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Induction of aryl hydrocarbon (benzo[a]pyrene) hydroxylase in two human hepatoma cell lines.

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LA THÈSE A ÉTÉ MICROFILMÉE TELLE QUE NOUS L'AVONS REÇUE
INDUCTION OF ARYL HYDROCARBON (Benzo[a]pyrene) HYDROXYLASE IN TWO HUMAN HEPATOMA CELL LINES

by

Yu Xiao Fei

A Thesis
Submitted to the Faculty of Graduate Studies
through the Department of Biological Sciences
in Partial Fulfillment of the Requirements for the Degree of

Master of Science

Windsor, Ontario, Canada
1986
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To my parents
THE INDUCTION OF ARYL HYDROCARBON (BENZO[a]PYRENE)
HYDROXYLASE IN TWO HUMAN HEPATOMA CELL LINES

by

Yu Xiao Fei

ABSTRACT

Two human, malignant hepatoma cell lines, Hep3B and HepG2, were studied with respect to the induction of aryl hydrocarbon hydroxylase (AHH) by polycyclic aromatic hydrocarbons. The mouse hepatoma cell line, Hepa cl-9, was used as a control. Using conditions established to produce maximum AHH activity in Hepa cl-9, the human cells were found to be inducible for 2,3,7,8-tetrachlorodibenz-
-p-dioxin (TCDD) but at levels 5 to 10-fold lower than Hepa cl-9. However, other inducers of AHH activity in Hepa cl-9 did not induce AHH activity in the human cells. To increase the level of induced AHH activity in human cells, several variables in the assay were modified including: a) inducer concentration, b) induction time, c) sample protein concentration, and d) reaction substrate concentration. Relative to Hepa cl-9, Hep3B and HepG2 required: a) 10-fold greater concentrations of inducer (e.g. 10 nM TCDD versus 1 nM TCDD), b) a 1.7-fold longer generation time (18 h versus 30 h), and c) higher concentrations of cellular protein in the AHH reaction mix. These changes resulted in 1 to 2-fold
higher levels of TCDD-induced AHH activity (units/mg protein) in Hep3B and HepG2. Levels of activity for other inducers was also higher than those obtained using murine assay conditions. Expressed in units/million cells the relative levels of TCDD-induced activity in Hep3B, HepG2 and Hepa cl-9 were: 1.0 : 1.0 : 1.24 respectively).

Phenotypic variation in levels of TCDD-induced AHH activity was examined in 13 first generation and 26 second generation clones of Hep3B and HepG2. A high degree of spontaneous variation was observed with five of the seven Hep3B clones and four of the six HepG2 clones demonstrating significantly lower or higher levels of TCDD-induced AHH activity relative to the parent cell lines. Significant variation was also observed when, for each Hep3B and HepG2, two clones having highest and lowest AHH activities were subcloned. In these second generation clones, seventeen of the twenty-six Hep3B and HepG2 subclones had either significantly higher or lower levels of TCDD-induced AHH activity relative to their respective first-generation parents. Although variation was observed, the mean activity of the clones was similar to the average activity of the parent regardless of the level of activity (i.e. high versus low) expressed by the parent cell line.

The high levels of variant clones and subclones in Hep3B and hepG2 indicate that variation is an inherited trait in these human cells. However, this variation occurs at frequencies $10^4$-$10^5$ higher than those expected for spontaneous mutation.
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<tbody>
<tr>
<td>Ah</td>
<td>Aromatic Hydrocarbon</td>
</tr>
<tr>
<td>AHH</td>
<td>Aryl Hydrocarbon Hydroxylase</td>
</tr>
<tr>
<td>BA</td>
<td>Benzantracene</td>
</tr>
<tr>
<td>BP</td>
<td>Benzo(a)pyrene</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DBA</td>
<td>1,2,3,4-Dibenzanthracene</td>
</tr>
<tr>
<td>GPO₄</td>
<td>0.25M potassium phosphate/30% glycerol</td>
</tr>
<tr>
<td>MCA</td>
<td>3-Methylcholanthracene</td>
</tr>
<tr>
<td>MEM</td>
<td>Alpha Minimal Essential Medium—not enriched</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic Aromatic Hydrocarbon</td>
</tr>
<tr>
<td>PB</td>
<td>Phenobarbital</td>
</tr>
<tr>
<td>TCDD</td>
<td>2,3,7,8-Tetrachlorodibenzo-p-dioxin</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>V</td>
<td>Variance</td>
</tr>
<tr>
<td>M</td>
<td>Mean of the Population</td>
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CHAPTER I

INTRODUCTION

Experimental studies have confirmed that polycyclic aromatic hydrocarbons (PAH) cause cancer in animals (reviewed in Okey, A.B. and Nebert, D.W., 1982). It has been further suggested that PAH also cause cancer in humans (Kellermann, G., et al., 1973; Kouri, R.E., et al., 1982). Current estimates indicate that large amounts of PAH such as benzo(a)pyrene and dioxin, enter the environment via combustion products and industrial wastes. Concern over the potential health problems resulting from environmental pollutants has led to intense efforts during the past fifteen years to define the mechanism(s) of PAH metabolism and its association with chemical carcinogenesis in humans.

The microsomal (cytochrome P-450) mixed function oxygenases (M.F.O.) together with other enzymes provide a detoxification mechanism by which cells can convert a wide variety of hydrophobic foreign compounds to more polar derivatives facilitating their elimination from the organism (Ishimura, Y., et al., 1976; Sato, R. and Omura, T., 1978; Estabrook, R.W. and Werringloer, J., 1979). The activity of some mixed function oxygenases is inducible by substrates such as: 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 3-methylcholanthrene (MCA), benzantracene (BA), dibenzanthracene (DBA) and phenobarbital (PB) — (Figure 1). These are gen-
Figure 1. Formulae of Some Inducers of Cytochrome P-450. Of these the 3-methylcholanthrene-type induce cytochrome P<sub>1</sub>-450 whereas the phenobarbital-type induces cytochrome P-450s other than cytochrome P<sub>1</sub>-450.
erally classified as MCA-type inducers and PB-type inducers.

Cytochrome P-450 is a general term representing a family of proteins. Cytochrome P₁-450, one form of P-450, is associated with aryl hydrocarbon hydroxylase activity (AHH) and is believed to be involved in carcinogenesis by altering the ratio of cytochrome P₁-450 to cytochrome P-450. Phenobarbital-type inducers induce P-450 other than P₁-450 and enable the cell to increase its rate of detoxification of hydrophobic compounds (Conney, A.H., 1967). However, MCA-type inducers induce an "activation" mechanism. The products of metabolic activation can be more reactive than the parent compound (i.e. more electrophilic) and thus bind to macromolecules such as DNA. These interactions are associated with the chemical's potential to cause drug toxicity, neoplasia and developmental malformations.

Because of the association of the P₁-450-related, AHH activity with cellular carcinogenesis (Kouri, et al., 1980), it has been suggested that the level of AHH activity could be used to reliably predict an individual's risk for developing PAH-induced cancers. However, the potential of this measurement has been restricted because of large variations; further methodological improvement for application of the assay is still required (Kouri, R.E. et al., 1982).
A. Polycyclic Aromatic Hydrocarbons In The Environment

The environment is polluted with thousands of chemicals and the number of newly synthesized chemicals increases each year. Evidence now suggests that a number of the chemicals that we are presently exposed to, either directly or indirectly, are mutagenic, carcinogenic and/or toxic (Gelboin, H.V. and Ts'O, P.O., 1981). Among these pollutants, the polycyclic aromatic hydrocarbons have been recognized as carcinogenic agents. There are at least 200 different PAH that can be identified in the air, soil, and water systems, as well as in our food products (Zedec, M.S., 1980; Tschirley, F.H., 1986). For example, PAH such as BP are found in cigarette smoke, broiled fish and charcoal-broiled beef (Conney, A.H., 1982). In 1977, investigators in the Netherlands reported that polychlorinated dibenzo-p-dioxins (PCDD) were present in fly ash from a municipal incinerator (Tschirley, F.H., 1986). An abundance of these PAH enter the environment each year: for example, as much as 1000 tons of BP has been reported to enter the environment each year (Gelboin, H.V. and Ts'O, P.O., 1981).

