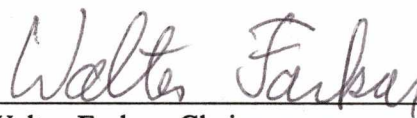


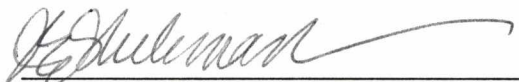
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I am submitting herewith a thesis written by Lisa Lenore Wessner entitled "Molecular Mechanisms Governing Lung Tumor Pathogenesis in Mice with Differing Susceptibility to Polycyclic Hydrocarbon-mediated Tumorigenesis." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Life Sciences.

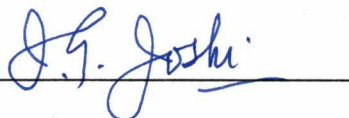


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We have read this thesis and
recommend its acceptance.



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Accepted for the Council:



Associate Vice Chancellor and
Dean of The Graduate School

**MOLECULAR MECHANISMS GOVERNING LUNG TUMOR
PATHOGENESIS IN MICE WITH DIFFERING
SUSCEPTIBILITY TO POLYCYCLIC HYDROCARBON-
MEDIATED TUMORIGENESIS**

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Lisa Lenore Wessner

August 1996

DEDICATIONS

This thesis is dedicated with love to:

My family, especially my parents,

Edwin L. Wessner

and

Eleanor B. Wessner

who support me every step of the way.

My best friends,

Jennifer Anderson

and

Kristine Jett

whose unconditional friendship is an inspiration.

The members of the UT Women's Rugby team

who have become my extended family;

you know who you are.

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Mere thank yous can never fully express my sentiments of gratitude toward those who have guided me over the past two years. I would, however, like to make a valiant attempt.

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ABSTRACT

A pharmacogenetic mouse model was utilized to determine the role of cytochrome P4501a1 expression on the formation of *Ki-ras* mutations in lung tumors following transplacental exposure to polycyclic aromatic hydrocarbons. A backcross between *Ah* responsive male B6D2F1 mice and nonresponsive female DBA mice resulted in a litter in which both responsive and nonresponsive fetuses resided in the same nonresponsive maternal environment. Pregnant mothers received a single ip injection of either 10 or 30 mg/kg of 3-methylcholanthrene or olive oil vehicle on day 17 of gestation. At the higher dose of 3-methylcholanthrene, the responsive offspring of both sexes had significantly ($P < 0.05$) higher incidences of lung tumors than their nonresponsive litter mates. The male responsive mice also exhibited a significantly increased liver tumor incidence over the nonresponsive mice at the $P < 0.05$ level. Administration of 10 mg/kg of 3-methylcholanthrene caused a very low incidence of lung tumors and did not result in the appearance of macroscopically visible liver tumors. Exons 1 and 2 of the *Ki-ras* gene were amplified from paraffin-embedded tissue samples. The polymerase chain reaction products were screened by allele-specific hybridization. Thirteen of 16 lung tumors (81%) exhibited point mutations in the 12th or 13th codon, including 7 tumors that contained GGT→GTT (GLY¹²→VAL¹²) transversions, 4 which exhibited GGT→TGT (GLY¹²→CYS¹²) transversions, and 2 which contained GGC→CGC (GLY¹³→ARG¹³) transversions. None of the tumors had mutations at codon 61. These results are consistent with a key role for *Cyp1a1* in modulating individual susceptibility to cancer formation through the formation of reactive intermediates that bind to DNA and result in activating mutations in key regulatory molecules.

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LIST OF ABBREVIATIONS

AHH	aryl hydrocarbon hydroxylase
AhR	aryl hydrocarbon receptor
ASO	allele-specific oligonucleotide hybridization
B6	C57BL/6 mouse strain
BPB	bromophenol blue
β NF	beta-naphthoflavone
BP	benzo[a]pyrene
BSA	bovine serum albumin
CYP	cytochrome P450
D2	DBA/2 mouse strain
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EROD	ethoxyresorufin <i>O</i> -deethylase
MC	3-methylcholanthrene
NADPH	nicotinamide adenine dinucleotide phosphate
PAH	polycyclic aromatic hydrocarbon
PCR	polymerase chain reaction
RNA	ribonucleic acid
SCLC	small cell lung cancer
SDS	sodium dodecyl sulfate
SSC	0.15 M sodium chloride/0.015 M sodium citrate
SSCP	single strand conformation polymorphism
<i>Taq</i>	<i>Thermus aquaticus</i>
T_M	melting temperature

CHAPTER I

INTRODUCTION

Rationale

Smoking has been an American vice and somewhat of an epidemic for decades. Unpublished data from the 1992 Current Population Survey conducted by the National Cancer Institute reported that 27.4% of all adult males in the United States smoked while 22.2% of all adult females smoked (1). In 1994, lung cancer accounted for 153,000 of all cancer deaths in the U.S. 94,000 of these deaths were males and 53,000 were women (1). Case control studies in humans as well as indirect studies monitoring lung cancer patterns in populations in relation to smoking habits provide evidence for smoking as a primary cause of lung cancer (2). The epidemiological studies linking smoking and lung cancer have been consistent across several studies, demonstrate a strong correlative relationship, the correct temporal sequence between exposure and disease, and the coherence of the association in a dose-response relationship. It is estimated that 90% of lung cancers are a direct result of cigarette smoking while indirect exposure to tobacco smoke is responsible for 30% of the cancer deaths in nonsmokers (1). Bacterial assays have analyzed tobacco smoke constituents for mutagenicity and have found certain fractions to contain a variety of polycyclic aromatic hydrocarbons (2). It is this fraction which contains the main tumorigenic activity associated with cigarette smoke. Considering the prevalence of the smoking habit in the United States and the fact that cigarette smoke contains many complete carcinogens, direct- and indirect-

acting tumor initiators, tumor promoters, and mutagens, research studies need to be designed to fully understand the ramifications of this smoking epidemic in the human population at the molecular level.

Statement of the Problem

Although it is known that cancer of the lung is the leading cause of cancer-related deaths among both women and men in the United States (1), few studies exist to correlate fetal exposure to environmental contaminants with subsequent middle age lung tumor incidence. In one such study, it was shown that the tumor incidence for several organ sites in adults was increased as a result of transplacental or early passive childhood exposure to a smoking environment regardless of whether the mother or father smoked (3). Several studies, cited below, have shown fetal tissues to be more sensitive to the toxic and carcinogenic effects of chemical and physical carcinogens than are adult tissues. Epidemiological studies, also cited below, suggest that exposure to cigarette smoke during pregnancy may result in the development of cancer in the offspring, indicating that the gestating embryo and fetus may be at greater risk than the adult population for the toxic and carcinogenic effects of chemical exposures. It is necessary to elucidate factors mediating tumor formation in the fetal compartment by environmental carcinogens and to determine how chemical exposure is modulated by individual genetic composition. Therefore, research efforts should be designed to determine risk factors modulating individual susceptibility to carcinogenic agents, potential risks of environmental contaminants to the fetal population, and the pathogenesis of lung tumor formation following *in utero* exposure to carcinogenic agents.

Specific Aim of the Study

This study utilizes a pharmacogenetic mouse model system for human lung tumorigenesis in order to provide data demonstrating how genetics and environmental factors interact in determining individual susceptibility to lung tumors. A bioassay component determined the tumor yield resulting from transplacental exposure to the polycyclic aromatic hydrocarbon (PAH), 3-methylcholanthrene (MC), in strains of mice with differing inducibility for *Cyp1a1*. It has previously been shown that the sensitivity of the fetus to *in utero* carcinogen exposure correlates with their metabolic phenotype (4, 5). This portion of the project provided documentation supporting the importance of the metabolic capacity of the fetus in determining tumor susceptibility as well as provide a sufficient amount of lung tumor tissue to allow for a comprehensive analysis of the *Ki-ras* protooncogene, which has been implicated in lung tumor pathogenesis.

At the molecular level, the lung tumor tissue generated from the bioassay was probed in order to analyze the mutational spectrum produced in the *Ki-ras* gene, particularly at codons 12, 13, and 61, following transplacental exposure of mice *in utero* to carcinogenic agents. Examination of the *Ki-ras* oncogenic locus by the polymerase chain reaction (PCR) and allele-specific oligonucleotide (ASO) hybridization illustrated specific patterns of oncogene mutations in the tumor model. The study considers whether differences in the metabolic capacity toward MC results in differences in the mutational spectrum produced by MC. With the dual components of this project, the bioassay and molecular oncogene analysis, the data produced illustrates the influence individual genetics has in susceptibility to cancer as a result of *in utero* exposure to environmental chemicals.

CHAPTER II

REVIEW OF THE LITERATURE

Cytochrome P450 Monooxygenase

The cytochrome P450 monooxygenase system represents a family of hemoproteins possessing catalytic oxidative activity toward a vast array of endogenous and exogenous substrates, including drugs, steroids, fatty acids, and environmental contaminants such as chemical carcinogens and mutagens. Recombinant DNA techniques have allowed the identification of over 70 distinct cytochrome P450 genes in various species and, to date, eight major mammalian gene families have been identified (6). Currently, two hypotheses have been proposed to explain how P450 enzymes are able to oxidize such a broad range of substrates. The general consensus in the past has been that overlapping substrate specificity of P450 isozymes accounts for all diversity seen in the P450-mediated reactions (7). An emerging hypothesis suggests many organisms have the genetic capacity to produce as many distinct forms of P450 as there are inducers (7). As a new chemical enters the system, a new drug-metabolizing enzyme is mobilized in response. To date, insufficient evidence has been obtained to allow a clarification as to which hypothesis is correct. Since the majority of phase I oxidative reactions are catalyzed by cytochrome P450, this enzyme system is worthy of in depth study.

Exposure, *in vivo*, of animals, humans, or cell cultures to certain substrates of this microsomal monooxygenase enzyme system results in the induction of specific P450 forms,

a phenomenon most often associated with the CYP1-4 families (8, 9). Some of the most extensively studied substrates of the P450 enzyme system are the PAHs, ubiquitous chemicals found as a result of the combustion of coal, air pollution, cigarette smoke, and charcoal-cooked foods. Figure B1 shows one of the most extensively studied PAHs, 3-methylcholanthrene, and its metabolism by P450. Exposure to polycyclic hydrocarbons results in the induction of *Cyp1a1* and *1a2* (7, 10-12) followed by enzyme-mediated metabolic conversion of the PAH compound by *Cyp1a1* to more polar molecules for rapid elimination from the body. However, metabolism by *Cyp1a1* also results in a toxification process whereby the parent PAH is converted into a reactive, ultimately carcinogenic compound. Intermediates created from the metabolism of the parent compound are positively charged electrophiles which react at the negatively charged nucleophilic centers of nucleic acids. This covalent binding of reactive electrophiles to critical macromolecules, such as DNA, within the cell may be one of the necessary requirements for the initiation of chemical carcinogenesis.

Cyp1a1 induction is mediated by the *Ah* receptor, which is a soluble cytoplasmic protein product of the *Ah* gene locus that binds PAHs as well as halogenated aromatic compounds. This particular receptor has been shown to be a member of the β -helix-loop-helix superfamily of transcription factors (13-15). Members of this superfamily are characterized by the presence of a region of homology, of approximately 250 amino acids, known as the PAS domain. In the *Ah* receptor, this domain has been shown to play a role in the agonist binding process (13). A PAH inducer, such as MC, is transferred passively across the cell membrane where it demonstrates highly specific binding to the cytosolic *Ah* receptor.

