH receptor are some of the cytochrome P450 superfamily. These enzymes were among the first discovered that were regulated by substrate induced genomal regulation, in organisms other than micro-organisms.

The cytochrome P450s are a large group of haem containing enzymes with mixed function monooxygenase activity. On the basis of their gene sequence, these proteins have been grouped into a superfamily. There are 13 separate gene families and hundreds of cytochrome P450 isozymes are known of in mammals alone. The cytochrome P450 isoforms are thought to have evolved over the past 400 million years to metabolise endogenous substances as well as the vast range of foreign chemicals ingested by organisms.

The major group of cytochrome P450 isozymes studied for their role in the elimination of xenobiotic chemicals from the body, are the hepatic microsomal enzymes. The enzyme system is a protein complex consisting of (in mammals); a flavoprotein, NADPH-cytochrome P450-oxidoreductase and cytochrome P450 units, which are embedded in the smooth endoplasmic reticulum (microsomes) with the substrate binding site facing the cytosol. The highest concentration of drug metabolising enzymes are found in the liver and small intestine, mainly to metabolise compounds that are ingested. Other drug metabolising P450 enzymes are found in the kidney, lung and brain but at much lower concentrations. [70-72]

Drug-metabolising enzymes make up the first four families, CYP1-4. A further five families are involved in steroid biosynthesis, one of which CYP11, is located in the mitochondria of the adrenals. Other families have been found in bacteria, yeast and insects.

Endogenous chemicals metabolised by the cytochrome P450 enzymes are fatty acids, prostaglandins, steroids and ketones. Exogenous compounds, such as carcinogens and drugs include; polycyclic aromatic hydrocarbons, nitrosamines, hydrazines, arylamines, codeine, caffeine and cyclosporine.

The metabolic process of the microsomal cytochrome P450 involves the insertion of a single oxygen atom, derived from O_2, into the substrate. These

reactions convert lipophilic substances to more hydrophilic compounds that can be more readily excreted from the body. Cytochrome P450 mediated reactions include; oxidative and reductive dehalogenation; N-hydroxylation, N-oxidation, oxidative deamination; S-, N-, and O-dealkylation and aliphatic and aromatic hydroxylation.[70]

1.4.5 Role of the Aromatic Hydrocarbon Receptor in Porphyria

The involvement of the AH receptor and cytochrome P450 enzymes in experimental porphyria is historically linked to the disease's induction by polychlorinated aromatic hydrocarbons: TCDD and PCBs which are classic inducers of cytochrome P450 isozymes, acting via the AH receptor as described.[4] As HCB can also induce the same P450 isozymes induced by TCDD, it has been suggested it does this via the AH receptor as well.[73] However, HCB, does not have the usual structure of an AH receptor ligand. These compounds are usually planar, with at least three or four halogenated positions in a lateral configuration. There is also at least one unsubstituted position and the structure should fit into a rectangular box, approximately 6x13Å. Binding studies *in vitro* and *in vivo* in rats and mice, have shown that HCB can compete with TCDD, therefore could be a weak agonist for the receptor. [60]

Demonstration of the AH receptor's involvement in experimental porphyria would help to explain the mechanism of porphyria induction and also the difference in susceptibility to porphyria by inbred strains of mice. It might also mean that possession of a high affinity receptor could be a genetic predisposition required in humans.

To show whether it was involved, a study on inbred mice congenic for the *Ahr* locus, was carried out to determine whether AH phenotype was linked to the development of experimental porphyria in mice. C57BL/6J mice congenic for Ahr^b or Ahr^d alleles, mice were given a single dose of iron in conjunction with HCB in the diet for 17 weeks. Mice congenic for the Ahr^b allele were found to have elevated urinary porphyrins after 7 weeks and uroporphyrin concentrations increased to 200-fold above normal after 15 weeks. The Ahr^d congenics

eventually became porphyric but uroporphyrin levels did not rise until after 13 weeks of treatment and then only rose to a six times the normal level.[74] The results of this experiment suggested that AH receptor genotype correlated with the development of HCB and iron induced porphyria, although previous work with inbred strains that possessed different variations of the *Ahr* gene, had shown an incomplete correlation [6]. However, even if the *Ahr* gene was partly involved, this hypothesis does not help to explain the importance of iron in the mechanism.

1.4.6 Role of the Cytochrome P450 Isozymes 1A1, 1A2 and 2B in Porphyria

Most aspects of toxicology involving cytochrome P450 induction are directed at the bioactivation of reactive and genotoxic molecules. Certain xenobiotics induce their own metabolism through cytochrome P450 activation and this can result in the production of high energy intermediates, for example epoxides, which can attack and damage DNA and RNA thus causing cell damage and possibly leading to cancers. However, the poor metabolism of HCB and TCDD make it unlikely that a reactive metabolite of these chemicals is responsible for the porphyria.[5,70,75]

Investigation of isozymes of the hepatic microsomal cytochrome P450 system induced by HCB in rats and in AH-responsive C57BL/6J mice, found that CYP1A1, CYP1A2 and CYP2B1 were upregulated. [73]

The induction of CYP2B isozymes is a common phenomenon caused by phenobarbital and lower chlorinated benzenes. Thus, HCB is known as a 'mixed-type inducer' as it can induce both the dioxin inducible enzymes as well as phenobarbital inducible enzymes. However, the induction mechanism of phenobarbital induced isozymes is not as well characterised as that of the dioxin induced enzymes. Recent studies of phenobarbital inducible enzymes in mice has suggested that induction may depend on a target gene and a complex regulatory pathway.[76]

HCB was found to induce CYP1A2 to a greater extent than the other isozymes. Induction of CYP1A2 has often been suggested as one of the factors governing

development of HCB-induced porphyria in rodents. CYP1A2 is reported to catalyse uroporphyrinogen to uroporphyrin *in vitro* and this reaction appears to be inhibited by the antioxidant ascorbic acid *in vivo*. [77,78] It has been hypothesised that this reaction could be the causative agent for porphyrin accumulation and could be responsible for S-PCT symptoms. Increases in synthesis and activity of CYP1A2 with development of porphyria in numerous rodent studies seems to support this theory. [8,10,77,78]

Induction of CYP1A2 is thought to be regulated through mechanisms other than (or as well as) the AH receptor. It has been demonstrated that induction can be dependent on the shape of the inducing chemical, with planar molecules inducing CYP1A2 in over globular-shaped molecules. Tricyclichydrocarbons are another class of compound that were also found to induce CYP1A2 preferentially over other isozymes.[79-81]

It has also been suggested that CYP1A2 could be regulated by transcriptional or post-transcriptional mechanisms and increases in CYP1A2 mRNA are thought to be due to stabilisation of the mRNA as well as transcriptional activation.[70]

Recently, increased expression of CYP1A2 mRNA has been reported in induced AH knock-out mice. It is thought that this induction, which is not mediated by the AH receptor, may be activated by chemicals with particular structures, such as tricyclic hydrocarbons. But it is still not known whether this 'induction' is caused by a novel mechanism or stabilisation of the mRNA. [82]

There is a known allelic variant of the *cyp1a2* gene discovered between C57BL/6J and DBA/2 strains. The two alleles differ at three bases, one of which causes an amino acid substitution, close to a conserved region and the active site. [83] It is plausible that this difference or polymorphism could be the source of the genetic variance in susceptibility to porphyria induction in mice. However there are no polymorphisms reported in human CYP1A2 which could serve as a predisposing gene. Although there is a difference in activity of CYP1A2 to metabolising substances such as caffeine, it is possible that this is caused by other genes, regulating the transcription or translation of CYP1A2 rather than a genetic mutation or polymorphism in the gene itself. [84]

Another process of cytochrome P450 mediated toxicity is postulated to be the production of oxidants, or oxygen radicals. Poorly metabolised compounds such as TCDD and HCB interacting with a cytochrome P450 could somehow uncouple the electron transfer that takes place between cytochrome P450 and cytochrome P450 NADPH-dependant reductase, leading to the formation of oxygen radicals in the form of a superoxide anion. This radical can spontaneously dismutate to hydrogen peroxide and in the presence of a transition metal such as iron, can react to produce the extremely reactive hydroxyl radical. [5,75] This theory has been proposed as a possible mechanism for the production of an inhibitor of UROD. Hydroxyl radicals are known to cause damage to biomolecules and it has been demonstrated that hydroxyl radicals can oxidise uroporphyrinogen to the unmetabolised uroporphyrin or to further uncharacterised products that could act as powerful inhibitors of UROD. [85]

1.5 The Involvement of Iron in Porphyria

1.5.1 Introduction

Iron is required by the cell for many essential functions such as oxygen and electron transport and mitochondrial energy metabolism. It is able to regulate many redox reactions as it can exist in at least two oxidative states, Fe^{2+} (ferrous) and Fe^{3+} (ferric).

Iron is absorbed in the gut by a mechanism that is still unclear. There is no mechanism to excrete iron, so it is lost primarily by haemorrhage or sloughing of intestinal cells. Insufficient iron absorption, leading to anaemia, is the most common disorder of iron metabolism whereas iron overload conditions are much rarer. Increases in iron levels can occur as a result of inheriting haemochromatosis, or from blood transfusions, increased oral intake and liver diseases such as alcoholic cirrhosis.

The majority of iron is contained as haem, in haemoglobin. The remainder is associated with proteins and iron-sulphur clusters. The protein ferritin, forms a

shell which contains the main cellular reservoir of ferric iron. It is also stored as an iron-protein aggregate, haemosiderin, which can increase in concentration in iron overload disorders. The main locations of ferritin and haemosiderin are the liver spleen and bone marrow.

Ferric iron is transported, via the plasma to cells, complexed to the protein transferrin. This complex is absorbed into the cell by receptor mediated endocytosis via the transferrin receptor, where it must be reduced to ferrous iron to be utilised. Absorption of iron into the cell is proposed to be controlled by translational regulation of transferrin receptor and ferritin mRNA (described in section 1.1.3) and an intracellular iron pool that is able to control its own concentration. In the cell, iron is complexed to protoporphyrin and ferritin to keep it in a nontoxic form, otherwise in conjunction with oxygen, it could catalyse the generation of damaging oxygen radicals.[11,15,86]

1.5.2 Toxicity of Iron

Toxicity from iron occurs when it exists in a catalytic state or as 'free' iron. This is only known to occur in rare instances of severe iron overload when serum transferrin is saturated but has been hypothesised to exist in the cell. *In vitro*, 'free' iron can occur when Fe^{2+} is chelated by low molecular weight biomolecules such as citrate, ADP, ATP or GTP. In this state, the bound ferrous iron is thought to be able to catalyse the production of reactive oxygen species from hydrogen peroxide by the Fenton reaction:

Fenton Reaction $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$

In the cell, certain enzyme reactions, processes such as inflammation, and uncoupling of cytochrome P450s can produce superoxide anions ($O_2^{\cdot-}$). Superoxide is able to mobilise iron from ferritin and haemosiderin by reducing Fe^{3+} to Fe^{2+}. Oxidation of unstable Fe^{2+} back to Fe^{3+} by hydrogen peroxide could lead to the formation of hydroxyl radicals or the formation of other radicals including ferryl and peroxyl radicals, all of which can cause cell damage by lipid

peroxidation and alterations in DNA structure causing strand breakages and mutations.[75,87-89]

1.5.3 Exacerbation of Porphyria by Iron

Iron increases the severity of both S-PCT in humans and the experimental porphyria induced in rodents by TCDD or HCB. The human condition is treated by phlebotomy or with iron chelating drugs for iron depletion, whether it is in excess or not, which produces a clinical and biochemical remission. Furthermore, administration of iron to a patient in remission can lead to a relapse. [1,2,90]

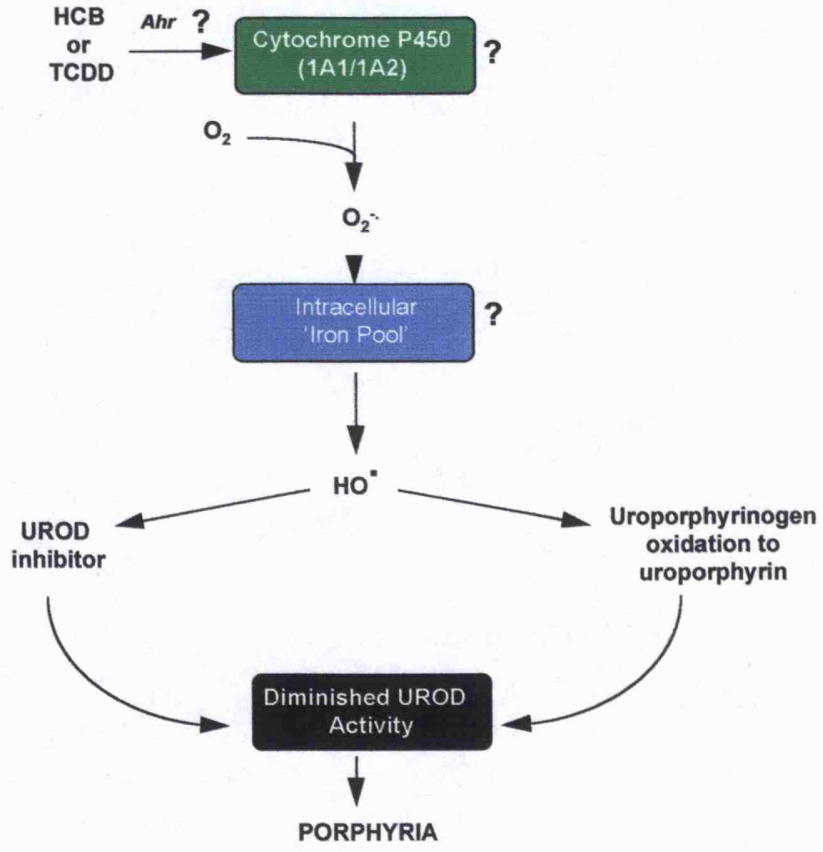
The exacerbation of porphyria by iron has been reported in many studies with rodents treated with HCB or TCDD. Mice were made iron deficient by bleeding and fed a low iron diet failed to develop porphryia after treatment with TCDD, thus iron depletion in the experimental condition also appears to have a protective effect.[57] Iron can also potentiate the development of an experimental porphyria with chemicals such as β-napthoflavone, 20-methylcholanthrene and the haem precursor 5-ALA, which do not produce the disease by themselves. [5,91] In fact, a single dose of iron alone can induce porphyria in certain susceptible mice such as the C57BL strains.[8,89]

For most of these cases it has been proposed that this synergistic action of iron, enhances the development of porphyria by interacting with oxygen metabolites, produced from induced cytochrome P450 metabolism, which could promote the oxidation of uroporphyrinogen to the nonmetabolised form uroporphyrin or catalyse the production of an inhibitor to UROD.[5]

Although most patients with S-PCT have some siderosis and serum iron levels can be elevated, iron levels are not usually as high as those seen in iron overload disorders such as haemochromatosis, some cases have normal or even low iron stores. Therefore it appears that S-PCT and its experimental counterpart is not a diseases of iron overload but of iron mobilisation or metabolism, localised to the liver, that may alter the availability of catalytic (Fe^{2+}) iron. Along with the other factors already covered in this chapter, such as the AH receptor and cytochrome P450 enzymes, this aspect of the disease is

also potentially a site for genetic variation, which could contribute to the overall susceptibility to this particular porphyria. The diagram below (Figure 1.6) summarises these interaction in the proposed mechanism of UROD inhibition leading to experimental porphyria.

Figure 1.6 Flow-diagram summary of the hypothesised interactions required for a reduction in UROD activity



HCB or TCDD stimulates its own metabolism by the cytochrome P450 system, via the AH receptor (*Ahr*).It is believed that poor metabolism of these chemicals leads to an uncoupling of the electron transfer in the catalytic cycle of the enzymes, which might produce reactive oxygen metabolites ($O_2^{\cdot-}$). These in turn may react with iron in the hepatocyte, producing hydroxyl radicals ($HO^{\cdot}$) that could inhibit UROD by either catalysing the production of an inhibitor or cause the oxidation of uroporphyrinogen to the non-metabolised uroporphyrin.[5]
The question mark (**?**) signifies interactions that might be genetically influenced.

1.6 Genetic Susceptibility In Experimental Porphyria

1.6.1 Genetic Variation to Iron and Chemically-Induced Porphyria

The ability of iron to increase the rate of development and severity of experimental porphyria has led to some doubt over the role of the AH receptor and the cytochrome P450s. Previously, AH-responsiveness had been thought of as the only genetic difference between strains, leading to susceptibility to induction of porphyria. [7]

In a study of inbred mouse strains given a single subcutaneous dose of iron and fed HCB in the diet, induction of porphyria did not correlate completely with the AH receptor phenotype of each strain. C57BL/10ScSn mice with the Ahr^{b-1} allele (an AH-responsive strain) were the most sensitive to treatment and some induction of porphyria was noticed in BALB/c and AKR strains but none in the DBA/2 strain. Although BALB/c is AH-responsive it produced a much weaker effect than expected. However AKR, an AH-nonresponsive strain with the Ahr^{d} allele, responded to iron and HCB unlike the other AH-nonresponsive strain DBA/2. [6]

Similarly, an incomplete correlation between iron and TCDD-induced porphyria and the AH receptor was reported. Five mouse strains; A2G, BALB-c, C57BL/10, AKR and DBA/2 were treated. The AH responsive strains; A2G, BALB/c and C57BL/10ScSn and also the nonresponsive strain AKR developed porphyria to varying extents.[7]

In addition to studying the susceptibility to iron and TCDD induced porphyria these strains, the inheritance of the disorder was followed through F_1 and F_2 intercrosses and backcrosses bred from AKR and DBA/2 mice. These strains displayed the highest and lowest response to treatment respectively, although both were AH-nonresponsive. As susceptibility to porphyria was inherited through the F_1 and F_2 generations, it was concluded that induction of porphyria indeed had a genetic rather than an environmental basis. The phenotypes of the F_2 intercross exhibited a range of susceptibilities that could be explained if more than one gene was involved.[7]

Where experimental porphyria is induced by polycyclic chlorinated chemicals in AH responsive mice, there is substantial evidence for the involvement of the *Ahr* locus and cytochrome P450 enzymes, especially CYP1A2. However, in the experiment described above, the developed in mice produced from a cross of two AH nonresponsive strains, so was unlikely to have involved the *Ahr* locus. This would imply that other loci were responsible for the susceptibility of these strains to the action of iron and TCDD. [7]

1.6.2 Molecular Genetic Analysis of Multigene Disorders

The evidence from the human condition and the experimental models suggests that susceptibility to this particular porphyria could be a multigene disorder. However, the genes responsible or their mode of inheritance are unknown. A great deal has been learnt about possible candidate genes through experiments with inbred mouse strains. This strongly suggested that a combination of this knowledge with a molecular genetic analysis of an experiment, based on the type described in the previous section, might prove useful to begin the search for these genes. This has become the basis for this thesis.

In order to find these genes, a genetic mapping procedure has been used which compares the inheritance of the porphyric phenotype in mice, along with chromosomal regions.

This type of mapping, known as linkage analysis was first used in 1913, by Sturtevant for mapping genes in *Drosophila*.[c1] Since then linkage mapping has been used to map genomes of numerous organisms, from yeast and nematode worms, to dogs and humans. Although individual genes themselves are not known, their positions in the genome can be deduced by following the pattern of inheritance of a genetic 'marker'. Markers in close proximity to the gene of interest are inherited along with the gene. The further away from the gene they are increases the chance of recombination happening between them, thus separating them.

Gene mapping in species such as *Drosophila*, maize and yeast was possible because a large number of genetic markers were available and also there was the ability to breed a controlled cross, to study the inheritance of these markers.

Until recently this technique was impossible for mapping human or even mouse genes on a similar scale because of a lack of genetic markers and in finding suitable pedigrees in the case of humans. [92,93]

A key breakthrough in mapping came with the discovery of variations in DNA sequences between individuals known as a polymorphism. These differences could be detected by restriction digest and southern blotting and were called restriction fragment length polymorphisms (RFLP). RFLPs could be followed through a pedigree and used to produce linkage maps. However, RFLPs are rare and also time consuming to analyse.[93,94]

A better source of DNA polymorphism became available when variations in simple sequence repeats were discovered. These sequences known as microsatellite DNA, are present in most eukaryotic genomes and show a high degree of polymorphism between individuals.[c2] Microsatellite DNA consists of a simple unit of sequence (for instance CA) which can be repeated from 14-40 times. The length of the microsatellite is unique to an individual or an inbred mouse strain. This makes microsatellite DNA ideal for mapping purposes. They are also easily screenable by PCR, a procedure that can even be automated.[93,94] Information on microsatellites is widely available and can be obtained from the Internet.[95] Currently over 7,000 microsatellites have been assigned to the mouse genome, spaced at an average of 0.2cM or every 400kb. [96]

Now that fine resolution maps for the mouse genome are available, genetic mapping of complex traits is possible. Several hundred human genes have been cloned. Many of these have been found by positional cloning or 'reverse genetics', in which the biochemical defect is not known and the gene has had to be mapped initially by linkage analysis. Most of these disorders are caused by a single gene and so follow simple Medelian inheritance patterns. However, more inherited human disorders are being found that are caused by more than one gene, or are polygenic. Examples of such diseases are; susceptibility to heart disease, hypertension, diabetes, cancer and infection.[92]

Hundreds of inbred mouse strains have been bred over the past century, these mice include mutants for many single gene loci or have genetic variations in

physiology, development and behaviour. By finding an inbred strain that models a human condition, it is possible to find the gene in the mouse which is a much easier process than mapping genes in humans. It may also be possible to find the human homologues of such genes, if a sufficient similarity exists between them. [92]

To locate the genes responsible for a complex trait, such as susceptibility, a cross between two strains is required, one of which must be susceptible to the trait and one resistant to it. This technique is commonly used to find quantitative trait loci (QTL) in mice. These are areas of a chromosome which may contain one or more susceptibility genes and are found by comparing inherited genotype and phenotype of mice produced in an F_2 intercross or a backcross, with the original inbred strains. [92,97]

The principle behind this type of genetic mapping, involves firstly producing an F_1 cross between the two strains with differing susceptibilities. The parent strains are homozygous as they are inbred, so each strain is genetically identical. The resulting F_1 hybrid mice are heterozygotes, carrying one chromosome from each parent. An F_2 generation is produced from mating F_1 mice. These mice have a mixture of parental genes, as the F_1 chromosomes have undergone recombination and genes from the original strains are segregated.

The susceptibility of the F_2 generation must then be scored. There is usually a continuous range in susceptibilities, from those which display parental phenotypes to those with an intermediate response. This range is due to separation of the susceptibility genes and the mixture of which has been inherited by each individual F_2 mouse.

The F_2 mice can then be genotyped with microsatellite DNA markers. If markers are chosen that are polymorphic, that is they differ in length between the two parental strains, the F_2 mice can be genotyped by amplifying across the repeat by polymerase chain reaction (PCR).

To find chromosomal areas that may contain a susceptibility gene, two groups of F_2 mice selected as having the highest or lowest susceptibilities are genotyped. Inheritance of each marker should follow a 1:2:1 ratio, of one of

each homozygote allele and two heterozygote alleles. This ratio is assumed when random segregation of two codominant alleles occurs. If a marker was linked to a gene causing susceptibility, this ratio would be upset. Mice in the high group would inherit, predominantly, alleles from the susceptible parent and *vice versa* in the low groups. The statistical significance of the deviation is tested with the χ^2 test.[92,98,99]

1.6.3 Aims

The aim of this project was to find a genetic component for the susceptibility to porphyria induced by HCB and iron-overload, in a cross between the strains C57BL/10ScSn (BL/10) (susceptible) and DBA/2 (resistant). A second experimental cross was bred from SWR and DBA/2 strains, to search for susceptibility genes in a model where iron alone induces porphyria.

As well as a systematic search through the genome with microsatellite DNA markers, some candidate genes were also tested in these models. The influence of the *Ahr* gene in the BL/10xDBA/2 F_2 cross was examined by the use of an RFLP which distinguished between the Ahr^b and Ahr^d alleles.[67] The cytochrome P450 isozymes CYP1A1 and CYP1A2 were also investigated by expression of their mRNA and enzyme activities, as well as genetic analysis.

One chromosome, 17, was examined in more detail as it is the hypothetical location of the mouse homologue of the the human haemochromatosis gene.[100,101] Together with the microsatellite linkage analysis, the affect of this region on the development of porphyria was studied in the congenic mouse strain BL/10.D2n, which contains the DBA/2 H-2 complex (and thus the resistant form of any hypothetical genes located within this region) on a C57BL/10ScSn background.

Currently many human inherited disorders are being mapped in the mouse, using this a similar approach. This system has allowed the analysis of complex traits such as susceptibility to epilepsy, diabetes, cancers, hypertension, pain, multiple sclerosis, alcohol and drug addiction. [102-113] However, there are few studies of a similar nature in the field of toxicology but by applying this technique to experimental porphyria might help to gain some insight into the mechanism of this disorder.

2 - Materials and Methods

A list of the suppliers of the chemicals, reagents and commercial kits used, is given in Appendix III, unless stated. All the inbred mouse strains and some F_1 hybrids and backcrosses bred from C57BL/10ScSn/Ola and DBA/2/Ola strains, were purchased from Harlan Ltd, Bicester, UK. Mice were maintained in negative pressure isolators at approximately 21°C with 12 hours light/12 hours dark cycles. All the mice were fed on Rat and Mouse 1 maintainance diet (RM1), unless indicated.

2.1 Animal Protocols

2.1.1 Induction of Porphyria by HCB and Iron in C57BL/10ScSn and DBA/2 Mouse Strains

Iron in conjunction with the compound hexachlorobenzene (HCB) causes an experimental porphyria in C57BL/10ScSn mice similar to the human disease sporadic-porphyria cutanea tarda. In order study the inheritance of this disorder in mice and to find any predisposing genes, an F_2 intercross was bred from C57BL/10ScSn and DBA/2 (a resistant strain) parents.

Animals and Breeding Protocol:

C57BL/10ScSn/Ola, DBA/2/Ola, F_1 hybrid and backcrosses to each strain, were purchased at 7-10 weeks old.

F_2 mice were bred from the following matings:

Female C57BL/10ScSn were mated with male DBA/2mice, to produce 10 pairs of (C57BL/10ScSnxDBA/2) F_1 hybrid mice. These were subsequently mated to produce approximately 100 male (BL10xDBA/2)F_2 hybrids (Group 1).

Female DBA/2 were mated with male C57BL/10ScSn mice, to produce 10 pairs of (DBA/2xBL/10) F_1 mice which were subsequently used to breed approximately 100 male (D2xBL10)F_2 hybrids (Group 2).

Only male mice were kept for the study, females were culled after weaning.

Reagents:

- Iron as iron-dextran (100 mg Fe and 100 mg dextran/ml solution)
- HCB (organic analytical grade) with no traces of TCDD or related compounds detectable by mass-spectrometry.[6]

Treatment:

Seven to ten weeks after weaning, male mouse strains and hybrids were given a single subcutaneous injection of iron-dextran (600mg/kg). After three days, mice were fed on mantainance diet mouse RM1 containing HCB (0.02% HCB in 2% corn oil) for seven weeks. [6] Mice were killed by a schedule 1 method. Livers were immediately frozen in liquid nitrogen for analysis of porphyrin concentration, cytochrome P450 enzyme activity and RNA preparation. Tails were saved for DNA preparation.

2.1.2 Induction of Porphyria by Iron Overload in SWR and DBA/2 Mouse Strains

Iron alone can cause experimental porphyria in the mouse strain SWR but not in DBA/2. The haem precursor 5-aminolaevulinic acid (5-ALA) administered in the drinking water can enhance the rate at which porphyria develops. [8] An F_2 intercross was bred from these strains in order to find chromosomal regions that might contain genes responsible for this variation between the two strains.

Animals and Breeding Protocol:

SWR/Ola and DBA/2/Ola strains, were purchased at 7-10 weeks old. F_2 generation mice were bred from the following matings:

F_2 generation - Set I

6 pairs of SWR females and DBA/2 males were mated to produce F_1 pairs.

14 pairs of F_1 mice were mated to produce approximately 100 F_2 males .

F_2 generation - Set II

10 pairs of SWR females were mated with DBA/2 males, to produce F_1 pairs .

25 pairs of F_1 mice were mated to produce approximately 100 F_2 males.

Reagents:

- Iron as iron-dextran (100 mg Fe and100 mg dextran/ml solution)
- 5-aminolaevulinic acid solution (2mg/ml)

Diet:

Set I mice were fed on RM1 maintenance diet and set II fed RM3 breeder diet. The diet was changed to find out if it affected the development of porphyria. (Chapter 5) The main difference between the diets being that the breeder diet was slightly richer, with a high protein and nutrient content.

Treatment:

Male mice, strains and hybrids 7-10 weeks after weaning, were given a single subcutaneous injection of iron-dextran (600mg/kg), then after three days administered 5-aminolaevulinic acid (5-ALA) as their drinking water (2mg/ml) for five weeks, to induce porphyria. 5-ALA was provided fresh every 2-3 days and drinking bottles protected from light to minimise degradation.

Animals were killed by a schedule 1 method. On culling, livers were removed and immediately frozen in liquid nitrogen for porphyrin analysis and DNA preparation.

2.1.3 Induction of Porphyria in BL/10.D2n Congenic Mice

BL/10.D2 congenic mice have a DBA/2 H-2 complex bred onto a C57BL/10 background and were used to study the influence of any H-2 linked genes on the development of porphyria. Mice were induced by HCB in the presence of iron overload (experiment A) or by iron overload alone (experiment B).

Animals:

Male C57BL/10ScSn and DBA/2 strains were puchased at 7-10 weeks old.

Reagents:

- Iron as iron-dextran (100 mg Fe and 100 mg dextran/ml solution)
- HCB (Organic analytical grade) with no traces of TCDD or related compounds detectable by mass spectrometry.[6]

A - Induction of porphyria by HCB and iron-overload in C57BL/10ScSn and BL/10.D2n mouse strains

Male mice of each strain; C57BL/10ScSn and BL/10.D2n, at 7-10 weeks after weaning, were induced for experimental porphyria with iron and HCB. [6] Treated mice were dosed subcutaneously with iron-dextran (600mg/kg) and

after three days were fed RM1 diet containing HCB (0.02% in corn oil) for seven weeks. Controls from each strain were fed RM1 for seven weeks. Mice were killed by a schedule 1 method and livers saved for porphyrin analysis.

B - Induction of porphyria by iron-overload in C57BL/10ScSn and BL/10.D2n mouse strains

Male mice from strains; C57BL/10ScSn, DBA/2 and BL/10.D2n, at 7-10 weeks old, were induced for experimental porphyria by iron overload alone.[8] Treated mice were dosed subcutaneously with iron-dextran (600mg/kg). All mice, including controls, were fed diet RM1 for six months. Mice were killed by a schedule 1 method and livers retained for porphyrin analysis.

2.1.4 Induction of Cytochrome P450 Isozymes in C57BL/10ScSn and DBA/2 Mice with β-Nathoflavone and Phenobarbital

To compare cytochrome P450 induction in the strains and crosses dosed with HCB, a control experiment in which the strains C57BL/10ScSn and DBA/2, were induced with classical P450 inducing compounds; β-napthoflavone and phenobarbital was used to induce the cytochrome P450 isoenzymes 1A1, 1A2 and 2B1.

Animals:

Male mouse strains C57BL/10ScSn and DBA/2, were purchased at 7-10 weeks old.

Reagents:

- β-Napthoflavone was dissolved in corn oil (10mg/ml) by warming.
- Phenobarbital was prepared from sodium phenobarbitone (5mg/ml) dissolved in sterile saline.

Treatment:

At approximately 7-10 weeks of age, three groups of male mice of both strains were given intraperitoneal injections of corn oil, β-napthoflavone and phenobarbital, at a concentration of 100mg/kg. The phenobarbital dosed group received two doses of 5mg/ml on two consecutive days. Animals were killed by

a schedule 1 method, after two days. Livers were removed and frozen immediately in liquid nitrogen for microsome preparation.

2.2 Analysis of Hepatic Porphyrin Concentration

To determine whether the mouse strains or crosses had developed porphyria after treatment with either HCB and iron, or iron and 5-ALA, the concentration of hepatic porphyrins was measured by a specrsfluorimetric method based on the matrix method of Grandchamp *et al.* [114]

Reagents:

- Acid mixture: 1:1 (v/v) Absolute ethanol:1N perchloric acid prepared fresh for each assay.
- 100nM Standard Solutions of coproporphyrin, uroporphyrin and protoporphyrin: 100nM solution of coproporphyrin was prepared from a 5μg standard vial of coproporphyrin dissolved in 0.1N HCl.

Porphyrin Assay:

Frozen livers were defrosted if necessary and stored on ice prior to homogenisation. Approximately 0.5g of liver was used to make a 20% homogenate in distilled H_2O. Liver samples were homogenised with an Ultraturrex homogeniser.

0.5 ml of homogenate was diluted 1:10 with the acid mixture, to a volume of 5ml. Homogenates were kept on ice until added to the acid mixture and kept in the dark as much as possible.

The homogenate/acid mixture was centrifuged at 1200g for 15 minutes at 4°C.

3 ml of the supernatant was decanted and used for fluorimetric analysis. The porphyrin analysis was performed on a Perkin Elmer Luminescence Spectrometer (LS 50B).

Initially, the fluorescence emission of three 100nM standard solutions of coproporphyrin, uroporphyrin and protoporphyrin were measured to obtain values for a matrix required for subsequent calculation of each porphyrin in the sample. (Table 2.1) A standard solution of 100nM coproporphyrin was used to check the calibration of the matrix prior to all assays thereafter.

The fluorescence of the supernatants was recorded at three different excitation and emission wavelengths (Table 2.1) using a computer program supplied by Perkin Elmer. The acid mixture was used as a blank and for sample dilution. Samples with a high porphyrin content commomly had to be diluted 100-fold.

Table 2.1 Fluorescence of coproporphyrin, uroporphyrin and protoporphyrin standard solutions (100nM)

Excitation λ (nm)	Emission λ (nm)	Fluorescence of Standard Solutions 'Matrix'		
		Copro	Uro	Proto
400	595	448.7	69.5	69.5
405	595	406.3	99.7	99.7
410	605	168.0	164.1	164.1

To determine the concentration of each porphyrin in the mixture from the supernatant, the fluorescence readings from each sample were multiplied by the inverse of the matrix formed by the three standard solutions.

$$\begin{pmatrix} C_c \\ C_u \\ C_p \end{pmatrix} = \begin{pmatrix} \text{inverse of} \\ \text{Matrix} \end{pmatrix} \begin{pmatrix} F_1 \\ F_2 \\ F_3 \end{pmatrix}$$

Where C_c, C_u and C_p represent the concentrations of coproporphyrin, uroporphyrin and protoporphyrin, respectively and $F_{1\text{-}3}$ are the fluorescence readings of the sample

The porphyrin concentrations were calculated, using an Excel spreadsheet, by multiplying the sample fluorescence data with the inverse of the standard solution fluorescence matrix .

2.3 Preparation of Microsomes from Mouse Liver

Microsomes were prepared by ultracentrifugation of the 9,000 g supernatant (S9 fraction) produced from liver sample homogenates.

Reagents:

- 0.25M Sucrose
- 10mM Tris.HCl (pH 7.4), 154mM KCl
- 10mM Tris.HCl (pH 7.4)

Protocol:

Approximately 0.5g of mouse liver was used for each preparation. A 20% homogenate was prepared in 0.25M sucrose, the tissue was homogenised thoroughly using an Ultraturrex homogeniser.

Homogenates were centrifuged at 9,000g for 30 minutes at 4°C. The supernatants (S9 fraction) were decanted into clean ultracentrifuge tubes (Beckmann) and balanced with sucrose, before centrifuging at 200,000g for 30 minutes at 4°C, in a Beckmann Optima ultracentrifuge.

The lipid layer on the surface of the supernatant (cell cytosol) was removed by suction, before the supernatant was decanted from the pellet. The microsome pellets were resuspended *in situ* in 10mM Tris.HCl pH 7.4, 154mM KCl, using the homogeniser and centrifuged again, as above.

The final microsome pellets were resuspended in 1ml of 10mM Tris.HCl pH7.4 as before. 250μl aliquots of the microsomes were snap frozen in liquid nitrogen and stored at -80°C.

2.4 Protein Estimation

The protein content of microsomes was estimated using the Bicinchoninic Acid Protein Assay (Sigma) adapted for microtitre 96-well plates.

Reagents:

- Protein determining reagent: 50:1 (v/v) Bicinchoninic acid solution:(4%) Copper(II) sulphate pentahydrate

- Bovine serum albumin (1mg/ml) standard vials.

Protocol:

Samples for analysis were diluted 1:10 with distilled H_2O. 10µl of each aliquoted in 96-well plate along with a distilled H_2O blank. (11 samples per plate, 8 replicates)

Bovine serum albumin was used to prepare a set of concentrations from 0.1 to 1mg/ml in distilled H_2O, which were then diluted 1:10 and aliquoted in 10µl aliquots as above.

The required amount of protein determination reagent was prepared (about 20ml per plate). 200µl of protein determining reagent was added per sample and the reagents mixed by aspirating before being incubated at 37°C for 1 hour.

Absorbance was measured at 540nm using a Labsystems iEMS microtitre plate reader.

A standard curve was plotted from the BSA concentrations, which was used to calculate the protein content of the microsome samples.

2.5 Estimation of Non-Haem Iron in Tissue

Non-haem iron in liver samples was estimated using a method based on the method of Torrence and Bothwell. [115]

Reagents:

- Acid reagent: 20% Trichloroacetic acid/ 6N HCl
- Chromogenic reagent: Bathophenanthrolinedisulphonic acid (1mg/ml), 1% Mercaptoacetic acid (10mg/ml).
- Colour reagent: 100ml of 50% sodium acetate 10ml chromogenic reagent.
- Iron standard solution (1mg/ml)

Protocol:

To 150µl of 20% liver homogenate in a 0.5ml microfuge tube (prepared as in the porphyrin assay protocol) an equal volume of acid reagent was added and then mixed by votexing and incubated at 65°C overnight. Each sample was

assayed in duplicate and a distilled H_2O blank included. Samples were centrifuged at 10,000g for 5 minutes.

A set of standard concentrations from 5 to 25 μg/ml was prepared by diluting the iron standard solution in distilled H_2O.

100μl of each sample's supernatant, blank and standard were diluted 1:10 with colour reagent in a final volume of 1ml and incubated at room temperature for 10 minutes before measuring absorbance at 540 nm. Samples should have an optical density between 0.1 to 1 OD units.

NB: For iron loaded tissue, 20μl supernatants were diluted 50-fold into a final volume 1ml, with 1:1 distilled H_2O:acid reagent. 100ml of the dilute sample was then dilute 1:10 with the colour reagent. This is necessary to keep the absorbance in scale.

Iron concentrations in μg iron per gram liver were calculated by subtracting the blank reading from the sample before reading from the iron standard curve.

2.6 Hepatic Microsomal *O*-Dealkylase Fluorimetric Assay

Activity of liver microsomal cytochrome P450 isozymes 1A1, 1A2 and 2B were measured by a direct fluorimetric assay . Enzyme activity was measured as the rate of O-dealkylation of 7-ethoxyresorufin yielding resorufin. 7-Ethoxyresorufin was used as a substrate of CYP1A1 activity], 7-methoxyresorufin as a substrate of CYP1A2, and 7-benzyloxyresorufin and 7-pentoxyresorufin as substrates for CYP2B(1). [116-119]

Reagents:

- 100mM Tris.HCl (pH8.0) filtered through a 0.2μm filter.
- 30mM Resorufin stock, dissolved in dimethyl-sulphoxide (DMSO).
- Substrate stocks: 0.6mM 7-ethoxyresorufin
  1.5mM 7-benzyloxyresorufin
  0.6 mM 7-methoxy-resorufin
  3.0mM 7-pentoxyresorufin

All substrates were dissolved in (DMSO).

- 3.2mM β–NADPH prepared fresh and stored on ice at all times.

Assay:

100μl of microsome suspension, stored on ice during the assay, and 10μl of substrate were added to 3ml of tris buffer in an acrylic fluorimeter cuvette.

The reaction was started by stirring in 100μl of NADPH (100μM final concentration) and was run at approximatley 25°C. A Perkin Elmer luminesence spectrophotometer (LS 50B) was used to record the rate of increase in fluorescence.