The dioxins represent another class of toxic PAH compounds found in the environment and are formed as by-products of the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). The general awareness that TCDD is a potential hazard to health
and the environment arose in 1970 (reviewed in Tscharley, F.H., 1986). Since then there has been a steady accumulation of information about the sources of TCDD, its environmental fate and its toxic effects. Moreover, TCDD has been specifically identified in soil and dust from numerous locations including soot from the chimneys of wood furnaces and residues in river fishes (Sun, M., 1983). Studies with rodents indicate that TCDD, and PAH in general, enter the animal's vascular system and concentrate initially in the liver (Zedeck, M.S., 1980). Subsequently, the PAH, or its metabolic product, becomes involved in the initiation step of carcinogenesis or in affecting the immune system or producing mutagenic and teratogenic effects.

B. The Metabolism of Polycyclic Aromatic Hydrocarbons

The mixed function oxygenases (M.F.O.) are a family of enzymes that metabolize both exogenous (eg. drugs, pesticides) and endogenous (eg. fatty acids, hormones) substrates (Lu, A.Y., et al., 1970, 1972). It contains three functional components: a) a phospholipid fraction; b) a flavoprotein called NADPH cytochrome c(P-450) reductase; and c) a heme protein called cytochrome P-450. This enzyme system catalyzes the insertion of one atom of molecular oxygen into the substrate to produce epoxides, or arene oxides (Nebert, D.W. and Thorgeirsson, S.)
1977). Depending on which form of cytochrome-P-450 is involved, the metabolites can be either more toxic to the cells, more excretable from the cells, or both (Gelboin, H.V. and Ts'o, P.O., 1981). Cytochrome P<sub>450</sub> is a form of cytochrome P-450 associated with the formation of toxic intermediates which are highly electrophilic and capable of covalent binding to nucleic acids and proteins.

Aryl hydrocarbon hydroxylase activity—taken to be a measure of cytochrome P<sub>450</sub>—is induced by the MCA-type PAH. According to the general model, in the metabolism of these PAH, the PAH first undergoes epoxide formation at the K-region or non K-regions (Fig. 2). Addition of oxygen at the K-region is catalyzed by cytochrome P-450 associated monoxygenases whereas addition of oxygen at the non K-regions is catalyzed by cytochrome P<sub>450</sub> monoxygenase(s). Subsequently, the epoxides may convert to phenols by nonenzymatic rearrangement of the epoxides. Phenols can be conjugated with glutathione by soluble transferases to produce water-soluble, excretable products, or further metabolized by monoxygenases to produce diol-epoxides. The epoxides can be K-region and non K-region epoxides, however the glutathione conjugates that have been identified as PAH metabolites have been primarily non K-region epoxides. Epoxides can also be converted to dihydrodiols which result from the hydration of
Figure 2. Pathway for the Metabolism of an Aromatic Ring in a Polycyclic Hydrocarbon. An oxygen is inserted in the molecule by monoxygenases to produce epoxides. The epoxides can convert into phenols through non-enzymatic rearrangement, then phenols or epoxides can be conjugated with glutathione to be excreted from the body, or the epoxide can be converted to a dihydrodiol by epoxide hydase to become a diol-epoxide. The latter can then undergo further metabolism. (from Harry et al, 1981).
simple epoxides by epoxide hydrase (Zedeck, M.S., 1980). The
dihydriodiols can be further metabolized by oxidation at sites in
the molecule adjacent to or distant from the diol grouping.

In a given cell, both cytochromes P-450 and P$_1$-450 can
exist at the same time. Thus the ratio of P$_1$-450 to P-450 deter-
mines the extent of metabolic activation in the cell and subse-
sequently the number of toxic intermediates (Nebert, D.W. and Thorgeisson,
S.S., 1977). In the case of benzantracene there are five forms
of dihydrodiol (Fig. 3). Of these, the 5,6-dihydrodiol, the
K-region dihydrodiol, is least present in animals in which
cytochrome P$_1$-450 is predominant in metabolism (Thakker, D.R.,
et al., 1976). All five forms of dihydrodiol are believed to
be mutagenic and carcinogenic (Gelboin, H.V. and Ts'O, P.O.,
1981). However, the further metabolites of these dihydrodiols, the
diol-epoxides, are usually more mutagenic and carcinogenic as in
the case of benzo(a)pyrene metabolism (Nebert, D.W. and Thorgeisson,

C. Carcinogenic Role of Polycyclic Aromatic Hydrocarbons

Studies during the past decade have identified one of the
isomeric metabolites of BP. 7,8-dihydroxy-9-10-epoxy-7,8,9,10-
tetrahydrobenzo(a)pyrene, as the ultimate carcinogenic species
Figure 3. Five Forms of Dihydrodiol of Benzanthracene. Benzanthracene can form five dihydrodiols: the K-region dihydrodiol, 5,6-dihydrodiol, and the four non-K-region dihydrodiols: 3,4-dihydrodiol, 1,2-dihydrodiol, 8,9-dihydrodiol and 10,11-dihydrodiol. All five dihydrodiols of BA are mutagenic and carcinogenic. The four non-K-region dihydrodiols of BA are in greater amounts in animals in which P₄₅₀ is dominantly involved in the metabolism.
to DNA formed in cells in tissue culture show elution profiles on Sephadex 2H-20 columns different from adducts formed with microsomal enzymes (Sims, P. and Grover, P.L., 1974). Phenol metabolite-derived adducts are prominent when microsomes catalyze the reaction, whereas the majority of binding adducts formed in cells are derived from 7,8-dihydroxybenzo(a)pyrene 9,10 oxide. This might be attributable to the elimination of free phenols in cells by glucuronidation. It has been suggested that there is a substantial decrease in the covalent binding of BP to DNA in comparison with the metabolites of BP. This has been taken for evidence that the metabolites of BP are more carcinogenic than BP itself.

D. Model for Induction of Aryl-Hydrocarbon Hydroxylase

The model for induction of AHH activity by PAH has been established using tritiated 2,3,7,8-tetrachlorodibenzo-p-dioxin as the radioligand (Greenlee, W.F. and Poland, A., 1979), since TCDD is a potent inducer of AHH activity and demonstrates specific, high-affinity binding to the Ah receptor. The current model (Fig.4) (reviewed in Greenlee, W.F. and Neal, R.A., 1985) postulates that a PAH interacts with a specific cytosolic receptor and then translocates into the nucleus, in a time-and-temperature dependent step (Okey, A.B., et al., 1980). Following translocation, the
Figure 4. Hypothetical Scheme for the Induction of Cytochrome P₄₅₀ (Aryl Hydrocarbon Hydroxylase Activity). After "activation", the ligand-receptor complex translocates into the nucleus to interact with specific acceptor sites on the chromatin. This results in the induction of a "pleiotypic response" which includes increased levels of Cytochrome P₄₅₀ (Okey, A.B. and Nebert, D.W., 1982).
PAH: receptor complex interacts with the Ah gene complex and stimulates transcription and translation of AHH associated structural genes. There is an excellent correlation between the kinetics of accumulation of the DNA-binding PAH: receptor complex and the appearance of P₁-450 mRNA (Tukey, R.H., et al., 1982). More recent evidence has indicated that increases in mRNA are due to transcriptional activation of the P₁-450 and P₃-450 genes and occur within several hours of treatment with a single dose of inducer (Gonzalez, F.J., et al., 1984). Since both the P₁-450 and P₃-450 genes are located on mouse chromosome 9 (Tukey, R.H., et al., 1984) and exhibit strikingly similar intron-exon patterns (Gonzalez, F.J., et al., 1984) it is postulated that they are regulated by the same receptor (Shioko, K., et al., 1984).

