INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

Université d'Ottawa · University of Ottawa

Pharmacogenetic Analysis Of Variable Drug Metabolism Among Inbred Mouse Strains

By

William L. Casley

Thesis submitted to the School of Graduate Studies and Research, University of Ottawa, in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Ottawa-Carleton Institute of Biology

Doctor of Philosophy (1999), University of Ottawa
(Biology)

Title: Pharmacogenetic Analysis of Variable Drug Metabolism Among Inbred Mouse Strains

Author: William L. Casley M.Sc. (University of Western Ontario, 1992)

Supervisor: Dr. Thomas W. Moon, Professor, Department of Biology, University of Ottawa

Abstract

Susceptibility to acetaminophen-induced hepatotoxicity was found to vary widely in an outbred colony of Swiss Webster mice based on differences in serum levels of the hepatic enzyme alanine aminotransferase among males. A selective breeding program produced inbred mouse strains which were either susceptible (APS strain) or nonsusceptible (APN strain) to the hepatotoxic effects of acetaminophen. Hepatic enzyme activities associated with the cytochrome P450 isoform CYP1A2 showed a statistically significant increase in APS *versus* APN mice. Further examination of hepatic *Cyp1a1* and *Cyp1a2* gene expression revealed that mRNA and specific protein levels were significantly elevated in animals from the susceptible group. The co-segregation of elevated basal gene expression for both *Cyp1a* subfamily genes in animals selected for susceptibility to acetaminophen-induced hepatotoxicity suggested a common heritable basis for regulation of basal expression.

Caffeine 3-demethylation can be used as an index of CYP1A2 activity *in vivo* and was found to co-segregate with acetaminophen susceptibility in APS mice. Serum levels of caffeine and the 3-demethylated metabolite were compared among six common inbred strains (A/J, P/J, BALB/cJ, C3H/HeJ, AKR/J and SWR/J) and the APN strain. Significant variations were found between a number of different strains, including the APN strain, indicating a genetic basis for variation in this trait. Hepatic *Cyp1a2* gene expression levels were significantly higher in C3H/HeJ, relative to APN male mice. The striking differences observed between the APN and C3H/HeJ mice suggested that these strains would be suitable for a genetic analysis of the factors affecting caffeine 3-demethylation and, by extension, its use as an index of CYP1A2 expression.

Conflicting data from caffeine assays have suggested a uni-, bi- or tri-modal distribution of

CYP1A2 activity in human populations, although no linkage of a genetic polymorphism affecting basal expression to the CYP1A2 locus has been demonstrated. In order to investigate the genetic determinants of variable caffeine 3-demethylation in the mouse, quantitative phenotypic data, in the form of caffeine 3-demethylation indices, were obtained for the progeny of an F_2 intercross of C3H/HeJ X APN. The phenotypically extreme animals were genotyped at marker loci which achieved complete coverage of the genome. Interval mapping was employed to search for statistically significant linkages between trait data and marker genotypes across the genome. Two statistically significant quantitative trait loci (QTLs) were mapped to chromosomes 1 and 9, and statistically suggestive linkage was found for a QTL on chromosome 4. The QTL on chromosome 9 showed highly significant linkage to the *Cyp1a2* gene, while no obvious candidate genes known to be involved in caffeine metabolism have been mapped to chromosomes 1 or 4.

Pharmacogenetic studies of variable xenobiotic metabolism in humans and experimental animal models have historically focused on monogenic polymorphisms of the enzymes of metabolism and detoxification. Methods to permit genetic analysis of complex quantitative traits have not previously been applied to the study of variations in drug metabolism.

Dedication

This work is dedicated to Laura Jean Casley, for her faith and support.

Acknowledgments

I wish to gratefully acknowledge the contributions and forbearance of my coworkers and collaborators in the Therapeutic Products Programme Research Services Division of Health Canada. I thank Drs. William Wilson and Larry Whitehouse for their commitment in support of my ambitions. Special thanks are due Allan Menzies, without whose tireless efforts and wise counsel this work would not have been possible. I am grateful to Dr. Thomas Moon for his guidance and advice as my thesis supervisor.

"Rem tene; verba sequentur"

Cato The Elder, *Ars Rhetorica I*

Table of Contents

Abstract iii
Dedication v
Acknowledgements vi
Table of Contents vii
List of Original Publications x
List of Figures xii
List of Tables xiv
List of Abbreviations xv

Chapter 1. General introduction 1

1.1. Xenobiotic metabolism 1

1.2. Conjugation reactions 2
1.2.1. Glucuronidation 2
1.2.2. Sulfation 3
1.2.3. Acetylation 3
1.2.4 Glutathione 4

1.3. Cytochrome P450 6
1.3.1. The *CYP1A* subfamily 14
1.3.2. The *CYP2* family 20
1.3.3. The *CYP3* family 23

1.4. Pharmacogenetics 25

1.5. Genetic analysis in murine models of human disease 37

1.6. Aims of the present study 41

Chapter 2. Identification of cytochrome P450 variation between inbred mouse strains selected for susceptibility to acetaminophen-induced hepatotoxicity 46

2.1. Introduction 46

2.2 Materials and methods 51
2.2.1. Animals 51
2.2.2. Standards and reagents 51
2.2.3. Acetaminophen phenotyping 52
2.2.4. Selection and breeding of animals 52
2.2.5. Microsomal assays 53
2.2.6. Caffeine phenotyping 54

2.2.7. RNA isolation 54
2.2.8. cDNA probes 55
2.2.9. RNA slot blots 55
2.2.10. Semi-quantitative RT-PCR 56
2.2.11. Quantitation of PCR products by HPLC 57
2.2.12. Western blotting and immunodetection 58
2.2.13. Statistical analyses 59

2.3. Results 59
2.3.1. Susceptibility to acetaminophen-induced hepatotoxicity 59
2.3.2. Segregation of microsomal activities 62
2.3.3. Comparison of CYP1A2 and CYP1A1 mRNA expression 62
2.3.4. Immunoassay of CYP1A microsomal protein content 68
2.3.5. Caffeine metabolism 68

2.4. Discussion 73

Chapter 3. Differences in caffeine 3-demethylation activity among inbred mouse strains: A comparison of hepatic *Cyp1a2* gene expression between two inbred strains 82

3.1. Introduction 82

3.2. Materials and methods 89
3.2.1. Animals 89
3.2.2. Standard and reagents 90
3.2.3. Caffeine phenotyping 90
3.2.4. 3-methylcholanthrene treatment 91
3.2.5. Microsomal assays 91
3.2.6. Western blotting and immunodetection 92
3.2.7. RNA isolation and cDNA synthesis 93
3.2.8. Duplex PCR 93
3.2.9. Quantitation of PCR products by HPLC 94
3.2.10. RNA slot blots 96
3.2.11. Statistical analyses 96

3.3. Results 97
3.3.1. Caffeine 3-demethylation in inbred strains 97
3.3.2. Liver microsomal activities in APN and C3H/HeJ mice 97
3.3.3. Immunoblot analysis of liver microsomal cytochrome P450 proteins 103
3.3.4. Semi-quantitative RT-PCR analyses of CYP1A1 and CYP1A2 mRNA levels 106
3.3.5. Slot blot analysis of UGT1*06 and GSTYa mRNA levels 112

3.4. Discussion 112

Chapter 4: Identification of quantitative trait loci affecting caffeine metabolism by genome-wide interval mapping of a C3H/HeJ X APN F_2 intercross 118

4.1. Introduction 118

4.2. Materials and methods 119
4.2.1. Crosses 119
4.2.2. Statistical analysis of phenotypic data in crosses 122
4.2.3. Genomic DNA extraction 123
4.2.4. PCR conditions 124
4.2.5. Selection of markers for a genome-wide scan 125
4.2.6. Analysis of amplified STRs 125
4.2.7. Resolution of STR amplicons using polyacrylamide/urea slab gel electrophoresis 126
4.2.8. Detection of amplicons using ^{32}P-end labeled PCR primers 126
4.2.9. Detection of amplicons using agarose gel electrophoresis and ethidium bromide staining 127
4.2.10. Detection of amplicons using a $(CA)_{12}$ oligoprobe 128
4.2.11. Detection of amplicons using capillary electrophoresis and CCD capture of laser- induced fluorescence 129
4.2.12. Genotypic data analysis 130
4.2.13. Construction of a genetic linkage map 131
4.2.14. Interval mapping of linkage to the caffeine 3-demethylation index trait 131

4.3. Results 132
4.3.1. Analysis of C3-D index phenotypic data from crosses 132
4.3.2. Genotyping STR markers 133
4.3.3. Genetic linkage map 133
4.3.4. Interval mapping 140

4.4. Discussion 151

Chapter 5. General Discussion 162

5.1. Elevated basal CYP1A expression in mice selected for differences in susceptibility to acetaminophen-induced hepatotoxicity 162

5.2. Genetic analysis of variation in caffeine 3-demethylation activity between inbred mouse strains 165

5.3. Conclusions 169

List of References 172

List of Original Publications

Casley, W.L., Menzies, J.A., Whitehouse, L.W., and Moon, T.W. Detection of quantitative trait loci affecting caffeine 3-demethylation in a genome wide scan of C3H/HeJ X APN F_2 intercross mice. *Drug Metab. Disp.* (Submitted)

Jurima-Romet, M., Casley, W.L., LeBlanc, C.A. and Nowakowska, M. Dextromethorphan O-demethylation is catalyzed by a CYP2D6-like enzyme in pig liver. *Toxicol. in vitro* (Submitted)

Otto, D.M.E., Sen, C., Casley, W.L. and Moon, T.W. (1999) Regulation of cytochrome P4501A metabolism by glutathione. *Pharmacol. Toxicol.* **84**:201-210.

Casley, W.L., Menzies, J.A., Girard, M., Larocque, L., Mousseau, N., Whitehouse, L.W. and Moon, T.W. (1997) Differences in caffeine 3-demethylation activity among inbred mouse strains: A comparison of hepatic *Cyp1a2* gene expression between two inbred strains. *Fundam. Appl. Toxicol.* **40**: 228-237.

Casley, W.L., Menzies, J.A., Mousseau, N., Girard, M., Moon, T.W. and Whitehouse, L.W. (1997) Increased basal hepatic expression of *Cyp1a1* and *Cyp1a2* genes in inbred mice selected for susceptibility to acetaminophen-induced hepatotoxicity. *Pharmacogenetics* **7**: 283-293.

Jurima-Romet, M., Neigh, S., and Casley, W.L. (1997) Induction of cytochrome P450 3A by retinoids in rat hepatocytes. *Hum. Expt. Toxicol.* **16**: 198-203.

Jurima-Romet,M., Foster, B.C., Rode, A., Casley, W.L., Vloshinsky, P. Huang, H. and Geertsen, S. (1997) CYP2D6-related oxidation polymorphism in a Canadian Inuit population. *Can. J. Physiol. Pharmacol.* **75**: 165-172.

Otto, D.M.E., Sen,C.K., Casley, W.L. and Moon T.W.(1997) Regulation of 3,3',4,4'-tetrachlorobiphenyl-induced cytochrome P450 metabolism by thiols in tissues of rainbow trout. *Comp. Biochem. Physiol.* **117C**: 299-309.

Otto, D.M.E., Sen,C.K., Casley, W.L. and Moon T.W.(1996) Gluthathione Regulates 3,3', 4, 4' - Tetrachlorobiphenyl Induced Cytochrome P450 Metabolism: Evidence for a Cross-Talk Between the Two Major Detoxication Pathways. *Biochem. Mol. Biol. Int'l.* **38**:1127-1133.

Jurima-Romet, M., Casley, W.L., Neu, J.M. and Huang, H.S. (1995) Induction of CYP3A and associated terfenadine N-dealkylation in rat hepatocytes cocultured with 3T3 cells. *Cell Biol. Toxicol.* **11**: 313-327.

Geertsen, S., Foster, B.C., Wilson, D.L., Cyr, T.D. and Casley, W.L. (1995) Metabolism of methoxyphenamine and 2-methoxyphenamine in P4502D6-transfected cells and cell preparations. *Xenobiotica* **25**:895-906

Casley, W.L., Allon, M., Cousin, H.K. Ting, S.S., Crackower, M.A., Hashimoto,L., Cornelis, F. Beckmann,J.S., Hudson, A.J. and Ebers, G.C.(1992) Exclusion of linkage between hypokalemic periodic paralysis (HOKPP) and 3 candidate loci. *Genomics* **14**: 493-494.

Abdalla, J.A., Casley, W.L., Hudson, A.J., Murphy, E.G., Armstrong, H.A. and Ebers, G.C. (1992) Linkage analysis of candidate loci in autosomal dominant myotonia-congenita. *Neurology* **42**: 1561-1564.

Abdalla, J.A., Casley, W.L., Cousin, H.K. , Hudson, A.J., Murphy, E.G., Cornelis, F.C., Hashimoto, L. and Ebers, G.C. (1992) Linkage of Thomsen's Disease to the T-Cell receptor beta (TCRB) locus on chromosome 7q35. *Am. J. Hum. Genet.* **51**: 579-584.

Ebisuzaki, K., Casley, W.L., Griffiths, A. and Wheaton, L. (1991) Temporal mapping of the differentiation pathway of the murine erythroleukemia cell. *Cancer Res.* **51**: 1668-1673.

List of Figures

Figure	Description	Page
1.1	Reaction pathway for cytochrome P450-mediated monooxygenation	8
1.2	The molecular basis of genotyping by short tandem repeat (STR) markers	42
2.1	Acetaminophen metabolism	47
2.2	Serum alanine aminotransferase (ALT) levels after acetaminophen dosing	60
2.3	Liver microsomal enzyme activities	63
2.4	CYP1A1 and CYP1A2 relative mRNA expression levels in APN and APS mice	65
2.5	Correlation of CYP1A2 with CYP1A1 relative mRNA expression	69
2.6	Immunoblot analysis of P450 isoform protein expression	71
2.7	Caffeine metabolism in male and female mice from inbred colonies selected for nonsusceptibility or susceptibility to acetaminophen-induced hepatotoxicity	74
2.8	Correlation of CYP1A2 relative mRNA expression with caffeine-3-demethylation activity	76
3.1	Metabolic pathways of caffeine in humans	83
3.2	Comparison of caffeine 3-demethylation activity among different inbred strains	98
3.3	Liver microsomal activities in male APN and C3H/HeJ mice	101

Figure	Description	Page
3.4	Microsomal CYP1A activities after 3-MC treatment	104
3.5	Immunoblot analysis of liver microsomal proteins from APN and C3H/HeJ mice	107
3.6	Semi-quantitative RT-PCR comparison of CYP1A RNA levels between APN and C3H/HeJ mice	109
3.7	Slot blot analyses of GSTYa and UGT1*06 mRNA levels in APN and C3H/HeJ livers	113
4.1	Parental mouse strains for genetic analysis	120
4.2	Distribution of Caffeine 3-demethylation (C3-D) indices among progeny of different crosses	134
4.3	Raw data obtained with different genotyping methods	138
4.4	Log likelihood (LOD) plots for each linkage group in the genome-wide scan of the APN X C3H/HeJ F2 intercross	141
4.5	Linkage group scans for chromosomes containing quantitative trait loci (QTLs) comparing genetic models for the APN allele	152

List of Tables

Table	Description	Page
1.1	Cytochrome P450 genes in human and mouse	12
3.1	Oligonucleotides used in semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of hepatic cytochrome P450 CYP1A mRNA levels	95
3.2	Statistical comparisons of caffeine 3-demethylation activity between inbred mouse strains	100
4.1	Statistical data for C3-D indices in genetic crosses	136
4.2	Genomic coverage obtained with the APN X C3H/HeJ marker set	147
4.3	Comparison of maximum Log likelihood (LOD) scores for different genetic models at proposed quantitative trait loci (QTLs)	150
5.1	Candidate loci involved in caffeine metabolism	167

List of Abbreviations

1,3,7-X: 1,3,7-trimethylxanthine

1,7-X: 1,7-dimethylxanthine

3-MC: 3-methylcholanthrene

ADH: alcohol dehydrogenase

AFMU: 5-acetylamino-6-formylamino-3-methyluracil

AHH: aryl hydrocarbon hydroxylase

ALDH: aldehyde dehydrogenase

ALT: alanine aminotransferase

APN: acetaminophen nonsusceptible inbred mouse strain

APS: acetaminophen susceptible inbred mouse strain

BSO: L-buthionine-[S,R]-sulfoximine

C3-D: caffeine 3-demethylation

cDNA: complementary deoxyribonucleic acid

EROD: ethoxyresorufin *O*-deethylase

G6PD: glucose-6-phosphate dehydrogenase

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GSH: reduced glutathione

GSSG: oxidized glutathione

GST: glutathione S-transferase

hnRNA: heterogeneous nuclear ribonucleic acid

HPLC: high performance liquid chromatography

List of Abbreviations

LOD: log likelihood

MEIQX: 2-amino-3,8-dimethyl-3 H-imidazo[4,5-f]quinoxaline

MGD: Mouse Genome Database

mRNA: messenger ribonucleic acid

MROD: methoxyresorufin *O*-demethylase

NAC: *N*-acetylcysteine

NAPQI: *N*-acetyl-*para*-benzoquinone imine

NAT: *N*-acetyltransferase

PAGE: polyacrylamide gel electrophoresis

PAH: polycyclic aromatic hydrocarbon

PAPS: 3'-phosphoadenosine-5'phosphosulfate

PXR: pregnane X receptor

PCR: polymerase chain reaction

QTL: quantitative trait locus

RFLP: restriction fragment length polymorphism

RT-PCR: reverse transcription-polymerase chain reaction

STR: short tandem repeat

TCDD: tetrachlorodibenzo-*para*-dioxin

UGT: uridine diphosphate-glucuronosyltransferase

XRE: xenobiotic responsive element

Chapter 1

Chapter 1: General introduction

1.1: Xenobiotic metabolism

The co-evolution of plants and herbivores has led to the evolution of detoxification pathways to deal with exposure to toxins. The advent of industrialization has led to an increased exposure to xenobiotics in human and other animal populations in the form of industrial effluents and contamination of food webs. In addition, the emergence of synthetic pharmaceuticals and food additives has led to concerns over the toxicological implications of exposures to these xenobiotics. There has been a continuous expansion in research directed toward addressing the processes by which organisms deal with environmental insults. Since the 1950s, significant advances have been made in understanding the biochemistry and the biology of the metabolism of xenobiotics. Most organisms, from bacteria to mammals, have evolved similar systems for the detoxification and clearance of xenobiotics (reviewed in: Nebert and Gonzalez, 1987). In general terms, the detoxification of xenobiotics proceeds by the biotransformation of a compound to increase its hydrophilicity in order to facilitate its clearance by excretion. These activities can be divided into two broad functional categories, known as phase I and phase II biotransformations.

Phase I reactions include hydrolysis, reduction and oxidation reactions which introduce new functional groups. Addition of one or more of these groups may impart an increase in water solubility, or the introduced functional group may serve as the reaction site for subsequent conjugations with endogenous compounds in Phase II reactions. Phase II reactions include acetylation, sulphation, glucuronidation and glutathione conjugation reactions. The various transferase enzymes involved in phase two reactions have broad substrate specificities. Phase II

reactions impart a significant increase in hydrophilicity and often result in excretion without further biotransformation (Benet *et al*, 1996).

1.2: Conjugation reactions

1.2.1: Glucuronidation

Glucuronidation is a major detoxification pathway in most mammalian species and is an important factor in the metabolism of many important drugs in humans. The process requires the cofactor UDP-glucuronic acid and is mediated by microsomal UDP-glucuronosyltransferases (UGTs). In intact cells, UGTs are embedded in the endoplasmic reticulum where they interact with both lipophilic xenobiotics and the activated metabolites generated by phase I reactions (Axelrod *et al*, 1958; Miners and Mackenzie, 1991). Glucuronidation occurs principally in the liver, but can also occur in kidney, spleen, intestine, skin, brain and intestinal mucosa. Hepatic glucuronidation can lead to excretion via the urine or bile, depending on the physical properties of the parent compound. Like most xenobiotic transforming pathways, glucuronidation is also involved in the metabolism of endogenous compounds such as thyroid and steroid hormones (Tephly *et al*, 1989). Multiple forms of UGT were first identified in the rat on the basis of distinct substrate classes with different developmental profiles and responses to different exogenous inducers of activity (Mackenzie *et al*, 1989). Four classes of UGT activity were proposed. The molecular biology of the UGT enzyme family was investigated using the congenitally jaundiced Gunn rat strain (Bosma *et al*, 1994). In this strain, the impairment of glucuronidation affected three of the four functional classes of UGT activity, while the genetic analysis of the defect suggested a single locus underlying the phenotype. Subsequent cloning and expression studies

demonstrated that there are two UGT families in the rat. There are 4 UGT1 enzymes which are the product of alternative gene splicing whereas the 4 members of the UGT2 class are each the products of distinct genes. It is the UGT1 gene that is mutated in the Gunn rat, accounting for the pleiotropic effects on glucuronidation. The organization of UGT enzymes in humans is similar to the situation in rats, with group 1 enzymes being the product of alternative splicing from a single gene, and group 2 enzymes each arising from unique genes. There are at least 12 different UGTs in humans, with different patterns of tissue and developmental expression (Burchell *et al*, 1995).

1.2.2: Sulfation

Sulfation produces a significant increase in the hydrophilicity of most lipophilic xenobiotics through the formation of a sulfuric acid ester moiety on the parent compound. Sulfation is mediated by a class of cytosolic enzymes called sulfonyltransferases. Sulfation requires the cofactor 3'-phosphoadenosine-5'phosphosulfate (PAPS) and involves the transfer of SO_3^- to the parent compound, usually, but not exclusively, at the hydroxyl group of a phenol or aliphatic alcohol (Klaassen and Boles, 1997). Consequently, sulfation can occur both in the unmodified parent compound, depending on its structure, and following phase I biotransformation. This activity is found mainly in the liver, kidney and intestine, but also in lung, brain and platelets (Paulson *et al*, 1990). In addition to facilitating the clearance of xenobiotics, sulfation is involved in the metabolism of thyroid and steroid hormones. Sulfation has also been implicated in the biotransformation of some procarcinogens, such as safrole, to reactive carcinogenic metabolites (Boberg *et al*, 1983).

1.2.3: Acetylation

Acetylation is a conjugation reaction which has been implicated in both adverse drug

reactions (Spielberg, 1996) and susceptibility to environmental carcinogens in humans (Hengstler *et al*, 1998). *N*-acetylation is a major route of metabolism for compounds containing aromatic amine or hydrazine groups. Acetylation proceeds through the transfer of the acetyl group from acetyl-CoenzymeA to the nitrogen of the amine or the hydrazine functional group via an *N*-acetyltransferase (NAT) enzyme. NAT*s* are also capable of mediating some *O*-acetylations (Hanna et al, 1982). The ability of *N*-acetylation reactions to facilitate excretion through increased water solubility is strongly dependent on the structure of the parent compound. In some cases, *N*-acetylation can actually reduce the clearance of the metabolite by competing with phase I reactions (Hein, 1988). NAT activity is found in the liver as well as extra hepatic tissues of most mammals, with the interesting exception of dogs (Vatsis *et al*, 1995). There are two NAT enzymes that have been cloned in humans and three in mice (Grant *et al*, 1992). NAT enzymes have broad substrate ranges with considerable overlap. Most compounds that are *N*-acetylated in humans show biphasic reaction kinetics, reflecting the differing affinities of the compound for each of the NAT enzymes (Grant *et al*, 1991). NAT1 is an extra hepatic cytosolic enzyme that is expressed in many different tissues, while NAT2 expression is limited to the liver and intestinal epithelium. Genetic variation in *N*-acetylation has been well documented in humans as the basis for a number of adverse drug reaction phenotypes. Variable capacity for *N*-acetylation of drugs has been attributed to expression polymorphisms of the NAT2 gene. This polymorphism has also been implicated in susceptibility to aromatic amine procarcinogens (King, 1997).

1.2.4: Glutathione

Glutathione is the principal cellular reducing sink for reactive electrophiles, including those arising from hyper oxygenation of tissue or oxidative metabolism of xenobiotics. Glutathione is a

tripeptide of L-γ-glutamyl-L-cysteinyl-glycine. The tripeptide is synthesized through the formation of the L-γ-glutamyl-L-cysteine via γ-glutamylcysteine synthetase, followed by addition of glycine to the dipeptide via glutathione synthetase. Glutathione is conjugated to reactive electrophiles either through the catalytic action of a family of enzymes known as glutathione S-transferases (GSTs), or non-enzymatically through spontaneous reactions (Meister, 1989). GSTs are a complex family of homo- or heterodimeric enzymes for which multiple subunit genes have been identified in humans and rodents (Tu *et al*, 1984; Pickett *et al*, 1987; Awasthi *et al*, 1994; Bammler *et al*, 1994). Both cytosolic and microsomal GSTs have been identified with xenobiotic conjugating activities. In the mouse and rat, the GSTYa subunit gene has been shown to be inducible as part of the aromatic hydrocarbon inducible gene battery (Friling *et al*, 1990: Ding and Pickett, 1985). Glutathione and GSTs are highly abundant in the cell and are an essential detoxification pathway in the protection of cellular proteins and DNA from electrophilic attack (Shan *et al*, 1990). Genetic deficiency in GST activity has been linked to susceptibility to smoking-induced cancers (London *et al*, 1994). Reactive electrophiles may occur in parent xenobiotic compounds, or more frequently, result from phase I oxidative metabolism. Following glutathione conjugation, the cysteinyl conjugate of the parent compound is either excreted from the liver in the bile or further modified through sequential action of γ-glutamyltranspeptidase, aminopeptidase and NAT to produce a mercapturate which is then excreted via the urine (Skiles *et al*, 1991). Glutathione is essential to the maintenance of the redox balance in the cell. Reduced glutathione (GSH) can react directly to reduce reactive oxygen species, with the concomitant formation of oxidized glutathione (GSSG). In the mammalian liver, in the absence of oxidative stress, the ratio of GSH to GSSG is greater than 100:1. The enzyme glutathione reductase

recovers GSH from GSSG and is essential to the maintenance of the GSH/GSSG redox balance in cells under oxidative stress (Meister, 1995).

The importance of glutathione in the protection of the cell from electrophilic attack was first demonstrated using the specific γ-glutamylcysteine synthetase inhibitor L-buthionine-[S,R]-sulfoximine (BSO). BSO treatment effectively decreases the glutathione content of tissues and cells, both *in vivo* and *in vitro* by irreversibly inhibiting the rate limiting step in glutathione synthesis (Meister, 1991). Mice treated with BSO were significantly more sensitive to the hepatotoxic effects of acetaminophen (Gomez *et al*, 1994). The toxicity of this drug results from the binding of an electrophilic metabolite to cellular macromolecules. Similarly, human lymphocytes, treated with BSO *in vitro*, were several fold more sensitive to the cytotxic effects of radiation (Dethmers and Meister, 1981). In humans, several genetic defects in glutathione metabolism have been identified. These defects, in γ-glutamylcysteine synthetase (Konrad *et al*, 1972), glutathione synthetase (Mohler *et al*, 1970) or glutathione reductase (Loos *et al*, 1976), all affect the ability of cells to maintain the cellular pool of glutathione. In the first two conditions, cellular glutathione levels are low, and severe pathologies are associated with these conditions. In the case of deficient glutathione reductase levels, red blood cell glutathione levels are normal, in the absence of oxidative stress, suggesting that glutathione reductase is not essential to the maintenance of glutathione levels under normal redox conditions (White *et al*, 1994).

1.3: Cytochrome P450

Phase I reactions include hydrolysis reactions mediated by carboxylesterase, peptidase or epoxide hydroxylase enzymes. Metabolism through reduction reactions includes dehalogenations,

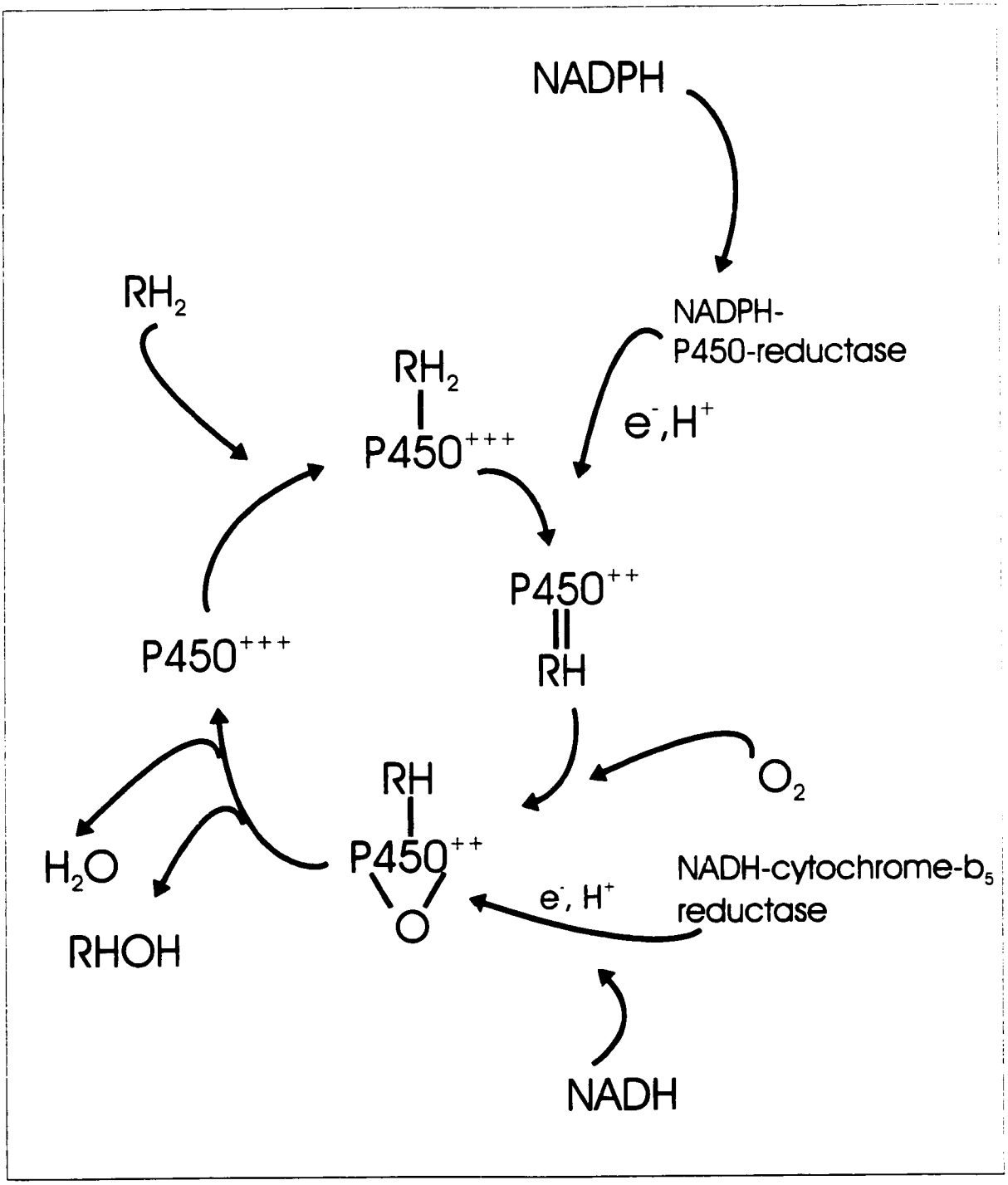
carbonyl, disulfide or quinone reductions as well as reduction of azo- or nitro-groups. The most extensively studied phase I reactions are monooxygenation reactions mediated by the mixed - function oxidase system, more commonly referred to as the cytochrome P450 enzymes. The cytochrome P450 enzymes collectively catalyse a diverse range of biotransformations of a variety of structurally unrelated substrates. These reactions include epoxidations, – and O-dealkylations, S-oxidations and hydroxylations. In addition, a given xenobiotic may act as a substrate for two or more of these reactions (Hollenberg, 1992). These enzymes were first identified as hemoprotein pigments in liver with an absorbance maximum of 450 nm after binding carbon monoxide (Omura and Sato, 1964). This fraction was subsequently identified as a collection of microsome-associated proteins with the capacity to mediate NADPH and O_2-dependent oxidations over a broad range of substrates (Lu and Coon, 1968). In the cell, cytochrome P450 enzymes are bound to the endoplasmic reticulum. The reaction mechanism for P450-mediated monooxygenation of a substrate involves the binding of the substrate to the P450 enzyme which has the heme iron in the oxidized (Fe^{3+}) state. The substrate range of a particular P450 isoform is dictated by the receptor properties of the substrate binding domain. The heme iron is then reduced by the action of NADPH cytochrome P450 reductase, using reducing equivalents from NADPH. Subsequent oxidation of the heme iron by molecular oxygen results in the transfer of one oxygen atom to the substrate, usually resulting in hydroxylation of the substrate. The second oxygen atom is reduced to H_2O (Fig.1.1) (Guengerich, 1993).

Biotransformation of a substrate by monooxygenation may introduce a reactive site for subsequent phase II conjugations or toxic reactions with cellular components. This enzyme superfamily is tremendously important in the study of xenobiotic metabolism, both in

Figure 1.1: Reaction pathway for cytochrome P450-mediated monooxygenation

A xenobiotic compound (RH_2) is bound by the substrate binding domain of a cytochrome P450 enzyme, which has the heme iron in the oxidized state (Fe^{3+}). NADPH-cytochrome P450 reductase uses NADPH to reduce the heme iron (Fe^{2+}) in the substrate enzyme complex. The complex is oxidized in the presence of molecular oxygen (O_2), and undergoes a conformational change. Reducing equivalents are transferred to the complex from cytochrome b_5 reductase, in the presence of NADH. One oxygen atom is transferred to the substrate, resulting in the release of a hydroxylated substrate, H_2O and the P450 enzyme in the oxidized heme iron state.

(Modified from Guengerich, 1993).


NADPH
NADPH-
P450-reductase
e⁻,H⁺
RH2
P450+++
RH2
P450++
RH
P450+++
RH
P450++
O
O2
H2O
RHOH
e⁻, H⁺
NADH-cytochrome-b5
reductase
NADH

environmental toxicology and pharmaceutical research. Early *in vitro* inhibition and enzyme kinetic studies suggested that a number of different enzymes were mediating the biotransformations observed in isolated liver fractions. Furthermore, it was observed that the capacity for the biotransformation of different substrates *in vitro* and *in vivo* could be induced by the pretreatment of experimental animals with different chemical inducers, some of which were also substrates for the induced oxidative capacity (Felton and Nebert, 1975). These results suggested that mammalian detoxification systems were both able to deal with a broad range of xenobiotic toxicants and were responsive to novel environmental exposures. The cytochrome P450s are also involved in the metabolism of endogenous compounds, including steroids (Porter and Coon, 1991). However, endogenous substrates have not been identified for all mammalian P450 isoforms. Classical biochemistry approaches were used to fractionate and reconstitute active detoxification systems from liver, which led to the conclusion that substrate specificities and inducible responses were mediated by the endoplasmic-reticulum-associated cytochrome P450 fraction of hepatic proteins (Lu and Coon, 1968).

Research in the area of cytochrome P450 has gained tremendous momentum since the advent of molecular biology. The tools of cloning and DNA sequence analysis have permitted an ordered classification of the P450 superfamily of enzymes into groups based upon sequence homology. Phylogenetic relationships have been studied to investigate the evolution of the P450 superfamily (Nebert and Gonzalez, 1987; Lewis *et al*, 1998). The ancestral P450 is thought to have emerged shortly after the beginning of terrestrial life in a prokaryotic life form, possibly as long as 3.5 billion years ago. The expansion of the P450 gene superfamily coincides with the emergence of terrestrial biota, approximately 400 million years ago, an observation that is

consistent with the hypothesis that the evolution of broad substrate detoxification systems was a response to the evolution of chemical defences against herbivores among plant species (Gonzalez and Nebert, 1990). The shared homology of isoforms within a given family has permitted the rapid identification of new related forms, with over 500 genes identified to date. Cytochrome P450 genes have been identified in prokaryotes (33 families), fungi (15 families), plants (22 families) and animals (31 families). At least 98 different gene families have been described to date, with one gene family (CYP51) common to all groups (Nelson *et al*, 1996). In humans and mice, two extensively studied species, 14 and 11 gene families have been identified, respectively. Within these families are 19 common subfamilies containing a total of 39 and 35 distinct genes, respectively (Table 1.1). This represents those genes that have been identified, and should not be considered a complete inventory of the P450 gene repertoire in either species. The nomenclature for cytochrome P450s is based on amino acid sequence homologies. By convention, P450s having 40% or less sequence homology are grouped into different families. Mammalian P450s within a family are grouped into the same subfamily if they show greater than 55% identity. P450 genes are identified by the italicized prefix *"CYP"*, except in mice. In accordance with mouse genome nomenclature rules, P450s in mice are identified by the italicized prefix *"Cyp"*. Designations for mRNA, cDNA and protein use non-italicized upper case characters in all species. Gene families are identified by a number following the prefix, and subfamilies are designated by a letter after the family number. Individual isoforms within a subfamily are designated by a number following the subfamily letter designation (Nelson *et al*, 1993). Hence two unique members of the "A" subfamily of gene family "1" are designated "*CYP1A1*" and "*CYP1A2*" in humans, and "*Cyp1a1*" and "*Cyp1a2*" in mice.

Table 1.1: Cytochrome P450 genes in human and mouse

Listing of cytochrome P450 genes identified by cDNA sequence analysis in humans and mice. Genes encoding enzymes with ≤ 40% deduced amino acid sequence identity are grouped into different families as designated by the numeral following the gene designation "*CYP*" (or "*Cyp*" in the mouse). Within a gene family, genes encoding ≤ 55% amino acid sequence identity are grouped into different subfamilies, as designated by the letter following the family numeral. Multiple genes within subfamilies are designated by numerals after the subfamily letter. From: Nelson *et al* (1996).

Gene Subfamily	Human Genes	Mouse Genes
1A	*CYP1A1, 1A2*	*Cyp1a1, 1a2*
1B	*CYP1B1*	*Cyp1b1*
2A	*CYP2A6, 2A7, 2A13*	*Cyp2a4, 2a5,2a12*
2B	*CYP2B6*	*Cyp2b9, 2b10, 2b13*
2C	*CYP2C8, 2C9, 2C18, 2C19*	*Cyp2c29*
2D	*CYP2D6*	*Cyp2d9, 2d10, 2d11, 2d12, 2d13*
2	*CYP2E1*	*Cyp2e1*
2F	*CYP2F1*	*Cyp2f2*
2J	*CYP2J2*	-
3A	*CYP3A3, 3A4, 3A5, 3A7*	*Cyp3a11, 3a13, 3a16, 3a25*
4A	*CYP4A9, 4A11*	*Cyp4a10, 4a12, 4a14*
4B	*CYP4B1*	*Cyp4b1*
4F	*CYP4F2, 4F3*	-
5A	*CYP5A1*	*Cyp5a1*
7A	*CYP7A1*	*Cyp7a1*
7B	-	*Cyp7b1*
8*	*CYP8*	-
11A	*CYP11A1*	*Cyp11a1*
11B	*CYP11B1, 11B2*	*Cyp11b1, 11b2*
17A	*CYP17A1*	*Cyp17a1*
19*	*CYP19*	*Cyp19*
21A	*CYP21A2*	*Cyp21a1*
21B	*CYP21B*	-
24*	*CYP24*	*Cyp24*
27*	*CYP27*	-
51*	*CYP51*	-

* Gene families for which no subfamilies have been designated

An understanding of the phylogenetic relationships of the P450 superfamily between species is important to toxicology. In choosing animal models for human toxicological studies, it is critical to understand the comparative capacities for xenobiotic metabolism. Similarly, in environmental toxicology, when comparing the effects of environmental insults between species, or developing biomarkers of exposure, it is necessary to understand the likelihood that different species share common P450 isoforms. For example, the inducible cytochrome P450 *CYP1B* subfamily has been identified in mammals, and is of interest as a biomarker of exposure to polycyclic aromatic hydrocarbons (PAHs) due to its expression in peripheral blood lymphocytes. This isoform has yet to be identified in non-mammalian vertebrates, but phylogenetic analysis suggests that this isoform likely emerged prior to the divergence of mammals (Lewis *et al*, 1998).

Cloning of P450 cDNAs has led to the development of specific antibodies and DNA sequence based methods for the quantitative analysis of specific gene expression *in vivo* and *in vitro*. Cloning of P450 genes has also led to isoform-specific expression of individual P450 cDNAs in transgenic lymphocytes *in vitro*, which in turn permits the study of isoform-specific substrate ranges and enzyme kinetics, including competitive drug interactions (Gonzalez *et al*, 1989).

In the context of pharmacology, the research focus has been on the cytochrome P450 families 1-3. These families include the liver microsomal isoforms which collectively account for the great majority of xenobiotic metabolism, including the metabolism of drugs.

1.3.1: The *CYP1A* subfamily

One of the most extensively studied subfamilies is the cytochrome P450 *CYP1A* group. In mammals, this subfamily consists of two members, *CYP1A1* and *CYP1A2*. Each of these have

been identified in all mammals examined to date. The substrate specificity, tissue distribution of basal expression and inducibility are similar, but not identical between humans and mice. CYP1A1 is an extra hepatic enzyme expressed mainly in lung, intestine, skin and placenta. Enzyme protein and mRNA levels are extremely low in uninduced livers in both mice and humans (Goldstein and Linko, 1984, Song *et al*, 1985, McKinnon *et al*, 1991). CYP1A2 expression is mainly hepatic, although expression of the rat and human orthologues in brain (Schilter and Omiecinski, 1993; Farin and Omiecinski, 1993; Morse *et al*, 1998), and the human orthologue in oesophageal mucosa (Lechevrel *et al*, 1999) have been detected. CYP1A2 may also be inducible in human duodenal mucosa (McDonnell *et al*, 1992). Because of this pattern of expression, CYP1A1 tends to be evaluated solely for its potential role in susceptibility to environmental carcinogens, whereas CYP1A2 has been studied extensively both in the context of drug metabolism, and with respect to the metabolism of environmental toxicants. Both enzymes are important in the activation of a number of environmental procarcinogens. CYP1A1 is principally involved in the metabolism of PAHs, whereas CYP1A2 metabolizes heterocyclic- and arylamines, as well as certain nitroaromatic compounds (Shimada *et al*, 1989). It had long been accepted in toxicology that some compounds, which were inactive in microbiological mutagenicity assays, could be converted to mutagens by pretreatment with liver fractions from metabolically competent species. It was further recognized that the capacity to mediate these biotransformations could be enhanced by pretreatment of animals with various inducers prior to the preparation of liver fractions. Different types of inducers were observed to enhance the bioactivation of different classes of procarcinogens (Conney *et al*, 1982). This demonstrated the toxicological significance of differences in the expression of different P450 isoforms. The ability to experimentally induce

large increases in a specific subset of activities, both *in vivo* and *in* vitro, made the CYP1A subfamily an excellent candidate for the study of the impact of environmental responsiveness of mammalian detoxification processes on susceptibility to xenobiotic-induced disease. CYP1A2 is involved in the metabolism of a number of important drugs, including the anti-neoplastic tamoxifen, the anti-arrhythmic verapamil, the blood thinning agent warfarin, caffeine and acetaminophen (Spatzenegger and Jaeger, 1995). The *O*-deethylation of the pro-drug phenacetin was used as a clinical *in vivo* probe for CYP1A2 activity until its drug status was revoked (Butler *et al*, 1989). Currently caffeine is the preferred *in vivo* probe, as discussed below.

The study of the murine *Cyp1a* subfamily was stimulated by the early observation that liver microsomes from mice pretreated with the aromatic hydrocarbon 3-methylcholanthrene (3-MC) had different substrate specificities and absorption spectra than those of microsomes from animals treated with other inducers of microsomal metabolism. Specifically, a generic activity known as aryl hydrocarbon hydroxylase (AHH) was induced several fold. Nebert and Gelboin (1969) demonstrated that the induction of AHH activity by 3-MC was strain- dependent in the mouse. 3-MC treatment of C57BL/6 inbred mice produced a 6-fold induction in AHH activity, while no increase was seen after treatment of DBA/2 mice. The oxidative activities induced by 3-MC were determined to be a result of the co-induction of two P450 enzymes, subsequently determined to be CYP1A1 and CYP1A2 (Guenthner and Nebert, 1978). CYP1A1 was determined to be responsible for AHH activity, while CYP1A2 was determined to be a broad spectrum arylamine hydroxylase. Subsequent genetic analysis of responsive and nonresponsive strains revealed that the responsiveness trait was inherited in a Mendelian dominant fashion (Gielen *et al*, 1972). The genetic locus *Ah*, for "Aromatic hydrocarbon responsiveness" was

designated. The 3-MC responsive allele was designated Ah^b (for C57BL/6) while the nonresponsive allele was designated Ah^d, for DBA/2. 2,3,7,8-tetrachlorodibenzo-*para*-dioxin (TCDD) was subsequently identified as a several thousand fold more potent inducer of the same response as 3-MC (Poland *et al*, 1976). At sufficient concentrations, TCDD could induce CYP1A1 expression in the 3-MC nonresponsive mouse strain DBA/2. This was a key observation in that it supported the hypothesis that DBA/2 mice retained functional AHH activity and that the nonresponsiveness must reside in a regulatory locus influencing CYP1A1 expression (Poland and Glover, 1973; Okey *et al*, 1979). This hypothesis was verified by the eventual discovery of an approximately 90 kDa protein with strain-dependent 3-MC and TCDD binding properties, designated the Aromatic Hydrocarbon Receptor (AHR) and assigned to the *Ah* locus, later renamed *Ahr* (Burbach *et al*, 1992). Cloning and expression of the various components of the TCDD induction pathway have produced a more complete picture of the process. The AHR is a cytosolic receptor which was identified as part of the part of the Per, ARNT, Sim (PAS) family of ligand- activated transcription factors based on sequence homology with domains of the Period (PER) and Single minded (SIM) gene products of *Drosophila*, and the ARNT protein described below. The PAS domain of the AHR protein is required both for protein-protein interactions as well as ligand binding. Receptors in this group also share a basic helix-loop-helix structural motif which is involved in heterodimeric binding and DNA binding (Denison and Whitlock, 1995). In the cytosol AHR is complexed with the heat shock protein HSP90 and a 37kDa protein identified as Ah receptor-interacting protein (AIP), in mice or ARA9 in humans. Upon binding of a ligand (such as the prototypical AHR ligand, TCDD), the AHR dissociates from HSP90 and translocates to the nucleus where it binds a second regulatory factor known as ARNT, for Aromatic

Hydrocarbon Receptor Nuclear Translocator. The nomenclature for ARNT was based on the assumption that ARNT binding to the AHR- ligand complex was required for translocation to the nucleus. More recent evidence suggests that nuclear translocation of ligand- bound AHR is independent of ARNT binding. The precise sequence of events for translocation and dissociation from HSP90 remain to be elucidated, although a widely held model involves the displacement of HSP90 by ARNT during AHR/ARNT dimerization, after nuclear translocation (Hankinson, 1995; Swanson and Yang, 1998). The dimerization of AHR with ARNT appears to be dependent on phosphorylation of the ARNT protein. A second phosphorylation event on AHR is required for DNA binding of the dimer. Cloning and sequencing of upstream sequences from TCDD-inducible *Cyp1a1* genes from mice and humans has led to the identification of conserved AHR/ARNT binding motifs. These sequences are known as xenobiotic responsive elements (XREs). Two XRE domains, have been isolated from the upstream region of the human *CYP1A1* gene. Binding of the AHR/ARNT dimer to these *cis*-acting sequences results in the transcriptional activation of the downstream gene (reviewed in Rowlands and Gustafsson, 1997). The transcriptional activation of this gene involves binding of other tissue-specific basal transcription factors, such as TFIIB, at the AHR/ARNT complex (Swanson and Yang, 1998).

While most studies of AH-mediated induction of gene expression have focussed on *Cyp1a1*, a number of genes are induced through this mechanism. Nebert (1989) coined the term "[Ah] gene battery" to encompass a number of xenobiotic metabolizing enzymes which are co-induced by TCDD. The criteria for inclusion in the [Ah] gene battery was later refined to those genes whose expression was both inducible by AH receptor ligands and repressible by CYP1A1 enzyme (Nebert *et al*, 1993). In addition to the genes encoding the phase I enzymes CYP1A1,

CYP1A2 and CYP1B1, the battery includes genes encoding GST Ya subunit (*GstYa*), cytosolic aldehyde-3-dehydrogenase (*Aldh-3*), NAD(P)H:Quinone oxidoreductase (Nmo_1) and UGT (*Ugt1*06*).

Recently, more attention has been paid to the transcriptional regulation of *Cyp1a2*. *Cyp1a2* is both constitutively expressed and inducible by TCDD in the liver. A single functional XRE-like sequence has been isolated from the upstream region of the human *CYP1A2* gene, and a second XRE-like sequence has also been identified, but AHR/ARNT binding studies with reporter constructs using this sequence indicate that it may be non-functional, at least with respect to AHR-mediated transcriptional activation (Chung and Bresnick, 1995;1997). XRE sequences have not been identified in the mouse *Cyp1a2* upstream region (Ikeya *et al*, 1989). Transfection experiments in mouse hepatoma cells, using the upstream sequence -1843 to +52 from the mouse *Cyp1a2* gene spliced to a reporter construct, demonstrated that this region was insufficient for both constitutive and TCDD inducible reporter expression (Owens and Nebert, 1990). The basal expression of *Cyp1a2* in liver appears to be dependent on *Ahr* gene expression. In *Ahr* (-/-) knockout mice, basal CYP1A2 mRNA expression is reduced by 85% (Fernandez-Salguero *et al*, 1995). Transactivation domains in the murine AHR protein have been identified which operate independently of ligand or ARNT binding (Ma *et al*, 1995). These results may lead to a re-evaluation of AHR as an orphan receptor. While no endogenous ligand has yet been demonstrated for AHR, it is clearly involved as regulatory factor in the basal transcription of *Cyp1a2*. In humans, a 259bp domain at -2352 of the 5' region of the gene is required for constitutive expression in the liver (Chung and Bresnick, 1995). This region consists of 3 protein binding sites, including a putative hepatic nuclear factor (HNF-1) binding site, which may confer the tissue-

specific expression of the gene. In addition, one of the binding domains appears to bind a repressor protein based on deletion analysis in reporter constructs (Chung and Bresnick, 1997). Collectively, these results indicate that basal CYP1A2 expression in both mice and humans is a complex, multifactorial regulatory process. Using *Ahr* (-/-) knockout mice, it has been clearly demonstrated that an AHR-independent induction mechanism exists for CYP1A2 but not for CYP1A1. Piperonyl butoxide and acenaphthylene induced increases in CYP1A2 and CYP1B1, but not CYPA1 steady state mRNA in *Ahr* (-/-) knockout mice (Ryu *et al*, 1996). CYP1A2 heterogeneous nuclear RNA (hnRNA), mRNA and protein were also found to be inducible by phenobarbital in *Ahr* (-/-) knockout mice (Corcos *et al*, 1998; Zaher *et al*, 1998a). Post-transcriptional stabilization of mRNA has also been proposed as a regulatory mechanism for the murine *Cyp1a2* gene (Pasco *et al*, 1988). Two nucleoproteins have been described which bind the 3' untranslated region of CYP1A2 mRNA with high affinity, and whose binding is affected by 3-MC (Raffalli-Mathieu *et al*, 1997). Interferon-mediated down-regulation of CYP1A1 and CYP1A2 mRNA appears to be a result of both decreased transcription and increased mRNA turnover (Delaporte and Renton, 1997). Because of the role of CYP1A2 in the bioactivation of environmental procarcinogens, and the observation of inter-individual differences in CYP1A2 expression among humans, there is considerable interest in understanding the environmental and/or genetic factors underlying the expression of this gene.

1.3.2: The *CYP2* family

The *CYP2* family is among the most diverse of the P450 gene families in humans and mice. It consists of 7 subfamilies in both species, of which 4 are involved in liver microsomal xenobiotic metabolism. These are the *CYP2A-E* (*Cyp2a-e* in mice) subfamilies (Nelson *et al*, 1996, Meyer,

1996). The *CYP2A* subfamily consists of 3 isoforms in both humans and mice. The *CYP2A* subfamily is a minor contributor to xenobiotic metabolism in humans, accounting for approximately 5% of total hepatic P450 protein content (Shimada *et al*, 1994). In humans, CYP2A6 is the predominant liver isoform, and has been characterized as a coumarin 7-hydroxylase. This activity is weakly inducible by dexamethasone or phenobarbital *in vitro* in primary human hepatocytes (Wrighton *et al*, 1996). In mice, there are three liver isoforms that are involved in coumarin 7-hydroxylation, and testosterone 7α- or 15α-hydroxylation. The mouse enzymes CYP2A4 and CYP2A5 differ by only a single amino acid substitution (Phe^{209}- Leu^{209}), and yet mediate different reactions with different substrates. CYP2A4 is the testosterone 15α-hydroxylase, whereas CYP2A5 is the coumarin 7-hydroxylase (Lindberg and Negishi, 1989). This example highlights the difficulty in making assumptions about the conservation of substrate specificities and favoured biotransformations between orthologues from different species.

The *CYP2B* subfamily was originally described as the phenobarbital-inducible class of P450s in early studies of induced drug metabolism in the rat. In mice, three isoform genes, *Cyp2b9, 2b10* and *2b13*, are expressed in liver. Both the basal expression and phenobarbital-mediated inducibility of these genes have been shown to have strong gender and strain determinants (Macleod *et al*, 1987). In humans, a single isoform, CYP2B6 is found in some, but not all, human livers, and is not inducible by phenobarbital. Where CYP2B6 is found to be expressed, it accounts for less that1% of hepatic P450 content, and its role in drug metabolism is uncertain (Shimada *et al*, 1994).