Fluorimeter Settings:

Excitation	λ/nm = 540nm	slit width 2.5 nm
Emission	λ/nm = 585nm	slit width 20 nm

A standard curve was prepared from a stock solution of resorufin [30mM] in DMSO. The final concentration of standards prepared were 0, 0.02, 0.04, 0.06, 0.08 and 0.1mM. Excitation and emission wavelengths were set as above. Two curves were prepared one with 100μl of microsomes and one without. It was observed that fluorescence was quenched by approximately 20% on addition of microsomes. This standard curve was used for subsequent calculations (unless less than 50μl of microsomes was used).

Rates of dealkylation (as pmol of resorufin formed/minute/mg of protein) were calculated from the standard curve, as the change in fluorescence is directly proporptional to the change in concentration of resorufin.

2.7 Isolation of Genomic DNA

Genomic DNA was prepared for microsatellite analysis by two main methods, phenol-chloroform extraction [120] or by QIAamp Tissue Kit (Qiagen).

2.7.1 Phenol-Chloroform Extraction of Genomic DNA from Mouse Liver

Reagents:

- SE buffer: 150mM NaCl, 100mM EDTA (pH 8.0), sterilised by autoclaving
- 10% SDS
- Proteinase K (20mg/ml)
- Phenol:chloroform:isoamyl alcohol (25:24:1)
- TE buffer (pH8.0): 10mM Tris.HCl (pH8.0), 1mM EDTA, sterilised by autoclaving.

Protocol:

Approximately 30mg of liver was homogenised with 600ml of SE buffer, in sterile 1.5 ml tubes. 60μl of 10% SDS and 6μl of proteinase K were added and the samples incubated overnight at 37°C or for 2-3 hours at 55°C. Samples were chilled on ice before extraction with 500μl phenol:chloroform:isoamyl alcohol, vortexed briefly and centrifuged in a bench microfuge at full speed for 2-3 minutes to separate the two phases. The aqueous layer was carefully removed to a clean tube without the debris. 500μl of chloroform:isoamyl alcohol (24:1) was used to extract any remaining phenol and removed as above. The aqueous layer was transferrred to a clean tube, 400μl of isopropanol added and the mixture shaken to precipitate the DNA. The precipitate was pelleted by centrifuging for 30 seconds, the supernatant removed and the pellet washed in 80% ethanol, before drying. DNA was resuspended in 200 -500μl of TE buffer. DNA yields were determined by UV spectroscopy. (Section 2.8)

2.7.2 Preparation of Genomic DNA from Mouse Tails Using the 'QIAamp Tissue Kit'

Reagents:

- QIAamp Tissue kit:
  - Buffer ALT
  - Reagent AL1
  - Buffer AL (Reagent AL2)
  - Buffer AW
  - Proteinase K (25mg)
  - QIAamp spin columns
- Absolute ethanol

Protocol:

Manufacturer's reagents were prepared, according to the protocol as follows. The lyophilised proteinase K was dissolved in 1.4ml of distilled H_2O to a concentration of approximately 18mg/ml. Buffer AL was prepared by decanting all of reagent AL1 into buffer AL (Reagent AL2) and mixed thoroughly by shaking. Then 210µl of absolute ethanol was added to 200µl of buffer AL. 52ml of absolute ethanol was added to buffer AW and mixed.

Two pieces of tail (equilibrated to room temperature) 0.4-0.6 cm in length were cut into small pieces and placed into a 1.5ml microfuge tube with 180µl of buffer ALT. 20 µl of proteinase K was added, the mixture vortexed and incubated in water bath at 55°C until the tail had completely lysed (about 2-3 hours). To disperse the sample, tubes were vortexed every 30 minutes.

Samples were centrifuged to pellet the bones and hair and 200µl of supernatant transferred to a new 1.5ml microfuge tube. 410µl of AL buffer/ethanol mixture was added and mixed thoroughly by vortexing.

The mixture was applied to a QIAamp spin column and centrifuged at 6000g for 1 minute. The filtrate was discarded and 500µl of AW buffer added to the column and centrifuged as above. This step was repeated, with a final 2 minute spin at full speed to remove all traces of ethanol.

The QIAamp columns were placed in new 1.5ml tubes for elution of the DNA in two extractions with 200µl of distilled H_2O preheated to 70°C. The tubes were centrifuged at 6000g for 1 minute to elute the DNA. DNA yields were determined by UV spectroscopy. (Section 2.8)

2.8 Determination of Nucleic Acid Yield and Purity

DNA and RNA yields and purity were determined by measuring absorbance of a dilute sample at 260nm and 280nm in quartz cuvettes.[120] The reading at 260nm allows calculation of the amount of nucleic acid present. An OD of 1 corresponds to 50µg/ml of double stranded (ds) DNA, 40µg/ml of RNA or single stranded (ss)DNA and 20µg/ml oligonucleotide.

The samples were diluted 1:100 in 1ml of distilled H_2O, which was also used as a blank.

Readings were taken at 260nm and 280nm, an absorbance between 0.1 and 1 was taken to be accurate, and samples were diluted when necessary.

Calculation: OD_{260nm} x dilution factor x 50 = µg/ml of ds DNA

Purity of a sample was determined by calculating the ratio of absorbance at 260nm to 280nm (260nm/280nm). Pure DNA or RNA was taken to have a ratio of 1.7 to 2.0.

2.9 Precipitation and Resuspension of Nucleic Acids

DNA, RNA or oligonucleotide samples which required purifying or concentration alteration were resuspended in a smaller volume, by precipitation with absolute ethanol and sodium acetate.[120]

Reagents:

- 3M sodium acetate, pH5.2 (DEPC-treated if required for use with RNA, section 2.11.1)
- Absolute ethanol

To the sample was added 3 volumes of absolute ethanol and mixed well by pipetteing. The 3M sodium acetate was added to a final concentration of 0.3M and mixed. The sample was the placed on ice for at least 30 minutes, before centrifuging for 5 minutes at 4°C, in a microcentrifuge at 12,000g. The supernatant was carefully removed avoiding disturbing the pellet, which was then resuspended in the required volume of either distilled H_2O, DEPC-treated H_2O or TE buffer.

2.10 Preparation of *cyp1a1* cDNA probe

The cDNA probe for *cyp1a1* cloned from the C57BL/6N mouse strain was puchased from ATCC (Rockville, Maryland, USA). The *cyp1a1* and *cyp1a2* nucleotide sequences are 68% homologous, with segements of high homology interspersed with regions of low homolgy, for this reason the probe was used to detect both *cyp1a1* and *cyp1a2* transcripts.[121,122] The probe was supplied as a freeze-dried culture of *E.coli* containg *cyp1a1* as a 1.2kb insert in a pUC18 plasmid.

Restoration of Culture Conditions

To extract the *cyp1a1* probe from the plasmid, a viable culture was first restored as a liquid culture, according to the suppliers protocol.[123]

Reagents:

- L-Broth; 10g bacto-tryptone, 5g bacto-yeast extract, 10g NaCl in 1litre of dH_2O, (pH 7.0) autoclaved to sterilie
- Ampicillin stock solution (50mg/ ml)

To restore the culture, 0.5ml of L-Broth was mixed with the pellet. This was then transferred asceptically to 5ml of the media containing 5μl of ampicillin to give a final concentration of 50μg/ml, in a sterile univrsal. The culture was incubated at 37°C in a shaking incubator, overnight.[120]

Plasmid DNA Extraction

pUC18 plasmid DNA was prepared from the liquid culture by the alkaline lysis method [120].

Reagents:

- Solution I; 50mM glucose, 25mM Tris.HCl (pH8.0), 10mM EDTA (pH8.0), autoclaved.
- Solution II; 0.2N sodium hydroxide,1% SDS, prepared fresh each time.
- Solution III; 60ml of 5M potassium acetate, 11.5ml of glacial acetic acid and 28.5ml of distilled H_2O.
- Phenol:chloroform:isoamyl alcohol (25:24:1) v/v.
- Absolute ethanol.
- 70% Ethanol.
- TE buffer (pH8.0); 10mM Tris.HCl (pH8.0),1mM EDTA, autoclaved.

Protocol:

The culture was transferred by sterile pasture pipette into a sterile 1.5ml microfuge tubes and pelleted by centrifugation at 12,000g in a microcentrifuge at 4°C in a cold room.

Supernatants were removed by careful pipetting, so as not to disturb the pellet. The pellets were then resuspended in 100µl of ice-cold solution I and vortexed. 200µl of ice-cold solution II was added and mixed by inverting the tube several times. Tubes were stored on ice after this step.

150µl of ice-cold solution III was added and the tubes vortexed to disperse the viscous lysate. The tubes were centrifuged at 12,000g for 5 minutes at 4°C. Supernatants were transferred to a clean tube and an equal volume of phenol:chloroform added (~500µl). The mixture was vortexed and centrifuged as above for 2 minutes. Supernatants were transferred to a clean tube.

The plasmid DNA was precipitated with 2 volumes of ethanol (room temperature) and vortexed. Tubes were left to stand at room temperature for 2 minutes, then centrifuged as before for 5 minutes.

The supernatant was discarded and the pellet left to drain by standing the tube upside down on tissue.

The DNA was washed in 1ml of 70% ethanol (4°C), and removed as above.

The pellet was allowed to air dry for about 10 minutes, before redissolving in 50µl of TE. Yield was determined by absorbance at 260 and 280nm. (Section 2.8)

Excision of *cyp1a1* cDNA from pUC18

The 1.2kb *cyp1a-1* insert was excised by digesting the plasmid DNA with the restiction enzyme Pst I. [120]

Reagents:

- Pst I (15units/μl) (Pharmacia)
- 10X One-Phor-All Plus restriction digest buffer (Pharmacia)
- RNAase 1A (1mg/ml) (Pharmacia)
- 0.5 EDTA (pH8.0) autoclaved

The following components were placed in a 1.5ml sterile microfuge tube: 20μl plasmid DNA (~2μg), 5μl of One-phor-all buffer, 5μl RNAase 1A, 1μl Pst I and 19μl of distilled H_2O.

Reactions were mixed by tapping the tubes and incubated at 37°C overnight before being stopped by addition of 1μl of 0.5M EDTA solution. The restriction digest products were separated by electrophoresis on a 0.6% low-melting point agarose gel. (Section 2.14.3)

Purification of *cyp1a1* 1.2kb insert

Once the 1.2kb insert band was excised from the gel, the agarose the cDNA insert was contained in was removed. As a cheaper alternative to a commercial kit, the band was purified by spinning through a sterile filtered tip. The excised band was placed in the top of a sterile 200ml filter-tip, with the very end of the tip removed with a sterile scalpel so as to fit a 1.5ml microfuge tube. The tip was then placed into a sterile 1.5ml microfuge tube and centrifuged at 1,000g for 10 minutes in a microcentrifuge, which pelleted the DNA whilst the agarose remained above the filter.[124]

2.11 Isolation of Total RNA from Mouse Liver

Total RNA was extracted from liver tissue either by ultracentrifugation through a caesium chloride gradient, [121] or by using 'RNAzol-B' RNA isolation solution. (BioTecx,USA)

All glass and plasticware was treated to remove RNAase. Non-disposable plasticware was treated with 3% H_2O_2 before use for 10-15 minutes. Glassware was treated with diethlypyrocarbonate (DEPC) by stirring a 0.1% solution in distilled H_2O overnight, then autoclaved to remove remaining DEPC.
Livers required for RNA preparation were removed as quickly as possible, snap frozen in liquid nitrogen and used immediately or stored at -80°C .

2.11.1 Total RNA Preparation by Ultracentrifugation

Reagents:

The following solutions did not requiring DEPC treatment:

- 4M guanidium thiocyanate/0.1M Tris.HCl (pH 7.5)
- β-Mercaptoethanol
- 70% ethanol (made with DEPC-treated distilled H_2O)

The remaining solutions were treated with 0.1% DEPC and stirred overnight, before autoclaving:

- 20% N-lauroylsarcosine (Sarcosyl)
- 5.7M Caesium Chloride, 0.1M EDTA (pH7.5) - the density should not exceed 1.7g/ml.
- distilled H_2O.

Protocol:

Approximately 0.5g of frozen liver was added to 7.5ml 4M GITC/0.1M Tris and 525μl β-mercaptoethanol in a sterile 50ml Falcon tube, then homogenised thoroughly with an Ultrturrex homogeniser which had been cleaned and rinsed in GITC/Tris solution to remove and RNAase. 200μl of 20% sarcosyl was added to give a final concentration of 5%. The homogenate was carefully layered over 4.5ml of 5.7M CsCl/0.1M EDTA. The gradients were prepared in clean thin wall polyallomer tubes. Samples were centrifuged at 150,000g in an SW40 ultracentrifuge rotor, for 18 hours at 20°C.
The RNA pellet at the bottom of the tube was removed after tipping off the supernatant and cutting off the last 2cm of the tube. The pellet was washed out in absolute ethanol and transferred to a sterile 1.5ml tube.

RNA was pelleted and washed for a second time in 70% ethanol. For long term storage, RNA was left under ethanol at -80°C.

For concentration determination (section 2.8) and further use the RNA pellet was resuspended in 0.5-1.0 ml DEPC treated H_2O. Resuspension usually required a freeze-thaw cycle as the concentration of RNA was high.

2.11.2 Total RNA Preparation using 'RNAzol B'

Reagents:

- 'RNAzol B'
- Chloroform
- Isopropanol
- 75% ethanol in DEPC treated H_2O

Manufacturer's Protocol:

100mg of liver tissue was homogenised in 2 ml 'RNAzol B', using an ultraturrex homogeniser, in a sterile, RNAase free tube. 0.2 ml of chloroform was added to the homogenate, the tube covers and shaken vigorously for 15 seconds, before placing on ice for 5 minutes. The suspension was centrifuged for at 12,000g for 15 minutes at 4°C, to separate it into two layers. The upper aqueous phase (approximately 1ml) was removed from the blue organic phase, by pipetting, and placed in a fresh tube. An equal volume of isopropanol was added to the sample, before placing on ice for a further 15 minutes and centrifuged again as above, to pellet the RNA. The supernatant was removed and the pellet washed in approximately 1ml of 75% ethanol by vortexing and subsequent centrifugation for 8 minutes at 7,500g at 4°C. The pellets were dried briefly in a vacuum drier, before resuspending in 200-500µl of DEPC-treated distilled H_2O depending on the amount of RNA. To resuspend very large quantities, the sample was incubated at 60°C in a water bath. Yields were determined by measuring absorbance at 260 and 280 nm.(Section 2.8)

2.11.3 Preparation of messenger RNA

Messenger RNA (mRNA) was purified from total RNA with Oligotex-dT latex beads. (Qiagen)

Reagents:

- Oligotex-dT suspension
- 2X Binding Buffer; 20 mM Tris.HCl (pH 7.5), 1M NaCl, 2 mM EDTA, 0.2% SDS,
- Wash Buffer; 10 mM Tris.HCl (pH 7.5), 150mM NaCl, 1mM EDTA
- Elution Buffer; 5 mM Tris.HCl (pH 7.5)

Solutions were treated with DEPC prior to making the final buffer. Tris.HCl was made with DEPC treated and autoclaved water. All solutions were autoclaved before use.

Protocol:

250μg of total RNA was added to DEPC treated water in an RNase-free 1.5 ml tube to give a volume of 250μl. 15μl Oligotex-dT suspension and 250μl 2X binding buffer were then added.

The reagents were mixed by gently flicking the bottom of the tube, then incubated at 65°C for 3 minutes, to disrupt any secondary structures. The sample was then left at room temperature for 10 minutes to allow hybridisation of polyA mRNA to the oligo-$(dT)_{30}$ latex beads.

The oligotex beads were pelleted by centrifugation at full speed in a microcentrifuge for 2 minutes and the supernatant carefully removed so as not to disturb the pellet.

The pellet was resuspended in 400μl of wash buffer and transferred onto a spin column. The column was centrifuged as above for 30 seconds and the eluate collected in a 1.5 ml RNase-free tube. This step was repeated with 400μl of wash buffer.The mRNA was eluted off the column in two 20μl volumes of preheated elution buffer (70°C).

Messenger RNA was quantified by absorbance at 260nm and 280nm (section 2.8) and stored at -80°C.

2.11.4 Northern Blotting

Total or mRNA was for northern hybridisations was fractionated on denaturing agarose gels containing formaldehyde. Gels were subsequently blotted to transfer the RNA to nylon membrane to be probed. [120]

Fractionation of RNA on Formaldehyde Containing Agarose Gels

Reagents:

- UltraPure agarose
- 3% Hydrogen peroxide
- 10X MOPS buffer: 0.2M MOPS, 80mM sodium acetate (pH7.0), 10mM EDTA, treated with 0.1% DEPC,
- Denaturing buffer (prepared fresh each time); 345µl formamide, 20µl formaldehyde (pH<4.0), 35µl 10X MOPS, 3µl ethidium bromide [10mg/ml],
- RNA loading buffer; 50% glycerol, 40mM EDTA, 0.2% bromophenol blue, 0.2% xylene cyanole, made with DEPC-treated distilled H_2O.
- RNA molecular weight ladder (9kb-0.24kb fragments)

Preparation of Agarose Denaturing Gels:

1% denaturing agarose gels were made by adding 57.7ml DEPC-treated water and 8ml 10X MOPS to 800mg of agarose and dissolved by boiling. 14.3ml of formaldehyde was added after the gel had cooled to below 60°C.

Gel casting trays, well combs, the electrophoresis tank and peristaltic pump tubing was cleaned with 3% H_2O_2 and rinsed in DEPC-treated distilled H_2O before use.

10µg of total RNA or 1µg of mRNA in a volume of 5.5µl was loaded per sample. RNA ladder was used as a size marker, 3µg of ladder was loaded per gel. Each sample (and the ladder) was denatured with 14.5 µl of denaturing buffer and

heated to 65°C for 15 minutes. Samples were chilled on ice and 3μl of loading buffer added prior to loading. Gels were either run overnight at 22V or for 4-5 hours at ~70V, with the buffer circulated by peristaltic pump.

Northern blotting of RNA to Nylon Membrane

RNA was transferred to nylon membrane immediately after electrophoresis by capillary action [120].

Reagents:

- Hybond-N nylon membrane (Amersham)
- 20X Standard Sodium Citrate (SSC): 3M sodium chloride, 0.3M sodium citrate

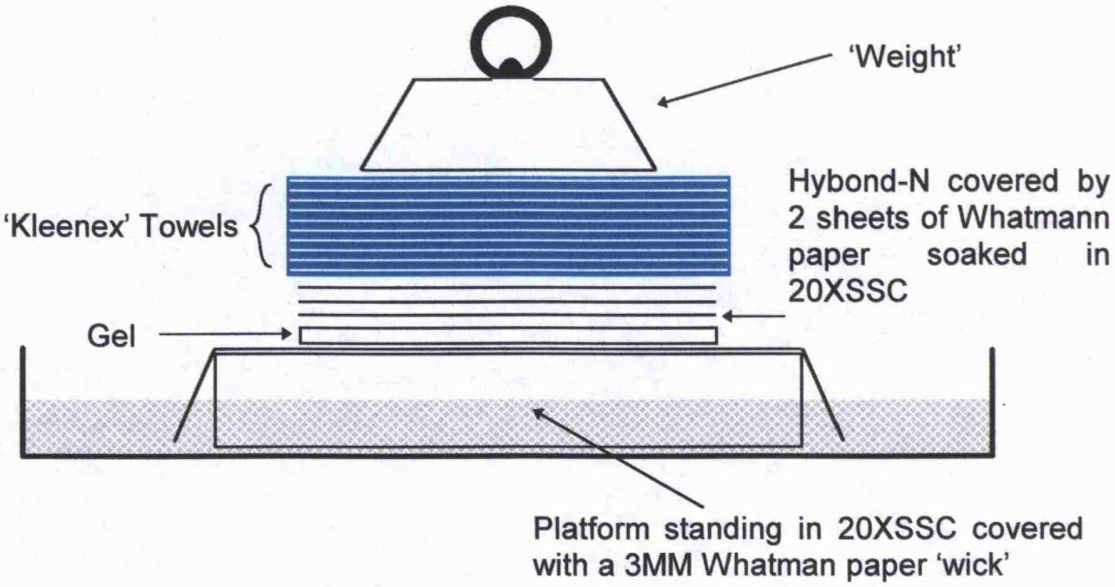
Protocol:

Gels were rinsed in DEPC-treated distilled H_2O and turned upside down to blot the flatter surface. The gel was placed on a platform covered with a square of 3MM Whatmann paper (Whatmann) to act as a wick and the platform was placed in a tray filled with 20X SSC. (Figure 2.1) The gel was covered with a piece of Hybond-N cut to the same size, then with two pieces of 3MM paper soaked in 20X SSC over the membrane. Kleenex paper towels are then stacked on top of the 'sandwich' covered with a glass plate and a heavy weight placed on top. This system draws up the SSC and RNA by capillary action.

Blots were left overnight, then dismantled and the RNA fixed to the membrane by cross-linking with UV irradiation or baking in an oven at 80°C for 2 hours.

Blots were stored dry at room temperature before hybridisation.

Figure 2.1 Diagram of Capillary Northern Blotting Apparatus



2.12 Preparation of cDNA and PCR probes and Hybridisation Conditions

Probes for hybridisation were labelled with α-^{32}P-dCTP either by random priming of the probe or by internal incorporation of the labelled nucleotide in a PCR product.[120]

2.12.1 Labelling of *cyp1a-1* probe by Random Priming

Reagents:

- 'Megaprime' random priming kit (Amersham)
  - Random hexamer primers
  - Labelling buffer
  - DNA polymerase I (Klenow fragment)
- α-^{32}P-dCTP, specific activty 3000mCi/mM (Amersham)
- 50X Denhart's reagents; 1% Bovine serum albumin, 1% polyvinylpyrrolidone, 1% Ficoll 400

- Prehybridisation solution: 50% formamide (deionised with mixed bead resin), 5X Denhart's reagent, 6X SSC
- 0.2M EDTA autoclaved
- NAP-5 sephadex columns (Pharmacia)
- TE buffer (pH8.0), autoclaved

Protocol:

Membranes were prehybridised in 15ml of prehybridisation solution for 2-4 hours at 42°C in a Techne hybridisation oven.

Approximately 25ng of cDNA in a 5μl volume, was required for labelling by random priming. To this was added 5μl of random hexamer primers (provided with the Megaprime Kit), in a sterile 1.5ml screw-top microfuge tube. The cDNA probe and primer mixture was denatured for 5 minutes by boiling in water bath, then cooled to room temperature to allow the primers to anneal. 10μl of labelling buffer, 23μl of distilled H_2O and 2μl (10 units) of DNA polymerase I (Klenow fragment) were added and finally, 5μl of α-^{32}P dCTP. The mixture was incubated at 37°C for 10 minutes and the reaction was stopped by addition of 5μl of 0.2M EDTA.

Non-incorperated isotope was removed by purification of the labelled probe through a NAP-5 column. The column was equilibrated with approximately 10ml of TE buffer before applying the probe. The probe was then eluted in 10 x 200μl aliquots of TE. The first few fractions with highest activity were pooled and the activity of 1μl of pooled probe measured by scintillation counting.

The probe was denatured by boiling for 5 minutes at 100°C, chilled on ice for 5 minutes, then $1x10^6$ cpm per ml of prehybridisation solution added. The hybridisation was carried out at 42°C overnight.

2.12.2 Incorporation of α-^{32}P dCTP by PCR into *Urod* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes

Probes were also required to detect *Urod* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs. The latter was used to check for equal loading and integrity of the RNA used in each northern blot. These probes were

labelled by internal incorporation of α^{32}P-dCTP into the amplified product by PCR.[125]

Reagents:

- PCR reagents:
  - 10X AmpliTaq PCR buffer
  - AmpliTaq DNA Polymerase
  - 10X nucleotide mix for cold reactions; 2mM dCTP, dATP dGTP dTTP.
  - 10X nucleotide mix for hot reactions; 0.125mM dCTP and 2mM of dATP dGTP, dTTP.
  - PCR primers (25 pmoles/μl)
- QIAquick Kit (PCR product purification kit)

All PCR buffers and solutions were made with autoclaved distilled water and prepared as cleanly as possible to avoid contamination with DNA.

Protocol:

Probes were made from a cDNA template (section 2.16). The target sequence was amplifyed by PCR and a blank control was included with distilled H_2O instead of cDNA to check for contamination. The reaction products were run out on a 0.8% agarose gel (section 2.13.3) and excised (section 2.10 p.53).

Approximately 20ng of this product (about 1μl) was used as a template for a second round of PCR, identical to that above except a hot dNTP mixture was used and 5μl of α-^{32}P-dCTP incorporated into the reaction. Hot PCR reactions were run on a Biometra Trio-block cycler.

The hot PCR product was purified using a QIAquick PCR purification kit. (Section 2.14 p.71). The probe was counted and denatured as above and $1x10^6$cpm per ml of prehybridisation solution added and hybridised overnight at 42°C.

Removal of nonspecificly hybridised probe from membranes

After hybridisation, nonspecifically hybridised probe was removed from the filter by successive washings in 50ml of SSC. The concentration of SSC used, the length of time and temperature of each wash depended on the probe. The progress of each wash was monitored with a hand-held Geiger counter, until

the blot reached approximately 10-50 counts per second. The washing conditions for the *cyp1a1* and GAPDH probes are given below. The conditions for the *Urod* probe are given in chapter 7.

Washing conditions for the *cyp1a1* cDNA probe

First and second wash; 2XSSC, 0.1% SDS for 15 minutes at room temperature.Third wash, 0.1X SSC, 0.1% SDS for 1 hour at 55°C.

Washing conditions for GAPDH probe

First and second wash; 2XSSC, 0.1% SDS for 15 minutes at room temperature. Final wash 1xSSC, 0.1% SDS for 45 minutes at 55°C.

Autoradiography

Membranes were autoradiographed by exposure to X-ray film (Amersham) at -80°C overnight (or longer depending on probe) or by exposing to Phosphorimager intensifying plates and analysed with ImageAnalysis software (Molecular Dynamics).

2.13 Microsatellite Analysis

Mouse MapPair PCR primers were purchased from Research Genetics, USA, the UK Human Genome Mapping Project (UK-HGMP) Resource centre, Hinxton, Cambridge, UK or synthesised at the Protein and Nucleic Acid Chemistry Laboratory (PNAC), University of Leicester.

Polymorphism data was obtained from the Mouse Genome Database (MGD) Mouse Genome Informatics, The Jackson Laboratory, Bar Harbor, Maine USA.[96,129,c3] Data on polymorphisms between SWR and DBA/2 microsatellites was supplied by Prof. Drinkwater, McArdle Laboratory for Cancer Research, Wisconsin, USA. A list of the microsatellites used can be found in Appendix I.

2.13.1 Polymerase Chain Reaction

Each marker or microsatellite was amplified for analysis by the polymerase chain reaction (PCR) on genomic DNA.[125]

Reagents:

Reagents for PCR were kept separately from other chemicals. All PCR buffers and solutions were prepared with autoclaved distilled water as cleanly as possible, to avoid contamination with DNA.

- 10 X PCR buffer; 0.1mM Tris-HCl (pH8.3), 0.5mM KCl, 1% gelatin.
- 10 X Magnesium chloride, stocks of 10, 15, 20, 25 and 30mM.

Both of these solutions were filtered through a 0.2μm filter and autoclaved.

- 10 X dNTPs 2mM of each dGTP,dATP,dTTP and dCTP
- PCR primers (6.6μM)
- AmpliTaq DNA polymerase (5units/μl)
- Mineral oil

Protocol:

Mouse genomic DNA was diluted to 50ng/μl. For each amplification reaction 2μl of genomic DNA (100ng) was aliquoted into a sterile 0.5ml microfuge tube. A master mix of reagents was prepared consisting of 1μl of PCR buffer,1μl of magnesium chloride,1μl of deoxynuclotides 1μl of each primer and 0.25 units of Taq for each reaction. Autoclaved distilled water was added to the mix to give a total reaction volume of 10μl per sample. Sufficient master mix was prepared for the number of samples plus a few extra to allow for pipetting errors and to ensure consistancy of reaction components between samples. 8μl of this mixture was added to each sample.

Reactions were overlayed with a drop of mineral oil. Amplification was carried out using a Perkin Elmer 480 DNA thermal cycler. A drop of mineral oil was placed in each well in the heating block to aid heat transfer to the tube.

The thermal cycler was programed with the PCR profile below. The first step ensure that the template DNA suficiently denatured. The second step, repeated 30 times, allows denaturing of the double stranded, amplified product and annealing of the primers to continue a next round of amplification. No elongation step at 72°C was required for most reactions, as the time taken to heat from 55°C to 94°C was sufficient to allow elongation of smaller products. This also shortened the length of time of the entire process.

Basic program:

Step 1 : 94oC for 5 minutes

Step 2 : 94oC for 30 seconds for 30 cycles

55oC for 45 seconds

For markers with allele size differences of less than 10bp, one of each pair of primers was 5' end labelled with γ^{33}P-dATP (section 2.13.2) to allow the products to be separated on denaturing acrylamide gels and visualised by autoradiography. For isotopically labelled or 'hot' reactions Step 2 was repeated for 25 cycles.

Most primer pairs required a magnesium concentration of 1.5mM and annealed at 55°C. Where it was necessary to improve the amplification of a marker a magnesium titration was carried out and the annealing temperature was altered. The magnesium chloride concentration titration required amplifying template DNA with a range of concentrations from 1.0 mM to 3.0 mM to select. Where necessary, the annealing temperature was was raised or lowered to decease the nonspecific annealing of primers and avoid nonspecific amplification or to improve amplification of the product.

2.13.2 5' End - Labelling of Primers

For markers which required isotopically labelling, the forward primer of each primer pair was 5' end-labelled' with γ^{33}P-dATP.[120]

Reagents:

- T4 Polynucleotide Kinase (T4PNK) 10 units/µl (United States Biochemicals)
- 10X Reaction buffer: 0.5M Tris.HCl (pH7.6), 100mM $MgCl_2$, 100mM β-mercaptoethanol (United States Biochemicals)
- Enzyme dilution buffer: 50mM Tris.HCl (pH8.0) (United States Biochemicals)
- γ-^{33}P-dATP specific activity 3000mCi/mmol (Amersham)

Protocol:

100 pmol of forward primer (oligonucleotide) to be labelled in a volume of 16μl was mixed with 2.5μl 10X reaction buffer and 2.5μl of autoclaved distilled H_2O and the reaction mix placed on ice. 1μl of T4PNK was diluted 1:8 with dilution buffer supplied and 2μl added to the reaction. Finally 3μl γ-^{33}P dATP to give a final volume of 25μl. The reaction was incubated for 1 hour at 37°C, heated to 65°C for 10 minutes to inactivated the enzyme.

0.5μl of labelled primer was added per reaction to the PCR mixture, to give a final primer concentration of 3.8μM per reaction. Amplification of 'hot' reactions were carried out in a Biometra 'Trio Block' thermal cycler under the conditions described above.

2.13.3 Gel Electrophoresis

PCR products were resolved by electrophoresis on agarose or denaturing acrylamide gels for analysis depending on the size difference between alleles.[120]

Agarose Gels

Reagents:

- Nusieve or Metaphor agarose (FMC)
- 5X Tris Borate/EDTA electrophoresis buffer (TBE): 450mM Tris.Borate EDTA (pH8.0)
- Ethidium bromide (10mg/ml)
- ΦX174 DNA ladder (HaeIII restriction fragments) (Gibco)
- 6X loading buffer: 50% glycerol, 0.02% xylene cyanole and 0.02% bromophenol blue

Protocol:

Nusieve or Metaphor agarose was used for resolving PCR products, Metaphor agarose could be used to resolve up to around 8-10 base pair differences between allele sizes clearly. Agarose was weighed out according to the percentage and volume of the gel required. Gels of 2-3.5% were required to

resolved most microsatellites with allele sizes of 10bp difference. The agarose was dissolved in 0.5X TBE, by boiling in a microwave.

The flask was cooled to hand hot, before ethidium bromide was added to give a final concentration of 0.5μg/ml. The gel was cast into a prepared gel bed and a well comb inserted. Gels were left to cool for 30 minutes to an hour at room temperature or chilled.

2μl of loading buffer was added to each sample and to 0.5μg of ΦX174 DNA ladder. Samples were loaded, with molecular size ladder in the first lane, and electrophoresed in 0.5XTBE running buffer for 2-3 hours at 100V or until the bands were resolved.

Gels were photographed on polaroid film P53 and P55 where a negative was required.

Acrylamide Gels

Reagents:

- Sequagel 20% acrylamide gel concentrate and Sequagel diluent
- 5XTBE buffer
- 10% ammonium persulphate (APS)
- N,N,N',N'-tetramethylethylenediamine (TEMED)
- 5% Dichlorodimethylsilane in hexane
- Fixer: 1litre prepared with 5% Methanol and 5% acetic acid with distilled H_2O.
- Formamide loading buffer: 95% formamide, 20mM EDTA, 0.02% xylene cyanole and 0.02% bromophenol blue

Protocol:

Radioactively labelled products were separated on 6% denaturing polyacrylamide gels. Gels were cast on large plates (Gibco BRL S2 tanks) to resolve 40 samples on one gel. Plates were cleaned with 70% IMS and one plate was treated with the 5% silane solution, to prevent the gel from adhering to both plates when they were separated. Plates were assembled with 0.4mm spacers and clamped with large bull-dog clips.

70ml of gel was prepared from 16.6ml of gel concentrate, 42.2ml of diluent, 14ml of 5XTBE. Polymerisation was catalysed by addition of 600µl of 10%APS and 25µl of TEMED. The gel was cast horizontally (avoiding air bubbles) the comb inserted and left to set for an hour or overnight. If the gel was poured the day before use the ends of the gel were wrapped in damp tissue, covered with cling film to prevent the gel from drying out.

Gels were run in 1 X TBE buffer and pre-warmed to ~60°C before loading by running at a set power of 60W for an hour. Samples were denatured with 2µl formamide loading buffer and heated to 95°C for 5 minutes, then chilled on ice before loading. Gels were electrophoresed at a set power of 65W for 1 to 3 hours depending on product size.

The plates were then separated, leaving the gel adhered to one plate where it could be fixed. The fixing solution was poured onto the gel and left for approximately 10 minutes, before carefully tipping off the gel. The gel was removed from the plate by blotting on to 3MM Whatmann paper and then covered with saran wrap and dried on a gel drier at 80°C for 45 minutes.

Gels were exposed to X-ray film and autoradiographed for 24 hours or longer if required, at room temperature.

2.13.4 Analysis of *Ahr* Receptor Restriction Fragment Length Polymorphism

A restriction fragment length polymorphism (RFLP) was reported that described a *Eco*47III restriction site polymorphism between C57BL/6 and DBA/2 strains.[67] This RFLP was used to follow the inheritance of the responsive and nonresponsive alleles of the *Ahr* gene in the BL/10 x DBA/2 F_2 crosses. It was also used to check the SWR *Ahr* allele which is reported to be the Ahr^d type.[65]

PCR primer sequences, OL72 and OL111, for amplifying across exon seven of the *Ahr* gene RFLP are given below. [67]:

OL 72 5'-GGTTCGAATTTCCAGGATGG-3'

OL111 5'-CCACCCCAGGTACATGATGGAACC-3'

PCR protocol:

Amplification reactions were prepared essentially as described in section 2.13.1. 100ng DNA of genomic DNA was used as a template in a reaction mix containing; 1X AmpliTaq buffer (which contined 1.5mM $MgCl_2$), 0.1mM dNTPs, 10 pmoles of each primer and 0.5 unit of AmpliTaq Polymerase per reaction. The final reaction volume was 20µl. Reactions were overlayed with mineral oil. Amplification conditions for the primers are given below:

Step 1 - 1 cycle	95°C for 5 min
Step 2 - 35 cycles	95°C for 1 min
	55°C for 1 min
	72°C for 10 seconds
Step 3 - 1 cycle	72°C for 15 mins

Reactions were amplified in a Perkin Elmer DNA thermocycler 480

Restriction Digest:

10µl of PCR product was aliquoted into sterile 1.5ml tubes A master mix containing; 2µl of 10X REact 3 buffer, 8µl of distilled H_2O and 2 units of *Eco*47III [5 units/µl] (Gibco), per reaction, plus extra to allow for pipetting errors, was made. The reactions were incubated at 37°C for 2-4 hours. Products were analysed on 3% metaphor agarose gels.(section 2.13.3)

2.13.5 Statistical Analysis of Microsatellite Inheritance

The inheritance of microsatellie alleles in the F_2 intercross follows that for codominant alleles. Parent strains carry two identical alleles for a particular microsatellite, as they are homozygotes. Heterozygote F_1 hybrids inherit one allele from each parent. Recombination occurs during meiosis in the F_1 gametes, so that F_2 mice receive a random mixture of parent alleles.

For any marker in an F_2 cross, the number of homozygote and heterozygote alleles will follow a ratio of 1:2:1, of homozygote alleles from parent 1:2 heterozygotes (with one of each allele) : homozygotes for allele 2 from the second parent.

In the high and low groups of mice selected for analysis a deviation from this ratio may imply that the marker is linked to a susceptibility gene if it were not inherited by chance.

To test this hypothesis, the statisitcal χ^2 test was used to analyse the genotype data produced from the microsatellite PCRs. The number of BL/10, DBA/2 and heterozygote alleles were counted for each microsatellite typed. Results were entered into an Excel spreedsheet and the χ^2 value calculated using the programs statistical package.

The p-value for this figure was given for 2 degrees of freedom. A p-value of <0.05 (95% confdence limits) is usually taken as there being less than a 5% chance that the marker was inherited along with a susceptibility gene at random. [98,99]

2.14 Synthesis of cDNA

Reverse transcription of mRNA to cDNA was carried out using a '1st-strand cDNA Synthesis' kit.(Clontech)

Reagents

- 1st-Strand cDNA Synthesis Kit (Clontech)
  - 5X Reaction buffer:250mM Tris-HCl (pH8.3), 375mMKCl, 15mMMgCl_2
  - Oligo$(dT)_{18}$ primer (20μM)
  - Random hexamer primer (20μM)
  - Moloney-Murine Leukemia Virus (Recombinant) (MMLV) Reverse transcriptase (200units/μl)
  - Recombinant RNase inhibitor (40units/μl)
  - dNTP mix, 10mM of each
  - control RNA (human placental total RNA)
  - DEPC-treated H_2O

Protocol

Tubes from the kit were thawed, spun down and placed on ice. All dilutions and additions were carried out on ice.

1μg of mouse mRNA and control total RNA were placed in sterile, RNase-free 0.5ml microcentrifuge tubes and DEPC-treated water added to give a volume of 12.5μl.

1μl of a 50:50 mix of random hexamer primers and oligo $(dT)_{18}$ primer (10 pmol of each) was added to the reaction, which was then heated at 70°C for 2 minutes then chilled rapidly on ice.

The following components were then added: 4μl of 5X reaction buffer, 1μl dNTP mix, 0.5μl RNase inhibitor, 1μl Moloney-Murine Leukemia Virus (MMLV) reverse transcriptase. This was prepared as a master mix when there was more than one sample for consistency between reactions and 6.5μl added to each sample. The contents of each tube were mixed by pipetting up and down, then incubated at 42°C for one hour.

Reactions were heat inactivated to stop cDNA synthesis and destroy any DNase activity, then spun down in a microcentrifuge.

The final reaction was diluted by addition of 80μl of DEPC treated water. cDNA was stored at -80°C.

PCR of Reverse Transcription Product (RT-PCR)

PCR from cDNA template was carried out according to the protocol supllied with the Clontech kit using AmpliTaq DNA polymerase and AmpliTaq 10X buffer.

Clontech Protocol:

5-10μl of the diluted cDNA sample was used in a 50μl reaction.

Reaction Components:

Sterile H_2O	36.6 μl
10X PCR buffer	5 μl
dNTP mix (10mM of each)	1 μl
Primers	2 μl
cDNA (1:100)	5 μl

AmpliTaq DNA Polymerase	0.4 units/μl
Total Volume	50 μl

Reactions were overlayed with mineral oil. Thermal cycling was carried out on a Perkin Elmer 480 DNA thermal cycler. Thermal cycling conditions depended upon the primers being used and are given below along with the sequences for the primers used in RT-PCR reactions. Products were electrophoresed on 2% Nusieve agarose.