Binding of the ligand to the receptor results in the translocation of the activated ligand-receptor complex into the cell nucleus, where this complex interacts with specific structural gene sequences that regulate the rate of *Cyp1a1* gene transcription (9, 12, 15, 16). The subsequent induction of drug metabolizing Cyp1a1 enzymes increases the detoxification pathways designed to eliminate the xenobiotic but also results in the formation of the reactive intermediates which bind covalently to nucleic acids. Initial studies in rodents elucidated the role of the *Ah* receptor in the regulation of *Cyp1a1* expression. Subsequent studies have demonstrated human *CYP1A1* to be under the same regulatory mechanisms found in rodents (17-21).

Cyp1a1 and Lung Tumorigenesis

Early studies utilizing skin-painting experiments in mice determined that the PAH fractions of cigarette smoke were the main carcinogenic fraction, thus demonstrating the need to focus on PAH-initiated chemical carcinogenesis (22). Subsequent studies identified covalent DNA damage in the form of benzo[a]pyrene (BP)-DNA adducts within pulmonary tissues of active cigarette smokers (23). A strong positive correlation between peripheral blood lymphocyte responsiveness to PAHs and incidence of lung cancer in the human population has been observed in studies and the rationale for this association was that individuals who were highly inducible for the CYP1A1 isozyme more readily and efficiently activated the promutagens and procarcinogens in cigarette smoke (24). Other studies have noted statistically significant elevations of CYP1A1 enzyme levels in pulmonary and peripheral blood lymphocytes from patients with lung cancer (25). Research from several laboratories, utilizing tissues from lung cancer patients of both smokers and nonsmokers, has

identified elevated levels of *CYP1A1* expression as a possible risk factor for lung cancer in humans (24, 26, 27). Normal lung tissues samples from smokers showed *CYP1A1* gene expression at the mRNA level in 89% of the cases whereas none was detected in nonsmokers. This was some of the first evidence that *CYP1A1* gene expression occurs *in vivo* in normal pulmonary tissue from active cigarette smokers, which supports a positive correlation between active cigarette smoking and the induction of *CYP1A1* gene expression (28).

In addition, epidemiological studies have suggested a link between maternal exposure to carcinogenic agents, particularly PAHs and nitrosamines, and increased incidence of childhood cancers in the offspring (29-31). A dose-response relationship was seen between the number of cigarettes smoked per day during pregnancy and cancer risk in offspring (30). The overall risk for cancer in these children exposed to 10 or more cigarettes per day during pregnancy increased by 50%, particularly in non-Hodgkin lymphoma, acute lymphoblastic leukemia, and Wilms' tumor (29-31). The tumor incidence for several organ sites in adults was also found to be increased as a result of transplacental or early passive childhood exposure to a smoking environment (3). PAH-DNA adducts have been detected in fetal tissues from nonsmoking mothers, demonstrating that these environmental toxicants can cross the placental barrier and damage DNA in the human fetal population (32). PAH adducts to both DNA (33) and hemoglobin (34) in placental and fetal tissues were found to be higher in mothers who smoked during their pregnancy, and the levels of BP-DNA adducts were found to correlate with higher levels of aryl hydrocarbon hydroxylase (AHH) activity in the placentas of pregnant women who either smoked or were exposed to environmental toxicants (35-39). Clearly, the potential risks to the fetus of mutagenic and carcinogenic damage by

environmentally prevalent toxicants warrants further investigation.

Despite the evidence from studies cited above suggesting an apparent correlation between the levels of CYP1A1-dependent metabolism of PAHs and the incidence of lung cancer, the degree to which *CYP1A1* expression actually modulates susceptibility to lung cancer in the human population as well as the molecular mechanisms regulating this effect are not well understood. Many studies are limited by the typical problems encountered when working with human tissues. A high degree of inter-individual variability in drug metabolic activity makes significant statistical comparisons difficult. The range of genetic variability in the human population also complicates studies. Several genetic loci, in addition to P4501A1, including specific alleles of P4502D6 and glutathione *S*-transferase M1 null genes, may also contribute to individual lung cancer susceptibility (40). In order to eliminate these complicating variables existing within the human population, laboratories have turned to a mouse model system to further delineate the role played by *CYP1A1* in lung tumorigenesis.

The pharmacogenetic mouse model system is a particularly good paradigm in which to model lung tumorigenesis in humans. It has been shown that humans contain an orthologue of the mouse *Cyp1a1* gene that is 80% related to the mouse amino acid sequence (41-43). Also, the expression of the human *CYP1A1* gene is regulated by the same mechanisms existing in mouse systems. Binding of a PAH to the *Ah* receptor results in the transcriptional increase of *CYP1A1* RNA expression and a corresponding increase in translated protein (8, 41-43). Mouse and human tissues have been shown to produce the same type of DNA adducts following exposure to PAHs and the subsequent metabolism by the CYP1A1 isozyme (44). Therefore, mice and humans both respond to PAH exposure by

increasing levels of *CYP1A1* RNA and protein resulting in an increase in hydrocarbon metabolism and an increase in mutagenic DNA adducts. An additional link between the human and mouse *CYP1A1* systems is that activation of the *Ki-ras* oncogene has been implicated in both human lung tumors and chemically-induced mouse lung tumors (45-48). Clearly the similarity in molecular mechanisms regulating the expression of human and mouse *CYP1A1* genes makes the mouse model a useful paradigm for studies of the role of the enzyme in human lung cancer.

Mouse Model System

One of the first reports on *in utero* toxicity associated with genetic differences in PAH metabolism utilized C57BL/6 (B6) genetically responsive mice and nonresponsive AKR (AK) mice (49). This study observed how the allelic differences at a single genetic locus that controls the rate of teratogen metabolism can be correlated with teratogenesis. Doses of BP, between 50 and 300 mg/kg given at day 7 or 10 of gestation, created more *in utero* toxicity in responsive (Ah^bAh^d) mice than in nonresponsive (Ah^dAh^d) mice. The results obtained showed that stillborns, resorptions, and malformations were four times greater in the genetically responsive B6 mice than in the genetically nonresponsive AK mice (49). This is explained by the fact that the (Ah^bAh^d) responsive fetuses have greater amounts of inducible Cyp1a1 isozymes and therefore higher levels of toxic BP metabolites making them more prone to toxic effects than (Ah^dAh^d) fetuses. It is important to note that the allelic differences associated with teratogenesis were dependant on the maternal environment. If the mother was nonresponsive, then the (Ah^bAh^d) fetuses exhibited more stillbirths, resorptions, and enhanced Cyp1a1-mediated covalent binding of the BP metabolites to fetal protein and DNA.

The same effect could not be detected in responsive mothers, presumably because of the enhanced BP metabolism in the maternal tissues thereby protecting the developing fetuses. These results demonstrated conclusively that allelic differences at a single genetic locus, the *Ah* gene locus, can result in differences in susceptibility to *in utero* toxicity and congenital defects (49). This data also determined that subsequent studies on fetal susceptibility to PAHs should be conducted in nonresponsive mothers in order to fully monitor the effects of carcinogens on the developing fetus.

In an attempt to expand upon the initial study indicating that allelic differences affect *in utero* toxicity and teratogenesis (49), numerous laboratories have utilized the pharmacogenetic mouse model developed by Thomas *et al.* (50) and Gielen *et al.* (51) to study the role of *Cyp1a1* gene induction on tumor susceptibility. C57BL/6 mice are genotypically inducible, or responsive (Ah^bAh^b), to PAH-mediated induction of members of the *Cyp1a1* gene family whereas the DBA/2 (D2) strain is noninducible, or nonresponsive (Ah^dAh^d), due to a low affinity binding form of the *Ah* receptor (52). Inducibility of the *Ah* locus is inherited as an autosomal dominant trait, thus a cross between the parental B6 and D2 strains results in a litter consisting of the hybrid genotype (Ah^bAh^d) where all of the offspring are phenotypically responsive. A further backcross between the responsive B6D2F1 generation (Ah^bAh^d) and the nonresponsive D2 mice yields a F2 generation litter in which half of the fetuses are responsive and half are nonresponsive. Since fetal mice with different induction abilities reside within the same maternal environment, one can determine how individual genetic differences in drug metabolic capacity observed in the fetal compartment can influence the incidence and mechanism of tumor causation in the transplacentally-treated

offspring. This allows for the elimination of many of the variables that are introduced when separately treating animals from different strains. An additional benefit in utilizing this particular pharmacogenetic mouse model system lies in the fact that both inbred B6 and D2 strains are resistant to lung tumor formation (53). Thus, as in human lung carcinogenesis, these two strains of mice produce lung tumors only in response to environmental chemical exposure. This model mimics environmental and genetic factors that influence the formation of human lung tumors.

Several studies utilizing the pharmacogenetic mouse model have provided data on the extent of transplacental carcinogenesis following exposure to MC. One such study was conducted to determine the extent of neonatal toxicity in backcrossed mice following nonresponsive maternal exposure to MC (4). In this study, females were exposed by oral route to either 7, 21, or 63 mg/kg/day of MC on days 15, 16, and 17 of gestation. A dose-dependant toxic response was obtained at doses of tested MC, including number of litters surviving to term, litter size at birth, survival to weaning, and weight gain. Offspring with the *Ah^b* responsive phenotype exhibited a higher incidence of mortality during the neonatal period than did nonresponsive offspring. The correlation between *Ah* phenotype and neonatal toxicity indicated the responsive offspring had higher levels of neonatal toxicity than nonresponsive offspring within the same exposure group.

A companion study to that described above was designed to determine if the *Ah* genotype influenced tumorigenesis in offspring exposed to MC during gestation (5). The backcrossed mice were exposed by gavage to the same MC treatments as above, and necropsies showed the lung to be the major site of neoplasms in 6 month old adult offspring

following *in utero* exposure to MC. A dose-related response was seen in all MC groups for percent of nodule-bearing animals, percent of adenoma-bearing animals, mean number of nodules per animal, and mean nodular size per animals. The correlation in this study between *Ah* phenotype and tumorigenesis showed that nonresponsive offspring had a higher incidence of nodules and adenomas than responsive offspring, suggesting that *Ah* genotype influences transplacental carcinogenesis and subsequent tumorigenesis.

An additional study utilizing reciprocally backcrossed mice and a single high ip injection of MC ranging from 5 to 175 mg/kg on day 17 of gestation clearly demonstrated the influence of both the maternal and fetal metabolic phenotypes on the formation of lung adenomas and adenocarcinomas in transplacentally-treated offspring (54). B6D2F1 females were mated with D2 males and B6D2F1 males were mated with D2 females creating an experimental group which utilized both responsive and nonresponsive mothers carrying both responsive and nonresponsive fetuses. A maximum number of tumors was seen at 100 mg/kg MC in responsive mothers and 30 mg/kg in nonresponsive mothers. Within the same dose group, the responsive offspring of both sexes were more susceptible to lung tumor initiation than their nonresponsive litter mates regardless of the phenotype of the mother. The percentage of responsive mice exhibiting tumors tended to be 2- to 3-fold higher than the nonresponsive littermates of the same sex, demonstrating that the incidence of lung tumors correlates positively with the responsive phenotype of the fetus. Lung tumors were generally more prevalent in male versus female offspring suggesting sex serves as an additional determinant of fetal susceptibility to lung tumorigenesis. In addition, liver tumors in male responsive mice were also significantly higher than in noninducible male mice and there was

a dose-dependant increase in liver tumors in male responsive progeny up to 100 mg/kg of MC. The offspring of the nonresponsive D2 mothers tended to have higher tumor incidence than the progeny of the responsive mothers, suggesting that, because of the lower metabolic/detoxification capacity of the D2 mothers, their fetal compartments were exposed to higher levels of the carcinogen. These results confirmed that responsiveness is a significant factor in determining fetal risk for both lung and liver tumorigenesis.