The *CYP2C* subfamily is important to human drug metabolism. There are 6 members of this subfamily with varied substrate specificities affecting many pharmacologically important

drugs. This subfamily collectively contributes about 25% of the total hepatic P450 content (Shimada *et al*, 1994). CYP2C8, for example, shares an overlapping substrate range with other CYP2C isoforms but appears to be the only CYP2C isoform involved in the metabolism of the anti-neoplastic agent Taxol, through 6-hydroxylation (Rahman *et al*, 1994). The CYP2C9 and 2C10 isoforms differ by two amino acids in primary sequence, and appear to have completely overlapping substrate ranges (Wrighton *et al*, 1996). This is in dramatic contrast with the sequence/substrate specificity relationship described earlier for murine CYP2A4/ 2A5. Human CYP2C9 and 2C19 have been extensively studied. These isoforms are major contributors to the metabolism of important drugs such as nonsteroidal anti-inflammatories and a number of antidepressants, and both show considerable genetic variation in expression. This genetically polymorphic expression has been shown to have significant clinical implications for the applications of a number of drugs. In the mouse, a single hepatic CYP2C isoform, CYP2C29, was originally characterized as an aldehyde oxygenase involved in the metabolism of the psychoactive drug mescaline (Watanabe *et al*, 1995). More recently, 4 new murine *Cyp2c* subfamily members have been cloned, all of which appear to be involved in arachadonic acid metabolism (Luo *et al*, 1998).

The *CYP2D* subfamily has a single member, *CYP2D6*, in humans , but at least 5 isoforms in the mouse (Nelson *et al*, 1996). CYP2D6 contributes approximately 2 % of the total microsomal P450 protein content in human liver (Shimada *et al*, 1994), but because of its substrate specificity, is nonetheless a very important enzyme in drug metabolism. CYP2D6 is involved in the metabolism of a number of β-adrenergic blocking agents, anti-arrhythmia drugs, antidepressants and neuroleptic drugs (Gonzalez and Idle, 1994). In addition, *CYP2D6* expression

is highly polymorphic in humans, and has been extensively studied in the context of genetic predisposition to adverse drug reactions (Daly *et al*, 1996). Dextromethorphan *O*-demethylation has been used as an *in vivo* and *in vitro* probe in identifying CYP2D6 metabolic polymorphisms (Kerry *et al*, 1994; Crespi *et al*, 1995).

The *CYP2E* subfamily consists of 1 member in most mammals, with the exception of rabbits where a second isoform is found. The *CYP2E1* gene produces an orthologous enzyme in both humans and mice, with similar substrate ranges and expression patterns. CYP2E1 has been extensively studied in toxicology due to its major role in the metabolism of halogenated alkanes to toxic reactive intermediates (Guengerich *et al*, 1991). CYP2E1 is also the principal P450 involved in the metabolism of carbon tetrachloride, acetone and ethanol. This enzyme is also important to drug metabolism in that it is principally responsible for the metabolism of the muscle relaxant chlorzoxazone, the widely used antipyretic analgesic acetaminophen, the xanthine drug theophilline and, to a lesser extent, caffeine (Gu *et al*, 1992; Kharasch and Thummel, 1993; Snawder *et al*, 1994). Chlorzoxazone is preferentially, although not exclusively, 6-hydroxylated by CYP2E1, and this drug has come to be applied as a clinical metabolic probe for this activity (Jayyosi *et al*, 1995; Ono *et al*, 1995). Ethanol and acetone are inducers of this activity. The induction of CYP2E1 appears to be somewhat unique among inducible P450s in that increased expression is mainly a result of stabilization of the enzyme, although transcriptional activation has also been suggested (Ingelman-Sundberg *et al*, 1993).

1.3.3: The *CYP3* family

The *CYP3A* subfamily is highly expressed in both human and mouse livers. In human liver this subfamily collectively accounts for at least 40% of the total P450 protein content (Shimada *et*

al, 1994). The human CYP3A7 isoform is principally a foetal enzyme, although its expression has been detected in adult liver. CYP3A5 is not uniformly expressed between individuals, and has been detected in only approximately 25% of adult livers. The CYP3A4 isoform in humans is one of the most important drug metabolizing enzymes, due to its substrate range and high capacity for biotransformation resulting from its relative abundance (Schuetz *et al*, 1994). CYP3A4 is the primary metabolizer of the corticosteroid cortisol, the antifungal ketoconazole, the anti-neoplastics etoposide and cyclophosphamide, the immunosuppressant cyclosporin A, and the antibiotic erythromycin (Wrighton *et al*, 1996). This is by no means a comprehensive list of CYP3A4 substrates, but does serve to illustrate the broad substrate range of this enzyme. In addition, CYP3A4 is involved in the metabolism of endogenous steroid hormones, and plays a role in the metabolism of acetaminophen and caffeine. The expression of this enzyme is highly inducible by a number of drugs, of which dexamethasone is the model inducer (Molowa *et al*, 1986). Recently, the pregnane X receptor (PXR) has been identified as the nuclear receptor which mediates induction *via* binding to response elements in the CYP3A4 promoter region (Kliewer *et al*, 1998; Lehmann *et al*, 1998). A great deal of attention has been paid to CYP3A4-mediated drug metabolism as the basis for possible adverse drug reactions. The induction of this enzyme by clinically relevant drugs as well as the possibility for competitive inhibition during multidrug therapeutic regimens, owing to its broad substrate range, has resulted in a number of pharmacological anomalies linked to the action of this enzyme (Ciummo and Katz, 1995). Testosterone 6β-hydroxylation and erythromycin *N*-demethylation have been used as *in vitro* probes for this activity, although the absolute specificity of these reactions to CYP3A4 has not been demonstrated (Mäenpää *et al*, 1993).

In mice, there are at least 4 members of the *Cyp3a* subfamily. These are the *Cyp3a11*, *3a13*, *3a16* and *3a25*. Of these, the CYP3A11 enzyme is the predominantly expressed isoform in liver. This isoform is also more inducible by dexamethasone and other classic *Cyp3a* inducers than the other enzymes of this subfamily. Both CYP3A11 and CYP3A13 have been shown to mediate the biotransformation of aflatoxin B1, an important dietary procarcinogen, to its toxic metabolite in transgenic cell lines expressing these cDNAs (Yanagimoto *et al*, 1997).

1.4: Pharmacogenetics

The term "pharmacogenetics" was first used in the literature by Vogel (1959) and was defined as "the study of the role of genetics in drug response". As biochemical and molecular studies in drug metabolism proceeded, it became clear that the detoxification systems affecting drug metabolism were essentially the same as those influencing the detoxification of environmental toxicants. Some authors have proposed the term "ecogenetics" to distinguish studies on genetic variation in response to environmental toxicants from those affecting drug metabolism (Brewer, 1971, Motulsky, 1991; Nebert and Carvan, 1997). Kalow (1996) argued that such distinctions were trivial, and suggested that the literal interpretation of the Greek root "pharmakon" as a drug or poison would allow the application of the term "pharmacogenetics" to the study of genetic variation in the response to any xenobiotic. This broader definition of the term has been generally accepted. The first pharmacogenetic studies actually predate the use of this term. A classic human genetic polymorphism is the ability to taste phenylthiourea (Snyder, 1932), and this could be considered as the first formal study of a pharmacogenetic phenomenon.

A major goal in pharmacogenetics is to support the hypothesis that individual differences

in susceptibility to environmentally induced carcinogenesis or adverse drug reactions have a predictable genetic basis. One of the first studies to demonstrate ethnic variations in drug metabolism was the elucidation of the glucose-6-phosphate dehydrogenase (G6PD) polymorphism underlying adverse reactions to the anti-malarial drug primaquine. Individuals with low G6PD activity are at risk for primaquine-induced haemolytic anaemia when exposed to an environment with low oxygen tension, due to G6PD-related glutathione deficiency. This situation came to light in a peculiar convergence of circumstances when it was noted that soldiers in the WWII era, who were receiving mandatory doses of primaquine, suffered a high incidence of haemolytic anaemias when transported at moderate altitudes in unpressurized aircraft. The further observation that soldiers of African descent were more prone to this effect than Caucasians led to the eventual discovery of ethnic variation in the distribution of multiple G6PD-deficiency alleles (Carson *et al*, 1956). Another striking example of ethnic differences in xenobiotic metabolism was the characterization of alcohol intolerance, which is far more prevalent among orientals than Caucasians, as arising from the rapid formation, and/or poor clearance, of the toxic metabolite acetaldehyde. The conversion of ethanol to acetaldehyde was subsequently shown to be mediated by the polymorphic enzyme alcohol dehydrogenase (ADH), while clearance of acetaldehyde is mediated by aldehyde dehydrogenase (ALDH). There are three ADH genes and 10 ALDH genes identified in humans. Rapid metabolizer ADH alleles, and ALDH-deficiency alleles are both more prevalent in oriental *versus* Caucasian populations (vonWartburg *et al*, 1964; Harada *et al*, 1978).

Modern pharmacogenetic analysis of therapeutic drug metabolism has its roots in two salient examples of high incidences of adverse drug reactions when a therapeutic drug was widely distributed in the general population. The anti-tuberculosis drug isoniazid was found to cause a

pathological peripheral neuropathy in many patients when in general use in the 1940s. This adverse reaction was traced to poor clearance of the parent compound (Hughes, 1954). Genetic analysis revealed that isoniazid metabolism rates, specifically *N*-acetylation, were bimodally distributed in the population and that the "slow acetylator" phenotype segregated as a Mendelian recessive trait (Bonicke and Lisboa, 1957). Furthermore, it was determined that the frequency of the slow acetylator phenotype varied from approximately 10% in the Japanese population, through 50% in North American Caucasians, to greater than 90% in certain Mediterranean populations (Weber and Hein, 1985; Hickman and Sim, 1991). This phenotype polymorphism has subsequently been linked to allelic variation in the *NAT2* gene. At least 15 *NAT2* alleles have been identified in human populations, of which at least 5 corelate with the slow acetylator phenotype (Grant *et al*, 1997; Cascorbi and Roots, 1999). The slow acetylator phenotype, and its incidence in different populations, has become an important consideration in the development of new drugs and in the investigation of adverse drug reactions.

The anti-hypertensive drug debrisoquine was found to have caused an unusual number of adverse drug reactions after it was first released for prescription. A pharmacogenetic analysis revealed that the Caucasian population could be segregated into "poor metabolizers" and "extensive metabolizers" of the drug, with the former having at least 10-fold lower levels of the urinary 4-hydroxy metabolite of debrisoquine after therapeutic dosing (Mahgoub, 1977). Dangerous hypotension was occurring in poor metabolizers who were failing to clear the parent compound. This phenotypic variation was subsequently linked to polymorphic expression of the cytochrome P450 *CYP2D6* gene (Gonzalez *et al*, 1988). Poor metabolizers account for approximately 8% of the North American Caucasian population, while the poor metabolizer

phenotype is rare (less than 1%) in oriental populations (Bertilsson *et al*, 1992). This gene was determined to have multiple alleles, ranging from those with wild type levels of expression to null alleles with no functional CYP2D6 enzyme. An "ultrarapid metabolizer" phenotype has also been identified, which results from a gene amplification event at the *CYP2D6* locus (Daly *et al*, 1996). CYP2D6 is involved in the metabolism of a number of widely used prescription and non prescription drugs, now described as the "debrisoquine panel" on the basis of their common polymorphic metabolism.

The human cytochrome P450 CYP2C19 has also been implicated in polymorphic drug metabolism. Poor metabolizers of drugs such as *S*-mephenytoin, omeprazole, diazepam, amitriptyline and propranolol have been identified, and a common genetic basis for these phenotypes has been identified as allelic variation in the *CYP2C19* gene (Goldstein and de Morais, 1994).

The advent of molecular biology techniques has led to the cloning and sequencing of the genes underlying most known polymorphisms of drug metabolism. Genetic tests to identify allelic variants of most drug metabolizing enzymes have been developed, and the correlations of genotypes to phenotypes have been evaluated in developing individual genetic drug metabolism profiles to avoid adverse drug reactions (reviewed in: Lu, 1998; Housman and Ledley, 1998).

The genes described above were identified as candidate genes responsible for highly penetrant monogenic traits having straightforward Mendelian patterns of inheritance. The genetic analysis of susceptibility to xenobiotic-induced disease, typically involves less direct genotype to phenotype correlations. Cloning and genetic mapping of most human polymorphic xenobiotic metabolizing enzyme genes has permitted epidemiological analyses of the associations between

particular genotypes and susceptibility to disease. The human *NAT2* polymorphism has been associated with altered risk for a number of cancers. The slow acetylator phenotype resulting from NAT2 deficiency was associated with a lower risk for colon cancer, (Lang *et al*, 1994) but an increased risk for bladder cancer (Lower *et al*, 1979; Cartwright *et al*, 1982; Evans, 1989). In the context of susceptibility to bladder cancer, this risk is exacerbated in slow acetylators who were exposed to both arylamines and cigarette smoke. Cigarette smokers with no occupational exposures to arylamines were not at increased risk over fast acetylators (Brockmoller *et al*, 1996). Cigarette smoking was found to be a required cofactor in increasing the risk of breast cancer among post menopausal women who are slow acetylators (Ambrosone *et al*, 1996). These results indicate the multifactorial nature of susceptibility to the carcinogenic effects of xenobiotics. Individuals with intact CYP2D6 metabolism ("extensive metabolizers") appear to be at increased risk for stomach, bladder and liver cancers, presumably through the increased activation of environmental or dietary procarcinogens, *versus* poor metabolizers (Kaisary *et al*, 1987; Roots *et al*, 1992) . An increased risk for lung cancer in extensive metabolizers was also described among cigarette smokers (Bouchardy *et al*, 1996). Of those genetic loci with polymorphisms of greatest consequence to drug metabolism, only the *CYP2C19* locus has not yet been convincingly associated with increased cancer risk. Brokmöller *et al*, (1996) demonstrated a weak statistical association between altered CYP2C19 activity and human bladder cancer risk, in contrast to an earlier study by Branch *et al* (1995) in which no association between bladder cancer risk and altered *S*-mephentoin metabolism was found. Tsuneoka *et al* (1996) found a statistically significant increase in *CYP2C19* loss of function alleles in squamous cell carcinoma patients *versus* controls in a small study of the Japanese population.

There are also a number of polymorphically expressed xenobiotic metabolizing enzymes that are not major contributors to clinically significant overt adverse drug reactions, but which may contribute to susceptibility to environmental carcinogenesis. The phase II conjugation enzymes known collectively as the GSTs can be grouped into four distinct classes according to gene sequence. Of these, a genetic polymorphism in the *GSTM1* gene has been associated with susceptibility to lung cancer among smokers. Individuals homozygous for the null *GSTM1*0* allele are completely lacking GSTM1 activity. Cigarette smokers homozygous for *GSTM1*0* were found to be at greater risk for developing squamous cell or adenocarcinomas of the lung than smokers with functional GSTM1 (Ketterer *et al*, 1992; Hirvonen *et al*, 1993). This result is consistent with the knowledge that GST activity is involved in the detoxification of aromatic hydrocarbon and arylamine procarcinogens found in cigarette smoke. An association has also been demonstrated for increased risk of bladder cancers in individuals carrying both the *GSTM1* null genotype and a specific *CYP1A1* allele (Bell *et al*, 1993; Kato *et al*, 1995).

The *CYP2E1* locus has been investigated in the context of cancer susceptibility on the basis of its role in the metabolism of procarcinogen nitrosamines, benzene, and hydroxypyridine, a procarcinogenic constituent of cigarette smoke. Restriction fragment length polymorphisms (RFLPs) have been identified at this locus which correlate with altered expression (Hayashi *et al*, 1991; Hirvonen *et al*, 1993). Positive correlations have been established between decreased function alleles and lowered susceptibility to lung cancer (Uematsu *et al*, 1991, Persson *et al*, 1993). Recently, an RFLP corresponding to a 100bp insertion in the regulatory region of the *CYP2E1* gene in humans has been identified. The insertion allele is fairly prevalent in both Caucasian (6.9%) and African American populations (31%), and corresponds to an increased

inducibility of CYP2E1 activity in response to obesity or alcohol consumption (McCarver *et al*, 1998). Given the incidence of these two factors in the general population, any correlation between this genotype and increased cancer risk would be a most significant finding. As yet no such study has been reported.

The association between polymorphic expression of the *CYP1A* subfamily and susceptibility to environmentally-induced carcinogenesis has been extensively studied. This is due in part to the established role of the *CYP1A* gene subfamily in the activation of aromatic hydrocarbon and arylamine procarcinogens. Another reason for the intense interest in this group of enzymes stems from the extensive research into the induction of their expression via the AHR pathway, as described above. Essentially, the fact that these enzymes could be induced to high levels of expression by exposures to the very procarcinogens that they subsequently render toxic, made them intriguing candidates as carcinogenicity risk factors. Both CYP1A1 and CYP1A2 demonstrate polymorphic expression in the human population and among inbred rodent strains (Ikeya *et al*, 1989; Schweikl *et al*, 1993). The well described induction polymorphism (Nebert and Gelboin, 1969) which maps to the *Ahr* locus on chromosome 12 in inbred mouse strains (Cobb *et al*, 1987) stimulated the search for a similar polymorphism in humans. Interest in human AHR polymorphisms was due, in part, to the demonstrated correlation between *Ahr* genotype and susceptibility to carcinogenesis in the mouse. A genetic analysis of the Ahr^b and Ahr^d alleles revealed that the Ahr^d/Ahr^d genotype conferred resistance to subcutaneous 3-MC- induced tumours (Atlas *et al*, 1976). Experiments with a photoaffinity ligand for the Ah receptor and antibodies against the protein strains identified 4 common alleles in different inbred mouse strains identified as Ahr^d, Ahr^{b-1}, Ahr^{b-2} *and* Ahr^{b-3}. The Ahr^d allele encodes a protein of MW 104 kDa, as

does the Ahr^{b-2} allele. The Ahr^{b-1} and Ahr^{b-3} alleles encode proteins of 95kDA and 105kDa, respectively (Swanson and Bradfield, 1993). Each of the Ahr^{b} alleles encode a receptor protein with 10-20 fold higher affinity for the prototypical ligand, TCDD, than the Ahr^{d} gene product (Okey *et al*, 1989). DNA sequence analysis of the Ahr^{b-1} and Ahr^{d} alleles identified two sequence differences as potentially underlying the differences in ligand affinity. A $T_{2326}C$ polymorphism results in the substitution of a proline residue at amino acid 471 and may result in the disruption of α-helical secondary structure. A second polymorphism in the Ahr^{d} allele results in the loss of an Opal stop codon present in the Ahr^{b-1}, and extends the gene product by 43 amino acids and adds a potential *N*-glycosylation site (Chang *et al*, 1993). While a number of sequence polymorphisms have been identified in the human *AHR* gene, no strong correlation between sequence polymorphism and loss of ligand binding function comparable to the murine Ahr^{d} allele has been identified (Fujii-Kuriyama *et al*, 1995).

Toxicological studies demonstrated an increased susceptibility to tumours in lung and liver in Ahr^{b}/Ahr^{b} *versus* Ahr^{d}/Ahr^{d} mice, when exposed to various tumourogenic PAHs (Miller *et al*, 1990). Ames mutagenicity tests demonstrated an increased mutagenicity of a number of different PAHs in tests employing liver microsomes from PAH-treated *Ahr* responsive mouse strains (Felton and Nebert, 1975). These results supported the hypothesis that increased levels of CYP1A1 lead to increased susceptibility to PAH-induced carcinogenesis, and that functional polymorphisms of the Ah receptor could confer increased susceptibility by mediating induction of CYP1A1 levels in response to environmental exposures to PAHs. These results suggested that the human *AHR* gene could be a major cancer susceptibility locus. CYP1A1 and CYP1A2 enzyme protein content in human livers shows at least a 20-fold inter-individual variation, although these

differences were not shown to be associated with different *AHR* genotypes (Ikeya *et al*, 1989; Schweikl *et al*, 1993). An aromatic hydrocarbon responsiveness polymorphism was proposed on the basis of apparent high and low AHH induction among cigarette smokers (Kellerman *et al*, 1973). Although conflicting results were obtained in different studies, an increased susceptibility to carcinogenesis in a number of different tissues has been postulated for cigarette smokers with the putative high inducer phenotype (Emery *et al*, 1978; Paigen *et al*, 1979; Kouri *et al*, 1982) . *In vitro* assays using ^{3}H-TCDD to measure binding kinetics to AHR from different individuals showed a continuous 20-fold range in ligand binding affinities among humans (Gurtoo *et al*, 1977), and in *in vitro* assays exposing lympocytes to AHR ligands, a possible bimodal distribution of responsiveness was demonstrated (Catteau *et al*, 1995a). The human *AHR* gene was cloned and sequenced (Dolwick *et al*, 1993; Itoh and Kamataki, 1993) and recently, cytogenetically mapped to chromosome 7p15 (Micka *et al*, 1997). A number of genetic polymorphisms were identified at the human *AHR* locus, which permitted epidemiological studies of possible correlates between the incidence of various cancers and different *AHR* genotypes (Kawajiri *et al*, 1995; Nebert *et al*, 1996). To date, no significant association between *AHR* genotype and disease susceptibility has been demonstrated. A small pedigree analysis, using *in vitro* inducibility of CYP1A1 activity in lymphocytes, identified a weak genetic linkage association between a polymorphic marker genotype linked to the *AHR* locus, and the inducibility of CYP1A1 (Micka *et al*, 1997). Daly *et al* (1998) found a significant association between the G(1721)A *AHR* polymorphism and highly induced CYP1A1 activity in Caucasians.

Genetic polymorphisms at the *CYP1A1* locus were also explored as a possible basis for inter-individual variation in both AHR-mediated CYP1A1 inducibility, and susceptibility to

environmental carcinogens. The human *CYP1A1* gene was cloned and localized to chromosome 15 (Hildebrand *et al*, 1985a; Jaiswal *et al*, 1985). A number of genetic polymorphisms were identified at the human *CYP1A1* locus. An allele of an *Msp*I RFLP at the *CYP1A1* locus was found to be associated with increased basal and induced CYP1A1 mRNA and enzyme activity in a mitogen-activated lymphocyte assay (Landi *et al*, 1994) . This polymorphism was also found to be associated with susceptibility to lung cancer in a Japanese population (Kawajiri *et al*, 1990), and, combined with the null genotype of the GSTM1 locus, susceptibility to lung cancers among cigarette smokers (Nakachi *et al*, 1993). These findings appear to be peculiar to the Japanese population, where the frequency of the suspect *CYP1A1 Msp*1 allele is substantially higher than in European or North American populations. A more recent study showed that a single amino acid substitution polymorphism in *CYP1A1* exon 7 was associated with susceptibility to lung cancer among smokers in a Brazilian population, while the *Msp*1 polymorphism was not (Sugimura *et al*, 1995).

The human *CYP1A2* locus does not appear to have the same genetic heterogeneity found in the *CYP1A1* locus. Although there is substantial inter-individual variability in *CYP1A2* expression, few functional polymorphisms affecting gene expression have been identified. The sequence determined by Nakajima *et al* (1994) did differ from that previously reported by Quattrochi *et al* (1986) and Jaiswal *et al* (1986). An in-frame 3 base deletion in exon 7 in the Jaiswal *et al* (1986) sequence, was not confirmed in subsequent analyses. A C/T_{763} polymorphism in exon 7 suggested by the conflict between the Nakajima *et al* (1994) sequence and those of Jaiswal *et al* (1986) and Quattrochi *et al* (1986) was confirmed (Huang *et al* 1999), but did not alter the amino acid sequence of the translated protein. Recently, Huang *et al* (1999) identified a

$C_{2866}G$ mutation in exon 2 which caused a Phe_{21}- Leu_{21} amino acid change, in less than 1% of Chinese subjects. The frequency of this mutation in other populations, as well as its functional significance, has yet to be determined. A single nucleotide polymorphism in intron 1 has recently been reported (MacLeod *et al*, 1998; Sachse *et al*, 1999). A second point mutation , in the 5'-flanking region has also been identified in a Japanese population which affects binding of nuclear proteins (Nakajima *et al*, 1999). Both of these polymorphisms have been correlated to decreased inducibility, but not basal expression, of the gene, based on caffeine metabolite assays of CYP1A2 activity among smokers and nonsmokers. Whether the polymorphism in intron 1 is functionally significant or simply in linkage disequilibrium with the 5'-flanking region polymorphism has yet to be demonstrated. Ethnic and familial differences in *CYP1A2* expression have been postulated on the basis of phenotypic variations in the metabolism of theophylline (Miller *et al*, 1985), phenacetin (Shahidi, 1968), the heterocyclic arylamine procarcinogen 2-amino-3,8-dimethyl-3 H-imidazo[4,5-f]quinoxaline (MEIQX) (Yamazoe *et al* 1988) and caffeine (Grant *et al* 1983; Butler *et al* 1989). Caffeine metabolism is the most extensively used metabolic probe for CYP1A2 activity. A number of population studies employing caffeine metabolites provided evidence for a genetic polymorphism of CYP1A2 expression based on the determination of bi-modal or tri-modal phenotypic distributions. Fuhr and Rost (1994) found a bimodal distribution of the caffeine metabolism phenotype in both smokers and nonsmokers using plasma 1,7-dimethylxanthine/caffeine ratios. Butler *et al* (1992) found a trimodal distribution of urinary metabolite ratios in each of three geographically distinct populations. Tang *et al* (1994) proposed that confounding variables such as altered renal function could contribute to the apparent bimodal distribution of caffeine metabolite ratios seen in some studies. This argument was later supported

by Rostami-Hodjegan *et al* (1996). Other population studies with caffeine metabolites have reported uni-modal population distributions for this trait (Kalow and Tang. 1991; Vistisen *et al*, 1992). Catteau *et al* (1995b) failed to detect a significant genetic component for interindividual variations in urinary caffeine metabolite ratios in family studies. Nakajima *et al* (1994), using a probit analysis of urinary caffeine metabolites, reported a bi-modal distribution of CYP1A2 activity in both Japanese smokers and nonsmokers, and postulated the existence of genetic poor and extensive metabolizers. This group also examined *CYP1A2* genomic DNA sequences and concluded that no sequence variation could be found between their posited phenotypic groups.

Evidence for a *CYP1A2* expression polymorphism based on caffeine metabolism studies remains inconclusive. However, direct methods for quantitation of CYP1A2 expression confirm substantial interindividual variation. Ikeya *et al* (1989) and Schweikel *et al* (1993) demonstrated at least 15-fold interindividual variation in CYP1A2 mRNA from human liver samples, and a similar variation in CYP1A2 protein levels was confirmed by immunodetection methods (Schweikel *et al*, 1993).

The study of CYP1A2 expression polymorphisms in the human population is somewhat confounded by the environmental responsiveness of the expression of the gene. Since numerous environmental exposures, including foods, drugs, smoking and industrial pollutants may influence gene expression, it is extremely difficult to discriminate these factors from genetic variation in expression, especially when employing phenotypic assays which themselves are subject to confounding variables. Even the role of gender as a determinant of CYP1A2 expression is unclear. In the mouse CYP1A2 expression has not been extensively studied, except in the context of the genetic variation in AHR-mediated inducibility. The mouse does offer opportunity to

investigate CYP1A2 expression while controlling for some of the confounding variables which have plagued human studies. Such studies would depend on the availability of a suitable mouse model of genetic variation in CYP1A2 expression.

1.5: Genetic analysis in murine models of human disease

The laboratory mouse has a long history as an experimental tool in biochemistry, toxicology and genetics. Mice are a preferred model for biological research because of their size, fertility, short gestation period, physiological similarities with humans and tremendous genetic variation (Morse, 1981). Historically, mouse breeders have segregated many traits into inbred strains through selective breeding from genetically variable outbred populations (Staats, 1981). More recently, large scale mutagenesis programs coupled with rational phenotypic screening protocols have been initiated to produce new variants in an effort to close the "phenotype gap" between physiological variants of mice and analogous human diseases (Brown and Nolan, 1998). The genetics underlying a number of monogenic diseases of humans were established in mouse models (Wynshaw-Boris, 1996). The ability to control breeding schemes in mice to produce the most genetically informative crosses made the mouse a powerful tool in the application of classical and molecular genetics to the identification of pathological mutations. With the rapid development of the human genome initiative including the anticipated completion of the complete genomic DNA sequence within three years (Collins *et al*, 1998), and the availability of extensive genetic and physical mapping tools, the mouse may be supplanted as the organism of choice for the analysis of monogenic human diseases by reverse genetics. In toxicology, appropriate animal models remain essential, due to the ethical constraint against intentional exposure of human

subjects to toxic substances. While efforts to develop *in vitro* alternatives to whole animal toxicological testing continue to intensify, it is likely that cell culture models developed for this purpose will depend heavily on the extensive genetic and biochemical data derived from the source animals.

The mouse remains the model of choice for the study of complex polygenic diseases and toxicological studies of disease susceptibility resulting from complex genetic and environmental interactions (Goodfellow and Schmitt, 1994; Frankel, 1995). There are three main reasons for this. The use of an animal model allows experimental control of environmental variables. Environmental carcinogenesis in humans is usually the result of the subtle effects of a lifetime of low level exposures, coupled with lifestyle variations. It is often difficult, if not impossible, to effectively control for these important, but often unknown, variables in case-control studies in humans. Another tremendous advantage of the mouse is the ability to manipulate the genome through the development of transgenic lines. In particular, the introduction of loss of function, or knockout, transgenic mice, is proving to be a very valuable new tool in toxicology (Nebert and Duffy, 1997). A number of knockout mice have been developed which are of interest to toxicologists. Mice with loss of function in proinflammatory cytokines, haematopoietic growth factors or their receptors, tumour suppressor and proto-oncogenes have been extensively studied to elucidate the roles of these gene products in inflammatory responses and cancer susceptibility (Ryffel, 1997). Several knockout lines have been developed in which genes directly affecting xenobiotic metabolism have been deleted. These include the cytochrome P450 *Cyp2e1* (Lee *et al*, 1996), *Cyp1a2* (Pineau *et al*, 1995; Liang *et al*, 1996) and *Cyp1b1* (Buters *et al*, 1999) as well as components of cytochrome P450 induction pathways including the *Ah* receptor locus (Fernandez-

Salguero *et al*, 1995; Schmidt *et al*, 1996; Mimura *et al*, 1997), the aromatic hydrocarbon receptor nuclear translocator (*Arnt*) (Kozak *et al*, 1997; Maltepe *et al*, 1997), and the peroxisome proliferator receptor subtype, *Pparα*, which controls induction of the cytochrome P450 *CYP4A* subfamily (Lee *et al*, 1995). *Cyp1a2* knockout mice have been used to elucidate the role of this enzyme in chemically-induced porphyria (Sinclair *et al*, 1998), TCDD induction of CYP1A1 (Liang *et al*, 1997), as well as the metabolism of caffeine (Buters *et al*, 1996), acetaminophen (Zaher *et al*, 1998b; Tonge *et al*, 1998) and zoxazolamine (Liang *et al*, 1996). *Ah* (-/-) knockouts have been used to investigate AHR-mediated and AHR-independent induction of *CYP* genes, transcriptional regulation of basal CYP1A2 expression as well as the role of AHR in organ and immune system development (Fernandez-Salguero *et al*, 1995; Schmidt *et al*, 1996). *Arnt* (-/-) is an embryonic lethal knockout, suggesting a critical role for this gene product in prenatal development, but precluding any study of the effects of the loss of function in adults (Kozak *et al*, 1997; Hankinson *et al*, 1997; Maltepe *et al*, 1997). With the advent of new technologies permitting the development of conditional knockouts in which loss of function is delayed until an appropriate stage of development, it may become possible to overcome embryonic lethality in this and other cases (Nebert and Duffy, 1997). A third major advantage of the mouse in genetic analysis lies in the development of experimental and statistical methods to genetically map loci contributing to a quantitative continuous phenotypic trait. While these methods can be employed in humans (Goodfellow and Schmitt, 1994), the mouse offers tremendous advantages in terms of generating large numbers of progeny from controlled crosses in order to obtain strong statistical associations between a locus and the trait under study. This approach is particularly useful in assessing susceptibility phenotypes, which typically have a multifactorial genetic component.

Lander and Botstein (1989) proposed a method, termed "maximum likelihood interval mapping", which permitted the linkage mapping of genetic loci underlying continuous, quantitative, multigenic traits without the requirement for prior knowledge of the location or function of candidate genes. Interval mapping can be applied across the entire genome to test for the statistical likelihood of associations between a quantitatively continuous phenotype and the corresponding genotypes among intercross or backcross progeny. This method was not widely applied in murine genetics when it was first proposed due to the relative scarcity of mapped genetic markers in the mouse genome and the technical difficulty involved in genotyping large numbers of markers, most of which were of the RFLP type. Genotyping RFLP markers was a labour intensive and expensive process ill-suited to the type of high-throughput analysis required for the Lander and Botstein method. The advent of short tandem repeat (STR) microsatellite markers (Weber and May, 1989) solved the technical limitation of rapid genotyping. STR markers are widely distributed sequences containing multiple tandem repeats of di-, tri- or tetranucleotide repeats. They can be detected in sub-microgram quantities of total genomic DNA through the application of the polymerase chain reaction (PCR), a method which is well suited to high throughput analysis. STR markers are amplified using unique PCR primer sequences which flank the core repeat sequence and confer the genomic regional specificity of the marker (Fig.1.2). Microsatellite sequences tend to be highly polymorphic between inbred strains and are inherited in a co-dominant fashion, permitting informative genotyping between most mouse strains. The interval mapping approach is dependent on a relatively dense distribution of markers across the genome. An intensive effort to map STR markers in the mouse genome was completed in 1996, resulting in a dense genetic linkage map of STR markers (Dietrich *et al*, 1996). With the

completion of this map it became technically feasible to map loci contributing to complex phenotypes with continuous quantitative trait values. Traits involving susceptibilities to disease or predisposition to pathological behaviours were particularly amenable to this approach in mice. These phenotypes show incomplete penetrance and variable expressivity and thus require large sample sizes to achieve statistical significance. A number of complex trait loci were mapped using this approach, including loci contributing to autoimmune disorders (Sundvall *et al*, 1995), obesity (Taylor and Phillips, 1996), susceptibility to stroke (Rubattu *et al*, 1996) and behavioural traits such as anxiety (Wehner *et al*, 1997), and predisposition to alcohol abuse (Crabbe *et al*, 1998).

The identification of the monogenic *Ahr* genetic polymorphism led to extensive use of responder and nonresponder strains in the elucidation of the role of the *Ah* gene battery in drug metabolism and susceptibility to environmental carcinogenesis, as previously described. More recently, quantitative trait analysis methods have been employed to investigate the genetic determinants which underlie the observed strain variation in susceptibility to carcinogenesis among strains with the same *Ahr* genotype (Fijneman *et al*, 1996). This approach has permitted the identification of other loci which may contribute to human genetic variation in susceptibility.

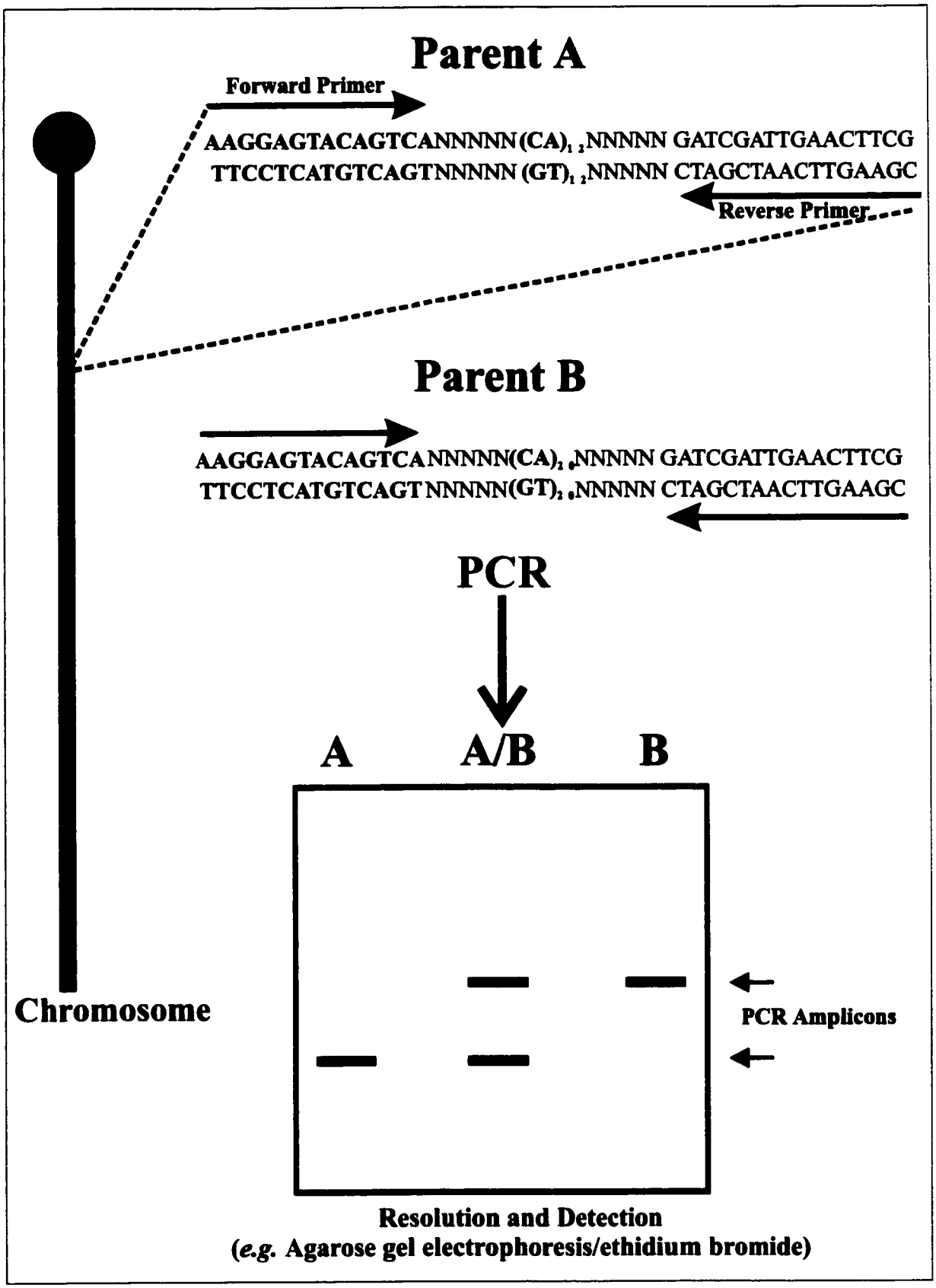
1.5: Aims of the present study

The overall aim of the study was to test the hypothesis that the genetic determinants of variation in drug metabolism among inbred mice could be identified with respect to their individual contribution to the observed variation and mapped to defined regions of the genome using an interval mapping strategy.

The aims of the work described in chapter 2 were to characterize the inbred mouse strains

Figure 1.2: The molecular basis of genotyping by short tandem repeat (STR) markers

STR markers are anchored to specific locations within chromosomes by site-specific sequences which flank the di-, tri-, or tetranucleotide repeat core sequences. In this hypothetical example, the core sequence is a (CA) tandem dinucleotide repeat. These specific sequences are used to generate primer oligonucleotides for use in a PCR amplification of the STR. Amplicons are resolved by any of a number of methods, including gel electrophoresis, and amplicons corresponding to the maternal and paternal alleles of the marker are detected and scored.


Parent A
Forward Primer
AAGGAGTACAGTCANNNNN(CA)1,2NNNNN GATCGATTGAACTTCG
TTCCTCATGTCAGTNNNNN(GT)1,2NNNNN CTAGCTAACTTGAAGC
Reverse Primer
Parent B
AAGGAGTACAGTCANNNNN(CA)2,6NNNNN GATCGATTGAACTTCG
TTCCTCATGTCAGTNNNNN(GT)2,6NNNNN CTAGCTAACTTGAAGC
PCR
A
A/B
B
Chromosome
PCR Amplicons
Resolution and Detection
(e.g. Agarose gel electrophoresis/ethidium bromide)

derived from an outbred Swiss Webster colony on the basis of differential susceptibility to acetaminophen- induced hepatotoxicity and to test the hypothesis that specific enzyme activities involved in the metabolism of acetaminophen were differentially expressed between the derived inbred strains. Acetaminophen has been extensively studied in toxicology as a model drug for cytotoxicity induced through bioactivation and subsequent covalent modification of cellular macromolecules. The purpose of this study was to define a model of susceptibility to an adverse drug reaction in the mouse based on differential expression of cytochrome P450 genes in order to provide genetic evidence for a common basis for the regulation of cytochrome P450 CYP1A subfamily. A further goal of this study was to evaluate different biochemical assays for their ability to discriminate between these inbred strains and provide a phenotyping assay for analysis of differential cytochrome P450 expression in mice.

The work described in chapter 3 was aimed at testing the hypothesis that there is a significant genetic component in the variation in caffeine 3-demethylation among inbred mouse strains. Caffeine 3-demethylation, measured as the ratio of 1,7-dimethylxanthine *versus* caffeine in serum after oral administration of caffeine, was chosen as an index of cytochrome P450 CYP1A2 expression, based on the work described in chapter 2, as well as the extensive research employing this biomarker in mice and humans. A further goal of the work described in this chapter was to validate the prediction of differential CYP1A2 expression based on this assay between one of the inbred strains described in chapter 2 and a common inbred laboratory strain, C3H/HeJ. The final aim of this work was to identify inbred strains suitable for a subsequent genetic analysis of the factors underlying differential caffeine 3-demethylation.

Chapter 4 describes a genetic analysis of differential caffeine 3-demethylation between the

inbred strains APN, described in chapter 2, and C3H/HeJ. The purpose of this work was to test the hypothesis that caffeine 3-demethylation is a polygenic trait in the mouse. This hypothesis was tested using a quantitative trait analysis of caffeine 3-demethylation employing F_2 intercross mice derived from the APN and C3H/HeJ parental strains. This approach tests the likelihood of an association between the quantitative value of the phenotypic trait under study and specific regions of the genome defined by codominant markers of fixed position. This type of analysis makes no *a priori* assumptions about the number or position of loci affecting the trait, nor their relative contribution to the phenotypic variation. One aim of this study was to identify the number of loci which affect caffeine 3-demethylation and which are polymorphic between the parental strains. A second aim of this work was to map these loci, based on their positions in the mouse genome relative to fixed markers. A third aim of this work was to quantify the relative contribution that each hypothetical locus would contribute to caffeine 3-demethylation, a phenotypic marker which is widely used to define expression of a single gene, *Cyp1a2*.

The work presented here represents the first application of the interval mapping approach to characterize polygenic quantitative genetic variation in drug metabolism. Quantitative trait loci (QTLs) affecting variations in caffeine metabolism have implications both for pharmacogenetic studies of CYP1A2 expression, and in the identification of novel determinants of xenobiotic metabolism.

Chapter 2

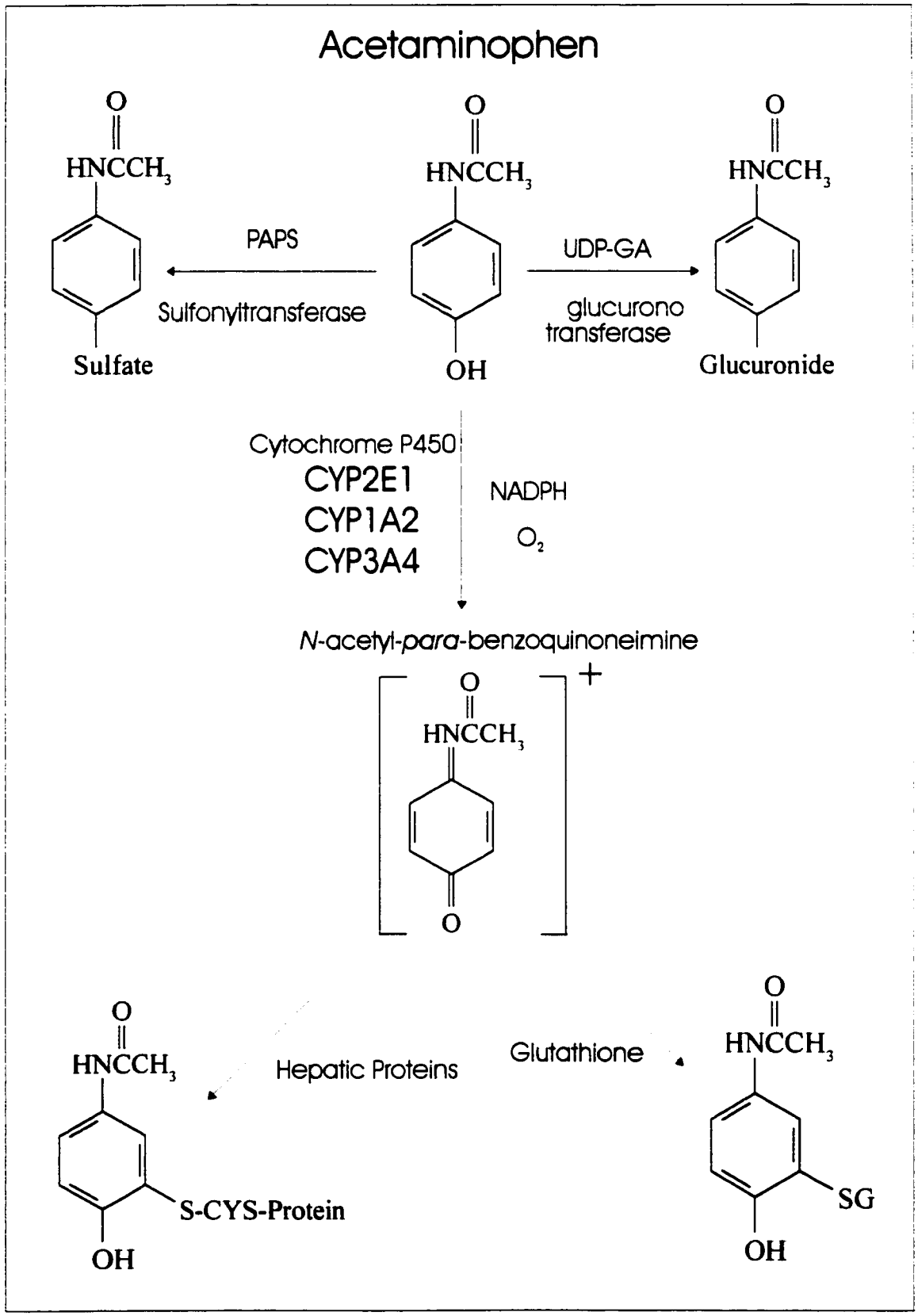
Chapter 2: Identification of cytochrome P450 variation between inbred mouse strains selected for susceptibility to acetaminophen-induced hepatotoxicity

2.1: Introduction

In the present study we have examined phenotypic variation in the hepatotoxic response to the analgesic and antipyretic drug acetaminophen in an outbred mouse population. The results and interpretations presented here were published in 1997 in the journal *Pharmacogenetics* as "Increased basal expression of hepatic *Cyp1a1* and *Cyp1a2* genes in inbred mice selected for susceptibility to acetaminophen-induced hepatotoxicity". The APN and APS inbred strains derived and characterized as described herein have been registered in the inbred mouse strains database of the Mouse Genome Database (MGD) (Blake, 1998).

Acetaminophen (*N*-acetyl-*p*-aminophenol) is a widely used analgesic and anti-pyretic drug that has been studied extensively as a test compound in modelling cytotoxicity induced by reactive electrophiles (Burcham and Harman, 1991; Bruno *et al.* 1992; Dalhoff and Poulsen, 1993; Donnelly *et al.* 1994; Gomez *et al.* 1994; Hongslo *et al.* 1994). The metabolism of acetaminophen has been well established, primarily in rat, mouse and human studies (Fig. 2.1). Species variations in sensitivity to acetaminophen and routes of metabolism have been identified, both *in vitro* and *in vivo*, with mice being a relatively sensitive species (Holme and Søderlund, 1986; Gregus *et al*, 1988). Acetaminophen is principally metabolized by conjugation to glucuronide and sulfate, through the action of the phase II microsomal UGT and cytosolic sulfonyltransferase enzymes. A small proportion of the therapeutic dose is metabolized through cytochrome P450 mediated oxygenation to the reactive electrophilic intermediate, *N*-acetyl-*para*-benzoquinone imine (NAPQI) (Dahlin *et al*, 1984). The limited amount of NAPQI that is

Figure 2.1: Acetaminophen metabolism Acetaminophen is principally detoxified by phase II conjugation reactions. A sulfate moiety is transferred to acetaminophen by sulfonyltransferase, in the presence of PAPS. Glucuronidation occurs through the action of UGTs in the presence of uridine diphosphate-glucuronic acid. A small proportion of the therapeutic dose is metabolized by the cytochrome P450 isoforms CYP2E1, CYP1A2 and CYP3A (CYP3A4 in humans) to a reactive electrophilic intermediate *N*-acetyl-*para*-benzoquinone imine (NAPQI). This reactive metabolite is detoxified by spontaneous reaction with GSH. In the case of overdose, or where glutathione levels are depleted, NAPQI can react with protein sulfhydryls, causing potentially cytotoxic covalent acetaminophen-protein adducts (from: Hinson *et al*, 1995).


Acetaminophen
HNCCH3
PAPS
Sulfonyltransferase
Sulfate
OH
UDP-GA
glucurono transferase
Glucuronide
Cytochrome P450
CYP2E1
CYP1A2
CYP3A4
NADPH
O2
N-acetyl-para-benzoquinoneimine
Hepatic Proteins
S-CYS-Protein
Glutathione
SG

generated at the therapeutic dose is normally detoxified by nonenzymatic reaction with glutathione, to form an acetaminophen-glutathione conjugate (Powis *et al*, 1984; Shen *et al*, 1992). Acetaminophen hepatotoxicity is postulated to arise when excess NAPQI is generated after saturation of the phase II detoxification systems. In humans, this occurs in cases of overdose, or where the glutathione status of an individual has been compromised (Spielberg, 1985; Birge *et al*, 1990). NAPQI is presumed to induce cytotoxicity through the formation of acetaminophen-protein adducts, primarily at cysteinyl residues (Matthews *et al*, 1996). Acetaminophen hepatotoxicity is presumed to depend on the formation of adducts with specific cellular proteins, since the patterns and extent of protein adduct formation arising from methylated isomers of acetaminophen do not result in similar levels of hepatotoxicity (Birge *et al*, 1989). Using anti-acetaminophen/protein thiol antiserum, a number of groups identified 58kDa and 44kDa hepatic proteins as the major targets of acetaminophen conjugation (Bartalone *et al*, 1989; Birge *et al*, 1991). Hoivik *et al* (1996) used an anti-p58 antibody to establish an inverse correlation between p58 content and non-p58 protein modification in mice, supporting the hypothesis of p58 as the preferred target of NAPQI in the hepatocyte.

A number of studies have indicated that inhibition of mitochondrial respiration is an important component of acetaminophen-induced cytotoxicity, suggesting that enzyme proteins of the respiratory chain may be important targets of NAPQI (Burcham and Harman, 1990,1991; Donelly *et al*, 1994). Changes in hepatocellular protein phosphorylation patterns have also been observed after acetaminophen intoxication, and a protein phosphatase has been suggested as a possible target (Bruno *et al*, 1998). Acetaminophen has also demonstrated genotoxic effects in rodents, arising both from covalent binding of NAPQI to DNA as well as through inhibition of DNA repair systems (Shen *et al*, 1992; Hongslo *et al*, 1994). More recently, more than twenty

acetaminophen-modified proteins in mouse liver were identified using a combination of two-dimensional gel electrophoresis and mass spectrometry to identify labelled proteins after administration of radiolabelled acetaminophen (Qiu *et al*, 1998). The role of each of these target proteins, if any, in mediating the hepatotoxic effects of the drug have yet to be elucidated.

The role of cytochrome P450 monooxygenases in acetaminophen hepatotoxicity was identified by Potter *et al* (1973). Subsequent studies suggested that specific subsets of the P450 family of enzymes were involved. Steele *et al* (1983) showed that microsomes from β-naphthoflavone, but not phenobarbital pretreated rats showed increased activation of acetaminophen. Morgan *et al* (1983) compared the activity of six purified P450 isoforms from rabbit with respect to acetaminophen activation, while Harvison *et al* (1988) established the specific rat isoforms. Raucy *et al* (1989) identified CYP2E1 and CYP1A2 as the major isoforms contributing to the activation of acetaminophen in human liver microsomes *in vitro*. The involvement of human CYP3A4 was subsequently confirmed using purified CYP3A4 *in vitro* (Thummel *et al*, 1993). These observations were confirmed in studies using transgenic lymphoblastoid cells expressing single cytochrome P450 isoforms (Patten *et al*, 1993; Snawder *et al*, 1994). CYP1A2, CYP2E1, and CYP3A4 collectively accounted for greater than 90% of the detectable bio-transformation of acetaminophen in humans, based on *in vitro* immunoinhibition studies (Patten *et al*, 1993). The detailed understanding of acetaminophen metabolism makes it an excellent test compound for dissecting the genetic basis of aberrations in these metabolic pathways.

In the present study, basal cytochrome P450 gene expression has been studied in inbred mice selected for differences in susceptibility to acetaminophen-induced hepatotoxicity. Gene expression was characterized at the levels of enzyme activity, enzyme protein abundance and

steady state mRNA levels. We have also examined the co-segregation of pharmacogenetic parameters with the selection criterion of elevated serum alanine aminotransferase (ALT) activity after oral dosing with acetaminophen.

2.2: Materials and methods

2.2.1: Animals

Outbred Swiss-Webster mice were originally obtained from Connaught Laboratories (Toronto, ON) in 1968 and maintained as a closed penbred colony at the animal care facility of the Health Protection Branch of Health Canada (Ottawa, ON). These animals were designated HPB-white. Animals were fed irradiated Prolab 3000 rodent feed (Agway Inc. Portsmouth, RI, USA) and sterile water *ad libitum*, and housed on sterile Sanichip maple chip bedding (P.W.I. Industries, St. Hyacinthe, PQ). All animals were housed and cared for in accordance with Canadian Council on Animal Care guidelines.

2.2.2: Standards and reagents

Caffeine, 8-chlorotheophylline and chlorzoxazone were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 6-hydroxychlorzoxazone was from Salford Ultrafine Chemicals and Research Ltd. (Manchester, England). Acetanilide was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Resorufin, methoxyresorufin and ethoxyresorufin were purchased from Pierce (Rockford IL, USA). N-acetylcysteine (NAC)-acetaminophen metabolite conjugate was a generous gift of Dr. J. Sinclair, University of Vermont VA Medical Centre (Burlington VT, USA). All other chemicals were reagent grade or higher quality.