UROD primer protocol [126]

PCR primers sequence:

Primer A: 5'-TCAATTCTGTCGAAGCAGGCGTGAGTG-3'

Primer B: 5'-CTGGAAGAGCTGGCCCAGGCTGGCTATGAG-3'

A primer concentration of 25 pmol/μl of each was used per reaction.

PCR program:	Step 1	1 cycle	94°C x 5 mins
	Step 2	30 cycles	94°C x 1 mins
			63°C x 30 seconds
			72°C x 2 mins
	Step 3	1 cycle	72°C x 5 mins

GAPDH primer protocol [127]

PCR primers sequence:

Primer A: 5'-ACTGGTGTCTTCACCACCAT -3'

Primer B: 5'-TGCAGGGATGATGTTCTGG-3'

A primer concentration of 25 pmol/μl of each was used per reaction.

PCR program:	Step 1	1 cycle	94°C x 5 mins
	Step 2	30 cycles	94°C x 1 min
			55°C x 1 min
			72°C x 2 mins
	Step 3	1 cycle	72°C x 5 mins

2.15 Cycle Sequencing

The mouse *Urod* RT-PCR product produced (chapter 7) was sequenced by cycle sequencing using the Applied Biosystems system.

PCR Product Purification

PCR products to be sequenced were purified with a QIAquick PCR purification kit (Qiagen).

Reagents:

- QIAquick PCR purification kit
  - QIAquick spin columns
  - Buffer PB
  - Buffer PE (concentrate)
- Absolute ethanol

QIAquick PCR purification protocol:

Buffer PE concentrate was diluted by addition of 42ml of absolute ethanol to the bottle for all subsequent purifications. 5 volumes (250 μl) of buffer PB1 was added to the PCR reaction and mixed. The sample was applied to a spin column, centrifuged for 30 to 60 seconds at 10,000g in a microcentrifuge. The flow through was discarded and the spin column transferred to a clean 1.5ml tube.

The column was washed with 750 μl of buffer PE and centrifuged as above. The column was centrifuged again for 1 minute to remove residual buffer, then transferrred to another tube and the PCR product eluted in 50 μl of distilled H_2O.

Cycle sequencing protocol

Sequencing of purified PCR products was performed by using a Taq DyeDeoxy Terminator Cycle Sequencing kit. (Applied Biosystems) Approximately 2 μg of purified product was sequenced per reaction.

The following reagents were mixed together in a 0.5 mi microcentrifuge tube:

Premix buffer containing: (5X TACS Buffer, dNTP Mix; dyedeoxy A, T, G and C terminators and AmpliTaq DNA Polymerase)	9.5μl
Template (purified PCR fragment)	7.0 μl
Primer (3.2 pmol of forward or reverse)	1 μl
distilled H_2O	2.5 μl
Final Volume	20.0 μl

The reaction mix was overlayed with one drop of mineral oil.

Cycle sequencing was carried out in a Perkin Elmer 480 DNA thermal cycler.

The tubes were placed in the block preheated to 96°C with immediate thermal cycling (25 cycles) of step , according to the following program;

step 1 - 96°C x 30 seconds,
63°C x 15 seconds
60°C x 4 minutes

step 2 - 4°C soak

Sequence Product Purification

After cycling the extension products were purified by phenol:chloroform extraction, pror to loading onto an Applied Blosystems DNA Automated Sequencer.

Reagents:

- Chloroform
- Phenol:distillled H_20:chlorofrom mixture (68:18:14)
- 2M sodium acetate(pH4.5) autoclaved
- Absolute ethanol
- 70% ethanol

Protocol:

80μl of distilled H_2O was added to the reaction mixture and 100μl of chloroform, to dissolve the oil.

The terminators were extracted by addition of 100μl phenol:distilled H_2O:chloroform mix. The sample was vortexed and centrifuged and the upper aqueous phase removed.

The aqueous layer was extracted a second time as above, and the aqueous layer transferred to a clean 1.5ml tube. The extension products were then precipitated by adding 15μl of sodium acetate and 300μl ethanol. The mixture was centrifuged for 15 minutes at room temperature, the pellet washed with 70% ethanol and dried. The sequenced product was analysed on an automated sequencer at the PNAC Laboratory, University of Leicester.

3 - Microsatellite Analysis of Inheritance of Susceptibility to HCB and Iron Induced Porphyria

3.1 Summary

Certain inbred mouse strains are prone to experimental porphyrias implying there is an underlying genetic predisposition. There is also evidence for a genetic susceptibility in the human disease sporadic porphyria cutanea tarda. Current hypotheses predict that more than one gene is involved in this predisposition; candidates genes indicated from studies in mice include *Ahr*, *cyp1a1*, *cyp1a2* and possibly an unknown gene, perhaps one related to the human haemochromatosis gene, associated with iron metabolism.

Experimental uroporphyria can be induced in particular inbred strains by the chlorinated chemical hexachlorobenzene (HCB) and is exacerbated by iron.[6] Different strains of mice display a range of responses to this agents. One of the most susceptible strains reported is C57BL/10ScSn (BL/10) and the most resistant appears to be DBA/2. By breeding a second generation (F_2) intercross from these two strains, it seemed that it might be possible to dissect out the susceptibility genes responsible.[92,98,99]

Mice produced from the BL/10xDBA/2 cross were phenotyped by analysing their susceptibility to porphyria. Groups of the most susceptible and the most resistant mice were subsequently genotyped, by following the inheritance of parental microsatellite DNA markers. Markers from the BL/10 susceptible parent when inherited together with the highly susceptible phenotype, were assessed for the possibility that they were linked to a predisposing locus using the χ^2 test. The results from this analysis have indicated that there may be genes responsible for susceptibility to HCB and iron induced porphyria, located on chromosomes 12, 14 and 17.

3.2 Introduction

The sporadic form of human PCT is characterised by a depression in the activity of the haem biosynthetic enzyme, UROD. The activity of UROD is depressed only in the liver and this does not appear to result from a mutation in the enzyme or even to a loss in the concentration of the protein. [1-4,30,31] As not all individuals develop S-PCT after exposure to toxins such as chlorinated aromatic hydrocarbons or alcohol, it seems likely that a genetic predisposition is necessary for susceptibility. Experimentally, predisposition to the toxic affects of TCDD and HCB exists in some mouse strains and this can be used as a model for the human disease. [6,7]

Initially, susceptibility to porphyria in mice was believed to segregate solely with AH receptor phenotype [7,61]. Four alleles for this receptor are known. [65,66] Two of these, Ahr^{b-1} and Ahr^{d}, confer responsive and nonresponsive phenotypes, respectively. The AH receptor is responsible for mediating the transcription of particular hepatic cytochrome P450 enzymes, which are also implicated in the hypothesised mechanism of producing an inhibitor of UROD. [5] Classically, susceptibile mice were thought to be AH-responsive with induction CYP1A1 and CYP1A2 after administration of compounds such as TCDD or 3-MC. Whereas treatment of resistant strains with the nonresponsive allele presented a poor response in CYP1A1 or CYP1A2 activity. Therefore these genes had become strong candidates.

However, the presence of iron in excess exacerbated the porphyrogenic effect of TCDD and HCB and in conjunction with these chemicals, was demonstrated to induce porphyria in some AH nonresponsive strains. [7]

The lack of complete correlation of AH phenotype with susceptibility to porphyria seemed to suggest that the *Ahr* gene was not an absolute requirement in the mechanism of porphyria.[6-8] Whether the *Ahr* gene is a prerequisite of this genetic variance or not, it is clear that other unknown genetic factors, possible involved in iron metabolism, are also required for susceptibility.

In order to investigate the porphyria susceptibility further, a technique was employed that is now commonly used to elucidate the genetic components of many complex genetic disorders. This entailed breeding an experimental cross between two strains, BL/10 and DBA/2. BL/10 is an AH responsive strain (Ahr^{b-1}) and susceptible to porphyria after treatment with HCB and iron. DBA/2 is resistant to the development of porphyria and is also an AH nonresponsive strain (Ahr^{d}).

By producing an F_1 hybrid and subsequently a second generation hybrid (F_2) from these two strains, the genes involved in causing this susceptibility should have been segregated by recombination of the F_1 parental chromosomes. The F_2 mice would inherit parent strain genes at random, so that some would receive a majority of susceptibility genes from the BL/10 strain, some would inherited a mixture of susceptibility and resistance genes and others would have mainly resistance genes, from the DBA/2 parent.

The hybrid mice were tested for a response to treatment with HCB and iron, in order to compare phenotype with genotype. Identification of chromosomal regions that might contain a predisposing gene was accomplished by screening two groups of F_2 mice which had the highest and lowest susceptibility to porphyria induction. To genotype the F_2 mice, microsatellite DNA markers were chosen, from the most current linkage maps available. [93, 96,129] The criteria for selecting markers was based on two main factors; first, that they were polymorphic between BL/10 and DBA/2 strains and secondly, where possible, alleles could be resolved by agarose gel electrophoresis.

3.3 Results

To map predisposing genes causing susceptibility to HCB and iron induced porphyria, crosses were bred from the susceptible strain C57BL/10ScSn and the resistant strain DBA/2. To analyse the mode of inheritance a (BL/10xDBA/2) F_1 hybrid was first bred from the two strains, as well as two backcrosses, produced by mating (BL/10xDBA/2) F_1 mice to either the BL/10 or

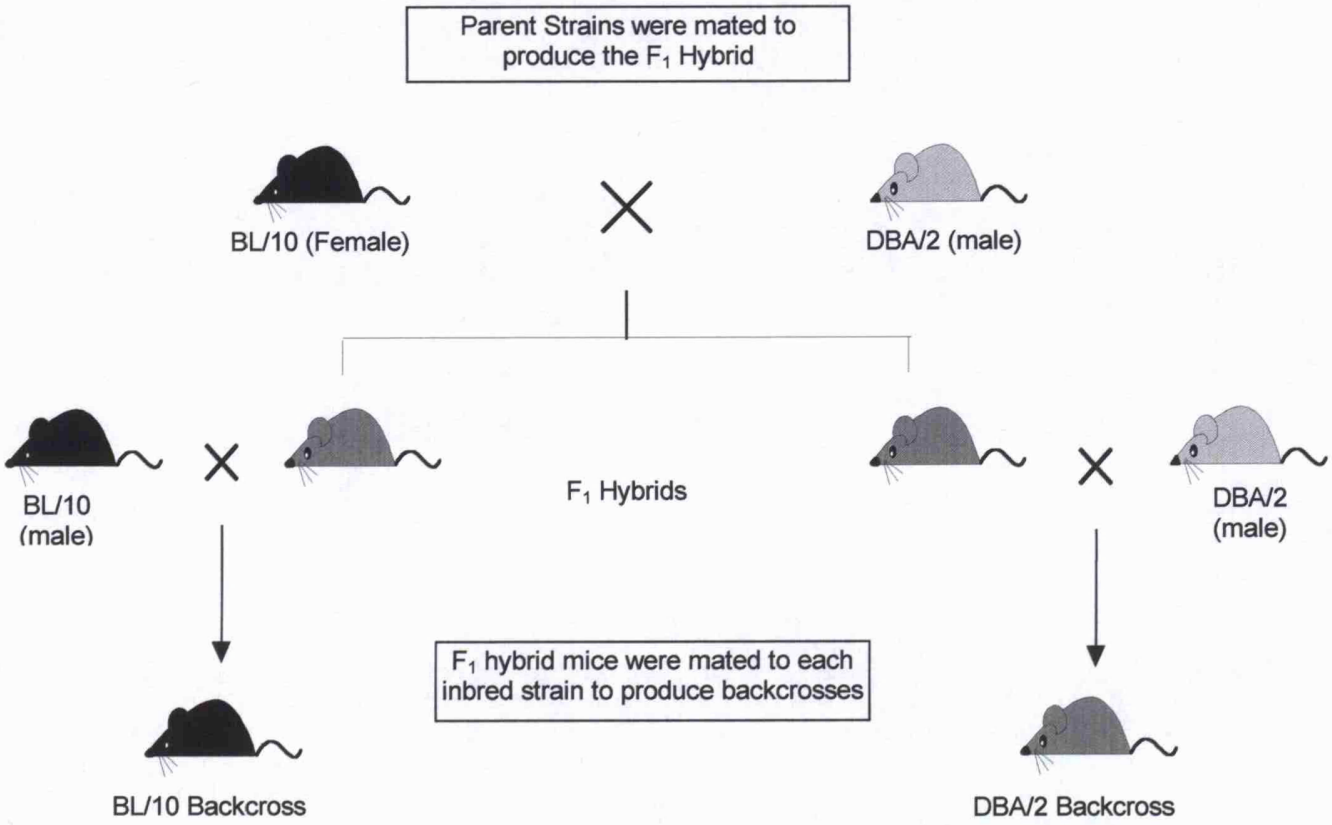
DBA/2 strains. The F_2 generation was bred from two reciprocal matings, of BL/10 females with DBA/2 males and DBA/2 females with BL/10 males.

Parental strains were dosed with iron and exposed to HCB to determine the extreme phenotypes. F_1 and backcrosses were treated similarly to try to determine whether the inheritance of susceptibility was dominant or recessive. Finally, on the basis of susceptibility to porphyria, the F_2 progeny were classified into two groups with extreme phenotypes. These groups were used for genomic DNA extraction and subsequent selective genotyping in order to identify regions of the mouse genome that were inherited along with the susceptibility trait.

Only male mice were used for this study, mainly as the experiment would have become too large if females were included. It was established previously, that female mice respond more slowly to HCB than males.[6] This is in contrast to rats, with this species females are markedly more susceptible than males. [128]

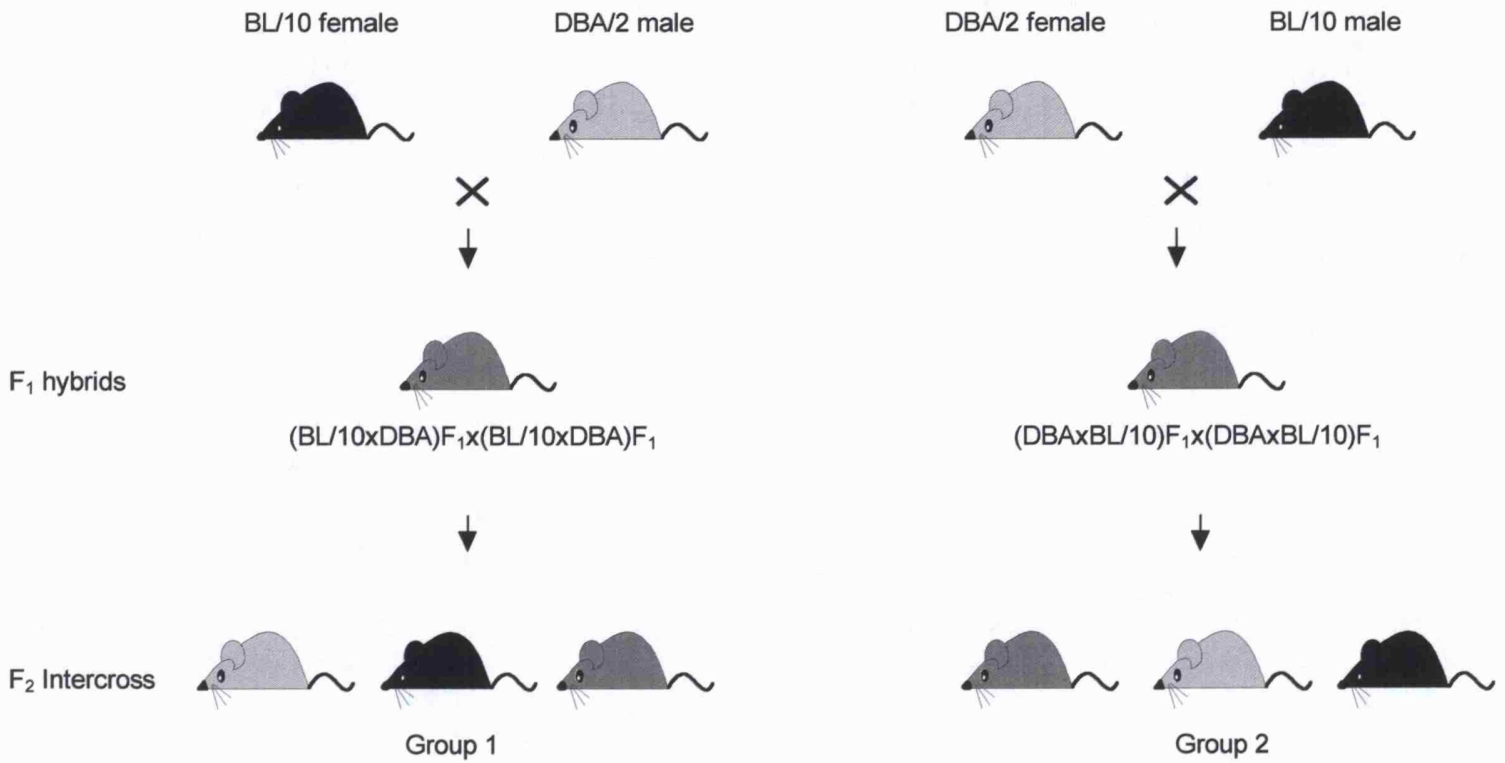
A summary of the breeding and treatment of the strains and crosses used is shown in Figure 3.1.

Figure 3.1 Summary of Breeding and Treatment of F_1 Hybrid and Backcross Mice



At 7-10 weeks after weaning, male F_1 hybrid, BL/10 backcross and DBA/2 backcrosses were dosed with iron-dextran. After three days they received diet containing HCB (0.02%) for seven weeks, after which mice were culled and hepatic porphyrin concentrations were determined.

Figure 3.2 Summary of breeding and treatment of F_2 intercross mice



Two groups of F_2 intercrosses were bred starting from sets of parents, with males and females of each strain, to allow for any sex linked genes. Mice were treated as stated previously, if susceptibility was markedly sex-linked, there would be a significant difference to development of porphyria between the two groups.

3.3.1 Induction of Porphyria in C57BL/10ScSn and DBA/2 Mouse Strains

Male mice from each parental strain were given iron (600mg/kg) as a single subcutaneous injection and fed a diet containing HCB (0.02%) for seven weeks. (section 2.1.1) At the end of this period, mice were killed and hepatic porphyrins levels were measured by a fluorimetric method which distinguished between uroporphyrin, coproporphyrin and protoporphyrin (section 2.2). The results of porphyria induction are shown in Table 3.1, as the mean uroporphyrin concentrations of each group.

Table 3.1 Hepatic uroporphyrin concentrations in C57BL/10ScSn and DBA/2 mouse strains after iron overload and exposure to HCB for seven weeks.

Strain	Treatment	Number	Mean Uroporphyrin Concentration (nmol/g liver) ± SEM
C57BL/10ScSn	-	5	0.1 ± 0.01
C57BL/10ScSn	iron	4	0.1 ± 0.1
C57BL/10ScSn	iron + HCB	5	398.7 ± 167.8*
DBA/2	-	5	0.1 ± 0.04
DBA/2	iron	4	0.1 ± 0.1
DBA/2	iron + HCB	4	0.1 ± 0.1

Hepatic uroporphyrin concentrations are the means from groups of mice; controls (-), iron overloaded (iron) and iron overloaded with exposure to HCB (iron+HCB).
* significantly different from untreated controls ($p<0.05$), analysed by Student-t test.

A massive accumulation of uroporphyrin, of nearly 400nmoles per gram of liver, occurred in the BL/10 mice treated with iron and HCB after seven weeks. This figure is 4,000-fold greater than that of controls and also of the identically treated DBA/2 strain. Such a substantial difference between the phenotypes of each strain used to produce a cross suitable for genetic mapping is important in order to accurately score the F_2 progeny.[98] However, there was a wide

variation in response in the BL/10 strain. Even though the mice were treated in the same manner and are genetically identically.

After this time period, iron alone did not have an effect on UROD activity in either strain, although it does cause porphyria by itself in C57BL/10ScSn mice after a prolonged period of at least 15 weeks.[89]

3.3.2 Induction of Porphyria in F_1 Hybrids, Backcrosses and F_2 Intercrosses

Crosses between BL/10 and DBA/2 strains were either purchased or bred to produce F_1 hybrid mice and F_1xBL/10 backcross and F_1xDBA/2 backcross mice. In addition, two sets of F_2 mice were bred from reciprocal matings of each strain, as previously decribed. A summary of the breeding and treatment of the crosses is given in Figure 3.1 and 3.2. Table 3.2 (p.86) shows the details of the crosses made and the number of male mice produced in each.

The male offspring produced in each cross were treated with HCB and iron (section 2.1.1) and after seven weeks, the mice were culled and the livers removed and immediately frozen in liquid nitrogen for subsequent porphyrin analysis, DNA, RNA and microsome preparation.

The mean hepatic uroporphyrin concentrations of each cross is shown in Table 3.3 (p.87). The uroporphyrin concentrations for all of the mice treated is shown graphically in Figure 3.3 (p.88) along with the original parent strains for comparison. Because of the extreme variations in the uroporphyria response, the data was transformed by taking logs of (uroporphyrin concentration+1). This enabled the data to be displayed on a linear scale.

This difference in respose to treatment can be seen in the parent strains, (Graph A). The inter-strain differences in within the BL/10 parents which causes the wide variation in the response in this group is also clear.

The F_1 hybrids, the BL/10 backcross and F_2 intercross (Graphs B-E) showed a wide variation in response to treatment, although there was a large number of low responding F_2 mice.

Both of the backcrosses analysed, displayed a 'skewed' distribution in uroporphyrin concentrations. The BL/10 backcrosses were predominantly of the

more susceptible phenotype, whereas there were hardly any response in the DBA/2 backcross, with only a few very weakly responding outliers. The difference between the backcrosses may be explained if one or more of the predisposing genes was partly dominant.[98]

The reason for the large number of low responding F_2 mice was unclear but this might be explained, in part, by an uncontrollable environmental effect. It is also possible that some of these mice may have responded if they were exposed to HCB for longer as the development of experimental porphyria has been shown to be exponential. [6] For consistency however, the seven week time period was maintained for induction in all strains and crosses, by iron overload and HCB.

Initially there appeared to be a significant discrepancy between the figures for the average and high responding uroporphyrin concentrations between the two F_2 groups. (Table 3.3). However after transforming the data, as previously described, the differences between the groups were no longer significant, so it was decided to combine the groups for the genetic analysis.

Overall, the distribution of responses in the 213 F_2 mice analysed, suggested that more than one gene was required for susceptibility to experimental porphyria.

Table 3.2 Table of Hybrids, Backcrosses and Intercrosses Bred for the Genetic Analysis of Suceptibility to HCB and Iron Induced Porphyria

Crosses	Generation	Number of Male Offspring
C57BL/10ScSn x DBA/2	F_1	31
(C57BL/10ScSnxDBA/2)F_1 X BL/10	Backcross	41
(C57BL/10ScSnxDBA/2)F_1 X DBA/2	Backcross	42
(C57BL/10ScSnxDBA/2)F_1 X (C57BL/10ScSnxDBA/2) F_1	F_2 [1]	117
(DBA/2xC57BL/10ScSn)F_1 X (DBA/2xC57BL/10ScSn) F_1	F_2 [2]	96

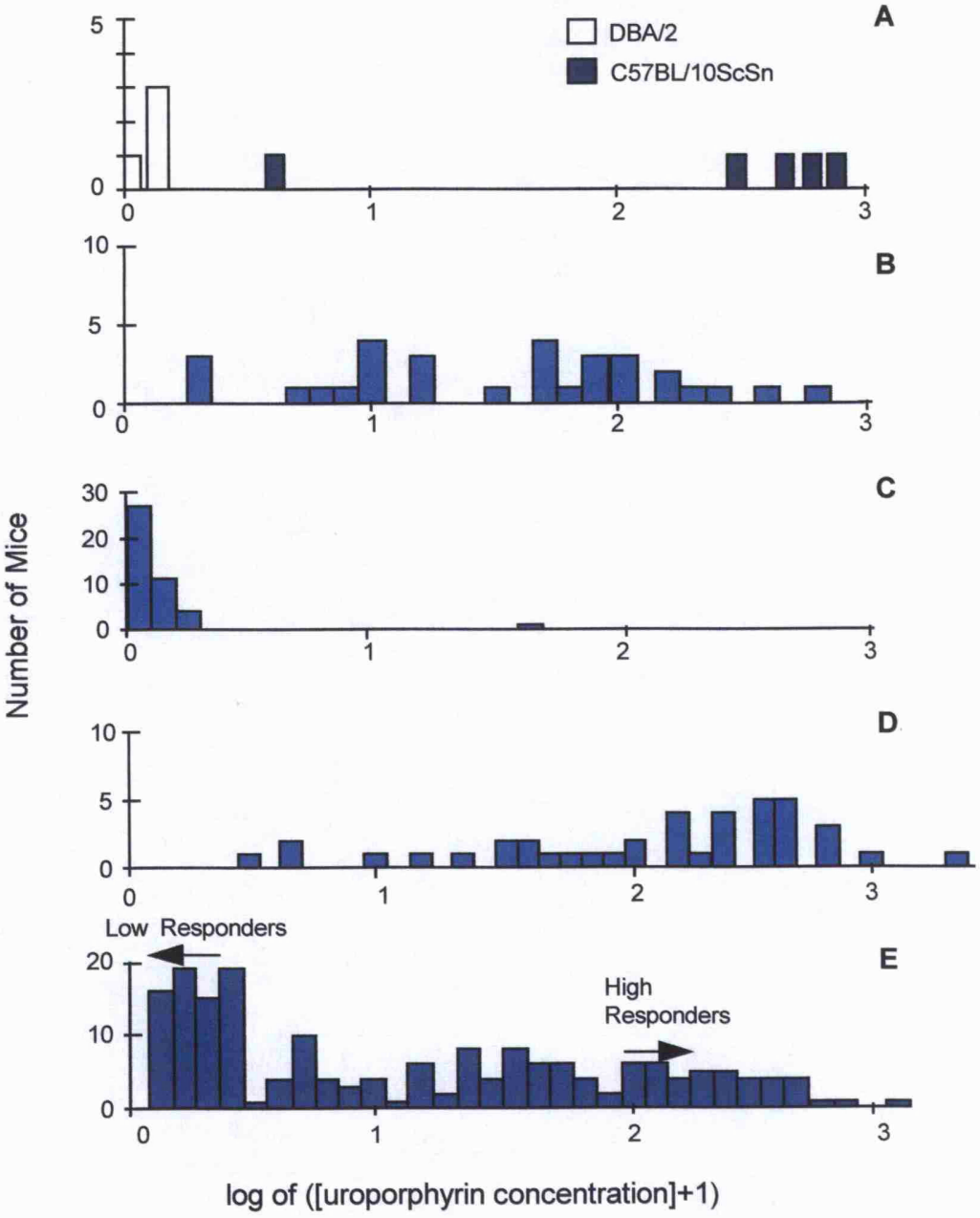
Table showing the crosses made between C57BL/10ScSn and DBA/2 mouse strains, and the numbers of male offspring produced.
The F_2 generation was bred from two pairings:
[1] Group 1; female C57BL/10ScSn and male DBA/2 parent strains
[2] Group 2; the reciprocal cross female DBA/2 and male C57BL/10ScSn strains.
The two groups were combined and a total of 213 F_2 males were produced for the study.

Table 3.3 Table of mean hepatic uroporphyrin concentrations in male F_1 hybrids, backcrosses and the F_2 intercross groups

Generation	Number of Mice	Mean Uroporphyrin Concentration (nmol/g liver) ± SD	Transformed Data ± SD
F_1	31	88.6 ± 132.6	2.0 ± 2.1
Backcross to BL/10	41	287.8 ± 421.9	2.5 ± 2.6
Backcross to DBA/2	42	1.0 ± 5.3	0.3 ± 0.8
F_2 Group 1	117	89.4 ± 182.0*	1.1 ± 1.0
High responders	25	360.8 ± 244.0	2.5 ± 0.3
Low responders	25	0.1 ± 0.02	0.04 ± 0.01
F_2 Group 2	96	40.1 ± 72.4*	0.9 ± 0.8
High responders	16	223.0 ± 155.6	2.3 ± 0.1
Low responders	16	0.2 ± 0.2	0.05 ± 0.02
F_2 (combined)	213	67.2 ± 145.2	1.0 ± 0.9
Low responders (groups 1&2 combined)	40	0.04 ± 0.05	0.04 ± 0.02
High responders (groups 1&2 combined)	40	293.4 ± 210.4	2.4 ± 0.3

*Group 1 and 2 untransformed data appeared to be significantly different ($p<0.05$ calculated by analysis of variance). After transformation no significant difference between the two F_2 groups.

Figure 3.3 Hepatic uroporphyrin concentrations measured in male C57BL/10ScSn and DBA/2 parent strains, and male F_1 hybrids, backcrosses and F_2 intercross mice after treatment with HCB and iron overload



A - C57BL/10ScSn and DBA/2 parent strains
B - F_1 hybrids
C - (BL/10xDBA/2)xBL/10 backcrosses
D - (BL/10xDBA/2)xDBA/2 backcrosses
E - combined F_2 groups, the arrows show the regions from where the high and low responding mice were selected.

3.3.3 Microsatellite Analysis

A selective genotyping method, based on the complex trait mapping technique developed by Lander and Botstein was applied in order to do a low density scan for susceptibility loci across the mouse genome.[89] This technique required genotyping only those mice in the F_2 cross which had a phenotype that differed substantially from the F_2 mean phenotype. It also assumed a normal, continuous distribution in the phenotype data.

As a very basic screen this method was adapted to give a first approximation of markers which may be linked to a predisposing gene. This approach is limited because the coverage of the genome is scant and would not detect linkage all predisposing loci. In particular, the difference in genetic length of each chromosome would mean that more than two markers would be required to detect linkage on larger chromosomes such as chromosome 1, than on smaller chromosomes such as 19.

Two groups of mice with extreme phenotypes were selected from the F_2 cross. These consisted of a 'high responding' group of 40 individuals, with hepatic uroporphyrin levels of 100 nmoles per gram of liver or higher and a 'low responding' group of 39 mice. These groups were chosen on the basis of a uroporphyrin concentration of less than 1 nmole uroporphyrin per gram of liver. The regions from where these groups were selected are shown on Graph E, of Figure 3.3.

Genomic DNA was extracted from tail or liver samples, by either phenol-chloroform extraction or with a commercial kit.(section 2.7)

The F_2 groups were typed with a set of markers selected from the Whitehead Institute/MIT Genome Centre mouse genome database,[93,96,129,c3] with two markers per chromosome in most cases.

Table 3.4 overleaf, lists the markers used, along with their amplification conditions. Figure 3.4 (p.92-93) shows the relative locations each marker in the genome.The position sof the microsatellite markers and candidate genes were taken from the mouse genome database. [93,96,129,c3]

Table 3.4 Table of microsatellites used to screen the BL/10xDBA/2 F_2 intercross and details of amplification conditions.

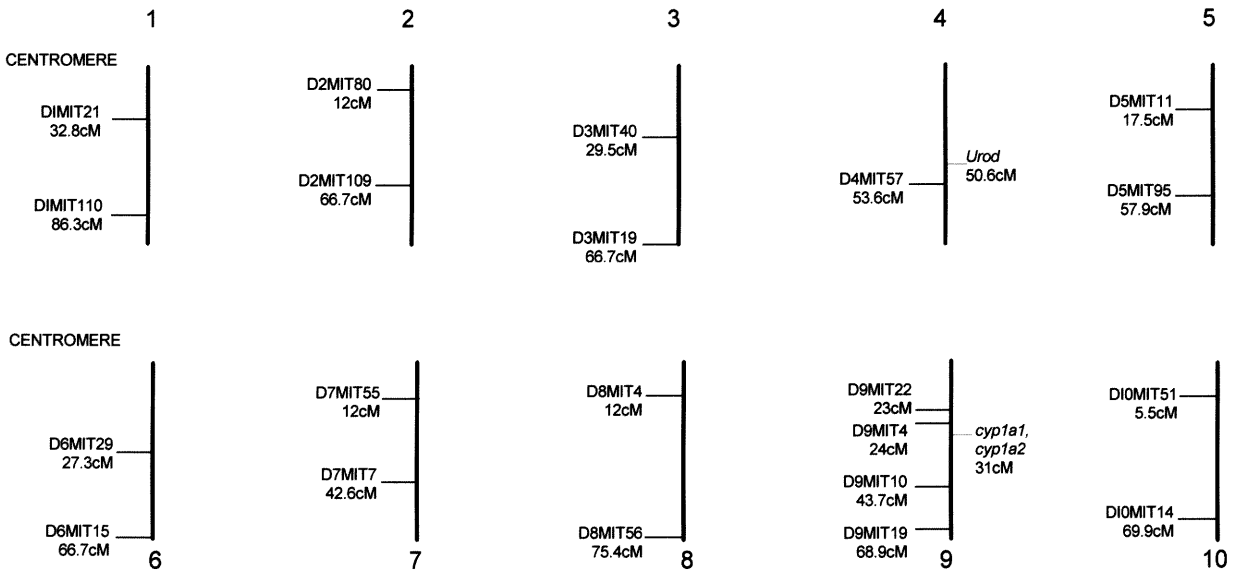
Marker	Chromosome	Allele Sizes (bp)		PCR Conditions	
		B	D	$[Mg^{2+}]$ mM	PCR profile†
D1MIT21	1	246	250	3.0	a*
D1MIT110	1	138	168	3.0	b
D2MIT80	2	192	180	3.0	a
D2MIT109	2	176	164	3.0	b
D3MIT19	3	160	176	1.5	d
D3MIT40	3	140	110	1.5	d
D4MIT57	4	128	136	3.0	b
D5MIT11	5	206	188	1.5	d
D5MIT95	5	116	130	2.0	a
D6MIT29	6	124	148	3.0	a
D6MIT15	6	260	195	1.5	b
D7MIT55	7	150	126	2.5	a
D7MIT7	7	79	86	2.0	a
D8MIT4	8	157	195	1.5	a
D8MIT56	8	160	182	1.5	a
D9MIT22	9	220	230	4.5	a
D9MIT4	9	124	138	4.5	a
D9MIT10	9	75	147	1.5	c
D9MIT19	9	102	89	3.0	b
D10MIT51	10	150	162	3.0	b
D10MIT14	10	192	182	1.5	a
D11MIT36	11	234	220	3.0	a
D12MIT2	12	132	149	3.0	a
Igh-v	*12*	148	<148	3.0	a
D13MIT35	13	190	182	2.5	a
plau	14	<188	188	3.0	a
D14MIT5	14	178	164	2.0	b
D15MIT12	15	150	160	2.0	b
D15MIT33	15	152	144	3.0	a
D16MIT4	16	132	123	4.5	a
D16MIT5	16	156	132	1.5	a
D17MIT16	17	118	106	1.5	a
D17MIT22	17	157	183	1.5	a
D17MIT41	17	204	220	2.0	a
D18MIT14	18	105	96	3.0	d
D19MIT19	19	208	210	1.5	a
D19MIT42	19	178	182	1.5	a*
DXMIT1	X	97	84	2.0	a

†PCR Profiles:
a: 55°C annealing temperature, 30 cycles of amplification
b: 60°C annealing temperature, 30 cycles of amplification

(Legend for Table 3.4 continued):

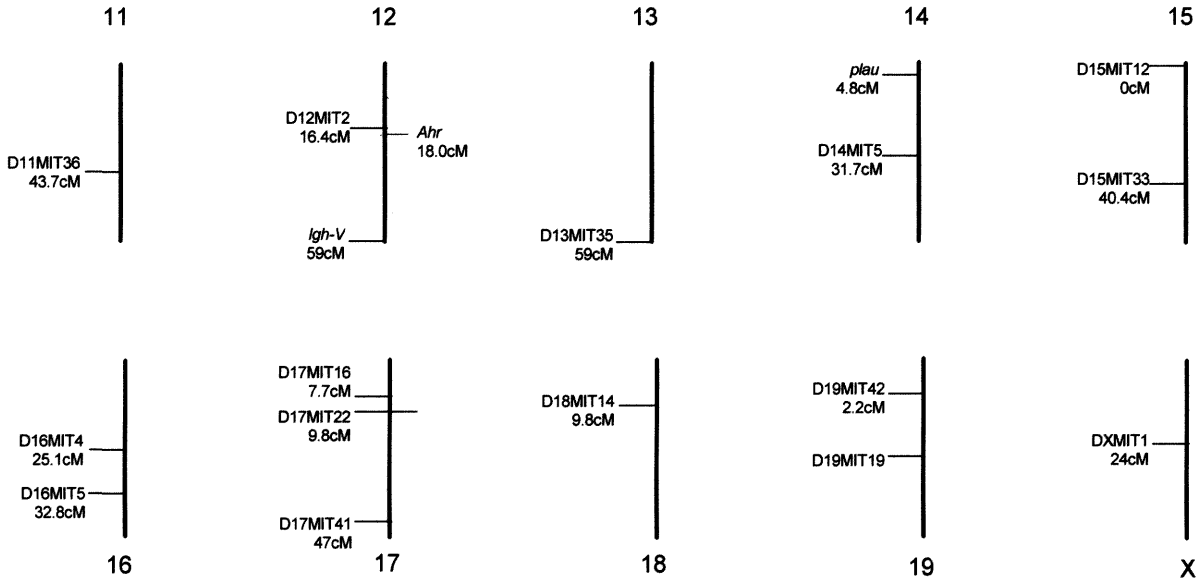
c: 59°C annealing temperature, 30 cycles of amplification
d:Touchdown Program; 2 minutes denaturing at 94°C, 1 minute annealing at X°C, 1 minute elongation step at 72°C. Annealing temperature starts at X = 70°C and drops by 2°C every two cycles to 50°C, followed by 20 cycles at 50°C annealing.[80,91]
* Primers were labelled with γ^{33}P-dATP, with 25 cycles of amplification and products were resolved by polyacrylamide gel electrophoresis.

Figure 3.3 Location of microsatellite markers in the mouse genome



The locations of each marker used for genotyping the (BL/10xDBA/2)F_2 groups are given in centimorgans (cM) from the centromere.[93,96,129,C3] Markers are given on the left and candidate loci on the right of each chromosome.

Figure 3.3 Location of microsatellite markers in the mouse genome (continued)



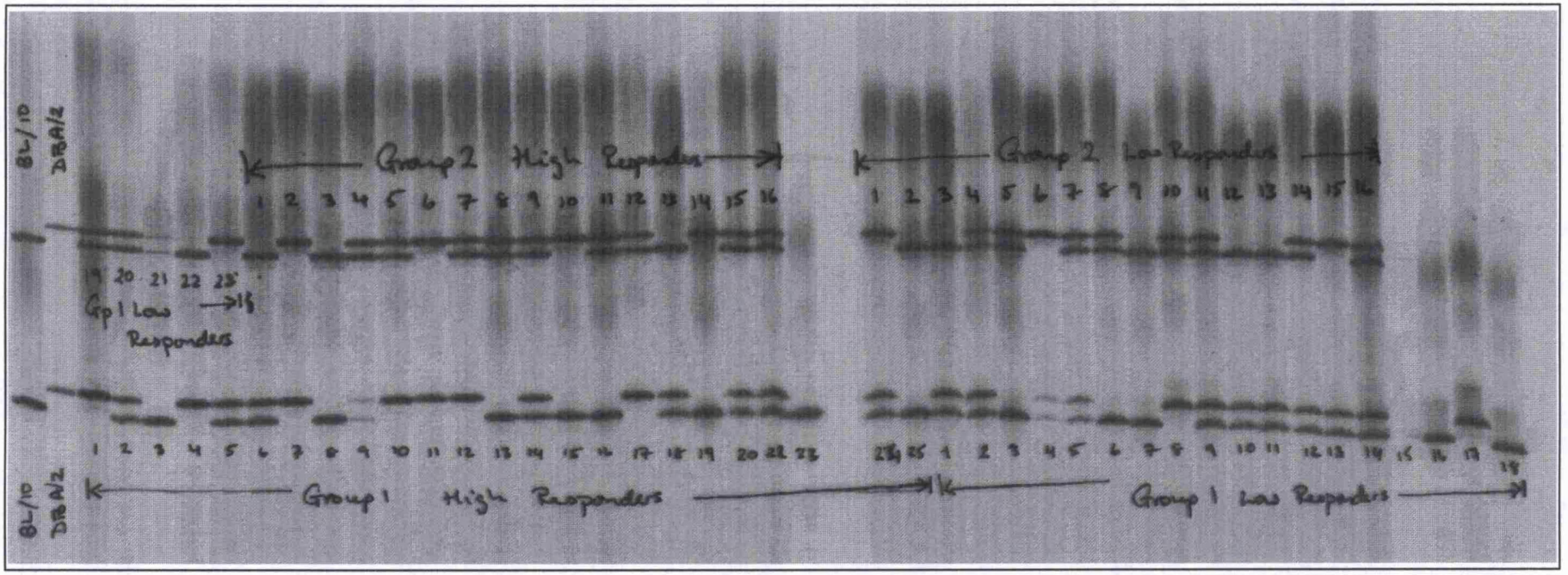
The locations of each marker used for genotyping the (BL/10xDBA/2)F_2 groups are given in centimorgans (cM) from the centromere.[93,96,129,C3] Markers are given on the left and candidate loci on the right of each chromosome.