These results have been confirmed at both the biochemical and molecular level. Studies by Miller *et al.* (55-57) found that *in utero* exposure to MC resulted in a dose-dependent induction of aryl hydrocarbon hydroxylase activity and *Cyp1a1* RNA expression in both the fetal liver and lung (55). The maternal phenotype appeared to play an important role in influencing the induction kinetics of both AHH activity and *Cyp1a1* RNA levels in the fetal compartment (56). In experiments utilizing reciprocal crosses between the two parental strains, the absolute levels of AHH activity and the relative induction ratios in fetal lungs from D2 mothers (D2B6F1) were very similar to those obtained in fetal tissues from B6 mothers (B6D2F1 fetuses) 24 hours after administration of MC. This implied that in both DBA/2 and C57BL/6 mothers, enough MC reached the fetus to cause a comparable induction of AHH activity. However, by 48 hours after injection of MC, the levels of both pulmonary and hepatic AHH activity of B6D2F1 fetuses had declined to nearly control values whereas in D2B6F1 fetuses, AHH activity remained elevated over the activity found in control tissue samples. Northern blot analysis indicated that the D2B6F1 fetuses generally showed a more sustained induction of *Cyp1a1* RNA over a longer time period than did the B6D2F1 fetuses. The metabolic and pharmacokinetic parameters of both mother and fetus combine to strongly

influence the susceptibility of the fetus to chemically-induced tumors, with the most susceptible combination being responsive fetuses gestating in a nonresponsive mother (40, 54, 56). Thus, a backcross between a hybrid male and a D2 nonresponsive mother was utilized in this study.

Molecular Oncology

Due to the complex mixture of PAHs in tobacco smoke, certain studies have focused on determining the DNA-binding characteristics of the PAH fraction responsible for the mutagenic properties of the mixture (58). ^{32}P -postlabeling is a nonspecific, sensitive procedure used to detect DNA adducts formed by a wide variety of compounds and has been used to monitor the complex pattern of DNA adducts *in vivo* from a coal-tar PAH mix on mouse skin. Results found a band of seven distinct DNA adduct patterns indicating that each specific carcinogen undergoes a unique covalent binding interaction, which suggested that this specific binding might influence the mutational spectrum seen in the DNA (58). The toxification process whereby reactive metabolites are created from enzymatic conversion of the parent compound followed by the covalent linkage of these activated intermediates with cellular macromolecules initiating the carcinogenic process has been studied extensively utilizing benzo[a]pyrene as a model for other PAHs to further understand the DNA-binding process.

It has been shown that the main metabolite responsible for the DNA-binding capabilities of the PAH compounds is the dihydrodiol epoxide (59). The metabolic activation of PAHs to the carcinogenic diol-epoxide involves two oxidations by Cyp1a1 isozymes as well as a hydrolysis of one epoxide by epoxide hydrolase. In the studies conducted on BP,

the epoxide with the greatest carcinogenic activity is the 7,8-diol-9,10-epoxide (59). This epoxide binds predominantly to the amino group of guanine in nucleic acids, although binding at both adenine and cytosine has been noted, and it inserts into the minor groove of DNA (10). It is this metabolite-DNA interaction which appears responsible in determining the mutational spectrum of various carcinogens. The binding of BP in the minor groove has been shown to lead to RNA polymerase errors during transcription leading to incorrect mRNA precursors as well as the slow recognition of mutational damage by the DNA repair system (10). The carcinogenic activity of reactive electrophiles appears to be a combination of the specific metabolite produced and where it inserts into the DNA helix. Studies began to focus on this DNA-binding specificity to further understand how the specific mutational spectrum is produced.

Recent advances in molecular oncology have demonstrated that the activation of protooncogenes and inactivation of tumor suppressor genes are commonly observed lesions in both human and experimentally induced rodent lung tumors (40, 45, 60). Mutational activation of the *Ki-ras* gene is found in approximately 30-50% of non-SCLCs in human patients (46-48). Additional studies utilizing larger patient populations have shown that the majority of *Ki-ras* mutations occur at the 12th codon with G→T transversions at the first or second base of the GGT wild-type sequence predominating (61, 62). Mutations at the 61st codon have been seen, although not as frequently, and tend to exhibit CAA→CGA transitions. These mutations seen in human *Ki-ras* genes are identical to those found in BP-induced mouse lung tumors. While mutation of this critical cell regulatory molecule has been implicated in the pathogenesis of human and rodent lung tumors, little work has been done

following *in utero* exposure to environmental toxicants. Considering the marked sensitivity of the fetus to tumor induction by chemical carcinogens and the long latency for actual tumor formation, fetal exposures to chemical carcinogens may account for a significant portion of tumors that occur later in adult life. Therefore, it is important to focus research on the molecular pathogenesis and etiology of lung cancer in order to further understand the interactions between environmental factors and genetics, such as differing metabolic capacity influencing individual susceptibility to lung tumorigenesis.

CHAPTER III

MATERIALS AND METHODS

Chemicals

Olive oil, MC, β NF, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, dicumarol, β -NADPH (reduced form), ethoxyresorufin, and resorufin were purchased from Sigma Chemical Co. (St. Louis, MO); the BioRad Protein Assay Kit II was obtained from BioRad Laboratories (Melville, NY); MDE gel solution was purchased from FMC Corp. (Rockland, ME); [γ P³²]ATP (~6000 Ci/mmol) was obtained from DuPont NEN Research Products (Boston, MA); and oligonucleotides were purchased from Oligos Etc. (Guilford, CT).

Animal and Treatment Protocols

C57BL/6, DBA/2, and B6D2F1 mouse strains (Charles River Laboratory, Wilmington, DE) were housed in a pathogen-free environment in plastic cages with hardwood shavings for bedding and allowed free access to food (Rat-Mouse-Hamster 3000) and water. A 12 hour fluorescent light/dark cycle was maintained. *Ah* responsive male B6D2F1 mice were mated with nonresponsive female D2 mice, resulting in a litter in which half of the fetuses had the responsive and half the nonresponsive phenotype. Females were checked for vaginal plugs the morning following mating and those which were positive were relocated to separate cages. Pregnant females were treated on day 17 of gestation (day 1 being the day when the vaginal plug was detected) with a single high ip injection of either 0.5 mL/35 gm

olive oil alone or a 10 or 30 mg/kg dose of MC dissolved in olive oil. After the pups were born, they were fostered immediately to untreated mothers, either D2 or B6, to avoid further carcinogen exposure through the mother's milk. Gloves were worn during the fostering process to avoid scenting the pups and newborn pups were rubbed with bedding from the foster cage to increase the likelihood of the foster mother accepting the new pups. Fostered pups were monitored for a 24 hour period to check for maternal rejection. Mice were raised to 12 months of age with no further treatments and were weighed monthly to monitor health and weight gain over the one year period. Mice exhibiting a significant weight loss and visible deterioration in health were euthanized prematurely. Forty eight hours prior to euthanizing the mice, they were given a single ip injection of 120 mg/kg of beta-naphthoflavone (β NF). β NF is a noncarcinogenic plant flavone that enters the cell passively and binds the cytosolic *Ah* receptor, thereby eliciting an inductive response. This chemical is administered in order to phenotype the mice by the levels of EROD catalytic activity.

The mice were euthanized by cervical dislocation and visible lung and liver tumors were counted. Visible liver tumors were hard, discolored masses ranging in size from approximately 0.3 to 1.5 cm. Visible lung tumors were seen as hard, white adhesions ranging from 0.1 to 0.7 cm. In some instances the entire lung was involved and differentiation between individual tumors was not possible. A portion of normal liver tissue was removed and processed for enzyme analysis as described below. Care was taken during the removal of lung and liver to avoid damaging the tissues and instruments were cleaned between each dissection to avoid contamination between individuals. Remaining lung and liver tissues were fixed overnight in 10% formalin. Two to three sections of formalin-fixed tissues, including

tumors if present, were trimmed to a size of 0.3 to 1.0 cm for paraffin embedding. Slides of tissue sections were prepared for histopathology analysis. Previous studies using this protocol have shown that treatment with MC produces adenomas and adenocarcinomas in the lung and hepatocellular adenomas and carcinomas in the liver (4). Lung histopathology on tissue samples from this study found the majority of lesions to be adenomas with a few mixed adenomas and adenocarcinomas.

Preparation of S9 Supernatant Fraction

Normal liver tissue samples (0.3-0.7 grams) were rinsed in 0.1 M chilled potassium phosphate buffer (pH 7.25) and homogenized in the same buffer (3.0-7.0 mL) for 30 seconds at medium speed with a Polytron Tissumizer. The Tissumizer was rinsed between each sample to avoid contamination between tissues. Supernatant fractions were isolated by centrifugation at $9000 \times g$ for 20 minutes at 4°C in a Sorvall RC-5B refrigerated superspeed centrifuge. Samples were stored at -70°C immediately following this process to avoid loss of enzyme activity in the S9 fraction.

Determination of Ethoxyresorufin O-deethylase Activity

EROD activity was determined by a modification of the assays described by Pohl and Fouts (63) and Lubet *et al.* (64). Metabolism of ethoxyresorufin is mediated by Cyp1a1, which catalyzes the oxidative conversion of ethoxyresorufin to resorufin, a fluorescent compound. Elevated Cyp1a1 enzyme levels produced as a result of β NF treatment result in increased production of resorufin due to Cyp1a1-mediated deethylase activity and thus increased fluorescence. One hundred μL of supernatant were added to a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 25 mM MgCl_2 , 5 mM glucose-6-phosphate, 1 unit/mL

of glucose-6-phosphate dehydrogenase, 10 μ M dicumarol, and 100 μ M NADPH. Each reaction was run in triplicate and started by the addition of 5 μ M ethoxyresorufin in a final volume of 2 mL and incubated for 5 minutes at 37°C in a shaking water bath. The reaction was stopped by the addition of 2.5 mL of methanol. Table A1 provides a brief summary of this protocol. The amount of resorufin formed was quantitated against a blank of pure methanol and a control of 100 μ L heat denatured supernatant on a Perkin-Elmer LS5B Luminescence Spectrometer (excitation wavelength, 522 nm; emission wavelength, 586 nm; excitation and emission slit widths, 10 nm). The concentration of a serially diluted standard solution of resorufin (1.250, 0.625, 0.250, 0.125 nmol/mL) was plotted against the average fluorescence value, resulting in a standard curve that was used to determine the deethylase activity present in the supernatant.