Standards for the high performance liquid chromatography (HPLC) quantitation of CYP1A1 and β-ACTIN PCR products were prepared by agarose gel purification of

authenticated PCR products using a silica gel binding system (QIAquick, QIAGEN, Chatsworth, CA, USA).

2.2.3: Acetaminophen phenotyping

Animals, deprived of food overnight, were dosed by oral gavage with a suspension of acetaminophen (15 mg per ml of 0.25% gum tragacanth, 150 mg/kg body weight). Blood was collected from the orbital sinus 24 hours after dosing using a heparinized glass capillary and centrifuged to separate serum. Serum ALT levels were determined on 1:5 diluted samples using the a-gent SGPT diagnostic kit and a VP Bichromatic Analyzer (Abbott Laboratories, Diagnostics Division, Irving, TX, USA) according to the manufacturer's instructions.

2.2.4: Selection and breeding of animals

Animals were selected for breeding from the outbred colony according to serum ALT levels after acetaminophen dosing as described above. Males with elevated ALT (> 1000U/L) were initially bred to siblings to generate an inbred colony. Subsequently, sensitive males were intermittently bred to cousins from the inbred colony to overcome inbreeding suppression before resuming sibling breeding. Males with the greatest ALT elevation after acetaminophen treatment from each generation were chosen for breeding. Females were chosen for breeding from families in which the male siblings showed the greatest ALT response, since females do not manifest the elevated ALT response. The acetaminophen-sensitive substrain was designated APS. An inbred colony of low responders was simultaneously established using the same breeding strategy. This substrain was designated APN (nonsusceptible). The mice which were chosen for microsomal activity assays, RNA and immunoassays were littermates of animals which had phenotyped as susceptible or nonsusceptible using the ALT assay. Naive (non-phenotyped) animals between the 10th and 17th generation of inbreeding were used in order to avoid confounding effects which

might otherwise arise from differential hepatotoxicity.

2.2.5: Microsomal assays

Animals were sacrificed by cervical dislocation followed by decapitation. Microsomes were prepared from freshly excised livers as previously described (Whitehouse and Paul, 1987) and stored at -80°C as pellets under a 50mM KCl pH 7.4 overlay until used. Protein concentrations were determined by the Biuret method using the Abbot Diagnostics total protein reagent with bovine serum albumin as the standard (Abbott Laboratories, Diagnostics Division, Irving, TX, USA). All HPLC analyses were carried out using a Beckman System Gold instrument (Beckman Instruments, Fullerton, CA, USA). Acetanilide 4-hydroxylase activity was determined according to the method of Liu *et al* (1991) by quantitation of 4-hydroxyacetanilide in the ethylacetate extract fraction after timed incubation of the microsomal suspension with acetanilide and NADPH. 3-hydroxyacetanilide (Aldrich Chemical Co., Milwaukee, WI, USA) was used as an internal standard. Chlorzoxazone 6-hydroxylation activity was measured as described by Peter *et al* (1990), using estriol (Searle Chemical inc. Chicago, IL, USA) as an internal standard. Testosterone 6-β-hydroxylation was determined as previously described (Whitehouse *et al.*, 1990). Conjugation of acetaminophen metabolite to NAC was determined by incubating microsomes for 30 minutes in a shaking water bath at 37°C with 0.5 mM acetaminophen, 0.9mM NADPH, 3mM $MgCl_2$ and 1mM NAC in 50mM KPO_4 buffer pH 7.4. The reaction was stopped by addition of 1.5 volumes of methanol. Products were stored at -20°C, then centrifuged to remove the alcohol-insoluble fraction. Supernatants were dried in a vacuum centrifuge (Oligoprep, Savant Instruments Inc., Farmingdale, N.Y. USA) without heating. Pellets were redissolved in 0.4 mL H_2O and 50 µL was injected for HPLC, using a 25cm, 5µm Spherosorb ODS2 column (Chromatography Sciences Co., St-Laurent, PQ) and a solvent system of 12%

acetonitrile in 50mM sodium phosphate, pH 2.2 at 1 mL/ minute. Eluate was monitored at 254nm.

Ethoxyresorufin O-deethylase (EROD) and methoxyresorufin O-demethylase (MROD) activities were determined according to Burke and Mayer (1983), using an Aminco DW-2a spectrofluorometer (Silver Springs MD, USA) set at λ_{ex} 510nm and λ_{em} 580nm to measure resorufin production. Substrates were dissolved in DMSO and incubated at room temperature with microsomes in suspension with NADPH in 50 mM KPO_4 pH 7.4 buffer.

2.2.6: Caffeine phenotyping

Animals with free access to food and water were dosed by oral gavage with an aqueous solution of caffeine (1mg/mL, 10 mg caffeine/kg body weight). Two hours later (± 5 min) approximately 0.2 mL of blood was collected from the orbital sinus under isoflurane-induced anaesthesia using a heparinized Microhematocrit Capillary Tube (Fisher Scientific, Pittsburg, PA. USA). The blood was centrifuged at 7420 x g for 10 min and 0.1 mL of serum was removed into a microtube to which 10 µL of 8-chlorotheophylline (100 µg/ml) internal standard was added. 15 µL of 20% trichloroacetic acid was added and the samples were cooled for 30 minutes at 4°C before a 10 minute centrifugation at 13,200 x g. 50 µL of deproteinized plasma diluted 1:1 with water was chromatographed on a 15-cm Inertsil 5μm, ODS2 column (Chromatography Sciences Co., St-Laurent, PQ) with the eluate monitored at 274 nm. A flow of 1 mL/min of 0.1 M Na_2PO_4(0.1% TEA pH 2.5 with H_3PO_4) : methanol : acetonitrile (78:14:7) was maintained. The area ratio of 1,7 dimethylxanthine/caffeine was calculated.

2.2.7: RNA isolation

Livers were excised immediately after sacrifice and approximately 100 mg slices were removed and quick frozen at -80°C. RNA was extracted from these tissue slices using the acid

guanidinium thiocyanate phenol chloroform method (Chomczynski and Sacchi,1987). Yield and purity were determined spectrophotometrically. Samples were stored in 0.2% sodium dodecylsulfate (SDS), 1mM EDTA at -80°C until used.

2.2.8: cDNA probes

A 400 base pair partial-length cDNA probe for murine CYP1A2 was prepared by amplifying a region of the mRNA from nucleotide 855 to nucleotide 1254 (Kimura *et al*, 1984a) by RT-PCR. The PCR product was cloned into the pCRII vector using the TA cloning system (Invitrogen, San Diego, CA, USA). The murine alpha-fetoprotein (serum albumin) cDNA probe was prepared by RT-PCR amplification of a 704 base pair region from nucleotide 28 to nucleotide 731 (Minghetti *et al*, 1985) and cloned as described above. Plasmid inserts from stock cultures were sequenced to confirm the identity of the clones. High specific activity probes were generated from gel purified inserts by labelling with α^{32}P-dCTP using a random priming system (Megaprime, Amersham Canada, Oakville, ON). Specificities of the probes were confirmed by unique bands generated after hybridization to northern blots containing 20 µg of total liver RNA from untreated and 3-MC-induced mice.

2.2..9: RNA slot blots

5 µg total liver RNA from each animal was denatured in 50% formamide, 7% formaldehyde at 65°C for 10 minutes, then transferred to a nylon membrane (HybondN, Amersham Canada, Oakville, ON) using a 48 well vacuum filtration manifold (Life Technologies, Burlington, ON) with 10X saline sodium citrate (SSC) as the transfer buffer. Probes were hybridized at 68°C using a rapid hybridization buffer (Quickhyb, Stratagene, La Jolla, CA, USA) containing 100 µg/mL sonicated herring sperm DNA (Boehringer Mannheim Canada, Laval, PQ). Blots were washed to a final stringency of 0.1 X SSC, 0.2% SDS at 62°C for 30 minutes, then

exposed to X-Ray film (XOMAT-AR, Kodak, New Haven, CT, USA) at -80°C for different exposure times. After appropriate exposures were made, the blots were stripped by boiling for 5 minutes in 0.1% SDS, exposed to confirm stripping, then rehybridized with the serum albumin cDNA probe using the same hybridization and washing conditions. 5µg of total liver RNA from rainbow trout and 3-MC-induced mouse were included as negative and positive hybridization controls, respectively. X-ray films were scanned using a laser densitometer (Molecular Dynamics, Sunnyvale, CA, USA), and autoradiographic signals were quantified using the scanner's image analysis software (ImagequantNT v 4.2). Multiple exposures were used to ensure that data used in the analysis were within the linear response range of the film.

2.2.10: Semi-quantitative RT-PCR

CYP1A1 and β-ACTIN target sequences were amplified from total liver RNA using Tth polymerase (Boehringer Mannheim Canada, Laval, PQ) for both the reverse transcription and PCR steps. Primers were selected to ensure unique priming of CYP1A1 by excluding regions of homology with CYP1A2 mRNA. In addition, primers were designed to span intron-exon boundaries in order to ensure that the predicted amplicon was exclusively the product of mRNA amplification. Primer sequences were : CYP1A1 upstream : 5'-GCCTCATGTACCTGGTAACCAAC-3', nucleotides 1099-1121, downstream: 5'-CCAATGGTCTCTCCGATGCACTT-3', nucleotides 1501-1479 (Kimura *et al*, 1987) and β-ACTIN upstream: 5'-GTGGGCCGCTCTAGGCACCA-3', nucleotides 25-44, downstream: 5'-CGGTTGGCCTTAGGGTTCAGGGGGG-3', nucleotides 269-245 (Alonso *et al*, 1986). Synthetic primers were obtained from Life Technologies (Burlington, ON). Reverse transcription was carried out in a 20µL reaction volume according to manufacturer's recommended conditions using 750nM of target-specific downstream primer, and varying amounts of template RNA. The

reaction was carried out at 70°C for 5 minutes followed by 25 minutes at 65°C. After incubation, samples were cooled to room temperature and immediately processed for PCR amplification. The PCR was set up by adding 30 μL of a master mix to the reverse transcription mix to produce final concentrations of 150nM of target-specific primers in a 50 μL reaction volume. Cycling conditions were: an initial denaturation of 1 minute at 94°C, followed by repeated cycles of 10 seconds at 94°C, 30 seconds at 65°C and 45 seconds at 72°C. The actual cycle number varied according to the experiment. All incubations were carried out in an MJ Research PTC 200 thermocycler (Watertown, MA, USA) using the calculated mode of temperature control. CYP1A1 and β-ACTIN targets were amplified in separate reaction vessels using RNA obtained from the same dilution series. Each reaction was performed in triplicate. Amplicons were sized against DNA size standards (100 base pair ladder, Life Technologies, Burlington, ON) by agarose gel electrophoresis. Unique amplicons of the predicted size of 403 base pairs (CYP1A1) and 245 base pairs (β-ACTIN) were obtained for each reaction. The identity of each amplicon was confirmed by specific hybridization with oligonucleotide probes complimentary to predicted internal sequences of the amplicons.

In order to establish reaction kinetics for each primer pair, triplicate tubes prepared from a common reaction mix were removed over increasing cycle numbers. Once the exponential phase of the reaction had been established with respect to cycle number for each primer pair, triplicate tubes were prepared with varying amounts of total RNA to establish the dynamic range of the assay at that cycle number.

2.2.11: Quantitation of PCR products by HPLC

Products from PCR experiments were analyzed by a HPLC system consisting of a 600E Multisolvent Delivery System coupled to a 717+ Autosampler (Waters Chromatography, Milford,

MA, USA) and a Spectroflow 783 Programmable Absorbance Detector (ABI Analytical, Kratos Division). Separation conditions were adapted from those reported by Zeillinger *et al* (1993). Modifications included optimization of the gradient profile, column temperature and pH. Separations were carried out on a Progel TSK DEAE-NPR 35 mm x 4.6 mm i.d. analytical column fitted with a guard column of the same support, 5 mm x 4.6 mm id (Supelco, Mississauga, ON). The analytical column was maintained at a constant temperature of 30°C. Mobile phase A was 25 mM Tris-HCl, pH 8.0, and mobile phase B was 25 mM Tris-HCl, pH 8.0, 1 M sodium chloride. Separations were effected using a linear stepwise gradient profile: 30%-55% B in 1 minute, 55%-65% B in 9 minutes, 65%-100% B in 0.5 minute, 100% B for 0.5 minute, 100%-30% B in 0.5 minute and 30% B for 8.5 minutes. The total run time was 20 minutes. The flow rate was 1 mL/minute and the absorbance was monitored at 260 nm. Samples were kept at 5°C in the autosampler. 15 µL of crude PCR products from CYP1A1 and β-ACTIN reactions were mixed and further diluted 1:1 with water. Samples were analyzed without prior purification. Products not analyzed within 12 hours were kept at -20°C. Duplicate injections (15 µL) were performed. The relative expression level of CYP1A1 mRNA for each sample was determined by calculating the ratio of the mean of triplicate CYP1A1 PCR products to that of the β-ACTIN products obtained within the exponential phase of the reaction and linear RNA input range.

2.2.12: Western blotting and immunodetection

Aliquots of equivalent protein content from liver microsomes of each of 12 males from the APN and APS groups were pooled and different amounts of total protein from each pool were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) according to Laemmli (1970), then transferred to nitrocellulose membranes (Biorad, Missisauga, ON). Blots were incubated for 2 hours with a rabbit polyclonal anti-rat CYP1A1/2 antibody (Human Biologics, Phoenix, AZ) or

rabbit polyclonal anti-rat CYP2E1 (Amersham Life Science, Oakville, ON). Antibody binding was detected by chemiluminescence using a biotinylated anti-rabbit IgG followed by a streptavidin-horseradish peroxidase conjugate (ECL, Amersham Life Science, Oakville, ON). After development, blots were exposed to Hyperfilm ECL (Amersham Life Science, Oakville, ON).

2.2.13: Statistical analyses

Groups were compared using the Mann-Whitney rank sum analysis to test for significant differences. Rank sum analyses were used due to the unequal variances seen in most groups. Correlation coefficients were calculated using the Spearman rank order analysis. Comparative analyses, correlations, means and standard deviations of the means were calculated using the SigmaStat software system (Jandel Scientific, San Rafeal, CA, USA).

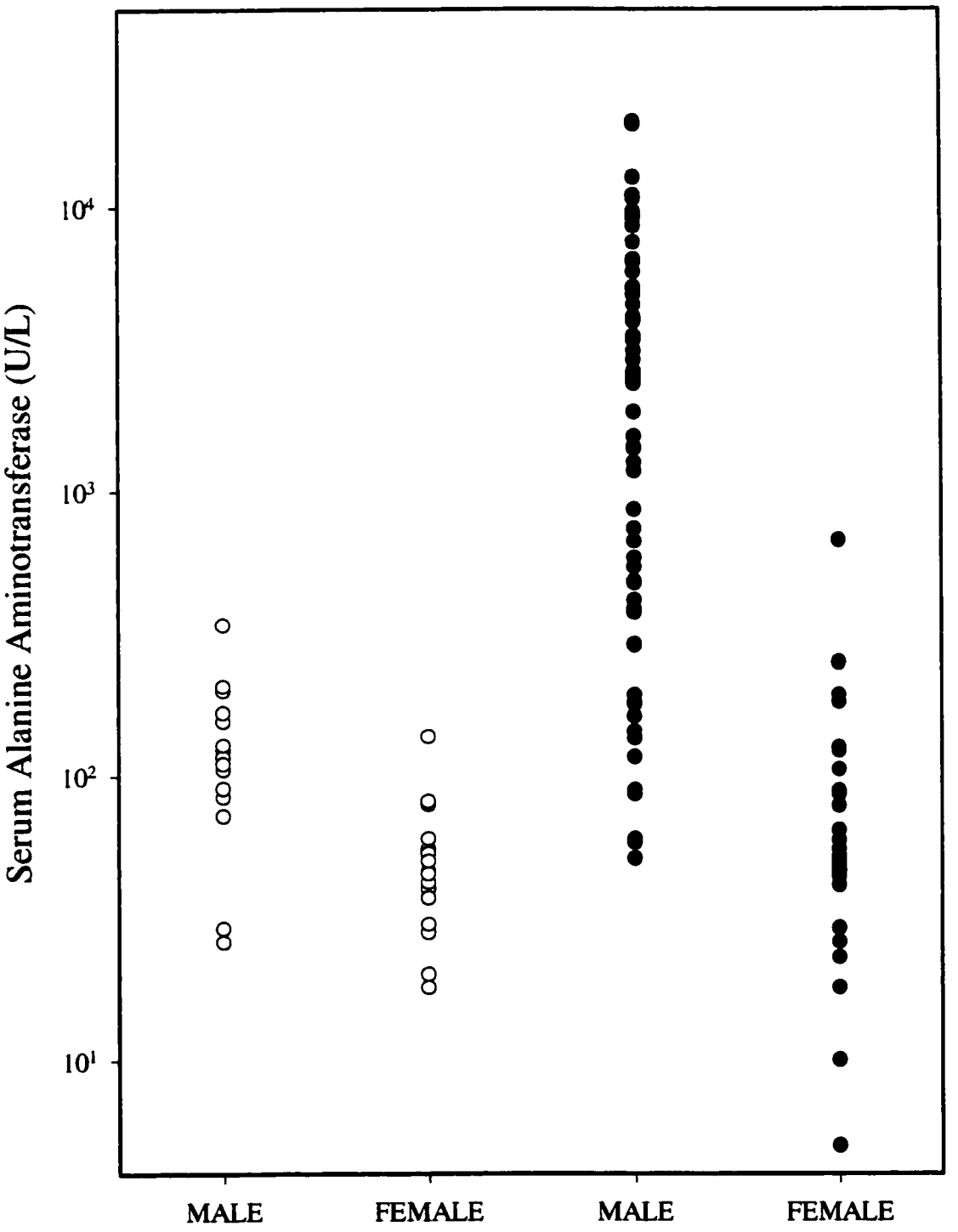
2.3:Results

2.3.1: Susceptibility to acetaminophen-induced hepatotoxicity

Testing of animals from the 6th to the 15th inbred generation showed a segregation of susceptibility to acetaminophen-induced hepatotoxicity between the two groups. The tenth generation of inbreeding showed a typical distribution of serum ALT response to acetaminophen exposure between the two groups (Fig. 2.2). Males from the nonsusceptible colony (APN) showed only rare sporadic elevations of serum ALT after acetaminophen treatment. Using the cut-off value of 1000 U/L serum ALT to define a susceptible response, 5 male mice of 248 tested (2.0%) in the APN group were susceptible over 10 generations of inbreeding. By contrast, 166 of 395 (42.0%) males from the susceptible (APS) colony were positive over the same period. No females from either the APN or APS colony were positive over 4 generations of testing (0/90 and 0/113, respectively). The nonsusceptible phenotype persisted in the susceptible colony from the

Figure 2.2: Serum alanine aminotransferase (ALT) levels after acetaminophen dosing

Serum ALT levels determined 24 hours after oral dosing with 150mg/kg acetaminophen in mice selected for nonsusceptibility (APN, ○) or susceptibility (APS, ●) to acetaminophen-induced hepatotoxicity. Results are from the tenth inbred generation. The APN male group is significantly different from APN females and APS males ($p<0.0001$). APN female and APS female groups do not differ significantly from one another ($p=0.203$) using a Mann Whitney rank sum test. APN: males n=16, females n=17, APS males n=66, females n=31.


Serum Alanine Aminotransferase (U/L)
10⁴
10³
10²
10¹
MALE
FEMALE
MALE
FEMALE

10th to the 17th generation at a frequency of approximately 60% in males.

2.3.2: Segregation of liver microsomal activities

Male mice from the APN and APS colonies were compared for several liver microsomal enzyme activities involved in the bioactivation of acetaminophen (Fig. 2.3). Microsomal preparations were assayed for cytochrome P450-associated activities including acetanilide 4-hydroxylation, testosterone 6-β-hydroxylation, chlorzoxazone 6-hydroxylation (Fig. 2.3A), and EROD and MROD (Fig. 2.3B). Of these, only assays associated with CYP1A-mediated activities (acetanilide 4-hydroxylation, EROD, MROD) showed significant differences between the two groups ($p < 0.0001$, $p < 0.0001$ and $p < 0.01$, respectively), with the APS mice having increased levels relative to APN animals in each case. Microsomal suspensions were also assayed for their capacity to conjugate acetaminophen with NAC, as a non-isoform-specific measure of the bioactivation of acetaminophen by cytochrome P450. Hepatic microsomes from the APS group showed a significantly ($p < 0.01$) greater capacity than those from the APN group (Fig. 2.3B).

2.3.3: Comparison of CYP1A2 and CYP1A1 mRNA expression

CYP1A2 mRNA levels were compared in 20 animals each from the APN and APS groups. Semi-quantitative data for CYP1A2 expression were obtained by laser densitometric scans of slot blot autoradiograms (Fig. 2.4A). CYP1A2 mRNA levels were expressed as the ratio of CYP1A2 over albumin signal intensities. CYP1A2 mRNA levels were determined to be significantly increased ($p < 0.0001$) in APS *versus* APN males (Fig. 2.4C). No significant difference was observed in the albumin band intensities between the APN and APS groups.

RT-PCR was chosen to analyse *Cyp1a1* gene expression levels due to its ability to quantitatively detect extremely low levels of gene expression, typical of basal expression of CYP1A1 in the liver. After 34 cycles of amplification the CYP1A1 assay produced a linear

Figure 2.3: Liver microsomal enzyme activities

Liver microsomal activities in mice from the acetaminophen nonsusceptible (APN, □), or susceptible (APS, ■) inbred colonies.

A: acetanilide 4-hydroxylation (n=12 for each group), testosterone 6β-hydroxylation (n=6) and chlorzoxazone 6-hydroxylation (n=12) activities.

B: Methoxyresorufin *O*-demethylase (MROD), (n=12), Ethoxyresorufin *O*-deethylase (EROD), (n=12) and NAC-acetaminophen (NAC-APAP) conjugation (right axis) (n=12) activities. Bars are mean values. Error bars indicate standard deviations, * : $p<0.0001$, ** : $p<0.01$, N.S: not significantly different using a Mann Whitney rank sum test.

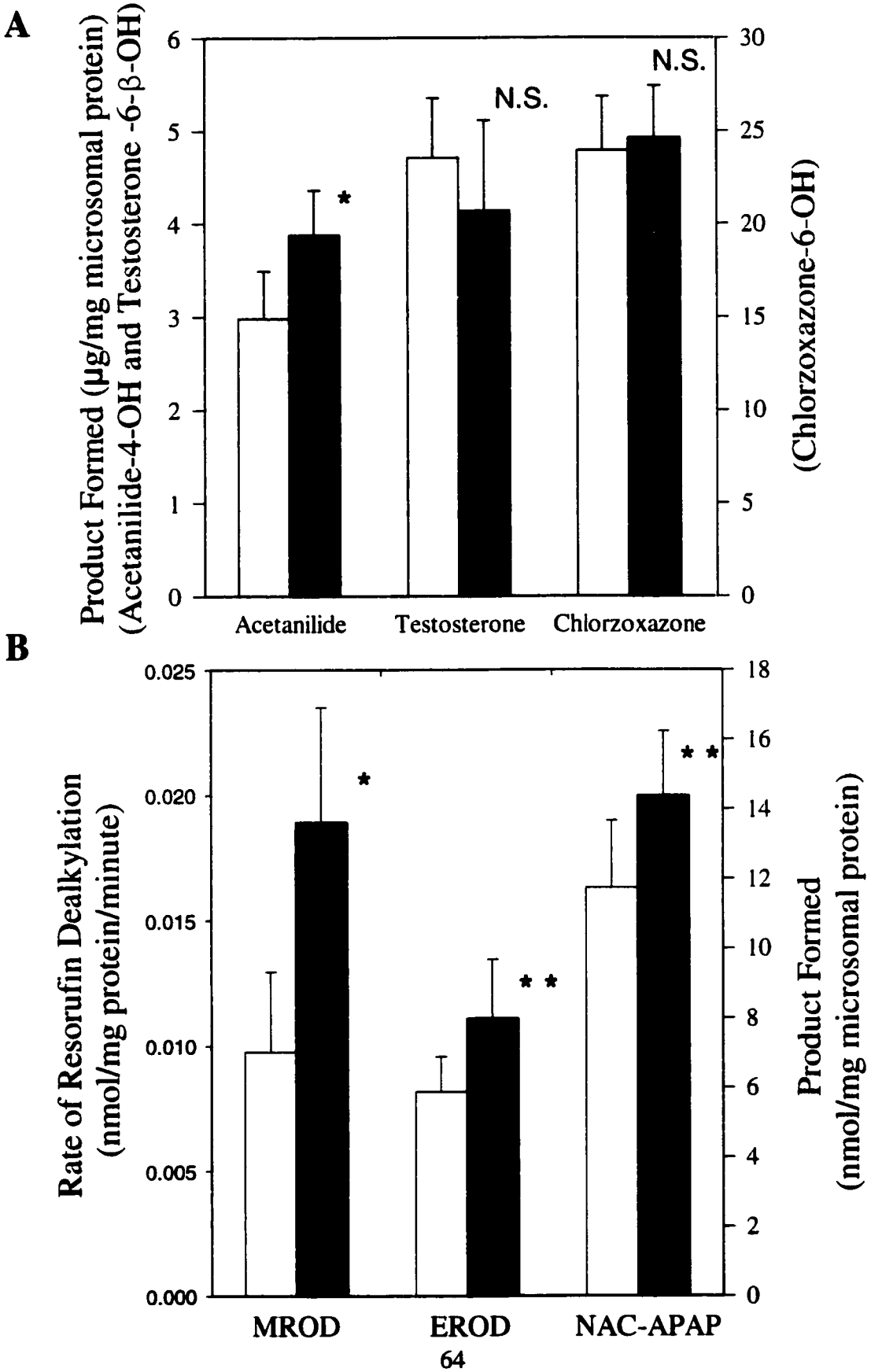
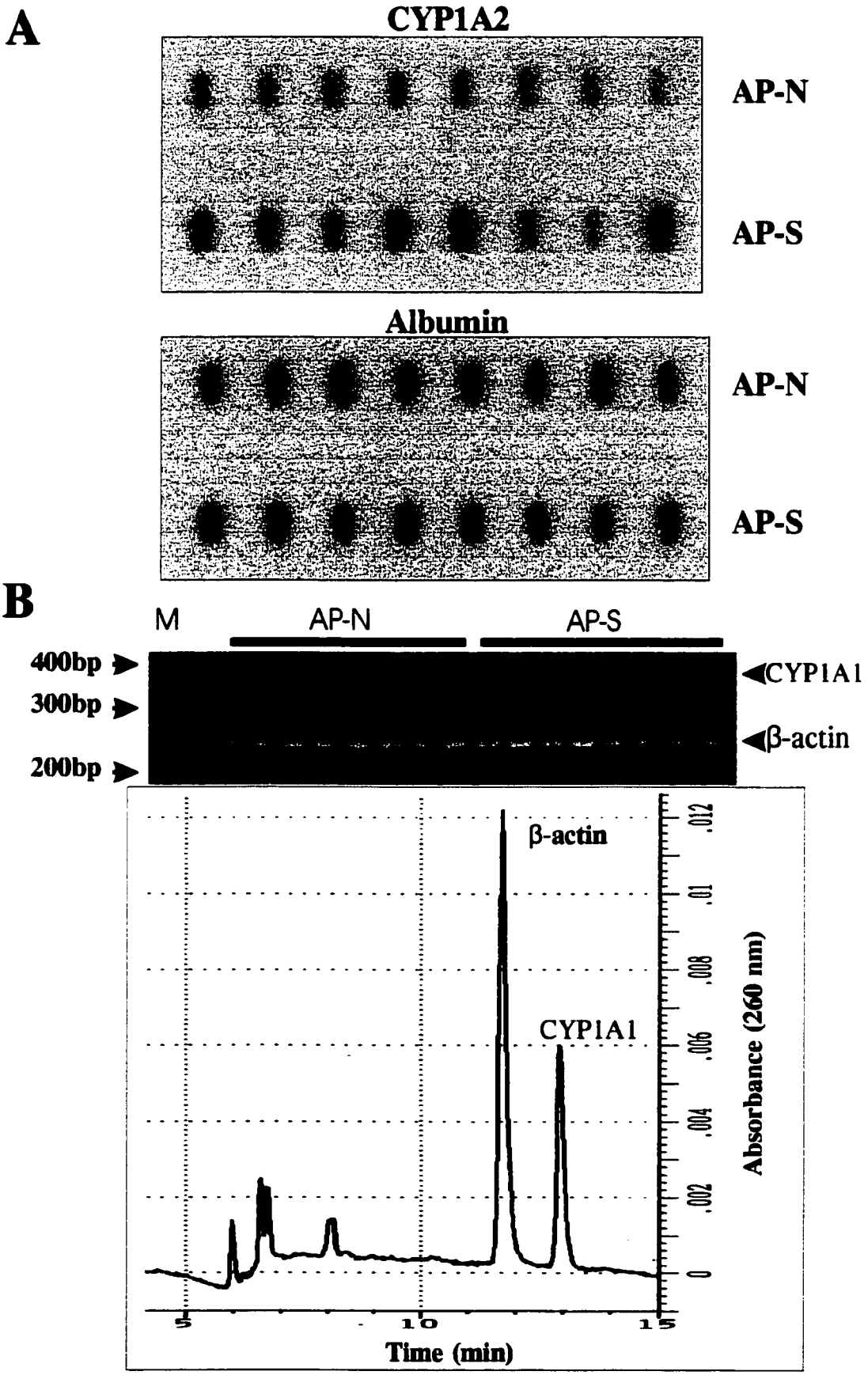
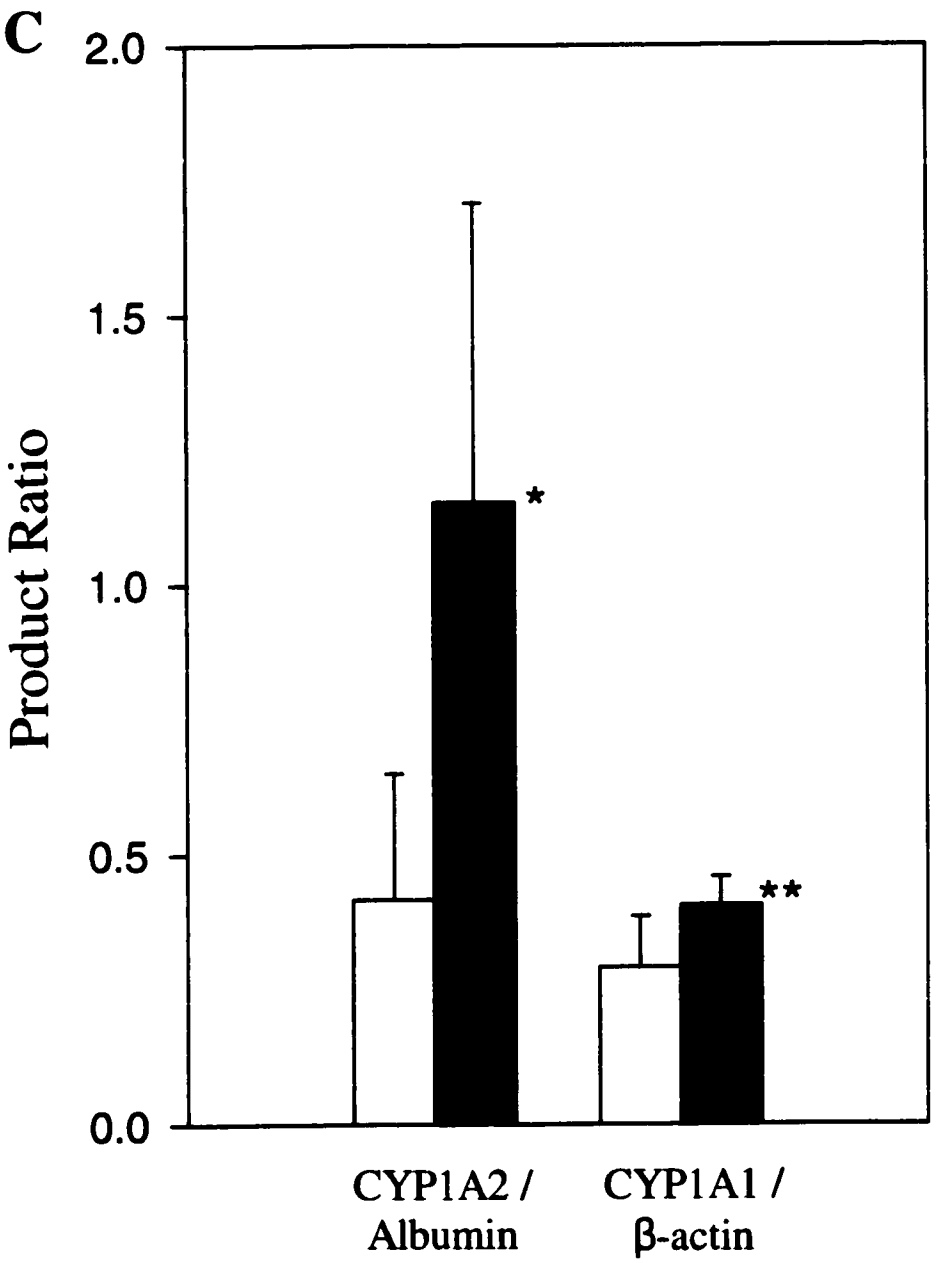

A
Product Formed (µg/mg microsomal protein)
(Acetanilide-4-OH and Testosterone -6-β-OH)
(Chlorzoxazone-6-OH)
N.S.
N.S.
*
Acetanilide
Testosterone
Chlorzoxazone
B
Rate of Resorufin Dealkylation
(nmol/mg protein/minute)
Product Formed
(nmol/mg microsomal protein)
*
* *
* *
MROD
EROD
NAC-APAP

Figure 2.4: CYP1A1 and CYP1A2 relative mRNA expression levels in APN and APS mice

A: Slot blots of total liver RNA (5μg/slot) from mice selected for nonsusceptibility (APN) or susceptibility (APS) to acetaminophen-induced hepatotoxicity. Blots were hybridized with a CYP1A2-specific cDNA probe, washed at high stringency, exposed to X-ray film then stripped and re-hybridized with an albumin-specific cDNA probe. Exposures were for 40 hours (CYP1A2) and 4 hours (Albumin). These autoradiographs represent 8 of the 20 animals from each group that were analysed on each blot. **B:** Agarose gel electrophoresis and HPLC separation of CYP1A1 and β-ACTIN-specific RT-PCR products from APN and APS mice. 5μL each of CYP1A1 and β-ACTIN PCR products were pooled, electrophoresed in a 2.5% agarose, 1X TBE gel, and visualized by ethidium bromide staining and UV fluorescence. DNA size standards (M) confirmed the predicted product sizes of 404 base pairs (CYP1A1) and 245 base pairs (β-ACTIN). 15μL of each PCR were pooled and resolved by HPLC for quantitation of specific products by UV absorbance at 260nm. **C:** Mean expression levels for CYP1A2 normalized to albumin by densitometric scanning of slot blot autoradiographs (n=20 for both groups) and CYP1A1 normalized to β-ACTIN by HPLC quantitation of RT-PCR products (APN: n=6, APS: n=8). □ APN, ■ APS. *: $p<0.0001$, **:$p<0.05$. Error bars represent standard deviations on the means.


A
CYP1A2
AP-N
AP-S
Albumin
AP-N
AP-S
B
M
AP-N
AP-S
400bp
300bp
200bp
CYP1A1
β-actin
β-actin
CYP1A1
Absorbance (260 nm)
0
.002
.004
.006
.008
.01
.012
5
10
15
Time (min)


C
2.0
1.5
1.0
0.5
0.0
Product Ratio
*
**
CYP1A2 / Albumin
CYP1A1 / β-actin

response from 200 to 500 ng of starting total liver RNA, whereas the β-ACTIN assay was linear from 20-100 ng of input RNA. Based on these data, CYP1A1 and β-ACTIN were assayed at 350 ng and 30 ng of input RNA, respectively. These conditions resulted in the production of discrete products when visualized by ethidium bromide staining after agarose gel electrophoresis (Fig. 2.4B). PCR products were accurately quantified by HPLC separation and UV absorbance detection, which provided excellent separation of the β-ACTIN and CYP1A1 products (Fig.2.4B). CYP1A1 mRNA levels were expressed as the ratio of CYP1A1-specific over β-ACTIN-specific PCR products. CYP1A1 mRNA levels were determined to be significantly elevated ($p< 0.05$) in males from the APS group (Fig. 2.4C). No significant difference was observed in β-ACTIN-specific PCR product levels between the two groups. The correlation between CYP1A1 and CYP1A2 mRNA expression levels was determined by pooling data obtained by RT-PCR and slot blot analyses from both APN and APS males. A significant positive correlation ($r= 0.644$, $p<0.02$) was observed between the expression of these two genes (Fig. 2.5).

2.3.4: Immunoassay of CYP1A microsomal protein content

Differences could be observed in the pooled microsomes from 12 APN or APS males. Increased CYP1A1 and CYP1A2, but not CYP2E1 signal intensities could be discerned on lumographs of these blots (Fig.2.6). Densitometric scanning of the lumographs indicated that the levels of CYP1A1 and CYP1A2 protein were approximately 5.5-fold, and 3-fold greater, respectively, in the APS *versus* the APN microsomal pools.

2.3.5: Caffeine metabolism

Caffeine 3-demethylation to 1,7-dimethylxanthine (paraxanthine) was measured by comparing the ratio of the serum levels of the metabolite to the parent compound in both males

Figure 2.5: Correlation of CYP1A2 with CYP1A1 relative mRNA expression

CYP1A2 expression was determined relative to albumin by densitometric scans of autoradiographs of slot blots of total RNA probed with specific cDNAs. CYP1A1 expression was determined relative to β-ACTIN by HPLC quantitation of specific products from RT-PCR amplification of total liver RNA. Both parameters were determined for each male from both acetaminophen nonsusceptible (APN, ○) (n=6) and sensitive (APS, ●) (n=8) groups. There is a significant positive correlation between these two parameters ($r=0.644$, $p<0.02$, $n=14$).

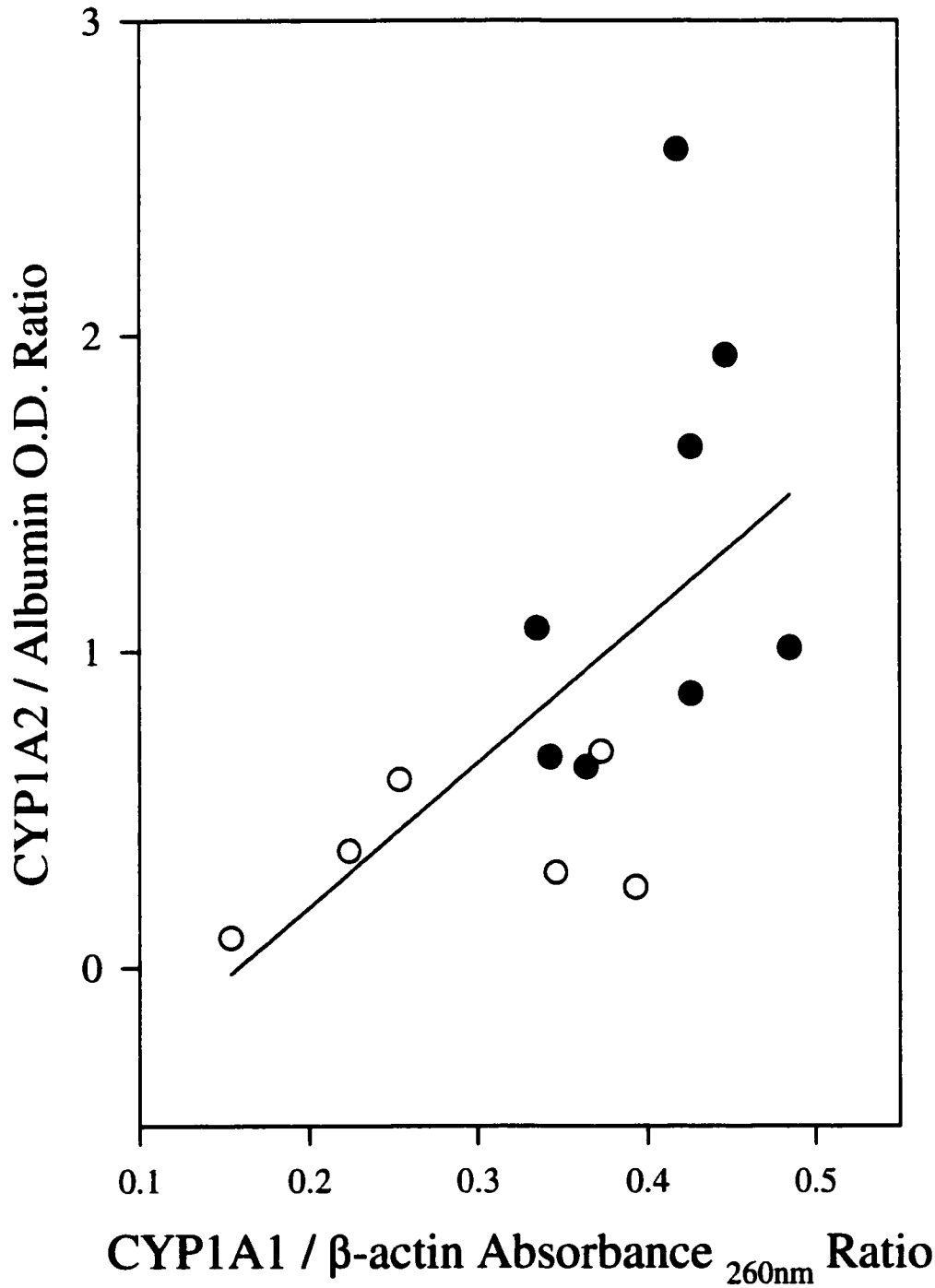
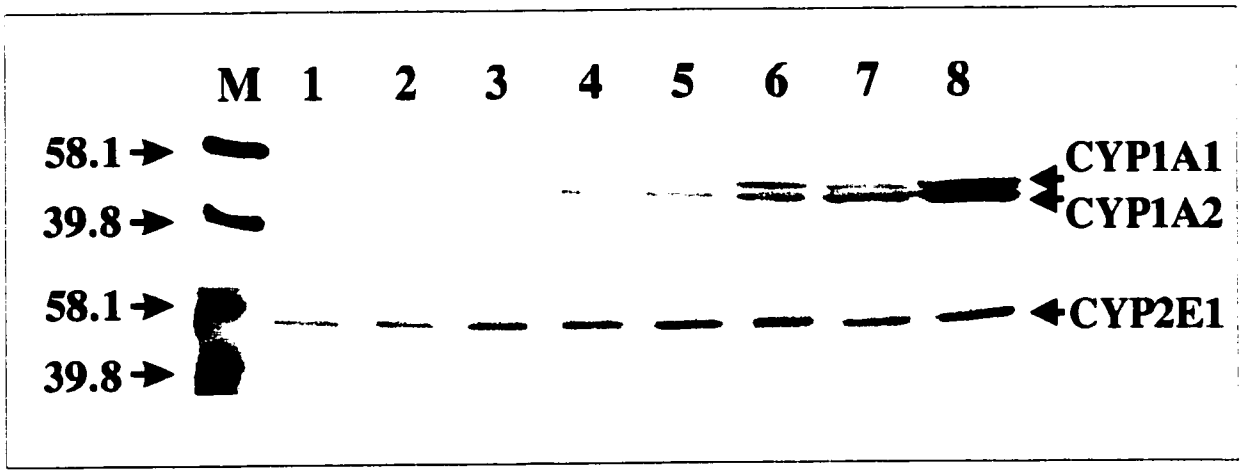

3
2
1
0
CYP1A2 / Albumin O.D. Ratio
0.1
0.2
0.3
0.4
0.5
CYP1A1 / β-actin Absorbance 260nm Ratio

Figure 2.6: Immunoblot analysis of P450 isoform protein expression

Microsomes from 12 male animals selected for nonsusceptibility (APN) or susceptibility (APS) to acetaminophen-induced hepatoxicity were normalized for protein content, then pooled. Varying amounts of pooled microsomes were separated by SDS-PAGE and transferred to blots for immunodetection by enhanced chemiluminescence using a biotin-streptavidin-horseradish peroxidase system. Odd numbered lanes: APN, Even numbered lanes: APS. Lanes 1 and 2: 2.5µg total protein (CYP1A1/2), 1.0µg (CYP2E1); lanes 3 and 4: 5.0µg (CYP1A1/2), 2.5µg (CYP2E1); lanes 5 and 6: 10.0µg (CYP1A1/2), 5.0µg (CYP2E1); lanes 7 and 8: 20.0µg (CYP1A1/2), 10.0µg (CYP2E1). M: Molecular weight standards (kDa).


M 1 2 3 4 5 6 7 8
58.1
39.8
CYP1A1
CYP1A2
58.1
39.8
CYP2E1

and females from the APN and APS groups. The ratio was significantly higher ($p<0.0001$) for the APS compared to the APN group for both genders (Fig. 2.7). There was also a statistically significant difference between APN males and females ($p< 0.0001$), but not between APS males and females ($p=0.45$). A statistically significant positive correlation ($r=0.626$, $p<0.005$) was observed between the *in vitro* parameter of CYP1A2 mRNA expression and the *in vivo* caffeine 3-demethylation data when animals from the APN and APS groups were considered together (Fig. 2.8).

2.4: Discussion

The data presented here demonstrate a co-segregation of microsomal activities and hepatic gene expression consistent with an elevation in both CYP1A1 and CYP1A2 activities in mice selected for susceptibility to acetaminophen-induced hepatotoxicity as defined by elevated serum ALT after exposure. The use of inbred laboratory mice allows for the control of environmental exposure to potential inducers as well as the equalization of nongenetic factors between the two groups under comparison.

Serum ALT is a well recognized marker of hepatic injury which correlates well to acetaminophen-induced hepatotoxicity (Hessel *et al*, 1996). The heterogenous response of males in the original outbred population to acetaminophen was segregated by selection into two populations with stable, heritable, acetaminophen susceptibility phenotypes. The limited but stable penetrance of the trait among males of the APS colony may be attributable to a number of factors. The selection scheme used females which were littermates of susceptible males, since females do not manifest acetaminophen susceptibility. This limits the ability to ensure segregation of the gene(s) responsible for the trait from the maternal donor. The trait itself is a measure of the

Figure 2.7: Caffeine metabolism in male and female mice from inbred colonies selected for nonsusceptibility or susceptibility to acetaminophen-induced hepatotoxicity

Serum caffeine and 1,7-dimethylxanthine levels were measured by HPLC 2 hours after oral dosing with 10mg/kg caffeine. APN (○) males: n=39, females: n=45. APS (●) males: n=38, females: n=31. *: $p<0.0001$.

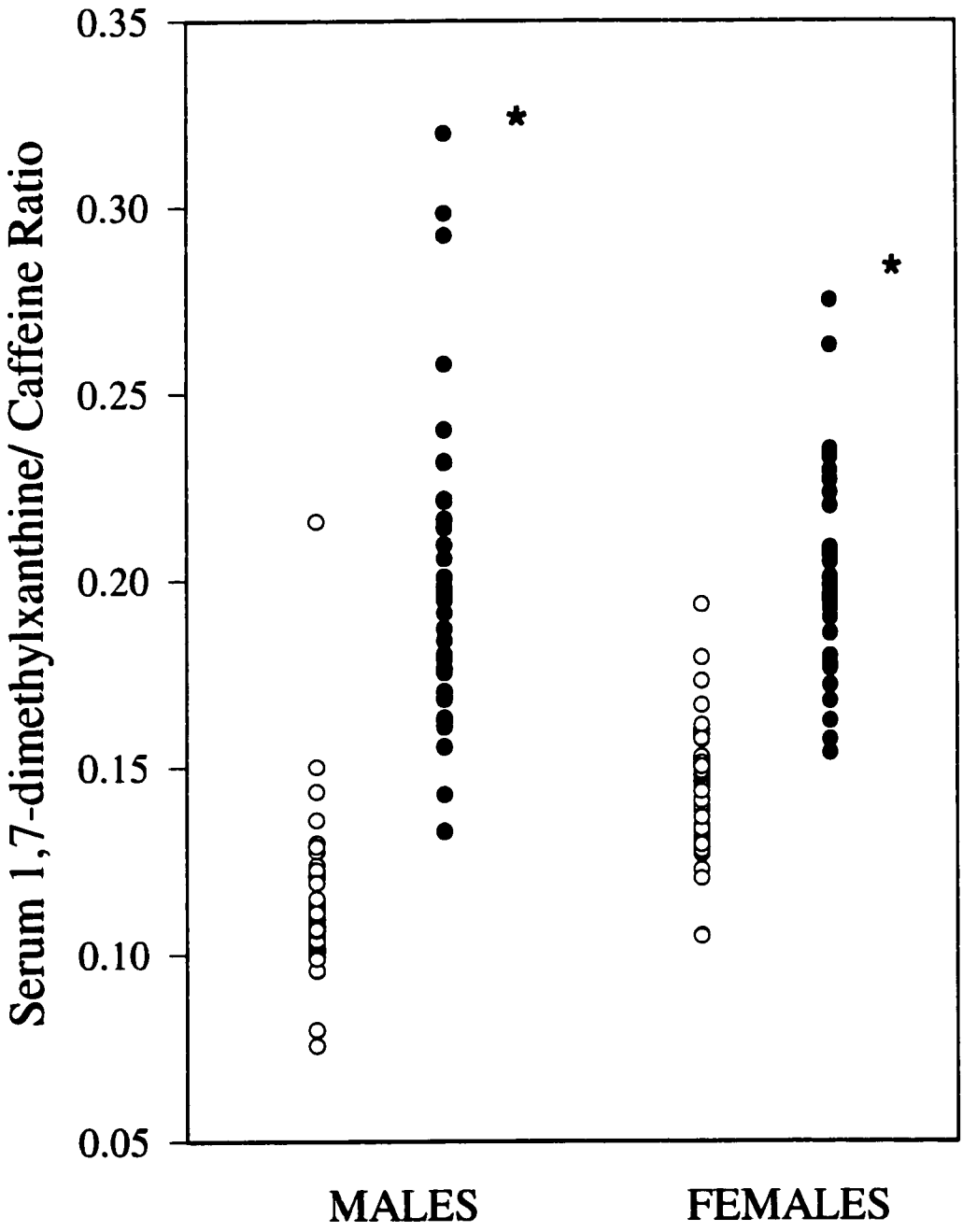
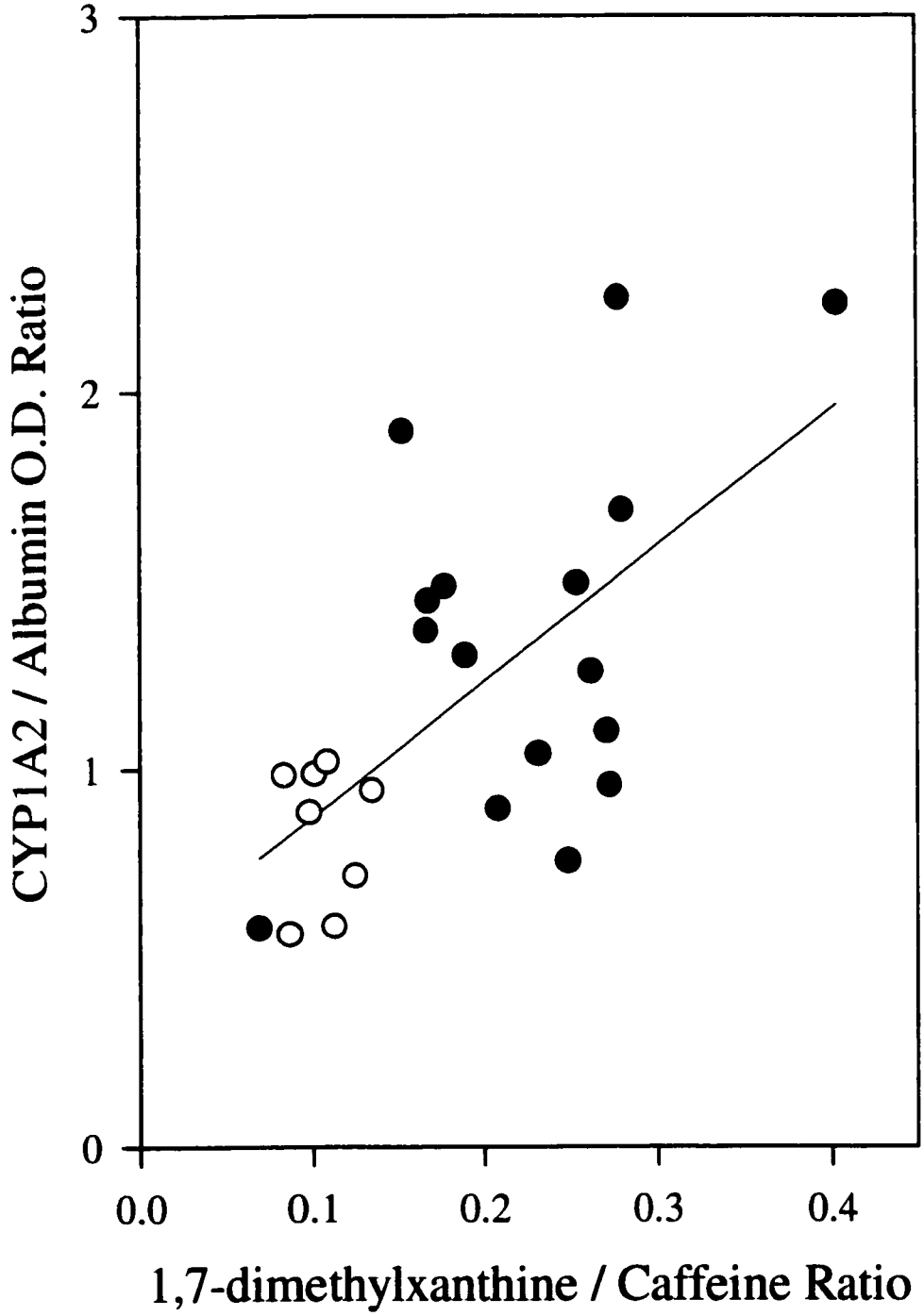

Serum 1,7-dimethylxanthine/ Caffeine Ratio
0.35
0.30
0.25
0.20
0.15
0.10
0.05
MALES
FEMALES

Figure 2.8: Correlation of CYP1A2 relative mRNA expression with caffeine-3-demethylation activity

CYP1A2 expression was determined relative to albumin by densitometric scans of autoradiographs of slot blots of total RNA probed with specific cDNAs. 1,7-dimethylxanthine /caffeine ratios were determined in serum 2 hours after oral dosing with 10mg/kg caffeine. Both parameters were determined in each animal from both acetaminophen-nonsusceptible (APN, ○) and susceptible (APS, ●) mice. There is a significant positive correlation between these two parameters in these animals. ($r=0.626$, $p<0.005$, $n=24$).