Microsatellites were amplified by PCR and most products were resolved by agarose electrophoresis. Markers with alleles less than 10 bases apart were amplified with $\gamma^{33}P$ end-labelled primers and resolved by acrylamide electrophoresis and autoradiography. A full description of how the agarose and acrylamide gels were prepared is given in section 2.13. Examples of amplified microsatellites, resolved by both acrylamide and agarose gels, are given in figure 3.5 a and b, p. 94 and 94a.

Approximately 10μl of each 'cold' reaction, containing loading dye was loaded onto the agarose gels, whereas only 2-3μl of isotopically-labelled product was loaded onto acrylamide gels.(Section 2.13) BL/10, DBA/2 or F_1 DNA samples were included on both agarose and acrylamide gels as controls. ΦX174 DNA molecular size markers, loaded in the first lane of each agarose gel, were used to gauge the approximate size of the bands. Gels were electrophoresed until the alleles were resolved and could be distinguished for genotyping.

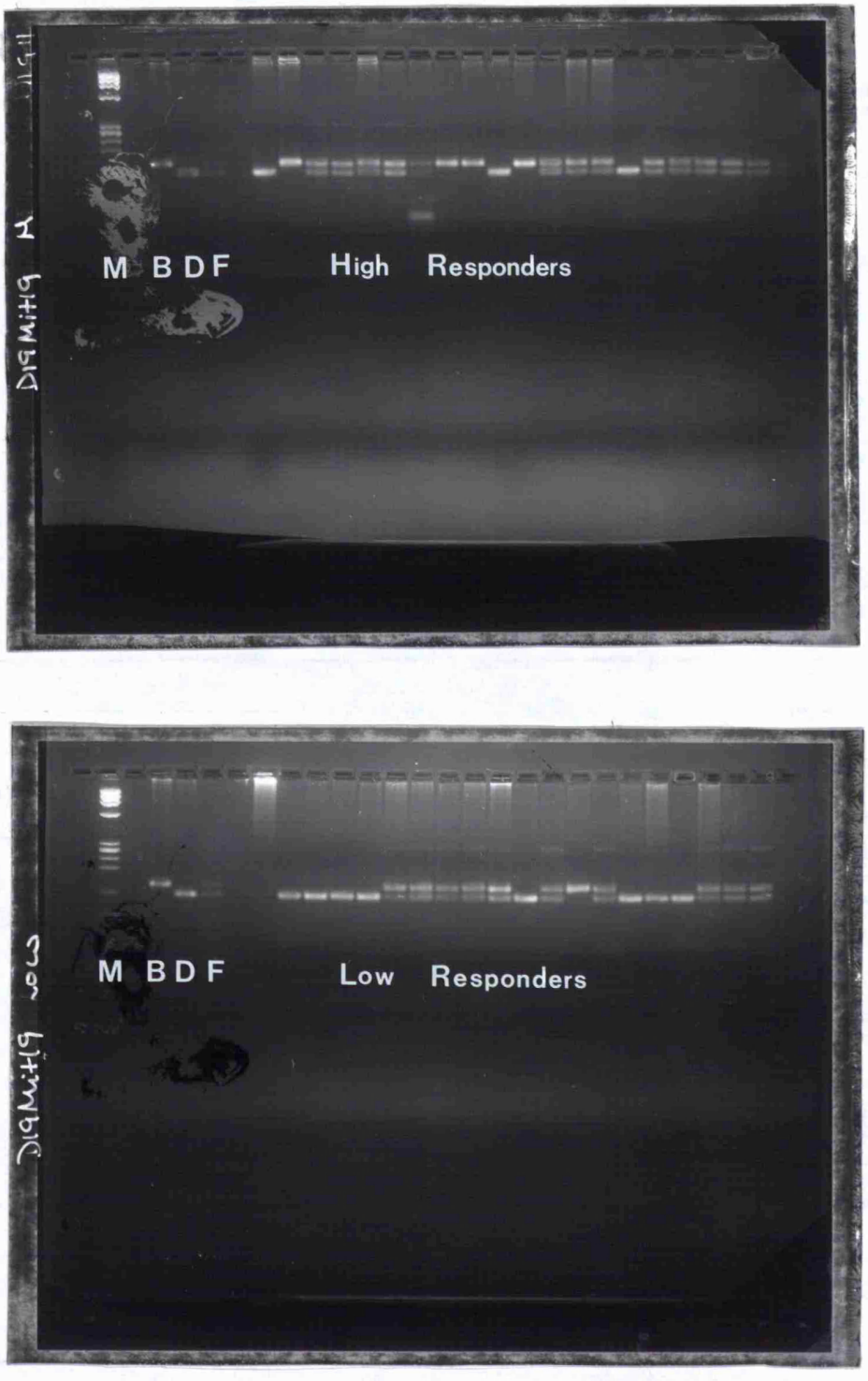
Each marker was genotyped by counting the number of BL/10 and DBA/2 homozygote alleles and heterozygote alleles, for both the high and low responding groups. These alleles were assigned the label B (for BL/10 homozygotes), D (for DBA/2 homozygotes) and H for BL/10:DBA/2 heterozygotes.

Figure 3.5 (a) Example of an amplified microsatellite marker (D1MIT110) resolved by polyacrylamide gel electrpohoresis



Microsatellite D1MIT110 was typed in both high and low respondoing groups. The PCR products were amplified with γ^{33}P-dATP 5' end labelled primer and resolved on a 6% denaturing polyacrylamide gel. To analyse a large number of samples on one gel, two sets of reactions or 'waves' were loaded. The homozygous parent strain products are labelled BL/10 and DBA/2. The numbered lanes represent the products for individual F_2 mice, which are a mixture of homozygotes and heterozygotes at this locus.

Figure 3.5 (b) Example of an amplified microsatellite marker (D19MIT19) resolved by agarose gel electrophoresis



M= ΦX174 Molecular size markers; B=BL/10; D=DBA/2; F=F_1 Hybrid

PCR products were resolved on a 2% metaphor agarose gel, using 0.5XTBE buffer. Bands were visualised by including ethidium bromide (0.5μg/ml) in the gel. Gels were photographed with polaroid camera, on P55 film.

A full description of the amplifications conditions used are given in section 2.15.1. Microsatellite primers and allele size information was obtained from the Mouse Genome Database or and the MRC-HGMP resource centre. C57BL/10ScSn allele sizes were taken from the C57BL/6 strain. The exact allele sizes for markers *Igh-V* and *plau* were unavailable as they had not been measured.[94]

Markers were always tested on strains and F_1 hybrid DNA to confirm they were polymorphic and codominant. A number of markers were found not to be polymorphic although reported to be so in the databases.

Non-polymorphic markers included; *Orm-1* from the HGMP, D11MIT100, D13MIT3, D18MIT36 and DXMIT10 from the Mouse Genome Database. Markers which were not codominant were D18MIT9 and D4MIT31. The size of the alleles for the chromosome 4 markers were also at least 100bp greater than the published sizes. This discrepancy which could be due to the differences in microsatellite sizes between the BL/6 and BL/10 strains, which are identical for all chromsomes, except chromsome 4.[c4]

The correlation between inheritance of alleles and susceptibility or resistance to porphyria was assessed by the χ^2 test. The number of homozygote and heterozygote alleles typed for each marker in the two groups, was compared with the 1:2:1 ratio of B:H:D alleles expected if the alleles were inherited randomly. χ^2 was calculated from the equation:

$$\chi^2 = \Sigma\,[(O-E)^2]/E$$

where O= observed number of alleles (either B, H or D) and E= the expected number for each alleles.

Where the χ^2 gave a p-value of less than 0.05, the inheritance of that marker with susceptibility to porphyria was assumed to be significant, and a possiblity that it may be linked to a predisposing gene. The results of this analysis are shown in Table 3.5(p.98-99).

The first observation from this analysis was that there was no correlation between the markers selected on chromosome 4 and susceptibility to the development of porphyria.

Chromosome 4 is the location of the the mouse *Urod* gene [126]. No mutations have been discovered in the human UROD gene sequence in any of the sporadic cases analysed and the result from this mouse study may reflect this too. Further analysis of the mouse *Urod* gene has been covered in chapter 7.

Chromosome 9 also showed no correlation with porphyria development. This chromosome is of especial interest as the cytochrome P450 isozyme genes, *cyp1a1* and *cyp1a2* are located here. Several markers were available to be tested but only one, D9MIT10, showed any correlation and this was only in the low responding group. Hence, the possiblity that a polymorphism in either of these isozymes is responsible for a genetic predisposition seems unlikely, unless the polymorphism occured in the enzymes' regulation mechanism.

Several other markers gave apparent correlation but these occured in only one response group. In some cases markers appeared highly significantly correlated, for example D3MIT40 and D7MIT55 but only because of a large number of BL/10 or DBA/2 alleles in both the high and low response groups. This could be because these markers were dominant not codominant in these strains. The most interesting results from this analysis indicated chromosomes 12, 14 and 17 as possible locations of susceptibility genes.

Chromosome 12 is the location of the *Ahr* locus and has always been associated with the development of chemically-induced porphyria in mice, due to the pharmacogenetic differences of strains, conferred by the Ahr^b and Ahr^d alleles. Microsatellite D12MIT2, showed a significant correlation (p=0.012) in the high response group, Further analysis of the *Ahr* locus was undertaken and is described in detail in chapter 4.

Chromosome 14 was the only chromosome to contain a marker (D14MIT5, p=0.001) that showed significant correlation in both response groups. The linkage found to this chromsome was particulary interesting as there are no obvious candidates for this chromosome.

Chromosome 17 is the location of the mouse histocompatibility or H-2 complex. This region has recently been reported to contain genes involved with iron metabolism. [101] This may even be the reason for significantly high correlation with susceptibility in the high responding group, although a similarly

high correlation was observed in the low responding group. A lack of significant correlation in the low responding group was observed on many occasions. The reason for this is unclear. It is possible that the samples selected for the 'low responding group' actually comprised mice with true, non-responding phenotypes and others that should have responded but due to some unknown envirnomental effect, failed to do so.

Table 3.5 Table of Genotyping Results from the BL/10xDBA/2 F_2 Intercross

Genotypes of the high and low response groups are given along with the calculated χ^2 and p-value for each microsatellite typed. B: BL/10 homozygote, H: heterozygote, D: DBA/2 homozygote. Chromosomes of interest are shaded.
* Significant correlation, $p<0.05$

Marker	Response to HCB and Iron	Genotype B	H	D	χ^2	p*
D1MIT21	High	11	18	11	0.4	0.819
	Low	11	21	6	1.7	0.420
D1MIT110	High	10	18	12	0.6	0.741
	Low	5	25	8	4.3	0.119
D2MIT80	High	10	21	9	0.2	0.928
	Low	5	12	22	20.6	0.000
D2MIT109	High	7	28	9	7.9	0.020
	Low	7	21	22	1.4	0.509
D3MIT19	High	2	14	3	4.3	0.113
	Low	6	7	7	1.9	0.387
D3MIT40	High	1	6	11	18.0	0.000
	Low	0	5	15	35.8	0.000
D4MIT57	High	6	26	14	3.6	0.168
	Low	7	18	6	0.9	0.647
D5MIT11	High	5	8	6	0.6	0.749
	Low	6	6	6	2.0	0.368
D5MIT95	High	10	21	9	0.2	0.928
	Low	1	23	15	11.3	0.004
D6MIT29	High	6	6	4	0.4	0.472
	Low	0	13	7	6.7	0.035
D6MIT15	High	6	5	9	5.9	0.052
	Low	4	12	4	0.8	0.670
D7MIT55	High	20	8	12	17.6	0.000
	Low	20	11	8	14.8	0.001
D7MIT7	High	7	8	5	1.2	0.549
	Low	5	11	4	0.3	0.861
D8MIT4	High	3	9	5	0.9	0.767
	Low	2	9	9	5.1	0.078
D8MIT56	High	7	9	4	1.1	0.577
	Low	5	10	5	0.0	1.000
D9MIT22	High	8	21	11	0.6	0.760
	Low	16	14	9	5.6	0.060
D9MIT4	High	8	21	11	0.6	0.760
	Low	16	16	7	5.4	0.067
D9MIT10	High	5	24	11	3.4	0.183
	Low	16	17	5	6.8	0.034

Table 3.5 (continued)

Marker	Response to HCB and Iron	Genotype B	H	D	χ^2	p-value* (p<0.05)
D9MIT19	High	8	22	10	0.6	0.741
	Low	13	18	7	2.0	0.658
D10MIT51	High	5	10	5	0.0	1.000
	Low	3	6	11	9.6	0.008
D10MIT14	High	3	11	6	1.1	0.577
	Low	5	8	5	0.2	0.895
D11MIT36	High	10	3	6	10.5	0.005
	Low	4	9	6	0.5	0.789
D12MIT2	High	17	18	4	8.9	0.012
	Low	9	18	12	0.7	0.707
Igh-v (12)	High	11	22	7	1.2	0.549
	Low	6	25	8	3.3	0.191
D13MIT35	High	4	9	6	0.5	0.789
	Low	2	10	8	3.6	0.165
plau (14)	High	8	12	0	7.2	0.027
	Low	6	8	6	0.8	0.670
D14MIT5	High	19	18	2	15.1	0.001
	Low	2	18	18	13.6	0.001
D15MIT12	High	7	7	6	1.9	0.387
	Low	3	10	7	1.6	0.449
D15MIT33	High	2	10	7	2.7	0.261
	Low	4	9	8	2.0	0.377
D16MIT4	High	4	9	7	1.1	0.577
	Low	7	11	2	2.7	0.259
D16MIT5	High	4	9	7	1.1	0.577
	Low	6	11	3	1.1	0.577
D17MIT16	High	19	17	4	12.2	0.002
	Low	5	24	10	3.4	0.186
D17MIT22	High	18	17	4	10.7	0.005
	Low	4	23	12	4.5	0.103
D17MIT41	High	7	25	7	3.1	0.212
	Low	6	21	10	1.5	0.463
D18MIT14	High	5	10	5	0	1.000
	Low	8	9	3	2.7	0.259
D19MIT19	High	4	13	3	1.9	0.387
	Low	1	10	8	5.2	0.074
D19MIT42	High	4	13	3	1.9	0.387
	Low	5	5	10	7.5	0.024
DXMIT1[†]	High	10	-	8	0.2	0.637
	Low	6	-	3	1.0	0.317

[†] χ^2 calculated with1d.f as X chromosome is hemizygous for each allele.

As the most promising results highlighted chromosome 17 and 14, further analysis with more markers selected along each chromosome were typed. This narrowed down the region of each chromosome where significant markers were present and the possible location of susceptibility loci.

The genotyping results of these chromosomes are shown in Table 3.6 and 3.7 overleaf. The positions of all the markers typed are shown in Figure 3.6 and 3.7 (p.99-100) along with a plot of the p-value calculated from the χ^2-test. Where the p-value of each marker typed crossed the 0.05 significance threshold (dashed line) is indicative of an significant correlation with susceptibility and possibly linkage to a predisposing gene.

Chromosome 14 Most of the extra marker typed showed highly significant correlation, in both susceptible and resistant groups in the case of D14MIT60. These markers span a region of approximately 30cM, which might be a target for future analysis.

Chromosome 17 This chromosome was typed with markers along the whole chromosome but significant correlation was found with markers in the vicinity of the H-2 complex. D17MIT49 which is located approximately 2cM telomeric from the H-2 complex gave a very high correlation. Again this region would be worth considering for further investigation, especially in the light of the recent finding of putative iron metabolism genes in the H-2 complex and the synteny between this region and the region where the human haemochromatosis genes has been mapped.[52,102]

Table 3.6 Chromosome 14 Microsatellites, PCR conditions and results of χ^2 analysis.

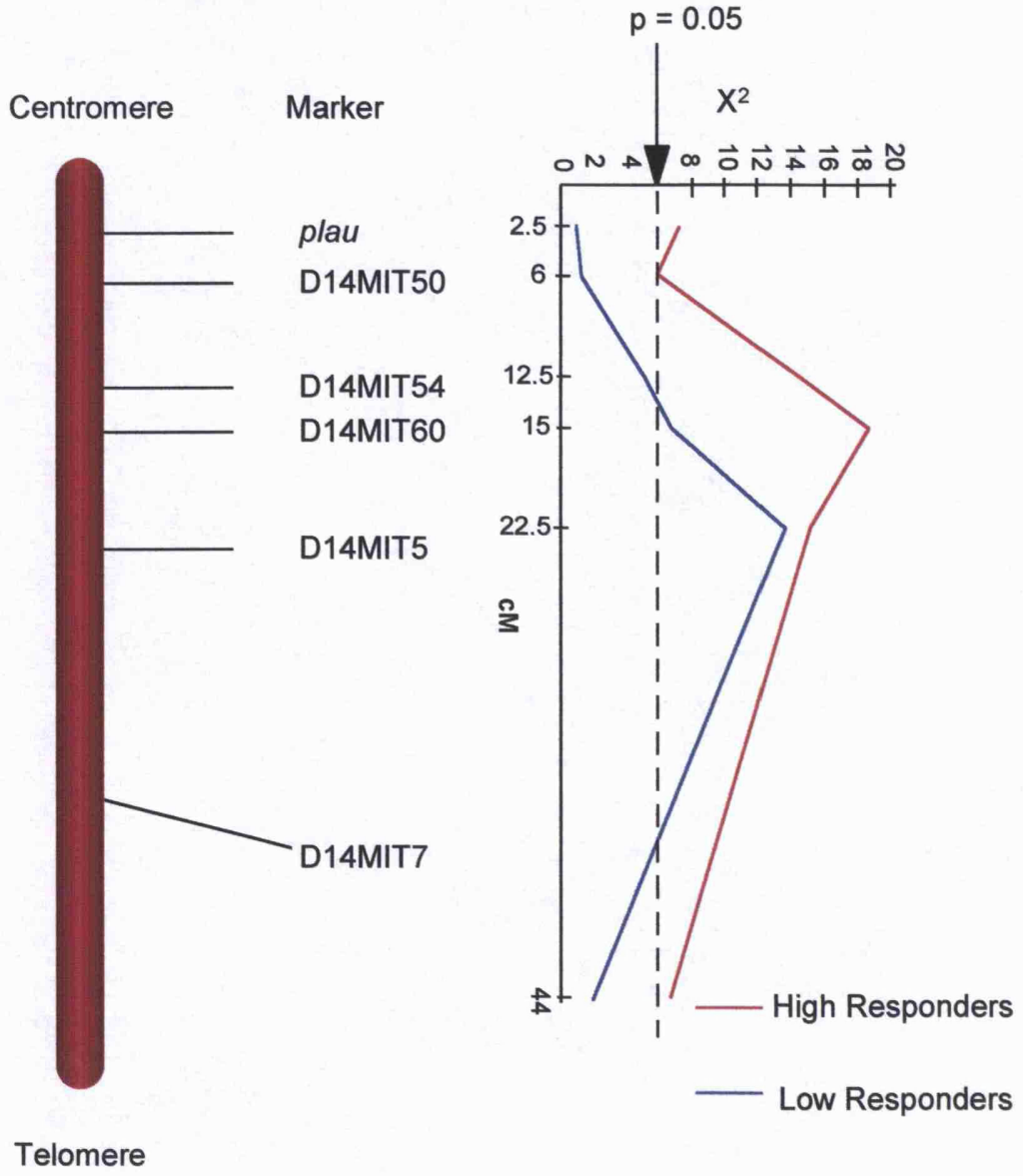
Marker	Allele Size		PCR Conditions		Genotyping			χ^2	p-value*
	B	D	$[Mg^{2+}]$ / Profile†		B	H	D		
D14MIT50	190	148	4.5mM	a	12	23	3	5.9	0.051[1]
					10	16	12	1.2	0.560[2]
D14MIT54	136	100	4.5mM	a	20	16	4	14.4	0.001[1]
					8	16	15	3.7	0.080[2]
D14MIT60	136	108	4.5mM	a	20	18	1	18.7	0.000[1]
					3	22	14	6.8	0.033[2]
D14MIT7	107	95	4.5mM	b	16	13	8	6.7	0.035[1]
					4	15	9	1.9	0.381[2]

Table 3.7 Chromosome 17 Microsatellites, PCR conditions and results of χ^2 analysis

Marker	Allele Size		PCR Conditions		Genotyping			χ^2	p-value*
	B	D	$[Mg^{2+}]$ / Profile†		B	H	D		
D17MIT30	150	160	1.5mM	a	12	11	1	10.3	0.006[1]
					2	15	6	3.4	0.181[2]
D17MIT24	142	126	1.5mM	a	19	17	12	6.1	0.047[1]
					5	24	4	6.9	0.032[2]
D17MIT49	250	224	1.5mM	a	23	13	4	23.0	0.000[1]
					6	18	10	1.1	0.589[2]
D17MIT20	185	178	2.0mM	a	12	20	6	2.0	0.368[1]
					5	21	11	2.6	0.270[2]

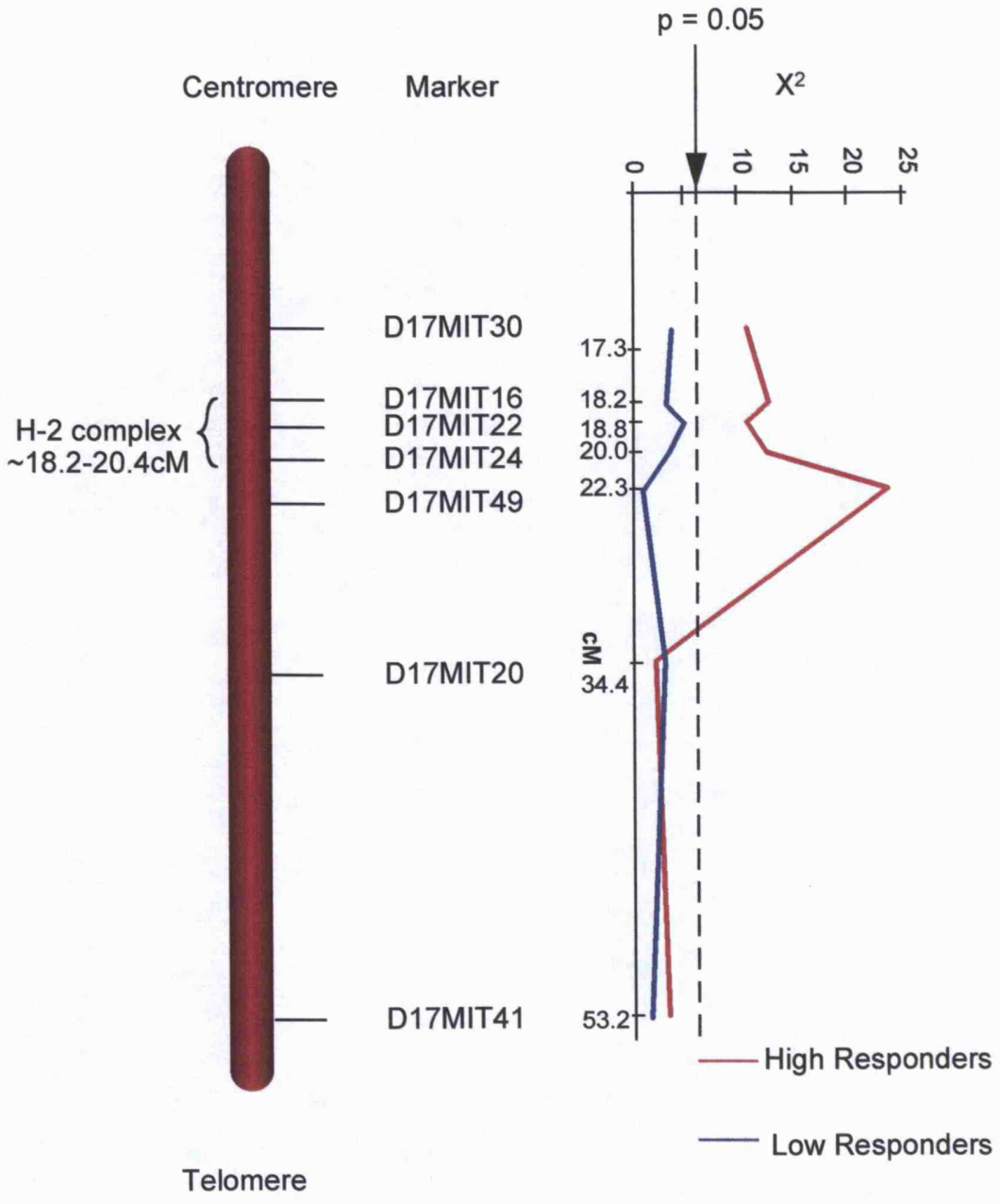
B: BL/10 homozygote, H: heterozygote, D: DBA/2 homozygote.
* Significant correlation where $p<0.05$
[1] High responding group
[2] Low responding group
† PCR profiles:
a: 55°C annealing temperature, 30 cycles of amplification
b: Touchdown program: 2 minutes denaturing at 94°C, 1 minute annealing at X°C, 1 minute elongation step at 72°C. Annealing temperature starts at X = 70°C and drops by 2°C every two cycles to 50°C, followed by 20 cycles at 50°C annealing.

Figure 3.6 Location of markers on chromsome 14 and correlation with susceptibilty to iron and HCB induced porphyria



The approximate location of the microsatellite markers selected for analysis on chromosome 14, given in centimorgans from the centromere. The graph to the right shows the correlation; calculated by χ^2-analysis, of each marker with susceptibility to experimental porphryia. The significant correlation threshold (where $p<0.050$ is marked by a dashed line.

Figure 3.7 Location of markers on chromsome 17 and correlation with susceptibilty to iron and HCB induced porphyria



The approximate locations (in centimorgans from the centromere) of the chromosome 17 microsatellite markers used to type the F_2 groups. The correlation of each marker with susceptibility is shown on the graph to the right, with the $p<0.05$ threshold marked. The region of the chromosome that contains H-2 loci is shown relative to the markers selected.

3.4 Discussion

The inheritance of experimental porphyria induced by polyhalogenated aromatic hydrocarbons in mice was assumed to be inherited along with AH responsiveness. [61] Interaction of the AH receptor with TCDD, 3-MC or even HCB upregulates the P450 isozymes CYP1A1 and CYP1A2, which have been implicated (especially CYP1A2 [77,78]) in the mechanism to depress UROD activity.[5]

However, iron is known to play an important role in both human sporadic porphyria cutanea tarda and experimental porphyria in rodents.[1-8] In man, treatment of S-PCT commonly involves the removal of iron.[34] There also appears to be a predominance in S-PCT patients to carry an allele of the haemochromatosis gene, which is responsible for severe iron overload in homozygotes.

In mice, the effect of dosing with iron-dextran at a concentration that makes the mouse iron overloaded, has the effect of exacerbating the porphyrogenic action of many chemicals including HCB. A genetic variation amongst inbred strains to the susceptibility to develop porphyria under these conditions has been reported, which does not always correlate with AH responsiveness and under some circumstances it may not be involved at all, as iron alone can cause experimental porphyria in C57BL/10ScSn and SWR strains, six months after a single dose.[8,89]

It has been proposed that there may be several genes responsible for the susceptibility of strains such as BL/10 to the action HCB and iron. In the work presented in this chapter, an experimental cross between the susceptible BL/10 mouse strain and the resistant DBA/2 strain was bred, as a first step towards finding these genes from their chromosomal location. The inheritance of susceptibility was also followed through an F_1 hybrid and backcrosses to both strains.

The nature of the inheritance of the susceptibility trait seemed unclear from the F_1 and backcrosses, but the overall trend appeared to be that it is dominant. From the range of susceptibilities in the F_2 generation, the porphyria would

appears to be caused by more than one gene which may interact in an additive fashion. There may be a mixture of dominant and recessive genes required for the full phenotype to be observed, which is probably coupled to an environmental effect. A much larger number of backcrosses to each strain would have been required to determine more accurately the exact mode of inheritance, by genotype as well as by phenotype.

The large number of F_2 mice that did not respond could be explained by an environmental effect that is difficult to control and this could be the reason for the inter-individual variation in the parental BL/10 mice treated. It is well known that even inbred mice held under 'apparently' identical conditions, they do not respond uniformly to a stimulus or treatment.

Another consideration may be the length of time of exposure to HCB. The increase in hepatic porphyrins has been shown to occur exponentially, with a significant rise not starting until after 3 to 4 weeks of treatment.[6] Therefore mice slightly behind others interms of the onset of porphyria, would appear to have significantly different porphyrin levels.

The effect of sex-linked genes has not been investigated in this study. Female BL/0 mice are known to be less sensitive to HCB and iron, [6] and a similar situation has been noticed in female mice treated with TCDD.[7] Female F_2 mice would need to be included to detect any sex-linkage.

The main results from the microsatellite analysis indicated three chromosomes of interest; 12, 14 and 17. Chromosome 12 is the location of the *Ahr* gene which encodes the AH receptor.[67] The inheritance and influence of this of this gene has been studied further in chapter 4. Chromosome 14 and 17 were selected for further study as they were the most likely locations for other genes predisposing to porphyria for several reasons.

Chromosome 14 held the only microsatellite markers that correlated significantly in both high and low responding groups whereas statistical significance in other markers only occured in the high responding group. The reason for this is unknown.

Further analysis of this chromosome, revealed markers with even greater correlation. (Figure 3.6) This area, spanning approximately 30cM, would be a

target area for further linkage analysis. One possible candidate gene, the hairless (*hr*) locus is located 39.6cM from the centromere and lies within this region. This candidate gene is involved in the epidermal toxicity of TCDD and is thought to be regulated by the AH receptor. A full description and discussion of this locus is given in chapter 8, p 168.

Chromosome 17 was selected as another promising location of a predisposing gene. This would be the hypothetical location of the mouse homologue of the human haemochromatosis gene. [100,101] Microsatellite markers close to the H-2 complex revealed this region to have a high correlation with inheritance of susceptibility, especially D17MIT49 which lies less than 2cM distal to this region. Recently, mice genes possibly involved in iron metabolism, have been located within the H-2 complex. One of which could be the mouse haemochromatosis gene or be involved with another unknown facet of iron metabolism.The effect of the H-2 region has been investigated in a series of experiments with mice congenic for the DBA/2 H-2 region in Chapter 6.

Interestingly, no linkage was detected on chromosome 4 where UROD is located.[126] In the human S-PCT, no mutations have been detected in the gene or promoter sequence.[30] Likewise, there was no convincing linkage to chromosome 9 where the *cyp1a1* and *cyp1a2* genes are located. These observations would imply that chromosomes 4 and 9 are not linked with susceptibility in this F_2 cross and it could be inferred that it is not a mutation or polymorphism in either the mouse *cyp1a1*, *cyp1a2* or *Urod* genes that accounts for the propensity of these mice to develop porphyria. All of these candidates genes have been analysed in more detail in chapters 4 and 7.

4 - Investigation of Candidate Genes Associated with Hexachlorobenzene and Iron Induced Porphyria

4.1 Summary

The analysis of the inheritance of microsatellite markers in the BL/10xDBA/2 F_2 intercross mice, indicated that chromosomes 12, 14 and 17 may be the location of genes predisposing BL/10 mice to experimental porphyria, although the nature of these genes is unknown.

As an alternative strategy, the influence of genes encoding proteins proposed to be involved in the mechanism of reduction in UROD activity were investigated.

The first candidate gene codes for the AH receptor, which is located on chromosome 12 [67] although its involvement in this mechanism is controversial. Some porphyrogenic agents bind to this receptor and induce cytochrome P450 1A1 and 1A2 activity. However, iron can override this mechanism by working synergistically with compounds such as TCDD and HCB to induce porphyria in certain AH nonresponsive mouse strains.

Segregation of the AH receptor was followed in the F_2 cross, by a restriction fragment length polymorphism (RFLP), that distinguished between the Ahr^{b-1} responsive allele and Ahr^{d} non-responsive allele. The result of genotyping the *Ahr* locus implied that the AH receptor was involved but was only significant in the high response group.

The other candidates investigated were the cytochrome P450 enzymes; CYP1A1, CYP1A2 and CYP2B which are implicated in the uncoupling mechanism thought to produce reactive oxygen species that could oxidise UROD substrates or produce an inhibitor to UROD.

Expression of CYP1A1 and CYP1A2 was analysed by northern blotting. CYP1A1 expression correlated with AH responsiveness in the BL/10 strain. It was also predominantly expressed in the high F_2 group but not the low group.

CYP1A2 expression was induced by HCB in both strains and also expressed equally in both the high and low response F_2 groups.

The results of the northern analysis were confirmed by assaying the hepatic microsomal P450 activity. CYP1A1, CYP1A2 and CYP2B activities were measured as O-dealkylase activities. Overall activity was induced by HCB in BL/10 mice and not in the DBA/2 strain. The only significant difference in activity between the two F_2 groups was in CYP1A1 activity, with a 2-fold difference between high and low responders.

The difference in CYP1A1 activity and expression suggests that this P450, and the AH receptor that regulates it, may be involved in the mechanism of HCB induced porphyria. However whether this activity, which is small compared with activities induced by β-napthoflavone and phenobarbital, is significant enough to produce the toxic effect is unknown.

4.2 Introduction

The microsatellite analysis highlighted three chromosomes which could contain genes predisposing BL/10 mice to experimental porphyria. The nature of these genes is not known and further genetic and molecular analysis would be necessary to elucidate them and their role in the mechanism of porphyria induction.

As well as the microsatellite analysis, several candidate genes that have been proposed to be involved in the mechanism of experimental porphyria induction were investigated.

Classically, compounds such as TCDD and HCB that cause a porphyria with the symptoms of S-PCT, are also inducers of the cytochrome P450 enzymes, especially CYP1A1 and CYP1A2, and also CYP2B1 in the case of HCB.[73] The cytochrome P450 enzymes are upregulated to metabolise these compounds by the AH receptor, to which TCDD and HCB (to a much lesser extent) are ligands.[60]

A proposed mechanism for the inhibition of UROD in experimental porphyria, involves the production of reactive oxygen species. These may be produced

when the cytochrome P450 NADPH-reductase/cytochrome P450 catalytic cycle is uncoupled as a result of the limited metabolism of the inducing compound, TCDD or HCB.[5,75]

At the genetic level, variation in the induction and expression of these enzymes, via the AH receptor, appears to be a plausible explaination for the difference in susceptibility to porphyria between the BL/10 and DBA/2 strains. There are two reasons for this; firstly, the AH receptor is known to have allelic variants in mice, two of which give the AH responsive and AH nonresponsive phenotypes in some mouse strains. [61] Secondly, a polymorphism in the sequence of a P450 enzyme could change the specificity of it's induction or metabolism of a compound. Polymorphisms in P450s occur at high frequencies in humans and are known to be associated with susceptibilities to cancer and toxicity of certain chemicals. [72] An allelic variant of CYP1A2 is known to exist in the DBA/2 strain.[83] This could alter the enzyme sufficiently to give the porphyria resistant phenotype of the DBA/2 mouse. The oxidation of uroporphyrinogen to uroporphyrin by rodent CYP1A2 has been demonstrated *in vitro* and is proposed as the basis for the build up of nonmetabolised porphyrin. [77,78] A less reactive alleleic variation of the *cyp1a2* gene in the DBA/2 mouse could be responsible for the resistance to induction of porphyia by compounds like TCDD and HCB.

However, segregation of AH receptor responsive alleles has been shown not to correlate completely with induction to porphyria.[6-8] In studies with TCDD, HCB and iron, the synergistic action between the polyhalogenated aromatic chemicals and iron, not only increases the severity of the porphyria but can also overcome the AH nonresponsive phenotype, for example in the strain AKR, which has the Ahr^d allele.[7] In addition, iron itself can induce porphyria in the AH responsive BL/10 mouse after six months [89] and also in the AH nonresponsive strain SWR [8]. Where no chemical inducers of the AH receptor were given, it seems unlikely that the *Ahr* gene could have a predisposing influence on the experimental induction mechanism in these cases.

To verify these hypotheses, the *Ahr* gene was typed in the F_2 intercross. Along with the *Ahr* gene, the two cytochrome P450 enzymes that it regulates were

examined in the F_2 intercross. The results of the microsatellite analysis did not show any substantial correlation with chromosome 9, where *cyp1a1* and *cyp1a2* are located. The closest marker to these loci, D9MIT10, gave a p-value of 0.034 only in the low responding F_2 group. If one of these enzymes carried a mutation or polymorphism causing the susceptibility to porphyria, a much higher correlation would be expected in both groups. As the *cyp1a1* or *cyp1a2* genes did not appear to be directly involved, the expression and activity of these enzymes was also examined.

4.3 Results

4.3.1 Ahr Genotyping of (BL/10xDBA/2)F_2 Mice

The BL/10xDBA/2 F_2 intercross was genotyped for the Ahr^{b-1} and Ahr^{d} alleles in order to check whether AH responsiveness correlated with susceptibility to HCB and iron induced porphyria. An RFLP which differentiated between the two alleles, was used to type the high and low groups used in the microsatellite analysis. The inheritance of the Ahr^{b-1} and Ahr^{d} alleles were analysed in the same way as the microsatellites markers, to search for correlation.

The *Ahr* RFLP used an *Eco*47III restriction site in exon 7, at nucleotide 752 (a AGCGCT to AGCACT conversion) and was originally used to distinguish between BL/6 and DBA/2 strains which carry the Ahr^{b-1} allele and the Ahr^{d} allele, respectively.[67]

Analysis of the RFLP was carried out by PCR, amplifying the region containing the restriction site, then digesting the product with *Eco*47III. Verification that BL/10 carried the same allele as BL/6 was required before typing the DNA samples in the high and low responding F_2 groups.

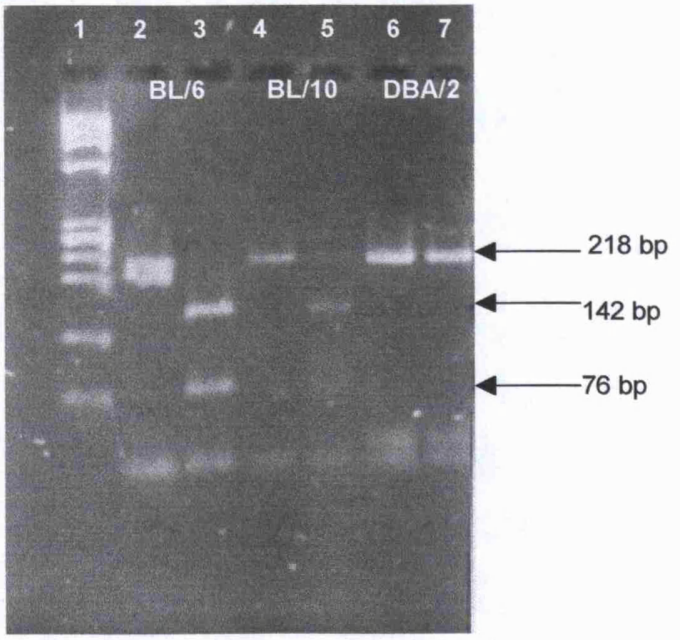
Primers OL72 and OL111 (section 2.13.4) were purified on NAP-5 sephadex columns (2.12.1 p.62, 0.5ml of primer was eluted in 1 x 1ml volume of TE).

The amplification reactions contained 100ng of genomic DNA, 1X AmpliTaq buffer, 0.1mM dNTPs, 10pmoles of each primer and 0.5 unit of AmpliTaq Polymerase in a final reaction volume of 20µl. Reactions were overlaid with mineral oil before thermocycling in a Perkin Elmer DNA thermocycler 480.