It is known that an animal's ability to respond to PAH in general is an inherited trait determined by its genetic makeup. The Ah gene complex, consisting of regulatory, structural and possibly temporal genes, controls the induction of cytochrome P₁-450 and other forms of P-450. Inbred strains of mice which exhibit elevated AHH activity after exposure to PAH are described as being responsive at the Ah locus. This trait, first distinguished in the prototype C57Bl/6 (B6) mouse, has been designated as the Ahᵇ allele. The nonresponsive trait, designated the Ahᵈ Allele, was initially characterized in DBA/2 (D2) mice. When genetic crosses
were performed between B6 and D2, mice, the
heterozygous (Ah^b/Ah^d) F_1 progeny all demonstrated the
responsive phenotype. Thus, responsiveness in this stain of
mice was expressed as a dominant trait (Nebert, D.W., et al.,
1981; Nebert, D.W. and Bigelow, S.W., 1982). Crosses with
other strains of mice and cells in culture, however, show that
the genetic model describing the AHH induction phenotype was
more complex than a single gene difference. Using the
fluorescence-activated cell sorter, a population of variant
mouse hepatoma cells was isolated. These variants, compared to
the wild-type cells, exhibited increased levels of AHH
activity when TCDD was used as the inducer (Jones, P.B.C., et
al., 1984). Cell fusion of the variant with the wild-type
suggested that the variant phenotype was expressed as a
co-dominant, rather than a dominant trait. Such studies
indicated that the genetic model describing AHH induction
involved several alleles.

E. Significance of Aryl Hydrocarbon Hydroxylase Activity

In mice, a correlation exists between genetically
regulated levels of inducible AHH activity and the risk of
PAH-induced leukemias and solid tumors (Kouri, R.E., et al.,
1980). The higher the inducibility, the greater the
susceptibility. The clinical application of this correlation
is of considerable interest because of the potential
for predicting an individual's risk for developing cancer. Recent clinical studies, using lymphocytes from patient donors, suggest a relationship between high levels of induced AHH activity and carcinomas of the lung and larynx (Kouri, R.E. et al., 1982; Levine, A.S., et al., 1984). However, the application of AHH activity levels for the assessment of an individual's risk has not been successful for two reasons. The first reason is that a positive correlation between AHH activity and the occurrence of cancer of some tissues studied in adult cancer patients is not always observed (Levine, A.S., et al., 1984). It has been suggested that cancer therapy, or cancer itself, might alter induction of AHH activity in mitogen-activated lymphocytes. The second reason is that there is a high degree of variation in measured levels of AHH activity within and between samples taken from the same individual at different times of the year. There has been considerable research into the source of the variation and studies in rat liver cell lines and human lymphocytes suggest the interaction of genetic, epigenetic and technical factors such as sample age, storage conditions, growth medium and time of assay (Forster-Gibson, C.F. and Dufresne, M.J., 1986). Despite these findings, the problem of variation in human samples and the failure to establish assay conditions to minimize these problems, prevents the application of AHH activity as an assessment tool.
This research makes use of two human hepatoma cell lines, Hep 3B and Hep G2, to address these two problems. Specifically:

1. What conditions permit the expression of maximum levels of inducible AHH activity in human cell lines?

and

2. Does clonal variation, observed in murine cell measurements, contribute to variation in levels of AHH activity in human cells?

In these studies, the mouse hepatoma cell line, Hepa cl-9, was used as a control. This cell line is the model cell system for the study of AHH induction (reviewed by Greenlee, W.F. and Neal, R.A. 1985).
CHAPTER II. MATERIALS AND METHODS

A. Cell Lines Used

Two human hepatoma cell lines, Hep3B and HepG2, and the control mouse hepatoma cell line, Hepa cl-9, were used in this study.

Hep3B and HepG2 were generously supplied by Dr. B. Knowles and Dr. D. Aden (Wistar Instute). These two cell lines were established from liver tumor biopsies obtained during extended lobectomies of a 15 year old Caucasian male from Argentina (1975) and an 8 years old black male from USA (1976) respectively. The cell lines established from these biopsies and cultured for several months were designated Hep3B and HepG2, respectively. Their morphological characteristics and epithelial cell shape were compatible with those of liver parenchymated hepatocellular carcinoma with a trabecular pattern. Ultrastructural examination of cells from the Hep3B indicates that the cells resemble previously described human and experimental liver cell carcinomas (Reviewed by David, P. Aden, et al, 1979). Hepa cl-9 is a clone of Hepa-1c1 which was derived from a transplanted hepatoma BW7756 originally produced in the C57 L/J (B6) mouse. This cell line demonstrates stable, high AHH inducibility and is well characterized (Okey and Dufresne, 1983, Dufresne and Dosescu, 1985).

Cell lines were maintained in alpha minimal essential
medium (Stanners, et al., 1971) supplemented with 10% fetal calf serum for both Hep3B and HepG2 and 5% fetal calf serum for Hepa cl-9. The medium also contained 50 μg of gentamicin per ml. Gentamicin is an antimycoplasmal agent.

B. Buffers and Solutions

The Bio-rad solution for protein determination contained 1 part of Bio-rad dye and four part of dH₂O (v/v) and was filtered with whatman #1 paper before use. The solution was made fresh every two weeks. Citrate saline contained 0.015 M trisodium citrate and 0.13 M potassium chloride (pH 7.8). Giemsa was made to 6% (v/v) in distilled water. Glycerol phosphate buffer (GOP₄) contained 0.25 M K₂HPO₄ and KH₂PO₄ in 30% glycerol (v/v) (Gielen and Nebert, 1971). Methylene Blue was made to 7x10⁻² M in 50% methanol. PBS refers to phosphate buffer saline which contains 27 mM KCl, 1.5 mM KH₂PO₄, 0.15 M Na₂PO₄·7H₂O and 0.14 M NaCl. Trypsin was made to 0.05% (w/v) in citrate saline. For Hepa cl-9, the 0.05% trypsin was diluted with citrate saline to 0.025% before use. Standard freezing solution contained 75% alpha medium, 15% fetal calf serum and 10% dimethyl sulfoxide (DMSO). Variations on this basic formula were used to improve cell viability if necessary. The freezing solution was stored at 4°C.

C. Cell Culture Techniques
Cultures were kept at 37°C in an atmosphere of 5% CO₂, 95% air and 100% relative humidity.

Subculturing: Cells were maintained in culture by plating 2-25 cm² flasks per line at approximately 1x10⁵ cells for Hep3B, 1.5x10⁵ for HepG2 and 2x10⁵ for Hepa-c19. For each flask of cells, when the cultures neared confluency, the medium was decanted and the cell surface was washed with warm 0.015 M citrate saline. The cells were treated with 0.025% trypsin for 3 minutes for Hepa c1-9 and 0.05% for 7 minutes for both Hep3B and HepG2, at 37°C. Trypsinized cells were collected into 5 mls of medium and the cell number was determined using a haemocytometer. Cells were then pelleted by centrifugation at 1500 rpm for 6 minutes. The pellet was resuspended to give the appropriate cell concentration, cells then were seeded into 2-25 cm² tissue culture flasks containing 5 mls of medium. When required, cells were also seeded into 100 mm tissue culture dishes and/or 80 cm² tissue culture flasks each containing 10 ml and 15 ml of medium respectively.

D. Biological Characterization of Cells in Culture

1). Efficiency of Plating: Cells were plated in each well of four-well Linbro's at densities of 5x10² cells for Hepa c1-9, and 3x10² and 2x10² cells for HepG2 and Hep3B. On day 5, the medium was decanted from each well, cells were washed with citrate saline and stained with methylene blue.
The number of colonies (i.e., clones consisting of greater than 20 cells) per well was then determined. The efficiency of plating is defined as the number of colonies formed, divided by the number of cells initially seeded, multiplied by 100.

2). Generation Time: The generation time of cells in culture was determined by seeding $10^5$ cells per 60 mm dish. Once plated, the number of cells in each of two dishes was determined every 12 h for 5–7 days. Cells were trypsinized, washed and counted as described under "Cell Culture Technique". The cell counts were plotted on a semi-logarithmic scale against the appropriate time point. The maximum number of cells per dish, defined as the saturation density and expressed as the number of cells per centimeter square can also be determined from this plot.

3). Chromosome Number: Cell cultures in the logarithmic phase of growth were incubated at $37^\circ$C for 2.5 h in the presence of 0.8 ug of colcemid per ml of fresh medium, and resuspended in 0.025 to 0.075 M KCl depending on the cell line at $37^\circ$C. Cells were pelleted, fixed in cold methanol : acetic acid (3:1), and stained with 6% Giemsa. At least 20 metaphase spreads were counted per cell line.