3
2
1
0
CYP1A2 / Albumin O.D. Ratio
0.0
0.1
0.2
0.3
0.4
1,7-dimethylxanthine / Caffeine Ratio

response to a relatively low dose of acetaminophen that was chosen to limit the impact of the phenotyping assay on animals required for breeding. A higher dose may have been more effective in identifying the extent of genetically determined susceptibility in the APS colony. In addition, the serum ALT response as a measure of hepatotoxicity may be dependent on a threshold of drug-induced hepatic damage. The relatively low dose of acetaminophen used in these experiments may not discriminate between animals with elevated, but sub-threshold, levels of drug-induced cytotoxicity, and those which are truly nonsusceptible.

Susceptibility to acetaminophen-induced hepatotoxicity in these animals at the phenotyping dose is a sex-determined trait as mentioned above. This gender effect is, at least in part, related to factors other than CYP1A2 expression, since elevated CYP1A2 activity can be detected in females of the APS population using caffeine-3-demethylation as an *in vivo* marker. Caffeine 3-demethylation, determined by a variety of metabolic ratios in serum, urine or saliva, is a recognized surrogate for CYP1A2 activity in humans (Kalow and Tang, 1993) and mice (Buters *et al*, 1996). Gender differences in acetaminophen toxicity observed in rats *in vivo* (Tarloff *et al*, 1996) and human primary cultured hepatocytes (Kane *et al*, 1995) were attributed to differences in phase II metabolism of this drug, specifically, to sex-determined variation in glucuronidation. Sex-related differences in acetaminophen toxicity have been demonstrated in C3H/HeJ mice, but these differences were correlated to sex differences in the renal expression of CYP2E1, apparently mediated by testosterone (Hu *et al*, 1993). No differences in hepatic CYP2E1 expression was detected in these mice, however, and differential toxicity was limited to the kidney.

To test whether changes in bioactivation of acetaminophen might be associated with the differences in sensitivity among male mice in the two populations, we examined the liver

microsomal capacity for metabolic activation of acetaminophen *in vitro* in the two groups. Metabolic activation in this context was defined as the ability of microsomal suspensions to mediate the conjugation of NAC to the NAPQI intermediate metabolite of acetaminophen, which is formed by the P450-mediated metabolism of the parent compound. Since there was a significant difference between the two groups for this parameter, we then examined microsomal activities associated with the P450 isoforms involved in acetaminophen metabolism. Chlorzoxazone 6-hydroxylation, testosterone 6-β-hydroxylation and acetanilide-4 hydroxylation are principally associated with CYP2E1, 3A and 1A2 activities, respectively (Peter *et al*, 1990; Whitehouse *et al*, 1990; Liu *et al*, 1991), although CYP1A2 activity does contribute to chlorzoxazone hydroxylation *in vitro* (Ono *et al*, 1995). Acetanilide 4-hydroxylation was the only activity that was significantly different between the two groups. This observation led to the examination of other microsomal activities associated with CYP1A2, namely, MROD and EROD (Burke and Mayer, 1983) both of which were significantly elevated in microsomal preparations from the APS group, relative to the APN group. Although EROD activity is mainly associated with CYP1A1 activity, especially at lower substrate concentrations, there is experimental evidence that CYP1A2 can mediate this dealkylation reaction in the absence of significant CYP1A1 activity (Tassaneeyakul *et al*, 1993).

In order to determine if increased microsomal EROD activity was indicative of increased CYP1A1 *versus* CYP1A2 activity, it was necessary to assay CYP1A gene expression in a highly isoform-specific manner. Basal hepatic CYP1A2 expression is sufficiently high that RNA levels could be accurately compared using a conventional blot hybridization method with an isoform-specific cDNA probe. Basal expression of CYP1A1 in the liver is, however, substantially lower than CYP1A2. This low level of expression, combined with the considerable sequence homology

between the two isoforms, makes it difficult to determine basal CYP1A1 levels by probe hybridization methods, without interference from CYP1A2 expression. In order to overcome these difficulties, we employed a semi-quantitative reverse transcription PCR (RT-PCR) method to compare CYP1A1 expression between the APS and APN groups. By selecting primer sequences which are specific to CYP1A1 mRNA, one can eliminate any contribution of CYP1A2 to the results. HPLC provided highly reproducible detection and quantitation of the amplified product. The results of these analyses indicated that both CYP1A1 and CYP1A2 mRNA levels are significantly elevated in the livers of APS relative to the APN mice. These findings are consistent with the observed increases in both EROD and MROD, although the semi-quantitative RNA analyses do not provide sufficient information to determine the contribution, if any, of CYP1A1 to the observed microsomal EROD activity. The co-segregation of elevated *Cyp1a1* and *Cyp1a2* hepatic gene expression is further supported by the significant positive correlation between CYP1A1 and CYP1A2 mRNA levels within animals. The positive correlation found here was consistent with that found in nonsmoking human subjects (Schweikl *et al*, 1993).

To support the data from the RT-PCR analysis, we examined the microsomal CYP1A protein content by immunoblot analysis. A polyclonal anti-rat CYP1A1/1A2 antibody was used which cross reacts with two 3-MC-inducible microsomal proteins in mice, which correspond to the predicted electrophoretic mobilities of the murine CYP1A1 and CYP1A2 under SDS-PAGE. In pools of microsomal protein from 12 animals from each of the APN and APS groups, there was a detectable increase in both CYP1A isoforms in the APS group. This indicates a consistent trend between mRNA levels, protein isoform content and enzyme activity.

In order to establish an *in vivo* correlate to *in vitro* data indicating differential CYP1A2 expression, caffeine metabolism was examined in the APN and APS groups. Caffeine 3-

demethylation, determined by a variety of metabolite ratios in serum, urine or saliva, is a recognized clinical surrogate for CYP1A2 activity in humans (Kalow and Tang, 1993). The role of CYP1A2 activity in caffeine metabolism in mice has been confirmed through the use of Cyp1a2 knockout mice (Buters *et al*, 1996). The expected segregation of increased caffeine 3-demethylation with acetaminophen sensitivity in APS mice was observed. In addition, a significant positive correlation between CYP1A2 RNA levels and 3-demethylation of caffeine was observed similar to that observed between caffeine 3-demethylation and anti-CYP1A2 immunoreactive protein content in human liver (Butler *et al*, 1989).

The use of isoform-specific *in vitro* biochemical assays demonstrated that the increased basal hepatic expression of P450 in mice selected for acetaminophen sensitivity was specific to the CYP1A isoforms, among those significantly involved in acetaminophen metabolism. The combination of immunochemical analysis of microsomal protein with hepatic mRNA analyses provided convincing evidence that elevated basal expression of both CYP1A isoforms is co-segregating with acetaminophen susceptibility.

Chapter 3

Chapter 3: Differences in caffeine 3-demethylation activity among inbred mouse strains: A comparison of hepatic *Cyp1a2* gene expression between two inbred strains

3.1: Introduction

This chapter describes a comparative analysis of the trait of caffeine 3-demethylation among different inbred mouse strains, and a detailed analysis of the differential expression of cytochrome P450 CYP1A2 between two strains. The results and interpretations presented in this chapter were published in 1997 in the journal *Fundamental and Applied Toxicology*. We had previously identified genetic covariation in the basal hepatic expression of both *Cyp1a* subfamily genes in two inbred mouse strains derived from a common outbred population by selection for differential susceptibility to acetaminophen-induced hepatotoxicity (see Chpt. 2; Casley *et al.*, 1997a). These results indicated the potential for genetic variations affecting basal *Cyp1a2* expression among inbred mice.

Phenotypic differences among inbred mouse strains can be exploited to identify genetic differences in the expression of genes underlying the trait under consideration. In order to investigate the possibility of genetic variation in basal CYP1A2 expression, a suitable phenotypic marker for CYP1A2 was required. Caffeine has been used as a metabolic probe for the assessment of CYP1A2 activity in mice (Buters *et al*, 1996) and is widely used for the assessment of CYP1A2 activity in humans (Rostami-Hodjegan *et al*, 1996). The determination of CYP1A2 expression is important both in clinical assessment of liver function or drug response, and also in epidemiological and pharmacogenetic studies. The metabolism of caffeine has been extensively studied (Fig. 3.1). Caffeine is a widely consumed stimulant present in many beverages,

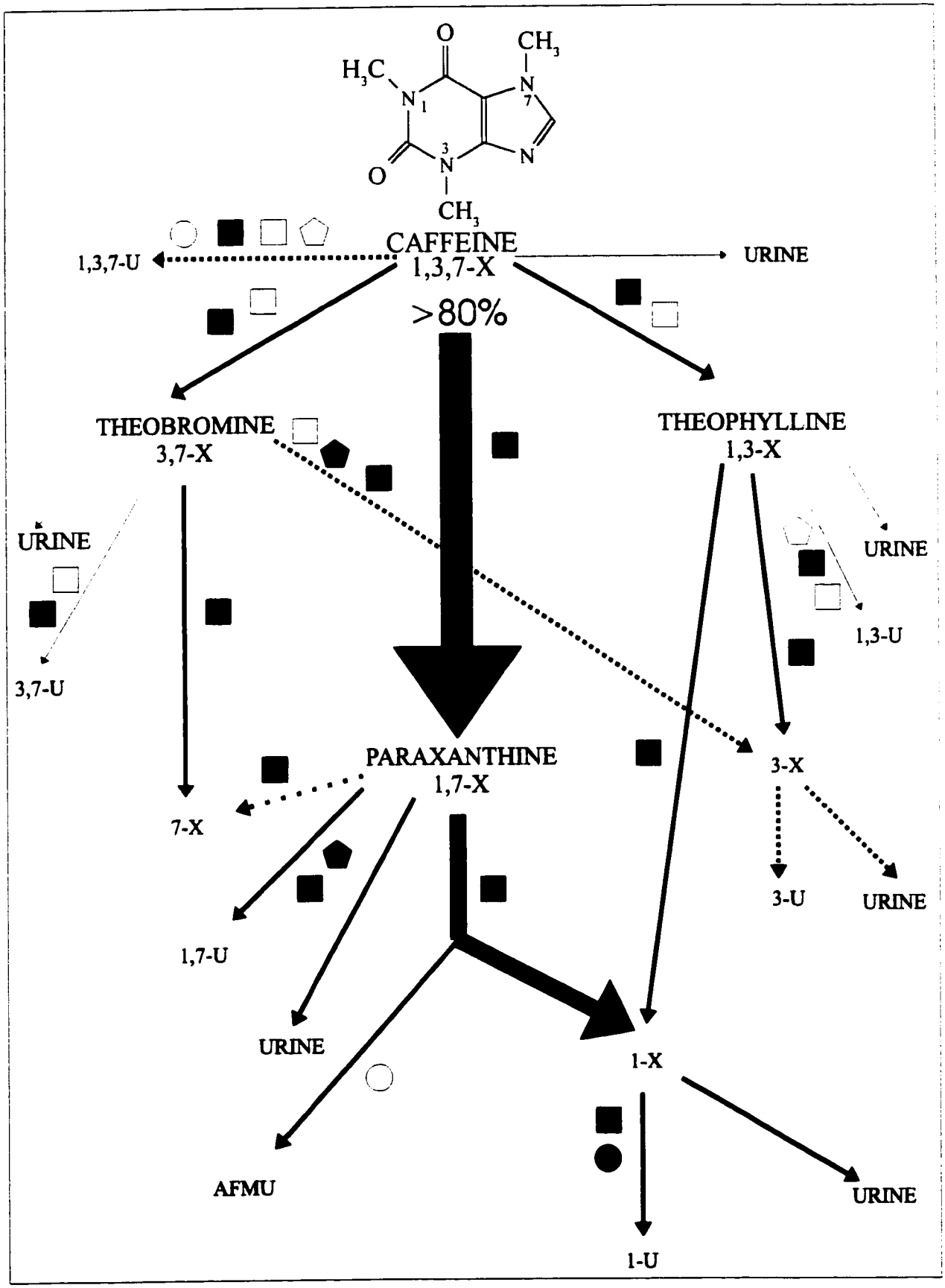
Figure 3.1: Metabolic pathways of caffeine in humans

The metabolic pathways of caffeine in humans, indicating the contributions of different drug metabolizing enzymes, as well as primary, secondary and tertiary metabolites. Dashed lines indicate minor pathways. Numbers refer to positions of methyl groups. abbreviations: U: urate, X: xanthine, AFMU: 5-acetylamino-6-formylamino-3-methyluracil

⬠ : CYP3A4, ■ : CYP1A2, □ : CYP2E1, ●: xanthine oxidase, ○: NAT2

⬟ : CYP2A6

(Modified from Rostami-Hodjegan *et al,* (1996)


CAFFEINE
1,3,7-X
1,3,7-U
URINE
>80%
THEOBROMINE
3,7-X
THEOPHYLLINE
1,3-X
URINE
3,7-U
URINE
1,3-U
PARAXANTHINE
1,7-X
3-X
7-X
3-U
URINE
1,7-U
URINE
1-X
AFMU
URINE
1-U

drug mixtures and foods including coffee, tea, cold remedies and chocolate. High levels of exposure can produce chronic or acute toxicity affecting psychomotor, cardiac and/or gastrointestinal symptoms but it is generally non toxic in the low doses associated with ingestion through food and drink. Caffeine has also become the *in vivo* metabolic probe of choice in the assessment of NAT and xanthine oxidase activities in humans (Kalow and Tang, 1993). Caffeine (1,3,7-trimethylxanthine) has a number of characteristics that make it well suited as a probe drug. It is rapidly absorbed after oral administration, shows single-compartment distribution throughout the total body water and low plasma protein binding. In addition, it is rapidly and extensively metabolized, almost exclusively in the liver, resulting in a short half-life for the parent compound in the blood (Benowitz, 1990). Caffeine and its metabolites can be quantitatively separated from plasma or urine by HPLC (Grant *et al*, 1983). Caffeine is primarily eliminated by 3-demethylation to paraxanthine (1,7-dimethylxanthine). It is this 3-demethylation, which accounts for at least 80% of the clearance of the parent compound, which serves as the basis for the CYP1A2 activity assay. Studies with specific P450 inhibitors established that the CYP1A2 isoform was almost exclusively responsible for the 3-demethylation to paraxanthine (Butler *et al*,1989; Berthou *et al*, 1991). Gu *et al* (1992) used recombinant cell lines engineered to individually express human P450 CYP1A2, 2A6, 2B7, 2E1, 3A4, and 3A5 cDNAs to determine the contributions of different isoforms to the metabolism of caffeine. These studies confirmed that almost 100% of caffeine 3-demethylation was attributable to CYP1A2, results that were later confirmed by Tassaneeyakul *et al* (1994a) using a similar approach with different transgenic cell lines. In order to exploit caffeine 3-demethylation as a clinical probe of CYP1A2 activity, relative concentrations of parent compound and primary metabolites could be employed. The ideal sample for a clinical assay is urine, which is preferable to blood in so far as the invasiveness of sampling is concerned.

However, all of the primary metabolites of caffeine are themselves extensively metabolised prior to excretion. Paraxanthine is a substrate for CYP1A2-mediated 1- and 7-demethylations and 8-hydroxylations as well as CYP2A6 mediated 8-hydroxylations and NAT2 transformation to 5-acetylamino-6-formylamino-3-methyluracil (AFMU) (Fig. 3.1). In addition, CYP1A2 as well as CYP2E1, mediate 1-demethylation and 7-demethylation of caffeine to produce theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine), itself a pharmacologically active compound used in the treatment of asthma. Since all of the N-demethylated secondary metabolites of caffeine are also themselves extensively metabolized, many clinical assays have focussed on the summed urinary metabolites. Since paraxanthine is both the major product and a substrate of CYP1A2 activity, any analysis of urinary metabolites must account for both the pleiotropic contribution of CYP1A2 activities and the non CYP1A2-based biotransformations. The ratio of (AFMU + 1-methylxanthine + 1-methylurate)/1,7-dimethylurate in overnight urine is the most widely used index of CYP1A2 activity calculated from urinary metabolites (Campbell *et al*, 1987; Rostami-Hodjegan *et al*, 1996). The numerator metabolites account for most, but not all CYP1A2-derived metabolites, while minimizing non CYP1A2- mediated products. The denominator, 1,7-dimethylurate was chosen on the basis of the empirical determination that this metabolite demonstrates the least interindividual variation as an index of initial caffeine dosage. Urinary metabolite assays have the advantage that they permit the most comprehensive exploitation of caffeine as a metabolic probe. 1-methylurate and/or 1-methylxanthine over AFMU ratios can be used to determine NAT2 activity (Grant *et al*, 1984) and the conversion of 1-methylxanthine to 1-methylurate has been used as a measure of xanthine oxidase activity (Kalow and Tang, 1991). A significant disadvantage of urinary metabolite assays is their sensitivity to alterations in renal function affecting clearance, a common event in many disease states (Tang *et*

al, 1994). One alternative to measurement of urinary metabolites, is the use of plasma or salivary samples. Fuhr and Rost (1994) demonstrated that single time point ratios of paraxanthine/caffeine from plasma or saliva taken less than 8 hours after oral dosing with caffeine, were highly correlated to total caffeine clearance and therefore CYP1A2 activity. They and others (Rostami-Hodjegan *et al*, 1996) demonstrated that this assay was not as affected by non- CYP1A2-related interindividual variation as urinary metabolite assays.

Historically, clinical evaluation of CYP1A2 activity employed the probe drug phenacetin. The *O*-deethylation of the analgesic phenacetin was considered to be specific to CYP1A2. Phenacetin is no longer used as a therapeutic agent in humans, however, and is therefore considered unsuitable as a noninvasive probe. Consequently, no case comparisons of *in vivo* phenacetin *versus* caffeine phenotyping of CYP1A2 are possible. The validation of the various caffeine metabolite assays as specific predictors of *in vivo* CYP1A2 activity was limited to relatively rare cases where liver biopsy samples were available to correlate specific *in vitro* assay results. In these cases assessment of CYP1A2 activity using caffeine correlated well to quantitative immunoassays of CYP1A2 enzyme protein content or quantitation of CYP1A2 mRNA (Kalow and Tang, 1993). Both caffeine 3-demethylation and phenacetin O-deethylation, as well as acetanilide 4-hydroxylation and MROD activities are used in *in vitro* assessments of CYP1A2 in microsomal preparations, and these too correlate well with *in vivo* caffeine based assessments (Tassaneeyukal *et al*, 1994a). Specific inhibitors of human CYP1A2 activity, such as furafylline produce the expected alterations in caffeine 3-demethylation activity. The loss of drug status for furafylline was due in part to episodes of caffeine toxicity resulting from the failure to clear dietary caffeine when taking the CYP1A2 inhibitor (Tassaneeyakul *et al*, 1994b) . Population studies using caffeine metabolites also demonstrate the bimodal distribution between

smokers and nonsmokers predicted by the Ah receptor-mediated induction of CYP1A2 by cigarette combustion products.

A more direct validation of caffeine as a probe drug for CYP1A2 was undertaken in mice. Mouse and human CYP1A2 have similar substrate specificities and primary amino acid sequence. Using *Cyp1a2* (-/-) knockout mice, Buters *et al* (1996) provided direct evidence for the role of CYP1A2 in caffeine metabolism in mice. These experiments demonstrated that the knockout mice showed an 8-fold decrease in the clearance of plasma caffeine, and concluded that CYP1A2 was accounting for 87% of the elimination of caffeine in wild type mice. This group further concluded that plasma clearance was a more accurate assessment of CYP1A2 activity than urinary metabolite ratios, given the contribution of the remaining complement of cytochrome P450 to the production of the urinary metabolites. The validity of plasma paraxanthine/caffeine ratios in short term spot tests in humans as demonstrated by Fuhr and Rost (1994), together with the role of CYP1A2 activity in caffeine clearance in mice demonstrated by Buters *et al* (1996), suggested that this assay would be valid for the assessment of CYP1A2 activity in mice. Single point plasma assays do have the advantage that they can be performed rapidly and easily, under reproducible assay conditions. These considerations make this approach well suited to the analysis of CYP1A2 activity in the mouse, where the influence of disease and dietary factors can be minimized. In situations where large numbers of animals must be examined, such as in genetic analyses, this approach would be especially useful. The laboratory mouse offers the opportunity to study genetic variation in gene expression in the absence of differential exposure to environmental inducers. We had previously employed a single point plasma caffeine metabolite assay to discriminate between our differentially acetaminophen-susceptible inbred strains, which showed less than two-fold differences in hepatic CYP1A2 activity, as measured by conventional

microsomal assays (see Chpt. 2; Casley *et al.*, 1997a).

In the present study, we have investigated the differences in caffeine 3-demethylation in order to test the hypothesis that significant heritable differences exist in this trait between inbred mouse strains. We further examined the correlation between the caffeine 3-demethylation phenotype and a number of specific biochemical and molecular markers of cytochrome P450 *Cyp1a2* gene expression in order to test the hypothesis that single point caffeine metabolite ratio phenotyping is suitable for evaluating the CYP1A2 expression status in a genetic analysis between candidate strains identified in this study.

3.2: Materials and methods

3.2.1: Animals

The APN strain was derived from outbred Swiss-Webster mice originally obtained from Connaught Laboratories (Toronto, ON) in 1968 and maintained as a closed penbred colony at the animal care facility of the Health Protection Branch of Health Canada (Ottawa, ON). The derivation of this strain has been described elsewhere (see Chpt 2.; Casley *et al.*, 1997a). Briefly, breeding pairs were selected as nonsusceptible to the hepatotoxic effects of acetaminophen at 150 mg/kg *p.o.* using serum ALT levels as a marker through the 20th generation of inbreeding. Animals used in the experiments described here are from the 21st generation of inbreeding. The inbred laboratory strains A/J, P/J, BALB/cJ, C3H/HeJ, AKR/J and SWR/J were obtained from Jackson Laboratory (Bar Harbor, ME, USA), and were maintained in the HPB facility for 3 weeks prior to caffeine phenotyping. Females were not synchronized for oestrus cycle prior to typing. Animals were fed irradiated Prolab 3000 rodent feed (Agway Inc., Portsmouth, RI, USA) and sterile water *ad libitum*, and housed on sterile Sanichip maple chip bedding (P.W.I.

Industries, St. Hyacinthe, PQ). All animals were housed and cared for in accordance with Canadian Council on Animal Care guidelines.

3.2.2: Standards and reagents

Caffeine, 8-chlorotheophyllline, 3-MC and chlorzoxazone were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 6-hydroxychlorzoxazone was from Salford Ultrafine Chemicals and Research Ltd. (Manchester, England). Acetanilide and 3-hydroxyacetanilide were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Estriol was obtained from Searle Chemical Inc. (Chicago, IL, USA). Resorufin, methoxyresorufin and ethoxyresorufin were purchased from Pierce (Rockford, IL, USA). NAC-acetaminophen metabolite conjugate was a generous gift of Dr. J. Sinclair, University of Vermont VA Medical Centre (Burlington, VT, USA). All other chemicals were reagent grade or higher quality.

Standards for the HPLC quantitation of cytochrome P450 CYP1A1 and CYP1A2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR products were prepared by agarose gel purification using a silica gel binding system (QIAquick, QIAGEN, Chatsworth, CA, USA).

3.2.3: Caffeine phenotyping

Animals at 7 weeks of age, with free access to food and water, were dosed by oral gavage with an aqueous solution of caffeine (1mg/mL, 10 mg caffeine/kg body weight). Two hours later (± 5 min) approximately 0.2 mL of blood was collected from the orbital sinus under isoflurane-induced anaesthesia using a heparinized Microhematocrit Capillary Tube (Fisher Scientific, Pittsburg, PA., USA). The blood was centrifuged at 7420 x g for 10 minutes and 0.1 mL of plasma was removed into a microtube to which 10 µL of 8-chlorotheophylline (100 µg/mL) internal standard was added. 15 µL of 20% trichloroacetic acid was added and the samples were

cooled for 30 minutes at 4°C before a 10 minute centrifugation at 13200 x g. 50 μL of deproteinized plasma, diluted 1:1 with water, was chromatographed on a 15 cm Inertsil 5μm, ODS2 column (Chromatography Sciences Company, St-Laurent, PQ) with the eluate monitored at 274 nm. A flow of 1 mL/minute of 0.1 M Na_2PO_4 (0.1% TEA pH 2.5 with H_3PO_4) : methanol : acetonitrile (78:14:7) was maintained. The area ratio of the 1,7 dimethylxanthine/caffeine peaks was calculated.

3.2.4: 3-methylcholanthrene treatment

Animals were injected I.P. with 3-MC (50 mg/kg as a 5 mg/mL solution in corn oil) once daily for 3 days. Controls were injected with corn oil only. On day 4 blood was drawn for caffeine phenotyping as previously described. On day 5 animals were sacrificed and microsomes prepared as described below.

3.2.5: Microsomal assays

Animals were sacrificed by cervical dislocation followed by decapitation. Microsomes were prepared from freshly excised livers as previously described (Whitehouse and Paul, 1987) and stored at -80°C as pellets under a 50 mM KCl pH 7.4 overlay until used. Protein concentrations were determined by the Biuret method using the Abbott Diagnostics total protein reagent with bovine serum albumin as the standard (Abbott Laboratories, Diagnostics Division, Irving, TX, USA). All HPLC analyses were carried out using a Beckman System Gold instrument (Beckman Instruments, Fullerton, CA, USA). Acetanilide 4-hydroxylase activity was determined according to the method of Lui *et al* (1991) by quantitation of 4-hydroxyacetanilide in the ethylacetate extract fraction after a 30 minute incubation of the microsomal suspension with acetanilide and NADPH. 3-hydroxyacetanilide was used as an internal standard. Chlorzoxazone 6-hydroxylation activity was measured as described by Peter *et al* (1990), using estriol as an

internal standard. Conjugation of reactive acetaminophen metabolite to NAC was determined by incubating microsomes for 30 minutes in a shaking water bath at 37°C with 0.5 mM acetaminophen, 0.9 mM NADPH, 3 mM $MgCl_2$ and 1 mM NAC in 50 mM KPO_4 buffer pH 7.4. The reaction was terminated by addition of 1.5 volumes of methanol. Products were stored at -20°C. Just prior to assay, products were centrifuged to remove the alcohol-insoluble fraction and supernatants were dried in a vacuum centrifuge (Oligoprep, Savant Instruments Inc., Farmingdale, N.Y. USA) without heating. Pellets were redissolved in 0.4 mL H_2O and 50 µL was injected for HPLC using a 25 cm, 5 µm Spherosorb ODS2 column (Chromatography Sciences Co., St-Laurent, PQ), and a solvent system of 12% acetonitrile in 50 mM sodium phosphate, pH 2.2 at 1 mL/ minute. Eluate was monitored at 254 nm. EROD and MROD activities were determined according to Burke and Mayer (1983), using an Aminco DW-2a spectrofluorometer (Silver Springs, MD, USA) set at λ_{ex} 510 nm and λ_{em} 580 nm to measure resorufin production. Substrates were dissolved in DMSO and incubated at room temperature with microsomes in suspension with NADPH in 50 mM KPO_4 buffer at pH 7.4.

3.2.6: Western blotting and immunodetection

Aliquots of equivalent protein content from liver microsomes of 6 males each from the APN and C3H/HeJ groups were separated by 12% SDS-PAGE according to Laemmli (1970), then transferred to nitrocellulose membranes (Biorad, Mississauga, ON). Blots were incubated for 2 hours with either rabbit polyclonal anti-rat CYP1A1/2 antibody (Human Biologics, Phoenix, AZ) or rabbit polyclonal anti-rat CYP2E1 (Amersham Life Science, Oakville, ON). Antibody binding was detected by chemiluminescence (ECL, Amersham Life Science, Oakville, ON).

3.2.7: RNA isolation and cDNA synthesis

Livers were excised immediately after sacrifice and approximately 100 mg slices were removed and quick frozen at -80°C. Total RNA was extracted from tissue slices using the acid guanidinium thiocyanate phenol chloroform method (Chomczynski and Sacchi, 1987). Total RNA was redissolved from ethanol precipitation in diethylpyrocarbonate-treated water, and incubated with 4 units of RNase-free DNase I (Ambion Inc., Austin, TX, USA) to remove any contaminating genomic DNA. After DNase treatment, samples were re-purified using a column binding system (RNeasy, QIAGEN, Chatsworth, CA, USA) and quantified by spectrophotometry. 1 μg of total RNA per reaction was converted to cDNA by incubating at 42°C for 1 hour with an oligo-dT primer and an RNase H deficient reverse transcriptase (Superscript II, Life Technologies, Burlington, ON) in a 20μL reaction volume.

3.2.8: Duplex PCR

PCRs were set up to co-amplify CYP1A1 or CYP1A2 cDNA sequences with the constitutively expressed GAPDH as an endogenous internal control sequence. Synthetic oligonucleotide primers were synthesized (Life Technologies, Burlington, ON) based on sequences chosen using PCR primer designing software (Primer 2, S&E Software State Line, PA, USA) to ensure specific and efficient co-amplification of target sequences (Table 3.1). In order to compensate for the significant differences in abundance of the target and control templates and ensure that both products could be assayed while in the linear phase of amplification, the addition of the control primers was delayed, as described by Kinoshita *et al* (1992). CYP1A1 and GAPDH sequences were co-amplified using 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 240 μM of each dNTP, 200 nM of each primer, and 3 units of Taq polymerase (Boehringer Mannheim, Laval, PQ), in a final volume of 50 μL. 4μL of 1/5 dilutions of the cDNA reactions

were used as templates. The reaction was set up in a volume of 45 μL without GAPDH primers, which were added in a volume of 5 μL after 14 cycles. Reaction conditions for the amplification of CYP1A2 and GAPDH were similar except that the concentrations of $MgCl_2$, dNTPs and Taq were 1.5 mM, 200 μM and 2.5 units/reaction, respectively. 2 μL of a 1/50 dilution of the cDNA reactions were used as templates and GAPDH primers were added after 4 cycles. Cycling conditions for both reactions were: 1 minute at 94°C, followed by cycles of 10 seconds at 94°C, 30 seconds at 65°C, and 30 seconds at 72°C in an MJ Research PTC 200 thermal cycler (Watertown, MA, U.S.A) run in calculated mode. Reactions were run for 35 cycles for CYP1A1/GAPDH and 30 cycles for CYP1A2/GAPDH. The linear ranges of both reactions for both cycle number and starting cDNA concentrations were determined empirically. Reactions were run in microwell plates using a film to seal the plate (Microseal A, MJ Research, Watertown, MA, U.S.A). This sealing film and the use of a multichannel pipettor permitted the rapid addition of GAPDH primers to all reactions simultaneously.

3.2.9: Quantitation of PCR products by HPLC

Products from PCR experiments were analyzed by an HPLC system consisting of a 600E Multisolvent Delivery System coupled to a 717+ Autosampler (Waters Chromatography, Milford, MA, USA) and a Spectroflow 783 Programmable Absorbance Detector (ABI Analytical, Kratos Division). Separations were carried out on a Progel TSK DEAE-NPR 35 mm x 4.6 mm i.d. analytical column fitted with a guard column of the same support, 5 mm x 4.6 mm id (Supelco, Mississauga, ON). The analytical column was maintained at a constant temperature of 30°C. Mobile phase A was 25 mM Tris-HCl, pH 8.0, and mobile phase B was 1 M sodium chloride in 25 mM Tris-HCl, pH 8.0. Separations were effected using a linear stepwise gradient profile: 30%-55% B in 1 minute, 55%-65% B in 9 minutes, 65%-100% B in 0.5 minute, 100% B for 0.5

Table 3.1: Oligonucleotides used in semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)analysis of hepatic cytochrome P450 CYP1A mRNA levels

Nucleotide sequences and position assignments are based on Genbank (Benson *et al.*, 1994) data as follows:

[a] Kimura *et al.*, 1984a, Genbank accession # K02588

[b] Kimura *et al.*, 1984b, Genbank accession # X00479

[c] Sabath *et al.*, 1990, Genbank accession # M32599

CYP1A1[a]	**Position**	**Product Size**
a) 5'-GCCTCATGTACCTGGTAACCAAC-3'	nt 1098-1120	403 bp
b) 5'-CCAATGGTCTCTCCGATGCACTT-3'	nt 1500-1478 (c)	
CYP1A2[b]		
c) 5'-ATGAGGAGCTGGACACGGTGGTT-3'	nt 1085-1107	396 bp
d) 5'-GCAGGATGGCTAAGAAGAGGAAG-3'	nt 1480-1458 (c)	
GAPDH[c]		
e) 5'-GGAGCCAAACGGGTCATCAT-3'	nt 383-402	251 bp
f) 5'-TCACGCCACAGCTTTCCAGA-3'	nt 633-614 (c)	

minute, 100%-30% B in 0.5 minute and 30% B for 8.5 minutes. The total run time was 20 minutes. The flow rate was 1 mL/minute and the absorbance was monitored at 260 nm. After amplification, reaction volumes were diluted 1:1 with water and duplicate injections of 25 μL were made. Samples were analyzed without prior purification. Samples were kept at 5°C in the autosampler prior to injection. Products not analyzed within 12 hours were kept frozen at -20°C.

3.2.10: RNA slot blots

5 μg total liver RNA from each animal was immobilized on a nylon membrane as previously described (see Chpt. 2; Casley *et al.*, 1997a). Probes for murine UGT1*06, and GAPDH were obtained by cloning specific PCR products from RT-PCR of mouse liver RNA. The GST Ya subunit (GSTYa) probe was the large PstI fragment of pGTB38, the cloned rat GSTYa, a generous gift of Dr. Cecil Pickett (Merck Frosst Canada Inc. Pointe Claire, PQ). This fragment shows 95% homology with the murine GSTYa sequence. Probes were labelled with ^{32}P by random priming (Megaprime, Amersham Life Science, Oakville, ON). The specificity of each of these probes was confirmed on northern blots of control and 3-MC-induced mouse liver RNA prior to their use on slot blots. X-ray films were scanned using a laser densitometer (Molecular Dynamics, Sunnyvale, CA, USA), and autoradiographic signals were quantitated using the scanner's image analysis software (ImagequantNT v 4.2). Multiple exposures were used to ensure that data used in the analysis were within the linear response range of the film.

3.2.11: Statistical analyses

Groups were compared using the Mann-Whitney rank sum analysis to test for significant differences. Rank sum analyses were used to account for the unequal variances seen in some groups. All analyses were done using the SigmaStat software system (Jandel Scientific, San Rafael, CA, USA).

3.3: Results

3.3.1:Caffeine 3-demethylation in inbred strains

1,7-dimethylxanthine (paraxanthine, 1,7-X) *versus* caffeine (1,3,7-X) ratios in serum 2 hours after oral dosing with 10 mg/kg caffeine vary widely among different inbred mouse strains. Values range from 0.12 for the APN strain to 2.92 for the SWR strain among males, and from 0.12 for the APN strain to 1.69 for the SWR strain among females (Fig. 3.2). Among the seven strains tested, 14 vary significantly from one another in males and 15 in females in 21 pairwise comparisons for each gender. There are also significant differences in this parameter between males and females of the same strain for 3 of the strains examined (Table 3.2). Females show consistently lower 1,7-X / 1,3,7-X ratios in the strains examined. In no case did females of a strain show higher levels than males. Among the 6 males and 6 females compared between the APN and C3H/HeJ strains, no overlap was observed in serum 1,3-X / 1,3,7-X ratios. There was at least a 10-fold difference in this value observed in comparing any two male animals from these strains.

Inducibility of microsomal enzyme activity was compared between both male and female mice for both APN and C3H/HeJ strains. Males were used in all subsequent comparisons between APN and C3H/HeJ strains in order to eliminate the possible increase in variation among females not synchronized for oestrus cycle. Basal microsomal enzyme activity assays, immunoassays and mRNA analyses were performed on samples from the same twelve mice.

3.3.2: Liver microsomal activities in APN and C3H/HeJ mice

Liver microsomal activities relevant to cytochrome P-450 CYP1A2 activity were examined in 6 male mice from the APN and C3H/HeJ strains. EROD and MROD activities were significantly higher, 5.9-fold and 2.8-fold, respectively, in the C3H/HeJ mice (Fig. 3.3A). EROD is typically associated with CYP1A1 activity in arylhydrocarbon-induced liver, but can be

Figure 3.2: Comparison of caffeine 3-demethylation activity among different inbred strains

Serum 1,7-xanthine / caffeine ratios determined 2 hours after an oral caffeine dose of 10 mg/kg body weight were compared between males and females from 7 different inbred strains. □ Males, ■ Females. Bars represent mean values for each group. Error bars indicate standard deviations. n=6 for all groups except SWR/J males, where n=5.

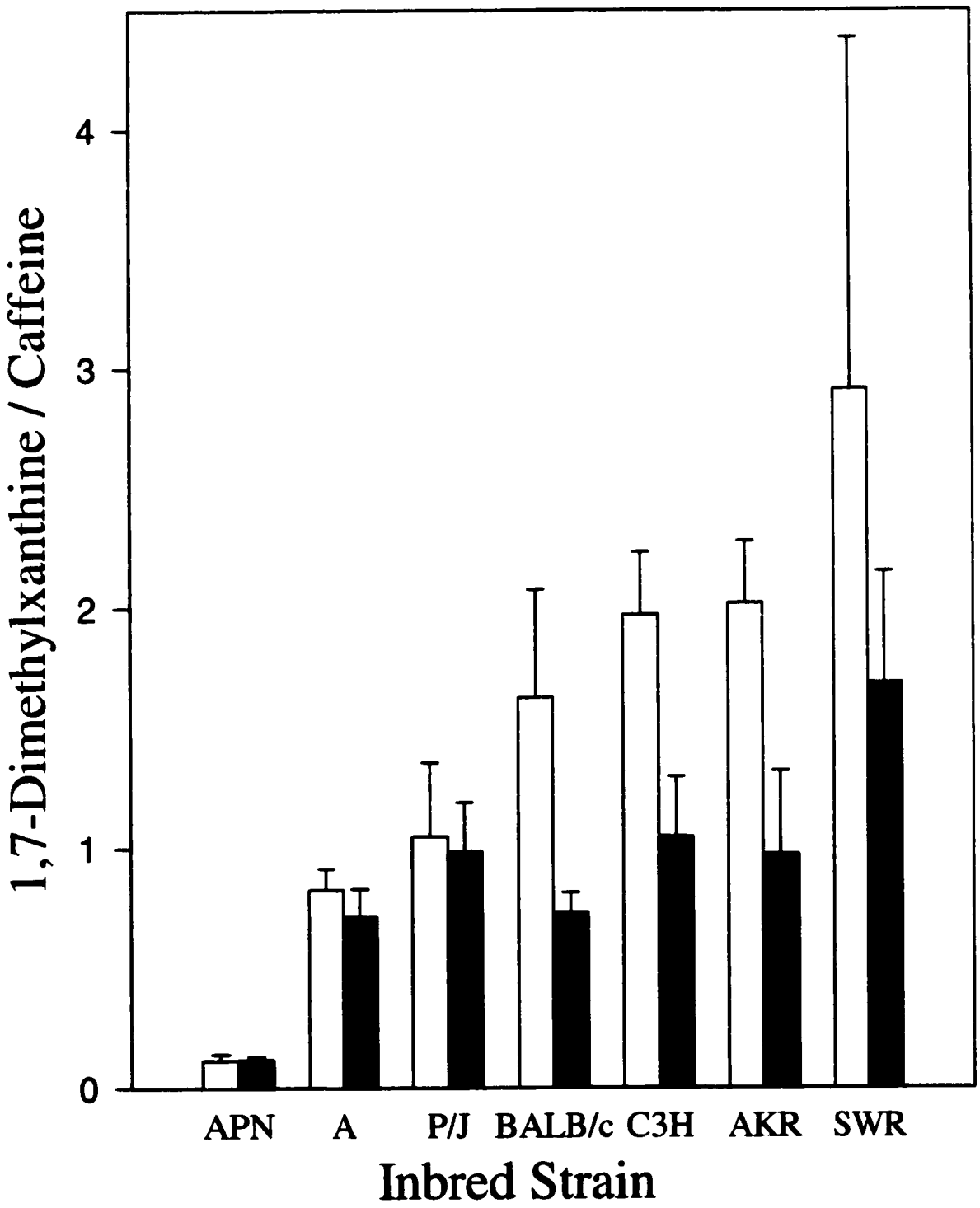

1,7-Dimethylxanthine / Caffeine
0
1
2
3
4
APN
A
P/J
BALB/c
C3H
AKR
SWR
Inbred Strain

Table 3.2: Statistical comparisons of caffeine 3-demethylation activity between inbred mouse strains

Serum 1.7-dimethylxanthine / caffeine ratios determined 2 hours after oral caffeine dosing were compared between males and females from 7 different inbred strains. Values in the shaded area are the results of pairwise comparisons between males of different strains. Values in the unshaded area are from pairwise comparisons of females from different strains. Values in the boxes are comparisons of males and females from the same strain. Groups were compared using the Mann-Whitney rank sum test to obtain p values. (N.S.: not significantly different, $p > 0.05$). n= 6 for all groups except SWR/J males where n=5.

	APN	A/J	P/J	BALB/cJ	C3H/HeJ	AKR/J	SWR/J
APN	N.S	$p < 0.005$	$p < 0.005$	$p < 0.005$	$p < 0.005$	$p < 0.005$	$p < 0.005$
A/J	$p < 0.005$	N.S.	N.S.	$p < 0.005$	$p < 0.005$	$p < 0.005$	$p < 0.005$
P/J	$p < 0.005$	$p < 0.05$	N.S.	$p < 0.05$	$p < 0.005$	$p < 0.005$	$p < 0.01$
BALB/cJ	$p < 0.005$	N.S.	$p < 0.02$	$p < 0.005$	N.S.	N.S.	N.S.
C3H/HeJ	$p < 0.005$	$p < 0.02$	N.S.	$p < 0.01$	$p < 0.005$	N.S.	N.S.
AKR/J	$p < 0.005$	N.S.	N.S.	N.S.	N.S.	$p < 0.005$	N.S.
SWR/J	$p < 0.005$	$p < 0.005$	$p < 0.005$	$p < 0.005$	$p < 0.02$	$p < 0.05$	N.S.

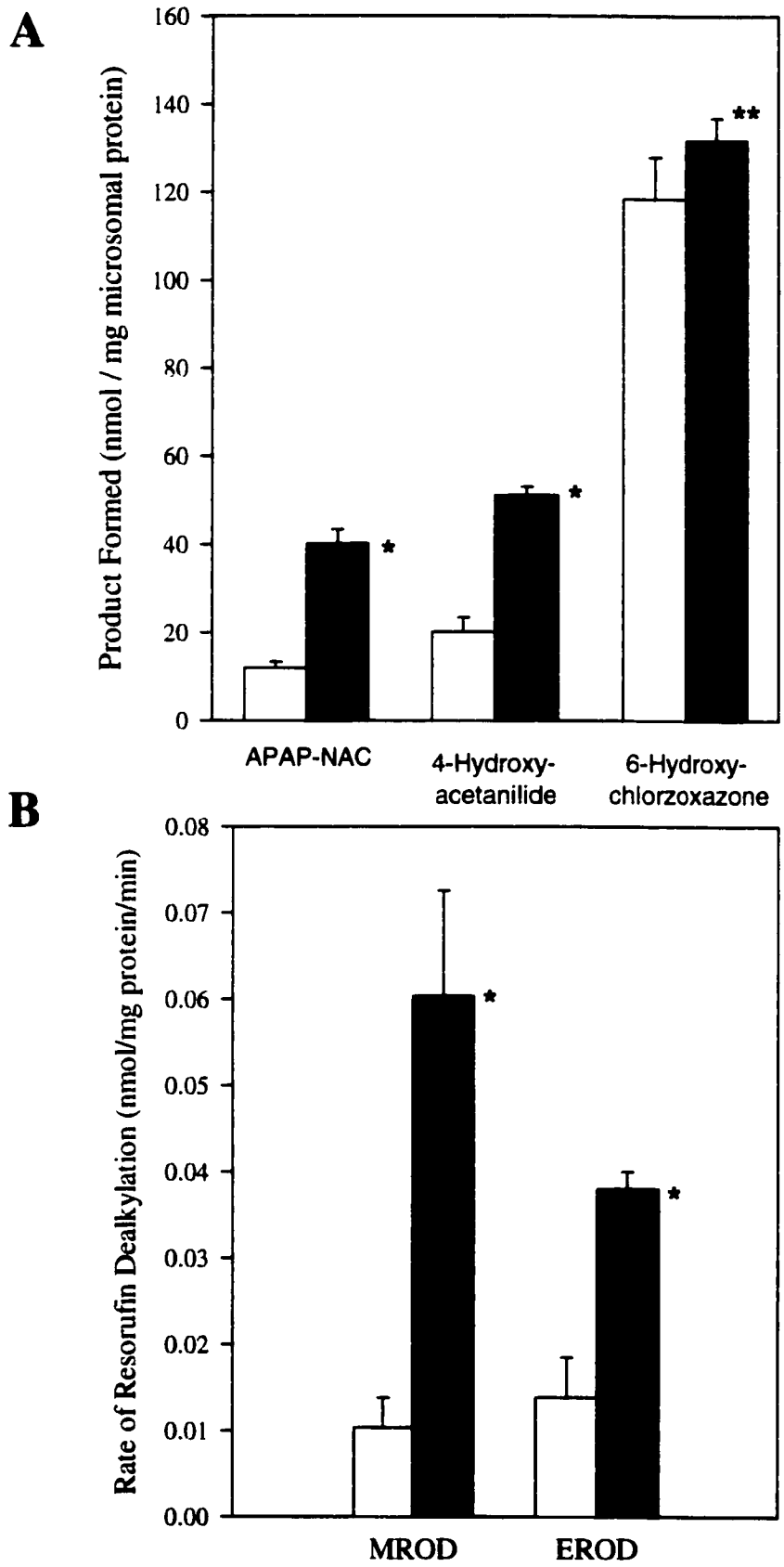
Figure 3.3: Liver microsomal activities in male APN and C3H/HeJ mice

A: NAC / acetaminophen conjugation (NAC-APAP), acetanilide 4-hydroxylation, and chlorzoxazone 6-hydroxylation activities. Product formation was measured after 30 minutes (NAC-APAP and 4-hydroxyacetanilide),or 45 minutes (6-hydroxychlorzoxazone).

B: Methoxyresorufin *O*-demethylase (MROD) and Ethoxyresorufin *O*-deethylase (EROD)

(□) APN, (■) C3H/HeJ. Bars indicate mean values. Error bars indicate standard deviations,

* : $p<0.005$, ** : $p<0.05$. n=6 for each group.


A
Product Formed (nmol / mg microsomal protein)
160
140
120
100
80
60
40
20
0
*
*
**
APAP-NAC
4-Hydroxy-acetanilide
6-Hydroxy-chlorzoxazone
B
Rate of Resorufin Dealkylation (nmol/mg protein/min)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
*
*
MROD
EROD

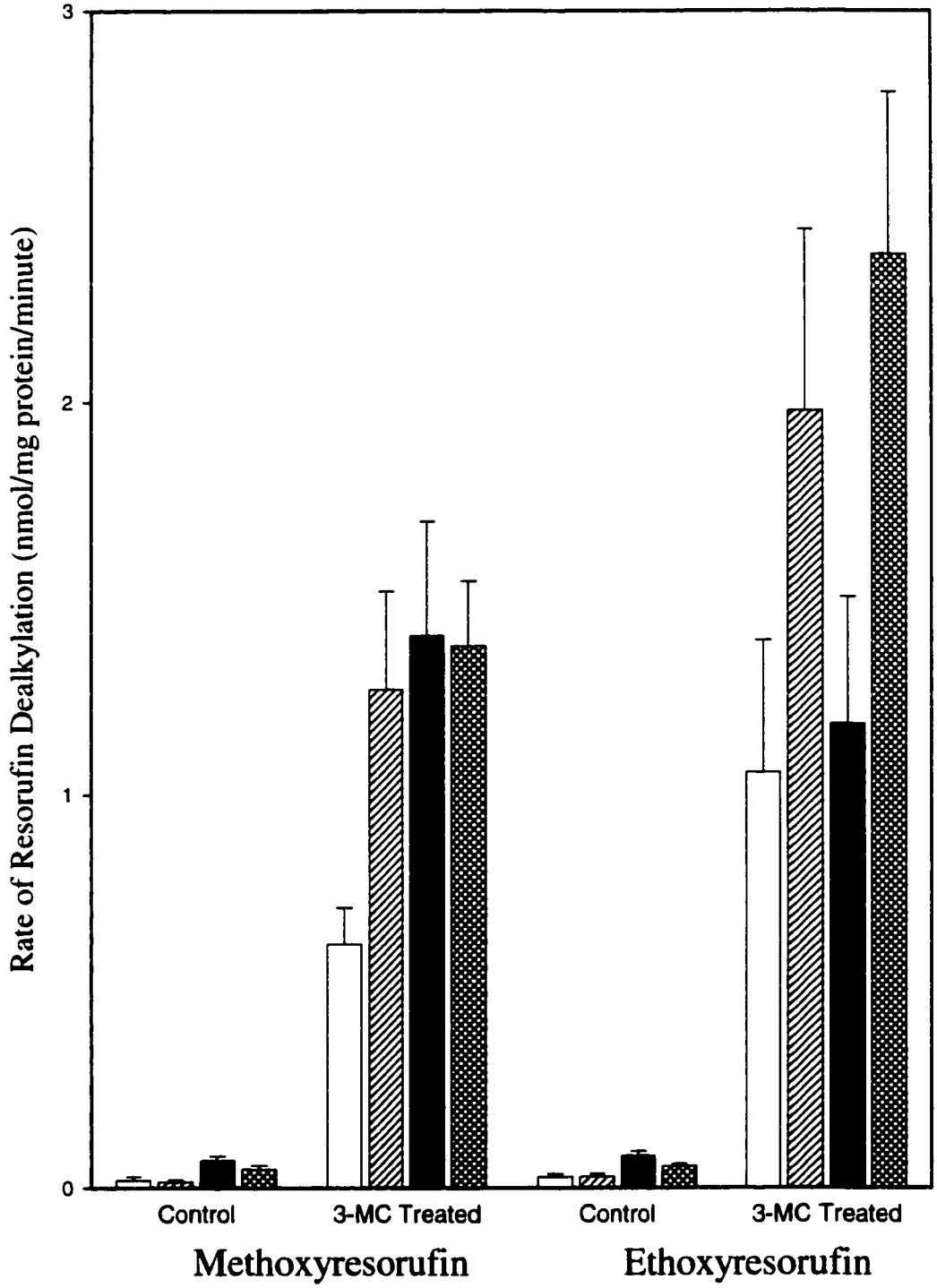
mediated by CYP1A2 in the absence of significant CYP1A1 activity, such as in uninduced liver tissue (Tassaneeyakul *et al*, 1993). Acetanilide 4-hydroxylation was 2.5-fold higher in C3H/HeJ mice, while there was a small but significant difference in chlorzoxazone 6-hydroxylation. The latter reaction is mainly mediated by CYP2E1 activity, although CYP1A2 can also catalyse this reaction in rat and human microsomes *in vitro* (Jayyosi *et al*, 1995; Ono *et al*, 1995). C3H/HeJ microsomes also showed a significant 3.3-fold higher capacity to activate acetaminophen to its reactive metabolite, as determined by the formation of a NAC conjugate (Fig. 3.3B). This reaction is mediated principally by the cytochrome P450 CYP1A2, CYP2E1 and CYP3A (Harvison *et al*, 1988; Patten *et al*, 1993). 3-MC treatment induced a significant increase from control values for EROD and MROD, of both males and females in APN and C3H/HeJ mice ($p<0.002$, Fig. 3.4). MROD was increased 32.6-fold and 90.6-fold in APN males and females, respectively, and 20.5-fold and 30.0-fold in C3H/HeJ males and females, respectively. EROD was increased 38.4-fold and 72.4-fold in APN males and females, respectively and 14.7-fold and 44.2-fold in C3H/HeJ males and females, respectively. The greater fold-induction in APN animals is due, at least in part, to the significantly lower basal activities in these animals. The absolute levels for MROD in 3-MC treated animals differed significantly between APN and C3H/HeJ males ($p<0.005$), but not between APN and C3H/HeJ females. Induced EROD values did not differ significantly between the two strains in either males or females. There was a significant gender difference in induced MROD levels in the APN, but not the C3H/HeJ, strain. Induced EROD levels showed a significant gender difference in both strains ($p<0.005$).

3.3.3: Immunoblot analysis of liver microsomal cytochrome P450 proteins

Liver microsome cytochrome P450 protein levels were compared using microsomal preparations from 6 male mice each for the APN and C3H/HeJ strains. CYP1A2 levels showed a

Figure 3.4: Microsomal CYP1A activities after 3-MC treatment

Microsomal activities in APN males (☐), APN females (◪), C3H/HeJ males (■) and C3H/HeJ females (⊠). Animals were treated with 50 mg/kg *I.P.* 3-MC once daily for three days. Microsomal activities were determined 5 days after the first treatment. Bars indicate mean values for each group. Error bars indicate standard deviations. n = 6 for each group. Statistical comparisons between different groups are made in section 3.3.2.