Amplification conditions consisted of a denaturing step; 95°C for 5 min, 35 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 10 seconds and a final elongation step at 72°C for 15 minutes. Afterwards, 10μl of each PCR reaction was digested with 2 units of *Eco*47III. The reactions were incubated at 37°C for 2 hours. Products were analysed on 3% metaphor agarose gels.(section 2.13.3)

Figure 4.1 shows the PCR products before and after digestion with BL/6, BL/10 and DBA/2 genomic DNA. The PCR gave a single band of 218 base pairs (bp). After 2 hours of digestion the single band was cleaved into two bands of 142bp and 76bp in the BL/6 and BL/10 samples. There was no digestion of the DBA/2 product. The BL/10 strain was cut showing that BL/10 has the Ahr^{b-1} allele. The DBA/2 strain, which has the Ahr^{d} allele does not have the restriction site, therefore was not cleaved.

Figure 4.1 Result of *Eco*47III restriction digest on C57BL/6, C57BL/10ScSn and DBA/2 *Ahr* exon 7 PCR product



PCR and restriction enzyme digest products of C57BL/6, C57BL/10ScSn and DBA/2, were resolved on a 3% agarose gel.
Lane 1) DNA ladder (ϕX174 fragments), 2) PCR 3) BL/6 product after digest, 4)BL/10 PCR product, 5) BL/10 product after digest, 6) DBA/2 PCR product, 7)DBA/2 product after digest.

The 40 highest and lowest F_2 DNA were typed according to this protocol. The results of which are shown in Table 4.1. Correlation of AH genotype with susceptibility to porphyria was calculated by the χ^2 test, after counting the number of Ahr^{b-1} and Ahr^{d} homozygotes and Ahr^{b-1} / Ahr^{d} heterozygotes.

The results of the χ^2 test gave a significant correlation between the Ahr^{b-1} allele and the high responding F_2 group ($p < 0.001$). However, there was no significant correlation between the low group and the Ah^{d} allele. A good correlation in both groups was expected, as the actual gene was genotyped, as opposed to a microsatellite marker which could be far enough away for recombination to take place. The reason for the lack of correlation with the gene in the low group is unknown. It may be that there is an alternative predisposing gene other than *Ahr* on chromosome 12 or because a number of loci are involved, reducing the effect of this locus. An alternative explaination might be that it were related to the actual F_2 samples chosen, as the large number of nonresponders may be an environmental and not genetic effect.

Table 4.1 Results of *Ahr* RFLP typing and χ^2 analysis on high and low responding BL/10xDBA/2 F_2 mice

F_2 Group	Genotype			χ^2	p
	Ahr^{b-1}	Ahr^{b-1} / Ahr^{d}	Ahr^{d}		
High Responders	19	17	4	12.2	0.001
Low Responders	7	18	13	2.0	0.368

Inheritance of *Ahr* alleles, Ahr^{b-1}(responsive) and Ahr^{d} (nonresponsive), was typed by an *Eco*47III restriction digest on a fragment of the *Ahr* gene amplified by PCR. Correlation of inheritance of responsive and nonresponsive alleles with susceptibility or resistance to experimental porphyria, induced by iron and HCB, was calculated by the χ^2-test. A significant level of correlation was assumed where $p<0.05$.

4.3.2 Expression of Hepatic CYP1A1 and CYP1A2 RNA

As susceptibility to HCB induced porphyria seemed to have some correlation with the AH receptor, the cytochrome P450 enzymes it regulates were analysed in both parent strains and some of the F_2 high and low responders.

If HCB were binding the AH receptor to upregulate CYP1A1 and CYP1A2, there would be an increase in the corresponding messenger RNAs, also the activities of each enzyme in the high responding F_2 group would be elevated as they predominately carry Ahr^{b-1} alleles. As HCB induces several types of cytochrome P450 in rodents, CYP2B1 a phenobarbital inducible P450 may also be upregulated.[73]

Upregulation of RNA expression for CYP1A1 and CYP1A2 was measured by northern blotting and hybridisation with a mouse *cyp1a1* cDNA probe. As *cyp1a1* and *cyp1a2* share a number of regions of homology this probe was used to detect both messages. [121,122] A probe for *cyp2b1* was not available.

Total RNA was prepared by caesium chloride gradient ultracentrifugation or a commercial kit, (section 2.11) from male BL/10 and DBA/2 untreated controls, iron overloaded and iron and HCB treated mice. Mice were exposed to HCB for 7 weeks, then killed by cervical dislocation and the livers removed and frozen immediately in liquid nitrogen. Total RNA was also prepared from the 10 highest and lowest responding F_2 mice.

Northern blots were prepared and probed with a ^{32}P labelled *cyp1a1* cDNA probe. (section 2.10,11 and 12) After exposure, the blots were reprobed with a ^{32}P labelled GAPDH PCR product, this hybridised to the constitutively expressed GAPDH RNA, which was used to confirm the integrity of the RNA and also the uniformity of sample loading.

The results of the hybridisation with both the *cyp1a1* probe and GAPDH probe are shown in Figures 4.2 and 4.3. The *cyp1a-1* probe hybridised to two bands of approximately 2.6 kb and 1.9 kb in length. These bands correspond to CYP1A1 and CYP1A2 respectively, the difference in length being due to a longer 3' untranslated region in *cyp1a-1* transcript.[132] The GAPDH probe hybridised to a message of approximately 1.2 kb.[127]

The main difference between the two parent strains was with CYP1A1 expression. The BL/10 mice treated with iron and HCB showed an increase in CYP1A1 message, whereas there was none was seen in the similarly treated DBA/2 mice.

No CYP1A1 was seen in the untreated or iron loaded controls of either strain, which suggested that it had been induced by HCB in the AH responsive strain only and therefore possibly via the AH receptor.

CYP1A2 transcripts were detected in controls and HCB treated mice as it is constitutively expressed. There appeared to be slightly less message in iron loaded mice, although the blot would require repeating and some quantitation would be necessary to confirm this. Expression of CYP1A2 was upregulated in the BL/10 strain and there was also a slight increase in the DBA/2 strain, which might imply that HCB was inducing CYP1A2 by a mechanism other than the AH receptor.

Figure 4.2 Expression of CYP1A1 and CYP1A2 messenger RNA in C57BL/10 mice

Upper Blot: Northern Blot of C57BL/10ScSn total RNA (10μg per lane) extracted from the liver of untreated controls (lanes 1-4), iron overloaded mice (lanes 5-8) and mice with iron overload and exposure to HCB [0.02%] for seven weeks (lanes 9-12). The lower band of approximately 1.9kb shows the CYP1A2 mRNA, the upper band of 2.6kb shows CYP1A1 mRNA.

Lower Blot: Hybridisation of the same blot with GAPDH probe.

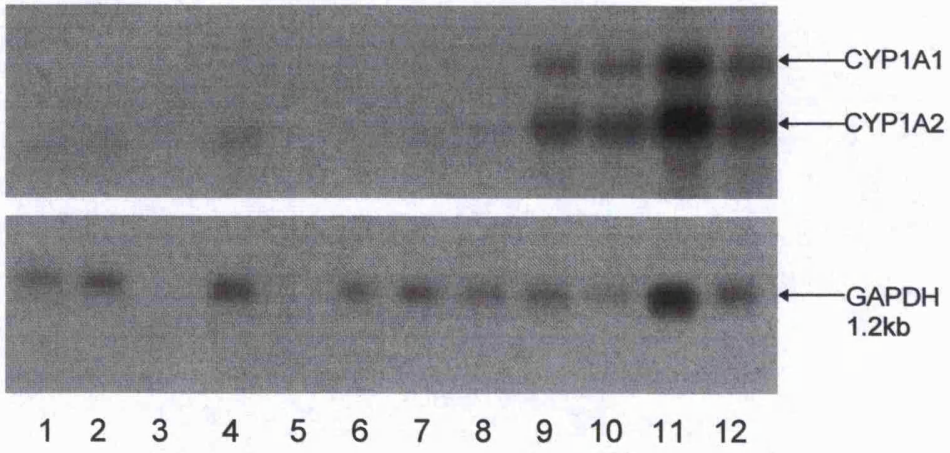
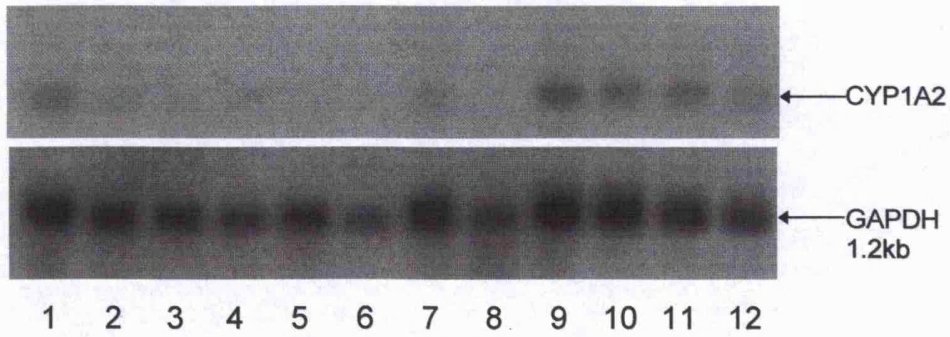


Figure 4.3 Expression of CYP1A1 and CYP1A2 messenger RNA in DBA/2 mice

Upper Blot: Northern Blot of DBA/2 total RNA (10μg per lane) extracted from the liver of untreated controls (lanes 1-4), iron overloaded mice (lanes 5-8) and mice with iron overload and exposure to HCB (0.02%) for seven weeks (lanes 9-12). The band at approximately 1.9kb represents CYP1A2 mRNA. No CYP1A1 message was detected.

Lower Blot: Hybridisation of the same blot with a GAPDH probe.



The F_2 blots show the expression of CYP1A1 and CYP1A2 in the first 10 mice from each group along with the subsequent GAPDH probing. Figure 4.4 shows blots of the high responding group and Figure 4.5 shows the low responding group. CYP1A1 is predominantly expressed in the high responding group but not in the low responding group. This result seems to confirm that AH responsiveness is required for susceptibility to HCB induced porphyria. The induction of CYP1A1 also agrees with the high proportion of Ahr^{b-1} alleles in the high responding group, suggesting that HCB is inducing CYP1A1 via the AH receptor. However, there is hardly any difference in expression of CYP1A2, which has been implicated in the mechanism of experimental porphyria.[77,78] If CYP1A2 was a required predisposing gene, much lower or no expression of this enzyme would be expected in the low responding F_2 group than the high responding group.

The results of the northern hybridisations were confirmed by measuring the activity of CYP1A1 and CYP1A2 in the same individuals.(section 4.3.3)

Figure 4.4 Expression of CYP1A1 and CYP1A2 messenger RNA in high responding F_2 mice

Upper Blot: Northern blot of high responding F_2 total RNA (10μg per lane) extracted from liver. All mice were iron overloaded and exposed to HCB [0.02%] for seven weeks. The lower band of approximately 1.9kb shows the CYP1A2 mRNA, the upper band of 2.6kb shows CYP1A1 mRNA.

Lower Blot: Hybridisation of the same blot with GAPDH probe,1.2kb mRNA band.

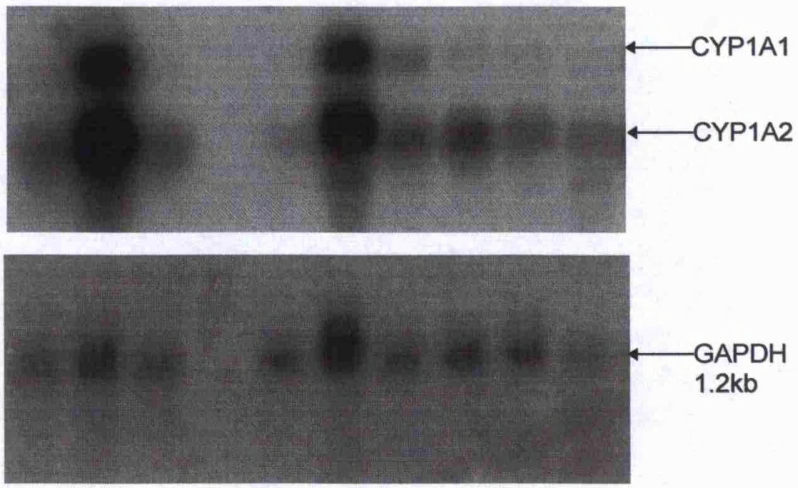
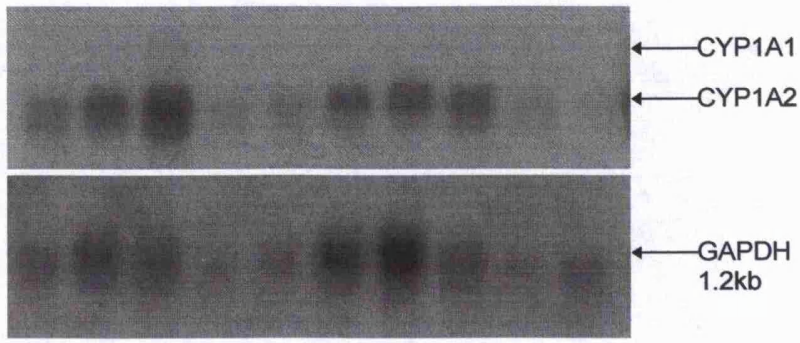


Figure 4.5 Expression of CYP1A1 and CYP1A2 messenger RNA in low responding F_2 mice

Upper Blot: Northern blot of low responding F_2 total RNA (10μg per lane) extracted from liver. All mice were iron overloaded and exposed to HCB [0.02%] for seven weeks. The lower band of approximately 1.9kb shows the CYP1A2 mRNA.

Lower Blot: Hybridisation of the same blot with GAPDH probe,1.2kb mRNA band.



4.3.3 Hepatic Cytochrome P450 Activity

From the results of genotyping the *Ahr* gene and expression of cytochrome P450 induction in the BL/10 and DBA/2 strains and F_2 mice, inheritance of AH responsiveness and induction of CYP1A1 appeared to correlate with susceptibility to experimental porphyria induced by iron overload and HCB.

The activity of hepatic microsomal CYP1A1, CYP1A2 and CYP2B1 were also measured in the parent strains and F_2 groups by a fluorimetric assay, to verify these observations.

As a comparative experiment, a set of each parent mouse strain was induced with β-napthoflavone and phenobarbital, to compare the activity of HCB induced microsomes, with those induced with these classical CYP1A1, CYP1A2 and CYP2B1 inducing compounds.

Male BL/10 and DBA/2 mice, around 7-10 weeks from weaning, were dosed by intraperitoneal injection with corn oil (as a control), β-napthoflavone and phenobarbital, (100mg/kg) (section 2.1.4). After 3 days mice were killed by cervical dislocation and livers were removed and immediately frozen in liquid nitrogen.

Microsomes were prepared from liver samples from the three treatment groups of each strain, and also from BL/10 and DBA/2 mice dosed with iron, iron and HCB and the first 10 individuals from the two F_2 groups. Microsomes were prepared by ultracentrifugation of the supernatant taken from the 9,000g spin of liver homogenates.(section 2.3)

The microsomal P450 activity was assayed by O-dealkylation of ethoxyresorufin, methoxyresorufin, benzyloxyresorufin and pentoxyresorufin as a measure of CYP1A1, CYP1A2 and CYP2B1 activities.(section 2.6)

The P450 activities of the strains treated with iron and HCB, and controls, are shown in graphical form in Figure 4.6 and the calculated rates are given in Table 4.2.

The activities of enzymes assayed in the mice treated with β-napthoflavone and phenobarbital are given in Table 4.3 (p.121) for comparison with HCB induced activity.

Figure 4.6 Bar chart of hepatic microsomal cytochrome P450 O-dealkylase activities in C57BL10/ScSn and DBA/2 mice after iron overload and exposure to HCB in the presence of iron for 7 weeks

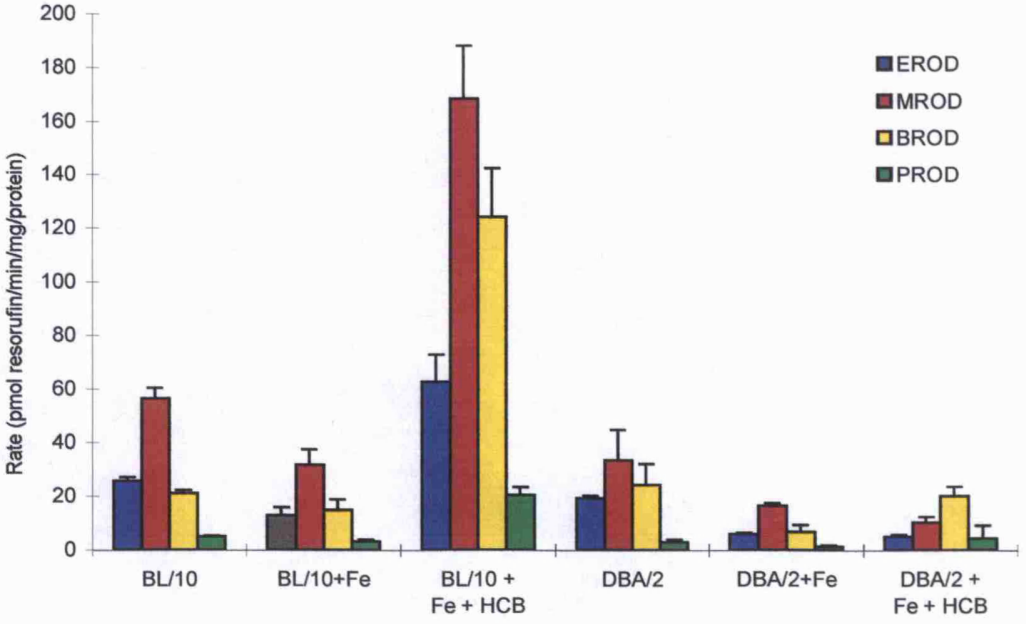


Table 4.2 Hepatic microsomal cytochrome P450 O-dealkylase activities measured in C57BL/10ScSn and DBA/2 strains after iron overload and exposure to HCB in the presence of iron for 7 weeks

Strain	Treatment	Assay Rate (pmoles resorufin formed/min/mg protein) ± SEM			
		EROD	MROD	BROD	PROD
C57BL/10	-	25.8±1.3	56.4±4.0	21.3±1.0	5.0±0.3
	iron	12.8±3.2*	31.7±5.8*	15.0±3.9	3.2±0.7
	iron+HCB	62.7±10.1*	168.5±19.5*	124.1±18.3*	20.4±3.0*
DBA/2	-	19.3±1.0	33.6±11.3	24.5±7.8	3.0±0.9
	iron	5.9±0.6*	16.6±1.0	6.8±2.6*	1.3±0.5
	iron+HCB	5.1±0.6*	10.4±2.1*	20.2±3.5	4.4±0.5

Rates given are the mean values of 4 individuals. Iron loaded mice (iron) received iron-dextran, HCB treated mice (iron+HCB) received iron and HCB in the diet, controls (-) were untreated. EROD was taken as a measure of CYP1A1 activity, MROD as CYP1A2 activity and BROD and PROD as CYP2B activity.

*Significantly different from untreated controls, ($p<0.05$) calculated by Student-t test .

Table 4.3 Microsomal cytochrome P450 O-dealkylase rates assayed from C57BL/10ScSn and DBA/2 livers following induction with β-napthoflavone and phenobarbital

Strain	Number of Mice	Treatment	Assay Rate (pmoles resorufin formed/min/mg protein) ± SEM			
			EROD	MROD	BROD	PROD
C57BL/10ScSn	3	Corn oil	24.0 ± 6.7	14.5 ± 4.0	56.5 ± 26.2	2.3 ± 0.6
	3	β–Napthoflavone	1385.0 ± 91.0*	866.0 ± 69.8*	340.6 ± 16.2*	23.2 ± 1.6
	3	Phenobarbital	65.6 ± 3.7*	60.7 ± 3.3*	1280.7±253.9*	35.2 ± 8.2*
DBA/2	3	Corn oil	3.1 ± 0.7	4.6†	8.1 ± 5.0	6.3 ± 1.0†
	3	β–Napthoflavone	6.9 ± 2.7	24.0 ± 8.5†	13.4 ± 2.8	1.9 ± 0.5†
	3	Phenobarbital	11.8 ± 1.8*	27.7 ± 6.0*	74.4 ± 3.8	8.6 ± 0.5

Mice were given intraperitoneal doses of corn oil (control), β-napthoflavone and phenobarbital [100mg/kg]. Hepatic P450 activity was measured as microsomal O-dealkylase activities. EROD was considered representative of CYP1A1, MROD as CYP1A2 and BROD and PROD as CYP2B.

* Significantly different ($p<0.05$) from corn oil control, anaylsed by Student t test.

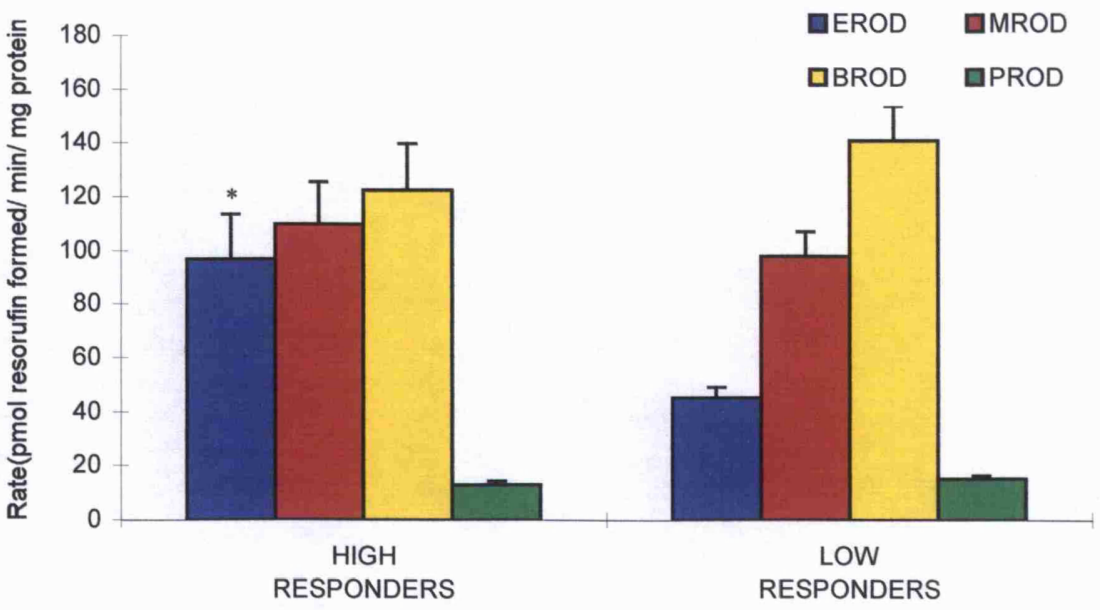
† Less than three samples.

As expected, the activity of all P450 isozymes measured were significantly increased in the BL/10 strain, after treatment with HCB and iron. The activities of CYP1A1 (EROD, 62.7 pmol/min/mg protein) and CYP1A2 (MROD, 168.5 pmol/min/mg protein) in the BL/10 strain were approximately 12 to 16-fold higher than the respective activities in the DBA/2 strain, (EROD; 5.1 pmol/min/mg protein, MROD; 10.4 pmol/min/mg protein) in agreement with *Ahr* genotypes. CYP1A2 had the highest activity of all and the contrast in activity between the two strains would appear to support the uroporphyrinogen oxidation hypothesis as the cause of the porphyria. However, in comparison to the enzyme activities induced by β-napthoflavone and phenobarbital the induction by HCB was minimal. After 7 weeks of continuous exposure, CYP1A1, CYP1A2 and CYP2B1 activities were 20-, 5- and 10- times lower, respectively, than after 3 days of a single dose of β-napthoflavone and phenobarbital.

Interestingly, P450 activities in both strains dosed with iron alone showed a slight reduction in activity, suggesting that iron overload alone can inhibit activity. (This reflects the possible reduction in mRNA seen in the northern blots)

The activities of the F_2 groups are shown in Figure 4.7 and Table 4.4. Overall there was little difference in activity between the two groups. The only significant difference is in CYP1A1 (EROD) activity, where there was a two-fold increase ($p<0.006$ calculated by ANOVA) in activity in the high response group over the low response group. Whether this difference in CYP1A1 activity was sufficient to cause the variation in susceptibility to experimentally induced porphyria seems unlikely. This result also corroborates the finding that there was no linkage with either *cyp1a1* or *cyp1a2* genes, therefore any genetic predisposition was not apparently caused by a polymorphism at these loci.

Figure 4.7 Comparison of the hepatic microsomal cytochrome P450 O-dealkylase activities of F_2 mice with high and low response to iron overload and exposure to HCB



*EROD activity was significantly different ($p<0.01$) between high and low groups

Table 4.4 Hepatic microsomal cytochrome P450 O-dealkylase rates in the high and low F_2 Groups

	Assay Rate (pmoles resorufin formed/min/mg protein) ± SEM			
Response Group	EROD	MROD	BROD	PROD
High	96.7 ± 17.1*	109.9 ± 15.5	122.6 ± 17.2	12.9 ±1.5
Low	45.3 ± 4.2	97.9 ± 9.3	141.0 ± 12.9	15.2 ± 1.3

Mice were selected from the highest and lowest groups responding to iron overload and exposure to HCB. Each groups contained 18 individuals and results were analysed by one way analysis of variance. EROD activity measured in the high responding group was significantly higher (*$p<0.01$) than the low responding group.

4.5 Discussion

Several candidate genes have been examined in the F_2 intercross between the susceptible BL/10 strain and resistant DBA/2 strain. This strategy was followed to confirm the results of the microsatellite analysis and to cover any shortfalls of the method because of the restricted number of markers and animals used.

The influence of the AH receptor was studied using an RFLP, to genotype the high and low responding F_2 groups. The responsive allele (Ahr^{b-1}) correlated well with susceptibility to porphyria ($p<0.001$), but there was no correlation between the AH nonresponsive allele and the low responding group. This result was expected to be clearer than using a microsatellite marker, as the *Ahr* gene itself was typed. However, it seem likely that AH responsiveness is a contributing factor when a compound which is both a ligand and porphyrogenic is administered.

One of the main roles of the AH receptor is to control expression of the cytochrome P450 enzymes CYP1A1 and CYP1A2. These enzymes have been suggested to produce the reactive oxygen species that in turn inhibit UROD by oxidising the substrate molecule or somehow damage the active site itself. There is also some evidence from *in vitro* studies, that CYP1A2 is capable of oxidising uroporphyrinogen to the nonmetabolised uroporphyrin.[77]

Any difference in expression or activity between strains and the two F_2 groups would uphold out this theory. Cytochome P450 induction was thus assessed qualitatively and quantitatively from northern blots and assaying microsomal O-dealkylase activity of both enzymes. The CYP2B1 isozyme was assayed also, as HCB can act as a mixed-type inducer, upregulating phenobarbital inducible P450 enzymes.

The northern blots showed that CYP1A1 was upregulated by HCB in BL/10 mice but not in DBA/2, agreeing with *Ahr* genotype. In the two F_2 groups, CYP1A1 was only induced significantly in the high responding group. This may be as a result of the higher proportion of Ahr^{b-1} alleles in this group found from the RFLP typing, thus susceptibility to chemically-induced porphyria may indeed require a responsive AH receptor.

CYP1A2 mRNA is constitutively expressed in mammalian liver [70] and so was detectable in both control mice as well as HCB treated strains and F_2 groups. The intensity of the CYP1A2 bands appeared to be increase after HCB treatment, implying that it upregulated the expression of CYP1A2 mRNA. in both mouse strains. and was also in all the high and low responding F_2 mice. However, there was no clear difference in CYP1A2 expression between the high and low responding F_2 groups, which suggested that induction of CYP1A2 was not a susceptibility factor.

After assaying the O-dealkylase activities of microsome prepared from HCB treated strains and F_2 mice, P450 induction was found to follow a similar pattern to mRNA expression. Enzyme activities of BL/10 mice were higher than DBA/2 for all enzymes assayed, agreeing with the AH responsiveness of this strain. The only significant difference between the two F_2 two groups was CYP1A1 activity, although overall the activities were very low in comparison to those measured in microsomes induced by β-napthoflavone or phenobarbital, even after seven weeks of continuous exposure.

Therefore the potential for these enzyme to be responsible for the variation in susceptibility to chemically-induced porphyria between BL/10 and DBA/2 strains, seemed unlikely.

5 - Susceptibility to Iron Induced Porphyria

5.1 Summary

The experimental porphyria studied in the previous two chapters was induced by the combined effect of iron overloading and the polyhalogenated aromatic hydrocarbon HCB. Iron overload itself is sufficient to cause a similar porphyria in C57BL/10ScSn and SWR strains.[8,89] The rate of development can be increased by including the haem precursor, 5-aminolaevulinic acid in the drinking water.[8] DBA/2 is resistant to this treatment as with the other inducing agents. This induction method was considered to be the simplest of all the mouse experimental porphyria models, thus a genetic analysis of an SWRxDBA/2 F_2 intercross was undertaken in an attempt to elucidate the predisposing genes.

However the effect of iron overload and 5-ALA in the SWR strain produced a much milder porphyria than the previous experiment and only a small number of susceptible F_2 mice were identified. This did not allow a full, statistically significant microsatellite analysis, so instead only the chromosomes indicated from the BL/10xDBA/2 cross were investigated. This limited analysis revealed chromosome 17 to be a probable location for a predisposing gene.

As no cytochrome P450 inducers were administered to this cross, no involvement of the *Ahr* locus or any cytochrome P450 isozymes was expected. Hepatic microsomal O-dealkylation activities were measured in ten of the highest and lowest F_2 mice to examine whether any difference in constitutive activity of CYP1A1 or CYP1A2 could account for the variation in susceptibility between the strains. No difference was found between the two groups in either enzyme. Therefore it appears unlikely that susceptibility to experimental porphyria, either induced by a polycyclic halogenated chemicals or iron, is caused by a difference in the induction or constitutive activity of the cytochrome P450 1A subfamily of enzymes.

5.2 Introduction

Iron overload alone will cause a experimental porphyria in certain inbred mouse strains, as well as potentiating the effect of the porphyrogenic chemicals that cause a porphyria similar to the human condition S-PCT.[3,4,6-8,40,89] The symptoms of iron-induced experimental porphyria are the same as the chemically-induced porphyria, showing a decrease in UROD activity and an accumulation of uroporphyrin, mainly in the liver. The experimental porphyria caused by iron overload has been assumed to be the simplest model for studying the mechanism of induction, as it does not appear to require involvement of the AH receptor or activation of the cytochrome P450 system.[8,89] In fact, induction of porphyria in mice by iron appears to have no relationship with *Ahr* genotype since one of the most responsive strains to this treatment, SWR, carries the Ahr^d or nonresponsive allele.[8] However, a genetic variation between some strains suggests that other predisposing gene(s) underlie this trait.

The haem precursor 5-aminolaevulinic acid (5-ALA) was found to accelerate the onset of porphyria caused by compounds such as 20-methylcholanthrene (20-MC), HCB and even iron alone.[91] By administering 5-ALA in the drinking water (2mg/ml) to iron loaded C57BL/6 mice also dosed with 20-MC caused a reduction in UROD activity, although by itself, 20-MC does not induce porphyria but does induce CYP1A1 and CYP1A2. It also noticed that 5-ALA could halve the time taken for porphyria to develop in iron and HCB treated C57BL/6 mice. As DBA/2 mice did not respond to treatment with 5-ALA and these chemicals, it was assumed that the response must be caused, in part, by cytochrome P450 1A1 and 1A2 activity.

However, a study on the effect of iron overload alone on twelve inbred strains, selected on the basis of *Ahr* genotype showed that there was no correlation with AH responsiveness. [8] The mice received a single dose of iron and after 25 weeks the most marked depression in UROD activity occurred in the strains C57BL/10ScSn and SWR which are AH responsive and AH nonresponsive, respectively. Further investigation revealed that iron and 5-ALA together could

induce experimental porphyria within 5 weeks, and that the variation in response again did not agree with *Ahr* genotype.[8] Two strains with the Ahr^{b-1} allele; C57BL/6J and C57BL/10ScSn and Ahr^{d} allele; DBA/2 and SWR, given a single dose of iron and administered 5-ALA in the drinking water for six weeks, found the SWR to be the most susceptible strain and DBA/2 the least, although both of these strains were AH nonresponsive.

One theory for the role of the cytochrome P450 enzymes in the mechanism of porphyria induction, has been that CYP1A2 could catalyse the oxidation of uroporphyrinogen to uroporphyrin. This has been demonstrated *in vivo* with CYP1A2 purified from hepatic microsomes of mice induced with 3-MC.[77] Although no difference in induced CYP1A2 activity was detected from the HCB experiment described in the previous chapter, it is possible that a variation in constitutive CYP1A2 activity could be a predisposing influence. CYP1A2 activity determined by microsomal methoxyresorufin O-dealkylase (MROD) assay, has been demonstrated to be slightly higher in SWR mice than in DBA/2, although this marginal difference seemed unlikely to be responsible for porphyria and does not explain how UROD is inhibited.[10]

Another proposition for the difference in susceptibility is a polymorphism in the *Alas-1* gene, whose activity could be affected by iron as happens with some porphyrogenic chemicals. [91] It is thought that administration of 5-ALA enhances the development of porphyria by providing surplus substrate for the inhibited UROD enzyme. Excess uroporphyrinogen would aggravate the disease by being converted to more inhibitor or being oxidised to the nonmetabolised uroporphyrin.[8,10] The administration of 5-ALA in the drinking water would overcome any difference in the biosynthesis of this precursor but it did not impede the variation in strain response.[8]

Recently, 5-ALA alone has been found to cause a porphyria in SWR mice and is also responsible for an accumulation of uroporphyrin in cultured hepatocytes.[10] Again a variation to 5-ALA alone exists between the SWR and DBA/2 strains. It is possible that an enhanced oxidation process is present in SWR mice, leading to a more rapid oxidation of uroporphyrinogen or formation of other products which could inhibit UROD. It has been proposed that 5-ALA

could act as an agent in free radical production and it has been demonstrated *in vitro*, that it can release iron from ferritin, although this would require a mechanism whereby iron still has a potentiating role in the development of this porphyria.

Therefore further investigation into this model of iron induced porphyria might help elucidate aspects of the mechanism involving susceptibility to free radical toxicity, haem synthesis and unknown genes encoding proteins involved with iron metabolism.

5.3 Results

An attempt was made to find an underlying genetic factor in the SWR strain to iron and 5-ALA induced porphyria, as was described with the BL/10 strain with HCB and iron treatment. An F_2 intercross was bred from SWR and DBA/2 parents. However, after breeding and treating over 400 mice, only 20 F_2 mice responded to treatment, so a full microsatellite analysis could not be undertaken.

Two small groups were selected from this F_2 cross on the basis of their response and the microsatellite analysis was only carried out on chromosomes which showed some correlation in the BL/10xDBA/2 cross, or that contained a candidate gene.

To establish whether any constitutive cytochrome P450 enzyme activity could be involved, hepatic microsomal O-dealkylase activities were assayed in the ten highest and lowest responding F_2 mice. CYP1A1 (EROD) and CYP1A2 (MROD) which have been implicated in the mechanism to depress UROD activity, were measured.

5.3.1 Induction of Porphyria by Iron and 5-ALA in SWR and DBA/2 Inbred Strains

Experimental porphyria was induced in male SWR and DBA/2 mice, at 7-10 weeks old. (2.1.2) Mice were given iron-dextran (600mg/kg) subcutaneously. After three days 5-ALA was added to their drinking water at a concentration of

2mg/ml which was then adjusted to pH6. As the 5-ALA solution was light sensitive it also had to protected from light. Control mice were given iron-dextran only. This regime was continued for 5 weeks before mice were culled and livers removed for analysis. The results of this induction are shown in Table 5.1 .

Table 5.1 Hepatic uroporphyrin concentrations in SWR and DBA/2 mouse strains after iron overload and exposure to 5-aminolaevulinic acid (pH6) for 5 weeks

Strain	Number of Mice	5-ALA	Uroporphyrin Concentration (nmol/g liver) ± SEM
SWR	5	-	0.01 ± 0.002
SWR	5	+	5.05 ± 3.76
DBA/2	5	-	0.004 ± 0.002
DBA/2	5	+	0.02 ± 0.004

All mice were dosed with iron and after three days two groups (+) received a solution of 5-ALA (2mg/ml) pH 6, as their drinking water. Mice were fed diet RM1 maintenance diet.

The uroporphyrin concentration in the livers of treated SWR mice were 50 times higher than control levels. Statistically there appears to be no significant difference between treated groups and controls, or even between strains. However, the difference in response between the two strains is clear, despite the variation in induction within the SWR strain, also no increase in uroporphyrin concentration in the DBA/2 strain was ever detected.

In this initial experiment, mice were fed RM1 maintenance diet and the 5-ALA solution was adjusted from pH3 to a more neutral pH, as the mice were reluctant to drink it as often as tap water (possibly because it was bitter tasting). However, stability tests showed that the 5-ALA in solution degraded quicker if it was adjusted to a more alkaline pH, so this was stopped. This experiment was repeated and the results of the porphyrin analysis are shown in Table 5.2. As a final precaution, the 5-ALA was replaced as often as possible, approximately every 2 to 3 days, so that mice were constantly

exposed to a fresh solution. The RM1 diet was changed from maintenance to RM3 breeder diet, which has been used in the original study that determined SWR to be the most susceptible to iron and 5-ALA induced porphyria.[8]

Table 5.2 Hepatic uroporphyrin concentrations in SWR and DBA/2 mouse strains after iron overload and exposure to 5-aminolaevulinic acid for 5 weeks

Strain	Number of Mice	5-ALA	Uroporphyrin Concentration nmol/g liver ± SEM
SWR	5	-	0.10 ± 0.01
SWR	5	+	98.30 ± 79.27
DBA/2	5	-	0.07 ± 0.01
DBA/2	5	+	0.07 ± 0.01

Mice were treated as before, except the 5-ALA solution was not adjusted to neutral pH and mice were fed on RM3 breeder diet.

In the second experiment, the porphyria in the SWR strain, was much more severe, with a mean uroporphyrin concentration of approximately 100 nmol per gram of liver. Although there was still a wide variation in response between individual mice, again no response was observed in the DBA/2 mice.

5.3.2 Breeding and Treatment of SWRxDBA/2 F_2 Hybrid Mice

To carry out a genetic analysis of this trait, an F_2 generation was bred from SWR and DBA/2 strains. The cross was produced as before with BL/10 and DBA/2 strains (Figure 3.2). SWR females were mated with DBA/2 males to produced an F_1 intercross, several pairs of which were subsequently mated to produce the F_2 generation.(section 2.1.2) A total of 439 F_2 males were bred and treated, in 13 batches.

Mice were treated as before with iron-dextran and 5-ALA for 5 weeks, with the same precautions taken to avoid rapid degradation of the 5-ALA. The final 100 F_2 mice bred, were given RM3 diet, the remainder were fed RM1.

Of the 13 batches treated, only six contained any that responded. This might have been a result of the variation seen in the SWR strain itself, which could

be an environmental effect. Otherwise the reason for the large number of low responding mice is not known. Analysis of non-haem iron concentrations in livers samples taken at random from these groups did not reveal insufficient iron dosing, so it was presumed that this was not the reason for the poor response. The problems with 5-ALA degradation were dealt with during the course of the experiment, but changing the solution more frequently and not adjusting the pH did not effect the overall outcome.

Table 5.3 shows the mean uroporphyrin concentrations of the F_2 crosses. The data is from the six batches of mice that contained a responding mouse. The first group of 104 mice were fed diet RM1 and the second group of 107 mice were fed diet RM3, but again there was no significant difference between the groups fed either diet. Groups of 20 high and low responding F_2 mice were selected for further analysis. Mice with a uroporphyrin concentration above 3 nmole per gram of liver were selected as high responders, and mice with less than 1 nmole per gram liver were chosen as low responders.

