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DEVELOPMENT OF HUMAN CELL LINES
CONTAINING CYTOCHROME P450IIB1

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Sheri Jeanine Batterman

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DEVELOPMENT OF HUMAN CELL LINES CONTAINING CYTOCHROME P450IIB1

By

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ABSTRACT

DEVELOPMENT OF HUMAN CELL LINES CONTAINING CYTOCHROME P450IIB1

By

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Cytochrome P-450 enzymes detoxify foreign chemicals in the body. Unfortunately, during detoxification, harmful intermediate compounds may be formed, some of which have been shown to bind to and mutate DNA, and therefore may play a major role as cancer initiators.

This project was designed to develop a human cell line which could be used to evaluate the presence of mutagenic compounds in foods. Two plasmids PMTP450IIB1 and PMMTVP450IIB1, which encoded for cytochrome P-450IIB1 (under the control of either a metallothionein or glucocorticoid promoter) and hygromycin resistance were constructed and transfected separately into MSU 1.1 cells. Hygromycin resistant cells were selected, established as cell lines, and tested for plasmid integration, cytochrome P-450IIB1 expression and enzymatic activity. Both cell lines showed integration of the transfected plasmids into the genomic DNA. Expression of cytochrome P-450IIB1 at the RNA level and the protein (enzymatic) level was undetectable.

This thesis is dedicated to those at Luther College who inspired me, especially Wendy Stevens, who was influential in my decision to study nutrition, and also to Dr. John Neill at Pioneer Hi-bred International for his generosity and patience while teaching me many useful molecular biology techniques.

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LIST OF ABBREVIATIONS

AhR: aromatic hydrocarbon receptor

AFB_1: aflatoxin B_1

depc water: diethyl pyrocarbonate water

DMSO: dimethyl sulfoxide

EDTA: ethylenediaminetetraacetic acid

GAPDH: glyceraldehyde phosphate dehydrogenase

G6P: glucose-6-phosphate

G6PDH: glucose-6-phosphate dehydrogenase

HEPES: N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid

LB broth: Luria-Bertani broth

MEM: modified Eagle's media

PBS: phosphate buffered saline

pMMTV: mouse mammary tumor virus promoter

pMT: metallothionein promoter

SDS: sodium dodecyl sulfate

SSC: standard saline citrate

TE: 10 mM Tris/1 mM EDTA

Tris: tromethamine

INTRODUCTION

Humans are constantly exposed to foreign compounds either through their diet or from environmental exposure. Approximately 80% of all human cancers are linked to both dietary and environmental factors (Doll and Peto, 1981). A careful review of the Doll and Peto manuscript by Bailey and Williams (1993) indicated that 35% of all cancers are influenced by dietary factors. Some examples of these dietary factors linked to cancer are natural components of foods, products resulting from food processing and metabolites from microbial growth. Significant environmental factors include pollutants, as well as products of medical, agricultural and industrial practices (Doll and Peto, 1981). Many of these foreign compounds (xenobiotics) which enter the body, are able to bind to DNA. This binding to DNA, if not repaired correctly, will mutate DNA, which increases the risk of cancer.

Although numerous environmental and dietary contaminants may enter our bodies, there are many protective mechanisms which prevent these contaminants from eliciting a toxic response such as DNA mutation. It has been demonstrated that minor dietary food constituents play a role in inhibiting or

preventing cancer through a variety of mechanisms. Some are blocking agents which prevent carcinogens from reaching or reacting with critical target sites. Others are suppressors which prevent evolution of neoplastic processes in cells that would otherwise become malignant (Wattenberg, 1992).

Another example of a protective mechanism against contaminants is through metabolism. There are a variety of metabolic reactions in animals which function to detoxify and enhance the excretion of foreign substances from the body. One key reaction for metabolism and detoxification of foreign substances is the cytochrome P-450 system. The details of this system are explained in the literature review section. The overall goal of this project was to incorporate an easily inducible cytochrome P-450 metabolic system into a human cell line that could be used as a bioassay to evaluate which dietary contaminants and cytochrome P-450 isozymes are involved in the bioactivation of promutagenic compounds such as aflatoxin B_1 (AFB_1) and benzo[a]pyrene to mutagenic compounds.

The specific objectives of the project were:

(1) Subclone the cytochrome P-450IIB1 cDNA into an expression vector.

(2) Expand the newly constructed plasmid and purify it.

(3) Transfect MSU 1.1 cells with PMTP450IIB1 and PMMTVP450IIB1 plasmids and select stable clones.

(4) Confirm that the PMTP450IIB1 (metallothionein promoter) and PMMTVP450IIB1 (glucorcorticoid promoter) plasmids had been incorporated into the genomic DNA of MSU 1.1 cells.

(5) Confirm that the cells lines express the full length, active cytochrome P450IIB1 protein.

LITERATURE REVIEW

Xenobiotic metabolism. When foreign chemicals enter the body, they are subjected to various metabolic reactions. These metabolic processes are called biotransformation and usually involve both cytosolic and microsomal enzymes. In general, the overall purpose of biotransformation is to alter the parent xenobiotic to a less toxic form that is more polar, and more easily excreted from the body.

There are numerous enzymes located in different cellular compartments which can biotransform xenobiotics in such a way as to make them more water soluble. These enzymatic reactions have been classified by pharmacologists and toxicologists as Phase I and Phase II metabolism (Dauterman, 1984; Hodgson and Dauterman, 1984; Nebert, 1979; Parke and Williams, 1969). Phase I metabolism is characterized by oxidation/reduction and hydrolysis reactions which play an important role in forming oxidative products or addition of hydroxyl groups. Phase II is involved in taking the foreign compound or Phase I-derived metabolites and linking it to an endogenous molecule to produce a polar conjugate.

Cytochrome P-450. The most important enzyme systems involved in Phase I reactions are the cytochrome P-450 containing monoxygenases. The cytochrome P-450 system is a complex membrane bound metabolic system that has been found to exist in most living organisms such as bacteria, yeasts, plants, insects, fish and mammals (Ioannides and Parke, 1990). In mammals, the cytochrome P-450 is found in nearly all tissues except striated muscle and erythrocytes (Guengerich, 1988).

In humans, cytochrome P-450 appears to be concentrated at specific sites which are involved in protecting the body from the effects of foreign compounds (Parke and Williams, 1969). For example, significant amounts of cytochrome P-450 are found in internal organs such as the kidney, lung, small intestine, which function to excrete foreign compounds from the body. The highest concentration of cytochrome P-450 is located in the endoplasmic reticulum in the cells of the liver. The liver also functions to detoxify or get rid of xenobiotics from the body. It has also been shown that skin contains moderate amounts of cytochrome P-450. The skin covers such a large area on the human body, that the total amount of cytochrome P-450 contained within it has potential to greatly contribute to the overall biotransformation processes of the body (Klaassen et al., 1986). This is a fact of particular importance to us since human skin cells are used in this research project.

The cytochrome P-450 monooxygenases are complex multiple component systems requiring molecular oxygen and nicotinamide-adenine dinucleotide phosphate (NADPH). The cytochrome P-450 system is made up of the two enzymes: (1) NADPH cytochrome P-450 reductase, and (2) a heme containing enzyme, cytochrome P-450. These enzymes are embedded in the phospholipid matrix of the endoplasmic reticulum. More than 75% of the reductase molecule is positioned above the lipid bilayer, while P-450 molecules are closely embedded in the endoplasmic reticulum membrane. Therefore electrons are easily transferred from NADPH via the reductase to cytochrome P-450 (Nebert, 1979, Guengerich, 1991). After the transfer of electrons, one atom of molecular oxygen combines with the substrate (drug, xenobiotic or endogenous molecule such as a steroid) through a heme molecule, and the other atom of oxygen forms water (Guengerich, 1993). The oxygenated substrate then dissociates, regenerating an oxidized form of cytochrome P-450. A general schematic diagram of this system is shown in Figure 1 (Coon et al., 1992). The oxygenated substrate is less harmful to the body and more easily eliminated. Thus, the ultimate function of this monooxygenase system or initial (Phase I) metabolism is to make foreign compounds more polar and therefore more water soluble so they can be readily metabolized by Phase II enzymes and excreted from the body (Dauterman, 1984; Parke and Williams, 1969).