Rate of Resorufin Dealkylation (nmol/mg protein/minute)
3
2
1
0
Control
3-MC Treated
Control
3-MC Treated
Methoxyresorufin
Ethoxyresorufin

pronounced difference between the two strains compared to CYP2E1 in lumographs of the western blots (Fig. 3.5). A mean 5-fold difference in CYP1A2 signals between strains was determined by densitometric scanning based on the analysis of two independently prepared blots

3.3.4: Semi-quantitative RT-PCR analyses of CYP1A1 and CYP1A2 mRNA levels

CYP1A1 and CYP1A2 cDNAs prepared by oligo dT-primed reverse transcription of total liver RNAs from 6 male APN and C3H/HeJ mice were co-amplified by PCR with GAPDH as an internal control. The co-amplification of CYP1A1 and GAPDH was determined to be linear for both products from 30 to 36 cycles of amplification, and from 2 to 5 μL of 1:5 diluted cDNA as starting material. The CYP1A2/GAPDH reaction was linear from 24 to 32 cycles for both targets and from 1 to 5 μL of 1:50 diluted cDNA as starting material.

Using 35 and 30 cycles of amplification, with 4 and 2 μL of template, respectively, for CYP1A1 and CYP1A2, PCR products were separated by 2.5% agarose gel electrophoresis and stained with ethidium bromide to confirm the specificity of the reactions (Fig. 3.6A). The authenticity of the products was confirmed by sizing against molecular markers as well as by probing of Southern blots with probes complementary to the predicted internal sequences of the amplicons. The reaction conditions produced products which could be resolved to baseline and accurately quantified by HPLC (Fig. 3.6B). The identity of the peaks obtained by HPLC of the PCR products was confirmed by comparing the retention times to authenticated gel-purified standards as well as by the loss of specific peaks when corresponding primers were omitted from the PCR amplification. The results of three independent PCR amplifications for each target indicate that significant variation can occur in the relative amplitude of each product peak between experiments, but that the proportional differences between samples is highly reproducible (Fig. 3.6C). For the CYP1A1 amplifications, differences between the two strains were found to be

Figure 3.5: Immunoblot analysis of liver microsomal proteins from APN and C3H/HeJ mice

Liver microsomal proteins from 6 APN and 6 C3H/HeJ male mice were separated by SDS-PAGE and transferred to nitrocellulose. Specific cytochrome P450 isoforms were detected by enhanced chemiluminescent immunodetection. Blots were prepared from 5 μg (CYP1A2) or 2.5 μg (CYP2E1) total microsomal protein per lane. M: biotinylated molecular weight standards. Numbers at right indicate the mass of markers in kDa. PC: positive controls from liver microsomes from rats induced with β-napthaflavone (CYP1A2) or acetone (CYP2E1).

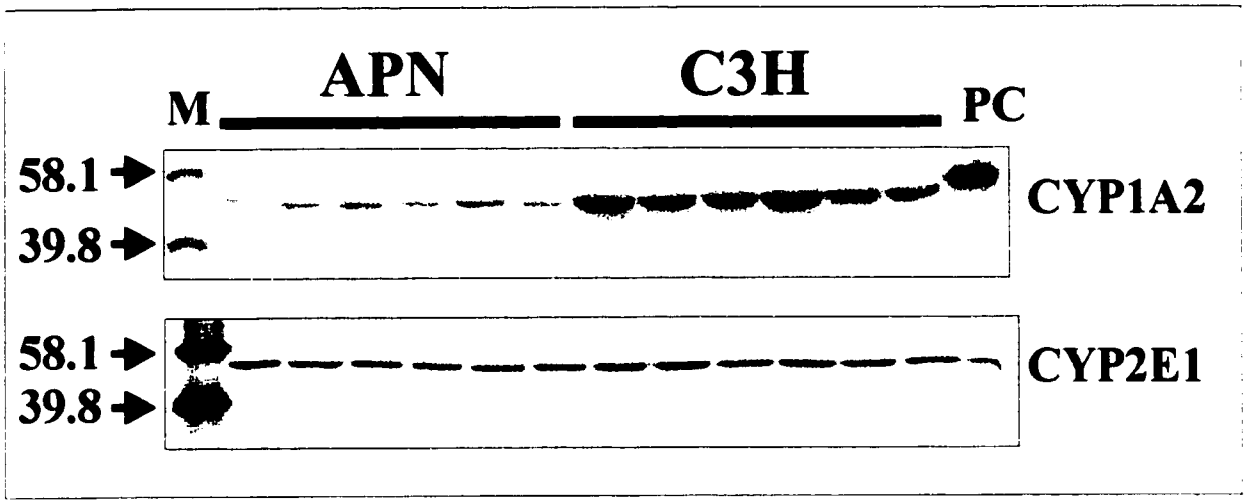
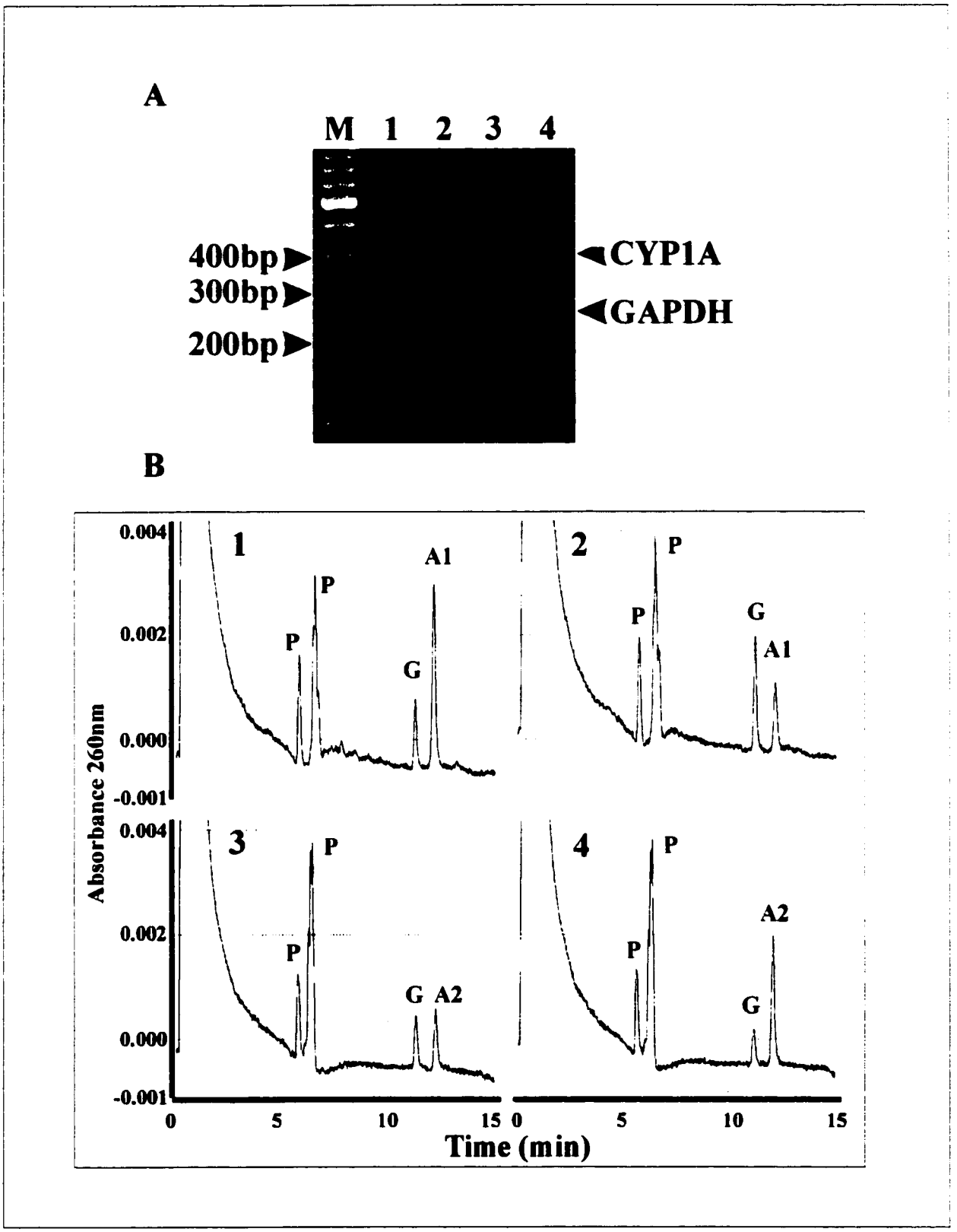
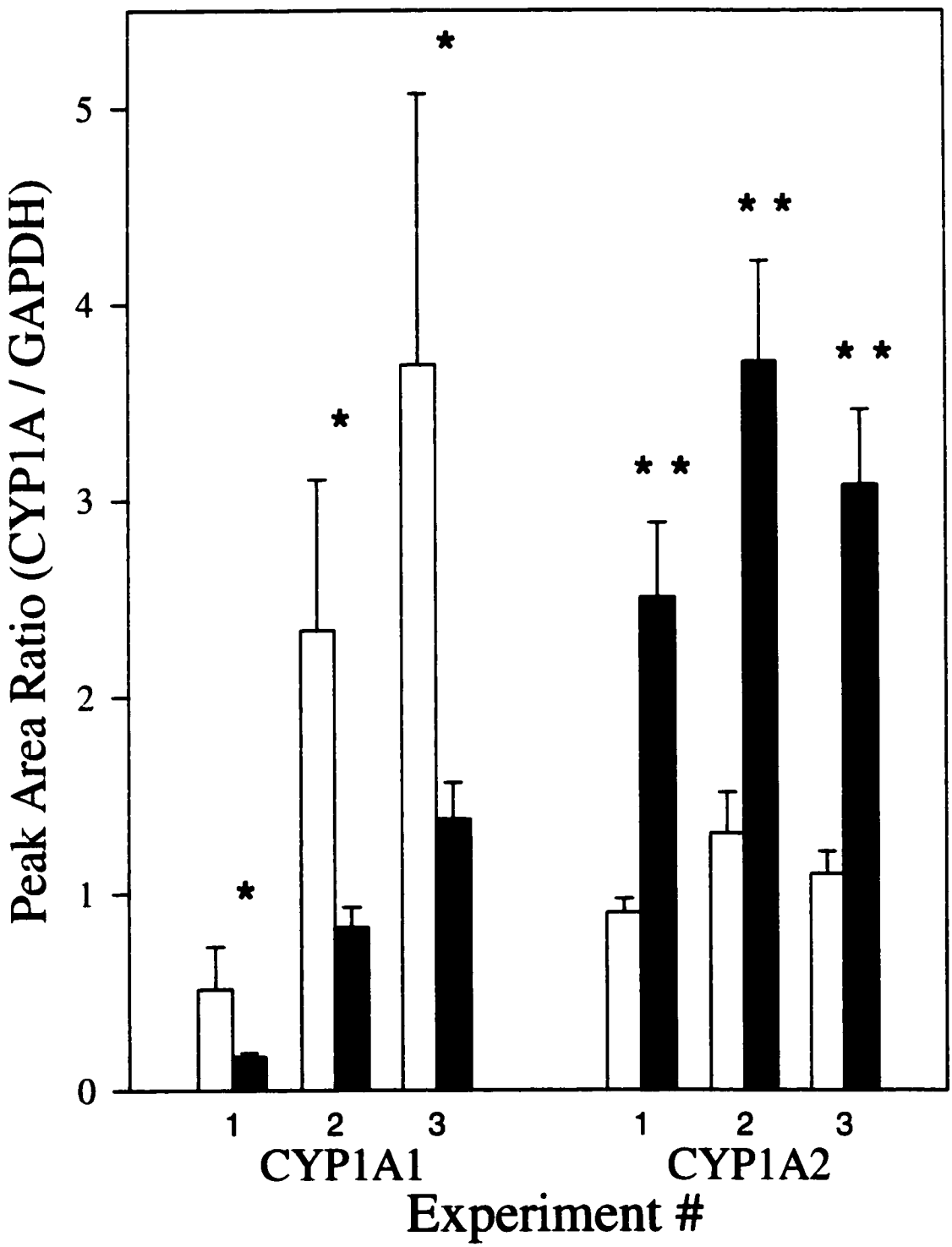

APN
C3H
M
PC
58.1
39.8
CYP1A2
58.1
39.8
CYP2E1

Figure 3.6: Semi-quantitative RT-PCR comparison of CYP1A RNA levels between APN and C3H/HeJ mice

CYP1A1 and CYP1A2 RNA levels in total liver RNA were determined relative to GAPDH RNA levels by co-amplification of oligo dT-primed cDNA with primers specific to GAPDH and either CYP1A1 or CYP1A2. PCR products were quantified by HPLC. **A**: Agarose gel electrophoresis of PCR products. cDNAs from 6 animals each from the APN and C3H/HeJ strains were amplified separately. PCR products from each strain were pooled and 10 μL of pooled products were loaded per lane. Lane 1: CYP1A1/GAPDH from APN pool. Lane 2: CYP1A1/GAPDH from C3H/HeJ pool. Lane 3: CYP1A2/GAPDH from APN pool. Lane 4: CYP1A2/GAPDH from C3H/HeJ pool. M: 100 bp size markers. **B**: Representative HPLC chromatograms of PCR amplifications from individual mice. 1 and 2 are CYP1A1/ GAPDH co-amplifications. 3 and 4 are CYP1A2/GAPDH co-amplifications. 1 and 3 are from an APN mouse liver cDNA. 2 and 4 are from a C3H/HeJ mouse. Peaks are identified as follows: P: PCR primers, G: GAPDH amplicon (251 bp), A1: CYP1A1 amplicon (403 bp), A2: CYP1A2 amplicon (396 bp). **C**: Comparison of PCR CYP1A amplicon levels normalized to GAPDH levels. Results of 3 amplification experiments each for CYP1A1 and CYP1A2 are shown separately. □: APN, ■: C3H/HeJ. Bars indicate mean values for each group. Error bars indicate standard deviations. *: $p < 0.01$, **: $p < 0.005$. n= 6 animals for each group in each experiment.


A
M 1 2 3 4
400bp
300bp
200bp
CYP1A
GAPDH
B
Absorbance 260nm
0.004
0.002
0.000
-0.001
1
2
3
4
P
G
A1
A2
0 5 10 15
Time (min)

C


Peak Area Ratio (CYP1A / GAPDH)
0
1
2
3
4
5
*
*
*
* *
* *
1
2
3
1
2
3
CYP1A1
CYP1A2
Experiment #

3.0-fold, 2.8-fold and 2.7-fold in three experiments. Similarly for CYP1A2, the differences were 2.8-fold in all three experiments. Negative controls (omitting cDNA) produced no detectable product peaks.

3.3.5: Slot blot analysis of UGT1*06 and GSTYa mRNA levels

The mRNA levels of two aromatic hydrocarbon-inducible genes were examined in total liver RNA from 6 males each from the APN and C3H/HeJ strains. No clear differences were observed in the signals obtained by slot blots probed with UGT1*06 or GSTYa-specific probes (Fig. 3.7A). Signal intensities normalized to GAPDH to account for RNA loading variation, were not significantly different for either target sequence between the two strains, although there was significantly more inter-animal variation in the expression levels of UGT1*06 (Fig. 3.7B).

3.4: Discussion

The results presented here demonstrate the influence of genetic factors on caffeine 3-demethylation activity and specifically on the basal hepatic expression of the *Cyp1a2* gene. The observed differences in 1,7-X / 1,3,7-X ratios in plasma may be attributable to factors other than CYP1A2 activity in the case of those strains other than APN and C3H/HeJ, since no other direct evidence was presented for differential CYP1A2 expression. However, the role of CYP1A2 in caffeine 3-demethylation in the mouse is substantial. Studies with CYP1A2 knockout mice have demonstrated that this activity accounts for 87% of the clearance of caffeine at a dose of 2 mg/kg in wild type mice and further concluded that whole blood elimination of the parent compound was more indicative of CYP1A2 activity than urinary metabolite ratios (Buters *et al*, 1996). We had previously demonstrated a significant positive correlation between this plasma metabolite ratio and CYP1A2 mRNA levels in two strains derived from the Swiss-Webster line (See Chpt. 2;

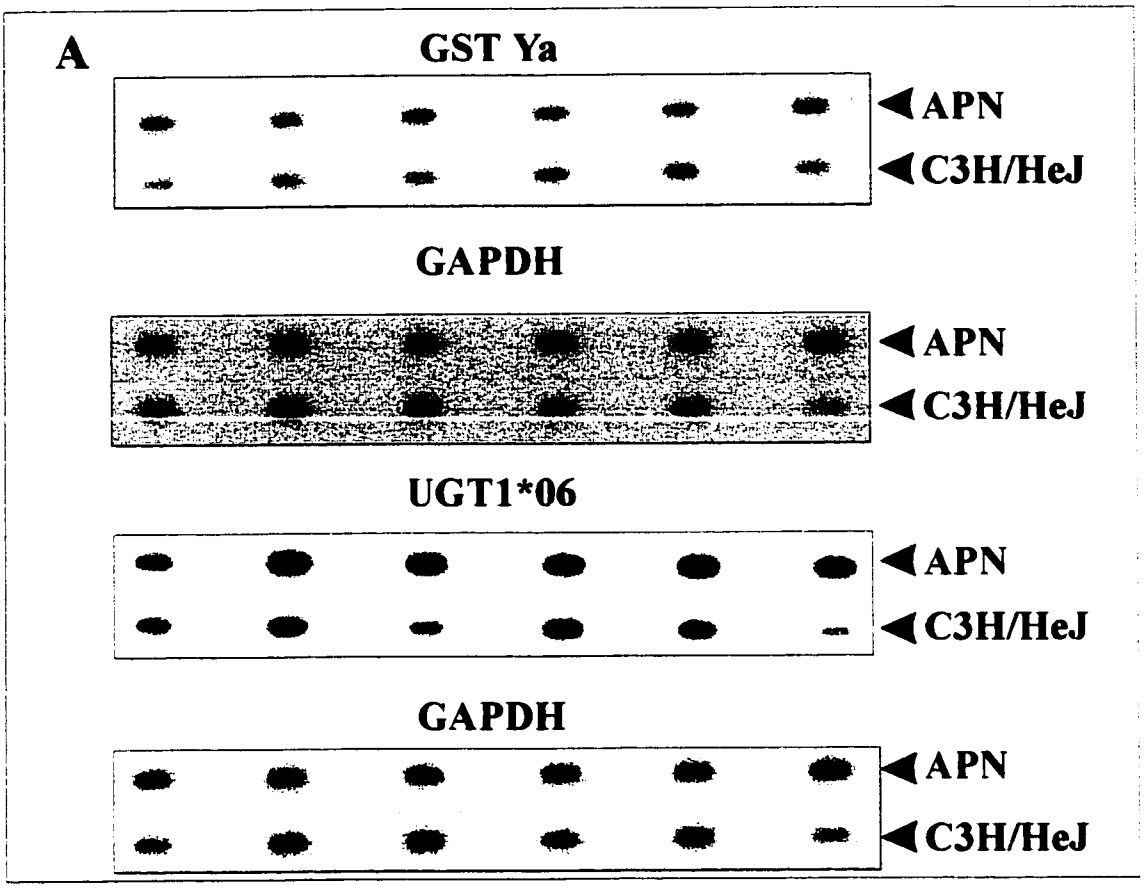
Figure 3.7: Slot blot analyses of GSTYa and UGT1*06 mRNA levels in APN and C3H/HeJ livers

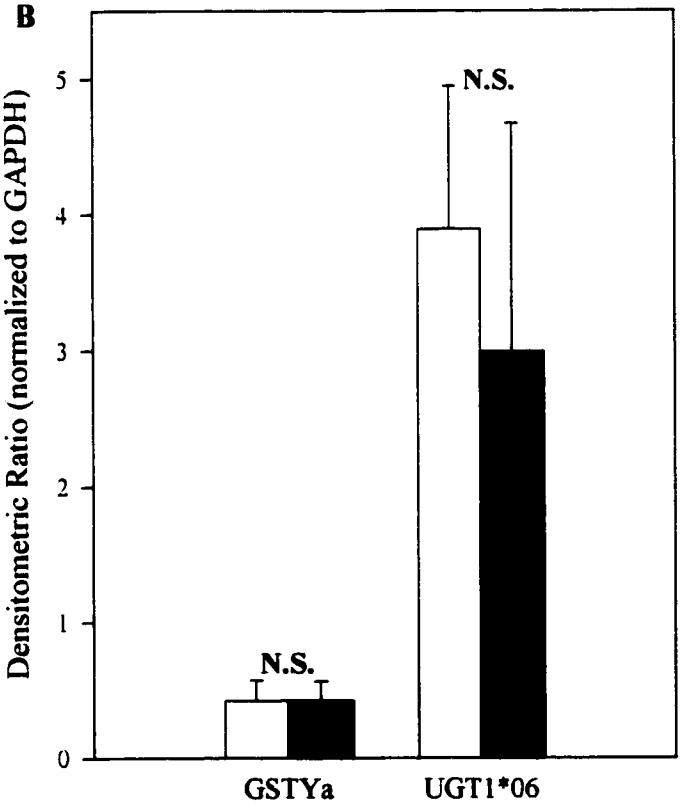
Total liver RNA from male APN and C3H/HeJ mice (5μg / well) was transferred to nylon membranes and probed with ^{32}P-labelled probes specific to the mRNAs of two aromatic hydrocarbon-inducible genes, *GstYa* and *Ugt*106*. Blots were exposed to X-ray film, stripped, re-exposed to confirm stripping, then hybridized with a probe for GAPDH mRNA.

A: Autoradiograms of GSTYa, UGT1*06 RNA levels. Below each is the same blot probed with GAPDH to serve as a control for variation in total RNA amounts between wells.

B: Comparison of RNA levels based on densitometric quantitation of autoradiographic signal intensities. Bars represent means for each group. Error bars indicate standard deviations.

□ : APN, ■: C3H/HeJ. N.S.: Not significantly different ($p > 0.05$), n = 6 for each group.


A
GST Ya
APN
C3H/HeJ
GAPDH
APN
C3H/HeJ
UGT1*06
APN
C3H/HeJ
GAPDH
APN
C3H/HeJ


B
Densitometric Ratio (normalized to GAPDH)
0
1
2
3
4
5
N.S.
N.S.
GSTYa
UGT1*06

Casley *et al*, 1997a). Differences in this parameter did accurately predict significant differences in CYP1A2 expression between these two strains. In the present study, a number of different biochemical and molecular markers for *Cyp1a2* gene expression were chosen to determine both the suitability of caffeine 3-demethylation as a phenotypic surrogate for *Cyp1a2* expression in these two strains, and to investigate the level at which this difference was manifested. The observed differences between the APN and C3H/HeJ strains with respect to CYP1A2 enzyme activity, protein levels and mRNA levels were consistent with the observed differences in plasma 1,7-X/1,3,7-X ratios, and indicate pre-translational differences in gene expression. The C3H/HeJ strain was chosen for more in-depth comparison to the APN strain due to its suitability for genetic analysis of the caffeine metabolism trait in crosses with APN. The C3H/HeJ strain differed significantly (more than 10 fold among males) from the APN strain with respect to the trait under study, and had the lowest intrastrain variance in the trait data among those strains tested. This is an important consideration in planning genetic analyses of quantitative traits. The AH-responsiveness phenotype for this strain has been established (Swanson and Bradfield, 1993), and genetic marker data are readily available (Dietrich *et al*, 1996). A comparison of the *in vitro* activation of acetaminophen suggests that the magnitude of the difference in CYP1A2 expression is pharmacologically relevant, since the P450-generated NAPQI metabolite of acetaminophen is the reactive intermediate which mediates the phase II metabolism and hepatotoxicity of the drug (Birge *et al*, 1990).

The observed gender differences in caffeine 3-demethylation are consistent with human studies in which a decrease in this activity is consistently observed in females relative to males (Relling *et al*, 1992; Horn *et al*, 1995; Carrillo and Benitez, 1996). The gender-dependent decrease in caffeine 3-demethylation is exacerbated in humans by the administration of estrogenic

hormones, such as contraceptive drugs (Bock *et al*, 1994). The present study has identified at least three inbred laboratory strains which might be suitable animal models for the investigation of this phenomenon.

In examining differences in CYP1A2 expression, it is important to identify the role, if any, of AHR-mediated induction of gene expression, since this property is polymorphic among different inbred mouse strains. Binding of ligands to the AH receptor induces transcriptional up-regulation of a number of phase I and phase II enzymes, collectively known as the *Ahr* gene battery. These include the UGT (*Ugt1*06*), the GST Ya subunit (*Gstya*), NAD(P)H:menadione oxidoreductase (*Nmo-1*), class 3 aldehyde dehydrogenase (*Aldh4*) genes, and both members of the *Cyp1a* gene subfamily, *Cyp1a1* and *Cyp1a2* (Nebert, 1989). It had been reported that caffeine could serve as an inducer of this battery in rats, when given twice daily for three days at 50 mg/kg (Goasduff *et al*, 1996). In mice, orally administered caffeine given over 10 days did induce benzo(a)pyrene hydroxylase activity, but only in the *Ahr*-nonresponsive strain DBA/2. The *Ah* responsive strain C57BL/6 showed no increase in metabolic activity after caffeine treatment (Ahokas *et al*, 1981). In our study, we demonstrated the responsiveness of both strains to a classic AHR ligand, 3-MC. Both the APN and C3H/HeJ strains showed marked induction of CYP1A1 and CYP1A2-mediated microsomal activities, indicating intact AHR-mediated induction pathways in both strains. Any differences in AHR-mediated induction were unlikely to influence the results of plasma caffeine/metabolite ratios established only two hours after caffeine administration.

Ahr genotype alone does not predict differences in plasma 1,7-X/1,3,7-X values. In the present study, these values were found to be similar between C3H/HeJ and AKR mice, the former being a responsive and the latter a nonresponsive strain (Swanson and Bradfield, 1993).

The inducer effects of caffeine might influence the analysis of CYP1A2 levels, even though these analyses were made 4 days after a single oral dose of 10 mg/kg. In order to account for this possibility, the mRNA levels of three other *Ahr* battery genes were examined. No significant differences were observed between the two strains for UGT1*06 or GSTYa mRNAs, while hepatic CYP1A1 mRNA levels were actually significantly elevated in the APN strain relative to the C3H/HeJ. These data indicate that differential induction via the AH receptor did not account for the observed differences in CYP1A2 levels.

Semiquantitative RT-PCR was used to assess CYP1A mRNA levels in order to permit accurate comparisons of the low abundance CYYP1A1 transcript in the uninduced liver. The consistent 2-3 fold increase in CYP1A1 mRNA in the APN liver relative to C3H/HeJ, suggests that the basal expression of this gene may also be subject to genetic polymorphism in the mouse.

The distinct differences in CYP1A2 expression between these two strains presents the opportunity for a genetic analysis of factors underlying the regulation of basal expression of this cytochrome P450 enzyme. The development of the APN strain, with its significant divergence from other strains with respect to CYP1A2 expression, offers a unique opportunity for further study. The validity of the 1,7-dimethylxanthine / caffeine plasma ratio as a probe for CYP1A2 activity in the mouse is significant since it will permit the rapid, high throughput, quantitative phenotyping required for such an analysis.

Chapter 4

Chapter 4: Identification of quantitative trait loci affecting caffeine metabolism by genome-wide interval mapping of a C3H/HeJ X APN F_2 intercross

4.1: Introduction

The work described in this chapter involves a quantitative genetic analysis of the variation in caffeine 3-demethylation between two inbred strains, and the genetic mapping of loci affecting this trait within the mouse genome. The work described in this chapter has been submitted for publication in the journal *Drug Metabolism and Disposition.*

In a previous study (see Chpt3; Casley *et al*, 1997b) we demonstrated interstrain variation in the phenotypic trait of caffeine 3-demethylation measured as the ratio of plasma 1,7-dimethylxanthine/caffeine 2 hours after oral dosing with caffeine. We refer to this trait as the caffeine 3-demethylation (C3-D) index. We further demonstrated that this phenotypic parameter predicted significant differences in cytochrome P450 *Cyp1a2* gene expression between the APN inbred strain developed in our laboratory (see Chpt 2; Casley *et al*, 1997a) and a common inbred laboratory strain, C3H/HeJ. These differences may be exploited in an analysis of the genetic determinants of variable caffeine metabolism in mice.

Classical genetic linkage mapping involves finding statistical associations between co-dominant molecular markers and discrete phenotypic classes, both of which follow Mendelian patterns of inheritance. Complex multigenic traits do not, however, follow classical Mendelian inheritance, but rather display continuous phenotypic variation in intercross progeny (reviewed in Avner, 1998). The genetic determinants of such complex phenotypes cannot be mapped by classical linkage approaches. One approach to mapping the genetic determinants of complex phenotypes is to test statistical associations between co-dominant markers and candidate genes.

This approach is limited by the requirement that rational candidate genes that might contribute to the phenotype can be surmised by some understanding of the biological mechanisms underlying the trait. In addition, it is further constrained by the requirement that candidate genes must have been mapped within the genome and genetic markers which are tightly linked to the candidate be available and informative in crosses between the parental strains (Ott, 1979).

Quantitative trait mapping has not previously been applied to variable drug metabolism. Pharmacogenetic studies have previously been confined to the analysis of allelic variants of monogenic traits (Nebert,1997). Novel genetic determinants affecting caffeine metabolism may be of significance in the disposition of other drugs, as well as the sensitivity to environmental toxicants, owing to the broad substrate range of many enzymes involved in the metabolism of caffeine.

In order to carry out the genetic analysis of a quantitative trait, it is necessary to have genetically homogeneous strains with significant differences in the trait under study. The APN and C3H/HeJ inbred mouse strains (Fig. 4.1) meet these requirements as previously determined (see Chpt. 3; Casley *et al*, 1997b). In the present study we have tested the hypothesis that caffeine metabolism, measured as the ratio of serum 1,7-dimethylxanthine/caffeine after an oral dose of caffeine, is a polygenic trait. We further hypothesize that the genetic loci affecting this trait could be mapped within the genome using a maximum likelihood interval mapping approach. In this chapter, we have undertaken such an analysis.

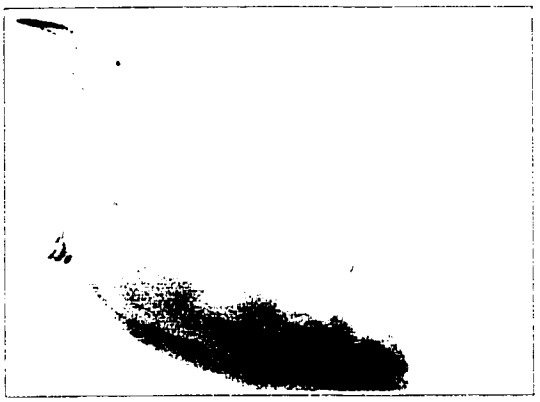
4.2: Materials and methods

4.2.1: Crosses

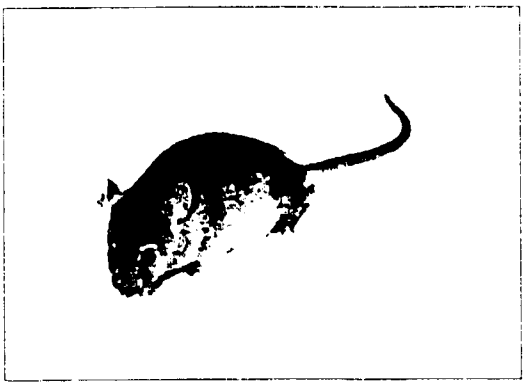
Males and females from the C3H/HeJ and APN parental inbred strains were phenotyped

Figure 4.1: Parental mouse strains for genetic analysis

Adult male mice from the APN and C3H/HeJ inbred strains used to produce F_2 intercross progeny for genetic analysis of variations in caffeine metabolism.



APN



C3H/HeJ

for caffeine metabolism as previously described (see Chpt. 3; Casley *et al*, 1997b). 7 APN females, 6 APN males, 7 C3H/HeJ females and 7 C3H/HeJ males were used in 14 breeding pairs to generate an F_1 of 153 animals. After caffeine phenotyping, 34 breeding pairs from the F_1 were used to generate 39 litters totaling 441 F_2 animals. F_2 animals were phenotyped for C3D index and tail clippings were collected for DNA extraction.

F_1 males were backcrossed to APN females in 4 breeding pairs to produce 35 offspring. In the reciprocal backcross, 4 F_1 females were bred with 3 APN males to generate 35 offspring. Likewise, reciprocal backcrosses to the C3H/HeJ parental strain were performed, producing 25 offspring from 3 C3H/HeJ females, and 53 offspring from 4 F_1 females, C3H/HeJ male breeding pairs. All backcross offspring were phenotyped for C3-D index. All animals were phenotyped at 7 weeks of age.

4.2.2: Statistical analysis of phenotypic data in crosses

Phenotypic data from crosses were compared by application of Students' t-test or the Mann-Whitney rank sum test to detect significant differences between groups. The rank sum test was employed to allow comparisons between groups within which the data were not normally distributed or had unequal variances. The Kolmogorov-Smirnov analysis was used to test for normality of distribution within groups. All analyses were done using the SigmaStat software system (Jandel Scientific, San Rafael, CA, USA). C3-D index phenotypic data from the F_2 males and females were log-transformed to obtain a normal distribution. In order to account for gender differences in analyzing F_2 data, the male and female data sets were merged by normalizing for gender differences. This was done by calculating the gender-specific means and standard deviations for each group after log-transformation, then subtracting the gender-specific mean from each value and dividing the result by the standard deviation of that group (see Taylor and

Phillips, 1996).

Heritability of the C3-D index trait was calculated from data obtained from phenotyping 6 different inbred strains, as described in chapter 3 (Casley *et al*, 1997b), according to Williams *et al*, (1996), using the formula:

$$h^2 = V_g / (V_g + V_e)$$

Where: h^2 = broad sense heritability

V_g = genetic variance (mean variance between inbred strains)

V_e = environmental variance (mean variance within inbred strains)

The number of genes segregating in the F_2 intercross was estimated separately for males and females according to the formula of Wright (1968):

$$N = [(1/k) \cdot (P_1 - P_2)^2] / V_g$$

Where: N = # of segregating loci

1/k = coefficient of difference between the square of parental means (k = 8 for an F_2 intercross)

P_1 , P_2 = phenotypic means of parental strains

V_g = genetic variance

V_g is calculated as : $V_g = V_{F2} - [¼ (V_{P1} + V_{p2}) + ½ (V_{F1})]$

4.2.3: Genomic DNA extraction

The genomic DNA from approximately 1 cm tail clippings from the 438 F_2 animals was extracted using a silica membrane spin column procedure (QIAamp, QIAGEN Inc., Mississauga, ON). Tail clippings were digested overnight with proteinase K at 50°C, and centrifuged for 2 minutes at 14,000 x g to remove any undigested material prior to application of the supernatant to

the column. Purified DNA was eluted in 10 mM Tris, pH 8.8, and stored at -20°C.

For genotyping, genomic DNA from the 92 phenotypically extreme F_2 males and females was diluted to approximately 20 ng/μL in 10 mM Tris, pH 8.8, and aliquoted into a 96 well plate master template. DNA from females of the parental strains and an F_1 female were included as positive controls, and a well containing 10 mM Tris, pH 8.8 only was included as a negative amplification control.

4.2.4: PCR conditions

STR markers were amplified in a 10 μL reaction containing 40 ng genomic DNA, 2.0 mM $MgCl_2$, 200 μM each of dATP, dCTP, dTTP and dGTP, 10 mM TrisHCl, pH8.3, 50 mM KCl, 0.25 units Taq DNA polymerase (Boehringer Mannheim Canada Inc., Laval, PQ) and 240 μM each of forward and reverse primers. Fluorescent dye-labeled and unlabeled primers were obtained from Research Genetics Inc. (Huntsville, AL, USA). 2 μL of genomic DNA was aliquoted into each well of a 96 well plate, preserving the configuration of the genomic DNA master plate, using an 8 channel manual pipettor. The plate was spun briefly to deposit the DNA at the base of the well, then allowed to dry for 2-24 hours at room temperature. 10 μL of PCR reaction mix, excluding genomic DNA, was pipetted into each well using an 8 channel pipettor. Sufficient master mix for 105 reactions was prepared to account for loss during pipetting. Plates were spun briefly, placed into a 96 well format thermocycler (PTC-200, MJ Research, Watertown, MA, USA) and amplified with the following conditions in calculated mode: 2 minutes at 94°C followed by cycles of 20 seconds at 94°C, 45 seconds at 55°C, 1 minute at 72°C, followed by a final extension of 5 minutes at 72°C, and an indefinite hold at 4°C. Samples to be analysed by ethidium bromide staining in agarose gels were amplified for 35 cycles. All others were amplified for 30 cycles. Samples were amplified without oil overlay using a heated lid in conjunction with a single-use

silicon blanket to seal the plates (Microseal A, MJ Research, Watertown, MA, USA).

4.2.5: Selection of markers for a genome-wide scan

All markers used in the genome-wide scan were part of the Whitehead Institute murine STR map (Dietrich *et al* 1996). No allele size data were available for any markers for the novel APN parental strain. Consequently, it was necessary to screen markers for polymorphism between the APN and C3H/HeJ parental strains. 410 unlabeled primer pairs comprising the Murine Screening set from Research Genetics Inc. (Huntsville, AL, USA) as well as 126 additional STR markers were initially screened by amplification of female F_1 heterozygote DNA and resolution of amplicons by electrophoresis in a 3.5% agarose gel with ethidium bromide staining as described below. Those markers that did not demonstrate bi-allelism in the F_1 on agarose gels, but were of interest due to their reported map positions, were resolved on 6% polyacrylamide/urea gels and detected using a $(CA)_{12}$ oligonucleotide probe as described below.

4.2.6: Analysis of amplified STRs

PCR amplicons from each of the 170 primer pairs used in the genetic analysis were analyzed to determine genotypes in one of four ways. Five markers were scored by 5' end-labeling of the forward primer from each pair with ^{32}P and resolving PCR products on 6% polyacrylamide/urea gels as described below. Nine markers were scored by electrophoresis of PCR products in a 3.5% agarose gel and staining with ethidium bromide as described below. Thirteen markers were scored by amplifying with unlabeled primers, resolving the products on 6% polyacrylamide/urea gels and detecting them with a ^{32}P labeled $(CA)_{12}$ oligo probe as described below. The remainder of the markers (143/170) were amplified using fluorescently labeled forward primers and were resolved and detected using a semi-automated capillary electrophoresis genetic analysis system as described below.

4.2.7: Resolution of STR amplicons using polyacrylamide/urea slab gel electrophoresis

Six % polyacrylamide/6M urea slab gels were prepared in 1X TBE buffer using a commercial preparation (Sequagel-6, National Diagnostics, Atlanta, GA, USA). Two 49-well, 0.4mm thickness gels were prepared for each 96 well plate, using two 24 well sharks tooth combs. One of the gel plates was treated with a water repellant coating (Rain-Away, Wynn's Canada, Mississauga, ON), to ensure that the gel would adhere to the opposite plate during disassembly of the plates. Gels were allowed to polymerize for at least 3 hours at room temperature, then were pre-run at a constant power of 60W per gel for 20 minutes using a sequencing gel electrophoresis system (Model S2, Life Technologies Inc., Mississauga, ON).

After amplification, PCR products were diluted 2:1 in the amplification plate with a gel loading buffer containing 98% deionized formamide, 10 mM EDTA pH 8.0, 0.025% xylene cyanol and 0.025% bromophenol blue. This mixture was denatured at 95°C, snap cooled in an ice water bath and 4 μL per well was loaded onto the gel. Eight μL of loading buffer was loaded into the spare rightmost lane of the gel in order to assist in maintaining the orientation of the gel in subsequent manipulations. Samples were electrophoresed for 2 to 3 hours at 60W per gel, depending on the size of the amplicons being resolved. After electrophoresis, gels were handled as described below, depending on the method of detection employed.

4.2.8: Detection of amplicons using ^{32}P-end labeled PCR primers

The forward primer from each PCR primer pair was labeled at the 5' end with ^{32}P by incubation of 15 pmol primer with [γ-^{32}P] ATP and T4 polynucleotide kinase according to Sambrook *et al* (1989). After labeling, the reaction was heated to 65°C for 10 minutes to inactivate the enzyme, then without further purification, mixed with a 5-fold excess of unlabeled forward primer and used in the PCR reaction at a total forward primer concentration of 200 μM.

After electrophoresis of the PCR products on 6% polyacrylamide/urea gels as described above, the gels were removed from the electrophoresis apparatus and allowed to cool briefly at room temperature before separation of the gel plates. The gel was transferred to 3mm Whatmann paper and wrapped in plastic film. Gels were exposed to X-ray film without drying for 1-24 hours to obtain autoradiograms for genotyping. Each lane was scored as either homozygous for either parental allele or heterozygous, using the position of autoradiographic bands from the parental strain and heterozygous F_1 control samples as a reference. Absolute sizes of parental alleles in base pairs were not obtained with this method. All autoradiograms scored using this method were negative for the negative control lane.

4.2.9: Detection of amplicons using agarose gel electrophoresis and ethidium bromide staining

PCR products amplified with unlabeled primers were mixed with 1/5 volume of 15% (W/V) Ficoll loading buffer, containing 0.25% bromophenol blue, in the amplification plate. Ten µL of this mixture was electrophoresed in a 3.5% agarose gel, in 1X TBE buffer. Both the gel and running buffer contained 0.5 µg/mL ethidium bromide. Gels were cast with 2 rows of 30 well combs per gel in a 10 (L) x 15 (W) cm gel format, and run in a wide-mini subcell apparatus for rapid resolution with minimum buffer volume (BioRad Inc., Mississauga, ON). Two gels were required for each 96 well amplification plate. Electrophoresis was carried out at 6 V/cm for 0.75 - 2 hours, depending on the sizes of the alleles being resolved. After electrophoresis, gels were illuminated by 310 nm UV light and photographed for subsequent analysis. Each lane was scored as either homozygous for either parental allele or heterozygous, using the position of bands from the parental strain and heterozygous F_1 control samples as a reference. Absolute sizes of parental alleles in base pairs were not obtained with this method, although DNA size standards were run

on these gels to confirm that the bands corresponding to the C3H/HeJ alleles approximately corresponded to the published size for these alleles. All gel photos scored using this method were negative for the negative control lane. Analysis of markers by this method was limited to those markers in which the APN and C3H/HeJ alleles differed in size by at least 10%.

4.2.10: Detection of amplicons using a $(CA)_{12}$ oligoprobe

After electrophoresis of the unlabeled PCR products on 6% polyacrylamide/urea gels as described above, the gels were removed from the electrophoresis apparatus and allowed to cool briefly at room temperature before separation of the gel plates. The gel was transferred to a dry nylon blotting membrane (HybondN, Amersham Life Science Inc., Oakville, ON) by gently rolling the membrane onto the gel, cutting away the excess gel, then lifting off the membrane with the gel adhering. The membrane was supported on one sheet of 3mm Whatmann paper, the gel covered with plastic film, and the DNA transferred to the membrane in a vacuum gel dryer without heat for 15 minutes as described by Heyer *et al* (1994). After transfer, DNA was bound to the membrane by UV cross linking and the gel removed from the membrane by gentle agitation in 2 x SSC buffer. The membranes were stored at room temperature.

Amplicons were detected using a custom synthesized $(CA)_{12}$ oligonucleotide probe (Life Technologies Inc., Mississauga, ON) directed against the common core dinucleotide repeat sequence of the markers as described by Morsch and Leibenguth (1993). The oligoprobe was 3' end-labeled using ^{32}P-dCTP and terminal deoxynucleotidyl transferase according to Sambrook *et al* (1989). After labeling, probes were purified using a silica gel spin column system (QIAquick, QIAGEN Inc., Mississauga, ON). 100 pmol of oligonucleotide per labeling reaction generated sufficient probe for 4 membranes. Membranes were pre-hybridized in glass hybridization tubes in a rotisserie oven at 42°C in a buffer containing 20% SDS (W/V), 50 PEG 8000 (W/V) and 0.5 M

phosphate buffer, pH 7.2. One quarter of the purified probe from a single labeling reaction was added to each membrane and allowed to hybridize overnight. After hybridization, the probe solution was poured off and the membranes were washed in the tubes at 42°C with 1 wash of 3 X SSC, 0.1% SDS, and two washes of 0.2X SSC, 0.1% SDS. After washing, the membranes were removed from the tubes, wrapped in plastic film and placed on x-ray film for 1 - 24 hours to obtain autoradiograms for genotyping. Each lane was scored as homozygous or heterozygous for either parental allele, using the position of autoradiographic bands from the parental strain and heterozygous F_1 control samples as a reference. Absolute sizes of parental alleles in base pairs were not obtained with this method. All autoradiograms scored using this method were negative for the negative control lane.

4.2.11: Detection of amplicons using capillary electrophoresis and CCD capture of laser-induced fluorescence

PCR amplifications were carried out using forward primers labeled at the 5' end with one of three fluorescent dyes: 4,7,2',7'-tetrachloro-6-carboxyfluorescein, 6-carboxyfluorescein, or 4,7,2',4',5',7'-hexachloro-6-carboxyfluorescein. Labeled primers were obtained from Research Genetics Inc. (Huntsville, AL, USA). After amplification, PCR products were diluted 5-fold to 20-fold, depending on signal intensity, and pooled with other reaction products from the same DNA, maintaining the 96 well plate format. Samples were pooled by selecting markers amplified with different dye labels, or markers amplified with the same dye labels whose alleles can be resolved from one another based on size differences. Typically, between 5 and 8 markers were pooled and analyzed in a single electrophoretic run. Diluted, pooled PCR products were further diluted 1:8 in 12 µL deionized formamide containing a DNA size standard labeled with a fourth fluorescent dye, N,N,N',N'-tetramethyl-6-carboxyrhodamine. The samples were denatured at

95°C in the 96 well plate format capillary electrophoresis sample tray, and quick chilled in an ice water bath.

Samples were analyzed by capillary electrophoresis with laser-induced fluorescence, using an Applied Biosystems Model 310 Genetic Analyzer (PE Biosystems, Mississauga, ON). Samples were run on a 47cm, 50µm i.d. capillary, using GeneScan™ POP4 polymer (PE Biosystems, Mississauga, ON), at 15kV constant voltage for 17 -22 minutes, depending on fragment sizes.

Raw data acquired during electrophoresis were exported to analysis software (GeneScan™ v2.1, PE Biosystems, Mississauga, ON) that determined fragment sizes against internal size standards. Data files generated by the GeneScan software were exported to a second analysis software (Genotyper™ v2.0, PE Biosystems, Mississauga, ON) that applied an algorithm to filter extraneous peaks common to the amplification of STRs, and identified peaks corresponding to one or both of the parental strain alleles in each sample. Peak assignments, in base pairs, were checked manually and exported in spreadsheet format.

4.2.12: Genotypic data analysis

All genotypes obtained from each of the methods described above were manually coded as "A", "B" or "H" for homozygous for the C3H/HeJ allele, homozygous for the APN allele, or heterozygous, respectively. Genotypes were entered into spreadsheet data files (QuattroPro v8.0, Corel Corp. Ottawa, ON) in which the genotypic data for each of the 170 markers used in the analysis were correlated to the phenotypic data for each of the 92 phenotypically extreme individuals of the F_2 . These files also included the complete phenotypic data for that portion of the F_2 whose individuals were not genotyped. These data were compiled into a text format suitable for importation into the genetic analysis software MapMaker 3.0b (Lander *et al,* 1987) and MapMaker QTL 1.1 (Paterson *et al,* 1988) for subsequent analyses.

4.2.13: Construction of a genetic linkage map

A genetic map of the mouse genome was constructed using the genotypic data obtained from 92 F_2 mice derived from an APN X C3H/HeJ intercross. The computer program MapMaker 3.0b (Lander *et al,* 1987) was used to assign markers into linkage groups using a two point linkage analysis with a minimum log likelihood (LOD) threshold score of 3 required to declare two markers as linked. Linkage groups were assigned to specific chromosomes by arbitrarily assigning one marker in the linkage group to a specific chromosome based on its published assignment (Dietrich *et al,* 1996).

Within each linkage group, most likely orders of markers were established by comparing the relative likelihoods of all possible orders of overlapping groups of 6 or more markers at one time. The genetic distance, in Haldane centimorgans, between markers for the most likely marker order was then calculated for each linkage group using a multipoint linkage analysis. Linkage groups, marker orders and map distances were compared to data from the MGD (Blake *et al,* 1998).

4.2.14: Interval mapping of linkage to the caffeine 3-demethylation index trait

LOD contour plots were obtained for each linkage group using the computer program MapMaker QTL 1.1 (Paterson *et al,* 1988). Stepwise LOD values were calculated at 2 cM intervals between markers. LOD plots were obtained from the gender merged F_2 phenotypic data employing genotype data from the 92 phenotypically extreme males and females. As well, gender-specific LOD plots using genotypic data from the 46 phenotypically extreme males or females of the F_2, with their respective log-transformed F_2 phenotypic data were tested. Where merged or gender-specific LOD plots exceeded a value of 2.0, additional markers, mapped to the region of maximal LOD score, were genotyped in an effort to increase and localize the maximal LOD

score. For LOD peaks exceeding 2.8, most likely peak locations, as well as 1 LOD confidence intervals were calculated for the position of a quantitative trait locus (QTL) predicted by the interval mapping data. A LOD score of 2.8 or greater was required to declare a putative QTL, and a score of 4.3 or greater was required to declare significant linkage to a QTL, as suggested by Lander and Kruglyak (1995). Where a QTL accounting for a significant fraction of the variation in the phenotypic data was identified, the contribution of this locus to the total phenotypic variation was fixed and the genome re-screened to search for other QTLs. Putative QTLs on chromosomes 1 and 9 were assessed for epistatic interactions in pairwise combinations according to the method of Peirce *et al* (1998), in which phenotypic data are compared for statistical differences between doubly homozygous and reciprocal singly homozygous classes for markers at or near the LOD peak for each QTL. Epistasis between all putative QTLs was also tested by fixing the contribution of each QTL and determining the LOD score for each of the other candidate linkage groups in turn.

4.3: Results

4.3.1: Analysis of C3-D index phenotypic data from crosses

Crosses of the two parental strains produced a F_1 population with gender-specific means that were significantly different from one another and from either of the parental strain gender-specific means. The gender-specific data for the F_1 backcross to the APN strain were significantly different from the F_1 and shifted toward the APN strain values. The gender specific data for the F_1 backcross to the C3H/HeJ strain were significantly different from the F1 values and shifted toward the C3H/HeJ strain values. The F_1 phenotypic data did not vary significantly from the F_2. Significant differences between male and female progeny were found in all the crosses

undertaken. These data are summarized in Fig. 4.2 and Tables 4.1 A-C.

Broad sense heritability (h^2) was determined across 6 inbred strains, for males and females independently. Heritability of the C3-D index trait was not calculated from the strains used to generate the F_2, owing to the selection of one parental strain (APN) using a trait, acetaminophen-induced hepatotoxicity, correlated to the trait under consideration. h^2 was determined to be 0.56 in males and 0.44 in females.

The number of segregating loci in the F_2 intercross was estimated as 4.3 for females and 3.4 for males according to the formula of Wright (1968).

A comparison of the progeny of reciprocal crosses used to generate the F_1 indicated the possibility of either sex linkage, a maternal effect, or both. F_1 males derived from C3H/HeJ dams (n=27, $\bar{x} = 0.619 \pm 0.124$) were significantly different from F_1 males derived from APN dams (n=44, $\bar{x} = 0.689 \pm 0.114$) by Mann-Whitney rank sum test ($p<0.01$). This is consistent with a sex linkage effect. However, F_1 females from C3H dams (n=25, $\bar{x} = 0.418 \pm 0.070$) also differed significantly ($p< 0.005$) from F_1 females from APN dams (n=57, $\bar{x} = 0.372 \pm 0.076$), suggesting a possible maternal effect.

4.3.2: Genotyping STR markers

Using the methods described above, alleles from either parental strain could be easily resolved for each of the 170 markers used in the analysis (Fig. 4.3). Occasional spot failures of the PCR did occur in some plates, but at least 86 of 92 possible genotypes were obtained for each marker.

4.3.3: Genetic linkage map

From the 536 STR markers screened, 170 were selected based on their informativeness in the experimental cross, and their map positions. Genotypes for these 170 STR markers from 92 F_2

Figure 4.2: Distribution of Caffeine 3-demethylation (C3-D) indices among progeny of different crosses

C3-D indices were calculated from serum caffeine and 1,7-dimethylxanthine levels 2 hours after oral dosing with 10mg/kg caffeine. Each point represents the C3-D value for a single animal within each class. Horizontal lines indicate means for each group. APN, C3H/HeJ: parental strains; F_1: progeny of parental cross; F_1 X APN: progeny of backcross of F_1 to APN parental strain; F_1 X C3H/HeJ: progeny of backcross of F_1 to C3H/HeJ parental strain; F_2: progeny of F_1 self cross. Males (♂) and females (♀) were considered separately.

Statistical comparisons between groups are made in Table 4.1.