Protein content was determined by the Bradford assay (65) and again each reaction was run in triplicate to ensure accuracy of values. S9 supernatant fractions, prepared as described above, were diluted in 0.1 M potassium phosphate buffer (pH 7.25) to a 1/25 ratio. Protein reagent dye from the BioRad Protein Assay Kit II was diluted in distilled, deionized water as instructed to a 1/5 ratio. In a test tube, 100 μ L of each S9 fraction was combined with 5 mL of diluted protein reagent. Tubes were incubated for 10 minutes at room temperature. Ultraviolet absorption was then measured at 595 nm against a blank of potassium phosphate buffer and a control of 100 μ L potassium phosphate buffer in the appropriate 5 mL of protein reagent dilution. Table A2, as in table A1, is a general protocol summary. The amount of BSA protein standard (50, 25, 10, 5 μ g) was plotted against the corresponding 595 nm absorbance value creating a standard curve used to determine the

amount of protein (mg) in the S9 fractions. Final EROD activity was calculated as amount of resorufin formed per minute per amount of protein and expressed as pmol/min/mg.

Amplification of Paraffin-embedded Tissues

Small sections (5 to 10 μm in width) of paraffin-embedded lung tissue from 16 tumors and untreated parental mice were cut from the paraffin blocks with a fresh razor blade. Excess paraffin was trimmed away from the embedded tissue to enhance the extraction process. Sections were prepared under a PCR hood that was UV-irradiated before each cut and new gloves were worn for each section to avoid cross-contamination. The sections were deparaffinized in 1.5 mL microcentrifuge tubes with two rounds of xylene incubations for 30 minutes at room temperature followed by two rinses with 100% ethanol (66). Tissue and residual paraffin were pelleted by a 5 minute centrifugation after each incubation. Xylene and ethanol were carefully removed from tubes with a clean, plugged Pasteur pipette. Following paraffin extraction the microcentrifuge tubes were covered with sections of Parafilm with holes poked in the top to avoid contamination and samples were dried in a vacuum desiccator. Samples were incubated overnight at 37°C with 200 $\mu\text{g/mL}$ of Proteinase K in digestion buffer (50 mM Tris-HCl pH 8.0/ 0.1 mM EDTA/ 0.5% Tween20). Proteinase K was then inactivated by incubation at 95°C for 8 minutes. A 2 μL aliquot of this lysate was subjected to PCR using the Perkin-Elmer GeneAmp PCR Reagent Kit. In addition, each amplification reaction included procedure controls (designated PC) which were taken through the entire experimental protocol, including mock extractions with xylene and ethanol, to check for cross-contamination. Negative controls (NEG) were PCR reactions that lacked any template DNA and served as buffer controls. Reactions were carried out in 0.5 mL microcentrifuge

tubes under the UV-PCR hood according to the manufacturer's instructions in 10 mM Tris-HCl (pH 8.3)/2.5 mM MgCl₂/50 mM KCl containing 200 μ M of each of the four nucleotide triphosphates. Amplimers to the DNA sequences to be amplified were added at a final concentration of 0.2 μ M of each primer and 1-2 units of *Taq* DNA polymerase were added in a final reaction volume of 50 μ L. The reaction mixture was overlaid with an equal volume of mineral oil and the samples amplified by the standard protocol of Saiki *et al.* (67) using an Ericomp Twin-Block System. The reaction mixtures were denatured for 2 min at 94°C and then subjected to 40 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 2 min, and elongation at 74°C for 2 min followed by a prolonged elongation step for 7 min at 74°C. In some experiments, a second round of amplification was performed using 2 μ L of PCR product from the first reaction. In this case, the annealing and extension times were decreased to 1 min each and the number of cycles reduced to 30. The amplimers for the murine *Ki-ras* gene were as described (68) and are shown in Table A3.

5' DNA Terminus Labeling

Commercially synthesized twenty base pair oligonucleotides to the 12th, 13th, and 61st codons of the mouse *Ki-ras* gene (Clontech, Palo Alto, CA) were used to screen for single base pair mutations; oligomers were end-labeled using the BRL 5' DNA Terminus Labeling System (Bethesda Research Laboratories, Gaithersburg, MD). Three μ L (3 pmol) of oligonucleotide was combined with 11 μ L distilled, deionized water in a 0.5 mL microcentrifuge tube and heated at 65°C for 5 minutes. The probe was spun down in a microcentrifuge to collect condensation and chilled briefly on ice. Five μ L of 5 X forward reaction buffer containing 300 mM Tris-HCl (pH 7.8)/75 mM 2-mercaptoethanol/50 mM

MgCl₂/1.65 μ M ATP, 5 μ L of [γ P³²]ATP (6000 Ci/mmol, 10 mCi/mL), and 1 μ L (10 units) of T4 polynucleotide kinase were added to bring the final reaction volume to 25 μ L and then incubated for 30 minutes at 37°C in a water bath. Labeled probe was purified from unincorporated nucleotides using a BRL NACS Prepac column and an elution buffer of 1 M NaCl in a final volume of 600 μ L. Duplicate 1 μ L aliquots of radiolabeled probe were placed in scintillation vials to determine the radioactivity. The specific activity (cpm/pmol) of the labeled probes were approximately 10⁷ cpm/pmol.

Allele-specific Oligonucleotide Hybridization

A Nytran membrane filter (Schleicher and Schuell, Keene, NH) was cut to fit a Schleicher and Schuell Minifold II slot blot apparatus, pre-wet in DEPC-water for 30 minutes, and then equilibrated in 10 X SSC for 10 minutes. Five μ L of the PCR products were added to 195 μ L of DEPC-water. These samples were heat-denatured for 10 minutes at 95°C and chilled briefly on ice before adding 190 μ L of diluted PCR product to individual slot blot wells. The membrane was dried at room temperature for 20 minutes and amplified DNA products were fixed to the membrane by UV crosslinking. The filters were prehybridized at 37°C for at least 1 hour with 5 X SSC buffer (pH 7.0)/50 mM sodium phosphate buffer (pH 7.0)/5 X Denhardt's solution/0.5% SDS detergent/100 μ g/mL salmon sperm DNA, which, along with the Denhardt's solution, serves to block non-specific DNA binding sites. The filters were hybridized in the same buffer containing 1-5 $\times$ 10⁶ cpm/mL of the specific 20 base pair oligonucleotide that had been 5' end-labeled with P³² to a specific activity of approximately 10⁷ cpm/pmol. Following hybridization, the filters were washed under stringent reaction conditions which consisted of two initial 10 minute washes at 37°C with

5 X SSC/0.1% SDS buffer to remove any residual salts bound to the filter followed by 3 x 20 minute washes at 3°C below the T_M for each particular oligonucleotide probe with 1 X SSC/0.1% SDS, which increases the hybridization stringency to ensure only tight binding of fully matched probes remain bound to the DNA (69). Table A4 lists each distinct oligonucleotide probe with its corresponding melting temperature and final wash temperature. The blot was then autoradiographed for eight hours.

Single Strand Conformation Analysis of the *Ah* Receptor

For those mice which died prior to the administration of β NF, SSCP was utilized to screen for base differences between (*Ah^bAh^d*) and (*Ah^dAh^d*) mice. Nucleotide differences in the B6 and D2 *Ah* receptor genes give rise to alterations in the sequence of the aromatic hydrocarbon receptor, resulting in differences in ligand-receptor affinity and, therefore, responsiveness between the two strains. The B6 *Ah* receptor was shown to have an affinity for agonist 10-25 times greater than the D2 receptor (50). A total of ten nucleotide differences exist between the B6 and D2 strains, five of which are silent. Of the five remaining differences, a nucleotide change causing a leucine residue in the B6 strain to be replaced by a proline in the D2 is likely to disturb the secondary structure of the *Ah* receptor, resulting in the changes in agonist-receptor affinity (70). PCR amplification of a specific segment, segment C, produces a 313 base pair fragment from exon 10 that differs at two base pairs between the mice. At position 2303 in fragment C, an [A] base in the B6 mouse is replaced by a [G] in the D2 mouse while at position 2326 a [C] is replaced by a [T]. It is this second base difference which causes the leucine residue in the B6 strain to be replaced by a proline in the D2 strain; these two base pair differences make this segment useful in probing

for band shifts on a high resolution gel (70). An initial PCR was run on normal lung tissue samples from D2, B6, and phenotypically unknown mice using lysates from paraffin-embedded tissue. The lysate was prepared as described above and 2 μ L were added to 48 μ L PCR reaction mixture containing the amplimers to the AhR shown in Table A3. The PCR mixture was modified from that described initially in that the $MgCl_2$ concentration was increased to 4 mM. Three separate PCR reaction mixtures of varied $MgCl_2$ concentrations of 1.5, 2.5, and 4.0 mM were run to ensure that the optimal magnesium ion concentration was utilized. The reaction mixtures were denatured for 2 minutes at 94°C followed by 40 cycles of denaturation for 1 minute at 94°C, annealing for 2 minutes at 55°C, and extension for 2 minutes at 72°C followed by a final extension for 7 minutes at 72°C.

A second round of PCR was performed with 2 μ L of PCR products from the initial reaction using the same amplimers. The amplimers were 5' end-labeled, added to an 18 μ L PCR mixture, and amplified as described above except that only 20 cycles were used and the annealing and elongation times were adjusted to 1 min. Two μ L aliquots of the radioactive PCR products were added to 20 μ L of stop buffer containing 95% formamide/10 mM NaOH/0.025% BPB/0.025% xylene cyanol and heat denatured for 5 min at 95°C, then chilled on ice. Four μ L of this mixture were electrophoresed in a nondenaturing 0.5 X MDE gel at 25 W in a cold room for 4 hours using a 0.6 X TBE running buffer. The gel was vacuum dried and autoradiographed.

Sequencing

Although it was not within the scope of my research, lung tumor tissues were sequenced by others in the laboratory to confirm the mutations identified by ASO for five of

the mouse lung tumors. The products of the initial PCR reactions were reamplified as described above and cloned using the TA Cloning Kit (Invitrogen Corp., San Diego, CA) according to the manufacturer's instructions. The PCR DNA was ligated directly into the pCRII cloning vector and used to transform competent *E. coli* bacteria. The transformed bacteria were grown overnight and solid white colonies picked for further analysis by gene sequencing. Plasmid DNA from the transformed bacteria was purified on a Qiagen column (Qiagen Inc., Chatsworth CA), then sequenced using the *Sequenase* DNA Sequencing Kit (USB, Cleveland, OH). The cloned PCR product was added to 20 mM Tris-HCl (pH 7.5)/10 mM MgCl₂/25 mM NaCl containing the sequencing primer and heat denatured. *Sequenase* enzyme and [α S³⁵]dATP were added and the mixture divided into 4 aliquots and added to microcentrifuge tubes which contained either the A, G, C, or T extension-termination mixes (each mix consisted of the 4 dNTPs plus the dideoxy dNTP form of one of the dNTPs). The reaction was incubated for 5 minutes and then stopped by addition of 95% formamide/20 mM EDTA/0.05% bromophenol blue/0.02% xylene cyanol FF. The reaction mixture was heat denatured for 5 minutes at 95°C and cooled on ice. The samples were loaded onto a 8% polyacrylamide sequencing gel (FMC Corp., Rockland, ME) containing urea and electrophoresed for several hours at approximately 1700 Volts. The gels were then dried and autoradiographed for 24-72 hours. The subcloned PCR products were sequenced on both strands using primers to the pCRII vector supplied by the manufacturer.

Statistics

The data were analyzed by a statistician for the effects of varied doses of carcinogen treatment, sex of mice, and phenotype of mice. The interactions among these factors were

examined for statistical significance by an analysis of the variance using the Fisher test at the $P < 0.05$ level of significance.