4). Cell Staining: Cell cultures could be stained at different stages of cell culture with 6% Giemsa or $7 \times 10^{-2}$ M Methylene Blue. The old medium was decanted, cells were washed with citrate saline then stained with Methylene Blue. For Giemsa staining, cultures were washed with citrate
saline, then treated with 95% ethanol, dried and finally stained with 6% Giemsa for 15 minutes.

5). Storage of Cells and Cell Pellets: When cell cultures were near confluency, the medium were decanted and the cells were washed, trypsinized and collected by centrifugation then resuspended in solution at a final concentration of $10^6$ cells per ml. Two ml of this cell solution were dispersed into each vial (from Gibco). The frozen cells were stored at −80°C in the Revco and/or −196°C liquid nitrogen. When frozen cells were thawed their viability was determined by efficacy of plating as previously described. Cell pellets for assay of aryl hydrocarbon hydroxylase activity were dried by absorbing the surplus supernatant with tissue paper. The dried pellets were then stored at −80°C. AHH assay results were not affected if the pellets were assayed within two weeks.

Cell Cloning

When cells were in logarithmic growth, their medium was decanted. The cell surface was washed with warm citrate saline and the cells treated with trypsin for 3-7 minutes at 37°C. Trypsinized cells were collected into fresh medium and counted using a haemocytometer. Cells were pelleted by centrifugation at 1500 rpm for six minutes. The pellet was resuspended and a dilution series was set up such that ten ml of each of 100, 10 and 5 cells per ml of growth medium was
obtained. Ninety-six well Linbro plates were then plated with 4 drops per well of the 5 and 10 cells per ml concentrations. Three wells of each Linbro plate were plated with 4 drops of the 100 cells per ml concentration as a growth control. All wells were screened within 24 hours to identify wells containing one cell. These wells were surveyed for colony growth (i.e. clones) over a period of up to three weeks. Individual clones were then trypsinized and transferred into plasticware of increasing area until they could be maintained in 25 cm$^2$ flasks and frozen.

F. Aryl Hydrocarbon Hydroxylase Assay

(Modified from Nebert and Gielen, 1972) Cells were plated at 1.2 X 10$^6$ cells for Hepa cl-9, 1 X 10$^6$ for HepG2, and 0.8 X 10$^6$ for Hep3B, per 100 mm culture dish. After approximately 3 days in culture, when the cells were in logarithmic growth just before confluency, the medium was decanted from each dish. The cells were then washed with citrate saline (0.015 M trisodium citrate, pH 7.8) at 37°C and medium containing the appropriate concentration of inducing agent in solvent (dimethyl sulfoxide or acetone), solvent only, or no solvent or agent was added. Eighteen h later (modified to 30 h for the human cell lines), cells were washed with cold PBS and scraped with a rubber policeman into cold PBS. Cells were washed 3 times in PBS and centrifuged
between each wash at 200 X g for 6 min. The final pellet was either frozen at -80°C or assayed for AHH activity immediately. For the assay, the pellet was suspended in 200 to 500 ul (per plate) of glycerol phosphate buffer. One hundred ul of the cell suspension was added to 900 ul reaction mixture containing 0.1 M MgCl$_2$, 0.36 mM NADPH, 0.42 mM NADH, and 0.7 mg bovine serum albumin per ml in 0.2 M tris buffer (PH 7.5). At 15-second intervals, 50 ul of 2 mM benzo(a)pyrene was added to each sample and each sample was incubated for 20 minutes at 37°C in a shaking water bath. The reaction was stopped by the addition of 3 ml of cold hexane:acetone (3.25:1), followed by another 10 minutes incubation at 37°C. One ml of the organic phase was extracted for 20 seconds with 3 ml of 1 N NaOH (the organic phase is the upper layer). After agitating for 30 seconds, the lower phase was taken and put into a 12 X 75 mm glass test tube to read. Fluorescence was then determined using a Turner Model 430 spectrofluorometer at an excitation wavelength of 396 nm and an emission wavelength of 522 nm. One unit of AHH activity is defined (Gielen and Nebert, 1971) as the amount of enzyme catalyzing per minute, at 37°C, the formation of hydroxylated product causing fluorescence equivalent to that of 1 pmol of 3-hydroxybenzo(a)pyrene recrystallized standard.

Routine standardization was accomplished using quinine sulfate in 0.1 N H$_2$SO$_4$ (Nebert, 1978). There is a direct linear relationship between fluorescence of quinine sulfate in H$_2$SO$_4$ and 3-OH-BP.
The calculation of AHH units from fluorescence units:

AHH Unit (3-OH-BP / min / mg protein / ml) =

Fluorescence Units X 22.5
20 (min) X mg Protein / ml

Protein concentration in mg / ml was determined by the method of Bradford (Bradford, M.M., 1976). Briefly, a standard curve was determined for each experiment by using a series of given concentrations of gammaglobulin protein standard (from Bio-rad) in 5 ml of Bio-rad dye:

<table>
<thead>
<tr>
<th>(x) protein standard(ul)</th>
<th>100</th>
<th>70</th>
<th>50</th>
<th>30</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>0</td>
<td>30</td>
<td>50</td>
<td>70</td>
<td>90</td>
</tr>
</tbody>
</table>

These standards were read against 5 ml dye on a spectrophotometer at a wavelength of 595 nm. The control value (the dye only) was subtracted from each standard to get a series of net O.D. values (Y). These values were plotted against the standard protein concentrations (x) to obtain the standard curve:

\[ Y = mx + b \]
To obtain the protein concentration of the unknown, 100 ul sample was read against 5 ml dye to get an O.D. value. The blank (dye only) was subtracted from the sample to get net sample O.D. value. The final protein concentration of a sample was calculated according to following formula:

\[
\text{protein concentration (mg/ml)} = \frac{(\text{sample reading} - \text{blank}) - b}{m \times 100}
\]

G. Information About Statistics

All the statistical tests and calculations were performed according to the following concepts and formulae (Alder H.L., et al., 1972, and Stephen A.B., 1977).

1). Variance: To find the variance of a distribution, the mean M was first determined, the mean was then subtracted from each value \((X - M)\) to obtain a distribution deviation. These were squared \((X - M)^2\), making the signs positive. The sum of these squared deviations then is divided by \(N\) which is the number of a group of values; \(N - 1\) represents the degree of freedom; \(S\) represents variance:

\[
S = \frac{\sum (X - M)^2}{N - 1}
\]
2). Standard Deviation (SD) and Standard Error (SE):

\[
SD = \sqrt{\frac{\sum (X - M)^2}{N - 1}}
\]

\[
SE = \frac{SD}{\sqrt{N}}
\]

3). Student Test (t test): When the number of observations is less than 30, the distribution shows a "t" distribution other than a normal distribution. According to this, a "t" test was used for the statistical analysis in this thesis to estimate the possibilities of "X" population belonging to "Y" population.

The value of t was determined from the equation:

\[
t = \frac{\bar{X} - \bar{Y}}{\sqrt{\left(\frac{(nx - 1) SD_x^2 + (ny - 1) SD_y^2}{nx - ny - 2}\right)\left(\frac{1}{nx} + \frac{1}{ny}\right)}}
\]

where \(\bar{X} = \frac{X}{nx}\) and \(\bar{Y} = \frac{Y}{ny}\)
CHAPTER III

RESULTS

A. Growth Parameters of Hep3B, HepG2 and Hepa cl-9

Before the two human hepatoma cell lines were assayed for
AHH activity and cloned, they were characterized, together with
the mouse hepatoma cell line, with respect to their growth
parameters. These results are presented in Table 1. The gener-
ation time for HepG2, 16.5 h, is slightly lower but comparable
to that for the control mouse cell line, Hepa cl-9, 18.5 h. The
human Hep3B line, on the other hand grows much more slowly than
either HepG2 or Hepa cl-9 with a generation time of 30 h. With
respect to size, Hep3B cells are also much larger than either
HepG2 or Hepa cl-9 and exhibit the lowest saturation density—
5.56 x 10^4/cm^2 compared to 1.14 x 10^5/cm^2 for HepG2 and 1.56 x 10^5/
cm^2 for Hepa cl-9. All three cell lines demonstrate a relatively
low efficiency of plating when compared to muscle cell lines
or other tissue cell lines in culture. This is partly due to
the concentration-dependent character of hepatoma cells in
general, when seeded at low concentrations (i.e., less than
10^4/ml) as required for E.O.P. determinations. At seed concentra-
tions greater than this, viability was always greater than 80%.
Finally, with respect to average chromosome number, all three