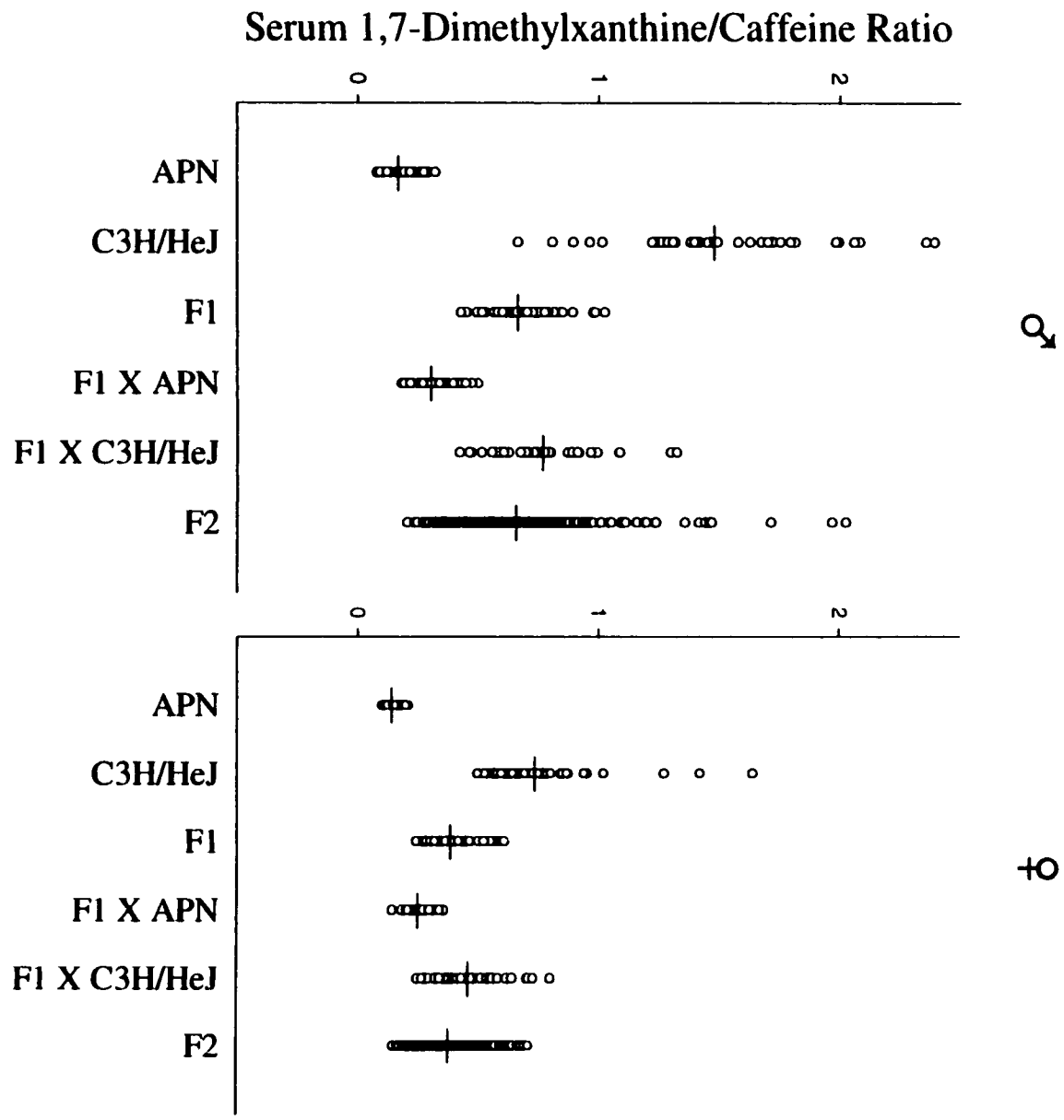

Serum 1,7-Dimethylxanthine/Caffeine Ratio
0
1
2
APN
C3H/HeJ
F1
F1 X APN
F1 X C3H/HeJ
F2
♂
0
1
2
APN
C3H/HeJ
F1
F1 X APN
F1 X C3H/HeJ
F2
♀

Table 4.1: Statistical data for C3-D indices in genetic crosses

A: Means and standard deviations were calculated for parental, F_1, F_2 and backcross groups. Values were calculated separately for males and females.

B: Statistical comparisons between all groups generated from parental mice, F_1 and F_2 intercrosses. All comparisons were made using the Mann-Whitney rank sum test. Shaded areas indicate groups with $p \geq 0.05$.

C: Statistical comparisons of backcross mice with all other groups. Shaded areas indicate comparisons between males, unshaded areas indicate comparisons between females, double lined boxes indicate comparisons between males and females.

A

♂	n	$\bar{x}$	σ	♀	n	$\bar{x}$	σ
APN	36	0.188	0.071	APN	37	0.15	0.032
C3H	30	1.464	0.279	C3H	30	0.667	0.111
F_1	71	0.663	0.122	F_1	82	0.386	0.077
F_1 x APN	42	0.303	0.086	F_1 x APN	28	0.249	0.048
F_1 x C3H	37	0.766	0.215	F_1 x C3H	40	0.462	0.145
F_2	219	0.655	0.298	F_2	220	0.379	0.118

B

	APN ♀	C3H ♂	C3H ♀	F_1 ♂	F_1 ♀	F_2 ♂	F_2 ♀
APN ♂	P = 0.013	p<0.001	-	p<0.001	-	p<0.001	-
APN ♀	-	-	p<0.001	-	p<0.001	-	p<0.001
C3H ♂	-	-	p<0.001	p<0.001	-	p<0.001	-
C3H ♀	-	-	-	-	p<0.001	-	p<0.001
F_1 ♂	-	-	-	-	-	p = 0.083	-
F_1 ♀	-	-	-	-	-	-	p = 0.249
F_2 ♂	-	-	-	-	-	-	p<0.001

C

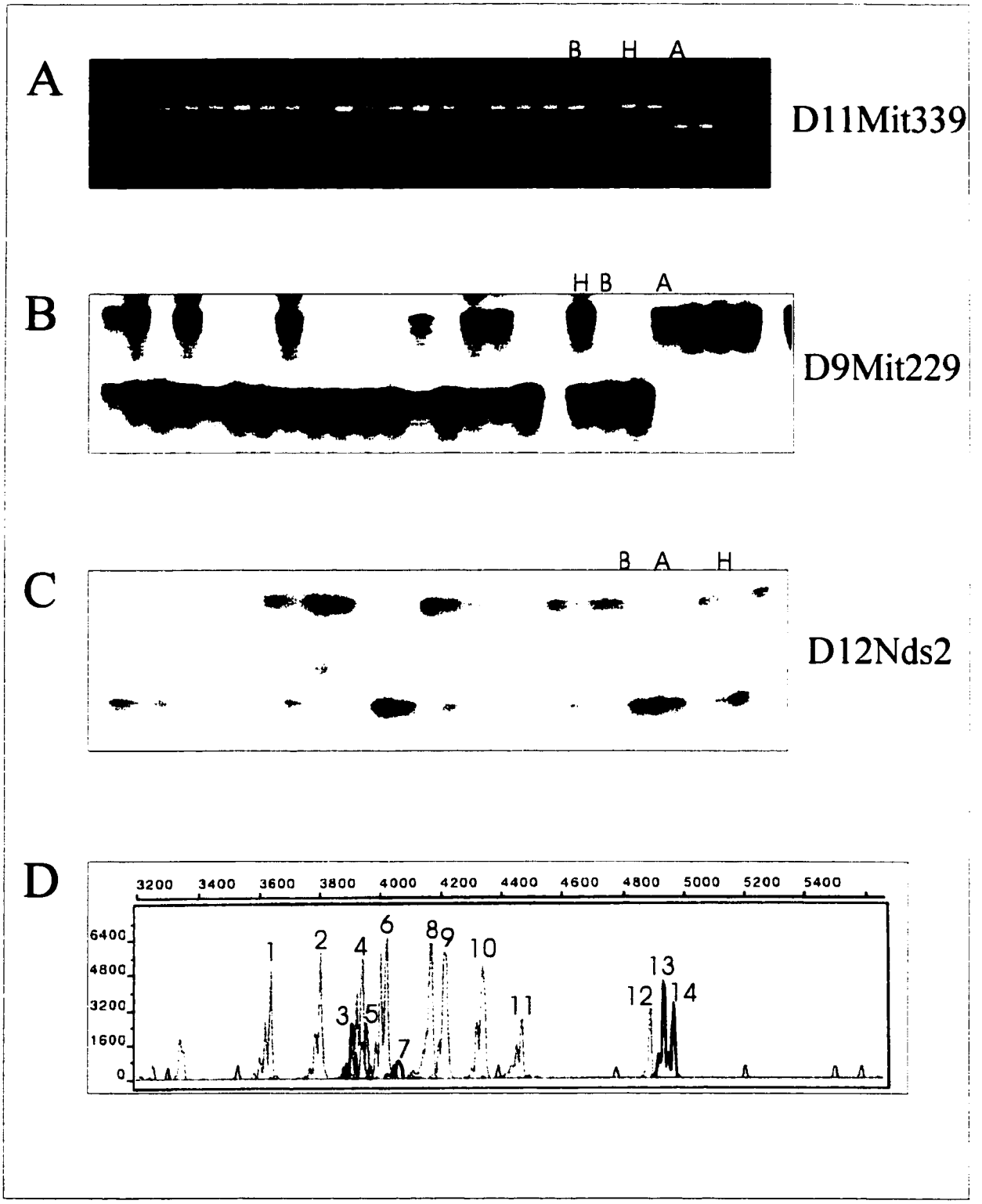
	APN	C3H	F_1	F_1 x APN	F_1 x C3H
F_1 x APN	p<0.001	p<0.001	p<0.001	p = 0.011	p<0.001
F_1 x C3H	p<0.001	p<0.001	p = 0.014	p<0.001	p<0.001
APN	-			p<0.001	p<0.001
C3H		-		p<0.001	p<0.001
F_1			-	p<0.001	p = 0.011

Figure 4.3: Raw data obtained with different genotyping methods

STR microsatellite markers were genotyped by resolving APN and C3H/HeJ-derived alleles by 4 different methods. Samples of raw data from each of these methods are shown. For panels A-C the letters A, B and H represent examples of the C3H/HeJ homozygote, the APN homozygote and the C3H/HeJ/APN heterozygote allelic patterns, respectively. **A:** PCR products for marker D11Mit339 from 24 individual mice resolved by electrophoresis in a 3.5% agarose gel and staining with ethidium bromide for UV fluorescence.

B: ^{32}P-end labeled PCR products for marker D9Mit229 from 24 individual mice, resolved on a 6% polyacrylamide/urea sequencing gel and detected by autoradiography of the undried gel.

C: PCR products for marker D12Nds2 from 28 individual mice resolved on a 6% polyacrylamide /urea sequencing gel and detected by blotting the DNA onto a nylon membrane and probing with a ^{32}P-labeled probe directed against the core repeat sequence of the STR, followed by autoradiography. **D:** Electropherogram of fluorescently labeled PCR products from 7 different chromosome 13 markers for one individual mouse, resolved by capillary electrophoresis through a polymer filled capillary, and detected by charge-coupled device capture of laser-induced fluorescence. Peaks 1,2,5,8, and 11: 6-carboxyfluorescein labeled. Peaks 3,7,13 and 14: 4,7,2',-4',5',7'-hexachloro-6-carboxyfluorescein labeled. Peaks 4,6,9 10 and 12: 4,7,2',7'-tetrachloro-6-carboxyfluoroscein labeled..Peak 1: D13Mit191 C3H/HeJ homozygote. Peaks 2 and 5: D13Mit207 heterozygote, Peaks 3 and 7: D13Mit186 heterozygote, Peaks 4 and 6: D13Mit230 heterozygote, Peaks 8 and 11: D13Mit16 heterozygote, Peaks 9 and 10: D13Mit35 heterozygote, Peaks 13 and 14: D13Mit204 heterozygote, Peak 12: non genotype-specific product generated in D13Mit230 amplification. Red peaks are internal size standards from 75 to 350 base pairs.


A
B H A
D11Mit339
B
H B A
D9Mit229
C
B A H
D12Nds2
D
3200 3400 3600 3800 4000 4200 4400 4600 4800 5000 5200 5400
6400
4800
3200
1600
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14

progeny (46 male and 46 female) were used to sort markers into linkage groups based on a transitive two point analysis. Markers were organized into 20 linkage groups at a minimum LOD score of 3.0 required to declare linkage between markers. The linkage groups obtained in this way corresponded to the chromosomal assignments for these markers reported by Dietrich *et al* (1996) and confirmed in the 1998 chromosome committee reports in the MGD (Blake *et al*, 1998). Most likely marker orders determined using the MapMaker 3.0b program agreed with those published in the latest chromosome committee reports. Map distances between markers were generally similar to reported values, with some exceptions. Most notable was a substantial compression of genetic distance between markers D6Mit123 and D6Mit149 on chromosome 6. The reported map distances for these markers was 17cM, while the present experiment determined a distance of 2.4cM on duplicate genotyping. Two markers, D7Mit215 and D8Mit114 were found to be unlinked with respect to the other markers on their assigned chromosomes after duplicate genotyping and were excluded from the analysis.

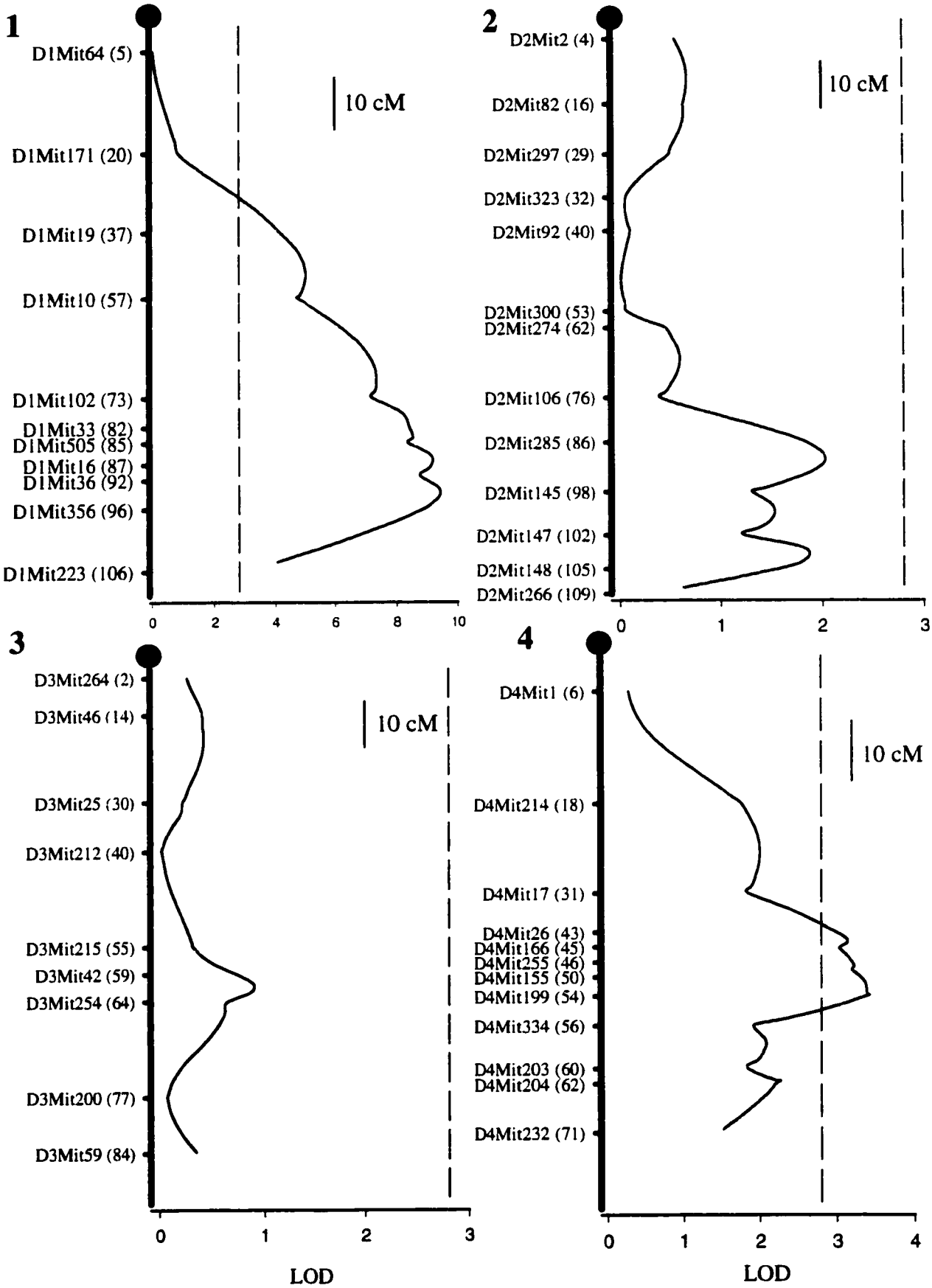
Chromosome assignments and map distances between markers are presented in Fig. 4.4. The selected screening set was used to obtain coverage of the genome at approximately a 13cM interval, as well as achieve more dense coverage in regions of interest. The genomic coverage achieved with this mapping set is summarized in Table 4.2.

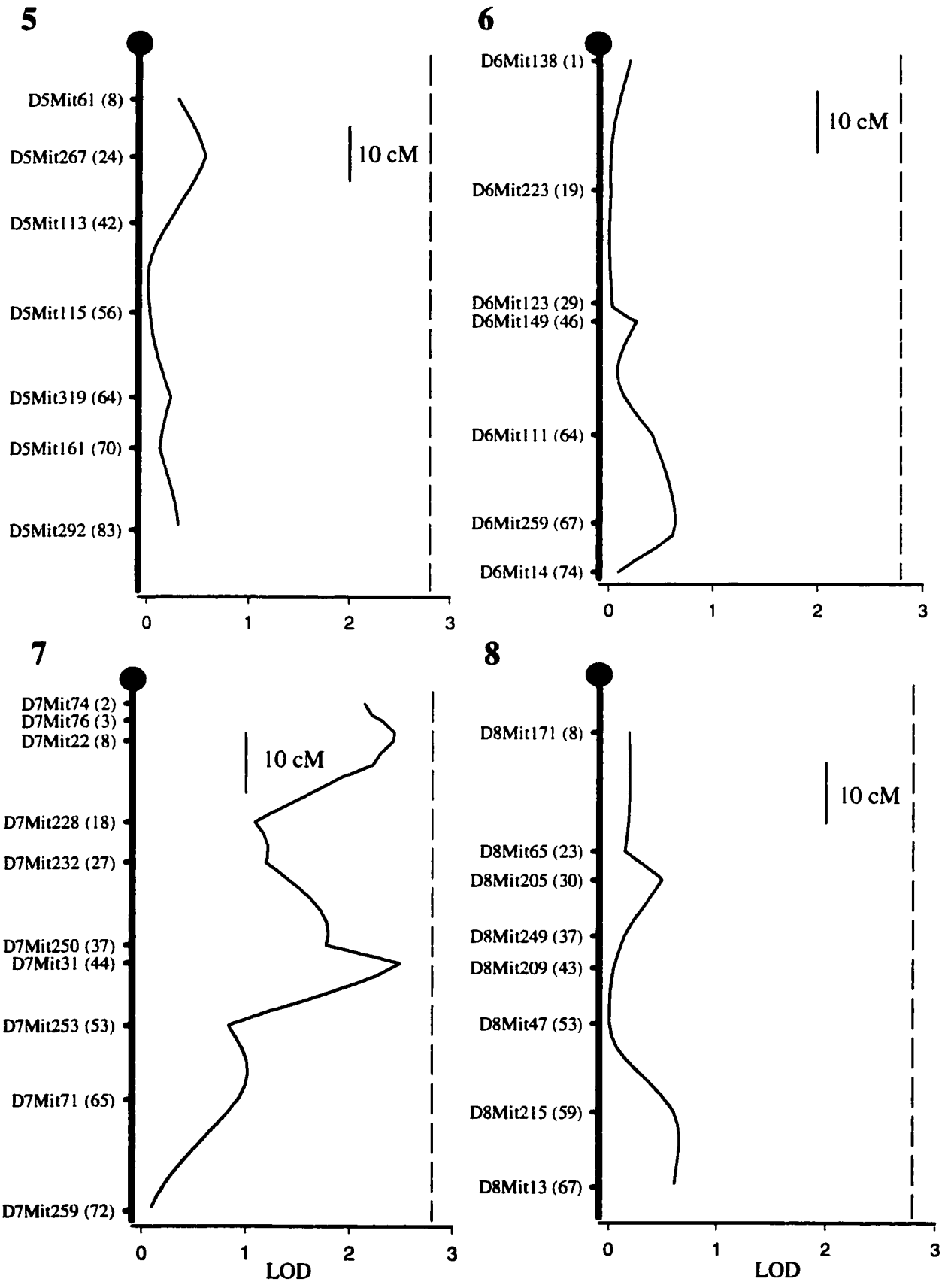
4.3.4: Interval mapping

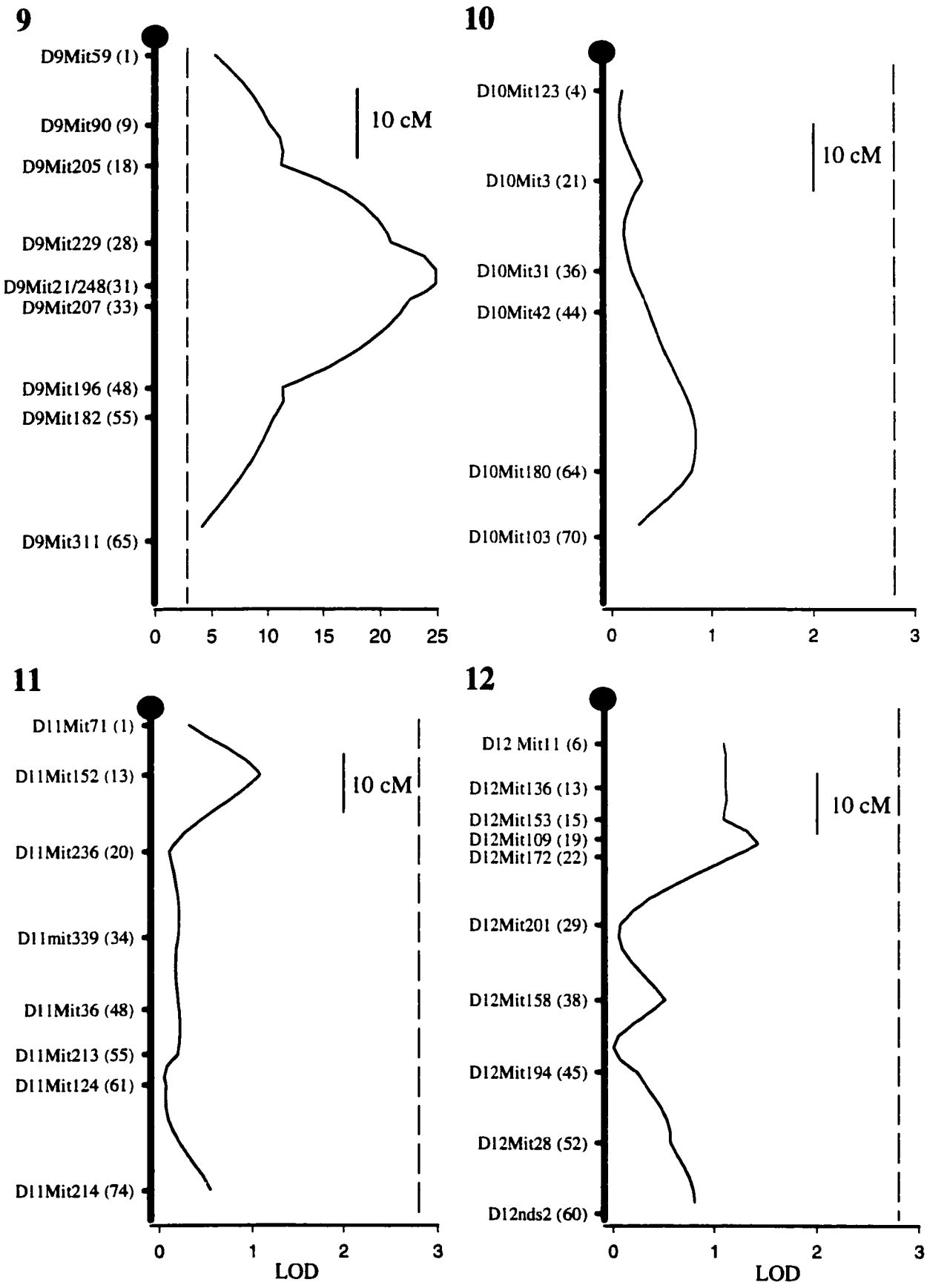
LOD plots were calculated for each linkage group using MapMaker QTL 1.1 (Paterson *et al*, 1988). LOD plots were calculated for gender merged, log transformed male and log transformed female data sets. The X chromosome was not included in gender merged data sets. A LOD plot was calculated for this chromosome using Log-transformed female data. Linkage to the X-chromosome in the male data set was calculated by testing for significant differences in

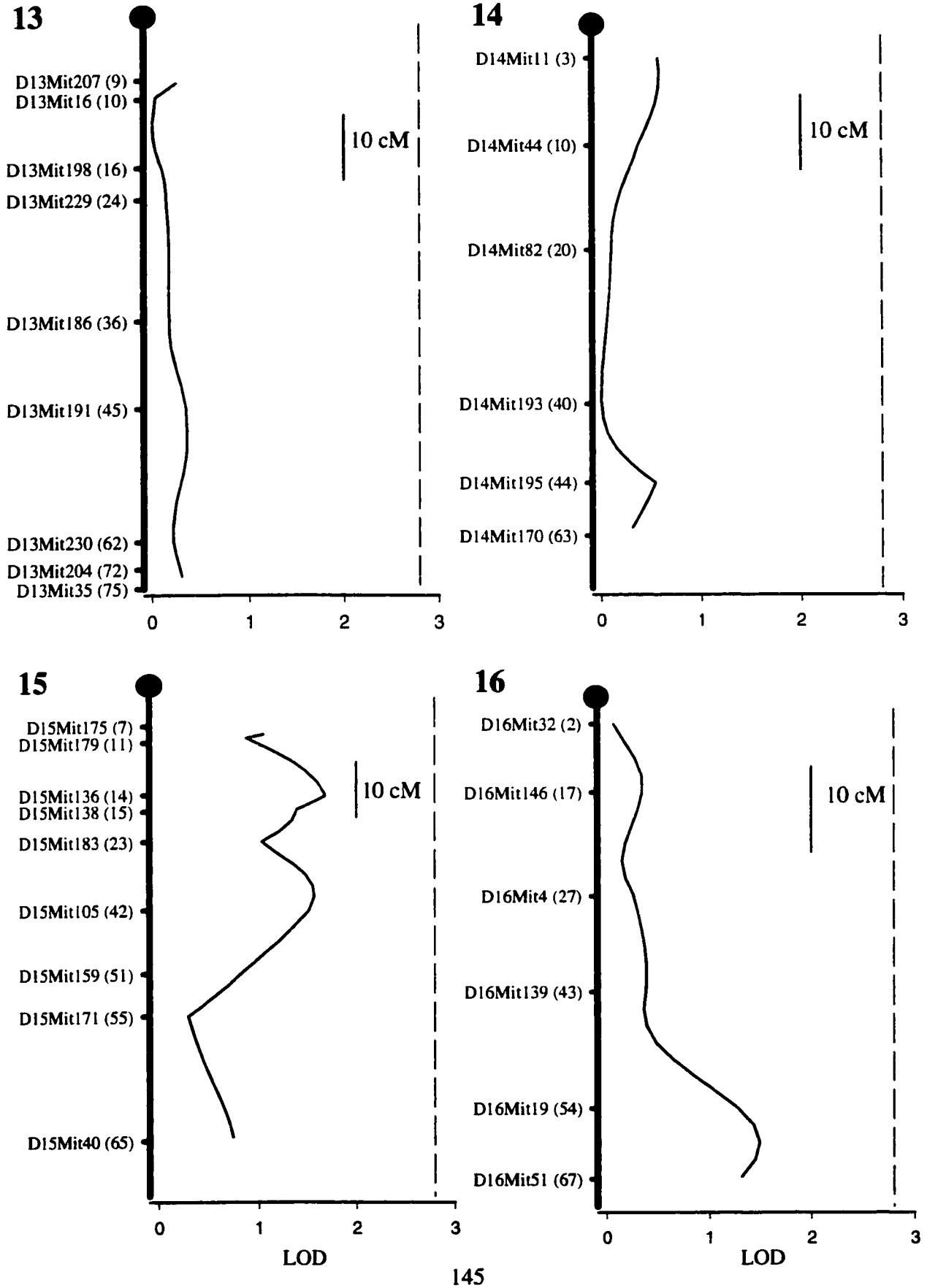
Figure 4.4: Log likelihood (LOD) plots for each linkage group in the genome-wide scan of the APN X C3H/HeJ F_2 intercross

LOD plots were obtained from log transformed, gender merged serum 1,7-dimethylxanthine/caffeine phenotypes from 438 F_2 animals and genotypic data from the 92 phenotypically extreme animals using MapMakerQTL 1.1 (Paterson *et al*, 1988). LOD values were plotted at 2 cM intervals. Chromosome numbers are indicated to the upper left of each plot. Distances between markers are drawn to scale and based on the values obtained in constructing the linkage map from 92 F_2 animals using MapMaker 3.0b (Lander *et al*, 1987). Markers are identified to the left of each chromosome with the published chromosome committee marker position from the MGD (Blake *et al*, 1998) in parenthesis. Chromosomes are not drawn to scale with respect to one another. The vertical solid line in the upper right of each plot represents a genetic distance of 10 centimorgans for that plot. The vertical dashed line in each plot indicates a LOD value of 2.8 .


1
D1Mit64 (5)
D1Mit171 (20)
D1Mit19 (37)
D1Mit10 (57)
D1Mit102 (73)
D1Mit33 (82)
D1Mit505 (85)
D1Mit16 (87)
D1Mit36 (92)
D1Mit356 (96)
D1Mit223 (106)
10 cM
0 2 4 6 8 10
2
D2Mit2 (4)
D2Mit82 (16)
D2Mit297 (29)
D2Mit323 (32)
D2Mit92 (40)
D2Mit300 (53)
D2Mit274 (62)
D2Mit106 (76)
D2Mit285 (86)
D2Mit145 (98)
D2Mit147 (102)
D2Mit148 (105)
D2Mit266 (109)
10 cM
0 1 2 3
3
D3Mit264 (2)
D3Mit46 (14)
D3Mit25 (30)
D3Mit212 (40)
D3Mit215 (55)
D3Mit42 (59)
D3Mit254 (64)
D3Mit200 (77)
D3Mit59 (84)
10 cM
0 1 2 3
LOD
4
D4Mit1 (6)
D4Mit214 (18)
D4Mit17 (31)
D4Mit26 (43)
D4Mit166 (45)
D4Mit255 (46)
D4Mit155 (50)
D4Mit199 (54)
D4Mit334 (56)
D4Mit203 (60)
D4Mit204 (62)
D4Mit232 (71)
10 cM
0 1 2 3 4
LOD


5
D5Mit61 (8)
D5Mit267 (24)
D5Mit113 (42)
D5Mit115 (56)
D5Mit319 (64)
D5Mit161 (70)
D5Mit292 (83)
10 cM
0
1
2
3
6
D6Mit138 (1)
D6Mit223 (19)
D6Mit123 (29)
D6Mit149 (46)
D6Mit111 (64)
D6Mit259 (67)
D6Mit14 (74)
10 cM
0
1
2
3
7
D7Mit74 (2)
D7Mit76 (3)
D7Mit22 (8)
D7Mit228 (18)
D7Mit232 (27)
D7Mit250 (37)
D7Mit31 (44)
D7Mit253 (53)
D7Mit71 (65)
D7Mit259 (72)
10 cM
0
1
2
3
LOD
8
D8Mit171 (8)
D8Mit65 (23)
D8Mit205 (30)
D8Mit249 (37)
D8Mit209 (43)
D8Mit47 (53)
D8Mit215 (59)
D8Mit13 (67)
10 cM
0
1
2
3
LOD


9
D9Mit59 (1)
D9Mit90 (9)
D9Mit205 (18)
D9Mit229 (28)
D9Mit21/248(31)
D9Mit207 (33)
D9Mit196 (48)
D9Mit182 (55)
D9Mit311 (65)
10 cM
0 5 10 15 20 25
10
D10Mit123 (4)
D10Mit3 (21)
D10Mit31 (36)
D10Mit42 (44)
D10Mit180 (64)
D10Mit103 (70)
10 cM
0 1 2 3
11
D11Mit71 (1)
D11Mit152 (13)
D11Mit236 (20)
D11mit339 (34)
D11Mit36 (48)
D11Mit213 (55)
D11Mit124 (61)
D11Mit214 (74)
10 cM
0 1 2 3
LOD
12
D12 Mit11 (6)
D12Mit136 (13)
D12Mit153 (15)
D12Mit109 (19)
D12Mit172 (22)
D12Mit201 (29)
D12Mit158 (38)
D12Mit194 (45)
D12Mit28 (52)
D12nds2 (60)
10 cM
0 1 2 3
LOD


13
D13Mit207 (9)
D13Mit16 (10)
D13Mit198 (16)
D13Mit229 (24)
D13Mit186 (36)
D13Mit191 (45)
D13Mit230 (62)
D13Mit204 (72)
D13Mit35 (75)
10 cM
0
1
2
3
14
D14Mit11 (3)
D14Mit44 (10)
D14Mit82 (20)
D14Mit193 (40)
D14Mit195 (44)
D14Mit170 (63)
10 cM
0
1
2
3
15
D15Mit175 (7)
D15Mit179 (11)
D15Mit136 (14)
D15Mit138 (15)
D15Mit183 (23)
D15Mit105 (42)
D15Mit159 (51)
D15Mit171 (55)
D15Mit40 (65)
10 cM
0
1
2
3
LOD
16
D16Mit32 (2)
D16Mit146 (17)
D16Mit4 (27)
D16Mit139 (43)
D16Mit19 (54)
D16Mit51 (67)
10 cM
0
1
2
3
LOD

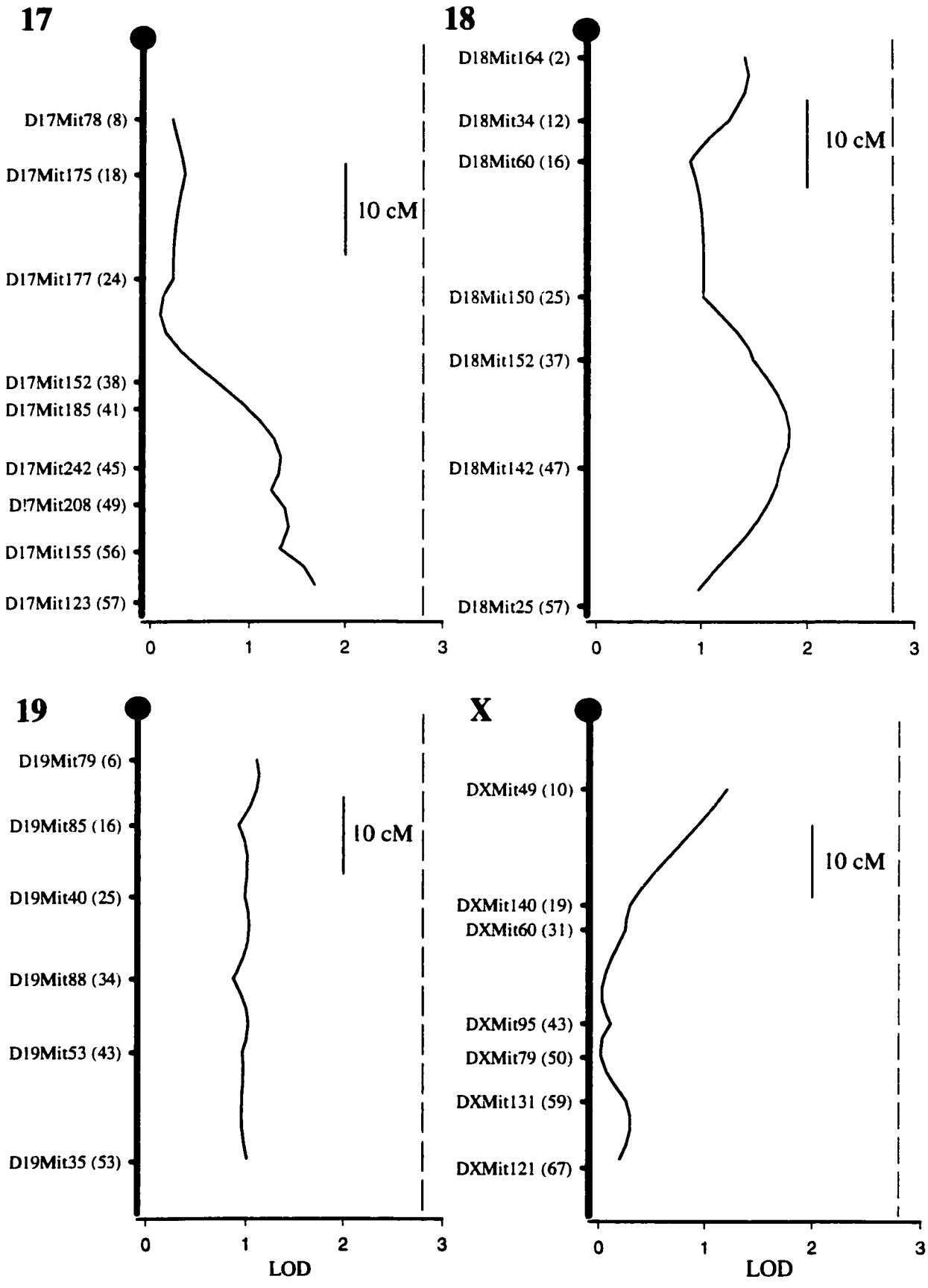

17
D17Mit78 (8)
D17Mit175 (18)
D17Mit177 (24)
D17Mit152 (38)
D17Mit185 (41)
D17Mit242 (45)
D!7Mit208 (49)
D17Mit155 (56)
D17Mit123 (57)
10 cM
0
1
2
3
18
D18Mit164 (2)
D18Mit34 (12)
D18Mit60 (16)
D18Mit150 (25)
D18Mit152 (37)
D18Mit142 (47)
D18Mit25 (57)
10 cM
0
1
2
3
19
D19Mit79 (6)
D19Mit85 (16)
D19Mit40 (25)
D19Mit88 (34)
D19Mit53 (43)
D19Mit35 (53)
10 cM
0
1
2
3
LOD
X
DXMit49 (10)
DXMit140 (19)
DXMit60 (31)
DXMit95 (43)
DXMit79 (50)
DXMit131 (59)
DXMit121 (67)
10 cM
0
1
2
3
LOD

Table 4.2: Genomic coverage obtained with the APN X C3H/HeJ marker set

Chromosomal assignments and mean marker intervals were determined using MapMaker 3.0b (Lander *et al*, 1987). Chromosome lengths were taken from the 1998 chromosome committee reports in the MGD (Blake *et al*, 1998). Genomic coverage and maximum coverage intervals were calculated using reported chromosome lengths and reported marker positions for the most proximal and distal markers relative to the top and bottom of the linkage group, respectively.

[a] 100% coverage interval is the largest interval required to obtain 100% coverage within a linkage group

[b]X chromosome data calculated from females only.

Chromosome	Length (cM)	# Markers	Mean Interval (cM)	Coverage within 10cM of Marker (%)	Largest Interval (cM)	100% Coverage Interval[a] (cM)
1	110	11	9.4	100	19.6	9.8
2	109	13	9.5	100	17.3	8.7
3	95	9	11.6	97.7	20.7	10.9
4	84	12	7.2	96.4	18.9	13
5	94	7	12.2	98.9	16.4	11
6	75	7	10.6	98.5	21.1	10.6
7	74	10	8.1	100	18.6	9.3
8	72	8	9.9	100	19.8	9.9
9	74	10	7.3	100	18	9
10	80	6	11.6	95	24	12
11	79	8	9.6	100	18.3	9.2
12	60	10	8.5	100	12.6	6.3
13	75	9	9.7	99.3	20.5	10.3
14	70	6	10.6	99.1	20.6	10.3
15	75	9	9.1	96.4	22.7	11.4
16	70	6	8.3	100	13.6	6.8
17	58	9	6.3	100	11.5	5.8
18	58	7	8.3	100	15.9	8
19	60	6	9.4	100	14.4	7.2
X[b]	74	7	8.6	100	16.1	8.1
Genome	1546	170	-	99	24	13

phenotypic data associated with the "A" *versus* "B" genotype at each X-linked marker using the Mann Whitney rank sum test. The minimum p value obtained was p = 0.035 at marker DXMit140. A p value less than 0.0016 is considered as the threshold for "suggestive linkage" (Lander and Kruglyak, 1995). LOD scores exceeding 2.0 were obtained in the initial genome scan for chromosomes 1, 2,4,7 and 9 for the gender merged data set. Additional LOD peaks greater than 2.0 were obtained for chromosome 15 ($LOD_{max} = 2.1$) and chromosome 17 ($LOD_{max} = 2.8$) in the male data set, and for chromosome 16 ($LOD_{max} = 2.2$) in the female data set. The maximal LOD value for chromosomes 2 and 4 fell below 2 in the gender-specific data sets. Further analysis with increased marker density in the region of these LOD peaks did not increase the recalculated LOD values beyond 2.8, the threshold for suggestive linkage proposed by Lander and Kruglyak (1995), for chromosomes 2, 7,15 or 16. The results of the genome scan for QTLs are summarized in Fig. 4.4.

The LOD peaks for Chromosomes 1 and 9 exceeded the threshold proposed for significant linkage (Lander and Kruglyak, 1995) in the merged data set. The LOD peak for chromosome 4 fell between the thresholds for suggestive and significant linkage. In the gender-specific data sets, the LOD peaks for chromosomes 1 and 9 exceeded the threshold for significant linkage in males. In females, the LOD peak for chromosome 9 exceeded the threshold of significance, while the LOD peak for chromosome 1 fell between the thresholds for suggestive *versus* significant linkage.

The QTL postulated for chromosome 1 accounted for 15.9% of the phenotypic variance in the merged data set, while QTLs on chromosome 4 and 9 accounted for 5.4% and 41.3% of the phenotypic variance, respectively. These results are summarized in Table 4.3A.

The mode of inheritance for each postulated QTL was tested by constraining the genetic

Table 4.3: Comparison of maximum Log likelihood (LOD) scores for different genetic models at proposed quantitative trait loci (QTLs)

A: LOD values were obtained for linkage to serum 1,7-dimethylxanthine/caffeine ratios using phenotypic data from 438 APN X C3H/HeJ F_2 animals and genotypic data from the 92 (gender-merged) or 46 (gender-specific) phenotypically extreme mice. Phenotypic data were log-transformed for each gender group, and merged as described above. LOD peaks and contributions to variance were calculated using MapMaker QTL 1.1 (Paterson *et al*, 1988).

B: The contribution of the APN allele to the phenotype of serum 1,7-dimethylxanthine/caffeine ratios was constrained as dominant, recessive or additive and compared to LOD values for an unconstrained model. The analyses were performed with MapMaker QTL 1.1 (Paterson *et al*, 1988).

A

Chromosome	Gender Merged	Phenotypic Variance Explained (%)	Males	Phenotypic Variance Explained (%)	Females	Phenotypic Variance Explained (%)
1	9.39	15.9	6.35	22.3	3.58	11.3
4	3.4	5.4	1.75	-	2.02	-
9	25.01	41.3	12.84	41.5	12.67	39.2

B

Chromosome	LOD maximum (Unconstrained)	LOD Maximum (Dominant)	LOD Maximum (Recessive)	LOD Maximum (Additive)
1	9.39	6.29	6.77	9.34
4	3.4	3.09	1.61	3.15
9	25.01	16.97	13.77	24.8

model for the APN allele as contributing to the phenotype in a dominant recessive or additive fashion, then comparing the LOD score for linkage to an unconstrained model. The results of these analyses are summarized in Table 4.3B and Fig. 4.5.

Epistatic interactions between putative QTLs were examined by testing for a significant decrease in LOD score when the effect of one QTL on phenotypic variance is fixed and the data are re-examined for the effects of the second QTL on the residual variance *versus* the LOD score for each QTL considered in isolation. No evidence of epistasis was observed for any of the combinations tested. Because of the major contribution of the QTLs on chromosomes 1 and 9 to the phenotypic variation in the F_2, these QTLs were fixed and the entire genome re-scanned in an attempt to identify new areas of significant linkage. No new LOD peaks exceeding a value of 2.0 were detected. Epistasis between QTLs on chromosomes 1 and 9 could not be excluded by comparing the phenotypic data for different genotypic classes at markers D9Mit248 and D1Mit356. The phenotypic means for the double homozygote (C3H/C3H at both loci, n=16) were not significantly different from either the single homozygous class C3H/C3H for D9Mit248, C3H/APN for D1Mit356, (n= 13) by Students' t-test (p=0.349) or the single homozygous class C3H/APN for D9Mit248, C3H/C3H for D1Mit356 (n =8) (p=0.165).

4.4: Discussion

The results presented here demonstrate that at least two genetic loci contribute to the variation in the continuous quantitative phenotypic trait of serum 1,7-dimethylxanthine ratios after oral caffeine dosage in an APN X C3H/HeJ F_2 intercross. The data further support the mapping of these loci to limited regions of the mouse genome. Statistically significant linkages of loci to the trait were established on chromosomes 1 and 9, and statistically suggestive linkage was

Figure 4.5: Linkage group scans for chromosomes containing quantitative trait loci (QTLs) comparing genetic models for the APN allele

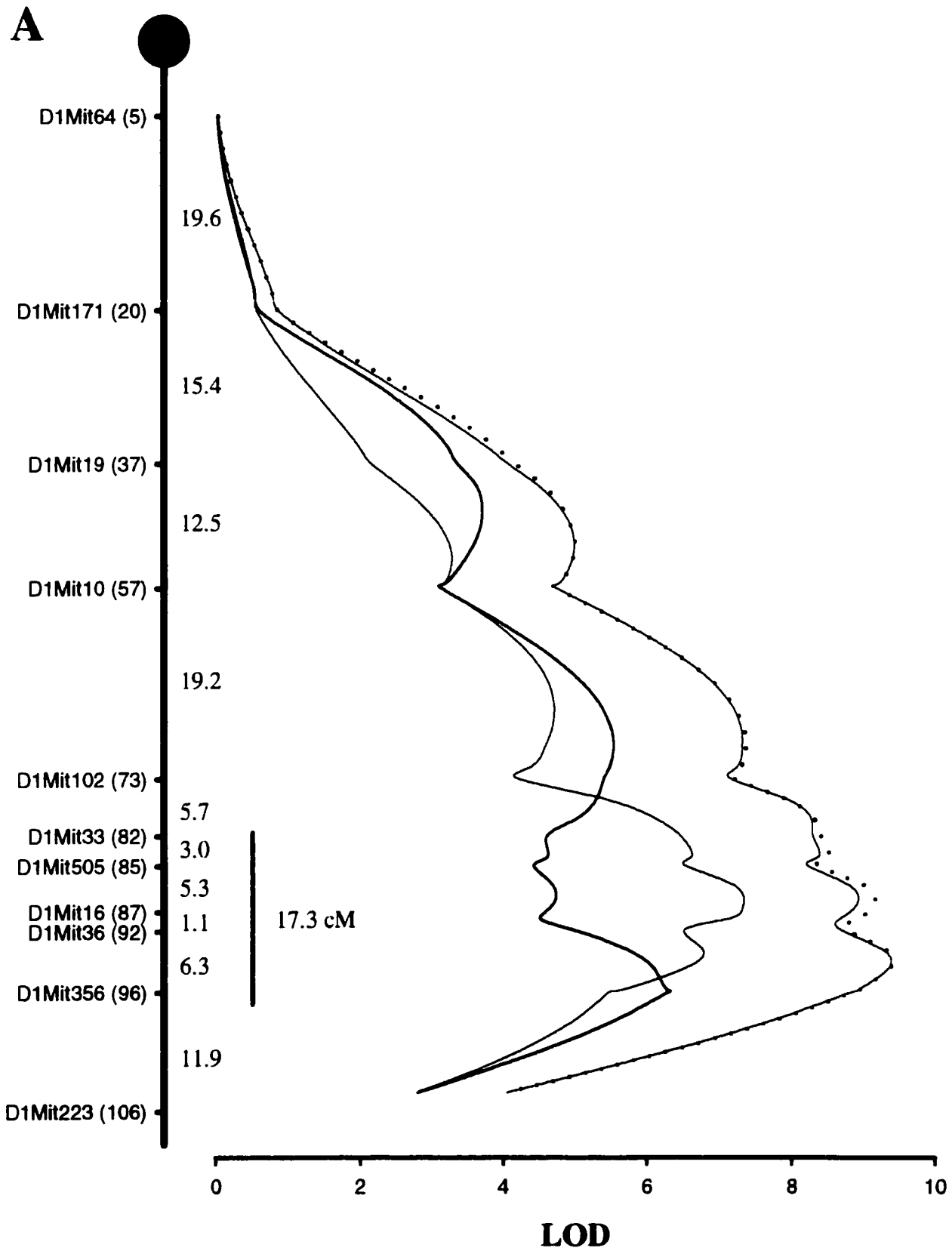
Linkage group scans were performed on chromosomes containing LOD peak values greater than 2.8 using MapMaker QTL 1.1 (Paterson *et al*, 1988). The contribution of the APN allele to the phenotype of serum 1,7-dimethylxanthine/caffeine ratio was constrained as dominant (—), recessive (—) or additive (—). LOD values obtained with constrained models were compared to an unconstrained model (····). The 1-LOD confidence interval for the position of the QTL, determined for the unconstrained model in each linkage group is indicated by the vertical line to the right of the chromosome. The length of this interval, in centimorgans is indicated. Markers are indicated to the left of the chromosome, with reported map positions in parenthesis. Map intervals between markers, obtained using MapMaker 3.0b (Lander *et al*, 1987), are indicated to the right of the chromosome.

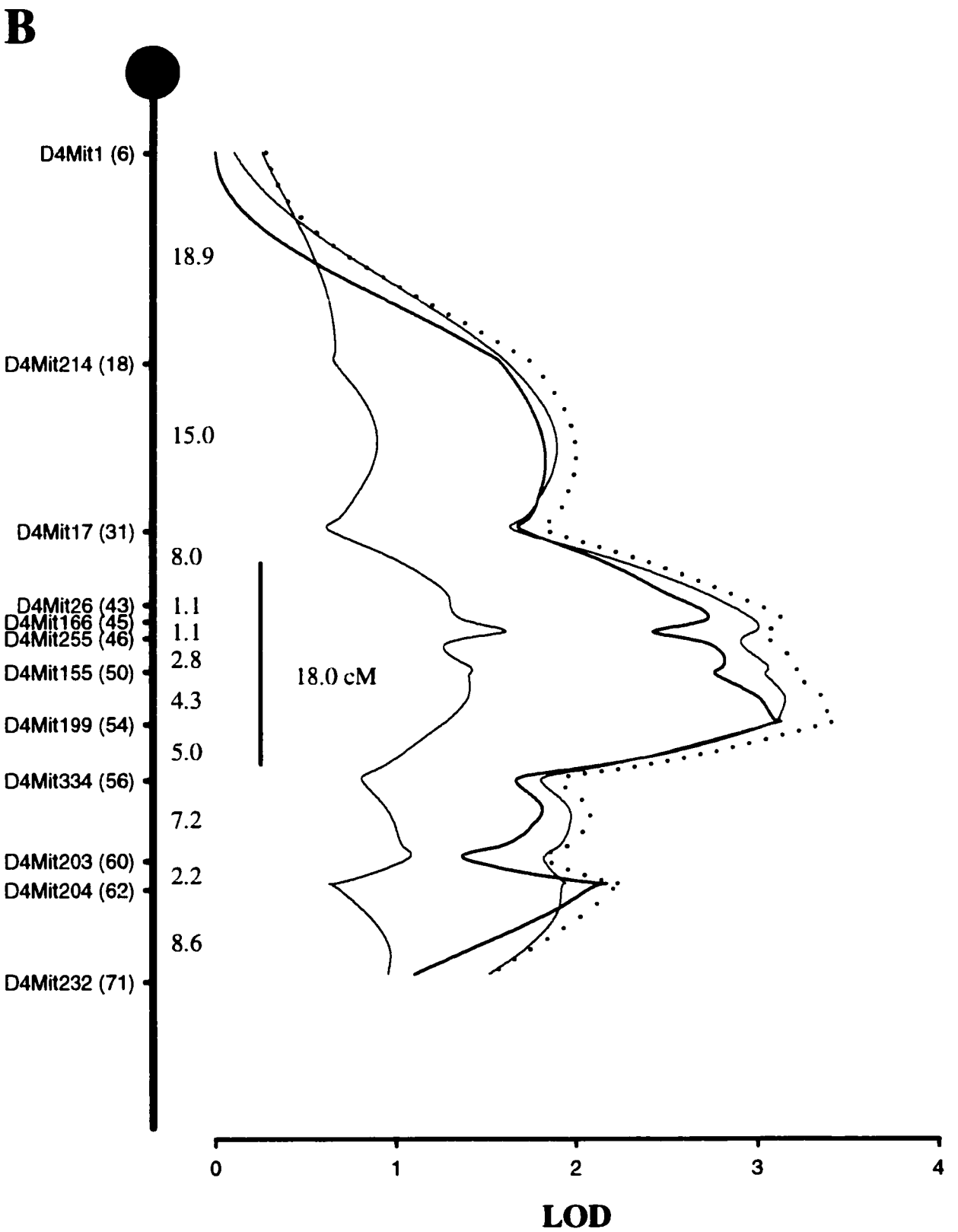
A: Chromosome 1

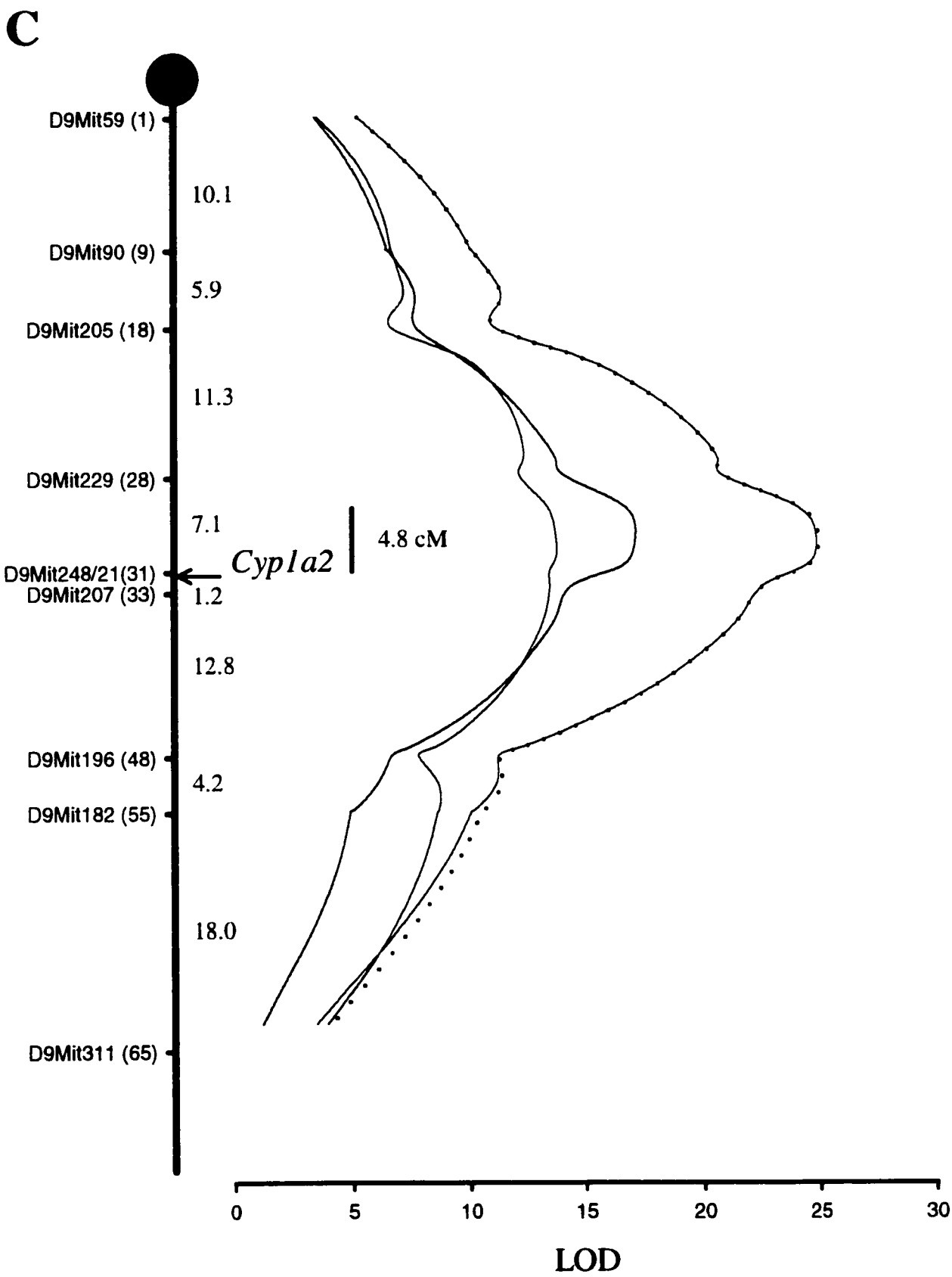
B: Chromosome 4

C: Chromosome 9.