CHAPTER IV

RESULTS

Introduction

Previous studies conducted by Anderson *et al.* (54) utilized the pharmacogenetic mouse model system to demonstrate the role of metabolic phenotype in modulating tumor susceptibility following *in utero* exposure to MC. These and subsequent studies (54-57) implicated both the fetal and maternal levels of *Cyp1a1* as a key determinant influencing tumor incidence in the offspring. Results from these experiments demonstrated that a single high ip injection of MC to either responsive or nonresponsive mothers resulted in a higher incidence of both lung tumor and liver tumors in the progeny of nonresponsive mothers. Since fetuses residing in a nonresponsive mother were the most susceptible to MC-mediated tumor induction, the experiments described in this report utilized a backcross between hybrid B6D2F1 males and D2 females. Thus, both responsive and nonresponsive fetal mice resided within the same nonresponsive maternal environment.

Animal Treatments

Pregnant D2 mice were treated with a single dose of either olive oil vehicle, 10, or 30 mg/kg of MC on day 17 of gestation. The offspring were born 3 days later and immediately foster-nursed to untreated B6 or D2 mothers. The mice were housed for 12-13 months with no additional treatment until 48 hr prior to cervical dislocation, when the mice received a single ip injection of 120 mg/kg of β NF. Mice were weighed monthly during their lifespan

to monitor their health. Treatment of mice with MC had no adverse effect on total body weights in either male or female mice at either dose (Figure B2). Weight gain increased normally with age in all dose groups. Although male mice weighed slightly more than females, within the same sex neither dose group deviated greatly from the vehicle-treated control suggesting that the doses of MC employed did not cause any overt toxicity.

Phenotype

The metabolic phenotype was determined by two distinct methods. The majority of the mice in this study, 164 of the 169 total individuals assayed, were phenotyped by determining the level of EROD activity. This is a highly sensitive, accurate, and easily reproducible fluorimetric assay designed to monitor levels of Cyp1a1 isozymes. Liver samples taken immediately following cervical dislocation were homogenized to produce a S9 supernatant fraction which could be stored up to six months at -70°C with no significant loss of enzyme activity. The EROD assay is based upon the oxidation of ethoxyresorufin to a fluorescent compound, resorufin, by Cyp1a1. A high level of fluorescence indicates a high level of Cyp1a1 isozymes produced in response to PAH exposure. Individuals with high levels of resorufin formation were labeled as responsive while those individuals with low levels of formation were nonresponsive.

Table A5 summarizes the actual numerical results obtained from the EROD activity assay. It was found that the EROD activity segregated distinctly into those mice that had an activity below 200 pmol/min/mg protein and those that were above that number, with the ranges for the nonresponsive mice and responsive mice being 22-162 and 239-2508 pmol/min/mg protein, respectively. On average, responsive mice of both sexes in all

treatment groups exhibited a statistically significant 10- to 15-fold higher level of EROD activity compared to their nonresponsive counterparts.

During the course of phenotyping the mice, we observed no significant difference in EROD activity in the nonresponsive mice between males and females or between different doses of MC. However, in the responsive mice there was an inverse correlation between induced EROD activity and the dose of MC administered during pregnancy (Table A5). These differences in EROD activity were significant for males across all 3 dose groups and for females between the 30 mg/kg dose and the 0 and 10 mg/kg doses ($P < 0.05$). No difference in EROD activity between the sexes of responsive mice was seen.

Although further studies would be required in order to determine if this effect is due to the *in utero* exposure to MC or some other factor, it is important to note that other studies have found similar results. Results obtained by Anderson *et al.* (54) supported those found in this study suggesting a correlation between Cyp1a1 activity and carcinogen doses. Such results as these have been attributed to genetic imprinting. In a study conducted by Anderson *et al.* (71), either responsive or nonresponsive pregnant mice were injected on day 17 of gestation with a single dose of MC ranging from 5 to 175 mg/kg. The pups were raised to nine or thirteen months of age, euthanized, and assayed for liver MC metabolism. Results showed that exposure of fetal mice to a single dose of MC permanently altered the ability of liver enzymes to metabolize MC; both positive and negative imprinting occurred (71). The mechanisms governing these imprinting actions have not been elucidated; however, sufficient evidence has been collected to suggest this may be a consequence of fetal exposure to toxic chemicals (72-74).

The five mice in this study which died prior to β NF injection could not be phenotyped with the EROD assay. In these instances the mice were phenotyped utilizing a SSCP analysis of the AhR gene. The SSCP technique detects point mutations in genes following amplification of a specific region of DNA. Single base mutations can alter the secondary structure of the fragment in the nondenaturing gel, which produces a visible shift in its mobility and, therefore, creates a unique banding pattern for each fragment. A 313 bp fragment, referred to as fragment C by Chang *et. al.* (70), was amplified from exon 10 of the AhR gene. There are two bases which differ between the B6 and D2 mice in this region at positions 2303 and 2326. As shown in Figure B3, SSCP analysis of PCR products from B6 and D2 mice produce distinctly different band patterns on a nondenaturing 0.5 X MDE gel. When the DNA from the embedded lung tissue of mice 6 and 15 were included in this analysis, the phenotypes of the mice could be easily determined. Mouse 6 contained bands present in both the B6 and D2 mice, and would therefore have the hybrid (Ah^bAh^d) genotype whereas the band pattern of mouse 15 resembled that of the noninducible D2 mice and would therefore be nonresponsive.

Analysis of Tumor Incidence

It has been previously mentioned that this mouse model is a particularly useful paradigm for human lung cancer since the inbred B6 and D2 strains are resistant to lung tumor formation (51). Therefore, any incidence of lung tumor formation in the offspring studied can be attributed to the transplacental exposure to MC. None of the vehicle-treated mice exhibited macroscopically visible tumors in the lungs of either sex (Table A6). At the high dose of MC, both male and female responsive mice had a significantly higher incidence

of lung tumors than did their nonresponsive litter mates ($P < 0.05$). None of the nonresponsive mice in the 10 mg/kg dose group exhibited lung tumors, but a low incidence of tumor formation was observed in the responsive mice of both sexes.

Vehicle-treated female mice also did not exhibit macroscopically visible liver tumors although a very low incidence of spontaneous liver tumors (7-8%) was observed in oil-treated male mice (Table A7). None of the mice, either male or female, treated at the 10 mg/kg dose of MC exhibited liver tumor formation.. There was a marked difference in tumor susceptibility between male and female mice, with males demonstrating a 6- to 9.5-fold higher incidence of liver tumors than female mice ($P < 0.05$). A similar trend was seen in results obtained from an earlier study (54). Male responsive mice had a significantly higher incidence of liver tumors than nonresponsive males ($P < 0.05$) whereas female mice had a very low tumor incidence that did not differ between responsive and nonresponsive mice.

Molecular Analysis of Lung Tumors

Sixteen tumors isolated from eleven mice were examined for activating point mutations in the *Ki-ras* gene, which has been implicated in a large percentage of human lung adenocarcinomas (46-48). All of the tumors examined, which were picked at random, were classified as adenomas. Table A8 lists these eleven mice with their corresponding catalytic activities for EROD and phenotype. Two of the mice in this study died before we were able to inject them with β NF for the purpose of phenotyping them by the EROD assay. These mice were therefore phenotyped utilizing SSCP.

The DNA isolated from paraffin-embedded tumor tissue was amplified by PCR, then blotted to nylon Nytran membranes for ASO analysis. The 12th, 13th, and 61st codons of

the *Ki-ras* gene were screened with 20 base pair oligonucleotide probes. Each codon was tested with at least 6 different mutant oligomers, which differed from the wild type sequence by only one base pair (Table A4). Figure B4 shows that the PCR products from the 16 tumors examined all hybridized to the wild type probe for codons 12 and 13. The signal obtained with the wild type probe tended to be the strongest indicating that normal cells were probably interspersed throughout the tumor tissue. In addition, some tumors exhibited only faint signals with the mutant probes, which suggests only a portion of the cells in the tumor tissue contained the mutant allele. In the tumors examined in the molecular analysis, tumors 21 and 32-1 were adenocarcinomas, tumors 15-1, 16-2, and 29-1 were mixed adenomas, while the remaining nine tumors were pulmonary adenomas representing an early stage in tumor progression.

Thirteen of the 16 tumors (81%) screened by ASO exhibited varied levels of binding to mutant probes to the 12th or 13th codons of exon 1 (Figure B4). Three separate amplification reactions were performed with the tumors and accompanying procedure and negative controls to ensure that the results were not due to either low background binding or a misincorporation error by the *Taq* polymerase. The only exception to this was with four tumor lysates (tumors 16-1, 16-2, 21, and 22) which did not have enough material to do more than one amplification. Tumors were considered as positive for a specific mutation only if binding to the oligonucleotide was observed in all three experiments. Faint background binding not appearing consistently throughout all experiments were excluded from consideration. ASO results found 7 tumors had GGT-GTT (GLY¹²-VAL¹²) transversions, 4 exhibited GGT-TGT (GLY¹²-CYS¹³) transversions, and 2 harbored GGC-CGC

(GLY¹³→ARG¹³) transversions. One of the tumors from a mouse containing the mutation CYS¹² (tumor 16-1) also appeared to contain a second mutation at this locus, resulting in a GGT→GAT (GLY¹²→ASP¹²) transition. The appearance of two different mutations occurring simultaneously within the same tumor at the *Ki-ras* gene locus has been reported by others (61). After the first amplification, PCR products from the second exon all bound to the wild type probe to the 61st codon; however, none of the tumors exhibited mutations at the 61st codon. Since there was no evidence of a mutation at this codon, there was no need to perform additional PCR reactions.

Sequencing analysis was done by other members of the lab in order to verify the results of the ASO. Five of the amplified tumor tissues were subcloned using the TA Cloning Kit. At least seven bacterial colonies were expanded and the isolated plasmids were sequenced. Two plasmid clones with the mutant sequence had to be identified in order to fully confirm the ASO results. Four of the five tumors examined contained the same base pair mutation sequence as seen in the ASO results (Figure B5). From the seven clones examined for tumor 16-1 that contained both a CYS¹² and ASP¹² mutations, two mutant colonies were found for the CYS¹² but only one was found for the ASP¹². This result is consistent with the ASO results which showed the CYS¹² mutation to be predominate based on its signal intensity (Figure B4). The tumor from mouse 34 exhibited a faint signal consistent with the CYS¹² mutant oligomer during each round of ASO, but did not show up during sequence analysis. The CYS¹² mutant signal from the ASO was faint relative to the wild type signal (Figure B4) so it is possible that the number of cells containing this particular mutation was too low to be detected by sequence analysis.

The same types of mutagenic lesions were seen in both responsive and nonresponsive mice with eight of the ten tumors from responsive mice and five of six tumors from nonresponsive mice containing activating point mutations at the first or second base of codon 12. Two tumors from the responsive female mouse 32 had a G→C transversion at the first base of codon 13. This lesion was not seen in any of the tumors from the nonresponsive or male mice, but the sample size was too small to draw any definitive conclusions. In addition, the GGT→TGT mutation at codon 12 was found only in male mice, while both sexes exhibited the GGT→GTT mutation. Six male mice accounted for nine of the tumors observed in the ASO analysis. In these male mice, four tumors contained the CYS¹² and three the VAL¹² mutations, while two males had the wild type sequence at all three codons. Five female mice were examined and four of their seven tumors demonstrated the VAL¹² and two the ARG¹³ mutations, with one mouse containing the wild type sequence. There is no obvious reason for the different mutational spectra between male and female mice since there was no significant difference in metabolic capacity for Cyp1a1 between the male and female mice (Table A6). The results seem to suggest that differences in metabolic phenotype can influence individual susceptibility to chemical carcinogens in a quantitative, but not qualitative, manner.