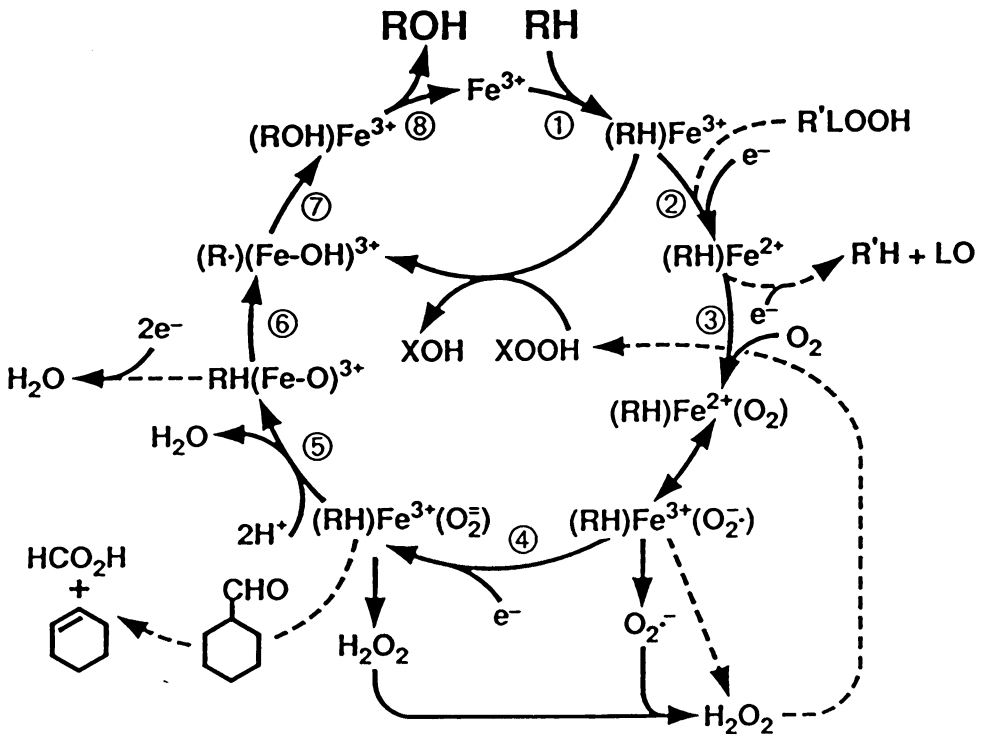


Figure 1. The mechanism of action of P450. (Coon et al., 1992)

Fe represents the heme iron atom in the active site, RH a substrate, and ROH the corresponding monooxygenation product. R'LOOH represents a lipid hydroperoxide and R'H and LO represent the corresponding reduction products (alkane and oxoacid, respectively). XOOH represents a peroxy compound that serves as an alternate oxygen donor to molecular oxygen.

Besides forming oxidative products and hydroxyl groups from foreign compounds, cytochrome P-450 may also be involved in altering xenobiotics by forming a non polar (methyl or ethyl) group to make the chemical more polar and thus expose or add functional groups (Hollenberg, 1991). These functional groups give Phase II enzymes a site for attachment and allow the foreign compounds to undergo Phase II metabolism, which links an endogenous molecule to a functional group (either present or produced by Phase I metabolism), making a highly polar conjugate. Examples of these endogenous conjugation molecules are: glucuronic acid, sulfate conjugates and glutathione (Bock et al., 1990). These water soluble conjugates are easily excreted from the body via the urine and bile (Nebert, 1979). In general, the Phase I reactions are the limiting reactions, whereas Phase II reactions, present in the cytosol of the cell, are in excess. Therefore changes in Phase I metabolism will increase overall detoxification of xenobiotics (Hodgson and Dauterman, 1984).

Bioactivation. Unfortunately, Phase I oxidative metabolism, may in some cases metabolize the chemicals to more reactive intermediates, which are significantly more toxic. This process is termed "metabolic activation" or "bioactivation" (Ioannides and Parke, 1990). For example, polyhalogenated alkanes can accept electrons to become radical anions that fragment into carbon-centered free radicals upon cleavage of

the carbon-halogen bond. These free radicals can damage macromolecules and have toxic effects on the cell. Another type of bioactivation is by chemical alteration to a reactive unstable intermediate. An example of this type of biactivation is when aflatoxin B_1 (AFB_1) is metabolized by cytochrome P-450IIIA4 to become the intermediate AFB_1-2,3-oxide, which is known to form a DNA adduct, with N-7 position of guanine (Faletto and Gurtoo, 1989). Another example of chemical alteration to a bioactivated intermediate is when benzo[a]pyrene is metabolized by cytochrome P-450IA1 and epoxide hydrolase to benzo[a]pyrene 7,8,9,10 diol-epoxide, which is an extremely potent mutagen that binds to DNA causing cellular damage (Bailey and Williams, 1993). The pathways of bioactivation and detoxification in aflatoxin and benzo[a]pyrene can be seen in Figure 2 (Cheeke and Shull, 1985; Bailey and Williams, 1993).

Highly reactive, harmful intermediates formed during bioactivation play an important role in binding to macromolecules (protein, RNA and DNA), and may be responsible for mediating various toxic effects. Reactive or harmful intermediates which bind to cellular DNA can cause mutations. These DNA mutations may be the initiating event(s) which lead to uncontrolled cellular growth and potential to produce malignant cells.

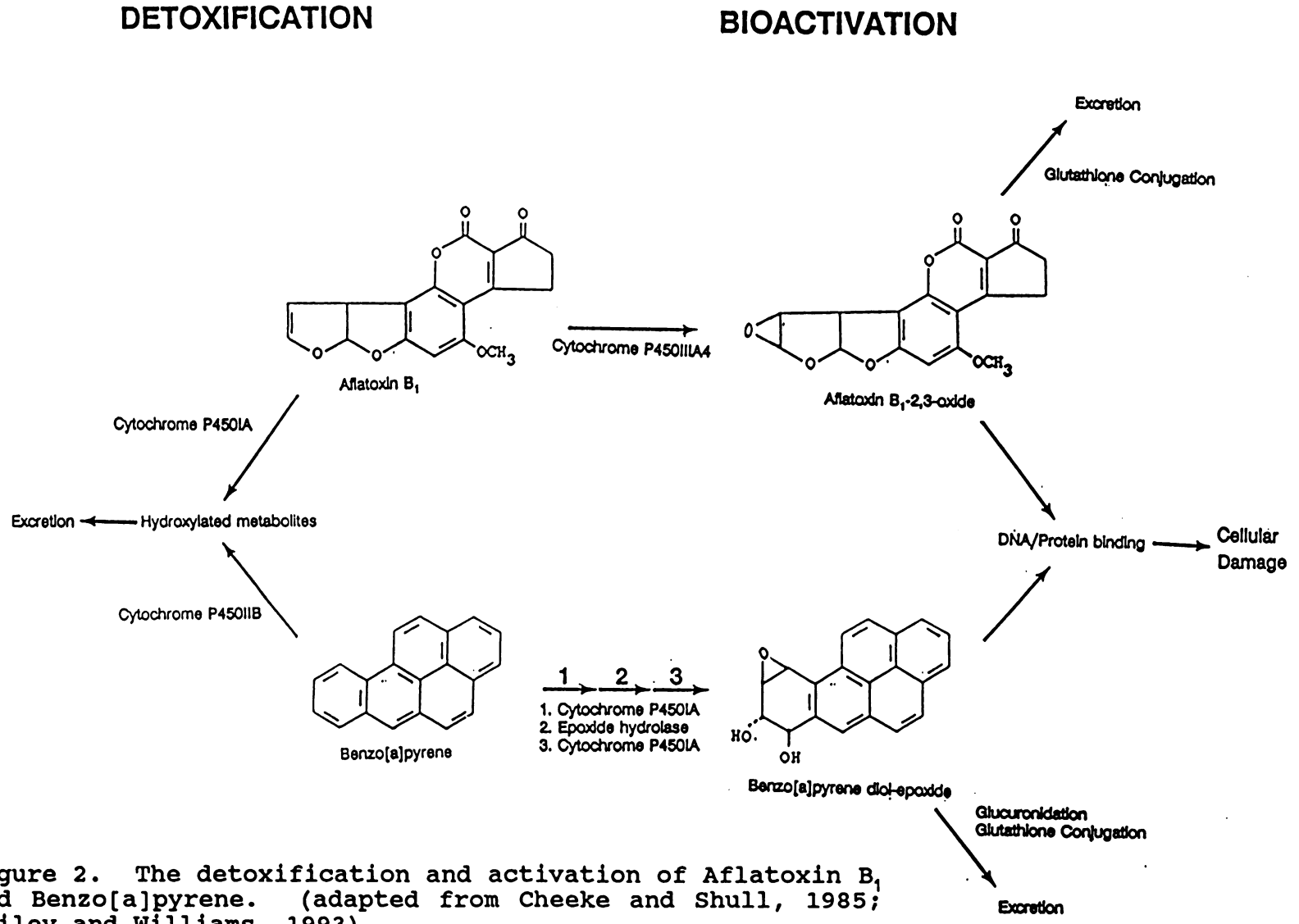


Figure 2. The detoxification and activation of Aflatoxin B_1 and Benzo[a]pyrene. (adapted from Cheeke and Shull, 1985; Bailey and Williams, 1993).