The reported map position of the *Cyp1a2* locus is indicated by an arrow to the right of chromosome 9.


A
D1Mit64 (5)
19.6
D1Mit171 (20)
15.4
D1Mit19 (37)
12.5
D1Mit10 (57)
19.2
D1Mit102 (73)
5.7
D1Mit33 (82)
3.0
D1Mit505 (85)
5.3
D1Mit16 (87)
1.1
D1Mit36 (92)
6.3
D1Mit356 (96)
11.9
D1Mit223 (106)
17.3 cM
0
2
4
6
8
10
LOD


B
D4Mit1 (6)
18.9
D4Mit214 (18)
15.0
D4Mit17 (31)
8.0
D4Mit26 (43)
D4Mit166 (45)
D4Mit255 (46)
1.1
1.1
2.8
D4Mit155 (50)
18.0 cM
4.3
D4Mit199 (54)
5.0
D4Mit334 (56)
7.2
D4Mit203 (60)
D4Mit204 (62)
2.2
8.6
D4Mit232 (71)
0
1
2
3
4
LOD


C
D9Mit59 (1)
10.1
D9Mit90 (9)
5.9
D9Mit205 (18)
11.3
D9Mit229 (28)
7.1
4.8 cM
Cyp1a2
D9Mit248/21(31)
D9Mit207 (33)
1.2
12.8
D9Mit196 (48)
4.2
D9Mit182 (55)
18.0
D9Mit311 (65)
0
5
10
15
20
25
30
LOD

established to a locus on chromosome 4, according to the criteria for linkage proposed by Lander and Kruglyak (1995).

The heritability of the phenotype under study was assessed by comparing the variation attributable to non-genetic *versus* genetic factors, using phenotypic data from multiple inbred mouse strains. Variability within strains was assessed as non-genetic, or environmental (V_e), while variance between strains was assessed as genetic (V_g). Non-genetic variance was considered without segregation of variance into experimental (technical) *versus* developmental factors. Variance due to genetic factors was considered as total genetic variation, without segregating this component into additive, dominance or epistatic factors. This analysis assesses heritability in the broadly defined sense, as the heritable proportion of the total phenotypic variance (Hartl, 1981). It is an evaluation of the heritability of the C3-D index trait among different strains within the species and does not address the heritability of the trait in the specific cross described here. The conventional assessment of heritability between two strains in an intercross experiment compares phenotypic variance between genetically homogeneous mice (the parental and F_1 animals) *versus* the variance in the genetically heterogeneous animals (the F_2) (Falconer, 1981). This type of analysis was not possible in the current experiments, since the analysis of variance contributing to heritability estimates is dependent on the absence of selection for the trait under analysis in the parental strains. In the case of the APN mice, selection for inbreeding was based on serum ALT levels after acetaminophen dosing. Selection on the basis of relative acetaminophen susceptibility likely constituted selection for low caffeine 3-demethylation activity, based on the correlation established for these two traits (see Chpt 2; Casley *et al*, 1997a). Using broad sense heritability, it was established that at least 40% of trait variation was attributable to genetic factors in females, and at least 50% in males, across different inbred mouse strains. This genetic contribution is

sufficient to suggest that the trait would be amenable to analysis using quantitative genetic methods (Williams *et al*, 1996). Analysis of the phenotypic data from the F_1 intercross and reciprocal backcross mice showed that the trait segregated in a manner consistent with multiple genetic factors acting in an additive fashion. Phenotypic means in the backcross progeny that are intermediate to the parental and F_1 phenotypes, as seen in the current experiments are consistent with multiple genetic factors acting in an additive fashion (Falconer, 1981).

The suitability of the APN and C3H/HeJ strains as parental lines for genetic analysis of an F_2 intercross was also assessed by estimating the number of genetic factors influencing the trait variation between the two strains, using the method of Wright (1968). This method depends on certain assumptions that typically cannot be tested prior to undertaking the interval mapping analysis. These include the assumptions that each genetic factor is contributing equally to the trait, that each locus is contributing additively (uniform dominance effects across loci) without epistatic interactions and that each allele is contributing to the trait in the quantitative direction of the parental strains deviation from the F_1 mean. That is, that all alleles from the parental strain having the higher phenotypic value, contribute additively to increase the phenotypic value. Any violation of these assumptions leads to an underestimation of the number of loci contributing to the trait (Paterson *et al*, 1988; West *et al*, 1994). Based on these considerations, the determination that at least 3 factors were segregating in the present cross suggested that quantitative trait mapping would be feasible.

The STR markers used to construct the genetic linkage map and perform the genome wide scan for linkage were a subset of the Whitehead Institute map of over 6000 codominant markers (Dietrich *et al*, 1996). Although no allele size or polymorphism data were available for these markers for the novel APN inbred strain, the utility of 170 of the 536 markers in the APN X

C3H/HeJ intercross, determined empirically, was consistent with the degree of polymorphism found for these markers between any two inbred laboratory strains (Dietrich *et al*, 1994).

The genome-wide scan for genetic linkage to the C3-D trait was limited to the phenotypically extreme 10.5 % of the F_2 animals for which both phenotypic data and genetic material were available. Selectively genotyping the phenotypic extremes permits a substantial reduction in the labour and materials required to complete the scan without a major loss in sensitivity of the method (Lander and Botstein, 1986), since those individuals that deviate most significantly from the mean of the group contribute most of the genetic information to the linkage analysis. Using this limited approach, which still required in excess of 15,000 individual genotyping assays to complete, the current study identified three loci that collectively account for over 60% of the variation in the F_2 phenotypic data.

The statistical criteria used to assert significant linkage between a locus and a quantitative phenotype have been the subject of much discussion as the genetics community attempts to reach a consensus on the reporting of quantitative trait analysis results (reviewed in Avner, 1998). While a simple pairwise test of linkage between genotypic and phenotypic classes at a single marker may result in a distribution of alleleotypes that differs significantly from the expected at a probability of $p< 0.05$, a genome scan typically consists of hundreds of such comparisons. Thus deviations from expected distributions that would occur by chance at a frequency of 1 in 20, would occur several times in a typical genome scan. Lander and Kruglyak (1995) proposed multiple levels of significance in reporting quantitative trait analysis results. LOD values of 2.8, corresponding to a p value of 0.0016, would be expected to occur by chance once in a genome scan of the mouse using an infinitely dense linkage map. This LOD value was thus proposed as the threshold for suggestive linkage between a phenotype and a locus. Significant linkage was

proposed for loci exceeding a LOD value of 4.3, corresponding to a p value of 0.000052. This value corresponds to a LOD score which would be obtained by chance once in every 20 genome scans, and is therefore functionally equivalent to the widely acceptance standard of significance of p=0.05, applied in single experiments. The term "confirmed linkage" is reserved by these authors for instances where significant linkage at the same locus is confirmed in one or more independent crosses. Applying these stringent criteria to the present analysis, two loci, on chromosomes 1 and 9 were found to be significantly linked to the C 3-D phenotype, while a third locus, on chromosome 4, showed suggestive linkage. The gender differences previously observed in a number of different inbred mouse strains for this trait (see Chpt. 3; Casley *et al*, 1997b) persisted in all crosses tested in the present study. An analysis of F_1 reciprocal cross progeny did not exclude the possibility of a sex linked locus or loci influencing this trait, although a pointwise analysis of genotype segregation in males for X-linked markers failed to identify any significant linkages. Similarly, interval mapping along the X-chromosome in females did not identify any suggestive linkages. In order to test for any gender-determined genetic effects, the F_2 data was segregated according to gender and the genome re-scanned for linkage. This approach produced an additional LOD peak score of 2.8 on chromosome 17 among males, however, this was not considered indicative of suggestive linkage, since this type of *post hoc* segregation of data requires an increase in the threshold for significance (Melo *et al*, 1996; Taylor and Phillips, 1996).

Interestingly, the LOD score peak previously identified in the gender merged data set were significantly different between males and females for the peak on chromosome 1, but not on chromosome 9. The peak identified on chromosome 4 in the gender merged data, fell below a LOD score of 2 when males and females were considered separately. These results suggest that

the genetic determinant underlying the locus on chromosome 1 contributes more to the phenotypic variation seen in males than in females. This locus is, therefore, a good candidate to explain some or all of the gender differences seen in caffeine metabolism. When different genetic models were considered for the manner in which the APN allele at the QTL on chromosome 1 contributed to the phenotype, strictly dominant or recessive models were excluded, while an additive model was consistent with the data. This analysis does not, of course, address the mechanistic basis by which the gender determined effect is acting.

The 1-LOD support interval for the QTL identified on chromosome 9 includes marker D9Mit21. This marker resides less than 1 kilobase from the transcription start codon for the *Cyp1a2* gene (Hildebrand *et al*, 1985; Dietrich *et al*, 1996). The mapping of the QTL to the interval encompassing *Cyp1a2*, combined with the molecular data previously obtained for expression differences for this gene between the two parental strains (see Chpt. 3; Casley *et al*, 1997b) and the evidence for the major role of this gene product in caffeine 3-demethylation in the mouse (Buters *et al*, 1996) make *Cyp1a2* an extremely good candidate for the gene underlying this QTL.

The suggestive linkage obtained for the locus on chromosome 4 was also tested for its most likely mode of inheritance. In this case, a recessive model for the inheritance of the APN allele was excluded, however, both the dominant and additive models are plausible. The presence of one or more APN alleles acting in a dominant fashion with respect to the C3H/HeJ counterparts is consistent with the shift in phenotypic mean sen in the F_1 X APN backcross progeny, relative to the F_1 And parental means (West *et al*, 1994).

No evidence of epistatic interactions between the significant QTLs could be inferred from the data, although the pairwise comparisons of phenotypes for different genotypes at these loci

were not inconsistent with such interactions (Pierce *et al*, 1998). It is not possible, given the existing data, to distinguish between models in which the loci identified in this study act independently through different points in the metabolic pathways affecting 1,7-dimethylxanthine and caffeine levels, or epistatically, affecting metabolite ratios indirectly through their effects on *Cyp1a2* expression.

Chapter 5

Chapter 5: General Discussion

5.1: Elevated basal CYP1A expression in mice selected for differences in susceptibility to acetaminophen-induced hepatotoxicity

The work described above characterized genetically distinct inbred mouse strains with respect to their susceptibility to acetaminophen-induced hepatotoxicity, and differential expression of drug metabolizing enzymes of the cytochrome P450 CYP1A subfamily. In addition, the observation that lower caffeine 3-demethylation activity segregated with the APN strain provided an opportunity to test for genetic variation in this trait among mouse strains. The APN strain proved to be highly distinctive when tested against other inbred lines. The derivation of the APN strain was, consequently, a critical step in the subsequent quantitative genetic analysis of caffeine metabolism.

Elevated CYP1A2 activity may not be the determining factor in acetaminophen susceptibility in the APN and APS animals. Experimental evidence suggests that CYP2E1 is the more important isoform in acetaminophen-induced hepatotoxicity in mice although CYP1A2 is involved in the metabolism of the drug (Hu *et al*, 1993; Snawder *et al*, 1994; Emeigh Hart *et al*, 1995). Thomsen *et al* (1995) used specific CYP1A2 and CYP2E1 inhibitors to demonstrate that CYP2E1 is primarily responsible for acetaminophen-induced hepatotoxicity in the mouse. Studies with *Cyp2e1* (-/-) knockout mice showed a significant increase in resistance to acetaminophen-induced hepatotoxicity in mice lacking functional CYP2E1 (Lee *et al*, 1996). More recent studies with *Cyp1a2* (-/-) *Cyp2e1* (-/-) double knockout mice demonstrated a dramatic, but not complete, resistance to the hepatotoxic effects of the drug (Zaher *et al*, 1998b). Subsequent analysis of *Cyp1a2* (-/-) single knockout mice did not show a significant change in the susceptibility to

acetaminophen induced hepatotoxicity, relative to wild type mice (Tonge *et al*, 1998). While these results suggest a minimal role for CYP1A2 in mediating the hepatotoxicity of this drug in the presence of functional CYP2E1, this study was extremely limited in size, and was further confounded by intraperatoneal administration of the drug, which produces significantly different metabolism from oral dosing. It is important to note that we have demonstrated only an association, but not a causal relationship, between elevated basal CYP1A2 expression and susceptibility to acetaminophen-induced hepatotoxicity.

Breeding selection on the basis of sensitivity to acetaminophen might impose a significant selection pressure for increased CYP1A2 expression, but not the other isoform of the CYP1A family, CYP1A1. Metabolism of acetaminophen by purified CYP1A1 has been demonstrated *in vitro* (Myers *et al*, 1994), but the data do not support a significant role for this isoform in acetaminophen metabolism *in vivo*. The minimal contribution of CYP1A1 to acetaminophen metabolism suggests that our breeding scheme would select to a far lesser degree for CYP1A1 over-expression. The co-segregation of increased basal expression for both CYP1A isoforms is therefore more likely to be a consequence of selection for a factor or factors which influence(s) the expression of both isoforms. A common basis for regulation of basal expression is supported by studies in human liver in which positive correlations have been observed for CYP1A1 and CYP1A2 mRNA expression levels in individuals (Schweikl *et al*, 1993).

We propose that the co-segregation of susceptibility to acetaminophen-induced hepatotoxicity with the elevated hepatic expression of both *Cyp1a* subfamily genes implies a common basis for both traits which does not necessarily depend on a causal role for increased CYP1A expression in the drug-induced hepatotoxicity. The correlated elevation of both CYP1A subfamily isoforms may also be due to the tight genetic linkage of these loci. *Cyp1a1* and *Cyp1a2*

have been mapped to the same region of chromosome 9 in the mouse (Hildebrand *et al*, 1985). Both are localized to a region 31 cM from the top of the chromosome 9 linkage group. If both genes have polymorphic expression, and the high expression alleles are fortuitously linked in the founder population, it is possible that both high expression alleles remained linked during selection for the high expression *Cyp1a2* allele on the basis of its contribution to the selected phenotype of susceptibility to acetaminophen-induced hepatotoxicity. In this case, co-segregation of elevated expression of both CYP1A1 and CYP1A2 would not be a consequence of the segregation of a common regulatory factor. A lack of DNA sequence heterogeneity in the founder population, and consequently between the APN and APS strains, at marker loci precludes a molecular genetic analysis to determine if segregation of CYP1A expression between APS and APN strains is occurring at the *Cyp1a* locus. Consequently, the model of selection for regulatory factor influencing the expression of both CYP1A isoforms *versus* the selection of high expression alleles due to linkage effects cannot be readily distinguished.

The role of glutathione in the detoxication of acetaminophen suggests a potential common basis for the co-segregation of traits reported here. Members of the *Ahr* gene battery are sensitive to induction by oxidative stress (Nebert *et al*, 1990; Vasiliou *et al*, 1995b; Xiao *et al*, 1995). Shertzer *et al* (1995) demonstrated that changes in cellular thiol status resulting from inhibition of glutathione synthesis induced expression of some members of the *Ahr* gene battery *in vitro*. Induction was limited to CYP1A1 among the CYP1A subfamily, using hepa-1 cells although this does not preclude responsiveness of CYP1A2 to oxidative stress *in vivo*, since CYP1A2 expression is extinguished in this cell line. Otto *et al* (1996; 1999) demonstrated *in vivo* induction of CYP1A by BSO-induced glutathione depletion in fish. A genetic alteration in glutathione metabolism could produce a chronic oxidative stress which might alter the expression of both

Cyp1a genes while also adversely affecting the animals' ability to detoxify the reactive electrophilic metabolites of acetaminophen. Alternatively, chronic elevation of CYP1A expression might result in increased oxidative stress in the absence of a drug challenge, resulting in a decrease in available reducing equivalents from glutathione. Shertzer *et al* (1998) reported that TCDD exposure produces a prolonged oxidative stress and concomitant decrease in reduced glutathione levels in mice, although this study did not demonstrate a mechanistic connection between enzyme induction and oxidative stress.

5.2: Genetic analysis of variation in caffeine 3-demethylation activity between inbred mouse strains

Variation in caffeine metabolite ratios is well documented in humans, although the genetic basis, if any, for such variation is unclear. By comparing C3-D indices among inbred mouse strains, we have established a strong genetic component for the variation in this trait in mice. Inbred mouse strains permit the determination of the genetic component of variation in a quantitative trait, based on the assumption of genetic homogeneity within, but not between strains. This permits the segregation of genetic and non-genetic components of any observed variation. The APN strain derived as described above (see Chpt. 2; Casley *et al*, 1997a) is distinct from the other strains tested for its unusually low caffeine 3-demethylation activity. The derivation of this strain permitted the subsequent genetic analysis of caffeine 3-demethylation that may not have been possible with existing mouse strains. The results of this study have identified two loci with a high level of confidence, and a third locus with somewhat lower confidence, that contribute to the genetic variation in caffeine metabolism seen between APN and C3H/HeJ inbred

mice. Of these loci, one co-localizes with a strong candidate gene, *Cyp1a2*. Of the two other loci identified on chromosomes 1 and 4, neither maps to a region which co-localizes with any of the genes thus far identified as being involved in caffeine metabolism (Table 5.1). It is important to note that in any cross between inbred strains, only those loci that are polymorphic between the strains can be detected in a quantitative interval mapping approach (Lander and Botstein, 1989). In other words, genetic loci, regardless of the magnitude of their contribution to the C3-D index, would not be detected in this cross if their expression were not polymorphic between the APN and C3H/HeJ strains. Intercrosses between different inbred strains may or may not confirm the QTLs detected in the present cross, or might reveal new QTLs associated with the phenotype for this reason. Confirmatory linkage for QTLs should ideally be performed among different strains than those used to make the initial determination of linkage, but this approach is sometimes limited by the lack of polymorphism in the gene underlying the QTL among different strains (Lander and Kruglyak, 1995).

The contribution of a QTL to the phenotype under study can be confirmed by generating congenic strains in which the chromosomal region containing the QTL from one parental strain is introduced onto the genetic background of the other parental strain through a series of backcrosses. The classical approach to the development of congenic strains requires at least 10 backcross generations to ensure the homogeneity of the genetic background of the recipient strain (Flaherty, 1981). More recently, marker assisted selection techniques, also known as "speed congenics" permit the introgression of marker defined chromosomal regions into a recipient background in approximately half as many backcross generations as classical methods (Markel *et al*, 1997; Wakeland *et al*, 1997). A congenic strain containing the donor QTL on a recipient genetic background should produce a quantitative change in the phenotype consistent with the

Table 5.1: Candidate loci involved in caffeine metabolism

LOD maxima, obtained in a genome wide scan, for the regions defined by markers flanking the map position of candidate loci chosen on the basis of their potential roles in caffeine metabolism. Marker D9Mit21 is located less than 1kb from the transcription start site of the *Cyp1a2* locus.

Locus	Description	Map Position	Local LOD Maximum
Cyp1a1 / 1a2	Cytochrome P450 CYP1A2	Chromosome 9[a] 31cM	D9Mit21 LOD = 23.35
Cyp2e1	Cytochrome P450 CYP2E1	Chromosome 7[b] 68.4cM	D7Mit71 - D7Mit259 LOD = 0.93
Cyp3a	Cytochrome P450 CYP3A	Chromosome 5[c] 82cM	D5Mit115 -D5Mit61 LOD = 0.13
Cyp2a	Cytochrome P450 CYP2A	Chromosome 7[d] 6.5cM	D7Mit76 - D7Mit22 LOD = 2.45
Nat1/2	N-acetyl transferases	Chromosome 8[e] 31cM	D8Mit205 - D8Mit249 LOD = 0.50
Xdh	Xanthine dehydrogenase	Chromosome 17[f] 45.3cM	D17Mit152 - D17Mit155 LOD = 1.33
Ahr	Aromatic hydrocarbon receptor	Chromosome 12[g] 18cM	D12Mit109 - D12it172 LOD = 1.42
Arnt	Ahr nuclear translocator	Chromosome 3[h] 47.9cM	D3Mit212 - D3Mit215 LOD = 0.31

[a]Hildebrand *et al*, 1985; [b]Gonzalez *et al*, 1990; [c]Simmons *et al*, 1985; [d]Miles *et al*, 1990; [e]Mattano *et al*, 1988; [f]Cazzaniga *et al*, 1994; [g] Cobb *et al*, 1987; Johnson *et al*, 1993.

contribution of that QTL. This approach is sometimes confounded by the presence of other modifier loci in the recipient background with strain specific effects that were not detected during the initial screening for QTLs in the intercross analysis, owing to their small individual contributions to the phenotype (Threadgill *et al*, 1995). Another difficulty in assessing the individual contribution of a single QTL in a congenic strain, is the possibility of undetected epistatic interactions with other major QTLs. In the absence of the effector or regulatory locus polymorphism from the donor strain, the isolated QTL may have little or no effect on the phenotype in the recipient background. The possibility of epistatic interactions between QTLs can be addressed by the development of multiple congenic strains in which congenic strains containing single QTLs are crossed to generate a strain containing two or more QTLs. The phenotypes of these strains can then be compared to those of the single QTL strains to assess interactions between the loci (Fijneman *et al*, 1998). In the present case, the development of congenic strains for the C3-D index QTLs identified on chromosomes 1 and 9 would be an appropriate approach to determining interactions between these loci. The possibility of a locus on chromosome 1 which modifies *Cyp1a2* gene expression is a tantalizing one. Such a modifier locus could potentially explain the paradoxical variation in *Cyp1a2* gene expression in the absence of genetic polymorphisms at that locus, both in mice and humans. The QTL identified on chromosome 4 would be difficult to assess in a congenic strain, owing to the relatively small contribution of this locus to the overall phenotypic variation within the F_2 population. Genes underlying QTLs which are minor components of variation are extremely difficult to resolve, owing to the likely phenotypic similarity between congenic strains carrying different alleles of the QTL.

The development of congenic strains is also an important step in the eventual identification of the gene underlying a QTL. The mapping resolution obtained by interval mapping of an F_2

intercross is not sufficient to permit any direct attempt at position cloning of genes responsible for the QTL. The QTL identified on chromosome 9 has 1-LOD interval resolution of 5cM. This unusually tight resolution still spans approximately 10Mb of DNA, given average rates of recombination in the region (Darvasi, 1997). A map resolution of approximately 0.5 cM is typically required to provide a reasonable likelihood of success in positional cloning. A 5cM span might be expected to contain more than 250 genes, based on the assumption of 50,000 to 100,000 genes in the mouse genome. Historically, when the genes underlying QTLs have been putatively identified, it has been on the basis of linkage of the QTL to a previously mapped gene (Rikke and Johnson, 1998). In these limited cases, a rational model can be proposed which would explain the connection between the known gene function and the phenotype used to derive the QTL. In the case of the chromosome 9 QTL, there is an obvious candidate gene, *Cyp1a2*, within the 1 LOD mapping interval. The strength of this candidate gene, being the principal enzyme of caffeine 3-demethylation, justifies a direct comparison of the coding and regulatory sequences of this gene between the parental strains. This approach is facilitated by the availability of genomic sequence for this gene, including substantial upstream and downstream non-coding sequence.

5.3: Conclusions

The APS and APN strains derived in these studies are novel mouse strains which carry genetic variations imparting differential sensitivity to acetaminophen-induced hepatotoxicity. The data presented here comprise the first genetic evidence that, in the absence of a disease state or exposure to environmental inducers, there may heritable factors which co-regulate the basal expression of both the *Cyp1a1* and *Cyp1a2* genes in the mouse. Alternately, the APN and APS inbred mouse strains may represent the segregation of novel expression polymorphisms of both

members of the *Cyp1a* gene subfamily.

The comparative analysis of caffeine 3-demethylation between different inbred mouse strains permitted the assessment of the genetic component of the variation in this trait in mice. The genetic heterogeneity of caffeine metabolite ratios in humans has been the subject of much speculation, owing to their use as *in vivo* probes of CYP1A2 activity in humans. The results presented here demonstrate a significant genetic component for this trait in mice. These studies also validate a rapid plasma caffeine metabolite ratio as a predictive assay for CYP1A2 activity in this important animal model. Comparisons of *Cyp1a2* gene expression and C3-D indices between the APN strain derived in these studies, and the common laboratory strain C3H/HeJ, identified differences that permitted a novel quantitative genetic analysis of pharmacogenetic variation in drug metabolism.

The results of a genome-wide scan for genetic associations between defined regions of the mouse genome and quantitative variation in C3-D index confirmed the hypothesis that there were multiple genetic determinants underlying the variation in this trait. Human variation in caffeine metabolism has been studied in an effort to correlate such variation with differential CYP1A2 expression. Variable CYP1A2 expression has been proposed as conferring susceptibility to environmentally-induced carcinogenesis and adverse drug reactions. Consequently, caffeine metabolite ratios are considered an important epidemiological marker in these studies. The work presented here provides the first genetic evidence that a locus other than the *Cyp1a2* gene contributes significantly to this phenotype in an important animal model. As yet no genetic variation at the *CYP1A2* gene contributing to differences in basal expression has been identified in humans. The possibility of other loci acting in the 3-demethylation of caffeine, as demonstrated here in the mouse, may explain this apparent paradox.

The identification of a major QTL which co-localizes with the *Cyp1a2* locus in the mouse may lead to the first example of a genetic polymorphism affecting basal expression at this locus. The position and confidence interval of the major QTL on chromosome 9 do not exclude the possibility that loci other than *Cyp1a2* are responsible for the linkage observed. However, the mechanistic connection between CYP1A2 activity and caffeine 3-demethylation make *Cyp1a2* an excellent candidate gene for this QTL. The regulation of the constitutive expression of this gene is poorly understood in both humans and mice. The present study has provided ample genetic evidence to warrant further investigation into this problem, using the novel genetic resources generated in the course of these investigations.

The study of variations in drug metabolism and nonpathogenic responses to xenobiotics has historically been limited to the identification of polymorphisms affecting monogenic traits. The research described herein demonstrates the potential for quantitative genetic analysis in the mouse in elucidating complex pharmacogenetic variation.

Reference List

Ahokas, J.T., Pelkonen, O., Ravenscroft, P.J., and Emmerson, B.T. (1981). Effects of theophylline and caffeine on liver microsomal drug metabolizing mono-oxygenase in genetically AHH-responsive and non-responsive mice. *Res Comm Substance Abuse* **2**, 277-290.

Alonso, S., Minty, A., Bourlet, Y., and Buckingham, M. (1986) Comparison of three actin-coding sequences in the mouse; evolutionary relationships between the actin genes of warm-blooded vertebrates. *J Mol Evol* **23**, 11-22.

Ambrosone, C.B., Freudenheim, J.L., Graham, S., Marshall, J.R., Vena, J.E., Brasure, J.R., Michalek, A.M., Laughlin, R., Nemoto, T., Gillenwater, K.A., and Shields, P.G. (1996) Cigarette smoking, N-acetyltransferase 2 genetic polymorphisms, and breast cancer risk. *JAMA* **276**, 1494-1501.

Atlas, S.A., Taylor, B.A., Diwan, B.A., and Nebert, D.W. (1976) Inducible monooxygenase activities and 3-methylcholanthrene-initiated tumorigenesis in mouse recombinant inbred sublines. *Genetics* **83**, 537-550.

Avner, P. (1998) Complex traits and polygenic inheritance in the mouse. *Methods* **14**, 191-198.

Awasthi, Y.C., Sharma, R., and Singhal, S.S. (1994) Human glutathione S-transferases. *Int J Biochem* **26** , 295-308.

Axelrod, J., Inscoe, J.K., and Tomkins, G.G. (1958) Enzymatic synthesis of *N*-glucosyluronic acid conjugates. *J. Biol. Chem.* **232**, 835-840.

Bammler, T.K., Smith, C.A., and Wolf, C.R. (1994) Isolation and characterization of two mouse Pi-class glutathione S-transferase genes. *Biochem J* **298 (Pt 2)**, 385-390.

Bartolone, J.B., Beierschmitt, W.P., Birge, R.B., Hart, S.G., Wyand, S., Cohen, S.D., and Khairallah, E.A. (1989) Selective acetaminophen metabolite binding to hepatic and extrahepatic proteins: an in vivo and in vitro analysis. *Toxicol Appl Pharmacol* **99**, 240-249.

Bartolone, J.B., Birge, R.B., Bulera, S.J., Bruno, M.K., Nishanian, E.V., Cohen, S.D., and Khairallah, E.A. (1992) Purification, antibody production, and partial amino acid sequence of the 58-kDa acetaminophen-binding liver proteins. *Toxicol Appl Pharmacol* **113**, 19-29.

Bell, D.A., Taylor, J.A., Paulson, D.F., Robertson, C.N., Mohler, J.L., and Lucier, G.W. (1993) Genetic risk and carcinogen exposure: a common inherited defect of the carcinogen-metabolism gene glutathione S-transferase M1 (GSTM1) that increases susceptibility to bladder cancer. *J Natl Cancer Inst* **85**, 1159-1164.

Benet, L.Z., Kroetz, D.L. and Sheiner, L.B. (1996) Pharmacokinetics: The dynamics of drug absorption, distribution and elimination, in *Goodman and Gilman's The pharmacological basis of therapeutics. 9th ed.* (Hardman, J.G., Limbird, L.E., Molinoff, P.B., Ruddon, R.W. and Goodman Gilman, A. eds.) pp 3-28. McGraw-Hill, New York.

Benowitz, N.L. (1990) Clinical pharmacology of caffeine. *Annu Rev Med* **41**, 277-288.

Benson, D.A., Boguski, M., Lipman, D.J., and Ostell, J. (1994) GenBank. *Nucleic Acids Res* **22**, 3441-3444.

Bertilsson, L., Lou, Y.Q., Du, Y.L., Liu, Y., Kuang, T.Y., Liao, X.M., Wang, K.Y., Reviriego, J., Iselius, L., and Sjöqvist, F. (1992) Pronounced differences between native Chinese and Swedish populations in the polymorphic hydroxylations of debrisoquin and S-mephenytoin. *Clin Pharmacol Ther* **51**, 388-397.

Birge, R.B., Bartolone, J.B., McCann, D.J., Mangold, J.B., Cohen, S.D., and Khairallah, E.A. (1989) Selective protein arylation by acetaminophen and 2,6-dimethylacetaminophen in cultured hepatocytes from phenobarbital-induced and uninduced mice. Relationship to cytotoxicity. *Biochem Pharmacol* **38**, 4429-4438.

Birge, R.B., Bartolone, J.B., Hart, S.G., Nishanian, E.V., Tyson, C.A., Khairallah, E.A., and Cohen, S.D. (1990) Acetaminophen hepatotoxicity: correspondence of selective protein arylation in human and mouse liver in vitro, in culture, and in vivo. *Toxicol Appl Pharmacol* **105**, 472-482.

Birge, R.B., Bulera, S.J., Bartolone, J.B., Ginsberg, G.L., Cohen, S.D., and Khairallah, E.A. (1991) The arylation of microsomal membrane proteins by acetaminophen is associated with the release of a 44 kDa acetaminophen-binding mouse liver protein complex into the cytosol. *Toxicol Appl Pharmacol* **109**, 443-454.

Blake, J.A., Eppig, J.T., Richardson, J.E., and Davisson, M.T. (1998) The Mouse Genome Database (MGD): a community resource. Status and enhancements. The Mouse Genome Informatics Group. *Nucleic Acids Res* **26**, 130-137.

Blum, M., Demierre, A., Grant, D.M., Heim, M., and Meyer, U.A. (1991) Molecular mechanism of slow acetylation of drugs and carcinogens in humans. *Proc Natl Acad Sci U S A* **88**, 5237-5241.

Boberg, E.W., Miller, E.C., Miller, J.A., Poland, A., and Liem, A. (1983) Strong evidence from studies with brachymorphic mice and pentachlorophenol that 1'-sulfoöxysafrole is the major ultimate electrophilic and carcinogenic metabolite of 1'-hydroxysafrole in mouse liver. *Cancer Res* **43**, 5163-5173.

Bock, K.W., Schrenk, D., Forster, A., Griese, E.U., Mörike, K., Brockmeier, D., and Eichelbaum, M. (1994) The influence of environmental and genetic factors on CYP2D6, CYP1A2 and UDP-glucuronosyltransferases in man using sparteine, caffeine, and paracetamol as probes. *Pharmacogenetics* **4**, 209-218.

Bonicke, R., and Liboa, B.P. (1957) On the inherited basis of intraindividual constancy of isoniazid elimination in man (studies of monozygotic and dizygotic twins). *Naturwissenschaften* **44**, 314.

Boon, J.P., Sleiderink, H.M., Helle, M.S., Dekker, M., Vanschanke, A., Roex, E., Hillebrand, M.T.J., Klamer, H.J.C., Govers, B., Pastor, D., Morse, D., Wester, P.G., and Deboer, J. (1998) The use of a microsomal in vitro assay to study phase I biotransformation of chlorobornanes (Toxaphene (R)) in marine mammals and birds - Possible consequences of biotransformation for bioaccumulation and genotoxicity. *Comp Biochem Physiol* **121**, 385-403.

Bosma, P.J., Seppen, J., Goldhoorn, B., Bakker, C., Oude, E., Chowdhury, J.R., Chowdhury, N.R., and Jansen, P.L. (1994) Bilirubin UDP-glucuronosyltransferase 1 is the only relevant bilirubin glucuronidating isoform in man. *J Biol Chem* **269**, 17960-17964.

Bouchardy, C., Benhamou, S., and Dayer, P. (1996) The effect of tobacco on lung cancer risk depends on CYP2D6 activity. *Cancer Res* **56**, 251-253.

Branch, R.A., Chern, H.D., Adedoyin, A., Romkes-Sparks, M., Lesnick, T.G., Persad, R., Wilkinson, G.R., Fleming, C.M., Dickinson, A.J., and Sibley, G. (1995) The procarcinogen hypothesis for bladder cancer: activities of individual drug metabolizing enzymes as risk factors. *Pharmacogenetics* **5**, S97-102.

Brewer, G.J. (1971) Annotation: human ecology, an expanding role for the human geneticist. *Am J Hum Genet* **23**, 92-94.

Brockmöller, J., Cascorbi, I., Kerb, R., and Roots, I. (1996) Combined analysis of inherited polymorphisms in arylamine N-acetyltransferase 2, glutathione S-transferases M1 and T1, microsomal epoxide hydrolase, and cytochrome P450 enzymes as modulators of bladder cancer risk. *Cancer Res* **56**, 3915-3925.

Brockmöller, J., Cascorbi, I., Kerb, R., Sachse, C., and Roots, I. (1998) Polymorphisms in xenobiotic conjugation and disease predisposition. *Toxicol Lett* **102-103**, 173-183.

Brown, S.D. and Nolan, P.M. (1998) Mouse mutagenesis-systematic studies of mammalian gene function. *Hum Mol Genet* **7**, 1627-1633.

Bruno, M.K., Cohen, S.D., and Khairallah, E.A. (1992) Selective alterations in the patterns of newly synthesized proteins by acetaminophen and its dimethylated analogues in primary cultures of mouse hepatocytes. *Toxicol Appl Pharmacol* **112**, 282-290.

Bruno, M.K., Khairallah, E.A., and Cohen, S.D. (1998) Inhibition of protein phosphatase activity and changes in protein phosphorylation following acetaminophen exposure in cultured mouse hepatocytes. *Toxicol Appl Pharmacol* **153**, 119-132.

Burbach, K.M., Poland, A., and Bradfield, C.A. (1992) Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. *Proc Natl Acad Sci U S A* **89**, 8185-8189.

Burcham, P.C. and Harman, A.W. (1990) Mitochondrial dysfunction in paracetamol hepatotoxicity: in vitro studies in isolated mouse hepatocytes. *Toxicol Lett* **50**, 37-48.

Burcham, P.C. and Harman, A.W. (1991) Acetaminophen toxicity results in site-specific mitochondrial damage in isolated mouse hepatocytes. *J Biol Chem* **266**, 5049-5054.

Burchell, B., Brierley, C.H., and Rance, D. (1995) Specificity of human UDP-glucuronosyltransferases and xenobiotic glucuronidation. *Life Sci* **57**, 1819-1831.

Burke, M.D. and Mayer, R.T. (1983) Differential effects of phenobarbitone and 3-methylcholanthrene induction on the hepatic microsomal metabolism and cytochrome P-450-binding of phenoxazone and a homologous series of its n-alkyl ethers (alkoxyresorufins). *Chem Biol Interact* **45**, 243-258.

Buters, J.T., Tang, B.K., Pineau, T., Gelboin, H.V., Kimura, S., and Gonzalez, F.J. (1996) Role of CYP1A2 in caffeine pharmacokinetics and metabolism: studies using mice deficient in CYP1A2. *Pharmacogenetics* **6**, 291-296.

Buters, J.T., Sakai, S., Richter, T., Pineau, T., Alexander, D.L., Savas, U., Doehmer, J., Ward, J.M., Jefcoate, C.R., and Gonzalez, F.J. (1999) Cytochrome P450 CYP1B1 determines susceptibility to 7, 12-dimethylbenz[a]anthracene-induced lymphomas. *Proc Natl Acad Sci U S A* **96**, 1977-1982.

Butler, M.A., Iwasaki, M., Guengerich, F.P., and Kadlubar, F.F. (1989) Human cytochrome P-450PA (P-450IA2), the phenacetin O-deethylase, is primarily responsible for the hepatic 3-demethylation of caffeine and N-oxidation of carcinogenic arylamines. *Proc Natl Acad Sci U S A* **86**, 7696-7700.

Butler, M.A., Lang, N.P., Young, J.F., Caporaso, N.E., Vineis, P., Hayes, R.B., Teitel, C.H., Massengill, J.P., Lawsen, M.F., and Kadlubar, F.F. (1992) Determination of CYP1A2 and NAT2 phenotypes in human populations by analysis of caffeine urinary metabolites. *Pharmacogenetics* **2**, 116-127.

Campbell, M.E., Spielberg, S.P., and Kalow, W. (1987) A urinary metabolite ratio that reflects systemic caffeine clearance. *Clin Pharmacol Ther* **42**, 157-165.

Carrillo, J.A. and Benitez, J. (1996) CYP1A2 activity, gender and smoking, as variables influencing the toxicity of caffeine. *Br J Clin Pharmacol* **41**, 605-608.

Carson, P.E., Flanagan, C.L., Ickes, C.E., and Alving, A.S. (1956) Enzymatic deficiency in primaquine-sensitive erythrocytes. *Science* **124**, 484-485.

Cascorbi, I. and Roots, I. (1999) Pitfalls in N-acetyltransferase 2 genotyping. *Pharmacogenetics* **9**, 123-127.

Cartwright, R.A., Glashan, R.W., Rogers, H.J., Ahmad, R.A., Barham-Hall, D., Higgins, E., and Kahn, M.A. (1982) Role of N-acetyltransferase phenotypes in bladder carcinogenesis: a pharmacogenetic epidemiological approach to bladder cancer. *Lancet* **2**, 842-845.

Casley, W.L., Menzies, J.A., Mousseau, N., Girard, M., Moon, T.W., and Whitehouse, L.W. (1997a) Increased basal expression of hepatic *Cyp1a1* and *Cyp1a2* genes in inbred mice selected for susceptibility to acetaminophen-induced hepatotoxicity. *Pharmacogenetics* **7**, 283-293.

Casley, W.L., Menzies, J.A., Girard, M., Larocque, L., Mousseau, N., Whitehouse, L.W., and Moon, T.W. (1997b) Differences in caffeine 3-demethylation activity among inbred mouse strains: a comparison of hepatic *Cyp1a2* gene expression between two inbred strains. *Fundam Appl Toxicol* **40**, 228-237.

Catteau, A., Douriez, E., Beaune, P., Poisson, N., Bonaiti-Péllie, C., and Laurent, P. (1995a) Genetic polymorphism of induction of CYP1A1 (EROD) activity. *Pharmacogenetics* **5**, 110-119.

Catteau, A., Bechtel, Y.C., Poisson, N., Bechtel, P.R., and Bonaiti-Péllie, C. (1995b) A population and family study of CYP1A2 using caffeine urinary metabolites. *Eur J Clin Pharmacol* **47**, 423-430.

Cazzaniga, G., Terao, M., Lo, S., Galbiati, F., Segalla, F., Seldin, M.F., and Garattini, E. (1994) Chromosomal mapping, isolation, and characterization of the mouse xanthine dehydrogenase gene. *Genomics* **23**, 390-402.

Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**, 156-159.

Chung, I. and Bresnick, E. (1995) Regulation of the constitutive expression of the human CYP1A2 gene: cis elements and their interactions with proteins. *Mol Pharmacol* **47**, 677-685.

Chung, I. and Bresnick, E. (1997) Identification of positive and negative regulatory elements of the human cytochrome P4501A2 (CYP1A2) gene. *Arch Biochem Biophys* **338**, 220-226.

Ciummo, P.E. and Katz, N.L. (1995) Interactions and drug-metabolizing enzymes. *Am Pharm* **NS35**, 41-43.

Cobb, R.R., Stoming, T.A., and Whitney, J.B. (1987) The aryl hydrocarbon hydroxylase (Ah) locus and a novel restriction-fragment length polymorphism (RFLP) are located on mouse chromosome 12. *Biochem Genet* **25**, 401-413.

Collins, F.S., Patrinos, A., Jordan, E., Chakravarti, A., Gesteland, R., and Walters, L. (1998) New goals for the U.S. Human Genome Project: 1998-2003. *Science* **282**, 682-689.

Conney, A.H. (1982) Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G. H. A. Clowes Memorial Lecture. *Cancer Res* **42**, 4875-4917.

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

Crabbe, J.C., Belknap, J.K., Metten, P., Grisel, J.E., and Buck, K.J. (1998) Quantitative trait loci: mapping drug and alcohol-related genes. *Adv Pharmacol* **42**, 1033-1037.

Crespi, C.L., Steimel, D.T., Penman, B.W., Korzekwa, K.R., Fernandez-Salguero, P., Buters, J.T., Gelboin, H.V., Gonzalez, F.J., Idle, J.R., and Daly, A.K. (1995) Comparison of substrate metabolism by wild type CYP2D6 protein and a variant containing methionine, not valine, at position 374. *Pharmacogenetics* **5**, 234-243.

Dahlin, D.C., Miwa, G.T., Lu, A.Y., and Nelson, S.D. (1984) N-acetyl-p-benzoquinone imine: a cytochrome P-450-mediated oxidation product of acetaminophen. *Proc Natl Acad Sci U S A* **81** , 1327-1331.

Dalhoff, K. and Poulsen, H.E. (1993) Synthesis rates of glutathione and activated sulphate (PAPS) and response to cysteine and acetaminophen administration in glutathione-depleted rat hepatocytes. *Biochem Pharmacol* **46**, 1295-1297.

Daly, A.K., Steen, V.M., Fairbrother, K.S., and Idle, J.R. (1996) CYP2D6 multiallelism. *Methods Enzymol* **272**, 199-210.

Darvasi, A. (1997) Interval-specific congenic strains (ISCS): an experimental design for mapping a QTL into a 1-centimorgan interval. *Mamm Genome* **8**, 163-167.

Deguchi, T., Mashimo, M., and Suzuki, T. (1990) Correlation between acetylator phenotypes and genotypes of polymorphic arylamine N-acetyltransferase in human liver. *J Biol Chem* **265**, 12757-12760.

Delaporte, E. and Renton, K.W. (1997) Cytochrome P4501A1 and cytochrome P4501A2 are downregulated at both transcriptional and post-transcriptional levels by conditions resulting in interferon-alpha/beta induction. *Life Sci* **60**, 787-796.

Dethmers, J.K. and Meister, A. (1981) Glutathione export by human lymphoid cells: depletion of glutathione by inhibition of its synthesis decreases export and increases sensitivity to irradiation. *Proc Natl Acad Sci U S A* **78**, 7492-7496.

Dietrich, W.F., Miller, J.C., Steen, R.G., Merchant, M., Damron, D., Nahf, R., Gross, A., Joyce, D.C., Wessel, M., Dredge, R.D., Marquis, A., Stein, L.D., Goodman, N., Page, D.C. and Lander, E.S. (1994) A genetic map of the mouse with 4,006 simple sequence length polymorphisms. *Nat Genet* **7**, 220-245.

Dietrich, W.F., Miller, J., Steen, R., Merchant, M.A., Damron-Boles, D., Husain, Z., Dredge, R., Daly, M.J., Ingalls, K.A., O'Connor, T.J., Evans, C.A., DeAngelis, M.M., Levinson, D.M., Kruglyak, L., Goodman, N., Copeland, N.G., Jenkins, N.A., Hawkins, T.L., Stein, L., Page, D.C. and Lander, E.S. (1996) A comprehensive genetic map of the mouse genome. *Nature* **380**, 149-152.

Ding, V.D. and Pickett, C.B. (1985) Transcriptional regulation of rat liver glutathione S-transferase genes by phenobarbital and 3-methylcholanthrene. *Arch Biochem Biophys* **240**, 553-559.

Dolwick, K.M., Schmidt, J.V., Carver, L.A., Swanson, H.I., and Bradfield, C.A. (1993) Cloning and expression of a human Ah receptor cDNA. *Mol Pharmacol* **44**, 911-917.

Donnelly, P.J., Walker, R.M., and Racz, W.J. (1994) Inhibition of mitochondrial respiration in vivo is an early event in acetaminophen-induced hepatotoxicity. *Arch Toxicol* **68**, 110-118.

Emery, A.E., Anand, R., Danford, N., Duncan, W., and Paton, L. (1978) Aryl-hydrocarbon-hydroxylase inducibility in patients with cancer. *Lancet* **1**, 470-472.

Evans, D.A. (1989) N-acetyltransferase. *Pharmacol Ther* **42**, 157-234.

Falconer, D.S., Gauld, I.K., Roberts, R.C., and Williams, D.A. (1981) The control of body size in mouse chimaeras. *Genet Res* **38**, 25-46.

Farin, F.M. and Omiecinski, C.J. (1993) Regiospecific expression of cytochrome P-450s and microsomal epoxide hydrolase in human brain tissue. *J Toxicol Environ Health* **40**, 317-335.

Felton, J.S. and Nebert, D.W. (1975) Mutagenesis of certain activated carcinogens in vitro associated with genetically mediated increases in monooxygenase activity and cytochrome P 1-450. *J Biol Chem* **250**, 6769-6778.

Fernandez-Salguero, P., Pineau, T., Hilbert, D.M., McPhail, T., Lee, S.S., Kimura, S., Nebert, D.W., Rudikoff, S., Ward, J.M., and Gonzalez, F.J. (1995) Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor. *Science* **268**, 722-726.

Fijneman, R.J., de Vries, S.S., Jansen, R.C., and Demant, P. (1996) Complex interactions of new quantitative trait loci, Sluc1, Sluc2, Sluc3, and Sluc4, that influence the susceptibility to lung cancer in the mouse. *Nat Genet* **14**, 465-467.

Fijneman, R.J., van der Valk, M.A., and Demant, P. (1998) Genetics of quantitative and qualitative aspects of lung tumorigenesis in the mouse: multiple interacting Susceptibility to lung cancer (Sluc) genes with large effects. *Exp Lung Res* **24**, 419-436.

Flaherty, L. (1981) Congenic strains, in *The mouse in biomedical research.* (Foster HL, Small JD and Fox JG eds.) pp 215-221. Academic Press, New York.

Frankel, W.N. (1995) Taking stock of complex trait genetics in mice. *Trends Genet* **11**, 471-477.

Friling, R.S., Bensimon, A., Tichauer, Y., and Daniel, V. (1990) Xenobiotic-inducible expression of murine glutathione S-transferase Ya subunit gene is controlled by an electrophile-responsive element. *Proc Natl Acad Sci U S A* **87**, 6258-6262.

Fuhr, U. and Rost, K.L. (1994) Simple and reliable CYP1A2 phenotyping by the paraxanthine/caffeine ratio in plasma and in saliva. *Pharmacogenetics* **4**, 109-116.

Gielen, J.E., Goujon, F.M., and Nebert, D.W. (1972) Genetic regulation of aryl hydrocarbon hydroxylase induction. II. Simple Mendelian expression in mouse tissues in vivo. *J Biol Chem* **247**, 1125-1137.

Goasduff, T., Dréano, Y., Guillois, B., Ménez, J.F., and Berthou, F. (1996) Induction of liver and kidney CYP1A1/1A2 by caffeine in rat. *Biochem Pharmacol* **52**, 1915-1919.

Goldstein, J.A. and Linko, P. (1984) Differential induction of two 2,3,7,8-tetrachlorodibenzo-p-dioxin-inducible forms of cytochrome P-450 in extrahepatic versus hepatic tissues. *Mol Pharmacol* **25**, 185-191.

Goldstein, J.A. and de Morais, S.M. (1994) Biochemistry and molecular biology of the human CYP2C subfamily. *Pharmacogenetics* **4**, 285-299.

Gomez, M.R., Benzick, A.E., Rogers, L.K., Heird, W.C., and Smith, C.V. (1994) Attenuation of acetaminophen hepatotoxicity in mice as evidence for the bioavailability of the cysteine in D-glucose-L-cysteine in vivo. *Toxicol Lett* **70**, 101-108.

Gonzalez, F.J., Schmid, B.J., Umeno, M., Mcbride, O.W., Hardwick, J.P., Meyer, U.A., Gelboin, H.V., and Idle, J.R. (1988) Human P450PCN1: sequence, chromosome localization, and direct evidence through cDNA expression that P450PCN1 is nifedipine oxidase. *DNA* **7**, 79-86.

Gonzalez, F.J., Skoda, R.C., Kimura, S., Umeno, M., Zanger, U.M., Nebert, D.W., Gelboin, H.V., Hardwick, J.P., and Meyer, U.A. (1988) Characterization of the common genetic defect in humans deficient in debrisoquine metabolism. *Nature* **331**, 442-446.

Gonzalez, F.J., Liu, S.Y., Kozak, C.A., and Nebert, D.W. (1990) Decreased Hnf-1 gene expression in mice homozygous for a 1.2-centiMorgan deletion on chromosome 7. *DNA Cell Biol* **9**, 771-776.

Gonzalez, F.J. and Nebert, D.W. (1990) Evolution of the P450 gene superfamily: animal-plant 'warfare', molecular drive and human genetic differences in drug oxidation. *Trends Genet* **6** , 182-186.

Gonzalez, F.J. and Idle, J.R. (1994) Pharmacogenetic phenotyping and genotyping. Present status and future potential. *Clin Pharmacokinet* **26**, 59-70.

Goodfellow, P.N. and Schmitt, K. (1994) Human genetics. From the simple to the complex [news; comment]. *Nature* **371**, 104-105.

Grant, D.M., Tang, B.K., and Kalow, W. (1983) Variability in caffeine metabolism. *Clin Pharmacol Ther* **33**, 591-602.

Grant, D.M., Tang, B.K., and Kalow, W. (1984) A simple test for acetylator phenotype using caffeine. *Br J Clin Pharmacol* **17**, 459-464.

Grant, D.M., Blum, M., Beer, M., and Meyer, U.A. (1991) Monomorphic and polymorphic human arylamine N-acetyltransferases: a comparison of liver isozymes and expressed products of two cloned genes. *Mol Pharmacol* **39**, 184-191.

Grant, D.M., Blum, M., and Meyer, U.A. (1992) Polymorphisms of N-acetyltransferase genes. *Xenobiotica* **22**, 1073-1081.

Grant, D.M., Hughes, N.C., Janezic, S.A., Goodfellow, G.H., Chen, H.J., Gaedigk, A., Yu, V.L., and Grewal, R. (1997) Human acetyltransferase polymorphisms. *Mutat Res* **376**, 61-70.

Gregus, Z., Madhu, C., and Klaassen, C.D. (1988) Species variation in toxication and detoxication of acetaminophen in vivo: a comparative study of biliary and urinary excretion of acetaminophen metabolites. *J Pharmacol Exp Ther* **244**, 91-99.

Gu, L., Gonzalez, F.J., Kalow, W., and Tang, B.K. (1992) Biotransformation of caffeine, paraxanthine, theobromine and theophylline by cDNA-expressed human CYP1A2 and CYP2E1. *Pharmacogenetics* **2**, 73-77.

Guengerich, F.P., Kim, D.H., and Iwasaki, M. (1991) Role of human cytochrome P-450 IIE1 in the oxidation of many low molecular weight cancer suspects. *Chem Res Toxicol* **4**, 168-179.

Guengerich, F.P. (1993) The 1992 Bernard B. Brodie Award Lecture. Bioactivation and detoxication of toxic and carcinogenic chemicals. *Drug Metab Dispos* **21**, 1-6.

Guenthner, T.M. and Nebert, D.W. (1978) Evidence in rat and mouse liver for temporal control of two forms of cytochrome P-450 inducible by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Eur J Biochem* **91**, 449-456.