CHAPTER V

DISCUSSION

Polycyclic aromatic hydrocarbons are ubiquitous pollutants created from many of the processes of an industrialized society. These chemicals have attracted considerable attention for years due to their widespread occurrence and evidence linking PAHs with human cancers. Since lung cancer is the leading cause of cancer related deaths and 90% of these deaths can be attributed to the PAH fraction of cigarette or tobacco smoke, continued work in this area of research should be directed towards a greater understanding of the basic mechanisms governing the pathogenesis of lung neoplasms.

Numerous studies have indicated that the gestating embryo and fetus are at greater risk than the adult population for the toxic and carcinogenic consequences of exposure to such contaminants (29-31, 56). Epidemiological studies have seen an increased tumor incidence at several organ sites in adults as a result of transplacental exposure to a smoking environment (3) while PAH-DNA adducts have been detected in fetal tissues from nonsmoking mothers (32). These studies demonstrated that environmental contaminants, such as the PAHs, are able to cross the placental barrier and damage fetal DNA indicating a need to elucidate factors mediating tumor formation in the fetal compartment.

Since mouse and tumor tissues have been shown to produce the same type of PAH-DNA adducts (44), the pharmacogenetic mouse model system used in this study was an appropriate model of human lung tumorigenesis. As in humans, there is a genetic component

to the induction of lung tumors in mice. Large differences in the genetic make-up of the human population along with varied levels of environmental exposure make interpretation of data difficult. With the mouse model system, many variables are eliminated by exposing hybrid fetuses with differing Cyp1a1 capacity to MC *in utero*. Therefore, the model mimics the environmental and genetic components of lung tumor formation in the human population.

The tumor bioassay results from this study agreed with previous results from the Anderson *et. al.* (54) study which found a strong correlation between inducibility of the Cyp1a1 gene and lung tumor incidence. Both studies showed a significantly higher incidence of lung tumors in the responsive offspring of both sexes compared to their nonresponsive litter mates. In general, the percentage of mice with lung tumors was 2- to 3-times greater in responsive versus nonresponsive mice of the same sex. A key difference between this study and that of Anderson *et. al.* (54) was that this study utilized foster mothers to raise the hybrid pups in order to eliminate postnatal exposure to MC through the mother's milk. This could account for the much higher incidence of lung tumors in the nonresponsive male mice as seen in the Anderson group (75%) compared to the results obtained in this study (35%).

As with the lung tumors, liver tumor incidence in male responsive mice was significantly higher than in their nonresponsive counterparts. Female mice, as expected, showed a significantly lower incidence for liver tumor formation than did male mice. There is a marked difference between male and female mice in their pharmacological and toxicological response to xenobiotic agents (6) and numerous studies have indicated that male mice are more susceptible to hepatic injury than females. In one of the earliest such studies, C3H mice were injected with either estrogen or the androgen testosterone in order to monitor

their effect on hepatocarcinogenesis (75). The results suggested that estrogen injections in male mice can prevent the occurrence of hepatoma formation while testosterone injections may increase the occurrence of liver tumors in females. Subsequent studies supported the results indicating the presence of contrasting effects of androgens and ovarian hormones in liver tumorigenesis. One study administered the potent liver carcinogen urethan to newborn and infant mice (76). Within this study, gonadectomized male mice had a significantly lower incidence of liver tumor formation than did the male controls, while ovariectomized female mice had a higher incidence of tumor formation than did the controls. These results indicated that male sex hormones play a role in the promotion of liver tumor formation, while the female sex hormone estrogen acts to suppress the development of hepatomas. Additional studies found the same sex-dependent difference in liver tumor formation in mice exposed to the carcinogens ethylnitrosourea (77) and dimethylnitrosamine (78).

Most of the sixteen tumors examined in this study for activating point mutations were classified as adenomas, which are precancerous lesions. Eighty one percent of the tumors exhibited mutations at the first or second base of codons 12 and 13. This evidence supported previous data that has implicated the activation of the *Ki-ras* gene as an early event in lung tumorigenesis. In a study on a small human population, five of ten adenocarcinomas were found to be activated by point mutations in codon 12 (46), which further supported studies linking *Ki-ras* activation to subsequent lung tumor progressions. Eleven of the sixteen (69%) mutations in the mouse lung tumors were G→T transversions. This type of mutation corresponds with human lung tumors in which the G→T transversion was also predominant (62). In those instances where more than one tumor was isolated from the same mouse, it

was observed that the different tumors did not necessarily contain the same point mutation. Five mice accounted for ten of the tumors examined in this study with each mouse contributing a pair of different tumors. Tumors from three of the five pairs (mouse 16, 29, and 32) did contain the same base mutation, while two of the mice (mouse 6 and 15) contained mutations in one of their tumors, but lacked any mutation in the *Ki-ras* gene locus in the second tumor. This data suggests that factors beyond the metabolic capacity of the mouse also plays a role in determining the fate of carcinogen binding to DNA.

The molecular data produced in this study supports previous work indicating that the reactive electrophiles of MC produced by the Cyp1a1 enzyme bind mainly at guanine bases in DNA (79). Cyp1a1 isozyme activity in the mouse has been detected as early as days 10-11 of gestation (80) and has been shown to be inducible by day 12 (80, 81), but these isozymes are present only in fetal lung and liver tissues (80). Numerous forms of CYP known to exist in adult tissues are either absent or expressed only at low levels in fetal organs (40), including Cyp1a2, which is not present until day 17 of gestation and only at low levels when compared to Cyp1a1 levels (82). Fetal exposure to MC results in an inductive response whereby pulmonary AHH levels reach those seen in adult lungs following similar exposure to MC (57), indicating that the gene regulatory mechanisms responsible for an AHH inductive response are present in fetal pulmonary tissues early on in development. The existence of more definitive Cyp1a1 levels in the relative absence of other metabolic enzymes in the developing fetus allows for the determination of the role of *Cyp1a1* in modulating genetic damage. The data from this research supports previous studies in both mouse and human tissues demonstrating a link between the levels of *Cyp1a1* expression and individual susceptibility to

lung tumor formation.

Research has shown that chemical carcinogens elicit dose-dependent differences in the mutational spectrum produced (83-85). This study maintained a constant concentration of carcinogen while the enzyme levels differed due to the genetic crosses of the mice employed. It appears that the metabolic phenotype influences the amount of genetic damage caused by exposure to MC in a quantitative manner, but may not result in qualitative alterations in the types of mutations produced. There did not appear to be any differences in the types or locations of the mutations in responsive versus nonresponsive mice, although a female responsive mouse did contain a G→C transversion at the first base in codon 13, which was not seen in either nonresponsive or male mice. Male mice had G→T transversions at the first and second bases of codon 12, whereas female mice exhibited such a mutation at the second base only. These differences in mutational spectrum was not due to metabolic phenotype since there was no difference in the levels of EROD activity in responsive or nonresponsive mice of either sex at a given dose of MC (Table A5). Analysis of a larger population will be needed to determine the significance of these findings and the mechanistic basis for the reported differences.

CHAPTER VI

CONCLUSION

Earlier epidemiological studies along with the results from this thesis research have provided evidence linking elevated *CYP1A1* levels to lung tumor incidence in both human lung cancer patients and experimental animal models. The evidence suggests maternal exposure to carcinogenic chemicals, such as MC, impacts the developing fetus and influences subsequent lung tumorigenesis. The data demonstrate that environmental and genetic factors appear to combine to modulate an individual's response to toxic contaminants. While the complexity of genetic composition within the human population makes it difficult to monitor single factors modulating tumor formation, the pharmacogenetic mouse model eliminates this problem of compounding variables by producing a paradigm in which individual genetic differences in metabolic capacity can be observed. As in humans, the mouse strains utilized have a low incidence of spontaneous lung tumor formation and they develop lung tumors in response to chemical toxicants. The genetic differences between the two mouse strains have been clearly elucidated which allows for an accurate correlation between metabolic phenotype and its effect on lung tumor incidence. The benefits of continued work with this mouse model system extend far into the future. Transplacental exposure to toxicants during fetal development produces a high incidence of lung tumors making this system ideal for testing novel mechanism-based chemotherapeutic and chemopreventative agents. Numerous factors

induce lung tumorigenesis; by taking advantage of the versatility of the pharmacogenetic mouse model system we can begin to understand the earliest stages of tumor causation.

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APPENDICES

APPENDIX A

TABLES

TABLE A1:

Summary of EROD Assay Protocol

Reaction Mixture: 2.0 mL total volume

100 μ L	50 mM Tris-HCl buffer (pH 7.5)
100 μ L	25 mM Magnesium chloride
200 μ L	5 mM Glucose 6-phosphate
40 μ L	50 unit/mL Glucose 6-phosphate dehydrogenase in 5 mM sodium citrate (pH 7.5)
20 μ L	100 μ M NADPH 8.3 mg/mL in water made fresh daily
10 μ L	2 mM Dicumarol in 0.1 N sodium hydroxide
1420 μ L	Distilled water
100 μ L	S9 supernatant fraction
10 μ L	5 μ M Ethoxyresorufin 1 mg/4.1 mL in DMSO

↓

S9 fraction, glucose 6-phosphate dehydrogenase, dicumarol, and NADPH are kept chilled on ice. Ethoxyresorufin is added last to initiate reaction. Vortex to mix.

↓

5 minutes in a 37°C shaking water bath.

↓

Stop reaction by the addition of 2.5 mL 100% methanol.

↓

Final reaction volume is 4.5 mL.

↓

Pellet protein components by centrifugation.

↓

Assay fluorescence of remaining supernatant.

TABLE A2:**Summary of Determination of Protein Content Protocol**

100 μ L 1/25 dilution of S9 supernatant
in 0.1 M potassium phosphate buffer (pH 7.5)

+

5 mL 1/5 dilution of protein reagent dye
in distilled water

↓

Incubate at room temperature for 10 minutes

↓

Determine UV absorption at 595 nm

TABLE A3:**Oligonucleotide Primers**

Ki- <i>ras</i> codon 12/13	5' primer	5' - ATGACTGAGTATAAACTTGT -3'
Ki- <i>ras</i> codon 12/13	3' primer	5' - TCGTACTCATCCACAAAGTG -3'

Ki- <i>ras</i> codon 61	5' primer	5' - TACAGGAAACAAGTAGTAATTGATGGAGAA -3'
Ki- <i>ras</i> codon 61	3' primer	5' - ATAATGGTGAATATCTTCAAATGATTTAGT -3'

Ah receptor	5' primer	5' - CCCTTTCTCTCCCATATTGG -3'
Ah receptor	3' primer	5' - AGAGAGTGCAAGGTTACGT -3'

Primers for the Ki-*ras* and AhR gene were as described in Devereux *et. al.* (68) and Chang *et. al.* (70), respectively.