Induction. Another feature of the cytochrome P-450 system is that it is induced or "turned on" by a wide variety of chemicals to increase its specific enzymatic activity. Certain chemicals can induce many isoforms of cytochrome P-450 at once, while suppressing other forms at the same time. The mechanism of induction of most of the cytochromes P-450s is not well understood. Most induction processes are regulated at the transcriptional level, but it has also been observed that some chemicals have the ability to physically block degradation of certain P-450s. Also, some hepatic cytochrome P-450s are under strong hormonal regulation and are changed by alterations in the levels of androgens, which are also influenced by peptide hormones (Guengerich, 1988).

The mechanism of induction of cytochrome P-450IA1 has been extensively worked out. Briefly, cytochrome P-450IA1 is induced by halogenated aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) which are known to bind to the soluble, cytosolic receptor called the aromatic hydrocarbon receptor (AhR). The ligand-receptor complex translocates into the cell nucleus. This ligand-receptor complex binds to enhancer sequences upstream of the cytochrome P-450IA1 gene and increases pre-mRNA transcription from the gene. This pre-mRNA is processed to the mature mRNA and transported out to the cytoplasm where it is translated into new P450IA1 protein. Increased amounts of newly synthesized cytochrome P-450IA1 ultimately increases metabolism. A

diagram of the mechanism of induction of cytochrome P-450IA1 can be seen in Figure 3 (Denison, 1991).

Some examples of inducers which "turn on" the P-450 system, include phenobarbital (PB), 3-methylcholanthrene (3-MC), polychlorinated biphenyls (PCBs), isosafrole, isoniazid, ethanol and clofibrate; animals treated with these compounds show several fold increases in various cytochrome P-450 isoforms in liver microsomes (Ryan and Levin, 1990). Overall, certain chemicals can induce the cytochrome P-450 system, which increases the rate of metabolism and elimination of the specific compounds from the body.

Nomenclature for cytochrome P-450. To date, more than 200 distinct isoforms of cytochrome P-450 have been discovered (Coon et al., 1992; Nebert, 1993). Because there are so many cytochrome P-450s, the nomenclature used by researchers can be very confusing. Therefore, attempts have been made to categorize cytochromes based on their evolutionary relationships. Roman numerals placed directly after the P-450 represent distinct gene families. A family consists of those cytochrome P-450 who have protein sequences that are >40% homologous. Capital letters signify subfamilies which contain sequences that are >59% identical. Finally, Arabic numbers are used for individual genes (Nebert et al., 1989).

As stated above, more than 200 cytochrome P-450 isoforms exist (Nelson et al., 1993). Many of these isoforms have not

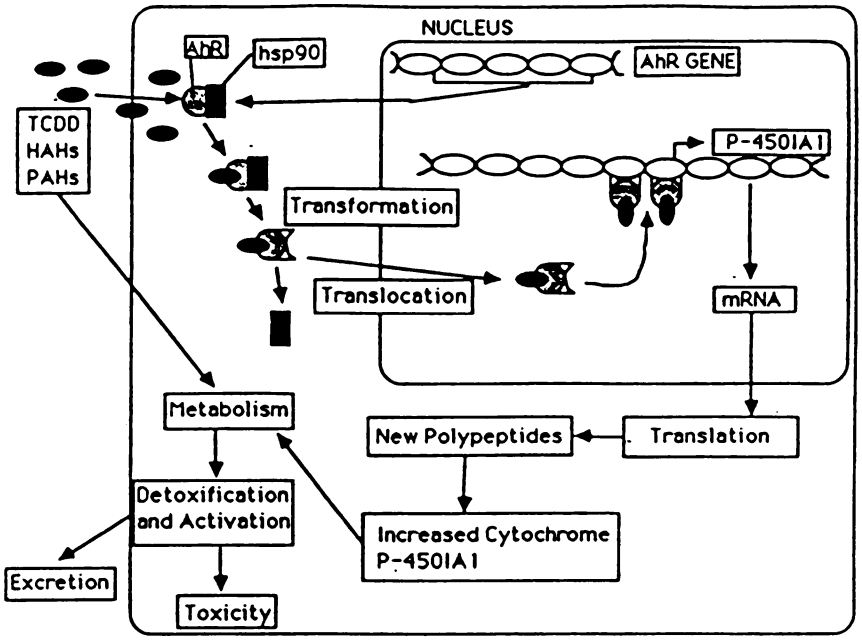


Figure 3. Induction of cytochrome P450 proteins by polycyclic aromatic hydrocarbons as mediated by the Aromatic Hydrocarbon Receptor (Denison, 1991).

been studied, characterized or even named yet. In this project we have specifically chosen to study cytochrome P-450IIB1 because it is one of the most well characterized isoforms with regard to its metabolic activity and detoxification of xenobiotics.

Cytochrome P-450IIB1. The cytochrome P-450IIB1 used in this study was from rat origin. This was chosen because humans are not known to have the IIB1 isoform of cytochrome P-450. Cytochrome P-450IIB1 is known to be induced by phenobarbital, which occurs primarily through an increase in transcription rate in the corresponding genes. It has also been speculated that cytochrome P-450IIB1 is capable of catalyzing the monooxygenation of a wide variety of foreign organic chemicals such as plant toxins and pesticides (Juchau, 1990).

Overall, as stated previously, cytochrome P-450 is a metabolic system which breaks down foreign compounds in order to eliminate them from the body. Unfortunately, in some instances, the metabolism of a foreign compound leaves harmful intermediates, which can damage or mutate DNA. Therefore it is critical to test foreign compounds or chemicals to see how mutagenic or damaging they are in a living system. Various assays have been designed for this purpose.

Mutagenic Assays. The *Salmonella*/mammalian microsomal mutagenic assay developed by Ames et al. (1975) has been the

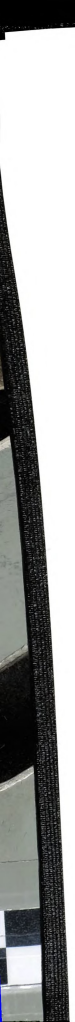
most widely used assay for research in the food industry as a means of determining the mutagenic potential of many environmental and dietary compounds (Maron and Ames, 1982). This assay, referred to as either the Ames assay or Ames test, is a bacterial based assay which uses a modified *Salmonella* strain (TA98, TA100). The modified strain has a mutation on the gene that codes for enzymes involved in the synthesis of histidine. Therefore, these *Salmonella* strains require histidine in order to grow. *Salmonella* are plated with minimal histidine to allow a lawn of bacteria. Only those bacteria that contain a mutation in the gene will revert back to wild type (reversion) and allow cell growth in the absence of histidine. Thus, only the *Salmonella* which have had DNA damage mediated by the test compound will form colonies. The greater the number of revertant colonies, the greater the mutagenic potential of the chemical (Hoffmann, 1982).

Salmonella is a prokaryote, and therefore has many unique cellular characteristics such as: 1) no nucleus 2) no introns 3) permeable cell membrane 4) poor repair mechanisms. These characteristics show that *Salmonella* DNA is unprotected, which allows for it to be easily damaged, and may give rise to false positives in the Ames assay (Ames and Gold, 1992). The accuracy of the Ames assay has been questioned due to the great number of conflicting results when compared to rodent *in vivo* carcinogenic assays. For example, chemicals that have been shown not to be carcinogenic in a number of

animal models, show up as being mutagenic in the Ames assay. It has been estimated that 50% of compounds testing positive in the Ames assay are not actually carcinogens (Ames and Gold, 1990b). Additionally, compounds found to be non-mutagenic in the Ames assay are carcinogenic in rodent models (Ames and Gold, 1990a). Although the Ames assay has been used extensively to evaluate mutagenic potential of a variety of compounds, the assay has certain limitations because it utilizes bacterial cells.

Due to the limitations of the Ames assay, it was thought that a eukaryotic system may be a more accurate indicator of mutagenicity of foreign compounds. Eukaryotic systems have greater means of protection such as a strong cell wall and a nucleus, as well as efficient DNA repair mechanisms, which may be more analogous to repair mechanisms in humans. Systems utilizing the enzyme hypoxanthine phosphoribosyl-transferase (HPRT) are commonly used to determine mutagenicity of foreign compounds in eukaryotic cells.