Gurtoo, H.L., Minowada, J., Paigen, B., Parker, N.B., and Hayner, N.T. (1977) Factors influencing the measurement and the reproducibility of aryl hydrocarbon hydroxylase activity in culutred human lymphocytes. *J Natl Cancer Inst* **59**, 787-798.

Hankinson, O. (1995) The aryl hydrocarbon receptor complex. *Annu Rev Pharmacol Toxicol* **35**, 307-340.

Hanna, P.E., Banks, R.B., and Marhevka, V.C. (1982) Suicide inactivation of hamster hepatic arylhydroxamic acid N,O-acyltransferase. A selective probe of N-acetyltransferase multiplicity. *Mol Pharmacol* **21**, 159-165.

Harada, S., Agarwal, D.P., and Goedde, H.W. (1978) Isozyme variations in acetaldehyde dehydrogenase (e.c.1.2.1.3) in human tissues. *Hum Genet* **44**, 181-185.

Hartl, D. (1981) *A primer of population genetics.* pp151-157, Sinauer Associates Inc., Sunderland

Harvison, P.J., Guengerich, F.P., Rashed, M.S., and Nelson, S.D. (1988) Cytochrome P-450 isozyme selectivity in the oxidation of acetaminophen. *Chem Res Toxicol* **1**, 47-52.

Hayashi, S., Watanabe, J., and Kawajiri, K. (1991) Genetic polymorphisms in the 5'-flanking region change transcriptional regulation of the human cytochrome P450IIE1 gene. *J Biochem (Tokyo)* **110**, 559-565.

Hein, D.W. (1988) Acetylator genotype and arylamine-induced carcinogenesis. *Biochim Biophys Acta* **948**, 37-66.

Hengstler, J.G., Arand, M., Herrero, M.E., and Oesch, F. (1998) Polymorphisms of N-acetyltransferases, glutathione S-transferases, microsomal epoxide hydrolase and sulfotransferases: influence on cancer susceptibility. *Recent Results Cancer Res* **154**, 47-85.

Hessel, G., De-Santi-Neto, D., and Collares, E.F. (1996) Correlation between the severity of acute hepatic necrosis induced by acetaminophen and serum aminotransferase levels in fasted and sucrose-fed rats. *Braz J Med Biol Res* **29**, 793-796.

Heyer, B., Schmidtke, J., and Stuhrmann, M. (1994) Analysis of microsatellite DNA using a non-radioactive method: blotting from polyacrylamide gel to nylon membrane using a gel dryer with subsequent hybridization to digoxigenin-labelled (dCdA)8 oligonucleotide probes. *Mol Cell Probes* **8**, 181-183.

Hickman, D. and Sim, E. (1991) N-acetyltransferase polymorphism. Comparison of phenotype and genotype in humans. *Biochem Pharmacol* **42**, 1007-1014.

Hildebrand, C.E., Gonzalez, F.J., Mcbride, O.W., and Nebert, D.W. (1985) Assignment of the human 2,3,7,8-tetrachlorodibenzo-p-dioxin-inducible cytochrome P1-450 gene to chromosome 15. *Nucleic Acids Res* **13**, 2009-2016.

Hildebrand, C.E., Gonzalez, F.J., Kozak, C.A., and Nebert, D.W. (1985) Regional linkage analysis of the dioxin-inducible P-450 gene family on mouse chromosome 9. *Biochem Biophys Res Commun* **130**, 396-406.

Hinson, J.A., Pumford, N.R., and Roberts, D.W. (1995) Mechanisms of acetaminophen toxicity: immunochemical detection of drug-protein adducts. *Drug Metab Rev* **27**, 73-92.

Hirvonen, A., Husgafvel-Pursiainen, K., Anttila, S., and Vainio, H. (1993) The GSTM1 null genotype as a potential risk modifier for squamous cell carcinoma of the lung. *Carcinogenesis* **14**, 1479-1481.

Hirvonen, A., Husgafvel-Pursiainen, K., Anttila, S., Karjalainen, A., and Vainio, H. (1993) The human CYP2E1 gene and lung cancer: DraI and RsaI restriction fragment length polymorphisms in a Finnish study population. *Carcinogenesis* **14**, 85-88.

Hoivik, D.J., Manautou, J.E., Tveit, A., Mankowski, D.C., Khairallah, E.A., and Cohen, S.D. (1996) Evidence suggesting the 58-kDa acetaminophen binding protein is a preferential target for acetaminophen electrophile. *Fundam Appl Toxicol* **32**, 79-86.

Hollenberg, P.F. (1992) Mechanisms of cytochrome P450 and peroxidase-catalyzed xenobiotic metabolism. *FASEB J* **6**, 686-694.

Holme, J.A. and Søderlund, E. (1986) Species differences in cytotoxic and genotoxic effects of phenacetin and paracetamol in primary monolayer cultures of hepatocytes. *Mutat Res* **164**, 167-175.

Hongslo, J.K., Smith, C.V., Brunborg, G., Søderlund, E.J., and Holme, J.A. (1994) Genotoxicity of paracetamol in mice and rats. *Mutagenesis* **9**, 93-100.

Horn, E.P., Tucker, M.A., Lambert, G., Silverman, D., Zametkin, D., Sinha, R., Hartge, T., Landi, M.T., and Caporaso, N.E. (1995) A study of gender-based cytochrome P4501A2 variability: a possible mechanism for the male excess of bladder cancer. *Cancer Epidemiol Biomarkers Prev* **4**, 529-533.

Housman, D. and Ledley, F.D. (1998) Why pharmacogenomics? Why now? *Nat Biotechnol* **16**, 492-493.

Hu, J.J., Lee, M.J., Vapiwala, M., Reuhl, K., Thomas, P.E., and Yang, C.S. (1993) Sex-related differences in mouse renal metabolism and toxicity of acetaminophen. *Toxicol Appl Pharmacol* **122**, 16-26.

Huang, J.D., Guo, W.C., Lai, M.D., Guo, Y.L., and Lambert, G.H. (1999) Detection of a novel cytochrome P-450 1A2 polymorphism (F21L) in Chinese. *Drug Metab Dispos* **27**, 98-101.

Hughes, H.B. (1954) Metabolism of isoniazid in man as related to the occurrence of peripheral neuritis. *Am. Ren. Tuberculosis* **70**, 266-273.

Ikeya, K., Jaiswal, A.K., Owens, R.A., Jones, J.E., Nebert, D.W., and Kimura, S. (1989) Human CYP1A2: sequence, gene structure, comparison with the mouse and rat orthologous gene, and differences in liver 1A2 mRNA expression. *Mol Endocrinol* **3**, 1399-1408.

Ingelman-Sundberg, M., Johansson, I., Yin, H., Terelius, Y., Eliasson, E., Clot, P., and Albano, E. (1993) Ethanol-inducible cytochrome P4502E1: genetic polymorphism, regulation, and possible role in the etiology of alcohol-induced liver disease. *Alcohol* **10**, 447-452.

Itoh, S. and Kamataki, T. (1993) Human Ah receptor cDNA: analysis for highly conserved sequences. *Nucleic Acids Res* **21**, 3578-3578.

Jaiswal, A.K., Gonzalez, F.J., and Nebert, D.W. (1985) Human dioxin-inducible cytochrome P1-450: complementary DNA and amino acid sequence. *Science* **228**, 80-83.

Jaiswal, A.K., Nebert, D.W., and Gonzalez, F.J. (1986) Human P3(450): cDNA and complete amino acid sequence. *Nucleic Acids Res* **14**, 6773-6774.

Jayyosi, Z., Knoble, D., Muc, M., Erick, J., Thomas, P.E., and Kelley, M. (1995) Cytochrome P-450 2E1 is not the sole catalyst of chlorzoxazone hydroxylation in rat liver microsomes. off. *J Pharmacol Exp Ther* **273**, 1156-1161.

Johnson, B., Brooks, B.A., Heinzmann, C., Diep, A., Mohandas, T., Sparkes, R.S., Reyes, H., Hoffman, E., Lange, E., and Gatti, R.A. (1993) The Ah receptor nuclear translocator gene (ARNT) is located on q21 of human chromosome 1 and on mouse chromosome 3 near Cf-3. *Genomics* **17**, 592-598.

Kaisary, A., Smith, P., Jaczq, E., McAllister, C.B., Wilkinson, G.R., Ray, W.A., and Branch, R.A. (1987) Genetic predisposition to bladder cancer: ability to hydroxylate debrisoquine and mephenytoin as risk factors. *Cancer Res* **47**, 5488-5493.

Kalow, W. and Tang, B.K. (1991) Use of caffeine metabolite ratios to explore CYP1A2 and xanthine oxidase activities. *Clin Pharmacol Ther* **50**, 508-519.

Kalow, W. and Tang, B.K. (1993) The use of caffeine for enzyme assays: a critical appraisal. *Clin Pharmacol Ther* **53**, 503-514.

Kalow, W. (1997) Pharmacogenetics in biological perspective. *Pharmacol Rev* **49**, 369-379.

Kane, R.E., Li, A.P., and Kaminski, D.R. (1995) Sulfation and glucuronidation of acetaminophen by human hepatocytes cultured on Matrigel and type 1 collagen reproduces conjugation in vivo. *Drug Metab Dispos* **23**, 303-307.

Kato, S., Bowman, E.D., Harrington, A.M., Blomeke, B., and Shields, P.G. (1995) Human lung carcinogen-DNA adduct levels mediated by genetic polymorphisms in vivo. *J Natl Cancer Inst* **87**, 902-907.

Kawajiri, K., Nakachi, K., Imai, K., Hayashi, S., and Watanabe, J. (1990) Individual differences in lung cancer susceptibility in relation to polymorphisms of P-450IA1 gene and cigarette dose. *Princess Takamatsu Symp* **21**, 55-61.

Kellermann, G., Shaw, C.R., and Luyten-Kellerman, M. (1973) Aryl hydrocarbon hydroxylase inducibility and bronchogenic carcinoma. *N Engl J Med* **289**, 934-937.

Kerry, N.L., Somogyi, A.A., Bochner, F., and Mikus, G. (1994) The role of CYP2D6 in primary and secondary oxidative metabolism of dextromethorphan: in vitro studies using human liver microsomes. *Br J Clin Pharmacol* **38**, 243-248.

Ketterer, B., Harris, J.M., Talaska, G., Meyer, D.J., Pemble, S.E., Taylor, J.B., Lang, N.P., and Kadlubar, F.F. (1992) The human glutathione S-transferase supergene family, its polymorphism, and its effects on susceptibility to lung cancer. *Environ Health Perspect* **98**, 87-94.

Kharasch, E.D. and Thummel, K.E. (1993) Identification of cytochrome P450 2E1 as the predominant enzyme catalyzing human liver microsomal defluorination of sevoflurane, isoflurane, and methoxyflurane. *Anesthesiology* **79**, 795-807.

Kimura, S., Gonzalez, F.J., and Nebert, D.W. (1984a) Mouse cytochrome P3-450: complete cDNA and amino acid sequence. *Nucleic Acids Res* **12**, 2917-2928.

Kimura, S., Gonzalez, F.J., and Nebert, D.W. (1984b) The murine Ah locus. Comparison of the complete cytochrome P1-450 and P3-450 cDNA nucleotide and amino acid sequences. *J Biol Chem* **259**, 10705-10713.

Kimura, S., Smith, H.H., Hankinson, O., and Nebert, D.W. (1987) Analysis of two benzo[a]pyrene-resistant mutants of the mouse hepatoma Hepa-1 P(1)450 gene via cDNA expression in yeast. *EMBO J* **6**, 1929-1933.

King, C.M., Land, S.J., Jones, R.F., Debiec-Rychter, M., Lee, M.S., and Wang, C.Y. (1997) Role of acetyltransferases in the metabolism and carcinogenicity of aromatic amines. *Mutat Res* **376**, 123-128.

Kinoshita, T., Imamura, J., Nagai, H., and Shimotohno, K. (1992) Quantification of gene expression over a wide range by the polymerase chain reaction. *Anal Biochem* **206**, 231-235.

Klaassen, C.D. and Boles, J.W. (1997) Sulfation and sulfotransferases 5: the importance of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) in the regulation of sulfation. *FASEB J* **11**, 404-418.

Kliewer, S.A., Moore, J.T., Wade, L., Staudinger, J.L., Watson, M.A., Jones, S.A., Mckee, D.D., Oliver, B.B., Willson, T.M., Zetterström, R.H., Perlmann, T., and Lehmann, J.M. (1998) An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *CELL* **92**, 73-82.

Konrad, P.N., Richards, F., Valentine, W.N., and Paglia, D.E. (1972) γ-Glutamyl-cysteine synthetase deficiency. A cause of hereditary hemolytic anemia. *N Engl J Med* **286**, 557-561.

Kouri, R.E., McKinney, C.E., Slomiany, D.J., Snodgrass, D.R., Wray, N.P., and McLemore, T.L. (1982) Positive correlation between high aryl hydrocarbon hydroxylase activity and primary lung cancer as analyzed in cryopreserved lymphocytes. *Cancer Res* **42**, 5030-5037.

Kozak, K.R., Abbott, B., and Hankinson, O. (1997) ARNT-deficient mice and placental differentiation. *Dev Biol* **191**, 297-305.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lander, E.S. and Botstein, D. (1986) Mapping complex genetic traits in humans: new methods using a complete RFLP linkage map. *Cold Spring Harb Symp Quant Biol* **51 Pt 1**, 49-62.

Lander, E.S., Green, P., Abrahamson, J., Barlow, A., Daly, M.J., Lincoln, S.E., and Newburg, L. (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics* **1**, 174-181.

Lander, E.S. and Botstein, D. (1989) Mapping mendelian factors underlying quantitative traits using RFLP linkage maps. *Genetics* **121**, 185-199.

Lander, E. and Kruglyak, L. (1995) Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. *Nat Genet* **11**, 241-247.

Landi, M.T., Bertazzi, P.A., Shields, P.G., Clark, G., Lucier, G.W., Garte, S.J., Cosma, G., and Caporaso, N.E. (1994) Association between CYP1A1 genotype, mRNA expression and enzymatic activity in humans. *Pharmacogenetics* **4**, 242-246.

Lang, N.P., Butler, M.A., Massengill, J., Lawson, M., Stotts, R.C., Hauer-Jensen, M., and Kadlubar, F.F. (1994) Rapid metabolic phenotypes for acetyltransferase and cytochrome P4501A2 and putative exposure to food-borne heterocyclic amines increase the risk for colorectal cancer or polyps. *Cancer Epidemiol Biomarkers Prev* **3**, 675-682.

Lechevrel, M., Casson, A.G., Wolf, C.R., Hardie, L.J., Flinterman, M.B., Montesano, R., and Wild, C.P. (1999) Characterization of cytochrome P450 expression in human oesophageal mucosa. *Carcinogenesis* **20**, 243-248.

Lee, S.S., Pineau, T., Drago, J., Lee, E.J., Owens, J.W., Kroetz, D.L., Fernandez-Salguero, P.M., Westphal, H., and Gonzalez, F.J. (1995) Targeted disruption of the alpha isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. *Mol Cell Biol* **15**, 3012-3022.

Lee, S.S., Buters, J.T., Pineau, T., Fernandez-Salguero, P., and Gonzalez, F.J. (1996) Role of CYP2E1 in the hepatotoxicity of acetaminophen. *J Biol Chem* **271**, 12063-12067.

Lehmann, J.M., Mckee, D.D., Watson, M.A., Willson, T.M., Moore, J.T., and Kliewer, S.A. (1998) The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J Clin Invest* **102**, 1016-1023.

Lewis, D.F., Watson, E., and Lake, B.G. (1998) Evolution of the cytochrome P450 superfamily: sequence alignments and pharmacogenetics. *Mutat Res* **410**, 245-270.

Liang, H.C., Li, H., McKinnon, R.A., Duffy, J.J., Potter, S.S., Puga, A., and Nebert, D.W. (1996) Cyp1a2(-/-) null mutant mice develop normally but show deficient drug metabolism. *Proc Natl Acad Sci U S A* **93**, 1671-1676.

Liang, H.C., McKinnon, R.A., and Nebert, D.W. (1997) Sensitivity of CYP1A1 mRNA inducibility by dioxin is the same in Cyp1a2(+/+) wild-type and Cyp1a2(-/-) null mutant mice. *Biochem Pharmacol* **54**, 1127-1131.

Lindberg, R.L. and Negishi, M. (1989) Alteration of mouse cytochrome P450coh substrate specificity by mutation of a single amino-acid residue. *Nature* **339**, 632-634.

Liu, G., Gelboin, H.V., and Myers, M.J. (1991) Role of cytochrome P450 IA2 in acetanilide 4-hydroxylation as determined with cDNA expression and monoclonal antibodies. *Arch Biochem Biophys* **284**, 400-406.

London, S.J., Daly, A.K., Cooper, J., Navidi, W.C., Carpenter, C.L., and Idle, J.R. (1995) Polymorphism of glutathione S-transferase M1 and lung cancer risk among African-Americans and Caucasians in Los Angeles County, California. *J Natl Cancer Inst* **87**, 1246-1253.

Loos, H., Roos, D., Weening, R., and Houwerzijl, J. (1976) Familial deficiency of glutathione reductase in human blood cells. *Blood* **48**, 53-62.

Lower, G.M., Nilsson, T., Nelson, C.E., Wolf, H., Gamsky, T.E., and Bryan, G.T. (1979) N-acetyltransferase phenotype and risk in urinary bladder cancer: approaches in molecular epidemiology. Preliminary results in Sweden and Denmark. *Environ Health Perspect* **29**, 71-79.

Lu, A.Y. and Coon, M.J. (1968) Role of hemoprotein P-450 in fatty acid omega-hydroxylation in a soluble enzyme system from liver microsomes. *J Biol Chem* **243**, 1331-1332.

Lu, A.Y. (1998) Drug-metabolism research challenges in the new millennium: individual variability in drug therapy and drug safety. *Drug Metab Dispos* **26**, 1217-1222.

Luo, G., Zeldin, D.C., Blaisdell, J.A., Hodgson, E., and Goldstein, J.A. (1998) Cloning and expression of murine CYP2Cs and their ability to metabolize arachidonic acid. *Arch Biochem Biophys* **357**, 45-57.

Mörsch, G. and Leibenguth, F. (1993) DNA fingerprinting of the roe deer, Capreolus capreolus L. *Comp Biochem Physiol [B]* **104**, 229-233.

Morse, H.C. (1981) The laboratory mouse-A historical perpective, in *The mouse in biomedical research.* (Foster HL, Small JD and Fox JG eds.) pp 1-16. Academic Press, New York.

Ma, Q., Dong, L., and Whitlock, J.P. (1995) Transcriptional activation by the mouse Ah receptor. Interplay between multiple stimulatory and inhibitory functions. *J Biol Chem* **270**, 12697-12703.

Mackenzie, P.I., Chowdhury, N.R., and Chowdhury, J.R. (1989) Characterization and regulation of rat liver UDP glucuronosyltransferases. *Clin Exp Pharmacol Physiol* **16**, 501-504.

Macleod, J.N., Sorensen, M.P., and Shapiro, B.H. (1987) Strain independent elevation of hepatic mono-oxygenase enzymes in female mice. *Xenobiotica* **17**, 1095-1102.

MacLeod, S., Tang, Y-M., Yokoi, T., Kamataki, T., Doublin, S., Lawson, B., Massengill, J., Kadlubar, F., and Lang, N. (1998) The role of a recently discovered genetic polymorphism in the regulation of the human CYP1A2 gene, in *Proceedings of the American Association for Cancer Research*, pp.396.

Mahgoub, A., Idle, J.R., Dring, L.G., Lancaster, R., and Smith, R.L. (1977) Polymorphic hydroxylation of Debrisoquine in man. *Lancet* **2**, 584-586.

Maltepe, E., Schmidt, J.V., Baunoch, D., Bradfield, C.A., and Simon, M.C. (1997) Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. *Nature* **386**, 403-407.

Markel, P., Shu, P., Ebeling, C., Carlson, G.A., Nagle, D.L., Smutko, J.S., and Moore, K.J. (1997) Theoretical and empirical issues for marker-assisted breeding of congenic mouse strains. *Nat Genet* **17**, 280-284.

Mattano, S.S., Erickson, R.P., Nesbitt, M.N., and Weber, W.W. (1988) Linkage of Nat and Es-1 in the mouse and development of strains congenic for N-acetyltransferase. *J Hered* **79**, 430-433.

Matthews, A.M., Roberts, D.W., Hinson, J.A., and Pumford, N.R. (1996) Acetaminophen-induced hepatotoxicity. Analysis of total covalent binding vs. specific binding to cysteine. *Drug Metab Dispos* **24**, 1192-1196.

McCarver, D.G., Byun, R., Hines, R.N., Hichme, M., and Wegenek, W. (1998) A genetic polymorphism in the regulatory sequences of human CYP2E1: association with increased chlorzoxazone hydroxylation in the presence of obesity and ethanol intake. *Toxicol Appl Pharmacol* **152**, 276-281.

McDonnell, W.M., Scheiman, J.M., and Traber, P.G. (1992) Induction of cytochrome P450IA genes (CYP1A) by omeprazole in the human alimentary tract. *Gastroenterology* **103** , 1509-1516.

McKinnon, R.A., Hall, P.D., Quattrochi, L.C., Tukey, R.H., and McManus, M.E. (1991) Localization of CYP1A1 and CYP1A2 messenger RNA in normal human liver and in hepatocellular carcinoma by in situ hybridization. *Hepatology* **14**, 848-856.

McKinnon, R.A. and Nebert, D.W. (1998) Cytochrome P450 knockout mice: new toxicological models. *Clin Exp Pharmacol Physiol* **25**, 783-787.

Meister, A. (1991) Glutathione deficiency produced by inhibition of its synthesis, and its reversal; applications in research and therapy. *Pharmacol Ther* **51**, 155-194.

Meister, A. (1995) Glutathione metabolism. *Methods Enzymol* **251**, 3-7.

Melo, J.A., Shendure, J., Pociask, K., and Silver, L.M. (1996) Identification of sex-specific quantitative trait loci controlling alcohol preference in C57BL/ 6 mice. *Nat Genet* **13**, 147-153.

Meyer, U.A. (1996) Overview of enzymes of drug metabolism. *J Pharmacokinet Biopharm* **24**, 449-459.

Micka, J., Milatovich, A., Menon, A., Grabowski, G.A., Puga, A., and Nebert, D.W. (1997) Human Ah receptor (AHR) gene: localization to 7p15 and suggestive correlation of polymorphism with CYP1A1 inducibility. *Pharmacogenetics* **7**, 95-101.

Miles, J.S., Moss, J.E., Meehan, R.R., and Wolf, C.R. (1990) Close linkage of the cytochrome P450IIA gene subfamily (Cyp2a) to Cyp2b and Coh on mouse chromosome 7. *Genomics* **7**, 445-448.

Miller, C.A., Slusher, L.B., and Vesell, E.S. (1985) Polymorphism of theophylline metabolism in man. *J Clin Invest* **75**, 1415-1425.

Miller, M.S., Jones, A.B., Park, S.S., and Anderson, L.M. (1990) The formation of 3-methylcholanthrene-initiated lung tumors correlates with induction of cytochrome P450IA1 by the carcinogen in fetal but not adult mice. *Toxicol Appl Pharmacol* **104**, 235-245.

Mimura, J., Yamashita, K., Nakamura, K., Morita, M., Takagi, T.N., Nakao, K., Ema, M., Sogawa, K., Yasuda, M., Katsuki, M., and Fujii-Kuriyama, Y. (1997) Loss of teratogenic response to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in mice lacking the Ah (dioxin) receptor. *Genes Cells* **2**, 645-654.

Miners, J.O. and Mackenzie, P.I. (1991) Drug glucuronidation in humans. *Pharmacol Ther* **51**, 347-369.

Minghetti, P.P., Law, S.W., and Dugaiczyk, A. (1985) The rate of molecular evolution of alpha-fetoprotein approaches that of pseudogenes. *Mol Biol Evol* **2**, 347-358.

Mohler, D.N., Majerus, P.W., Minnich, V., Hess, C.E., and Garrick, M.D. (1970) Glutathione synthetase deficiency as a cause of hereditary hemolytic disease. *N Engl J Med* **283**, 1253-1257.

Molowa, D.T., Schuetz, E.G., Wrighton, S.A., Watkins, P.B., Kremers, P., Mendez-Picon, G., Parker, G.A., and Guzelian, P.S. (1986) Complete cDNA sequence of a cytochrome P-450 inducible by glucocorticoids in human liver. *Proc Natl Acad Sci U S A* **83**, 5311-5315.

Morgan, E.T., Koop, D.R., and Coon, M.J. (1983) Comparison of six rabbit liver cytochrome P-450 isozymes in formation of a reactive metabolite of acetaminophen. *Biochem Biophys Res Commun* **112**, 8-13.

Morse, D.C., Stein, A.P., Thomas, P.E., and Lowndes, H.E. (1998) Distribution and induction of cytochrome P450 1A1 and 1A2 in rat brain. *Toxicol Appl Pharmacol* **152**, 232-239.

Motulsky, A.G. (1991) Pharmacogenetics and ecogenetics in 1991. *Pharmacogenetics* **1**, 2-3.

Myers, T.G., Thummel, K.E., Kalhorn, T.F., and Nelson, S.D. (1994) Preferred orientations in the binding of 4'-hydroxyacetanilide (acetaminophen) to cytochrome P450 1A1 and 2B1 isoforms as determined by 13C- and 15N-NMR relaxation studies. *J Med Chem* **37**, 860-867.

Mäenpää, J., Pelkonen, O., Cresteil, T., and Rane, A. (1993) The role of cytochrome P450 3A (CYP3A) isoform(s) in oxidative metabolism of testosterone and benzphetamine in human adult and fetal liver. *J Steroid Biochem Mol Biol* **44**, 61-67.

Nakachi, K., Imai, K., Hayashi, S., and Kawajiri, K. (1993) Polymorphisms of the CYP1A1 and glutathione S-transferase genes associated with susceptibility to lung cancer in relation to cigarette dose in a Japanese population. *Cancer Res* **53**, 2994-2999.

Nakajima, M., Yokoi, T., Mizutani, M., Shin, S., Kadlubar, F.F., and Kamataki, T. (1994) Phenotyping of CYP1A2 in Japanese population by analysis of caffeine urinary metabolites: absence of mutation prescribing the phenotype in the CYP1A2 gene. *Cancer Epidemiol Biomarkers Prev* **3**, 413-421.

Nakajima, M., Yokoi, T., Mizutani, M., Kinoshita, M., Funayama, M. and Kamataki, T. (1999) Genetic polymorphism in the 5'-flanking region of human *CYP1A2* gene: Effect on the CYP1A2 inducibility in humans. *J Biochem* **125**, 803-808.

Nebert, D.W. and Gelboin, H.V. (1969) The in vivo and in vitro induction of aryl hydrocarbon hydroxylase in mammalian cells of different species, tissues, strains, and developmental and hormonal states. *Arch Biochem Biophys* **134**, 76-89.

Nebert, D.W. (1989) The Ah locus: genetic differences in toxicity, cancer, mutation, and birth defects. *Crit Rev Toxicol* **20**, 153-174.

Nebert, D.W., Petersen, D.D., and Fornace, A.J. (1990) Cellular responses to oxidative stress: the [Ah] gene battery as a paradigm. *Environ Health Perspect* **88**, 13-25.

Nebert, D.W., Puga, A., and Vasiliou, V. (1993) Role of the Ah receptor and the dioxin-inducible [Ah] gene battery in toxicity, cancer, and signal transduction. *Ann N Y Acad Sci* **685**, 624-640.

Nebert, D.W. and Duffy, J.J. (1997) How knockout mouse lines will be used to study the role of drug-metabolizing enzymes and their receptors during reproduction and development, and in environmental toxicity, cancer, and oxidative stress. *Biochem Pharmacol* **53**, 249-254.

Nebert, D.W. and Carvan, M.J. (1997) Ecogenetics: from ecology to health. *Toxicol Ind Health* **13**, 163-192.

Nebert, D.W. (1997) Polymorphisms in drug-metabolizing enzymes: what is their clinical relevance and why do they exist?. *Am J Hum Genet* **60**, 265-271.

Nelson, D.R., Kamataki, T., Waxman, D.J., Guengerich, F.P., Estabrook, R.W., Feyereisen, R., Gonzalez, F.J., Coon, M.J., Gunsalus, I.C., Gotoh, O., Okuda, K., and Nebert, D.W. (1993) The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol* **12**, 1-51.

Nelson, D.R., Koymans, L., Kamataki, T., Stegeman, J.J., Feyereisen, R., Waxman, D.J., Waterman, M.R., Gotoh, O., Coon, M.J., Estabrook, R.W., Gunsalus, I.C., and Nebert, D.W. (1996) P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* **6**, 1-42.

Okey, A.B., Bondy, G.P., Mason, M.E., Kahl, G.F., Eisen, H.J., Guenthner, T.M., and Nebert, D.W. (1979) Regulatory gene product of the Ah locus. Characterization of the cytosolic inducer-receptor complex and evidence for its nuclear translocation. *J Biol Chem* **254**, 11636-11648.

Omura, T., and Sato, R. (1964) The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239**, 2370-2378.

Ono, S., Hatanaka, T., Hotta, H., Tsutsui, M., Satoh, T., and Gonzalez, F.J. (1995) Chlorzoxazone is metabolized by human CYP1A2 as well as by human CYP2E1. *Pharmacogenetics* **5**, 143-150.

Ott, J. (1979) Genetic linkage studies in man. *Transplant Proc* **11**, 1689-1691.

Ott, J. (1999) Methods of analysis and resources available for genetic trait mapping. *J Hered* **90**, 68-70.

Otto, D.M., Sen, C.K., Casley, W.L., and Moon, T.W. (1997) Regulation of 3,3',4,4'-tetrachlorobiphenyl induced cytochrome P450 metabolism by thiols in tissues of rainbow trout. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* **117**, 299-309.

Otto, D.M.E., Sen, C., Casley, W.L. and Moon, T.W. (1999) Regulation of cytochrome P4501A metabolism by glutathione. *Pharmacology and Toxicology* **84**, 201-210.

Owens, R.A. and Nebert, D.W. (1990) Expression of the chloramphenicol acetyltransferase (CAT) reporter gene by the murine Cyp1a-2 (cytochrome P3(450)) promoter in hepatoma cell cultures. *Biochem Biophys Res Commun* **172**, 1109-1115.

Paigen, B., Ward, E., Steenland, K., Havens, M., and Sartori, P. (1979) Aryl hydrocarbon hydroxylase inducibility is not altered in bladder cancer patients or their progeny. *Int J Cancer* **23**, 312-315.

Pasco, D.S., Boyum, K.W., Merchant, S.N., Chalberg, S.C., and Fagan, J.B. (1988) Transcriptional and post-transcriptional regulation of the genes encoding cytochromes P-450c and P-450d in vivo and in primary hepatocyte cultures. *J Biol Chem* **263**, 8671-8676.

Paterson, A.H., Lander, E.S., Hewitt, J.D., Peterson, S., Lincoln, S.E., and Tanksley, S.D. (1988) Resolution of quantitative traits into Mendelian factors by using a complete linkage map of restriction fragment length polymorphisms. *Nature* **335**, 721-726.

Patten, C.J., Thomas, P.E., Guy, R.L., Lee, M., Gonzalez, F.J., Guengerich, F.P., and Yang, C.S. (1993) Cytochrome P450 enzymes involved in acetaminophen activation by rat and human liver microsomes and their kinetics. *Chem Res Toxicol* **6**, 511-518.

Paulson, G.D., Feil, V.J., and McDonald, J.J. (1990) Metabolism of 1,3-di-(4-[N-(4,6-dimethyl-2-pyrimidinyl)sulphamoyl] phenyl)triazene (DDPSPT) in the rat. *Xenobiotica* **20**, 395-400.

Peirce, J.L., Derr, R., Shendure, J., Kolata, T., and Silver, L.M. (1998) A major influence of sex-specific loci on alcohol preference in C57Bl/6 and DBA/2 inbred mice. *Mamm Genome* **9** , 942-948.

Persson, I., Johansson, I., Bergling, H., Dahl, M.L., Seidegärd, J., Rylander, R., Rannug, A., Hëgberg, J., and Sundberg, M.I. (1993) Genetic polymorphism of cytochrome P4502E1 in a Swedish population. Relationship to incidence of lung cancer. *FEBS Lett* **319**, 207-211.

Peter, R., Böcker, R., Beaune, P.H., Iwasaki, M., Guengerich, F.P., and Yang, C.S. (1990) Hydroxylation of chlorzoxazone as a specific probe for human liver cytochrome P-450IIE1. *Chem Res Toxicol* **3**, 566-573.

Pickett, C.B. (1987) Structure and regulation of glutathione S-transferase genes. *Essays Biochem* **23**, 116-143.

Pineau, T., Fernandez-Salguero, P., Lee, S.S., McPhail, T., Ward, J.M., and Gonzalez, F.J. (1995) Neonatal lethality associated with respiratory distress in mice lacking cytochrome P450 1A2. *Proc Natl Acad Sci U S A* **92**, 5134-5138.

Poland, A. and Glover, E. (1973) Chlorinated dibenzo-p-dioxins: potent inducers of delta-aminolevulinic acid synthetase and aryl hydrocarbon hydroxylase. II. A study of the structure-activity relationship. *Mol Pharmacol* **9**, 736-747.

Poland, A., Glover, E., and Kende, A.S. (1976) Stereospecific, high affinity binding of 2,3,7,8-tetrachlorodibenzo-p-dioxin by hepatic cytosol. Evidence that the binding species is receptor for induction of aryl hydrocarbon hydroxylase. *J Biol Chem* **251**, 4936-4946.

Poland, A., Clover, E., Kende, A.S., DeCamp, M., and Giandomenico, C.M. (1976) 3,4,3',4'-Tetrachloro azoxybenzene and azobenzene: potent inducers of aryl hydrocarbon hydroxylase. *Science* **194**, 627-630.

Porter, T.D. and Coon, M.J. (1991) Cytochrome P-450. Multiplicity of isoforms, substrates, and catalytic and regulatory mechanisms. *J Biol Chem* **266**, 13469-13472.

Potter, W.Z., Davis, D.C., Mitchell, J.R., Jollow, D.J., Gillette, J.R., and Brodie, B.B. (1973) Acetaminophen-induced hepatic necrosis. 3. Cytochrome P-450-mediated covalent binding in vitro. *J Pharmacol Exp Ther* **187**, 203-210.

Powis, G., Svingen, B.A., Dahlin, D.C., and Nelson, S.D. (1984) Enzymatic and non-enzymatic reduction of N-acetyl-p-benzoquinone imine and some properties of the N-acetyl-p-benzosemiquinone imine radical. *Biochem Pharmacol* **33**, 2367-2370.

Puga, A., Nebert, D.W., McKinnon, R.A., and Menon, A.G. (1997) Genetic polymorphisms in human drug-metabolizing enzymes: potential uses of reverse genetics to identify genes of toxicological relevance. *Crit Rev Toxicol* **27**, 199-222.

Qiu, Y., Benet, L.Z., and Burlingame, A.L. (1998) Identification of the hepatic protein targets of reactive metabolites of acetaminophen in vivo in mice using two-dimensional gel electrophoresis and mass spectrometry. *J Biol Chem* **273**, 17940-17953.

Quattrochi, L.C., Pendurthi, U.R., Okino, S.T., Potenza, C., and Tukey, R.H. (1986) Human cytochrome P-450 4 mRNA and gene: part of a multigene family that contains Alu sequences in its mRNA. *Proc Natl Acad Sci U S A* **83**, 6731-6735.

Raffalli-Mathieu, F., Geneste, O., and Lang, M.A. (1997) Characterization of two nuclear proteins that interact with cytochrome P-450 1A2 mRNA. Regulation of RNA binding and possible role in the expression of the Cyp1a2 gene. *Eur J Biochem* **245**, 17-24.

Rahman, A., Korzekwa, K.R., Grogan, J., Gonzalez, F.J., and Harris, J.W. (1994) Selective biotransformation of taxol to 6 alpha-hydroxytaxol by human cytochrome P450 2C8. *Cancer Res* **54**, 5543-5546.

Raucy, J.L., Lasker, J.M., Lieber, C.S., and Black, M. (1989) Acetaminophen activation by human liver cytochromes P450IIE1 and P450IA2. *Arch Biochem Biophys* **271**, 270-283.

Relling, M.V., Lin, J.S., Ayers, G.D., and Evans, W.E. (1992) Racial and gender differences in N-acetyltransferase, xanthine oxidase, and CYP1A2 activities. *Clin Pharmacol Ther* **52**, 643-658.

Rikke, B.A. and Johnson, T.E. (1998) Towards the cloning of genes underlying murine QTLs. *Mamm Genome* **9**, 963-968.

Roots, I., Brockmöller, J., Drakoulis, N., and Loddenkemper, R. (1992) Mutant genes of cytochrome P-450IID6, glutathione S-transferase class Mu, and arylamine N-acetyltransferase in lung cancer patients. *Clin Investig* **70**, 307-319.

Rostami-Hodjegan, A., Nurminen, S., Jackson, P.R., and Tucker, G.T. (1996) Caffeine urinary metabolite ratios as markers of enzyme activity: a theoretical assessment. *Pharmacogenetics* **6**, 121-149.

Rowlands, J.C. and Gustafsson, J.A. (1997) Aryl hydrocarbon receptor-mediated signal transduction. *Crit Rev Toxicol* **27**, 109-134.

Rubattu, S., Volpe, M., Kreutz, R., Ganten, U., Ganten, D., and Lindpaintner, K. (1996) Chromosomal mapping of quantitative trait loci contributing to stroke in a rat model of complex human disease. *Nat Genet* **13**, 429-434.

Ryffel, B. (1997) Impact of knockout mice in toxicology. *Crit Rev Toxicol* **27**, 135-154.

Ryu, D.Y., Levi, P.E., Fernandez-Salguero, P., Gonzalez, F.J., and Hodgson, E. (1996) Piperonyl butoxide and acenaphthylene induce cytochrome P450 1A2 and 1B1 mRNA in aromatic hydrocarbon-responsive receptor knock-out mouse liver. *Mol Pharmacol* **50**, 443-446.

Sabath, D.E., Broome, H.E., and Prystowsky, M.B. (1990) Glyceraldehyde-3-phosphate dehydrogenase mRNA is a major interleukin 2-induced transcript in a cloned T-helper lymphocyte. *Gene* **91**, 185-191.

Sachse, C., Brockmöller, J., Bauer, S., and Roots, I. (1999) Functional significance of a C-A polymorphism in intron 1 of the cytochrome P450 CYP1A2 gene tested with caffeine. *Br. J. Clin. Pharmacol.* **47**, 445-449.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular cloning: A laboratory manual, 2nd ed.* Cold Spring Harbor Press, New York.

Schilter, B. and Omiecinski, C.J. (1993) Regional distribution and expression modulation of cytochrome P-450 and epoxide hydrolase mRNAs in the rat brain. *Mol Pharmacol* **44**, 990-996.

Schmidt, J.V., Su, G.H., Reddy, J.K., Simon, M.C., and Bradfield, C.A. (1996) Characterization of a murine Ahr null allele: involvement of the Ah receptor in hepatic growth and development. *Proc Natl Acad Sci U S A* **93**, 6731-6736.

Schuetz, J.D., Beach, D.L., and Guzelian, P.S. (1994) Selective expression of cytochrome P450 CYP3A mRNAs in embryonic and adult human liver. *Pharmacogenetics* **4**, 11-20.

Schweikl, H., Taylor, J.A., Kitareewan, S., Linko, P., Nagorney, D., and Goldstein, J.A. (1993) Expression of CYP1A1 and CYP1A2 genes in human liver. *Pharmacogenetics* **3**, 239-249.

Shahidi, N.T. (1968) Acetophenetidin-induced methemoglobinemia. *Ann N Y Acad Sci* **151**, 822-832.

Shan, X.Q., Aw, T.Y., and Jones, D.P. (1990) Glutathione-dependent protection against oxidative injury. *Pharmacol Ther* **47**, 61-71.

Shen, W., Kamendulis, L.M., Ray, S.D., and Corcoran, G.B. (1992) Acetaminophen-induced cytotoxicity in cultured mouse hepatocytes: effects of Ca(2+)-endonuclease, DNA repair, and glutathione depletion inhibitors on DNA fragmentation and cell death. *Toxicol Appl Pharmacol* **112**, 32-40.

Shertzer, H.G., Vasiliou, V., Liu, R.M., Tabor, M.W., and Nebert, D.W. (1995) Enzyme induction by L-buthionine (S,R)-sulfoximine in cultured mouse hepatoma cells. *Chem Res Toxicol* **8**, 431-436.

Shertzer, H.G., Nebert, D.W., Puga, A., Ary, M., Sonntag, D., Dixon, K., Robinson, L.J., Cianciolo, E., and Dalton, T.P. (1998) Dioxin causes a sustained oxidative stress response in the mouse. *Biochem Biophys Res Commun* **253**, 44-48.

Shimada, T., Martin, M.V., Pruess-Schwartz, D., Marnett, L.J., and Guengerich, F.P. (1989) Roles of individual human cytochrome P-450 enzymes in the bioactivation of benzo(a)pyrene, 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene, and other dihydrodiol derivatives of polycyclic aromatic hydrocarbons. *Cancer Res* **49**, 6304-6312.

Shimada, T., Yamazaki, H., Mimura, M., Inui, Y., and Guengerich, F.P. (1994) Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *J Pharmacol Exp Ther* **270**, 414-423.

Simmons, D.L., Lalley, P.A., and Kasper, C.B. (1985) Chromosomal assignments of genes coding for components of the mixed-function oxidase system in mice. Genetic localization of the cytochrome P-450PCN and P-450PB gene families and the NADPH-cytochrome P-450 oxidoreductase and epoxide hydratase genes. *J Biol Chem* **260**, 515-521.

Sinclair, P.R., Gorman, N., Dalton, T., Walton, H.S., Bement, W.J., Sinclair, J.F., Smith, A.G., and Nebert, D.W. (1998) Uroporphyria produced in mice by iron and 5-aminolaevulinic acid does not occur in *Cyp1a2*(-/-) null mutant mice. *Biochem J* **330**, 149-153.

Skiles, G.L., Smith, D.J., Appleton, M.L., Carlson, J.R., and Yost, G.S. (1991) Isolation of a mercapturate adduct produced subsequent to glutathione conjugation of bioactivated 3-methylindole. *Toxicol Appl Pharmacol* **108**, 531-537.

Snawder, J.E., Roe, A.L., Benson, R.W., Casciano, D.A., and Roberts, D.W. (1994) Cytochrome P450-dependent metabolism of acetaminophen in four human transgenic lymphoblastoid cell lines. *Pharmacogenetics* **4**, 43-46.

Snyder, L.H. (1932) Studies in human inheritance. IX. The inheritance of taste deficiency in man. *Ohio J. Sci.* **32**, 436-468.

Song, B.J., Gelboin, H.V., Park, S.S., and Friedman, F.K. (1985) Epitope-relatedness and phenotyping of hepatic cytochromes P-450 with monoclonal antibodies. *Biochem J* **231**, 671-676.

Spatzenegger, M. and Jaeger, W. (1995) Clinical importance of hepatic cytochrome P450 in drug metabolism. *Drug Metab Rev* **27**, 397-417.

Spielberg, S.P. (1985) Acetaminophen toxicity in lymphocytes heterozygous for glutathione synthetase deficiency. *Can J Physiol Pharmacol* **63**, 468-471.

Spielberg, S.P. (1996) N-acetyltransferases: pharmacogenetics and clinical consequences of polymorphic drug metabolism. *J Pharmacokinet Biopharm* **24**, 509-519.

Staats, J. (1981) Inbred and segregating inbred strains, in *The mouse in biomedical research.* (Foster HL, Small JD and Fox JG eds.) pp 177-210. Academic Press, New York.

Steele, C.M., Masson, H.A., Battershill, J.M., Gibson, G.G., and Ioannides, C. (1983) Metabolic activation of paracetamol by highly purified forms of cytochrome P-450. *Res Commun Chem Pathol Pharmacol* **40**, 109-119.

Sugimura, H., Hamada, G.S., Suzuki, I., Iwase, T., Kiyokawa, E., Kino, I., and Tsugane, S. (1995) CYP1A1 and CYP2E1 polymorphism and lung cancer, case-control study in Rio de Janeiro, Brazil. *Pharmacogenetics* **5**, S145-S148

Sundvall, M., Jirholt, J., Yang, H.T., Jansson, L., Engstr÷m, A., Pettersson, U., and Holmdahl, R. (1995) Identification of murine loci associated with susceptibility to chronic experimental autoimmune encephalomyelitis. *Nat Genet* **10**, 313-317.

Swanson, H.I. and Bradfield, C.A. (1993) The AH-receptor: genetics, structure and function. *Pharmacogenetics* **3**, 213-230.

Swanson, H.I. and Yang, J.H. (1998) The aryl hydrocarbon receptor interacts with transcription factor IIB. *Mol Pharmacol* **54**, 671-677.

Tang, B.K., Zhou, Y., Kadar, D., and Kalow, W. (1994) Caffeine as a probe for CYP1A2 activity: potential influence of renal factors on urinary phenotypic trait measurements. *Pharmacogenetics* **4**, 117-124.

Tarloff, J.B., Khairallah, E.A., Cohen, S.D., and Goldstein, R.S. (1996) Sex- and age-dependent acetaminophen hepato- and nephrotoxicity in Sprague-Dawley rats: role of tissue accumulation, nonprotein sulfhydryl depletion, and covalent binding. *Fundam Appl Toxicol* **30**, 13-22.

Tassaneeyakul, W., Birkett, D.J., Veronese, M.E., McManus, M.E., Tukey, R.H., Quattrochi, L.C., Gelboin, H.V., and Miners, J.O. (1993) Specificity of substrate and inhibitor probes for human cytochromes P450 1A1 and 1A2. *J Pharmacol Exp Ther* **265**, 401-407.

Tassaneeyakul, W., Birkett, D.J., McManus, M.E., Veronese, M.E., Andersson, T., Tukey, R.H., and Miners, J.O. (1994a) Caffeine metabolism by human hepatic cytochromes P450: contributions of 1A2, 2E1 and 3A isoforms. *Biochem Pharmacol* **47**, 1767-1776.

Tassaneeyakul, W., Birkett, D.J., Veronese, M.E., McManus, M.E., Tukey, R.H., and Miners, J.O. (1994b) Direct characterization of the selectivity of furafylline as an inhibitor of human cytochromes P450 1A1 and 1A2. *Pharmacogenetics* **4**, 281-284.

Taylor, B.A. and Phillips, S.J. (1996) Detection of obesity QTLs on mouse chromosomes 1 and 7 by selective DNA pooling. *Genomics* **34**, 389-398.

Tephly, T.R., Townsend, M., and Green, M.D. (1989) UDP-glucuronosyltransferases in the metabolic disposition of xenobiotics. *Drug Metab Rev* **20**, 689-695.

Thomsen, M.S., Loft, S., Roberts, D.W., and Poulsen, H.E. (1995) Cytochrome P4502E1 inhibition by propylene glycol prevents acetaminophen (paracetamol) hepatotoxicity in mice without cytochrome P4501A2 inhibition. *Pharmacol Toxicol* **76**, 395-399.

Threadgill, D.W., Dlugosz, A.A., Hansen, L.A., Tennenbaum, T., Lichti, U., Yee, D., LaMantia, C., Mourton, T., Herrup, K., and Harris, R.C. (1995) Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. *Science* **269**, 230-234.

Thummel, K.E., Lee, C.A., Kunze, K.L., Nelson, S.D., and Slattery, J.T. (1993) Oxidation of acetaminophen to N-acetyl-p-aminobenzoquinone imine by human CYP3A4. *Biochem Pharmacol* **45**, 1563-1569.

Tonge, R.P., Kelly, E.J., Bruschi, S.A., Kalhorn, T., Eaton, D.L., Nebert, D.W., and Nelson, S.D. (1998) Role of CYP1A2 in the hepatotoxicity of acetaminophen: investigations using Cyp1a2 null mice. *Toxicol Appl Pharmacol* **153**, 102-108.

Tsuneoka, Y., Fukushima, K., Matsuo, Y., Ichikawa, Y., and Watanabe, Y. (1996) Genotype analysis of the CYP2C19 gene in the Japanese population. *Life Sci* **59**, 1711-1715.

Tu, C.P., Lai, H.C., Li, N.Q., Weiss, M.J., and Reddy, C.C. (1984) The Yc and Ya subunits of rat liver glutathione S-transferases are the products of separate genes. *J Biol Chem* **259**, 9434-9439.

Uematsu, F., Kikuchi, H., Motomiya, M., Abe, T., Sagami, I., Ohmachi, T., Wakui, A., Kanamaru, R., and Watanabe, M. (1991) Association between restriction fragment length polymorphism of the human cytochrome P450IIE1 gene and susceptibility to lung cancer. *Jpn J Cancer Res* **82**, 254-256.

Vasiliou, V., Puga, A., Chang, C.Y., Tabor, M.W., and Nebert, D.W. (1995) Interaction between the Ah receptor and proteins binding to the AP-1-like electrophile response element (EpRE) during murine phase II [Ah] battery gene expression. *Biochem Pharmacol* **50**, 2057-2068.

Vasiliou, V., Reuter, S.F., Kozak, C.A., and Nebert, D.W. (1995) Mouse class 3 aldehyde dehydrogenases. *Adv Exp Med Biol* **372**, 151-158.

Vatsis, K.P., Weber, W.W., Bell, D.A., Dupret, J.M., Evans, D.A., Grant, D.M., Hein, D.W., Lin, H.J., Meyer, U.A., and Relling, M.V. (1995) Nomenclature for N-acetyltransferases. *Pharmacogenetics* **5**, 1-17.

Vistisen, K., Poulsen, H.E., and Loft, S. (1992) Foreign compound metabolism capacity in man measured from metabolites of dietary caffeine. *Carcinogenesis* **13**, 1561-1568.

Vogel, F. (1959) Moderne problem der Humangenetik. *Ergeb Inn. Med. Kinderheilkd* **12**, 52-125.

Von Wartburg, J.P., Bethune, J.L., and Vallee, B.L. (1964) Human liver alcohol dehydrogenase. Kinetic and physicochemical properties. *Biochemistry* **3**, 1775-1782.

Wakeland, E., Morel, L., Achey, K., Yui, M., and Longmate, J. (1997) Speed congenics: a classic technique in the fast lane (relatively speaking). *Immunol Today* **18**, 472-477.

Watanabe, K., Kayano, Y., Matsunaga, T., Yamamoto, I., and Yoshimura, H. (1995) 3,4,5-Trimethoxyphenylacetaldehyde, an intermediate metabolite of mescaline, is a substrate for microsomal aldehyde oxygenase in the mouse liver. *Biol Pharm Bull* **18**, 696-699.

Weber, W.W. and Hein, D.W. (1985) N-acetylation pharmacogenetics. *Pharmacol Rev* **37**, 25-79.

Wehner, J.M., Radcliffe, R.A., Rosmann, S.T., Christensen, S.C., Rasmussen, D.L., Fulker, D.W., and Wiles, M. (1997) Quantitative trait locus analysis of contextual fear conditioning in mice. *Nat Genet* **17**, 331-334.

White, C.W., Stabler, S.P., Allen, R.H., Moreland, S., and Rosenberg, A.A. (1994) Plasma cysteine concentrations in infants with respiratory distress. *J Pediatr* **125**, 769-777.

Whitehouse, L.W., and Paul, C.J. (1987). Use of a discrete diode array spectrophotometer for the quantitative and qualitative analysis of cytochrome P-450. *Mikrochim. Acta* **2**, 65-72.

Whitehouse, L.W., Pakuts, A.P., Paul, C.J., Mueller, R.W., and Thomas, B.H. (1990) Hepatic effects of ketoconazole in the male Swiss Webster mouse: temporal changes in drug metabolic parameters. *Can J Physiol Pharmacol* **68**, 1136-1142.

Williams, R.W., Strom, R.C., Rice, D.S., and Goldowitz, D. (1996) Genetic and environmental control of variation in retinal ganglion cell number in mice. *J Neurosci* **16**, 7193-7205.

Wright, S. (1968) *Evolution and the genetics of populations, Vol. 1, Genetic and biometric foundations*. pp373-420, University of Chicago Press, Chicago.

Wrighton, S.A., VandenBranden, M., and Ring, B.J. (1996) The human drug metabolizing cytochromes P450. *J Pharmacokinet Biopharm* **24**, 461-473.

Wynshaw-Boris, A. (1996) Model mice and human disease [news; comment]. *Nat Genet* **13**, 259-260.

Xiao, G.H., Pinaire, J.A., Rodrigues, A.D., and Prough, R.A. (1995) Regulation of the Ah gene battery via Ah receptor-dependent and independent processes in cultured adult rat hepatocytes. *Drug Metab Dispos* **23**, 642-650.

Yamazoe, Y., Abu-Zeid, M., Yamauchi, K., and Kato, R. (1988) Metabolic activation of pyrolysate arylamines by human liver microsomes; possible involvement of a P-488-H type cytochrome P-450. *Jpn J Cancer Res* **79**, 1159-1167.

Yanagimoto, T., Itoh, S., Sawada, M., and Kamataki, T. (1997) Mouse cytochrome P450 (Cyp3a11): predominant expression in liver and capacity to activate aflatoxin B1. *Arch Biochem Biophys* **340**, 215-218.

Zaher, H., Yang, T.J., Gelboin, H.V., Fernandez-Salguero, P., and Gonzalez, F.J. (1998a) Effect of phenobarbital on hepatic CYP1A1 and CYP1A2 in the Ahr-null mouse. *Biochem Pharmacol* **55**, 235-238.

Zaher, H., Buters, J.T., Ward, J.M., Bruno, M.K., Lucas, A.M., Stern, S.T., Cohen, S.D., and Gonzalez, F.J. (1998b) Protection against acetaminophen toxicity in CYP1A2 and CYP2E1 double-null mice. *Toxicol Appl Pharmacol* **152**, 193-199.

Zeillinger, R., Schneeberger, C., Speiser, P., and Kury, F. (1993) Rapid quantitative analysis of differential PCR products by high-performance liquid chromatography. *Biotechniques* **15**, 89-95.