TABLE A4:

ASO Final Wash Temperatures

Ki-ras/codon 12

ggagct GGT ggcgtcggcaa	$T_M = 66^\circ\text{C}$	Final wash = 63°C
AGT	$T_M = 64^\circ\text{C}$	Final wash = 61°C
TGT	$T_M = 64^\circ\text{C}$	Final wash = 61°C
CGT	$T_M = 66^\circ\text{C}$	Final wash = 63°C
GAT	$T_M = 64^\circ\text{C}$	Final wash = 61°C
GCT	$T_M = 66^\circ\text{C}$	Final wash = 63°C
GTT	$T_M = 64^\circ\text{C}$	Final wash = 61°C

Ki-ras/codon 13

ggagctggt GGC gtaggcaa	$T_M = 66^\circ\text{C}$	Final wash = 63°C
AGC	$T_M = 64^\circ\text{C}$	Final wash = 61°C
TGC	$T_M = 64^\circ\text{C}$	Final wash = 61°C
CGC	$T_M = 66^\circ\text{C}$	Final wash = 63°C
GAC	$T_M = 64^\circ\text{C}$	Final wash = 61°C
GTC	$T_M = 64^\circ\text{C}$	Final wash = 61°C
GCC	$T_M = 66^\circ\text{C}$	Final wash = 63°C

Ki-ras/codon 61

acagcaggt CAA gaggagta	$T_M = 60^\circ\text{C}$	Final wash = 57°C
GAA	$T_M = 60^\circ\text{C}$	Final wash = 57°C
AAA	$T_M = 58^\circ\text{C}$	Final wash = 55°C
CGA	$T_M = 62^\circ\text{C}$	Final wash = 59°C
CTA	$T_M = 60^\circ\text{C}$	Final wash = 57°C
CCA	$T_M = 62^\circ\text{C}$	Final wash = 59°C
CAT	$T_M = 60^\circ\text{C}$	Final wash = 57°C
CAC	$T_M = 62^\circ\text{C}$	Final wash = 59°C

The first sequence is the wild type for that particular codon. Capitalized, bold bases indicate the region of the probe with a single base change. T_M is the melting temperature. Final wash is 3°C below the T_M to ensure stringent binding between probe and DNA during ASO.

TABLE A5:

EROD Activity

	EROD activity in responsive mice	EROD activity in nonresponsive mice
Male		
<i>Control</i>	1276 ± 131 ^d	82 ± 7 ^a
<i>MC</i> (10 mg/kg)	896 ± 125 ^c	78 ± 6 ^a
<i>MC</i> (30 mg/kg)	506 ± 83 ^b	56 ± 7 ^a
Female		
<i>Control</i>	1156 ± 106 ^d	96 ± 11 ^a
<i>MC</i> (10 mg/kg)	897 ± 135 ^c	95 ± 9 ^a
<i>MC</i> (30 mg/kg)	697 ± 86 ^{b,c}	77 ± 8 ^a

EROD activity is expressed as pmol of resorufin formed/ min/ mg of protein and represents the arithmetic mean ± standard error. Values with different letters differ at the $P < 0.05$ level.

TABLE A6:

Lung Tumor Incidence

	% Responsive mice with tumors	% Nonresponsive mice with tumors
Male		
<i>Control</i>	0/14 = 0% ^a	0/13 = 0% ^a
<i>MC</i> (10 mg/kg)	1/16 = 6% ^a	0/14 = 0% ^a
<i>MC</i> (30 mg/kg)	13/16 = 81% ^d	7/20 = 35% ^{b,c}
Female		
<i>Control</i>	0/12 = 0% ^a	0/14 = 0% ^a
<i>MC</i> (10 mg/kg)	2/11 = 18% ^{a,b}	0/6 = 0% ^a
<i>MC</i> (30 mg/kg)	13/15 = 87% ^d	9/18 = 50% ^c

Tumor incidences with different letters differ at the $P < 0.05$ level.

TABLE A7:

Liver Tumor Incidence

	% Responsive mice with tumors	% Nonresponsive mice with tumors
Male		
<i>Control</i>	1/14 = 7% ^a	1/13 = 8% ^a
<i>MC</i> (10 mg/kg)	0/16 = 0% ^a	0/14 = 0% ^a
<i>MC</i> (30 mg/kg)	10/16 = 63% ^c	7/20 = 35% ^b
Female		
<i>Control</i>	0/12 = 0% ^a	0/14 = 0% ^a
<i>MC</i> (10 mg/kg)	0/11 = 0% ^a	0/6 = 0% ^a
<i>MC</i> (30 mg/kg)	1/15 = 7% ^a	1/18 = 6% ^a

Tumor incidences with different letters differ at the $P < 0.05$ level.

TABLE A8:

Summary of Molecular Analysis of Mouse Lung Tumors

Mouse number	Sex	EROD activity (pmol/min/mg)	Phenotype	Mutation
6-1	female	---	R	GTT
6-2	female	---	R	---
13	male	435	R	---
15-1	male	---	NR	---
15-2	male	---	NR	GTT
16-1	male	548	R	TGT/GAT
16-2	male	548	R	TGT
21	female	93	NR	GTT
22	female	678	R	GTT
28	male	60	NR	TGT
29-1	male	243	R	GTT
29-2	male	243	R	GTT
32-1	female	516	R	CGC
32-2	female	516	R	CGC
34	male	98	NR	TGT
40	female	110	NR	GTT

TABLE A8: (continued)

The sex, catalytic activity, phenotype, and the sequence found to be mutated in the exon are listed for each mouse examined. The numbers for the mice represent the animal number from the bioassay. When more than one tumor was examined from a given mouse, the tumors contained the additional designation “-X” where “X” is the tumor number. Phenotype is either responsive (R) or nonresponsive (NR). For the mutations, “---” denotes a tumor which exhibited the wild type sequence.

APPENDIX B

FIGURES

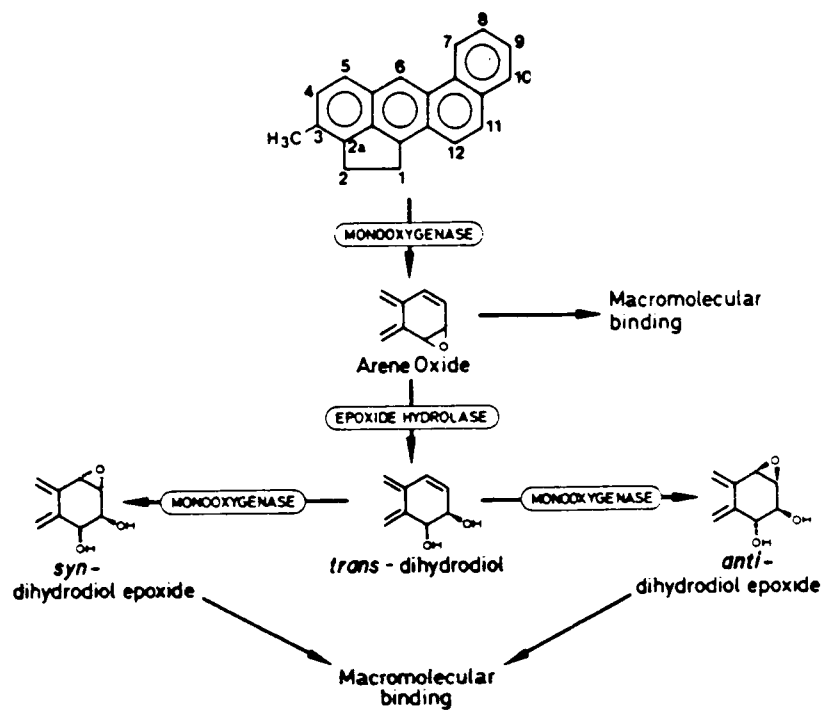


FIGURE B1:

Structure and Metabolism of 3-Methylcholanthrene

The structure of MC and its metabolic conversion into a dihydrodiol epoxide. Gelboin, H.V. (ed.) 1981. *Polycyclic Hydrocarbons and Cancer* Academic Press, New York. p 194.

FIGURE B2:**Lack of Effect of MC on Total Body Weight.**

Pregnant female DBA/2 mice were treated with either 0.5 mL/35 mg of olive oil (OO), 10 mg/kg of MC (LMC), or 30 mg/kg of MC (MC) on the 17th day of gestation. After birth, the neonates were foster nursed with untreated mothers to avoid further carcinogen exposure in the mother's milk. Mice were raised to 12 months of age with no further treatments and were weighed on a monthly basis.