30
Table 1. Growth Parameters

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Hepa cl-9</th>
<th>HepG2</th>
<th>Hep3B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Generation Time(^a) (hour)</td>
<td>18.5</td>
<td>16.5</td>
<td>30.0</td>
</tr>
<tr>
<td>Saturation Density(^b) ((x 10^5 \text{cells/cm}^2))</td>
<td>1.34</td>
<td>1.41</td>
<td>0.57</td>
</tr>
<tr>
<td>Plating Efficiency(^c) (percent)</td>
<td>26</td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td>Chromosome Number(^d) (average)</td>
<td>57</td>
<td>52</td>
<td>51</td>
</tr>
</tbody>
</table>

\(^a\) Generation time defined as the time it takes for an entire culture to double in number while in exponential growth.
\(^b\) Saturation density defined as the maximum number of cells/cm\(^2\) to which a given cell culture is capable of packing.
\(^c\) Plating efficiency defined as the number of cells that grow into colonies as compared to the number of cells initially seeded.
\(^d\) The number presented represents mean chromosome number where \(N = 20\).
cell line demonstrated aneuploidy with a stable modal chromosome number greater than the diploid number in either human (46) or mouse (42) primary cells.

B. Induction of AHH Activity Using Murine Conditions

In mouse or rat tissue and cell culture systems, TCDD is one of the most potent inducers of AHH activity reported in the literature (reviewed in Okey, A.B. and Neber, D.W., 1982). The results for the two human hepatoma cell lines presented in Table 2 are consistent with this observation. When Hep3B and HepG2 were assayed for AHH activity using inducer concentrations and induction times known to generate maximum levels of AHH activity in Hepa cl-9 (Dufresne, M.J. and Dosescu, J., 1985), the level of induced activity, in units/mg protein, in both human cell lines was consistently lower than that found in Hepa cl-9. The relative potency of the inducers, however, was similar in all cell lines with TCDD inducing the maximum induction response. Moreover, while the absolute values of AHH activity varied from experiment to experiment, the relative values remained the same. In terms of units/mg protein, activity was always greatest in Hepa cl-9 and least in Hep3B with these assay conditions (i.e. treatment of cells with indicated concentration of inducer for 18 h, followed by collection of cells, and assay).
Table 2. AHH Activity Of Hepa cl-9, Hep3B And HepG2
Under The Optimum Induction Conditions For Hepa-cl9

<table>
<thead>
<tr>
<th>CELL LINES</th>
<th>AHH Activitya (units/mg proteinb) (N = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 uM BAc</td>
</tr>
<tr>
<td>Hepa cl-9</td>
<td>13.6 ± 2.1</td>
</tr>
<tr>
<td>Hep3B</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>HepG2</td>
<td>1.3 ± 0.3</td>
</tr>
</tbody>
</table>

a Cells in logarithmic growth were incubated at 37°C in medium containing one of the inducers listed for 18 h and assayed for AHH activity. Induced specific activity was calculated by subtracting the activity for solvent treated cells from that for cells treated with solvent and inducing agent. One unit of AHH activity is defined as that amount of enzyme catalyzing, per minute at 37°C, the formation of hydroxylated products causing fluorescence equivalent to that of 1 pmol of 3-hydroxybenzo[a]pyrene recrystallized standard.  
b as described in "Materials and Methods"  
c BA = Benzantracene; BP = benzo[a]pyrene; MCA = 3-methylcholanthrene; TCDD = 2,3,7,8 tetrachlorodibenzo-p-dioxin.  
c Mean ± Standard Deviation
C. Establishment of AHH Assay Conditions for Human Cells

According to the results presented in Table 2, Hepa cl-9 is more inducible than either human cell line. However, since this assay was performed under conditions known to induce maximum AHH activity in murine cells (Nebert, D.W. and Gielen, J.E., 1972), the results might reflect a difference in assay conditions between human and murine assay conditions. To test this, several important factors in the general procedure were varied and the result on induced AHH activity measured; these included: inducer type and concentration, induction time, substrate concentration and sample protein concentration.

1) Effect of TCDD and MCA Concentration on AHH Activity

The effect of increasing concentrations of TCDD (0.01-100 nM) and MCA (0.01-100 uM) on levels of AHH activity in the human cells is presented in Figures 5 and 6 respectively. First, it can be seen that the concentration of either inducer required to induce maximum levels of AHH activity in the human cells is at least 10 fold greater than that required for the mouse Hepa cl-9 cell line. At concentrations lower than or greater than these optima AHH activity is lower. Moreover, regardless of the concentration of inducer tested, the relative order of induction remained Hepa cl-9 (results not shown) greater than HepG2 which, in turn,
Figure 5. Effect of TCDD Concentration on the Expression of AHH Activity in Hep3B (-●-) and HepG2 (-▲-).
The optimum TCDD concentration for the control mouse cell-line, Hepa cl-9, is indicated by a vertical arrow. Cells were treated with medium containing the appropriate concentration of TCDD for 18 h at 37°C then assayed as described in "Materials and Methods".
Figure 6. Effect of MCA Concentration on Expression of AHH Activity in Hep3B (○) and HepG2 (△). The optimum MCA concentration for the control mouse cell-line, Hepa cl-9, is indicated by a vertical arrow. Cells were treated with medium containing the appropriate concentration of MCA for 18 h at 37°C then assayed as described in "Materials and Methods".
is greater than Hep3B.

2) Effect of Induction time on Levels of TCDD-Induced AHH Activity

Since the human cell line Hep3B demonstrated a greater generation time than Hepa cl-9, the possibility that it required a longer exposure to inducer was examined. The effect of increasing induction periods on the expression of AHH activity induced with TCDD, in Hep3B and HepG2 is presented in Figure 7. These results demonstrate that induction time is critical in the expression of maximum levels of AH activity in Hep3B. However, an increased induction time is also required for HepG2 which has a shorter generation time than Hepa cl-9. Both Hep3B and HepG2 have optimal induction periods of 30 hours compared to the 16 hours routinely obtained for Hepa cl-9. It is interesting to note that at 18 hours, levels of AH activity in the human cells, particularly HepG2, is less than \( \frac{1}{3} \) that at the optimum times. Once again, regardless of the induction time, Hepa cl-9 demonstrated the highest level of induced AH activity, in units/mg protein, followed by HepG2 and finally Hep3B.

3) Effect of Various Inducers on Levels of AH Activity After 30 Hours of Exposure to the Inducer

Having established the requirement for a longer induction
Figure 7. Effect of Induction Period on Expression of AH activity in Hep3B (●) and HepG2 (▲). The optimum induction period for the control mouse cell-line, Hepa cl-9, is indicated by a vertical arrow. Cells were treated with medium containing 1 nM TCDD for various times at 37°C and assayed as described in "Materials and Methods".
time to induce maximum levels of AHH activity in Hep3B and HepG2, the effect of inducers of cytochrome P<sub>1</sub>-450, TCDD, MCA, BA, DBA and BP, and one inducer of cytochrome P-450 other than P<sub>1</sub>-450, PB was examined (Table 3). To minimize variation, the cells were assayed at the same time and under the same conditions. It is generally accepted that a cell is considered to be induced if the level of induced activity is at least 3-fold that obtained with solvent alone (Nebert, D.W. and Gieken, J.E., 1972). The greater the fold-induction, the greater the induction potential. In these studies, as with all AHH measurements in this research thesis, the levels of basal activity were consistently less than 0.5 unit/mg protein. This level of basal activity was invariant regardless of the assay condition and was therefore not directly included in the results. However, the activity levels presented were obtained by subtracting the basal levels of activity from the induced levels. Therefore the units presented reflect approximately one-half the actual fold-induction. In view of this, it can be seen that TCDD and MCA induce AHH activity, in the same relative order with respect to potency, in both Hep3B (13-fold and 4.5-fold respectively) and HepG2 (26-fold and 8-fold respectively). In HepG2, AHH activity is also induced by BA (5-fold). According to the 3-fold definition of inducibility, neither DBA nor BP induce AHH activity at the concentrations tested. As previously observed, levels of activity in units/mg protein,
Table 3. AH activity of Hep3B and HepG2 induced with different inducers