Briefly, the HPRT enzyme is coded for on the HPRT gene located on the X chromosome. Normal cells possessing HPRT activity will convert purine analogues substrates to their respective ribotides. When 6-thioguanine (6-TG) and 5-bromodeoxyuridine (5-BDURD) are used in the media and incubated with normal cells, death occurs. The reason for the cytotoxicity is because 6-TG and 5-BDURD are metabolized by the enzyme HPRT and these metabolites are incorporated into



the DNA. Incorporation of these nucleotides results in a high degree of DNA mutation and subsequent cell death. On the other hand, if the normal cells are incubated in media containing 6-TG and 5-BDURD with the addition of a mutagenic compound, the HPRT locus is mutated, which stops production the functional HPRT enzyme. If the HPRT enzyme is not functional, the 6-TG and 5-BDURD are not metabolized and can not be incorporated into DNA. Cell death does not occur, resulting in formation of colonies. The greater the mutagenicity of the compound being tested, the greater the number of colonies that will be formed (Albertini et al., 1982).

Although the utilization of the HPRT gene within a cell line is a common method for testing mutagenicity of foreign compounds, it has an important limitation: this system only detects mutations on one gene (HPRT) or locus. Toxic chemicals have the potential to mutate a wide variety of genes in the human body, which may go unmeasured by this system.

Although the Ames assay and the HPRT are two commonly used systems for evaluating mutagenic potential of foreign compounds, they both have limitations. These systems are not extremely accurate and they are not directly applicable to humans. Therefore a more accurate, reliable system is needed by researchers to better assess the potential of foreign compounds to alter DNA and cause cancer in humans.

The optimal cell system to test for mutagenic compounds should incorporate the following characteristics: 1) The cell system should be comprised of eukaroytic, mammalian cells, preferably human cells. 2) The cells should be easy to maintain in culture. 3) The cells need to have the ability to normally express cytochrome P-450 under certain growing conditions. 4) After coming into contact with mutagenic compounds, these cells should give a clearly visible, accurate indication of DNA damage in the form of a forward mutation. All of these characteristics are found in MSU 1.1 cells, which is why this particular cell line was chosen for the following study.

MSU 1.1 cells. MSU 1.1 is a human fibroblastic cell line obtained from Dr. J. McCormick at the Carcinogenesis Laboratory at Michigan State University (McCormick, and Maher, 1981; McCormick and Maher, 1985; Hurlin et al., 1989). The MSU 1.1 cells arose from a diploid neonatal foreskin derived cell line transfected with a plasmid containing a *v-myc* oncogene and a selectable marker for neomycin resistance (Yang et al., 1992). These cells have fibroblastic morphology and a stable, near diploid karyotype composed of 45 chromosomes, including two marker chromosomes (Fry et al., 1990). Except for their infinite life span, MSU 1.1 cells do not possess traits commonly associated with tumor derived malignant cells. MSU 1.1 cells have normal, unaltered morphology, and they are

dependent on growth factors. Additionally, MSU 1.1 cells will not grow large colonies in soft agar and they will not form tumors when injected into the nude mouse model (Wilson et al., 1990).

Like many other continuously growing cell lines, MSU 1.1 has low endogenous expression of cytochrome P-450s, thus they do not effectively metabolize xenobiotics (Doehmer et al., 1988). Therefore cytochrome P-450s need to be added to this system in order to activate metabolism. This can be accomplished by using molecular biology techniques to construct a plasmid which contains specific cytochrome P-450 DNA coding sequences. After this plasmid is transfected into the cells, the cytochrome P-450 mRNA can be expressed, and translated into active protein. Expression of the cytochrome P-450 mRNA can be controlled by an inducible promoter, which is induced or "turned on" by the addition of certain chemicals.

Once the MSU 1.1 cells are transfected with plasmids, such as those used in this study, potentially toxic compounds can be added to the cells. In the presence of mutagenic compounds, MSU 1.1 cells are transformed into rapidly growing cells which will form foci. These foci can easily be stained and counted. The number of foci formed should directly relate to the mutagenicity of the compound being tested. Foci that grow in soft agar, grow well in media without growth factors or form tumors after being injected into nude mice, are

thought to be malignant cells (McCormick and Maher; 1988, Yang et al., 1992).

Advantages of MSU 1.1. The development of a mammalian based mutagenic assay system such as the MSU 1.1 cell line has several advantages over both the Ames assay and the HPRT system. Often times the Ames assay results in false positives because the *Salmonella* bacteria have weak cell membranes and they lack a nucleus, which makes them extremely susceptible to mutagens. MSU 1.1 is a eukaroytic system, which means each cell has a nucleus, a strong cell membrane, and its genes have introns, all of which protect the DNA from the harmful effects of xenobiotics. Eukaroytic cells such as MSU 1.1 have better mechanisms by which to repair DNA than prokaryotes. Therefore, a toxic compound in the MSU 1.1 cell would have to pass through the plasma membrane, nuclear membrane, interact with chromatin structure and escape repair mechanisms before any effect could be observed in foci formation. Such defense mechanisms against xenobiotics exhibited by the MSU 1.1 system would mimic those occurring in other eukaryotic systems such as animals and humans.

The transfected MSU 1.1 cell line is also advantageous over the HPRT system because it does not require a toxic compound to damage only one specific gene, as in the HPRT system. The DNA within the MSU 1.1 cells can potentially be damaged at numerous undesignated places and the effects can

still be detected by the number of foci formed. Overall, the data obtained from the MSU 1.1 cells would be analogous to the detoxification system in the human body.

MATERIALS AND METHODS

Cell Culture. MSU 1.1 cells were routinely grown in modified Eagle's medium (MEM) (Sigma, St. Louis, MO) prepared with 10 % supplemented, defined bovine serum (Hyclone, Logan, Utah), 5 units/ml penicillin/ 5 μg/ml streptomycin (Sigma), and 5 μg/ml hydrocortisone (Sigma). Cells were maintained at 37°C in a humidified incubator with 5% CO_2. Cells were examined on a daily basis. Media were replaced every 48 hours, and when cells were confluent, the cultures were split.

Plasmid Construction. Plasmid PMMTVP450IIB1 (Figure 4) construction was done in the following manner: pSP450 oligo plasmid was cut with restriction enzymes NheI and SmaI to release a 2 Kb insert which contains the complete cytochrome P-450IIB1 cDNA sequence. After the plasmid was cut, it was run on an agarose gel to separate the bands. The band in the 2 kb region was removed and purified.

Purification was done as follows: The agarose containing the band was put into a 1 ml syringe with a yellow pipet tip affixed to the end. The contents were pushed through into a 1.5 ml eppendorf tube and 200 μl TE was also pushed through to remove remaining agarose. Next, 0.5 ml of water saturated

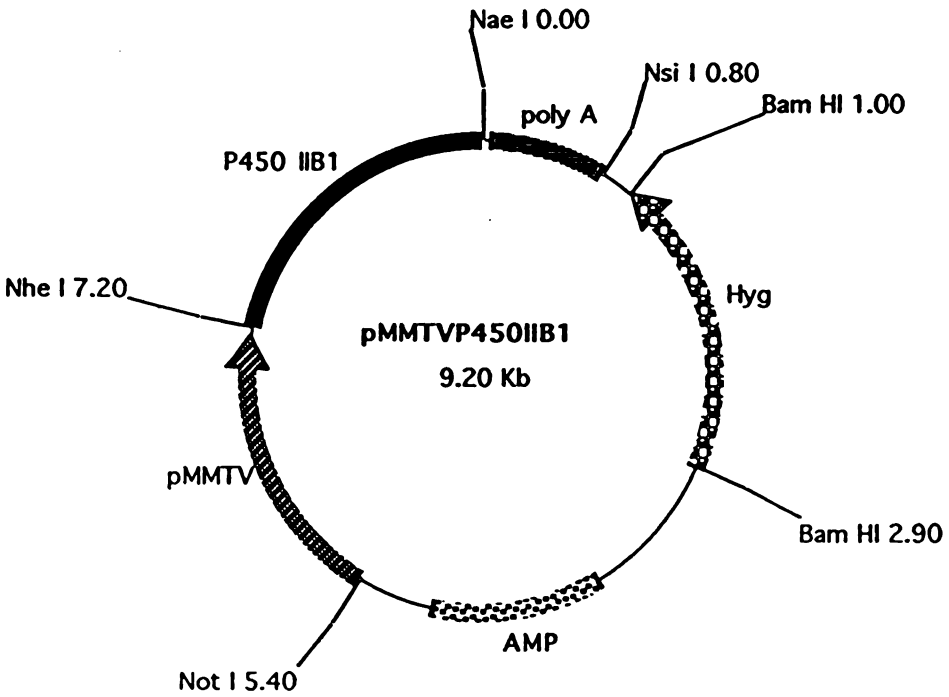


Figure 4. Plasmid map of pMMTVP450IIB1

The solid black area of the map is the 2000 Kb base pair insert (cytochrome P450 portion) from the pSP P450 oligo vector. The hyg portion represents the hygromycin B resistant gene, which allows for the selection of transfected cells. The AMP area represents the ampicillin resistant gene, which allows for this plasmid to be grown up in E. Coli. The pMMTV portion is the glucocorticoid promoter which turns on expression of cytochrome P-450 with the addition of glucocorticoids to the cell media. The other plasmid used, called PMTP450IIB1 is identical to the above figure, except it has a PMT promoter (which turns on expression of cytochromes with the addition of metals) in place of the PMMTV.

phenol (equilibrated in 0.1 M TE) was added, the tube was vortexed, and put into the -80°C freezer overnight. The following day, the tube was centrifuged at 12 K x *g* for 10 minutes. The top aqueous layer was collected and divided into 2 eppendorf tubes. Then 0.5 ml phenol/chloroform (50:50 v/v) was added to each tube, vortexed and centrifuged at 12 K x *g* for 3 minutes. The top layer was collected and ethanol-precipitated. Tubes were centrifuged at 12 K x *g* for 10 minutes and then washed with 70 % ethanol. After the wash, the tubes were dried in a Spin Vac (Savant, Farmingdale, NY), resuspended in water and quantitated at A_{260} on spectrophotometer (Cary 3E Varian Instruments, Sunnyvale, CA).