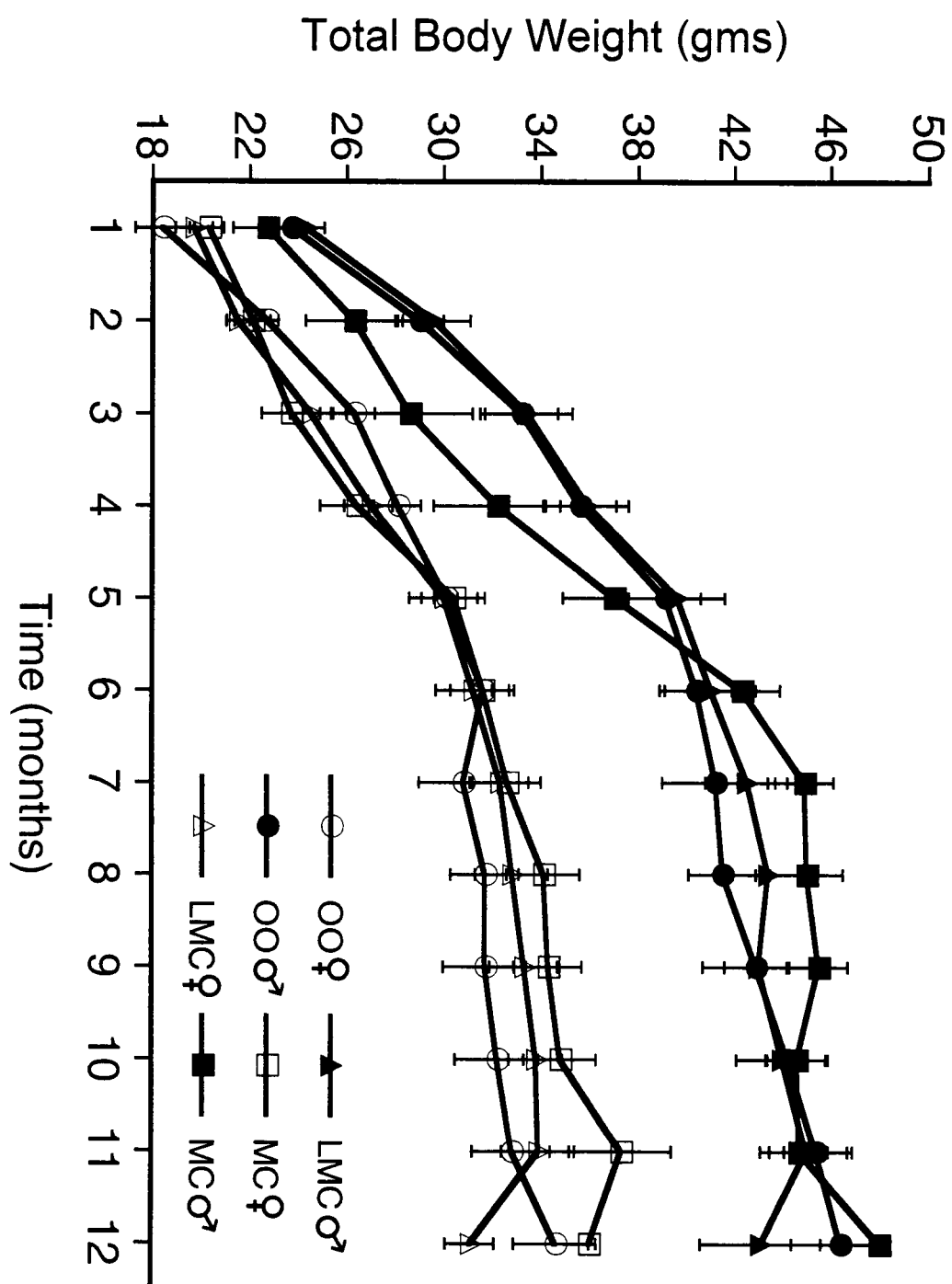


FIGURE B3:**SSCP Analysis of the AhR Gene.**

Paraffin-embedded lung tissue samples were deparaffinized and digested with Proteinase K. Two μL of lysate were amplified by the PCR technique for 40 cycles to produce a 313 bp fragment from exon 10 of the AhR gene. A 2 μL aliquot from this primary PCR was then reamplified for 20 cycles with 5' end-labeled primers. A 2 μL aliquot of labeled PCR products was added to 20 μL of 95% formamide/10 mM NaOH/0.025% bromophenol blue/0.025% xylene cyanol and heat denatured. Four μL of this mixture were electrophoresed in a nondenaturing 0.5 x MDE gel at 25 W in a cold room for 4 hr using a 0.6 x TBE running buffer. The gel was vacuum dried and autoradiographed for 6 hr. Samples include DNA from two individual B6 and two individual D2 mice, lung tissue from mice 6 and 15, a procedure control (PC) that was taken through all the experimental procedures but lacked any added DNA, and a negative buffer control (NEG) for the PCR reaction that also lacked added DNA template.

C57 C57 DBA DBA 6 15 PC Neg

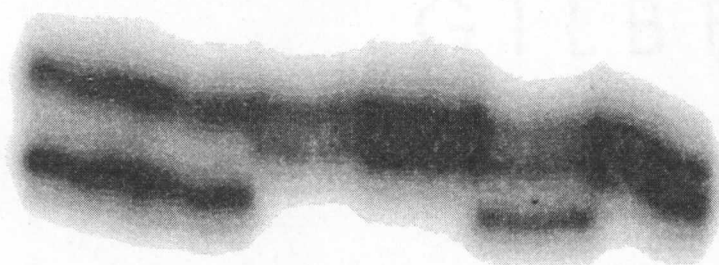


FIGURE B4:**ASO Analysis of Paraffin-embedded Tissue for Mutations in the *Ki-ras* Gene.**

Paraffin-embedded lung tissue samples were deparaffinized and digested with Proteinase K. Two μ L of lysate were amplified by the PCR technique for 40 cycles. The PCR products were blotted directly onto nylon membranes and probed with 20 base pair oligonucleotides to the 12th and 13th codons of the *Ki-ras* gene. The blots were autoradiographed for 8 hr. The first column marked C included all the positive and negative controls, including tissue from B6 and D2 mice as well as the procedure controls (PC) and negative buffer controls for the PCR reaction (NEG). Both of these latter controls lacked any added template DNA. The next two columns marked T contain the tumor DNAs which were (from top to bottom): *middle column*, 16-1, 16-2, 6-1, 6-2, 21, 13, 22, and 15-1; *last column*, 15-2, 28, 29-1, 29-2, 32-1, 32-2, 34, 40. The numbers identify the individual mice from the study. When more than one tumor was isolated from the same mouse, this was indicated by the designation "-X" where "X" is the tumor number.

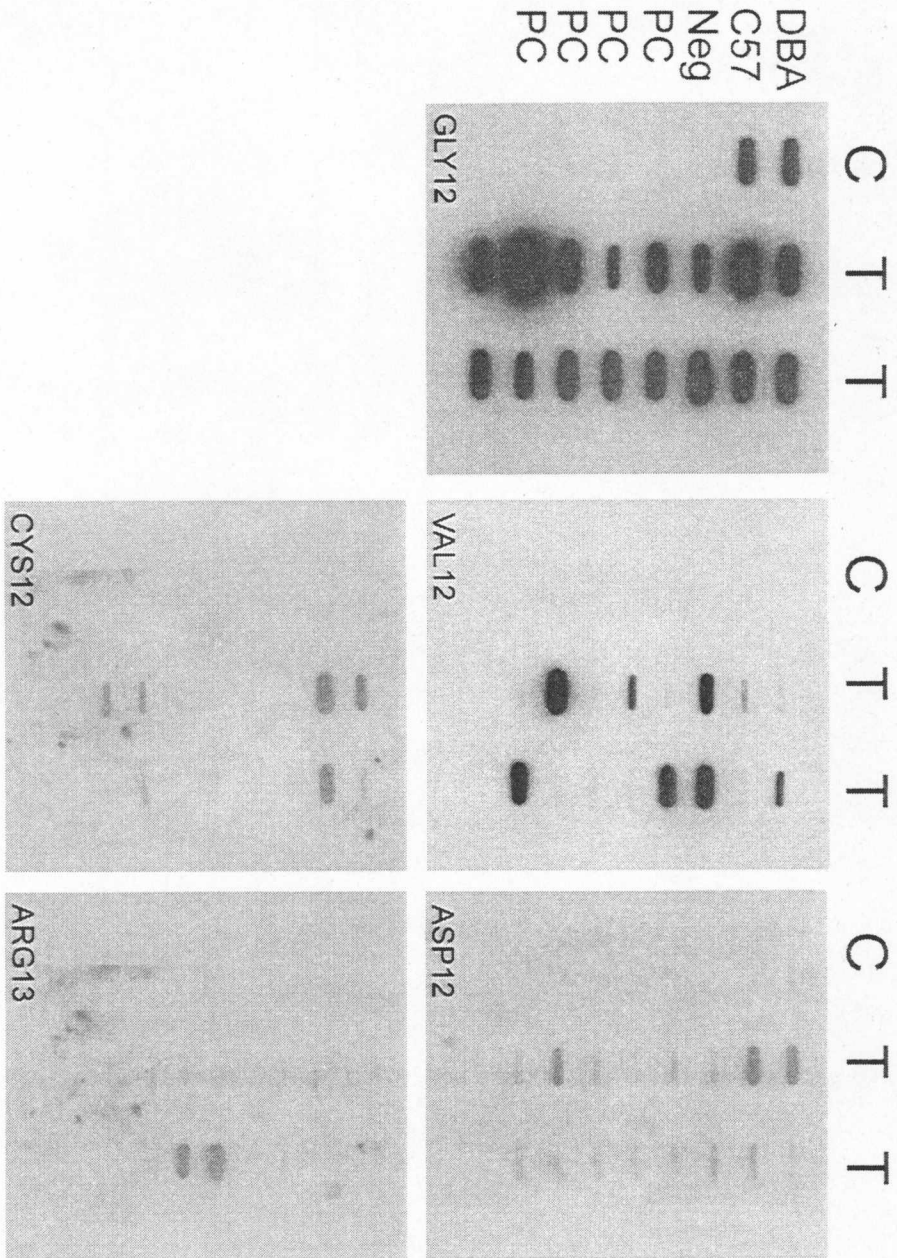
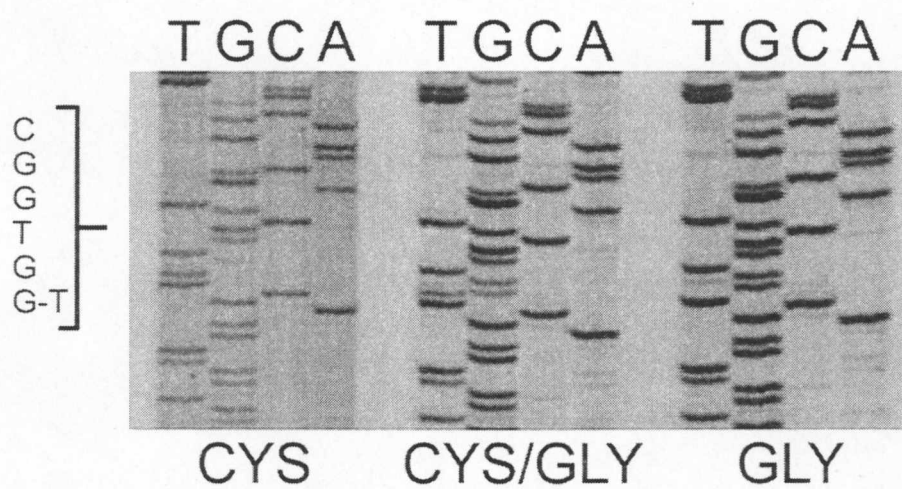
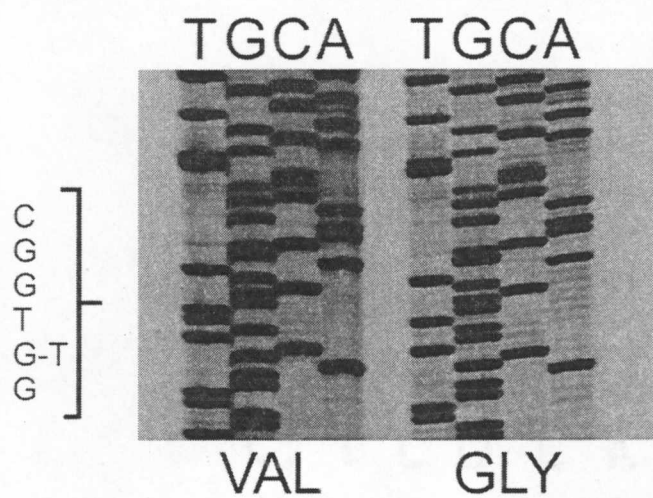


Figure B5:**Sequence Analysis of Specific Tumor Tissue**

PCR products from tumors 16-1 (bottom) and 21 (top) were subcloned using the TA Cloning Kit and sequenced with the *Sequenase* enzyme. The upper gel shows the results from two clones of tumor 21 that had the wild type (GGT) and mutated (GTT) sequences. The lower gel shows the results from three clones of tumor 16-1 that had the wild type, mutant (TGT), and a mixture of both sequences.



VITA

Lisa Lenore Wessner was born in Bartlesville, Oklahoma on June 29, 1972. She lived in Bartlesville up until her graduation from Bartlesville High School. During her younger years she spent her free time outside of school swimming for the Phillips 66 Splash Club and traveled extensively during her swimming career. She graduated from Bartlesville High School in May, 1990 and moved north to attend college. Her academic and swimming career continued at Ursinus College in Collegeville, Pennsylvania. She graduated from Ursinus College in May, 1994 with a Bachelor of Science in Biology. Following graduation, Lisa moved to Knoxville, Tennessee where she entered the Master's program in Life Sciences. Her research was spent under the direction of Dr. Mark Steven Miller and focused on molecular carcinogenesis. She officially received her Master of Science degree in August, 1996. During her time at the University of Tennessee Lisa gave up her swimming career and turned to rugby as her new sport.

Lisa's plans for her future include environmental conservation and restoration work. Her dream is to become actively involved in the conservation efforts undertaken at The Nature Conservancy in order to protect the fragile world she treasures.