<table>
<thead>
<tr>
<th>CELL LINE INDUCER</th>
<th>AHH ACTIVITY&lt;sup&gt;b&lt;/sup&gt; (units/mg prot.)</th>
<th>RELATIVE AHH&lt;sup&gt;c&lt;/sup&gt; ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCDD</td>
<td>10.61 ± 1.70&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.00</td>
</tr>
<tr>
<td>MCA</td>
<td>5.25 ± 0.81</td>
<td>0.49</td>
</tr>
<tr>
<td>Hep3B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBA</td>
<td>3.13 ± 0.97</td>
<td>0.29</td>
</tr>
<tr>
<td>BA</td>
<td>2.73 ± 0.84</td>
<td>0.26</td>
</tr>
<tr>
<td>BP</td>
<td>1.73 ± 0.57</td>
<td>0.16</td>
</tr>
<tr>
<td>PB</td>
<td>0.50 ± 0.07</td>
<td>0.06</td>
</tr>
<tr>
<td>TCDD</td>
<td>24.20 ± 4.34</td>
<td>1.00</td>
</tr>
<tr>
<td>MCA</td>
<td>7.00 ± 1.36</td>
<td>0.29</td>
</tr>
<tr>
<td>HepG2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBA</td>
<td>3.26 ± 0.98</td>
<td>0.13</td>
</tr>
<tr>
<td>BA</td>
<td>1.15 ± 0.41</td>
<td>0.05</td>
</tr>
<tr>
<td>BP</td>
<td>2.24 ± 0.78</td>
<td>0.09</td>
</tr>
<tr>
<td>PB</td>
<td>0.85 ± 0.07</td>
<td>0.03</td>
</tr>
</tbody>
</table>

<sup>a</sup> TCDD = 2,3,7,8-Tetrachlorodibenzo-p-dioxin, MCA = 3-Methylcholanthrene, BA = Benzantracene, DBA = 1,2,3,4-Dibenzanthracene, BP = Benzo[a]pyrene, PB = Phenobarbital.

<sup>b</sup> Cells in logarithmic growth were exposed to 10 nM TCDD and 10.0 µM of the other agents listed for 30 hours and AH activity was assayed as described under "Materials and Methods". The number of measurements was duplicate. The values given for specific AH activity were calculated by subtracting the activity for solvent treated cells from the activity of cells treated with solvent and one of the inducing agents.

<sup>c</sup> Relative AH activity obtained by comparing the AH activity induced by TCDD.

<sup>d</sup> The Standard Deviation (N = 4). (Mean ± S.D.)
are greater in HepG2 than Hep3B regardless of the inducer. Phenobarbital, representative of the class of inducers which do not induce cytochrome P<sub>450</sub>, did not induce AHH activity in either Hep3B or HepG2. This result is consistent with those previously reported for Hepa cl-9 (Dufresne, M.J. and Dosescu, J., 1985).

A comparison of results obtained for the human cells with those for Hepa cl-9 suggests that the characteristics of AHH induction with other inducers are similar in murine and human systems. However, the apparently greater inducibility of Hepa cl-9 suggests that the mouse cell line is more sensitive to all the inducers studies and may have higher levels of cytochrome P<sub>450</sub>-450 than Hep3B and HepG2. Data from Oliver Hanksinson’s laboratory (personal communication) suggest that P<sub>450</sub>-450 is the dominant P-450 in BP-induced Hepa lcl, the progenitor of Hepa cl-9. Lower levels of cytochrome P<sub>450</sub>-450 in the human cell lines would explain, at least in part, the requirement for at least 10-fold higher concentrations of inducers in these cells.

4) Effect of Sample Protein Concentration on Levels of TCDD-Induced AHH Activity

Many factors are known to influence the AHH assay
Figure 8. Effect of Sample Protein Concentrations on the Expression of AHH Activity in Hep3B (−▲−) and HepG2 (−●−). The optimum protein concentration for the control mouse cell-line, Hepa cl-9, is indicated by a vertical arrow. Cells were treated with medium containing 10 nM TCDD for 30 h at 37°C and assayed for AHH activity, with different sample protein concentrations, as described in "Materials and Methods".
as discussed in Chapter I. One important factor is the amount of sample protein in the reaction mix since this is the source of "enzyme". This is particularly important in this study since Hep3B is morphologically larger than HepG2 and Hepa cl-9 and also contains more total protein per cell. (This is discussed in Chapter IV, Table 9: from these data Hep3B contains approximately 187 pg protein; HepG2 contains 110 pg protein and Hepa cl-9 contains 58 pg protein, per cell.) Thus an equivalent amount of protein/ml does not necessarily reflect an equivalent amount of AHH associated protein per cell. In other words, more protein may be required for the human cell lines. Figure 8 shows the effect of protein concentration on levels of TCDD-induced AHH activity in Hep3B and HepG2. From the results it would appear that higher protein concentrations are required to induce maximum AHH levels in the human lines compared to Hepa cl-9. It is important to note that in previous assays, the level of protein for Hep3B has usually been lower than either HepG2 or Hepa cl-9. This is because the larger size and lower saturation density of Hep3B makes it difficult to obtain high protein yields. In view of this, it is possible that the 2-3.5 fold greater inducibility of HepG2 relative to Hep3B, reflects a technical rather than a biological difference. This possibility is supported and discussed in Chapter IV.
5) Effect of Benzo(a)pyrene Concentration in Reaction Mix

One unit of AHH activity is defined as the amount of enzyme catalyzing in one minute, at 37°C, the formation of product causing fluorescence equivalent to 1 pmole of 3-OH-BP. Since BP, the substrate is critical to the detection of activity, the amount of substrate used in the assay was examined. These results are presented in Figure 9 and demonstrate that the concentration of BP, 0.1 mM, routinely used is sufficient in the human cell lines. Maximum levels of induced AHH activity were routinely observed between 0.07 and 0.11 mM BP for all three cell lines.

D. The Expression of Induced AHH Activity in First Generation Clones of the Hep3B and HepG2 Cell Lines

1) Expression of TCDD-Induced AHH Activity

Having examined the expression of AHH activity in Hep3B and HepG2, these two "parent" cell lines were cloned as described in Chapter II. After daily examination of the over 500 clone wells for several weeks, seven clones of Hep3B and six clones of HepG2 were randomly selected and maintained as first generation clones. Hep cl-9 was again used as a control since clonal variation had been examined in this mouse hepatoma cell line previously (Forster-Gibson, C.J. and Dufresne, M.J., 1986).
Figure 9. Effect of Benzo(a)pyrene in Reaction Mix on the Expression of AHH Activity in Hep3B (●) and HepG2 (▲). The optimum BP volume for the mouse control cell-line, Hepa cl-9, is indicated by a vertical arrow. Cells were treated with medium containing 10 nM TCDD for 30 h at 37°C and assayed for AHH activity, as described in "Materials and Methods".
To minimize those factors, other than the cell's genetic make-up, that might cause variation in the clone's AHH activity, the assay conditions were controlled to give maximum levels of activity. Thus, the conditions applied, such as protein concentration, inducer concentration and induction time were those described previously: 10 nM TCDD and 30 h induction time for the human cell lines. The assays of all clones for each cell-line were synchronized by plating cells at the same time, harvesting at the same time, and freezing the harvested cells prior to assay. (Cells can be frozen for up to two weeks without affecting the levels of induced AHH activity.) Freezing cells permitted the simultaneous assay of a large number of samples. As another precaution, the number of subcultures each clone underwent in culture prior to assay was kept as close as possible. Slight variation in this parameter however is impossible to avoid since the clones were confluent at different times and the number of clones was too great to handle at the same time. The results of the AHH activity analyses are summarized in Figure 10. Clones of both Hep3B and HepG2 demonstrate variation in the expression of TCDD-induced AHH activity. The highest levels were reproducibly expressed in Hep3B cl-3 and HepG2 cl-2; the lowest levels were expressed in Hep3B cl-6 and HepG2 cl-5. When the average value for each
Figure 10. Expression of TCDD-Induced AH Activity in First Generation Clones of Hep3B and HepG2. Cells in logarithmic growth were incubated for 30 h with medium (37°C) containing 10 nM TCDD and AH activity was assayed as described under "Materials and Methods". "P" represents the parent.
clonal population was calculated (Table 4, lower panel) it was found to be close to the average value obtained in the parent. The average value for Hep3B clones for example, is 6.46 compared to a value of 7.10 units/mg protein for the Hep3B parent while the average value for HepG2 clones is 11.76 compared to 13.18 for the HepG2 parent. It is also interesting to note that the clonal variation (i.e. the range about the mean) value is greater than the standard deviation for the parent in both Hep3B and HepG2.