The cytochrome P-450IIB1 NheI and SmaI 2 kb insert was ligated into PMMTV-hyg vector (gift from Dr. J. McCormick). This ligation was performed by cutting the PMMTV-hyg vector with NheI and NaeI enzymes to generate ends which were compatable with the P450IIB1 insert. Briefly, this ligation was possible due to the fact that the cytochrome P-450IIB1 2 kb NheI end attaches to the NheI site of the PMMTV-hyg vector. The SmaI and NaeI generate blunt ends, which attach during the ligation procedure.

Ligation. The ligation of the cytochrome P-450IIB1 2 kb NheI and SmaI insert to the PMMTV-hyg vector involved using: 500 ng P450IIB1 insert and 200 ng PMMTV vector were mixed in 10.5 μl deionized water and heated at 65°C for 5 minutes. This

generated a 10:1 molar ratio of insert to vector. Then 1.5 µl 10x ligation buffer (0.66 M Tris pH 7.5, 50 mM $MgCl_2$ and 50 mM dithiothreitol), 1 µl 2 mM ATP and 1 µl T4 ligase (5 units/ml) (Boehringer Mannheim, Indianapolis, IN) were added. This mixture was mixed and incubated at room temperature for 16 hours.

The newly sub-cloned PMMTV-P450IIB1 expression vector was then transformed into competent *Escherichia coli* cells (DH5-α) (Seidman, 1989). The bacterial cells were then propagated overnight in Luria-Bertani (LB) broth at 37°C with vigorous shaking. The plasmid was purified using the alkaline lysis/ cesium chloride gradient procedure (Maniatis et al., 1989).

Construction of the other plasmid PMTP450IIB1 used in this study, was done previously by Dr. C.S. Mehigh. The PMTP450IIB1 plasmid was also propagated overnight in LB broth, but it was purified using Nucleobond Ax columns (The Nest Group, Southboro, MA).

Transfection. Transfection of the MSU 1.1 cells was done using the polybrene/dimethyl sulfoxide (DMSO) (Sigma) method (Morgan et al., 1985). Twenty-four hours before the transfection, MSU 1.1 cells were plated at 2 x 10^5 cells/100 mm diameter plate. The following day, normal growth media was removed from the plates. It was replaced with transfection media which consisted of 2 ml of MEM containing 30 µg/ml polybrene (hexadimethrine bromide) and 2 µg of plasmid DNA

(linearized with Not 1). Plates were incubated at 37°C for 6 hr. in a 5 % CO_2 humidified environment. Plates were gently agitated each hour during the 6 hour incubation to insure even dispersion of transfection media. Control transfection plate was prepared in the same manner, except no DNA was added to cells.

Following the incubation, transfection media was removed and the cells were subjected to a 4 minute shock treatment using MEM containing 30 % DMSO (freshly prepared) (Sigma). Cells were then rinsed twice with 0.15 M phosphate buffered saline (PBS) pH 7.4, and normal growth media was replaced.

Hirt Lysates. Forty-eight hours after transfection, Hirt lysates (Hirt, 1967) were collected in order to do a slot blot. Media were removed from the plates and cells were rinsed three times with PBS. One ml Hirt lysis buffer (10 mM Tris pH 7.5, 10 mM EDTA, 0.6% SDS) was added to each 100 mm plate of cells. Twenty minutes later, 250 μl 5 M NaCl was added to plates. Cells were then scraped from plates, pipetted into 1.5 ml eppendorf tubes and incubated at 4°C for 16 hours. The tubes were then centrifuged at 12 K x *g* for 20 minutes at 4°C. The supernatant was collected and extracted once with phenol/chloroform (50:50 v/v), followed by chloroform/isoamyl alcohol (24:1 v/v) extraction. The aqueous phase was collected, ethanol-precipitated, and centrifuged at

12 K x *g* for 10 minutes. The resulting pellet which contained the low molecular weight DNA, was resuspended in 50 µl water.

Varying amounts of the low molecular weight DNA, along with 20 µl each of NaOH (2 M) and ammonium acetate (2 M) were added to the slot blotter (Schleicher and Schuel, Keene, NH) which contained Hybond N, a non charged nylon membrane (Amersham, Arlington Heights, IL) The blot was probed with a ^{32}P (Dupont, Boston, MA) random prime labeled (Feinberg and Vogelstein, 1983) 1.9 Kb fragment which expresses hygromycin resistance from the PMMTVP450IIB1 plasmid. Varying amounts of the entire original PMMTVP450IIB1 vector were also used as positive controls and for quantitation purposes.

Selection for hygromycin resistance. Transfected MSU 1.1 cells were allowed to grow for 72-96 hours. The cells were then split 1:5 into 100 mm diameter plates. After the cells had attached to the plates (≈ 5 hrs.), hygromycin B (Sigma) was added to the media for a final concentration of 300 µg/ml. Throughout the rest of this thesis, hygromycin B will simply be referred to as hygromycin. Fresh media and hygromycin were replaced on the plates every three days. After 12-15 days, plates were carefully checked for the presence of hygromycin resistant cell colonies.

Hygromycin resistant colonies that were found were moved to 35 mm plates. Moving the colonies consisted of removing the media and wiping an area (≈ 3 cm. in diameter) around the

colony with a sterile cotton swab. Approximately 20 µl of 1x trypsin (Sigma) was pipetted directly onto the cluster, and left there for 30-45 seconds, or until the cells were round in shape instead of elongated. After the trypsin was removed, the cells were quickly resuspended in 50 µl MEM and transferred to 35 mm plates. Cells were grown in selective media containing 300 µg/ml hygromycin (final concentration) until they were confluent, upon which they were transferred to 60 mm plates and finally to 100 mm plates. The total amount of selection time in which hygromycin was in the media was approximately 3-5 weeks. Cells from several 100 mm plates were frozen in liquid nitrogen as described below.

Freezing cells consisted of removing media from the plates and placing 3 ml 1x trypsin directly onto the 100 mm plate. After approximately 45 seconds, when the cells became rounded, trypsin was removed, and the cells were resuspended in 1 ml of MEM containing 10% DMSO, 10 % supplemented defined bovine serum, 5 units/ml penicillin/ 5 µg/ml streptomycin and 5 µg/ml hydrocortisone. The 1 ml cell suspension was pipetted into a sterile cryogenic vial (Corning, Corning, NY) and placed in the -20°C freezer for 1 hour. The vial of cells was then placed in the -80°C freezer for 15 minutes and finally submerged into liquid nitrogen storage freezer.

Southern Analysis. Genomic (high molecular weight) DNA was extracted from transfected and non transfected cells by

dissolving them in a lysis buffer (10 mM tris pH 7.5, 50 mM EDTA, .1% SDS and 50 μg/ml proteinase K), and incubated overnight at 30°C (Maniatis et al., 1989). Solubilized DNA was extracted twice with phenol and once with chloroform/isoamyl alcohol (24:1 v/v) and dialyzed overnight at 4°C against 2 liters of 10 mM Tris/1 mM EDTA (TE). DNA yield was quantitated at A_{260} on a spectrophotometer. Approximately 15 μg DNA was cut with restriction enzymes, separated on a 0.7% agarose gel and transferred to non-charged nylon membrane (Hybond N-Amersham) using standard procedures (Maniatis et al., 1989). Three Southern blots were done using this genomic DNA. Two Southerns, were hybridized with a ^{32}P random prime labeled (Boehringer Mannheim) 1.9 Kb fragment which expresses hygromycin resistance from the original PMMTVP450IIB1 plasmid. The third blot was probed with a ^{32}P random prime labeled SV 40 A+ region of the PMMTVP450IIB1 plasmid.