Table 5.3 Mean hepatic uroporphyrin concentrations of the two (SWRxDBA/2)F_2 groups and of the high and low responding mice selected for genetic analysis

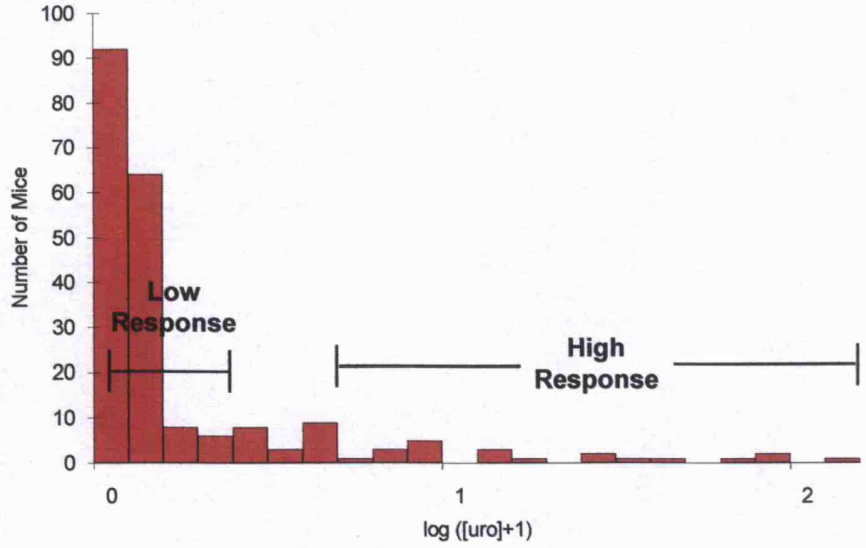
F_2 Group	Number of Mice	Mean Uroporphyrin Concentration nmol/g liver ± SD
Total F_2 mice (RM1)	104	3.9 ± 14.9
Total F_2 mice (RM3)	107	1.8 ± 7.4
High Responders	20	21.9 ± 28.6
Low Responders	20	0.1 ± 0.0

Mice were dosed with iron and received a 5-ALA solution (2mg/ml) as their drinking water for 5 weeks. The first figure is the mean uroporphyrin concentration for F_2 mice fed diet RM1, the second figure is for F_2 mice fed diet RM3. 'High' and 'Low Responders' refers to the 20 highest and lowest individuals taken from both F_2 groups.

Figure 5.1 shows the distribution of hepatic uroporphyrin concentrations among the remaining 211 mice used in the subsequent analysis. The data were

transformed as before by taking logs of (uroporphyrin concentration+1), although this did not produce a continuous or normal distribution in the data.

Figure 5.1 Distribution of hepatic uroporphyrin concentrations in 211 (SWRxDBA/2)F_2 intercross mice treated with iron overload and 5-ALA for 5 weeks



The regions indicated are where the high and low responding mice were selected from for further genetic analysis.

5.3.3 Microsatellite Analysis

As the treatment of the (SWRxDBA/2)F_2 mice did not produce a sufficient number of mice for a full analysis, similar to performed on the BL/10xDBA/2 cross. Only a selected number of chromosomes were typed in the highest and lowest responding mice. The chromosomes chosen typed were those already indicated by the previous analysis.

Genomic DNA was extracted from these individuals in order to be genotyped with a selection of microsatellite markers polymorphic between the two strains.(sections 2.7 and 2.13) Table 5.4 lists the marker used with allele sizes and PCR conditions. SWR microsatellites were not available in the public

databases but were supplied by Professor N Drinkwater from the MacArdle Laboratory for Cancer Research, Wisconsin, USA.

Table 5.4 Microsatellite Markers Selected for Genotyping High and Low Responding (SWRxDBA/2)F_2 mice

Marker	Chromosome	Allele Sizes (bp)		PCR Conditions	
		S	D	$[Mg^{2+}]$ mM	PCR Profile[†]
D4MIT2	4	172	187	1.5	b
D4MIT57	4	126	136	3.0	b
D9MIT31	9	128	112	4.5	a
D9MIT15	9	145	155	1.5	b
D12MIT2	12	132	149	3.0	a
D12MIT3	12	91	124	3.0	a
D14MIT5	14	178	164	2.0	b
D17MIT22	17	152	183	1.5	a
D17MIT49	17	>230	224	1.5	a

Data for microsatellite primers was obtained from the Mouse Genome Database.[96,129,c3]
PCR Profiles: (further details are given in section 2.13)
a: 55°C annealing temperature, 30 cycles of amplification
b: 60°C annealing temperature, 30 cycles of amplification

Chromosomes 4, 9 and 12 were typed as they are the locations of the *Urod*, *cyp1a* and *Ahr* genes, respectively. Chromosomes 14 and 17 were indicated in the BL/10xDBA/2 F_2 cross, as locations for possible porphyria susceptibility loci, although candidate genes for these chromosomes are not known. Two markers were genotyped per chromosome, in most cases, and the correlation between each marker with susceptibility was calculated with a χ^2 test, shown in Table 5.5.

Table 5.5 Results of genotyping the high and low responding (SWRxDBA/2)F_2 groups

Marker	Response to Iron and ALA	Genotype S	H	D	χ^2	p
D4MIT2	High	4	10	5	0.2	0.924
	Low	6	5	8	4.8	0.096
D4MIT57	High	5	12	2	2.3	0.323
	Low	4	9	7	1.1	0.577
D9MIT31	High	4	8	8	2.4	0.301
	Low	1	15	2	8.1	0.017*
D9MIT15	High	4	12	1	3.9	0.139
	Low	6	11	3	1.1	0.577
D12MIT2	High	8	9	3	2.7	0.259
	Low	3	12	5	1.2	0.379
D12MIT3	High	8	4	7	6.5	0.039*
	Low	2	15	3	5.1	0.078
D14MIT5	High	7	9	4	1.1	0.577
	Low	4	9	7	1.1	0.577
D17MIT22	High	9	1	10	16.3	0.000*
	Low	5	0	15	30.0	0.000*
D17MIT49	High	8	3	7	8.1	0.017*
	Low	4	6	9	5.2	0.074

*Siginificant correlation with susceptibility to iron induced porphyria ($p<0.05$).
S; SWR alleles, H heterozygote, D; DBA/2 alleles.

No correlation was found between markers on chromosomes 4 or 14. This would imply that a mutation or polymorphism in the *Urod* gene is not responsible for the predisposition to iron induced porphyria. One candidate gene for chromosome 14 is the hepatic ALA-synthase gene, *Alas-1*.[8,9,91] No correlation was found with the marker selected, which was correlated with susceptibility to HCB treated porphyria. Even if ALAS-1 was induced by iron loading, a variation in response to iron still present after administration of 5-ALA in solution.

As both strains were AH nonresponsive and are reported to have the Ahr^d form of the receptor, no linkage was expected with chromosome 9 or 12. The SWR *Ahr* gene was typed with the RFLP previously described (section 2.13.4) that distinguished between Ahr^{b-1} and Ahr^d alleles. Figure 5.2 shows the result of the PCR reaction and digest. As the amplified product was not cleaved, the

SWR alleles must possess the same sequence as the DBA/2 strain, at least in this region of the gene. [10]

Marker D9MIT31 appeared to correlate, although only in one group. These results could not be presumed to be significant until larger numbers of mice are typed.

The only markers that gave a significant result was D17MIT22 ($p<0.001$ in both groups) and D17MIT49, on chromosome 17. However, this result would also required verification with a larger number of mice.

Figure 5.2 Analysis of the *Ahr* RFLP in SWR and DBA/2 strains

Genomic DNA from strains SWR and DBA/2, was amplified with primers which spanned an *Eco*47III RFLP in exon 7 of the *Ahr* gene. The 218bp PCR products and restriction digest products were resolved on a 3% agarose gel.
Lane: 1) ϕX174 DNA ladder, 2) DBA/2 PCR product, 3) DBA/2 digest product, 4) SWR PCR product, 5) SWR digest product.
(A BL/6 positive control, originally included on this gel, was removed from the photograph, so unfortunately was unavaliable for this figure.)

5.3.4 Cytochrome P450 Activities of Susceptible and Resistant (SWRxDBA/2)F_2 Mice

Constitutive cytochrome P450 1A1 and 1A2 activities were measured in the high and low responding F_2 groups. The activities in the two F_2 groups from the (BL/10xDBA/2) cross only showed a marginal difference in CYP1A1 activity, assumed to be a result of the interaction between HCB and the AH receptor. With the iron and 5-ALA model, no AH receptor ligand or cytochrome P450 inducer was supplied, therefore levels were not expected to be increased. However, if a difference in constitutive expression existed between strains, this might prove to be an inherited susceptibility trait.

Hepatic microsomes were prepared from the first ten individuals, from the highest and lowest responding groups. (section 2.3) Activity of CYP1A1 and CYP1A2 were assayed as ethoxyresorufin (EROD) and methoxyresorufin (MROD) O-dealkylase activities.(2.6) The mean activity for each group is shown in Table 5.6.

Table 5.6 Hepatic microsomal O-dealkylase activities measured in (SWRxDBA/2)F_2 mice with high or low susceptibility to iron and 5-ALA induced porphyria

F_2 Group	Rate ± SEM (pmol resorufin formed/min/mg protein)	
	EROD	MROD
High Responders	7.4 ± 0.5	22.4 ± 1.5
Low Responders	7.0 ± 0.9	22.7 ± 3.2

Hepatic cytochrome P450 1A1 and 1A2 activities were measured by EROD and MROD activity, in 10 individuals F_2 mice with the highest and lowest response to treatment . As no P450 enzyme inducers were administered the activity seen in the EROD assay may reflect CYP1A2 activity.

There was no significant difference between either group for both enzymes. In fact, the rates were practically identical. The EROD and MROD activities for the high and low F_2 groups being approximately 7 and 22 pmoles resorufin/min/mg protein, respectively. The higher rate of CYP1A2 activity was expected as it is expressed constitutively in the liver.[10,70]

The lack of correlation between enzyme activity and susceptibility to iron-induced porphyria in this mouse model implies the cytochrome P450 1A isozymes, especially CYP1A2 are not inherited predisposing factors.

5.4 Discussion

A genetic variation exists between the inbred mouse strains SWR and DBA/2 to porphyria induced by iron overload alone. The onset of the disease can be greatly increased by administering the haem precursor 5-ALA in the drinking water.

The effect that iron and 5-ALA has on interrupting haem biosynthesis is much milder than that of chemicals such as HCB and TCDD. As these compounds are not involved, the iron and 5-ALA induction model is considered to be much simpler. However, it has been proposed that even this case is the result of more than one susceptibility gene.[8]

An F_2 intercross was bred from SWR and DBA/2 mice, in an attempt to carry out a genetic analysis on the same principles as the BL/10xDBA/2 intercross (chapter 3). Problems with reaching a sufficient number of responding mice meant that this analysis could not be accomplished to the same degree. Instead, the mice that responded (20 in total) were compared with an equal number that did not respond, by typing a number of microsatellites on chromosomes 4, 9, 12, 14 and 17. These chromosomes were chosen as they contained candidate genes or, in the case of 14 and 17, were highly significant in the BL/10xDBA/2 intercross.

The only significant result found from this analysis, was chromosome 17. This is the presumed location of an iron metabolising gene and possibly the mouse equivalent of the haemochromatosis gene and is thought to be involved in human S-PCT. [45] As this chromosome is common to both iron and chemical induction models, it would appear to be a strong candidate for the location of a susceptibility gene.

Chromosome 14 showed no correlation with susceptibility to iron induced porphyria, although it was implicated in the HCB experiment. However as a small number of animals were used it cannot not be ruled out completely.

On the other hand, the chances of either CYP1A1 or CYP1A2 having a predisposing influence are small. CYP1A2 is reported to be able to oxidise uroporphyrinogen to uroporphyrin *in vitro* [77] and there is a small difference in constitutive levels of activity between SWR and DBA/2 strains. [10] This difference was not inherited in the high and low responding F_2 groups. In fact, CYP1A1 and CYP1A2 activities were identical in both groups.

From this study the influence of only one chromosome, 17, appears to be required for susceptibility. Although a larger study would be needed to confirm any other candidates or find more unknown factors, it does corroborate the result from the HCB and iron experiment and implies that the iron and chemical induction mechanisms might be similar processes.

6 - Induction of Experimental Porphyria in BL/10.D2n Congenic Mice

6.1 Summary

The involvement of a gene (or genes) on mouse chromosome 17 in the induction of porphyria by HCB and iron has been investigated using the congenic mouse strain, BL/10.D2n. Chromosome 17 is the assumed location of the mouse haemochromatosis gene, as it contains the mouse equivalent of the HLA complex. Heterozygosity for a haemochromatosis allele has been suggested as a possible genetic predisposition for the human form of sporadic-PCT. The mouse homologue could be responsible for similar symptoms in the mouse. Recently, several genes encoding nonclassical HLA class I type proteins, that may be regulated by iron, have been mapped to chromosome 17 in the mouse.[101] These findings suggest that there could be a gene involved in iron metabolism coded for on this chromosome, making chromosome 17 a candidate location for a gene contributing to susceptibility to porphyria in mice.

BL/10.D2n mice are a congenic strain which have the DBA/2 H-2 complex on a BL/10 background, therefore any iron metabolising genes which are in the proximity of this complex and could be involved in experimental porphyria, will be the resistant (DBA/2) type. The influence of this gene on experimental porphyria, if it is at this location, was studied by inducing porphyria in congenic mice by exposure to HCB in the presence of iron and with iron overloading alone. Porphyria developed in the congenics treated with HCB and iron, whereas iron alone had a much smaller effect, suggesting that a gene responsible for susceptibility may be in the H-2 complex.

The extent of the DBA/2 region on the congenic chromosome 17 was examined with a series of microsatellite markers along the chromosome. Significant markers used in the analysis of the F_2 crosses were found to lie within the DBA/2 section of the congenic chromosome 17.

6.2 Introduction

It has been hypothesised that heterozygosity for hereditary haemochromotosis gene could be the genetic predisposition required for sporadic porphyria cutanea tarda.[45] As elevated iron stores and haemosiderosis are frequently observed in S-PCT, the involvement of an iron metabolism gene is thought to be important for susceptibility.

Recently, a gene has been cloned that is thought to be responsible for hereditary haemochromatosis in humans.[52] The gene codes for a novel nonclassical HLA class I protein, HLA-H, which is a membrane bound polypeptide, with three extracellular domains, including a β_2 microglobulin binding region, a membrane spanning region and a short cytoplasmic tail. Support for a defective class I MHC protein causing haemochromatosis has also come from studies with the β2 microglobulin knockout mouse which apparently accumulates iron as in the human condition.[101]

Several nonclassical MHC class I genes, including Q1, Q2 and two TL genes, mapped to chromosome 17, are thought to be involved in iron metabolism. These genes have a β-globin analogous promoter (β-GAP), this promoter that can work in either direction, promoting genes on both strands of DNA. The similarity to a β-globin promoter suggests a role for iron in the regulation of these genes. However, the mechanism by which the HLA-H protein is involved in iron overload in not known. It may be a receptor for an iron-binding ligand, it could be involved in signal transduction, regulation of a gene or genes controlling iron transfer in the plasma, or it may act with other components of the immune system involved with iron metabolism.

From the microsatellite analysis of inheritance of susceptibility to porphyria in both the BL/10xDBA/2 F_2 and SWRxDBA/2 F_2 intercrosses, chromosome 17 has a high chance of containing a predisposing gene. In particular, markers D17MIT16, D17MIT22, D17MIT24, which are all within the H-2 complex of genes were found to have a high correlation with susceptibility. As this chromosome was found to be common to both crosses, it is possible that it contains a gene which is involved with porphyria or iron-mediated toxicity. In

order to determine if a susceptibility gene definitely lay in the region of the H-2 complex, the susceptibility to HCB and iron induced porphyria of the congenic strain of mouse BL/10.D2n was studied.

Iron in excess potentiates the porphyrinogenic action of HCB in certain strains of mice and is especially effective at producing porphyria in BL/10 mice.[6] Susceptibility of BL/10 to porphyria is thought to be caused by several unknown predisposing genes and possibly also the AH receptor.

The BL/10.D2n strain is congenic for the DBA/2 H-2 complex, all the other chromosomes are derived from the BL/10 mouse. Thus any genes lying in the congenic region would be inherited from the DBA/2 strain and be the resistant type.

If susceptibility was partially controlled by a gene on this part of the congenic chromosome, it would effect the severity of the porphyria. If on the other hand, HCB or iron acted through a gene on chromosome 14 for example, the effect of the congenic region would be expected to be minimal. Experiments were conducted with both HCB and iron overload and iron alone. Iron overload alone in BL/10 mice will cause a moderate porphyria after six months [89] and it is possible that this induction requires the interaction of fewer genes. If a gene (or genes) in the congenic region of chromosome 17 was involved in iron metabolism, then induction by iron alone in the congenic strain would be decreased, with minimal or no porphyria at all.

The extent of the DBA/2 DNA in chromosome 17 was estimated with microsatellite markers. The microsatellites used for the linkage analysis were genotyped along chromosome 17 as this information would support the results from the F_2 intercrosses. If a gene lay in the congenic region that changed the susceptibility to HCB or iron induced porphyria, markers with a significant chance of being linked to a gene would have a DBA/2 genotype in the congenic strain.

6.3 Results

Experimental porphyria was induced in BL/10.D2n male congenic mice and controls by exposure to HCB and iron overload for seven weeks and also by iron overload alone for six months. The aim of these experiments was to investigate if a gene in the DBA/2 congenic region of chromosome 17 could affected susceptibility to experimental porphyria.

To assess the extent of the region covered by DBA/2 DNA on chromosome 17, microsatellite DNA was analysed by PCR. The genotype of each microsatellite was used as an indicator of whether the region was derived from BL/10 or DBA/2 DNA.

6.3.1 Induction of Porphyria by HCB and Iron Overload in BL/10.D2n Congenic Mice

Porphyria was induced in male mice by HCB and iron.(section 2.1.3-A). Mice were iron overloaded by subcutaneous injection with iron-dextran (600mg/kg), after 3 days they were fed a diet containing HCB (0.02%) for seven weeks. Controls of each strain were fed on RM1 maintenance diet for seven weeks. Results of the porphyrin analysis are shown in Table 6.1.

Table 6.1 Mean hepatic uroporphyrin and non-haem iron concentrations from BL/10.D2n and C57BL/10ScSn Strains treated with HCB and iron

Strain	Treatment	Number of Mice	Uroporphyrin Concentration (nmol/g liver) ± SEM	Non-Haem Iron (mg/g liver) ± SEM
C57BL/10ScSn	-	5	0.2 ± 0.04	0.06 ± 0.01
C57BL/10ScSn	HCB+iron	6	66 ± 37	1.2 ± 0.1
*(C57BL/10ScSn	HCB+iron	6	65± 26)	N/A
BL/10.D2n	-	4	0.3 ± 0.1	0.08 ± 0.01
BL/10.D2n	HCB+iron	5	547 ± 84	1.5 ± 0.1

Treated mice (HCB+iron) were given a single dose of iron and fed a diet containing HCB for seven weeks. Controls (-) were maintained on diet RM1 for seven weeks.

* Porphyrin analysis was repeated in the BL/10 group treated with HCB and iron with 1g of liver as opposed to 0.5g.

A uroporphyria had developed in both the BL/10 strain and the BL/10.D2n congenic mice, after seven weeks of treatment with HCB and iron overload. The extent of the porphyria in the BL/10 strain was much lower than expected. Results from previous experiments with HCB and iron on BL/10 mice have given uroporphyrin concentrations similar to those seen in the congenic strain (Table 6.2). As the porphyrin analysis used 0.5g of liver tissue, it was possible, due to the heterogeneity of porphyrin accumulation in the liver, that the samples taken for analysis were not representative of the total porphyrin content. To over come this problem, the analysis was repeated with 1g of tissue, however this did not alter the resulting porphyrin concentration.
Non-haem iron levels were measured in liver samples taken from the original mice BL/10 and BL/10.D2n treated with HCB and iron, to verify that they had been given a sufficient dose of iron-dextran to responded to treatment.

Table 6.2 Previous Induction of Experimental Porphyria by HCB and Iron Overload in C57BL/10ScSn Mice.

Strain	Treatment	Number of Mice	Uroporphyrin concentration (nmol/g liver) ± SEM
C57BL/10ScSn	iron	5	1.7 ± 2.6
C57BL/10ScSn	HCB+iron	4	379.9 ± 94.6

Mice were treated with iron or iron and HCB, as described above and hepatic porphyrins measured after seven weeks.

Overall, the response to HCB and iron in the congenic strain was typical of the experimental porphyria found in BL/10 mice. This would suggest that any gene in the congenic region of chromosome 17 does not affect susceptibility to HCB and iron in combination. However, if there is a gene in this region which is susceptible to iron alone, any effect it has may have been masked by the much more potent effect of HCB and iron working together.

6.3.2 Induction of Porphryia by Iron Overload alone in BL/10.D2n congenic mice

As the experimental porphyria induced by HCB may have been associated with genes on chromosome 12 or 14, the effect of chromosome 17 may have been overridden. As a moderate porphyria can be induced in BL/10 mice by iron alone, an experiment to assess the effect of iron overload in congenic mice was set up.

Male BL/10, DBA/2 and BL/10.D2n mice were dosed with iron-dextran (600mg/kg) by subcutaneous injection (section 2.1.3-B). After six months, the mice were culled and livers removed for porphyrin analysis, the results of which are shown in Table 6.3.

TABLE 6.3 Induction of Experimental Porphyria by Iron Overload in BL/10.D2n, C57BL/10ScSn and DBA/2 strains.

Strain	Treatment	Number of Mice	Uroporphyrin Concentration nmol/g liver ± SEM	Non-Haem Iron mg/g liver ± SEM
C57BL/10ScSn	-	5	0.1 ± 0.04	0.04±0.00
C57BL/10ScSn	iron	5	96 ± 35	2.0±0.2
DBA/2	-	5	0.06 ± 0.03	0.1±0.01
DBA/2	iron	5	0.07 ± 0.02	1.6±0.08
BL/10.D2n	-	5	0.13 ± 0.03	0.06±0.01
BL/10.D2n	iron	6	4.15 ± 3.03	1.5±0.1

Uroporphyrin concentrations are the mean for each group. Iron dosed mice (iron) and controls (-).

A moderate porphyria developed in the BL/10 mice after six months. No elevation of hepatic porphyrins was detected in the DBA/2 mice and only a minimal increase was seen in the congenic strain.

The much reduced porphyria produced in the BL/10.D2n mice is possibly caused by a gene within the congenic region. In this strain the gene would confer a resistance to induction as it is derived from the DBA/2 mouse. The slight elevation above control levels may be an environmental effect or could be due to a susceptibility gene at another locus.

6.3.3 Microsatellite Analysis of BL/10.D2n Chromosome 17

From the microsatellite analysis of BL/10xDBA/2 and SWRxDBA/2 F_2 intercrosses, markers with a high chance of being linked to a susceptibility gene lay in a region of approximately 5cM, between 18 to 23cM from the centromere. As the exact extent of the congenic region of chromosome 17 was not known, microsatellite markers were used for typing the congenic region.

Since the congenic strain had a much lower susceptibility to induction of porphyria by iron alone, it is possible that an iron metabolism gene causing a resistance induction lies within this stretch. If the extent of the congenic region was known, the position of the gene could be narrowed down further still and markers with a high significance that lay outside this region may be excluded, (unless there was a second gene involved situated on the same chromosome).

By genotyping congenic chromosome 17 with the same markers used in the F_2 analysis, the position of each marker relative to the congenic region could be assessed.

Genomic DNA was extracted from liver tissue from the congenic strain, by phenol-chloroform method. (section 2.7.1) PCR of 100ng genomic DNA from the congenic strain and BL/10 control DNA, was performed using the microsatellite primers listed, with amplification conditions, in Table 6.4. The PCR products were analysed by agarose gel electrophoresis (section 2.13.3).

The results of this genotyping are given in Table 6.5. The DBA/2 region of chromosome 17 was positioned from 17.3cM to at least 20cM from the centromere, with the markers available. Most of the significant markers from the F_2 analysis lie within this region except D17MIT49.

TABLE 6.4 List of microsatellite markers used to locate the DBA/2 derived region of chromosome 17 in the BL/10.D2n congenic region

Microsatellite Marker	PCR Conditions	
	$[Mg^{2+}]$mM	PCR Profile
D17MIT30	1.5	a
D17MIT16*	1.5	a
D17MIT22*	1.5	a
D17MIT24*	1.5	a
D17MIT49*	1.5	a
D17MIT20	2.0	a
D17MIT41	2.0	a

Microsatellite primer data was obtained from the Mouse Genome Database. [93,96,129,c3]
*Markers showed significant correlation ($p<0.05$) with susceptibility in the BL/10xDBA/2 F_2 intercross, calculated by χ^2 test .
PCR Profile:
a: 55°C annealing temperature, 30 cycles of amplification
b: 58°C annealing temperature, 30 cycles of amplification

Table 6.5 Genotypes of the BL/10.D2n congenic chromosome and extent of the DBA/2 congenic region

Microsatellite	Location from Centromere / cM	Congenic Genotype
D17MIT30	17.3	DBA/2
D17MIT16	18.2	DBA/2
D17MIT22	18.8	DBA/2
D17MIT24	20.0	DBA/2
D17MIT49	22.3	BL/10
D17MIT20	34.4	BL/10
D17MIT41	53.2	BL/10

Positions of microsatellite are approximate and taken from the Mouse Genome Database[93,96,129,c3]. Shaded areas represent markers which showed significant correlation with inheritance of susceptibility.

6.4 Discussion

Chromosome 17 was implicated as the location of a predisposing gene by the microsatellite analysis of inheritance of experimental porphyria in the F_2 intercrosses, bred from the susceptible strains BL/10 and SWR, and the resistant strain DBA/2. This chromosome is interesting to this study for two reasons. Firstly, it contains the mouse equivalent of the histocompatibility complex, the H-2 complex and is homologous to the region of human chromosome 6, where the haemochromatosis gene is located.[52,101,133] Secondly, there is also evidence for several genes involved in iron metabolism on the mouse chromosome 17. These genes code for non-classical class I type proteins, and have promoters similar to the β-globin gene, which is regulated by haem,[11] and have been proposed to be the mouse equivalent of the haemochromatosis gene. [101]

The link between these genes and experimental porphyria comes from studies of human S-PCT patients that have shown a higher incidence of heterozygosity for the haemochromatosis allele than the normal population. Homozygous carriers suffer from a serious iron overload disorder, and it is possible that heterozygous individuals could have sufficiently high iron levels to bring about S-PCT. [45]

Congenic mice have been used to find out if an H-2 linked gene could influence susceptibility to experimental porphyria by HCB and iron or iron alone. A congenic strain of mouse was available for this, which contained a DBA/2 H-2 complex on a BL/10 background.

An effect was shown in the iron overloaded congenic mice. Congenic mice given iron and HCB for seven weeks responded as well to treatment as the C57BL/10ScSn strain. However, in the iron overload experiment the extent of the porphyria in the congenic strain was less then 5% of the C57BL/10ScSn, which develops porphyria six months after being dosed.[89]

The reason for the lack of difference in response to HCB and iron induced porphyria may be due to the contribution of other genes, such as the *Ahr* on chromosome 12 or the chromosome 14 gene, which would still confer

susceptibility in the congenics, as they are derived from the BL/10 strain. This might imply that the chromosome 17 gene is a minor predisposing factor and as there is still a minimal induction with iron alone, that could be a second gene involved in this iron model or an environmental factor.

Finally, as the extent of the congenic region of chromosome 17 encompassed most of the highly significant DNA markers indicated from the BL/10xDBA/2 and the SWRxDBA/2 F_2 intercross. Therefore it appears that there is a porphyria susceptibility gene on chromosome 17, linked to the H-2 complex. The nature of the gene is unknown but is probably involved in iron metabolism and could even be the mouse haemochromatosis homologue.

7 - Uroporphyrinogen Decarboxylase

7.1 Summary

Uroporphyrinogen decarboxylase is the haem biosynthetic enzyme that converts uroporphyrinogen III to coproporphyrinogen III. Familial types of PCT are associated with an inherited mutation in the gene sequence. So far, no mutations have been found in the *Urod* sequence from sporadic PCT patients.[30] Therefore any genetic predisposition must be inherited from another locus. The actual defect in UROD activity in these cases is thought to be due to inactivation of the enzyme by an inhibitor or lack of substrate as a result of increased oxidation of uroporphyrinogen.

A 300bp fragment from the 3' end of the C57BL/10ScSn *Urod* gene was isolated by PCR, using primers originally designed to rat UROD.[126,134] The fragment was verified by cycle sequencing and comparison with the recently cloned C57BL/6 sequence.[135] The fragment was also used to probe a northern blot to try to examine *Urod* expression in the liver. No obvious differences in expression between BL/10 and DBA/2 strains, or mice with and without experimental uroporphyria, were detected.

7.2 Introduction

Uroporphyrinogen decarboxylase is the cytosolic enzyme that catalyses the removal of four carboxylic acid groups from uroporphyrinogen III to give the product coproporphyrinogen III (Figure 1.2, p.6). It has a molecular mass of approximately 42kDa and exists as a monomeric protein in all species so far examined except for chicken, where it appears to be a dimer with a mass of 72kDa. [1,32]

In humans UROD is encoded by a single gene and is located to chromosome 1. The gene is comprised of 10 exons spread over 3kb of DNA and the full length mRNA transcript is approximately 1.2kb. UROD has also been isolated

and cloned in several other species including *E Coli*, *Saccharomyces cerevisiae*, rat and, during the course of this work, mouse.[32,126,135,136]

There is a high degree of homology between species sequences. The human and mouse sequences being approximately 90% identical, and similarities with other species range from 59-68%. Several regions of highly conserved amino acid sequence exist between all species. These probably encode functionally important parts of the enzyme, suggesting that the structure of the protein has been conserved through evolution.[32,126]

Defective UROD activity is responsible for the most common porphyria, PCT. Despite its prevalence, PCT is the least well characterised of the porphyrias and little is known about the molecular genetics of the familial types. About 25% of PCT cases are familial (type II), the other 75% are sporadic (type I).[32]

Defects found in the UROD gene of familial PCT patients include a point mutation that decreases the stability of enzyme or a deletion of exon 6, the most common mutation, which produce defectively spliced mRNA. However, this deletion does not appear to always create a defective enzyme. Complex splice patterns have been found in the human UROD gene. Seven alternatively spliced UROD sequences, were found in normal individuals as well as familial PCT patients. However, these products were of low abundance and were probably produced as a by-product of failure of the normal splicing mechanism or post transcriptional modification. [137]

In familial PCT UROD activity is reduced to 50% in all tissues, and is caused by heterozygosity or compound heterozygosity for a mutation. Homozygotic mutations in UROD reduce activity to less than 10% and are thought to cause hepatoerythropoietic porphyria. [1]

In a study of ten unrelated patients with sporadic PCT no mutations were detected that could alter UROD activity. The promoter regions from six patients also showed no differences when compared with the promoter sequence from normal individuals.[30] This strongly suggested that if there was an inherited predisposition to sporadic PCT, it was not the UROD gene itself.

The concentration of immunoreactive UROD is also unchanged in patients with S-PCT [31] and in rodents with porphyria caused by exposure to the toxins

HCB or TCDD.[138] No quantitation of UROD mRNA from patients has been reported.

These observations suggested that a lack of UROD activity in cases of S-PCT in humans, or experimental porphyria in rodents, is not a result of a mutation but is probably inhibition or inactivation of the enzyme, which appears somehow to involve iron. Any genetic predisposition to this type of porphyria would require the influence of other loci.

In chapters 3 and 5, no linkage was detected between chromosome 4, (the location of the mouse *Urod* gene) and susceptibility to experimental porphyria. This finding agrees with what is already known about the UROD gene from human and experimental porphyrias. Further analysis of the mouse *Urod* gene was undertaken. A northern blot was probed using a fragment of BL/10 *Urod* cDNA, to show qualitatively, any differences in the expression of this gene in the livers of BL/10 and DBA/2 strains.

7.3 Results

The *Urod* mouse gene is located on chromosome 4, in a region syntenic with human chromosome 1.[126] The gene was located by linkage analysis using a genomic DNA PCR product as a probe, which was amplified using primers made to the rat sequence.[126,134] During the course of writing this thesis, the complete mouse *Urod* cDNA has been cloned from a C57BL/6 liver cDNA library and its position on chromosome 4 confirmed.

In order to detect any changes in expression of *Urod* in mice with experimental porphyria, a probe to the *Urod* gene was made using the published rat primers. The fragment amplified spanned the last 300bp at the 3' translated end and was confirmed by cycle sequencing and comparison with the C57BL/10 and rat sequence.

The 300bp product was subsequently used to probe to a northern blot by incorporating ^{32}P-labelled dCTP in the amplification reaction.

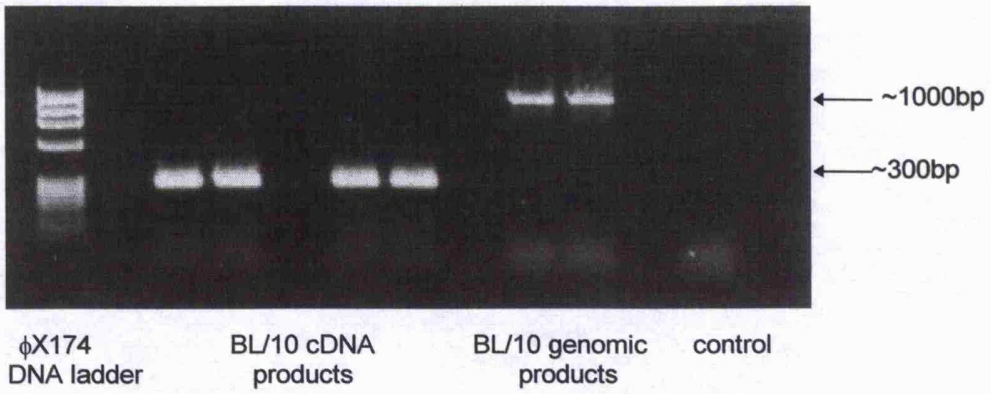
7.3.1 Amplification of Mouse *Urod* Fragment

Mouse genomic DNA was prepared from 30mg of liver tissue from C57BL/10ScSn mice and extracted by the phenol-chloroform method. (section 2.7.1) cDNA was prepared by reverse transcription of 1μg of mRNA with a Clontech cDNA synthesis kit (section 2.14). Total RNA was extracted from approximately 0.5g liver by the ultracentrifugation method (section 2.11.1) mRNA was prepared from 250μg of total RNA using oligotex-dT beads (section 2.11.3).

The reversed transcribed product and 100ng of genomic DNA were amplified with the published rat primers. (section 2.14)[126]

The predicted sizes of each products were 1,000bp for the genomic product and 300bp for the cDNA product. The amplified products were resolved by agarose gel electrophoresis and are are presented in Figure 7.1.

Figure 7.1. Amplification of mouse *Urod* cDNA and genomic DNA fragment



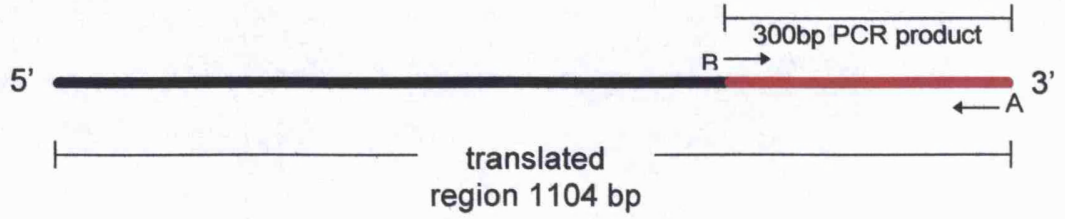
Duplicate samples of BL/10 cDNA and genomic DNA were amplified with primers taken from rat UROD sequence and products were resolved on a 2% agarose gel. The sizes of the cDNA and genomic products matched the predicted sizes of 300bp and 1,000bp, respectively.

7.3.2 Sequencing of *Urod* cDNA Product

To confirm that the product was *Urod*, the cDNA PCR product was sequenced by cycle sequencing. The 300bp product was cleaned with a Qiaquick spin column purification kit. (Section 2.14) The product was sequenced in both forward and reverse directions using the original amplification primers, with an ABI dye termination cycle sequencing kit. The position of the BL/10 sequence is shown in relation to the rest of the gene in Figure 7.2.

Approximately 2μg of template DNA was sequenced per reaction. (section 2.14) After sequencing, the products were purified and excess dye terminators were removed by phenol-chloroform extraction, precipitated in ethanol and dried under vacuum. (Section 2.14) The sequencing products were analysed on an ABI automated sequencer.

Figure 7.2 Position of the amplified mouse *Urod* fragment



The diagram above shows the position of the amplified and sequenced product in relation to the rest of the *Urod* coding sequence. A and B represent the position of the rat PCR primers used to amplify the fragment. Primer sequences A: 5'-TCAATTCTGTCGAAGCAGGCGTGAGTG-3', B:5'CTGGAAGAGCTGGCC CAGGCTGGCTATGAG-3'

The sequences from eight reactions, four from each primer, are given in Figure 7.3, p 155. Sequence data was downloaded from the automatic sequencer and analysed using 'Sequence Editor', Version 1.0.3 (Applied Biosystems), which displayed the data as text and chromatograms. The original chromatograms are given in Appendix II.

The first seventy bases of unreadable sequence, close to the primers, were removed. Sequence from primer A was reversed and complemented, using the editing program, as this sequence was in the reverse direction and complementary to the coding strand. The eight sequences were aligned using the 'multiple alignment' function of Sequence Editor.

Several ambiguities remained, such as extra bases (see 80-90 bp and 110-120 bp) and unresolved bases (depicted as an N). These were resolved by analysing and comparing the sequence data from the chromatograms.

Figure 7.4, p155a shows the consensus sequence, generated using Sequence Editor. The consensus sequence is shown in upper and lower case letters. Upper case indicated that the sequences were the same at that base. Lower case letters indicated that there were ambiguities between the sequences, however the most common base was displayed. Several bases were unresolvable and are given the standard code; s for C or G, r for A or G.

The sequence of the BL/10 fragment of *Urod* was compared with the DNA sequences of C57BL/6 [135] and the rat sequence.[134] C57BL/6 and rat sequences were obtained from Genbank.(c6) The three sequences were aligned using the program 'pileup', from the software package GCG (Genetics Computer Group). [c5] The alignment was displayed using the 'prettybox' function, which also indicated the sequence homology.

There were several highlighted differences between the BL/10 and BL/6 sequence. Most were the result of ambiguities in the BL/10 sequence, which could only be resolved by further sequencing.

Figure 7.3 Multiple alignment of *Urod* fragment raw sequence data

Sequences are aligned in a 5' to 3' direction, in blocks of 10 base pairs (numbers are given above each alignment box). Sequences from Primer A, (A1-4) have been reversed and complemented to align with the coding sequence from primer B.