The significance of the observed clonal variation (Table 4) in expression of AHH activity was statistically analyzed using the Student "t" test. In Hep3B clones, two clones; Hep3B cl-2 and Hep3B cl-3, demonstrated significantly higher levels of TCDD induced AHH activity than the parent (p < .005). Three clones demonstrated significantly lower AHH activity than the parent: Hep3B cl-4 (p < .001); Hep3B cl-6 (p < .001) and Hep3B cl-7 (p < .005). The AHH activity induced in Hep3B cl-1 and Hep3B cl-5 was not significantly different that the Hep3B parent.

With respect to the HepG2 clones, HepG2 cl-1 (p < .05) and HepG2 cl-2 (p < .005) demonstrated significantly higher; HepG2 cl-5 (p < .005) and HepG2 cl-6 (p < .001) demonstrated significantly lower; while HepG2 cl-3 and HepG2 cl-4 demonstrated similar levels of TCDD-induced AHH activity, relative to the HepG2 parent.
Table 4. The Analysis Of Variation In AHH Induction Of The First Generation Clones of Hep3B And HepG2

<table>
<thead>
<tr>
<th>CLONE #</th>
<th>AHH ACTIVITY^a (units/mg prot.)</th>
<th>p^b</th>
<th>CLONE #</th>
<th>AHH ACTIVITY (units/mg prot.)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.31 ± 1.22^c</td>
<td>&gt;.05</td>
<td>1</td>
<td>18.10 ± 3.11</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>2</td>
<td>12.20 ± 1.28</td>
<td>&lt;.005</td>
<td>2</td>
<td>19.43 ± 4.32</td>
<td>&lt;.005</td>
</tr>
<tr>
<td>3</td>
<td>13.23 ± 2.64</td>
<td>&lt;.005</td>
<td>3</td>
<td>12.67 ± 2.16</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>4</td>
<td>2.18 ± 0.32</td>
<td>&lt;.001</td>
<td>4</td>
<td>10.13 ± 3.09</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>5</td>
<td>4.67 ± 0.87</td>
<td>&gt;.05</td>
<td>5</td>
<td>4.10 ± 1.02</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>6</td>
<td>1.20 ± 0.08</td>
<td>&lt;.001</td>
<td>6</td>
<td>6.15 ± 1.43</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>7</td>
<td>2.48 ± 0.95</td>
<td>&lt;.005</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Average 6.46 ± 4.83^d  
Parent 7.10 ± 3.06^e

^a Cells were cloned, and prepared for AHH assay. Cells in logarithmic growth were incubated for 30 h in medium (37°C) containing 10 nM TCDD and assayed as described under Materials and Methods. The activity for solvent treated cells was substraction of the activity of cells treated with TCDD.

^b "t" test to estimate the possibility of each clones AHH activity in terms of the possibility Belonging to their parent population.

^c Mean ± Standard Deviation (N = 3).

^d Deviation of the Means.

^e The standard deviation of the parents from eight measurements.
2) Effect of Various Inducers on Levels of Induced AH Activity in the First Generation Clones of Hep3B and HepG2 Demonstrating Highest and Lowest Levels of TCDD-Induced Activity.

First generation clones of Hep3B and HepG2 showing the highest and lowest levels of TCDD-induced AH activity—Hep3B cl-3 and Hep3B cl-6 respectively and HepG2 cl-2 and HepG2 cl-5 respectively—were chosen for further testing with the inducers of cytochrome P1-450: TCDD, MCA and BA; and the inducer of cytochrome P-450 other than P1-450, PB. As previously indicated, all assays were conducted under conditions producing maximum levels of AH activity in human cells. The results for Hep3B and HepG2 first generation clones are presented in Figures 11 and 12 respectively.

In the Hep3B clones (Figure 11) all three cytochrome P1-450 inducers induced highest levels of AH activity in Hep3B cl-3. It is interesting to note that BA, which did not induce AH activity in the parent Hep3B cell line, does induce activity in the high activity clone indicating that the potential for BA-induction was present, but not detected, in the parent. Nevertheless, as observed with the parent, TCDD produced the maximum level of induction in the clone while PB resulted in no induction response. The results for the low activity Hep3B clone, Hep3B cl-6 parallel those observed in Hep3B cl-3 except
Figure 11. The Expression of Induced AHH Activity in the Two First Generation Clones of Hep3B with Several Inducers. Cells in logarithmic growth were incubated for 30 h with medium (37°C) containing 10 nM TCDD, 1 μM MCA, 1 μM BA or 1 μM PB and assayed as described under "Materials and Methods."
Figure 12. The Expression of Induced AHH Activity in the Two First Generation Clones of HepG2 with Several Inducers. Cells in logarithmic growth were incubated for 30 h with medium (37°C) containing 10 nM TCDD, 1 μM MCA, 1 μM BA or 1 μM PB and assayed as described under "Materials and Methods".
that BA at the concentration tested, did not induce AHH activity. This result is consistent with the results obtained in the parent Hep3B cell line and it is possible that low activity clones contributed to the BA nonresponsive phenotype of the parent cell line.

In the HepG2 first generation clones (Figure 12), the highest levels of AHH activity were observed with TCDD in both the high activity HepG2 cl-2 and low activity HepG2 cl-5 clones. The levels of TCDD-induced activity, however, were always highest in HepG2 cl-2. Benzantracene produced a low level of induction in HepG2 cl-2 but not in HepG2 cl-5 while PB did not induce activity in either clone. These results are consistent with those observed for the Hep3B clones and the expression of induced AHH activity in the parent HepG2 cell line. The results obtained with MCA, on the other hand, are somewhat confusing. Methylcholonanthrene is a fairly potent inducer of AHH activity in HepG2 and it would be logical to assume that this induction would be expressed at least in the high activity first generation clone HepG2, cl-2. However, in both the high and low activity clones, the level of MCA-induced AHH activity is lower than BA-induced activity and comparable to PB-induced activity. For whatever reason, therefore, MCA did not induce activity in the clones of HepG2. It is possible that this result represents an
artifact of the assay technique itself since even a small error in inducer concentration or substrate addition can be expected to affect AHH activity. If so, this artifact is reproducible. On the other hand, the apparently spontaneous loss of MCA-inducibility during cloning has been previously observed with the mouse embryo cells, C3H10T^5 cl-8 and C3H10T^5MCA cl-16 (Okey, A.B., et al., 1983). Further analyses are required to distinguish between these two alternatives.

E. The Expression of TCDD-Induced AHH Activity in Second Generation Clones of Hep3B and HepG2

Each of the four first generation clones: Hep3B cl-3, Hep3B cl-6, HepG2 cl-2 and HepG2 cl-5, were cloned to obtain second generation clones (i.e. subclones) of Hep3B and HepG2. These particular clones were selected for subcloning since they showed the expression of highest and lowest levels of induced AHH activities among the first generation clones. These levels can be easily distinguished. This makes analysis of variation in expression of AHH activity in the second generation clones relatively straightforward. In the absence of significant variation, for example, the subclones of the high activity Hep3B cl-3 clone should demonstrate activity that belongs statistically to the same population as the parent and so on.
A total of 26 subclonal populations - 6 for each of Hep3B cl-3 and Hep3B cl-6, and 7 for each of HepG2 cl-2 and HepG2 cl-5, were randomly selected from over 10,000 wells. With respect to analysis of the expression of AHH activity, this large sample size presented an even greater potential for technical variation than the first generation clones in that it was impossible to handle and assay all 26 subclones at the same time. On the other hand, for the purposes of statistical analyses, the sample size was necessary. To minimize technical variation, cells were grown, treated with inducer, harvested and frozen prior to assay. The cell pellets were then assayed over as short a time as possible. The results of these analyses are summarized in Figures 13 and 14. Subclones are designated by the parent clone, for example Hep3B cl-3, followed by the subclone number, -1, -2, -3 etc.