The prehybridization of the blot was done using 50% formamide, 3x SSC, 5x Denhardts (Maniatis et al., 1989) and 50 mM phosphate buffer. The hybridization was done in 50 % formamide, 3x SSC, 5 % dextran sulfate, 50 mM phosphate buffer, 5x Denhardts and .1% SDS. After the hybridization, the membrane was washed using a high stringency wash: 2x SSC, .1 % SDS for 1 hr. at 65°C and .1x SSC, .1% SDS for 15 minutes at 65 °C. The membrane was exposed to X-ray film (X-OMAT AR,

Eastman Kodak Co. Rochester, NY) with two intensifying screens at -80°C.

Northern Analysis. RNA expression from either the MMTV or MT promoter was induced 24 hours before harvesting the cells. This was accomplished through the addition of 75 μm $ZnSo_4$ (Sigma) to the PMTIIB1P450 transfected cells, and by the addition of 2.55 μM dexamethasone (Sigma) to the PMMTVIIB1P450 transfected cells.

Total RNA was isolated by a single step method (Chomczynski and Sacchi, 1987) using the RNA STAT-60 kit (Tel Test-"B", INC. Friendswood, TX). Cells were scraped from plates, and centrifuged at 1000 x *g* for 6 minutes. The pellet was resuspended in a guanidinium thiocyanate/phenol monophase solution. Then 0.2 volume of chloroform/isoamyl alcohol (24:1 v/v) was added to this solution and it was centrifuged at 12 K x *g* for 15 minutes to separate phases. The aqueous phase was precipitated with isopropanol and centrifuged at 7,500 x *g* for 5 minutes at 4°C. The resulting pellet was washed with 75% ethanol and resuspended in diethyl pyrocarbonate (depc) water. The RNA was then quantitated on a spectrophotometer.

RNA was denatured by heating at 65°C in formamide loading buffer, then electrophoresed through a 1.2% agarose gel that contained formaldehyde (Maniatis et al., 1989). The gel was then transferred to an uncharged nylon membrane (Hybond N, Amersham) as described by Maniatis (1989). The blot was

hybridized to a ^{32}P random prime labeled (GIBCO BRL) 2 Kb cytochrome P-450IIB1 fragment from the original insert. The blots were hybridized, as previously described for Southern blots. The Northern blots were washed under the following stringency: 2x SSC, 0.1% SDS for 1 hr. at 55°C and 0.3x SSC, 0.1% SDS for 15 minutes at 55°C. The membrane was exposed to X-ray film using two intensifying screens at -80°C.

Enzyme Assays. Transfected MSU 1.1 cells were induced for 24 hours before the assay was carried out. Induction was done on the PMTP450IIB1 cells by adding either $ZnSO_4$ or $ZnCl_2$ to a final concentration of 75 mM and 100 mM respectively and in the case of the PMMTVP450IIB1 cells by supplementing the media with 2.55 μM dexamethasone.

The day of the assay, cells cultures ($\approx 5x10^6$/100 mm plate) were trypsinized, resuspended in PBS, and centrifuged at 1000 x *g* for five minutes. The resulting cell pellet was resuspended in 1.3 ml of reaction buffer (178 mM HEPES, 9 mM $MgCl_2$, 25 mM G-6-P, 1 I.U. G6PDH, and 1 mg NADP). The cellular suspension was divided in 3 fractions: 480 μl was homogenized in a Dounce homogenizer (Fisher, Pittsburg, PA), 480 μl was used as whole cells and the remaining 340 μl was used to determine cellular protein concentration by the Bradford method (Bradford, 1976).

The assay was performed as follows: The suspensions of both homogenized and whole cells were pre-incubated at 37°C in

a rotating shaker at 160 rpm for 3 minutes. Then 1.25 nanomoles of 7-benzyloxyresorufin in DMSO was added (2.5 μM final concentration) and incubated for 10 minutes (Mayer et al., 1990). The reaction was terminated by the addition of 0.5 ml acetone and 2.5 ml 0.5 M Tris pH 9.8., followed by a 5 minute centrifugation at 1200 x *g*.

Fluorescence of the samples was measured using excitation and emission wavelengths of 535 nm and 585 nm respectively on a SLM Instruments fluorometer (SLM Instruments., Champaigne-Urbana, IL). Amount of resorufin (product) in the reaction was calculated using line constant data obtained from a standard curve of resorufin.

RESULTS AND DISCUSSION

Transfection Assessment. After extensive work in determining optimal transfection conditions for the MSU 1.1 cells, they were transfected using the polybrene/DMSO procedure. To evaluate the effectiveness of this procedure, DNA was blotted on to nitrocellulose membrane using a slot blotter. Figure 5 shows slot blot analysis of low molecular weight (plasmid) DNA from MSU 1.1 cells transfected with PMTP450IIB1 or PMMTVP450IIB1. The blot was hybridized to a ^{32}P random prime labeled 1.9 Kb fragment which expresses hygromycin resistance, obtained from the PMMTVP450IIB1 plasmid. The photograph of the slot blot shows strong signals from the sample lanes on the right, which contain low molecular weight DNA from the PMTP450IIB1 or PMMTVP450IIB1 transfected MSU 1.1 cells. The standards in left column, which are PMMTVP450IIB1 plasmid DNA, are utilized as a means of quantitating the amount of DNA from the transfected MSU 1.1 cells. From the slot blot, it appears as though many of the sample lanes loaded with 5-20 μl correspond to greater than 6.3 ng of the plasmid DNA. The 5-20 μl amounts loaded in the sample lanes represent only 2-10% of the final volume, so as much as 63 ng could have entered

the cells per plate. Compared to the original amount of plasmid DNA transfected, 63 ng seems to be a small amount, but it represents a tranfection efficiency as high as 2 %. Overall, the slot blot has intense signals for the loaded samples, which demonstrate that the polybrene/DMSO method used in this study was an effective method of transfection for MSU 1.1 cells.

The intense signals seen on the slot blot in Figure 5 were expected because generally, fibroblast cells such as the MSU 1.1 cells are easily transfected. Fibroblast cells are uniform size and shape, and they grow in a monolayer, which allows them to readily take up plasmid DNA, and therefore have a higher efficiency for transfection than many other mammalian cell lines (Morgan et al., 1986).

Southern Analysis. After it was determined that the transfection procedure was successful, the transfected cells were expanded and hygromycin resistant cells were selected. Several 100 mm plates of cells were then collected and Southern analysis was performed to see if the transfected MSU 1.1 cells had incorporated the PMTP450IIB1 and PMMTVP450IIB1 plasmids into their genomic DNA. Figure 6 is an autoradiograph of the Southern analysis done on high molecular weight (genomic) DNA from PMTP450IIB1 transfected MSU 1.1 cells. The blot was hybridized to a ^{32}P random prime labeled 1.9 Kb fragment, which expresses hygromycin resistance

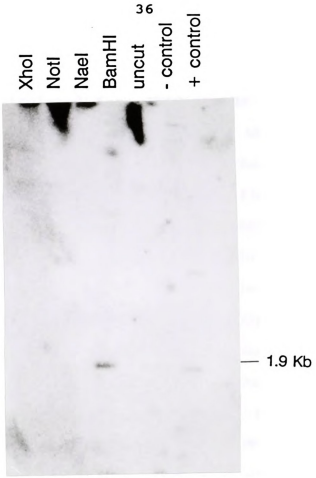


Figure 6. Southern analysis of high molecular weight DNA from PMTP450IIB1 transfected MSU 1.1 cells. Ten μg DNA was digested with various endonuclease restriction enzymes as indicated above, and hybridized with the ^{32}P random prime labeled 1.9 Kb fragment which expresses hygromycin resistance from the PMMTVP450IIB1 vector. DNA from untransfected MSU 1.1 cells cut with BamHI served as a negative control. DNA from a cell line (given to us by Ann Ryan in the M.S.U. Carcinogenesis laboratory) with a known 1.9 Kb BamHI hygromycin resistant region, was used as a positive control. The location of the expected 1.9 Kb fragments is indicated. The blot was exposed to X-ray film with two intensifying screens for 7 days.

obtained from the PMMTVP450IIB1 plasmid (Figure 4). This figure shows a single signal fragment of 1.9 Kb in the lane containing DNA isolated from PMTP450IIB1 transfected cells which had been cut with the restriction endonuclease enzyme BamHI. This signal is more intense than the positive control. From the Southern analysis as shown in Figure 6, it can be concluded that the transfected PMTP450IIB1 plasmid had integrated into the genomic DNA of the MSU 1.1 cells.