URO4RAW 09.04.1997 11:47am

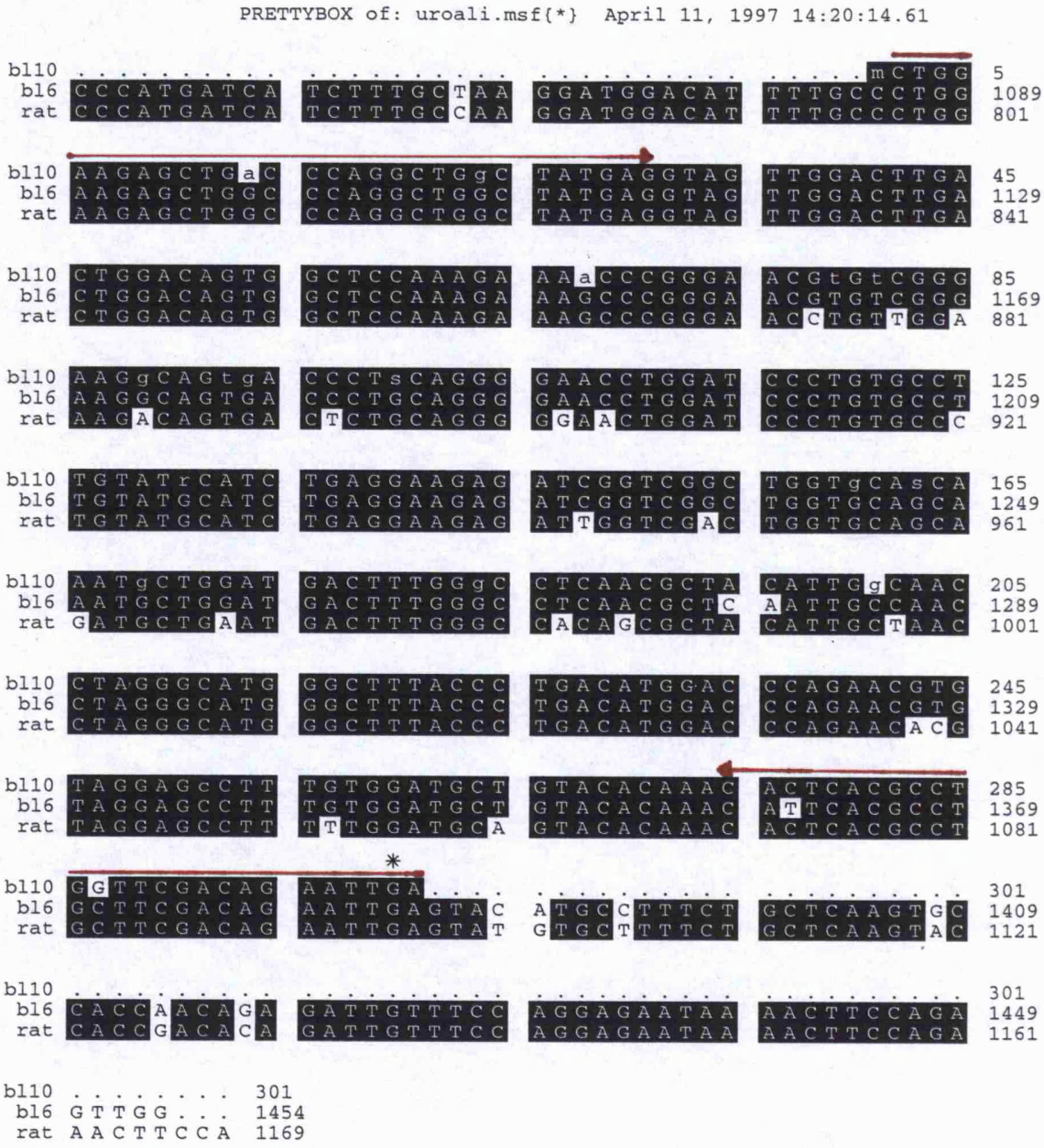
```
                                    10        20        30        40        50        60        70        80
 2    1... 80 28•B1.1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ------GGAA
 4    1... 80 29•B2.1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ------GGAA
 3    1... 80 30•B3.1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ------GGAA
 1    1... 80 24•B4.2  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ------GGAA
29£   1... 80 23•A1.2D ----ACTGGA AGAGCTGGCC CAGGCTGGCT ATGAGGTAGT TGGACTTGAC TGGACAGTGG CTCCAAAGAA ANCCCGGGAA
30£   1... 80 25•A2.2D ---TCCTGGA AGAGCTGACC CAGGCTGGCT ATGAGGTAGT TGGACTTGAC TGGACAGTGG CTCCAAAGAA AACCCGGGAA
31£   1... 80 26•A3.1D ----CCTGGA AGAGCTGACC CAGGCTGGCT ATGAGGTAGT TGGACTTGAC TGGACAGTGG CTCCAAAGAA AACCCGGGAA
32£   1... 80 27•A4.1D ---TACTGGA AGAGCTGACC CAGGCTGCCT ATGAGGTAGT TGGACTTGAC TGGACAGTGG CTCCAAAGAA AACCCGGGAA

                                    90       100       110       120       130       140       150       160
 2   81...160 28•B1.1  C-GTGNCGGG AAGGCAGNNA CCCTGCAGGG GTAACCTGGA TCCCTGTGCC TTGTATGCAT CTGAGGAAGA GATCGGTCGG
 4   81...160 29•B2.1  A-GTGTCGGG AAGGCAGNGA CCCTGCAGGG GNAACCTGGA TCCCTGTGCC TTGTATGCAT CTGAGGAAGA GATCGGTCGG
 3   81...160 30•B3.1  G-GGGNNGGG AAGGCAGAAA CCCTGCAGGG GTAACCTGGA TCCCTGTGCC TTGTATGAAT CTGAGGAAGA GATCGGTCGG
 1   81...160 24•B4.2  ACGNGNCGGG AAGGCAGTGA CCCTGCAGGG GTAACCTGGA TCCCTGTGCC TTGTATGCAT CTGAGGAAGA GATCGGTCGG
29£  81...160 23•A1.2D -CGTGTCGGG AAGTCAGTGA CCCTCCAGGG G-AACCTGGA TCCCTGTNCC TTGTATACAT CTGAGGAAGA GATCGGTCGG
30£  81...160 25•A2.2D -CGTGTCGGG AAGTCAGTGA CCCTCCAGGG G-AACCTGGA TCCCTGTGCC TTGTATACAT CTGAGGAAGA GATCGGTCGG
31£  81...160 26•A3.1D -CGTGTCGGG AAGTCAGTGA CCCTCCAGGG G-AACCTGGA TCCCTGTGCC TTGTATACAT CTGAGGAAGA GATCGGTCGG
32£  81...160 27•A4.1D -CGTGTCGGG AAGNCAGTGA CCCTCCAGGG G-AACCTGGA TCCCTGTGCC TTGTATACAT CTGAGGAAGA GATCGGTCGG

                                   170       180       190       200       210       220       230       240
 2 161...240 28•B1.1  CTGGTGCAGC AAATGCTGGA TGACTTTGGG CCTCAACGCT ACATTGCCAA CCTAGGGCAT GGGCTTTACC CTGACATGGA
 4 161...240 29•B2.1  CTGGTGCAGC AAATGCTGGA TGACTTTGGG CCTCAACGCT ACATTGNCAA CCTAGGGCAT GGGCTTTACC CTGACATGGA
 3 161...240 30•B3.1  CTGGTGCAGA AAATGTTGGA TGACTTTGGG CCTCAACGCT ACATTGGCAA CCTAGGGCAT GGGCTTTACC CTGACATGGA
 1 161...240 24•B4.2  CTGGTGCAGC AAATGCTGGA TGACTTTGGG CCTCAACGCT ACATTGGCAA CCTAGGGCAT GGGCTTTACC CTGACATGGA
29£ 161...212 23•A1.2D CTGGTCCACC AAATNCTGGA TGACTTTGGA CCTCAACGCT ACAATTCCAA CC
30£ 161...212 25•A2.2D CTGGTNCACC AAATNCTGGA TGACTTTGGA CCTCAACGCT ACAATTCCAA CC
31£ 161...212 26•A3.1D CTGGTGCACC AAATGCTGGA TGACTTTGGG CCTCAACGCT ACATTNCCAA CC
32£ 161...212 27•A4.1D CTGGTCCACC AAATNCTGGA TGACTTTGGA CCTCAACGCT ACATTGCCAA CC

                                   250       260       270       280       290       300       310       320
 2 241...309 28•B1.1  CCCAGAACGT GTAGGAGCCT TTGTGGATGC TGTACACAAA CACTCACGCC TGGTTCGACA GAATTGA--
 4 241...309 29•B2.1  CCCAGAACGT GTAGGAGCCT TTGTGGATGC TGTACACAAA CACTCACGCC TGGTTCGACA GAATTGA--
 3 241...309 30•B3.1  CCCAGAACGT GTAGGAGGCT TTGTGGATGC TGTACACAAA CACTCACGCC TGGTTCGNNA GAATTGATT
 1 241...309 24•B4.2  CCCAGAACGT GTAGGAGCCT TTGTGGATGC TGTACACAAA CACTCACGCC TGGTTCGACA GAATTGA--
29£ ---,--- 23•A1.2D <==
30£ ---,--- 25•A2.2D <==
31£ ---,--- 26•A3.1D <==
32£ ---,--- 27•A4.1D <==
```

Figure 7.4 Consensus sequence of BL/10 *Urod* fragment and alignment with C57BL/6 and Rat DNA sequences



The 'prettybox' function displays the alignment and homology (in black) between the three sequences. Differences between the sequences are highlighted in white. Primer sequences are indicated with by the red line and the stop codon with a star. Ambiguous bases; m = A or C, r = A or G, s = C or G.

7.3.3 Expression of *Urod* in C57BL/10ScSn and DBA/2 Strains

The expression of *Urod* RNA was analysed qualitatively by northern hybridisation. Total RNA was extracted from liver tissue taken from BL/10 and DBA/2 mice, either non-treated controls or induced for porphyria with HCB and iron (section 2.1.1), using a 'RNAzol-B'. (section 2.11.2) The RNA was fractionated on a formaldehyde containing agarose gel, transferred to Hybond-N nylon membrane by capillary blotting and fixed by baking at 80°C.(section 2.11.4)

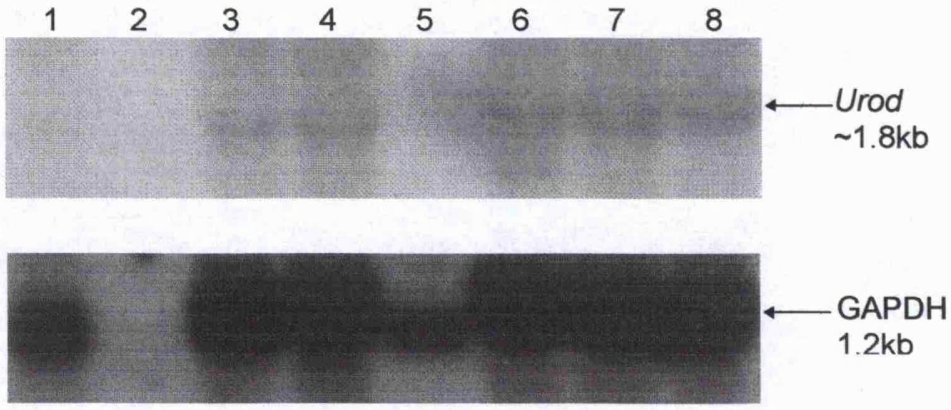
The *Urod* probe was produced according to the protocol in section 2.12. The 300bp PCR product was used as a template and amplified, with the inclusion of α^{32}P labelled dCTP. The blot was hybridised at 42°C overnight, and then washed to remove any non-specific hybridisation with two changes of 50ml of 2XSSC, 0.1% SDS, for 15 minutes at 55°C. The blot was then autoradiographed 2 days at -80°C.

The blot was subsequently reprobed with GAPDH (section 2.12.2) to check for degradation and even loading. Owing to the weak signal produced by this probe, (estimated with a hand-held monitor) the exposure time was significantly extended to give an adequate signal.

The result of the initial hybridisation is shown in Figure 7.5. The size of the transcript is approximately 1.8 kb, which is 700 bases longer than the coding sequence of approximately 1.1kb. However, the length of mouse *Urod* cDNA clones was reported to vary at the 5' untranslated end, the longest being 1.5kb.[135]

Although wo of the control RNAs (a BL/10 and a DBA/2 sample) degraded and a comparison with the some what overexposed GAPDH blot was difficult, there was no obvious difference in expression of *Urod* between the two strains, before or after treatment with iron and HCB. To verify this result, this blot would have had to be repeated. Fresh RNA samples would have been required, which unfortunately there was insufficient time to prepare.

Figure 7.4 Expression of *Urod* RNA in C57BL/10ScSn and DBA/2 liver after exposure to HCB and iron overload



Upper blot: Northern blot of C57BL/10ScSn (BL/10) and DBA/2 total RNA (10ug per lane) extracted from the livers of untreated controls and mice exposed to HCB (0.02%) for seven weeks in the presence of iron overload. Blot was exposed for 5 days.
Lanes 1 & 2 BL/10 controls, 3 & 4 BL/10 + iron + HCB, 5 & 6 DBA/2 controls, 7&8 DBA/2 + iron + HCB.
Lower blot: Hybridisation of the same blot with GAPDH probe, blot was exposed for two weeks.

7.5 Discussion

There are no reports of abnormalities in the UROD gene or promoter sequence from patients with sporadic PCT [30]. No change in enzyme concentration has been observed either in the human condition or in rodents made porphyric with TCDD or HCB. [31,138] Although the condition is characterised by a loss of activity in the hepatic enzyme, this must be caused by some other process and not a defective UROD gene.

A fragment of the mouse *Urod* gene was isolated by PCR, using primers derived from the rat sequence.[126] After confirmation that the 300 bp product was *Urod*, by sequencing and comparison with known sequences, the fragment was used to probe a northern blot of RNA extracted from the livers of BL/10 and DBA/2 mice.

Several of the bases in the BL/10 sequence did not match the published BL/6 sequence. These may be artefacts caused by infidelity of the DNA polymerase when copying the template. This could have also occurred during the cycle sequencing reaction which uses a *Taq* DNA polymerase. Alternatively, some of the variations might be genuine polymorphisms between the two strains but this could only be determined by further sequencing or sequencing the cloned *Urod* gene from a C57BL/10 cDNA library.

Expression of *Urod* was compared between BL/10 and DBA/2 strains, as well as mice induced for porphyria by iron overload with exposure to HCB. The blot showed no change in expression between treated and untreated animals and no difference in expression between strains. This agrees with the finding that UROD protein concentration does not change in experimental porphyria, so loss of hepatic UROD activity cannot be explained by insufficient transcription or translation of the gene.

Currently, the loss of activity in UROD is thought to be due to inhibition or the inactivation of the enzyme at the active site. Inhibitors of UROD have been reported that may possibly be formed from oxidised uroporphyrinogen.[85,139] The mechanism of how UROD is inhibited and why this only occurs in particular strains is unknown but presumably it is associated with a susceptibility to the

production of oxygen radicals (possibly catalysed by iron) in genetic predisposed individuals.

8 - General Discussion

In humans, PCT can be inherited as a mutation in the gene encoding the enzyme UROD or it can also be brought about by liver damage, through exposure to numerous agents such as polycyclic halogenated chemicals, oestrogenic drugs and iron but particularly through high alcohol consumption. However, as the sporadic disease is not found in all individuals exposed to these agents, a genetic predisposition has been proposed whereby only individuals with a particular set of 'susceptibility genes' would be prone to PCT after exposure to these triggering factors.[1-4]

The experimental porphyria induced in mice by HCB and other chemicals is characterised by a build up of uroporphyrin and heptacarboxy-porphyrins, similar inhibition of UROD and it is greatly accelerated by the pre-administration of iron.[6,40] These models reflect the important role of iron in the mechanism of the disorder and are considered to be of sufficient similarity to human S-PCT to merit their use as models for studying the mechanism of the development of this disorder.

In humans most patients have some degree of iron overload and treatment for iron depletion is the most successful method of controlling the symptoms of S-PCT.[1,34] As with humans, not all strains of mice will develop porphyria after exposure to these chemicals. A number of strains, BL/10, BL/6, AKR and SWR are susceptible to porphyria development after administration TCDD, HCB, the haem precursor 5-ALA, especially with cotreatment with iron or simply iron alone, whereas the strain DBA/2 appears to be very resistant.[6,7,8,10,89,91]

Many human illnesses are being recognised as having a genetic component, examples include; colon cancer, heart disease and hypertension. However these disorders are not thought to be the product of a single gene mutation but may be the result of several 'susceptibility' genes and possibly some environmental effects, which makes some individuals more susceptible than others.[92]

The reverse genetic approach of identifying a gene is to locate it initially by linkage analysis, rather than starting from a protein or gene sequence. This can be a laborious task, which is hindered in humans when there is a lack of large families with affected members to aid this type of analysis. The process becomes especially difficult when more than one gene is involved. However, by using a suitable mouse model for the disorder, it is possible to breed large numbers of mice in a short time period and proceed with mapping the responsible gene or genes in this species.

The project described in this thesis has employed a similar technique and appears to be one of the first uses of this approach in noncancer toxicology. Two 'model' strains, BL/10 which is susceptible to porphyria development after exposure to HCB in the presence of iron overload and SWR which develops a porphyria after iron overload and administration of 5-ALA, were used to breed F_2 intercrosses with the highly resistant strain DBA/2. These second generation offspring displayed a range of susceptibilities to porphyria induction as a result of inheriting the uroporphyria susceptibility and resistance genes from each 'grandparent' strain. The inheritance of these genes or loci, was examined by correlating the inheritance of microsatellite marker DNA from each strain with susceptibility to experimental porphyria. This approach could form the first step towards identifying the responsible 'uroporphyria susceptibility' genes and aid mechanistic studies.

The second approach to this project was to analyse the effect that candidate genes had on susceptibility to experimental porphyria. The genes included some of the cytochrome P450 mono-oxygenase enzymes and the AH receptor and were selected on the basis of their importance in the hypothesised mechanism of the development of the disorder and addressed the toxicological aspect of the disease.

The remainder of this chapter discusses the main findings of this work and the potential for future studies.

Inheritance of Porphyria

The major part of this study has concentrated on the development of experimental porphyria caused by the robust system of HCB and iron overload. [6] The inbred mouse strains C57BL/10ScSn (BL/10) and DBA/2 were used to breed F_1 and F_2 generations, as well as backcrosses to each strain. A second breeding project aimed to investigate the development of experimental porphyria in an SWR x DBA/2 F_2 intercross, when treated with iron and 5-ALA. However, with this model the response is much weaker than that caused by HCB and even more so in the F_2 crosses. Several hundred F_2 mice of this cross were bred and treated but this only produced a few samples suitable for analysis. This model was thought to be the simplest in terms of the mechanism of induction, as it does not obviously involve the cytochrome P450 mono-oxygenase system or the AH receptor. However, this has been the hardest experiment with which to obtain a successful result. To enable further genetic analysis of this trait, several hundred mice would need to be bred and treated, to obtain a sufficient number to study, which would have considerable financial and other implications.

The development of porphyria varied within groups of BL/10 and SWR mice in response to treatment with HCB or 5-ALA, although the DBA/2 strain was consistently resistant. This occurred despite the fact that the animals were genetically identical, purchased from the same breeder and treated in an identical manner. Irregularities in the iron-dextran dosing was considered and the liver non-haem iron concentrations were measured in some cases. A variation in the iron content was found but all of the mice appear to have received a sufficient dose to induce porphyria. The only other explanation could be that there are some unknown environmental factors involved which are difficult to control and this explanation would be consistent with the findings from other toxicological studies.[99]

The mode of inheritance of susceptibility to experimental porphyria could only be properly assessed in the BL/10xDBA/2 experiment, where F_1 hybrids and backcrosses were bred and treated with iron and HCB. The F_2 mice displayed the expected range in susceptibilities consistent with there being more than

one gene involved. Had only one gene been responsible for the effect a bimodal, rather than a continuous, distribution of uroporphyrin concentrations would have been expected. (Figure 3.3) [98,99] The response to HCB and iron in the F_1 and BL/10 backcross mice suggested that there was a dominant susceptibility trait, however no high responding mice were seen in the DBA/2 backcross. One possibility is that there are a mixture of dominant and recessive loci are involved and an interaction between these loci is necessary to achieve the fully susceptible phenotype. There is also the possibility that environmental factors are involved, as was stated previously, which were observed as the variation in the response of the inbred strains themselves to treatment.

It was also possible that these low responding strains might have responded more if their length of time of exposure to HCB or 5-ALA had been increased. The rise in hepatic porphyrins has been shown to increase at an exponential rate. Thus what may have been a 'low response' after 7 weeks of exposure to HCB (or 5 weeks for 5-ALA)may in fact induced a 'high response' after only a few more days. This might have also been the reason for the large number of low responding F_2 mice observed.

Only male mice were used in these studies, although there is a slight difference in the length of time taken for porphyria to develop between the sexes, with females taking longer to induce than males, there is no difference in the porphyria itself.[6] Female mice would have to be included to determine if any sex linked genes were involved. However, the BL/10xDBA/2 F_2 generation was bred from two reciprocal crosses of each strain and sex. Should a dominant sex-linked gene have been involved then a marked difference in the uroporphyrin concentrations would have been seen. Initially there was a small difference in the average uroporphyrin concentrations between the two groups, although there was a wide spread in the concentrations of both groups had both had individuals with very high uroporphyrin levels. (Table 3.2) For statistical purposes, however, the uroporphyrin concentration data were transformed to make the data fit a normal distribution and to compare the averages between all of the crosses. The transformed data showed no

difference in average concentrations between the two F_2 groups, so it was decide to combine them into one.

Microsatellite Linkage Analysis

The strategy for the genetic analysis was to identify the chromosomes where a 'susceptibility gene' might possibly be located. The microsatellite linkage analysis was undertaken mainly in the BL/10xDBA/2 F_2 intercross. A much smaller scale analysis of the SWRxDBA/2 F_2 cross was performed because only a few high responding mice were produced in this experiment. In this second cross only chromosomes 4, 9,12, 14 and 17, which were identified by the BL/10xDBA/2 cross, were typed.

Genetic mapping projects frequently involve processing hundreds of markers and DNA samples. As this was not feasible in this project, a simpler technique was employed. Instead of genotyping all 200 BL/10xDBA/2 F_2 mice, 40 of the highest and lowest responding mice were typed.[98] Each chromosome (all 19 autosomes and chromosome X) of each response group was genotyped initially with 2 microsatellite markers. The correlation between inheritance of a marker allele from the susceptible BL/10 parent in the high responding group, or a DBA/2 allele in the low responding group, was calculated. Using this technique three chromosomes; 12, 14 and 17 were identified as being the possible location of porphyria susceptibility genes. Only chromosome 17 showed some correlation with porphyria development in the SWRxDBA/2 cross.

Chromosome 4 and *Urod*

One observation from the genetic analysis of both crosses was that no linkage to chromosome 4, where the *Urod* gene is located was detected. The mouse *Urod* sequence was unknown until recently but during the course of this project the cDNA has been cloned and sequenced in the C57BL/6 strain.[135] As a polymorphic marker specific for the *Urod* gene was unavailable the inheritance of the gene itself could not be typed in either of the crosses produced. Using primers designed from the rat UROD cDNA sequence a 300 bp probe was constructed by PCR from BL/10 liver cDNA.[126] After sequencing, the BL/10

Urod fragment was found to have a 93% identity with the C57BL/6 predicted amino acid sequence. Subsequently this fragment was used to probe a northern blot of RNA extracted from liver tissue of treated and nontreated BL/10 and DBA/2 mice. This revealed a band of approximately 1.8kb (Figure 7.4), 200bp larger than the largest mouse *Urod* cDNA clone reported.[135] The coding sequence is known to be approximately 1.1kb and a long 5' untranslated region has been suggested as a possible explanation for the larger length of these cDNA clones. This might also be the reason for the size of the band on the blot. Although, there was no obvious difference in expression of *Urod*, a considerable number of studies would have to be undertaken to determine there was definitely no variation in expression between the strains or between porphyric and normal mice. More sensitive techniques such as quantitative RT-PCR could be used to quantitate the expression of *Urod* more accurately.

So far, no mutations in the human UROD cDNA or its promoter, have been found in S-PCT patients that could account for the loss of activity in the enzyme in the liver only. There is thought to be only one form of the UROD gene, unlike other haem biosynthetic enzymes (for example ALA synthase and PBG deaminase). Alternatively spliced transcripts of UROD have been reported in normal individuals and patients with familial PCT, which suggests that not all splice site mutations could be deleterious to the enzyme. Recently, the human genomic UROD sequence has become available. [25] Analysis of the complete gene sequence or promoter region in S-PCT patients, might reveal detrimental mutations or polymorphisms. However, there is some evidence to suggest that UROD enzyme concentration remains unchanged in both S-PCT and the experimental porphyria in rodents. These findings, together with the lack of any linkage to the mouse *Urod* gene, infer that the loss of activity of the enzyme in the mechanism of development of porphyria is probably by inhibition rather than as a consequence of mutation in the enzyme itself.

Chromosome 9 and Cytochrome P450 1A1 and 1A2

The second important candidate gene, or genes, not identified by the genetic analysis in both crosses, were the cytochrome P450 genes; *cyp1a1* and *cyp1a2*, located on chromosome 9. These enzymes have often been implicated from mechanistic studies on the development of porphyria in rodents.[3-5]

Cytochrome P450 isozymes 1A1, 1A2 and 2B1 are thought to be induced in rodents by HCB (to a much lesser extent than chemicals such as TCDD) to enhance their own metabolism and excretion from the body.[73] It is hypothesised HCB is poorly metabolised by these enzymes and that reactive oxygen radicals are produced by uncoupling of the cytochrome P450 mono-oxygenase system. These radicals might then be involved in the production of an inhibitor of UROD or in the oxidation of the uroporphyrinogen substrate.[5] An alternative proposition is that the isozyme CYP1A2, induced by HCB oxidises uroporphyrinogen substrate to non-metabolisable uroporphyrin. This would reduce the amount of substrate for UROD, hence decreasing its *in vivo* activity and causing a build up of uroporphyrin.[78] The genetic variation in susceptibility to porphyria between strains might then be due to a mutation, polymorphism or difference in the constitutive expression of one of these cytochrome P450 isozymes. However, as no linkage was detected, a polymorphism or mutation in *cyp1a1* or *cyp1a2* is unlikely to be responsible. These candidates genes, along with CYP2B1, were investigated further at the level of RNA expression and enzyme activity.

The expression of cytochrome P450 isozymes CYP1A1 and CYP1A2 were measured qualitatively by northern blot analysis. Total RNA and microsomes were prepared from the BL/10 and DBA/2 parent strains and 10 of the highest and lowest responding F_2 mice for this study. HCB was found to increase the expression of CYP1A1 and CYP1A2 in BL/10 mice but there was no increase in CYP1A1 message in DBA/2 mice although there was a slight increase in CYP1A2.

If susceptibility to HCB and iron induced porphyria depended on a variation in induction of either of these enzymes, then a difference in expression would have been seen between the two F_2 groups. A small difference in the

expression of CYP1A1 between the high and low responding group was detected, with more CYP1A1 mRNA in high responders, but the expression of CYP1A2 message was approximately the same in both groups.

As a more quantitaitve approach, the activities of CYP1A1, 1A2 and 2B1 isoenzymes were determined. The P450 enzyme activities, measured by O-dealkylation of alkoxyresorufin substrates, reflected the results of the northern blots. Again, activities of CYP1A1 and CYP1A2 were increased in BL/10 mice to a greater extent than in the DBA/2 strain. With the two F_2 groups, activities of these enzymes (and possibly others because of overlaps in substrate specificity) were nearly identical. (Table 4.5) The only statistically significant difference was seen in CYP1A1 (EROD) activty (Table 4.5 and Figure 4.5) where the rate of the high responders was twice that of the low responders. Again CYP1A2 activities were the same in each group, a result which is not in total agreement with the hypothesis that the oxidation of uroporphyrinogen by CYP1A2 accounts for strain susceptibility. As a lower rate of activity would have been expected in the low responding group.

Overall, the rates of CYP1A1, 1A2 and 2B1 activity were much lower than those measured after induction with the classical inducing compounds; β-napthoflavone and phenobarbital, after only 3 days using identical strains. These compounds do not cause porphyria by themselves, although in conjunction with iron overload they can induce experimental porphyria in mice, albeit at much higher doses than required with HCB or TCDD.[5,10] On this basis, if the cytochrome P450 isozymes were involved in the mechanism, their activities would be expected to be much higher than those observed after 7 weeks of continuous exposure to HCB, as it was assumed that the enzymes would be constantly induced.

It is possible that the iron somehow decreased the P450 activity. In fact, this was observed in the mice given iron alone compared to non-iron loaded mice. (Table 4.2) There may have also been some decrease in CYP1A2 expression, in the same iron loaded mice but further northern hybridisations or quantitative PCR would be required. Only by measuring the P450 activities in BL/10 mice

exposed to HCB and comparing these with the rates from HCB and iron loaded mice would confirm this.

In addition to the cytochrome P450 activities measured in the BL/10xDBA/2 cross, CYP1A1 and 1A2 activities (EROD and MROD) were also measured in the 10 highest and lowest responding SWRXDBA/2 F_2 mice, induced by iron overload and exposure to 5-ALA.

The enzymes rates were expected to be low as no inducing compounds was administered but a difference in the constitutive levels of these enzymes, (particularly CYP1A2) between the two strains, was considered as a possible cause of the variation in strain susceptibility. However, the rates for both isozymes in the two groups were identical. (Table 5.5)

The induction of CYP1A1 by HCB implies that it might have been induced to metabolise this compound, though it remains unclear if a two fold difference in CYP1A1 activity alone, could cause a porphyria in some mice and not others. It was also demonstrated, in the SWRxDBA/2 cross, that induction of this enzyme was not necessary for porphyria development. Neither was any correlation between CYP1A2 activity and susceptibility seen. Further work would be necessary to establish any correlation with CYP2B1 isozymes and susceptibility.

Chromosome 12 and the AH Receptor

As the cytochrome P450 enzymes did not appear to be directly involved in causing porphyria, their regulatory mechanism in terms of the AH receptor, was also examined. The AH receptor gene *Ahr* is encoded on chromosome 12, this was coincidentally the first chromosome observed to display some correlation with the development of porphyria in the initial microsatellite analysis of the BL/10xDBA/2 F_2 intercross.

The microsatellite results were corroborated by genotyping the *Ahr* locus itself in the BL/10xDBA/2 intercross using the RFLP which distinguished between Ahr^{b-1} (AH responsive) and Ahr^{d} (AH nonresponsive) alleles.[67] Obviously, this could not be used to type the SWRxDBA/2 cross as both strains carried Ahr^{d} alleles. A high correlation (p=0.001) was found between Ahr^{b-1} alleles in the

high responding group. This agreed with the results of the cytochrome P450 enzyme study, in which CYP1A1 (regulated by the AH receptor) was induced in BL/10 (AH responsive) mice and was found to be induced in the highest responding F_2 group.

Conversely, the *Ahr*^{d} allele did not correlate with low response to HCB and iron. The reason for this is unknown but one explanation might be that are several other susceptibility or resistance genes involved. The lack of the *Ahr*^{d} allele might not always mean that the individual is susceptible, as the genotype at other susceptible loci could confer resistance.

Although the *Ahr* gene appeared to be involved in the BL/10XDBA/2 intercross, it has been shown from past work that it is not essential to the development of experimental uroporphyria. Studies with TCDD have shown a lack of complete correlation between *Ahr* phenotype and development of porphyria, which was originally assumed to be the dominant genetic factor.[7] This lack correlation could be seen again in the SWRXDBA/2 cross where both strains were AH nonresponsive. The SWR strain still responds after treatment with iron overload and 5-ALA, albeit with a much weaker porphyria. The development of the response in this model does not have an obvious role for the *Ahr* gene.

Chromosome 14

The genetic analysis of the BL/10xDBA/2 F_2 cross showed chromosome 14 to have a high correlation with the development of HCB induced porphyria. The only known candidate gene on this chromosome is the hairless (*hr*) gene, which lies approximately 40cM from the centromere and nearly 20cM telomeric from the marker D14MIT5 which showed a high correlation (p=0.001) with susceptibility and resistance in both F_2 response groups. The *hr* gene has been demonstrated to be involved in the epidermal changes, such as hyperkeratosis, hyperplasia and sebaceous gland metaplasia caused by exposure to chemicals such as TCDD, in mice. These skin lesions are analogous to the human condition chloracne seen in individuals exposed to TCDD. [140]

The *hr* locus was found to interact with the *Ahr* to produce this response, from studies with congenic mice homozygous (*hr/hr*) for the hairless gene. Only mice

homozygous at this locus develop the skin lesions, it is not seen in *hr*/+ heterozygotes or the wild-type. The *Ahr* gene is known to be involved as chemicals which are known ligands for the receptor, will produce these skin disorders in HRS/J *hr*/*hr* mice, (which are also AH responsive). The skin lesion trait was found to segregate with the Ahr^b allele in crosses made between C57BL/6 and DBA/2 mice bred with the *hr*/*hr* locus. It has been proposed that the *hr* locus forms part of a battery of genes involved in epidermal cell proliferation and differentiation, which could also be regulated by the *Ahr* locus. It was assumed that the *hr* gene was only involved in the epidermal changes associated with TCDD toxicity. However, possession of the *hr* gene was found to correlate with the development of porphyria in mice. The Ah responsive strain, A2G congenic for *hr*/+ and *hr*/*hr* developed porphyria after treatment with TCDD, thus the effect of this gene product may be extended to hepatic toxicity. [140,141]

This locus had the opposite effect with iron induced porphyria, where *hr*/*hr* seemed to protect against porphyria more than *hr*/+ and the A2G wild type strain was the most susceptible to induction.[8]

Genotyping at this locus would be required to find out if it were involved in susceptibility to porphyria in the two experimental crosses from this study. However, as the BL/10 strain would be the wild-type at the *hr* locus, it would be assumed that this would be the porphyria 'resistant' form if HCB was acting in a similar way to TCDD. If on the other hand iron was affecting this gene then it would be a 'susceptibility' locus. Although chromosome 14 showed no correlation with susceptibility in the iron induced SWRxDBA/2 cross but as only 20 mice were typed in each group more SWRxDBA/2 F_2 mice would need to be typed to give a statistically significant result.

Another gene which might be involved in porphyria development is the hepatic form of ALA synthase (ALAS-1).[90,91] This *Alas-1* gene has not been cloned or located in the mouse. It has been suggested that iron alone could induce ALAS-1 as many porphyria inducing chemicals do. The presence of a polymorphism (or a mutation) which altered the induction of this enzyme would, in turn, alter the rate of formation of the haem precursor 5-ALA. As providing a

providing a surplus of 5-ALA seems to increase the block at UROD, upregulation of ALAS-1 by iron may produce a similar response. This would not have been detectable in the SWRxDBA/2 cross as these mice received the precursor 5-ALA, which would have concealed any upregulation of the enzyme. Even if an ALAS-1 polymorphism was a predisposing factor, it does not explain how UROD activity is reduced in certain strains. One possiblity is that 5-ALA could yield free radicals which can release iron as Fe^{2+} from ferrtin.[142] The mobilised Fe^{2+} could then take part in the catalysis of a UROD inhibitor or oxidation of the uroporphyrinogen substrate.

Chromosome 17

The final chromosome deteted by genetic analysis was chromosome 17. This was the only one which appeared to correlate in both experimental crosses. It has also been proposed to be the chromosomal location of a gene (or genes) involved in iron metabolism.[101] This was mainly due to it containing the H-2 complex, the mouse equivalent of the human HLA-complex and because iron was the common inducing factor in both crosses.[133] The gene, *HLA-H*, which has been found in over 80% of haemochromatosis sufferers, is thought to be a predisposing gene for S-PCT and an HLA class I protein.[52] Similarly, histocompatibility class I genes on the mouse chromosome 17 have been proposed to be involved in iron metabolism and could possibly include the mouse homologue of the haemochromatosis gene.[101]

The contribution of a susceptiblity gene within the H-2 complex on chromosome 17 was investigated further using the congenic mouse strain BL/10.D2n. Induction experiments were carried out with HCB and iron and iron overload alone. As this complex was of DBA/2 origin, any contributing locus was expected to be of the 'resistant' type, therefore a reduction or even a resistance to porphyria development was expected in both experiments.

However, only the iron treated congenic mice displayed the predicted response with a weak response of about 4% of BL/10 controls. The HCB and iron treated congenic mice responded to the same extent expected of the BL/10 strain. (Although in this experiment the response of the BL/10 controls treated with

iron and HCB was much lower than expected. This did not appear to be caused by low iron doses or even a mix-up between the two types of mice as DNA had been extracted from the congenic mice to genotype the congenic chromosome).

The porphyria which developed in the HCB treated congenic mice, and even the very weak response seen with iron only, might have been mediated through other loci such as that on chromosome 14, or even the AH receptor and the cytochrome P450 isozymes in the case of HCB induction.

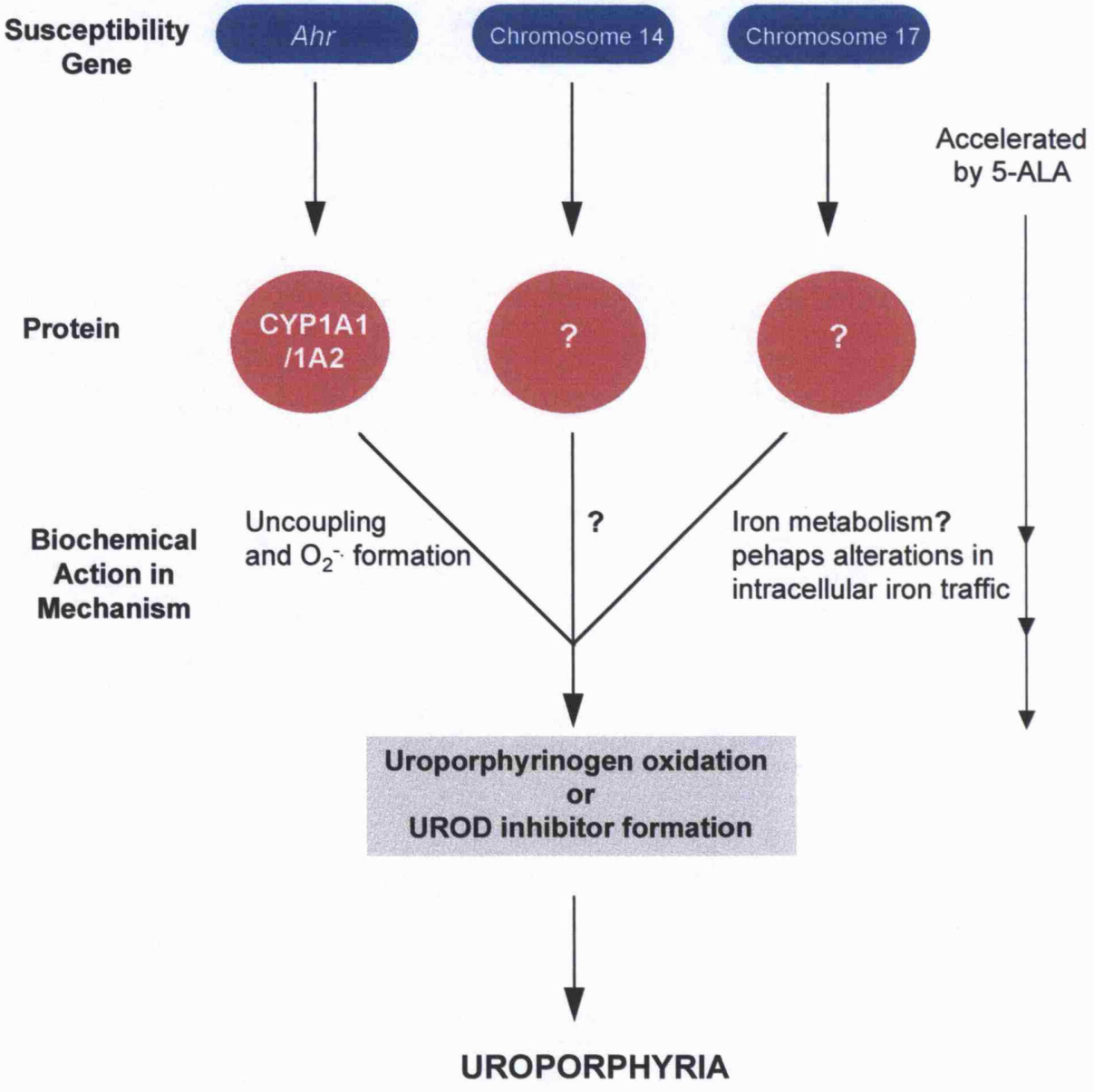
The linkage analysis approach used was unable to locate exactly the positions of each susceptibility loci. On chromosome 17, several markers were typed in order to find out if markers in the vicinity of the H-2 complex were more strongly correlated than others further away. The most significant markers lay 1-2 cM telomeric of the H-2 complex. (D17MIT49, significance $p<0.001$, was situated at 22.3cM). It appeared from the congenic induction experiments, that a susceptibility gene may lie within the H-2 region. However, from genotyping chromosome 17 with the microsatellite markers, the congenic region was found to span at least 3 cM, from 17.3 -20.2 cM. Therefore the congenic region might have covered the region where the susceptibility locus is located. The human gene *HLA-H*, was mapped 2-3cM telomeric from the HLA complex. Genotyping the two crosses with genetic markers homologous to those flanking the HLA-H gene, or the gene itself once it has been cloned, could help to prove if the mouse equivalent of the haemochromatosis gene is involved in the development of experimental porphyria. Nevertheless, functional studies are required to show how these histocompatibilty proteins are involved in iron overload or iron metabolism.

Summary

The flow-diagram (Figure 8.1) summarises the results of the investigation into the genetic basis of susceptibility to experimental porphyria caused by HCB and iron overload. Three possible genetic factors have been identified, the *Ahr* and cytochrome P450 isozymes CYP1A1 and CYP1A2, as well as unknown loci on chromosomes 14 and 17. Although no strong evidence was found to support

the involvement of the cytochrome P450 enzymes, this area still attracts much attention in toxicological related studies.