In addition, the lane of untransfected MSU 1.1 cells in Figure 6 shows no hybridization to the hygromycin resistance fragment, which serves as a negative control lane. This indicates that the untransfected MSU 1.1 cells do not contain genomic DNA with homologous sequences, to the hygromycin resistance gene. Since the untransfected MSU 1.1 cells do not contain hygromycin resistance, they will die in the presence of hygromycin, thus allowing the use of hygromycin resistance as a marker to select for transfected cells which contain the PMTP450IIB1 or PMMTVP450IIB1 plasmids.

During the selection procedure, transfected MSU 1.1 cells which contained the PMTP450IIB1 or PMMTVP450IIB1 plasmid with hygromycin resistance, grew well in the presence of hygromycin and were expanded to become stabily transfected cell lines. This indicated that the MSU 1.1 cells had been successfully transfected with the exogenous plasmid DNA. The MSU 1.1 cells which did not contain plasmid DNA, were not hygromycin resistant and did not survive in the presence of hygromycin.

As stated above, the data in Figure 6 show that at least the 1.9 Kb hygromycin resistant portion of the transfected plasmid has integrated into the genomic DNA of the MSU 1.1 cells. To demonstrate that more of the whole 9.2 Kb PMTP450IIB1 plasmid had integrated into the genomic DNA, another Southern was performed.

Figure 7 is an autoradiograph from Southern Analysis of high molecular weight (genomic) DNA from PMTP450IIB1 transfected MSU 1.1 cells which was hybridized with a SV+40 probe. In this figure, lane d, containing DNA from PMTP450IIB1 transfected cells which was digested with restriction endonuclease enzymes BamHI/Xho (double digest), exhibits the strongest signal at 5 Kb compared to lanes a and b which contain DNA from untransfected MSU 1.1 cells. This verifies that more than 5 Kb of the 9.2 Kb of the transfected PMTP450IIB1 plasmid had integrated into the genomic DNA of the MSU 1.1 transfected cells.

It was also necessary to confirm that the PMMTVP450IIB1 plasmid had become incorporated into the genomic DNA of the MSU 1.1 cells. Figure 8 is an autoradiograph of Southern analysis of high molecular (genomic) DNA from 3 plates of PMMTVP450IIB1 transfected MSU 1.1 cells hybridized to a ^{32}P random prime labeled 1.9 Kb fragment which expresses hygromycin resistance obtained from the PMMTVP450IIB1 plasmid.

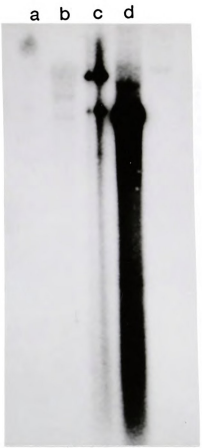


Figure 7. Southern analysis of high molecular weight DNA from untransfected MSU 1.1 cells and PMTP450IIB1 transfected MSU 1.1 cells. Ten μg DNA per sample was digested with various endonuclease enzymes and hybridized with the ^{32}P labeled SV 40A+ fragment from the original PMMTVIIB1 vector. Lanes: a) untransfected MSU 1.1 cells; b) untransfected MSU 1.1 digested with BamHI/XhoI (double digest); c) PMTP450IIB1 transfected MSU 1.1 cells uncut; d) PMTP450IIB1 transfected MSU 1.1 cells digested with BamHI/XhoI (double digest). This blot was exposed to X-ray film with two intensifying screens for 3 days.

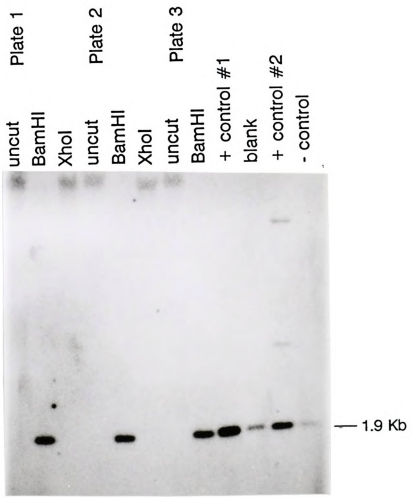


Figure 8. Southern analysis of high molecular weight DNA from three plates of PMMTVP450IIB1 transfected MSU 1.1 cells. Fifteen μg DNA was digested with the indicated endonuclease restriction enzymes, and hybridized with the ^{32}P random prime labeled 1.9 Kb fragment which expresses hygromycin resistance, from the PMMTVIIB1 vector (figure 4). DNA from PMTP450IIB1 transfected cells MSU 1.1 cells was used as positive control #1. DNA from a cell line with a known 1.9 Kb hygromycin resistant region was used as positive control #2. DNA from untransfected MSU 1.1 cells cut with BamHI was used as a negative control. The location of the expected 1.9 Kb fragments is indicated. The blot was exposed to X-ray film with two intensifying screens for 3 days.

In all 3 of the plates, the genomic DNA was extracted, and cut with various restriction enzymes before running the gel. The lanes in which the DNA from PMMTVP450IIB1 transfected MSU 1.1 cells was cut with BamHI showed hybridization to a single band of 1.9 Kb which corresponds to the size of the hygromycin resistance gene of the transfected plasmids. This demonstrates that the PMMTVP450IIB1 plasmid has integrated into the genomic DNA of the MSU 1.1 cells. The positive control #1 in Figure 8 showed a strong signal, as expected because it had previously exhibited a signal as seen in Figure 6. When the gel was loaded, some of the contents spilled over into the blank lane and the negative control lane, which explains why those lanes appear to have faint signals.

After proving that the plasmids PMTP450IIB1 and PMMTP450IIB1 had been integrated into the genomic DNA of the MSU 1.1 cells, it was concluded that the first step in the construction of the two cell lines PMTP450IIB1/MSU 1.1 and PMMTVP450IIB1/MSU 1.1 was successful. The next step was to determine if the incorporated plasmids would function to express mRNA and be translated into protein in the newly created cell lines as predicted.

Plasmids. It was predicted that the incorporated plasmids in the MSU 1.1 cell lines could be manipulated to turn "on" or turn "off" cytochrome P-450 activity by the addition of either metal ions to the MT promoter or glucocorticoids to the MMTV

promoter. This cytochrome P-450 activity in turn would then serve as the basis for expression of enzymes responsible for metabolic detoxification of foreign chemicals in the MSU 1.1 cells. It was thought that if known mutagenic compounds, such as aflatoxin B_1 or benzo[a]pyrene (which require metabolic activation), were added to this cell system, the expressed cytochrome P-450 isozymes would metabolically activate the compounds. The effects of such metabolic activation of these compounds would result in mutations in the DNA, and could be seen as foci formation (Yang et al., 1992). If the transfected plasmids were to function as anticipated, the construction of the plasmid is of key importance.

The plasmid PMMTVP450IIB1, as depicted in Figure 4, contains the following key components: (1) A gene which encodes for hygromycin resistance that allows for the selection of cells transfected with the plasmid, as described above for Figure 6. (2) A 2 Kb region which is encoded for cytochrome P-450IIB1 activity. (3) The mouse mammary tumor virus promoter (MMTV), which is able to regulate expression by administration of glucocorticoids. A certain region of this promoter contains a specific enhancer DNA sequence which responds to glucocorticoids. This DNA sequence, known as a glucocorticoid response element (GRE) allows the promoter to respond *in vivo* to glucocorticoids. GRE function is similar to enhancer elements which either increase or decrease transcriptional activity of promoters (Eisen, 1986).

Therefore the cDNA insert is able to be induced to express, in response to glucocorticoids. Thus the glucocorticoid, dexamethasone which was added to the media should have turned "on" expression of cytochrome P-450IIB1 activity in the transfected MSU 1.1 cells.

The other plasmid, PMTP450IIB1 is identical to PMMTVP450IIB1 as previously described, except it has a metallothionein (MT) promoter instead of the MMTV promoter. The PMTP450IIB1 plasmid makes it possible to inducibly regulate expression of the cytochrome P-450 cDNA in the MSU 1.1 cells by addition of metal ions to the tissue culture media. Thus $ZnSO_4$, which was added to the media, should have caused the promoter to turn "on" expression of the cytochrome P-450 activity in the PMTP450IIB1 transfected cells.

Northern Analysis. To induce or "turn on" the expression of cytochrome P-450 system, means creating cytochrome P-450 enzymes, which are proteins. To create new proteins, many processes are involved. Ribonucleic Acid (RNA) plays a key role in this process because it is involved in making specific amino acids which combine to make proteins that become the cytochrome P-450 enzymes. Therefore, to investigate whether the transfected plasmids are functioning as predicted and creating cytochrome P-450 enzymes, RNA can be examined, in a process called Northern analysis (Alwine et al., 1977).

Figure 9 represents Northern Analysis of RNA isolated from PMTP450IIB1 or PMMTVP450IIB1 transfected MSU 1.1 cells after induction with appropriate inducers (see methods section) and probed with the 2 Kb cytochrome P450IIB1 containing fragment from the PMMTVP450IIB1 plasmid. No signal can be seen in any of the lanes of the RNA from induced, transfected MSU 1.1 cells. The positive control of the Aroclor induced rat liver RNA exhibits an intense signal, which was expected because rat liver is known to express large amounts of cytochrome P-450IIB1.

The lack of signal in lanes with the RNA from induced, transfected cells suggests that cytochrome P-450IIB1 mRNA is not being properly expressed from the transfected cDNA. This could be due to a number of possibilities. First of all, the inducers could have been added to the cells at improper concentrations for inadequate time periods. A second possibility is that RNA is being degraded or broken down. Some RNA degradation could be seen in the photographs of gels loaded with RNA from induced, transfected MSU 1.1 cell lines (data not shown). A third possibility, is that the small amount of signal on the blot can not be detected by Northern Analysis. In similar experiments, researchers in the Carcinogenesis Laboratory at the MSU campus, have had to use other methods such as Polymerase Chain Reaction (PCR) in order to magnify signals from the RNA obtained from the MSU 1.1 cells (Wang, 1993). A final possibility as to why no signal

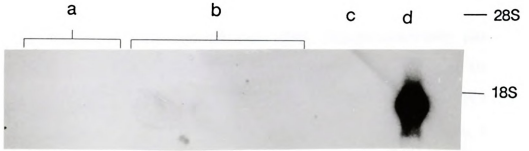


Figure 9. Northern analysis of RNA from induced PMTP450IIB1 transfected MSU 1.1 cells, and induced PMMTVP450IIB1 transfected MSU 1.1 cells. The blot was hybridized with the ^{32}P random prime labeled 2 Kb cytochrome P450IIB1 fragment from the PMMTVP450IIB1 plasmid. The blot was exposed to X-ray film with two intensifying screens for 2 days. The 28s and 18s bands are indicated. Five μg total RNA was loaded into each lane. Lanes: a) $ZnCl_2$ and $ZnSo_4$ induced PMTP450IIB1 cells; b) dexamethasone induced PMMTVP450IIB1 cells; c) MSU 1.1 cells; d) positive control: RNA from hepatocytes of Aroclor-1254 treated rat livers.

is seen from the induced, transfected MSU 1.1 cells in Figure 9 is that the technique used to transfer RNA from the gel to the blot was ineffective. So perhaps there is no RNA on the blot. After ruling out a number of other possible errors, we conducted experiments to determine the quality and amount of RNA transferred onto the blot.

Figure 10 shows Northern Blot Analysis of RNA from induced PMTP450IIB1 and PMMTVP450IIB1 transfected MSU 1.1 cells. The blot was hybridized with glyceraldehyde phosphate dehydrogenase (GAPDH), which is an enzyme that is constitutively expressed in many mammalian cells. In Lane 2, no signal was detected because the RNA was degraded, as seen from the ethidium bromide staining on the agarose gel (data not shown). The remaining lanes of RNA from PMTP450IIB1 and PMMTVP450IIB1 transfected cells show an consistent signal in all lanes, which indicates the following: 1) These cell lines express GAPDH. 2) The lanes were evenly loaded because the intense bands are of uniform size. 3) The RNA was in fact on the blot, which proves our technique for transferring RNA from gel to blot is highly accurate.

Overall, the results shown in Figures 5-8 show that the PMTP450IIB1 and PMMTVP450IIB1 plasmids have been integrated into the genomic DNA of the MSU 1.1 cells. Figures 9 & 10 give the indication that cytochrome P-450IIB1 is not being expressed at the message level through RNA. Therefore it was

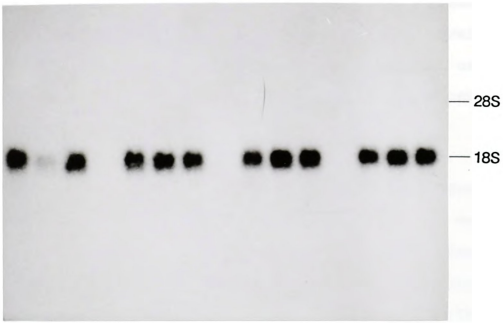


Figure 10. Northern analysis of RNA from induced PMTP450IIB1 transfected MSU 1.1 cells and 3 plates of induced PMMTVP450IIB1 transfected MSU 1.1 cells. Five μg total RNA was loaded into each lane. The blot was hybridized to a ^{32}P random prime labeled glyceraldehyde phosphate dehydrogenase (GAPDH) probe. The 28s and 18s bands are indicated. The blot was exposed to X-ray film with two intensifying screens for 2 days.

decided instead of using RNA as an indicator of cytochrome P-450IIB1 expression, that the proteins would be investigated.

Fluorometric Assay. A fluorometric assay was used to detect levels of cytochrome P-450 IIB1 protein in the induced transfected MSU 1.1 cells (described in the method section). The rational for this assay is that in the presence of metabolically active cells, (such as properly induced transfected cells expressing cytochrome P-450 IIB1), the added substrate 7-benzyloxyresorufin would be cleaved, forming resorufin, a fluorescent compound that can be quantified on a fluorometer. Thus the greater amount of fluorescence produced, the more cytochrome P-450IIB1 present.

The results obtained from this assay were so insignificant that they are not shown. After the induced PMTP450IIB1/ MSU 1.1 and PMMTVP450IIB1/MSU 1.1 cells were mixed with the substrate in the reaction tube, only minute amounts of light were detected on the fluorometer. The light emitted by the induced, transfected cells was the same as that of the untransfected MSU 1.1 negative control cells. The amount of light emitted by all the samples was almost non-existent, and not distinguished over that of background light. Therefore we concluded that our cell lines were not producing significant amounts of the active cytochrome P450IIB1 enzyme.

Recommendations. If this project is going to be pursued, it would be advisable to verify that the plasmid DNA had been integrated in the proper orientation into the genomic DNA of the MSU 1.1 cells. This information could be used in determining if the inducible promoters on the plasmids are functioning properly.

If the fluorometric assay is performed again, a greater number of cells should be used. Perhaps three to five 100 mm plates of cells per sample would provide enough active cytochrome protein for the reaction. Also it needs to be determined whether breaking open cell walls by sonication, or homogenization is beneficial in the detection of cytochrome enzymes. The fluorometric assay sensitivity needs to be assessed to determine if it can detect small amounts of the fluorescent compound resorufin, produced in the reaction.

SUMMARY AND CONCLUSIONS

The purpose of this project was developing a human cell line which could be used for detection of mutagenic compounds in foods. There is extensive literature documentation regarding the existence of hundreds of cytochrome P-450 isozymes, which act as a metabolic detoxification system by breaking down xenobiotics compounds that enter the human body. Thus, we attempted to develop a human cell line which could mimic the detoxification processes of cytochrome P450s in the human body.

The human fibroblast MSU 1.1 cell line used in this project does not express cytochrome P450 to any great extent, therefore, we introduced cytochrome P450IIB1 into the cells by using plasmid DNA. Two plasmids, PMTP450IIB1 and PMMTVP450IIB1 which encoded for inducible promoters, as well as cytochrome P450IIB1 activity were separately transfected into the MSU 1.1 cells. Slot blot analysis showed that the transfection was successful because adequate amounts of the plasmid DNA had entered into the cells. Furthermore, Southern analysis indicated that the transfected plasmid DNA had actually integrated into the genomic DNA of the MSU 1.1 cells.

The next stage of this project involved the addition of specific inducers to turn on the enzymatic machinery to produce cytochrome P450IIB1 enzymes. If the system was working properly, the cytochrome P450 activity should have been detected at the RNA level by Northern Analysis. The blot from our Northern Analysis did not show any signal, which led us to believe that the cytochrome P450IIB1 was not being properly expressed, or expressed in such small quantities that it was undetectable by Northern Analysis.

Since cytochrome P450IIB1 expression was not detectable at the RNA level, we attempted to show production of the cytochrome P450IIB1 at the protein level. This was done by combining induced cells with the substrate 7-benzyloxyresorufin. If the cells were metabolically active (expressing cytochrome P-450IIB1), the substrate would be cleaved, forming resorufin, a fluorescent compound which can be estimated on a fluorometer. The results we obtained from this assay were insignificant because the fluorometer was unable to detect fluorescence from either the induced or non-induced cell lines when mixed with the substrate.

Thus we concluded that the cytochrome P-450IIB1 was not being properly produced at the protein level either. Further work still needs to be done to figure out why cytochrome P-450IIA1 is not being expressed at the RNA level, and subsequently is not produced at the protein, or enzymatic level in our cell lines. Perhaps this may be accomplished by

determining if the inducible promoter is working properly, or by re-evaluating the sensitivity of our methods of detection of cytochromes.

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