Figure 8.1 Summary diagram of the proposed genetic influences involved in susceptibility to HCB and iron induced experimental uroporphyria in mice



Future Studies

The aim of this project has been to try to elucidate the genetic component of iron and chemically-induced porphyria in mice, as it has long been known that a susceptibility to this disorder exists between certain strains. The main benefit of such a study would be the further characterisation of the mechanism underlying this disorder.

Of the three candidate chromosomes identified from the microsatellite linkage analysis, one possible gene *Ahr* and the cytochrome P450 isozymes it controls, were studied in some detail. The possible contributing loci on chromosome 14 and 17 (that is *hr*, *Alas-1* and the mouse haemochromatosis homologue) could be studied in a similar fashion if or when markers for the cloned genes become available. Furthermore, it would be useful to analyse the cDNA sequences or even promoter regions for polymorphisms or expression differences, which might explain the reason for the variation in susceptibility to response between strains such as BL/10, SWR and DBA/2.

The use of recombinant inbred (RI) lines could be used to confirm the linkage analysis results from this study. RI mice have been bred from strains such as C57BL/6 (a uroporphyria susceptible strain) and DBA/2, known as the BXD series. The BXD RI strains were produced by inbreeding the F_2 progeny from a C57BL/6xDBA/2 cross, which established them as inbred strains themselves. There are 26 RI lines available and genetic mapping of these strains is on going and the data is generally available. Hence, as the genotype of these strains is already known, identification of susceptibility loci would be far simpler and the production of larger numbers of intercrosses and microsatellite analysis would be unnecessary. Only the induction of uroporphyria would be required and then a comparison of the phenotype of each line with its genotype should indicate the loci inherited from the susceptible C57BL/6 strain.[111]

If the susceptibility loci could not be identified or located from either of these techniques, it may be necessary to do further genetic mapping and possibly physical mapping, with mouse YAC libraries. Linkage analysis of a larger number of F_2 mice, with more markers spanning every chromosome or selected for gene rich regions of the genome, [143] could be used to establish markers

linked to a region of interest, using a computer program such as MAPMAKER.[98] This type of procedure for mapping complex traits is commonly used and programs for linkage analysis of nonparametric traits, that is where phenotyping might depend on grading the severity of a disease (for example by histological examination) and a normal distribution in this data does not exist, are now available.[144]

From this type of linkage analysis it would be possible to transfer to physical mapping of any region of interest highlighted, by screening mouse YAC libraries. However, as the genetic maps of the mouse genome are becoming denser (there is approximately one marker spaced every 400kb [96]) physical mapping should become easier. In fact in the future, physical mapping of mouse chromosomes should be possible just by identification of the YAC corresponding to the marker of interest.[133]

In due time, it would be hoped that the outcome from the future studies described, would help in understanding the mechanism that restricts the toxic response to iron or chemicals in particular strains of mice and that this may benefit similar studies in other species.

Appendix I

Table of microsatellite primer sequences and genetic map positions

Marker	Chromosome	Map position (cM)	Primer Sequence
D1Mit21	1	32.8	Left = CGCTGGACAATCTTATAATTGCA Right = TCGAATCCCAACAACCACAT
D1Mit110	1	86.3	Left = TTGGCATGTGTGTTCTGGAT Right = CCCCCAAATGAGCCTTTACT
D2Mit80	2	12	Left = TAGCCTACAGAGTGGACAGCC Right = CTAGGCTTTATGTAGCCTCTTTGC
D2Mit109	2	66.7	Left = CCTCTCCAATCAGTCACATGG Right = TTGTCTTACCTTCATCAGAGATGC
D3Mit40	3	29.5	Left = CAGCTGGTCTAACTATCCCCC Right = CCTTATTAAGTGCATGACCTTGC
D3Mit19[94]	3	66.7	Left = CAGCCAGAGAGGAGCTGTCT Right = GAACATTGGGGTGTTTGCTT
D4Mit2[94]	4	10.9	Left = GGATTTCTTGGGCACTCACA Right = GCACCAGTGACTTTACCCCA
D4Mit57	4	53.6	Left = ACCCTGTCTCAAAAATAACTCTGG Right = CATCTGTCCAGTCCCCATG
D5Mit11[94]	5	17.5	Left = GATCTTCCTACCTTCTTACCCAC Right = CATGATTTTATTTGGGGGG
D5Mit95	5	57.9	Left = TGTTCTTGTCCATGTCTGATCC Right = AACCAAAGCATGAAACAGCC
D6Mit29	6	27.3	Left = CTTCTTTACACCTGTATGGCACC Right = GGTTGGTCACTGCAGGAGTT
D6Mit15[94]	6	66.7	Left = CACTGACCCTAGCACAGCAG Right = TCCTGGCTTCCACAGGTACT
D7Mit55	7	12	Left = AACCCCAATGAGTCAATCATG Right = CAAGACATAGCAGACGACTGTACC
D7Mit7[94]	7	42.6	Left = ACTCAAAGGTTGTCCTGGCA Right = TGGTAGTGGTGGCTNCGGTG
D8Mit4[94]	8	12	Left = CCAACTCATCCCCAAAGGTA Right = GTATGTTCAAGGCTGGGCAT
D8Mit56	8	75.4	Left = ACACTCAGAGACCATGAGTACACC Right = GAGTTCACTACCCACAAGTCTCC
D9Mit22[94]	9	23	Left = ATTGCATAACACCCCCACAT Right = CAGTGCTTAACTGCTCAAATGC
D9Mit4[94]	9	24	Left = TGCTGAGCAAGCTATGAGGA Right = GACAGCCCATCACAGCTACA
D9Mit31	9	33.9	Left = TTACATGCATGGATGTACACATG Right = CCAGAATCTATGTCTGCTTGTATG
D9Mit10[94]	9	43.7	Left = TAACCAACCCTTCAAGGCAC Right = AATCCTTGGCTGAAGGGAAT
D9Mit15[94]	9	66.1	Left = TTCAGTCCAGTCTGGGGGTA Right = CCCCCAGTTTTGTTGTTTTG
D9Mit19[94]	9	68.9	Left = CCAAACACAACCCCTCAGAA Right = TCATGGCTTCAAGACTGCTT
D10Mit51	10	5.5	Left = CTTGAGCCTACGTGACCACA Right = TGCAGTCCCTCACACATATACA
D10Mit14[94]	10	69.9	Left = AGAGGGGACAAGGAGAGACC Right = AAGGTTTGGGTTCAGTTCCC
D11Mit36	11	43.7	Left = CCAGAACTTTTGCTGCTTCC Right = GTGAGCCCTAGGTCCAGTGA

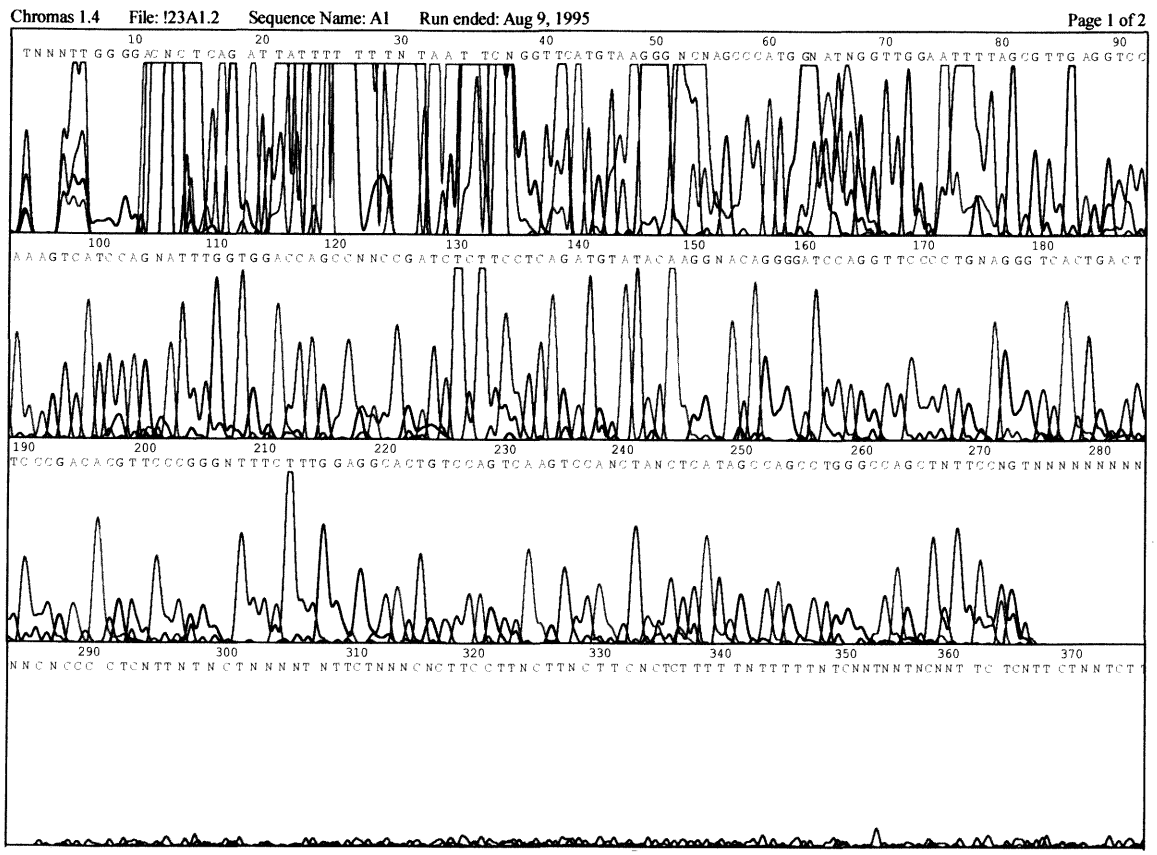
Marker	Chromosome	Map position (cM)	Primer Sequence
Igh-V [95]	12	59.0	Left = ACATGGTAATTTATGGGCAA Right = CTGGATACCTGCAATAGTAGA
D12Mit2 [94]	12	16.4	Left = ACACAGGCTAAAACATGGGC Right = GCATCTGTATTCCACAGGCA
D12Mit3 [94]	12	27.3	Left = TAAAGGGGTTTGCTTAAACA Right = ATGCCACTGAATGTCAAATT
D13Mit35	13	59	Left = GATTTTCCAGGTAAGTGGCG Right = CACATTCACTGTGAGTGCACA
plau [95]	14	4.8*	Left = GTCTTCCATGTGATGCTCCAC Right = AGGATTGGATGAACTAGTCTA
D14Mit50	14	9.8	Left = GAGGGGGAATCCTAGTGCTC Right = AGCAAAGCCCTATCCACATG
D14Mit54	14	19.7	Left = GAGCCATGGACAGAAAAAGTG Right = ATGTCCCTTTCCCTGGATTC
D14Mit60	14	24	Left = AGGCTGCCCATAAAAGGG Right = GTTTGTGCTAATGTTCTCATCTGG
D14Mit5 [94]	14	31.7	Left = CACATGAACAGAGGGGCAG Right = GTCATGAAGTGCCCACCTTT
D14Mit7 [94]	14	52.5	Left = AATGTATGGGCATGTGCGTG Right = GAGATAGTCAACCAAAACAA
D15Mit12	15	0	Left = ATGGACACCTGACACTGCAA Right = AAGGGCTTTTACCTGGGAAT
D15Mit33	15	40.4	Left = CAGGTTGTTAAAGGTGAGACTGG Right = CTGCATGTATGTACATGGTTGC
D16Mit4 [94]	16	25.1	Left = AGTTCCAGGCTACTTGGGGT Right = GAGCCCTCATTGCAAATCAT
D16Mit5 [94]	16	32.8	Left = CGGGGATCATCCCTAAAAAC Right = TCCCCAATTCCTCTTGTGTC
D17Mit30	17	6.6	Left = CAGGAGCCTGTATACCCACTG Right = CCTCTTTTGGCTTCTGTGGA
D17Mit16 [94]	17	7.7	Left = CCAGAAGACAGCATTCCACA Right = GTATGTCAGGGCTAGTTGACAGG
D17Mit22 [94]	17	9.8	Left = GGTAAGCATTAGATAGAGAG Right = TTATGATCTCCACACACGTG
D17Mit24 [94]	17	10.9	Left = ACCTCTCACCTCTCTCTGTG Right = TGGAGAGACGTCCTATGATG
D17Mit49	17	14.2	Left = TCTTAGAACTCACATCAATGCCA Right = TCCAGGGACCTTTTGTCTTG
D17Mit20	17	29.5	Left = AGAACAGGACACCGGACATC Right = TCATAAGTAGGCACACCAATGC
D17Mit41	17	47	Left = TGCTTGCTGCTTTCTCAGAA Right = GATCTGCCTGTCTCCTTAGTGC
D18Mit14 [94]	18	9.8	Left = GAGGTGATGTGGACACACTC Right = ACACAGCCTAGAATGCACGG
D19Mit19	19	35	Left = CCGTCTGTATAGTTCCTTTGCC Right = CCCTTGTGAGTGCCTGAATT
D19Mit42	19	2.2	Left = CCAAGGAAACTAGGGTATTTACAC Right = GCCTAAACCATTCTGTTGTAAGG
DXMit1 [94]	19	24	Left = CAAGCAACCGAGGAAGACAT Right = CAGGATGCTAATCACCCTGC

Genetic map positions were taken from the MIT F_2 intercross map [96], except for *plau* which was mapped in the Spretus backcross.[96]

Appendix II

C57BL/10ScSn *Urod* fragment original sequence data (Chapter 7)

Printout of sequence chromatograms A1-A4 and B1-B4. Sequence data for primer A has not been reversed and complemented.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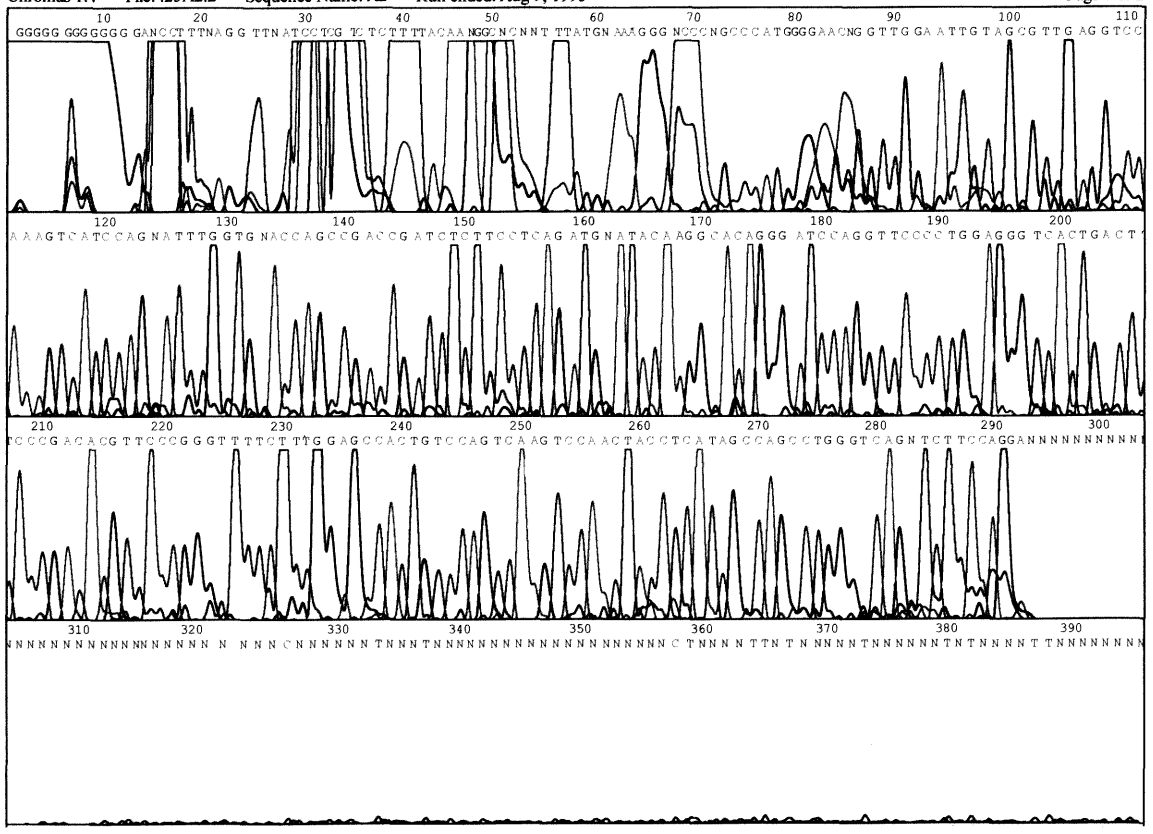
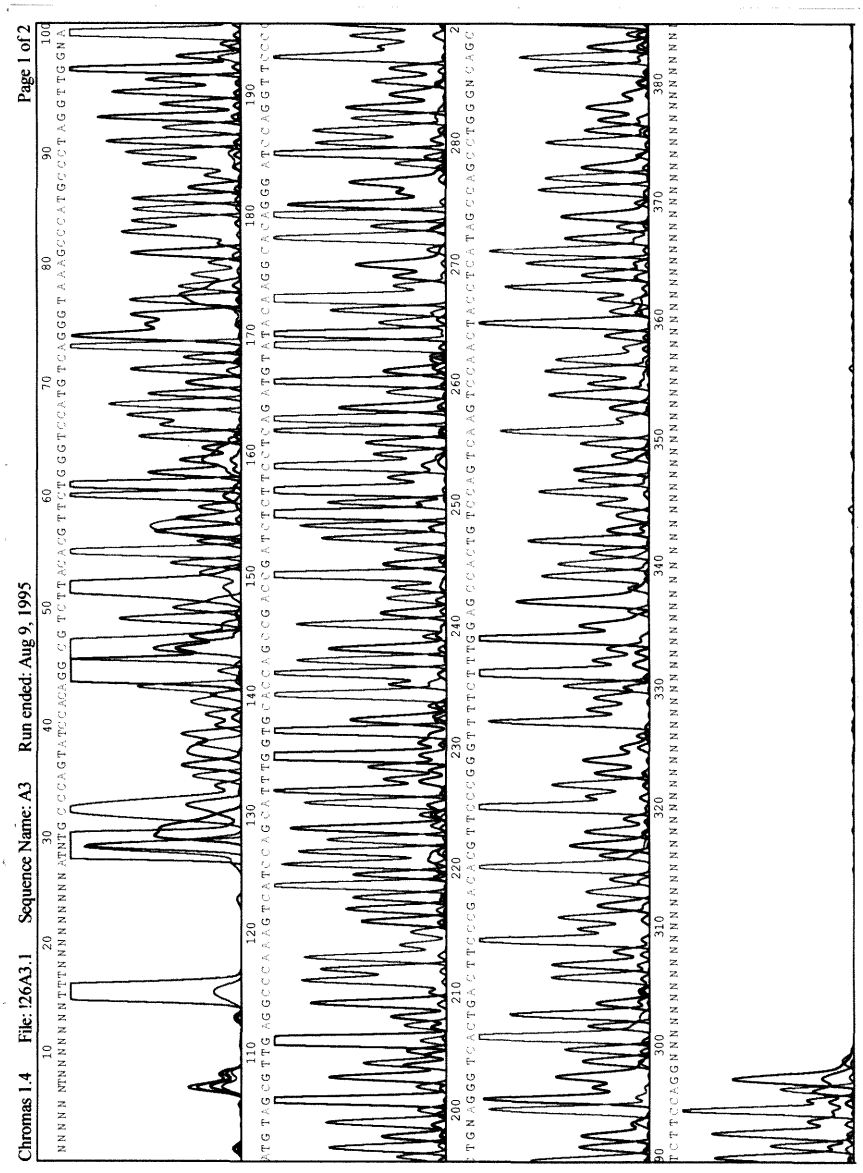
Page 1 of 2

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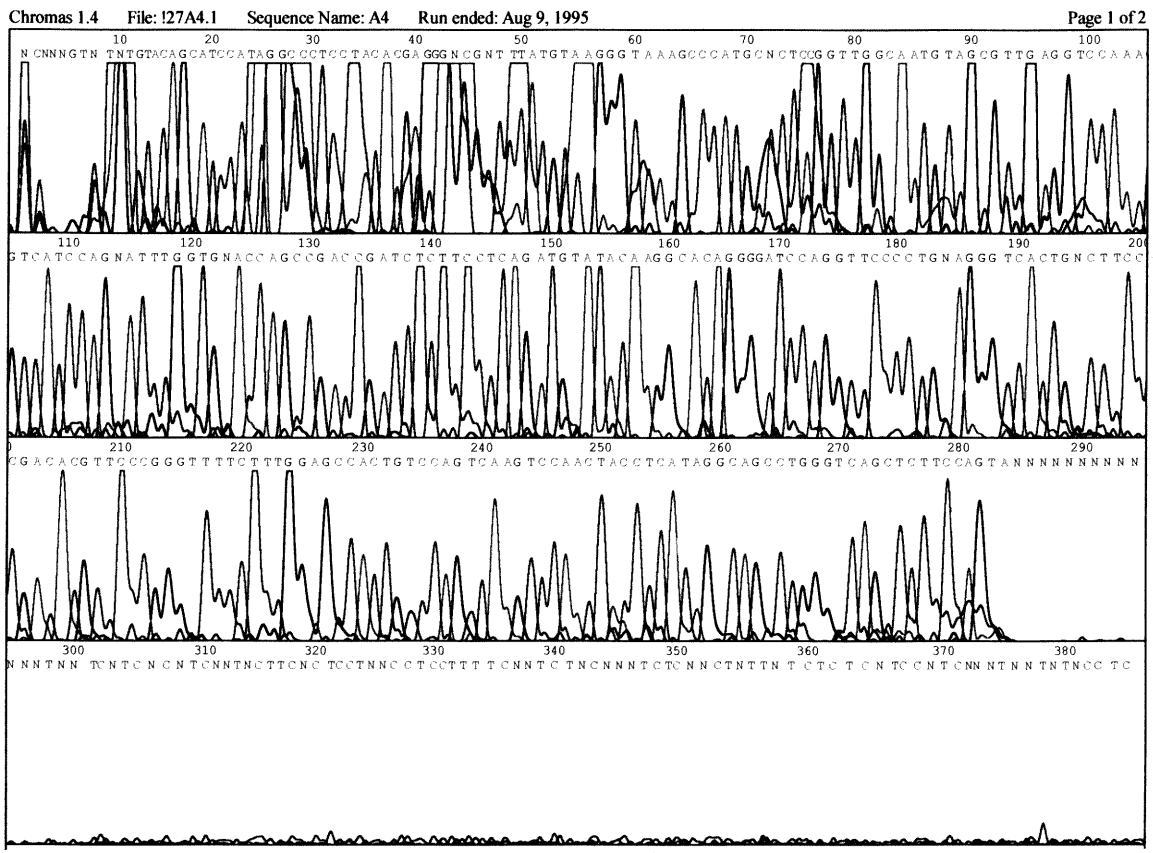
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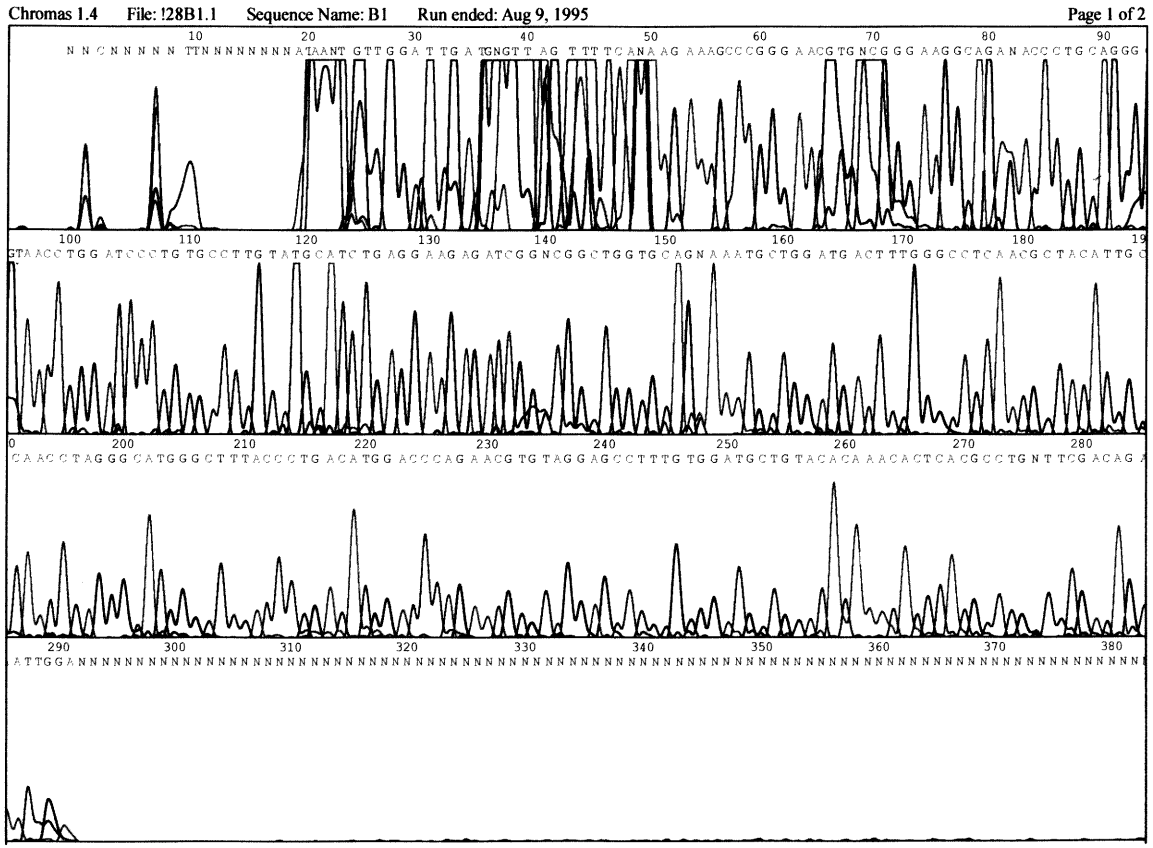
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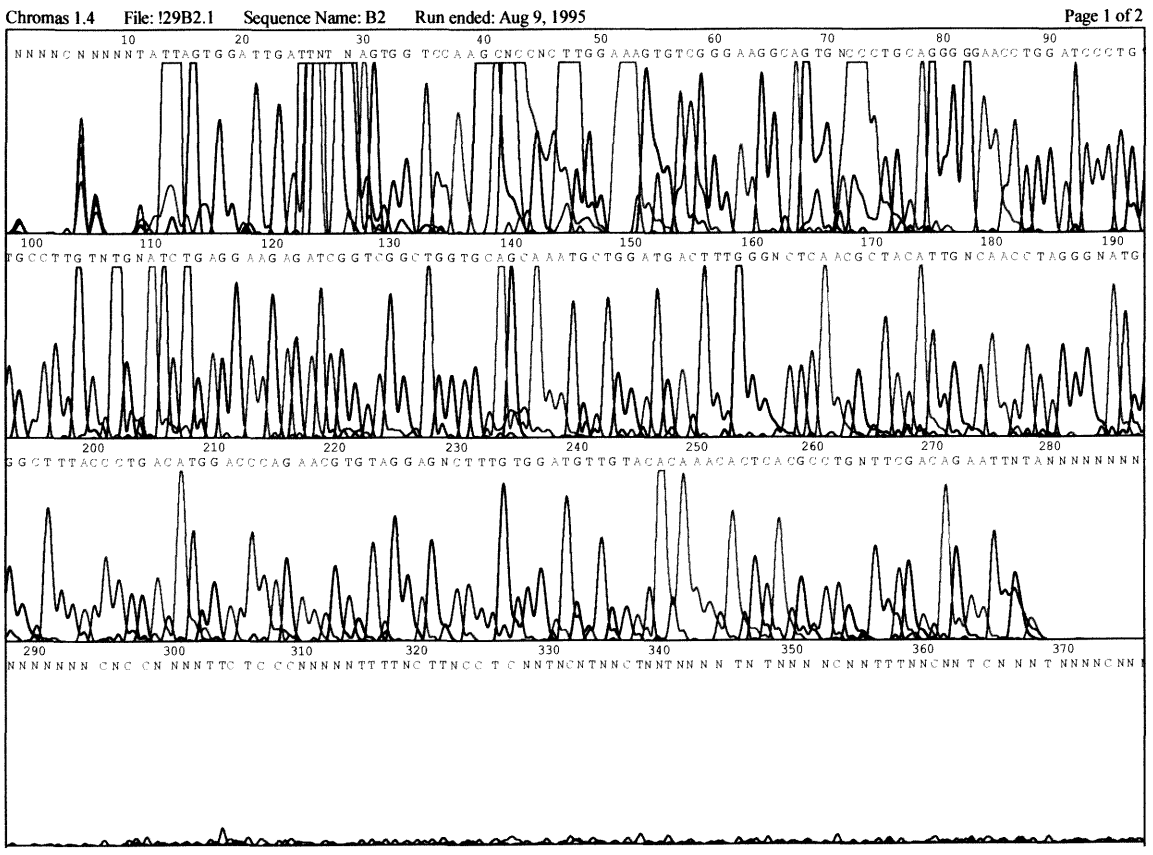
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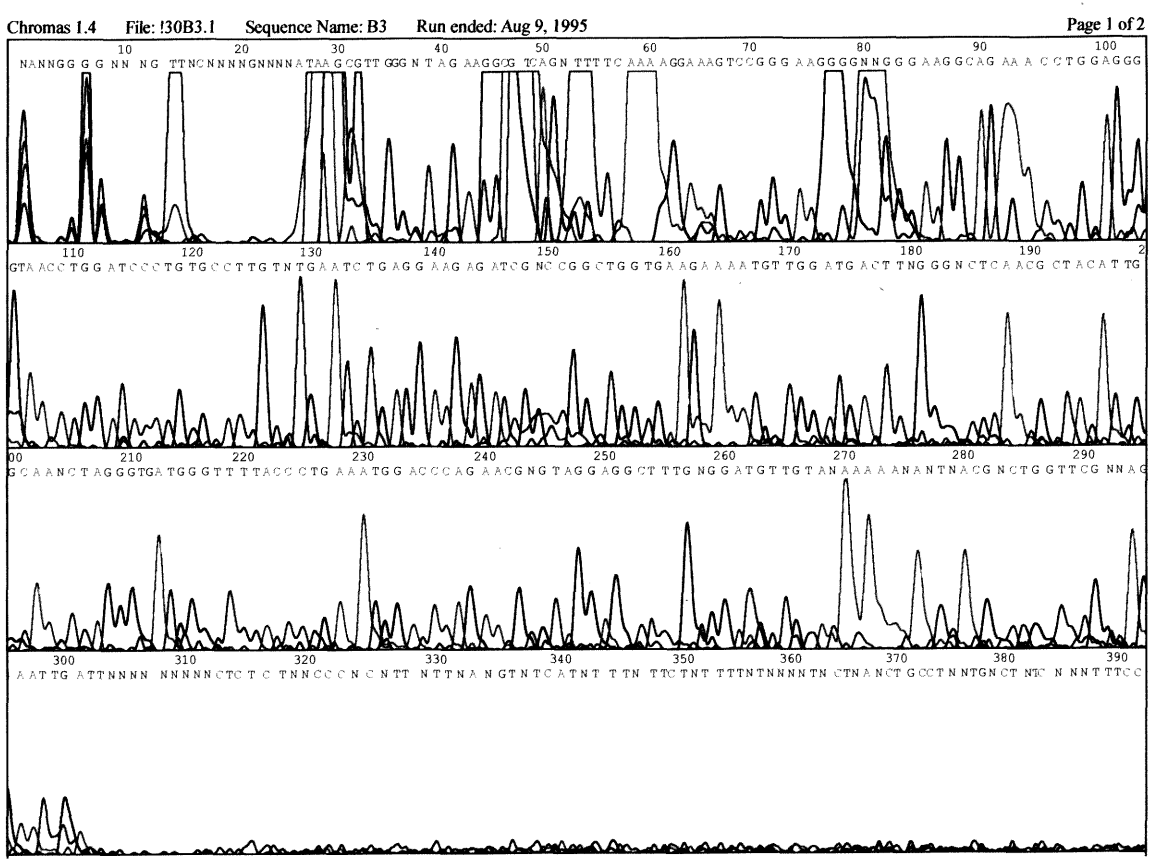
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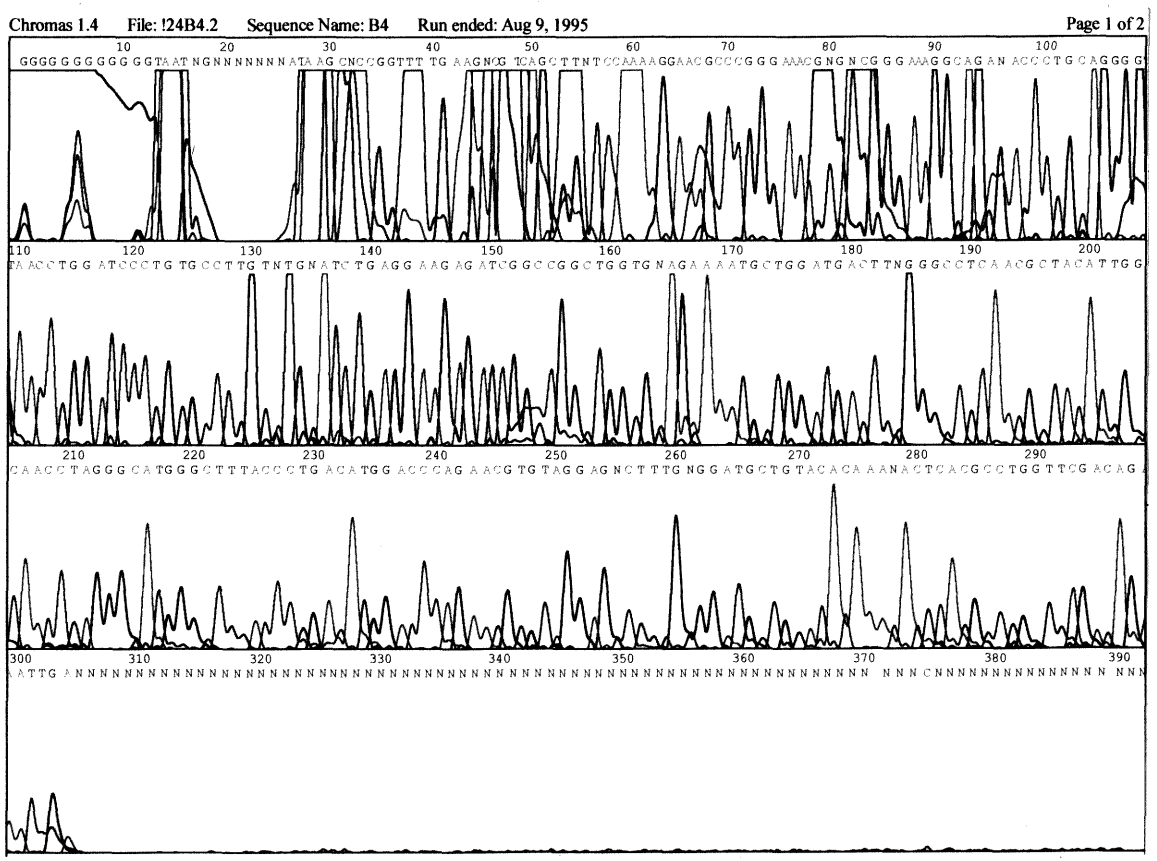
Chromas 1.4 File: !24B4.2 Sequence Name: B4 Run ended: Aug 9, 1995 Page 1 of 2

Appendix III

List of chemical reagents and commercial kits purchased, with suppliers details:

Amersham International plc, UK:
- α^{32}P-dCTP specific activity (3000Ci/mmol)
- γ^{33}P-dATP specific activity (3000Ci/mmol)
- Hybond-N nylon membrane
- T4 Polynucleotide Kinase and buffers (United States Biochemicals)

Applied Bioystems, Warrington, UK:
- AmpliTaq DNA Polymerase and Buffer (Perkin Elmer)
- Cycle Sequencing Dye Deoxy Terminators

Boehringer Mannheim UK (Diagnostics and Biochemicals) Ltd. Lewes, East Sussex, UK:
- Proteinase K
- Tris.HCl

Cambridge BioScicience, Cambridge UK:
- Ist Strand cDNA Synthesis Kit (Clontech)
- Phenol:Chloroform: Isoamylalcohol (25:24:1,v/v) (Amresco)

Fisher Scientific, Loughborough, UK:
- Chloroform
- Dimethylsulphoxide
- Formaldehyde
- Formamide
- Glacial acetic acid
- Hydrochloric acid (concentrated)
- Magnesium chloride
- Magnesium chloride
- Methanol
- Perchloric acid (70%)
- Potassium acetate
- Potassium chloride
- Sodium acetate
- Sodium citrate
- Sodium hydroxide (pellets)
- Trichloroacetic acid

Flowgen Instrumentation Ltd,Staffordshire, UK:
- Metaphor agarose (FMC)
- Nusieve agarose(FMC)

Sequagel concentrate and diluent (National Diagnostics)

Gibco Life Technologies, Inchinnan Business Park, Paisley, UK:
- ϕX174 (Hae III fragments) DNA ladder
- RNA ladder 0.24-9kb
- Eco47III and reaction buffer
- Ultrapure agarose

ICN Biomedicals, UK:
- Caesium chloride

Merck-BDH (UK):
- Ammonium persulphate
- Boric acid
- EDTA
- Hexachlorobenzne
- Sodium chloride
- Sodium dodecyl sulphate
- Sodium phenobarbitone
- Sucrose

Pharmacia, Herts, UK:
- 100mM dNTP solutions: dATP,dCTP,dGTP and dTTP
- NAP-5 sephadex columns
- Pst I and reactiob buffer
- RNA 1A (Bovine pancreatic)

Molecular Probes:
- 7-Methoxyresorufin

Sigma-Aldridge,Chemical Co, Poole, UK:
- 5-Aminolaevulinic acid
- 7-Benzyloxyresorufin
- 7-Pentoxyrsorufin
- 7-Ethoxyresorufin
- β-NAD(P)H
- β-Napthoflavone
- Bathophenanthrolinedisulphonic acid
- Bicinchoninic acid
- Bovine serum albumin (1mg/ml standard solution)
- Bovine serum albumin (powdered?)
- Bromophenol blue
- Copper sulphate II pentahydrate
- Coproporphyrin (5μg standard vials)
- Corn oil
- Dichlorodimethylsilane
- Diethylpyrocarbonate
- Ethidium bromide (10mg/ml sloution)
- Ficoll 400

Hydrogen peroxide (30%)
Iron standard solution (1 mg/ml)
Iron-dextran
Mineral oil
Mixed bed resin
MOPS
Phenol
Polyvinylpyrrolidone
Resorufin
N-Lauroyl sarcosine
N,N,N',N'-tetramethylethylenediamine (TEMED)
Xylene cyanole

Research Genetics, Alabama, USA:
Mouse MapPair Primers

Abbreviations

5-ALA	5-aminolaevulinic acid
AH	aromatic hydrocarbon
Ahr	AH receptor
ALAS	aminolaevulinic acid
ARNT	AH nuclear translocator
bp	base pair
cDNA	complementary DNA
cM	centiMorgan
CYP1A1	cytochrome P450 1A1
CYP1A2	cytochrome P450 1A2
CYP2B1	cytochrome P450 2B1
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
GAPDH	glyceraldehyde-3-phosphate dehydrogen-ase
GMP	guanosine monophosphate
HCB	hexachlorobenzene
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen complex
Hsp 90	heat shock protein 90
IRE	iron responsive element
IRE-BP	IRE-binding protein
kDa	kilo Daltons
M	molar
MHC	Major histocompatibility complex
MOPS	3-(N-morpholino) propanesulphonic acid
mRNA	messenger RNA
3 & 20-MC	methylcholanthrene
NADPH	nicotinamide adenine dinucleotide phosphate
PBG	porphobilinogen
PCB	polychlorinated biphenyl
PCT	Poprhyria cutanea tarda
RNA	ribonucleic acid
Taq	*Thermus Aquaticus*
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
UROD	uroporphyrinogen decarboxylase
XRE	xenobiotic responsive element

Amino Acids

A	Alanine
B	Asparagine
C	Cysteine
D	Aspartic acid
E	Glutamic acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Seronine
Q	Glutamine
R	Arginine
S	Seronine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine
Z	Glutamine or Glutamic acid
*	stop

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