As with the first generation clones, the second generation clones demonstrated variation in the expression of TCDD-induced AHH activity. In each case, whether the initial parent clone had high or low levels of induced AHH activity, their subclones expressed AHH activity both less than and greater than the parent clone. Specifically, Figure 13 shows that Hep3B cl-3-5 expressed the highest and Hep3B cl-3-3 expressed the lowest TCDD-induced AHH activity among the Hep3B cl-3 subclones; and Hep3B cl-6-5 expressed the highest and Hep3B cl-6-2-expressed the lowest activity among
Figure 13. The Expression of TCDD-Induced AH Activity in Second Generation Clones of Hep3B, the Subclones of Hep3B cl-3 and Hep3B cl-6. Cells in logarithmic growth were incubated for 30 h with medium (37°C) containing 10 nM TCDD and the AH activity assayed as described in "Materials and Methods". The AH Activity of the parent first generation clone is designated P.
the Hep3B cl-6 subclones. In HepG2 second generation clones (Figure 14) the subclones of highest and lowest TCDD-induced AHH activity were HepG2 cl-2-5 and HepG2 cl-2-3 respectively among the HepG2 cl-2 subclones; and HepG2 cl-5-5 and HepG2 cl-5-4 respectively among the HepG2 cl-5 subclones.

While each of the four first generation clones gave rise to subclones which demonstrated variation in the expression of AHH activity, the highest TCDD-induced AHH activity was reproducibly observed in subclones of the high activity first generation clones, specifically Hep3B cl-3 and HepG2 cl-2. Comparison of Hep3B cl-3 subclone results with those of Hep3B cl-6 subclones, and HepG2 cl-2 subclone results with HepG2 cl-5 subclone results suggests that this is a general pattern. The activities of those subclones from the high activity parent are generally higher than those from the low activity parent. This trend can be further seen if the average activity for each subclone population is compared to that for the parent (Tables 5, 6, 7, 8, bottom panels). As in the case of first generation clones, the average value for AHH activity for each subclone population is similar to that of the respective first generation clone parent. As a result, the average activity value for the subclones from the high activity first generation parent is higher than the average activity value for the subclones
Figure 14. Expression of TCDD-Induced AH Activity in the Second Generation Clones of HepG2, the Subclones of HepG2 cl-2 and HepG2 cl-5. Cells in logarithmic growth were incubated for 30 h with medium (37°C) containing 10 nM TCDD and AH activity was assayed as described under "Materials and Methods". The AH Activity of the Parent first generation clone is designated P.
from the low activity first generation parent in both Hep3B and HepG2. However, the clonal variation (i.e. range of AHH values about the mean) is closer to the standard deviation of the parent clones in the second generation clones than observed in the first generation clones (Table 4). The significance of this apparent trend requires further analysis at the level of later generations.

The statistical significance of the observed variation in TCDD-induced AHH activity among the second generation clones was analyzed by the Student "t" test. Of the six Hep3B cl-3 subclones (Table 5), Hep3B cl-3-5 (p < .01) and Hep3B cl-3-6 (p < .05) demonstrated higher; and Hep3B cl-3-1 (p < .01) and Hep3B cl-3-3 (p < .001) demonstrated lower TCDD-induced AHH activity compared to the Hep3B cl-3 first generation parent. Three of the six Hep3B cl-6 subclones expressed significantly higher (Hep3B cl-6-5, p < .01), or lower (Hep3B cl-6-1, p < .001; Hep3B cl-6-2, p < .001), levels of TCDD-induced AHH activity relative to the Hep3B cl-6 first generation parent. Although these subclones demonstrated significant variation from the parent, the degree of significance appears less than that observed with first generation clones. The general low levels of AHH activity obtained in Hep3B cells, however, may contribute to this since a somewhat higher degree of significance was obtained with HepG2
<table>
<thead>
<tr>
<th>SUBCLONE #</th>
<th>AHH ACTIVITY&lt;sup&gt;a&lt;/sup&gt; (units/mg prot.)</th>
<th>n&lt;sup&gt;b&lt;/sup&gt;</th>
<th>p&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.18 ± 1.04&lt;sup&gt;d&lt;/sup&gt;</td>
<td>(2)</td>
<td>&lt; .01</td>
</tr>
<tr>
<td>2</td>
<td>5.02 ± 1.42</td>
<td>(2)</td>
<td>&gt; .05</td>
</tr>
<tr>
<td>3</td>
<td>1.33 ± 0.50</td>
<td>(2)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>4</td>
<td>6.50 ± 0.76</td>
<td>(2)</td>
<td>&lt; .05</td>
</tr>
<tr>
<td>5</td>
<td>10.00 ± 0.85</td>
<td>(2)</td>
<td>&lt; .01</td>
</tr>
<tr>
<td>6</td>
<td>9.01 ± 2.20</td>
<td>(2)</td>
<td>&lt; .05</td>
</tr>
<tr>
<td>AVERAGE</td>
<td>6.02 ± 3.60&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PARENT</td>
<td>7.10 ± 2.16</td>
<td>(8)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Cells in logarithmic growth were incubated in medium (37°C) containing 10 nM TCDD for 30 hours and AHH activity was assayed as described under "Materials and Methods". The values for specific AHH activity were calculated by subtracting the activity for solvent treated cells from that for cells treated with solvent and inducing agent.

<sup>b</sup> Represents the number of measurements.

<sup>c</sup> Values from "t" test to estimate the value of each subclone in terms of the possibilities of belonging to the parent population.

<sup>d</sup> Mean ± Standard Deviation.

<sup>e</sup> Deviation of the Means.
### Table 6. Analysis Of TCDD-Induced AH Activity In Hep3B cl-6 Subclones

<table>
<thead>
<tr>
<th>SUBCLONE #</th>
<th>AH Activity&lt;sup&gt;a&lt;/sup&gt; (units/mg prot.)</th>
<th>N&lt;sup&gt;b&lt;/sup&gt;</th>
<th>P&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.34 ± 0.61&lt;sup&gt;d&lt;/sup&gt;</td>
<td>(2)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>2</td>
<td>1.27 ± 0.38</td>
<td>(2)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>3</td>
<td>4.31 ± 0.71</td>
<td>(2)</td>
<td>&gt; .05</td>
</tr>
<tr>
<td>4</td>
<td>2.70 ± 0.82</td>
<td>(2)</td>
<td>&gt; .05</td>
</tr>
<tr>
<td>5</td>
<td>5.13 ± 1.38</td>
<td>(2)</td>
<td>&lt; .01</td>
</tr>
<tr>
<td>6</td>
<td>2.99 ± 0.97</td>
<td>(2)</td>
<td>&gt; .05</td>
</tr>
<tr>
<td><strong>AVERAGE</strong></td>
<td>2.95 ± 1.65&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PARENT</strong></td>
<td>3.80 ± 1.06</td>
<td>(8)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Cells in logarithmic growth were incubated in medium (37°C) containing 10 mM TCDD for 30 hours and AH activity was assayed as described under "Materials and Methods". The values given for specific AH activity were calculated by subtracting the activity for solvent treated cells from that for cells treated with solvent and inducing agent.

<sup>b</sup> Represents the number of measurements.

<sup>c</sup> Values from "t" test to estimate the value of each subclone in terms of the possibilities of belonging to the parent population.

<sup>d</sup> Mean ± Standard Deviation.

<sup>e</sup> Deviation of the Means.
Table 7. Analysis Of TCDD-Induced AHH Activity In HepG2 cl-2 Subclones

<table>
<thead>
<tr>
<th>SUBCLONE</th>
<th>AHH ACTIVITYa (units/mg prot.)</th>
<th>N</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17.91 ± 3.07d</td>
<td>(2)</td>
<td>&lt; .05</td>
</tr>
<tr>
<td>2</td>
<td>5.13 ± 1.59</td>
<td>(2)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>3</td>
<td>3.95 ± 1.44</td>
<td>(2)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>4</td>
<td>7.74 ± 1.68</td>
<td>(2)</td>
<td>&lt; .01</td>
</tr>
<tr>
<td>5</td>
<td>19.12 ± 3.01</td>
<td>(2)</td>
<td>&lt; .01</td>
</tr>
<tr>
<td>6</td>
<td>9.06 ± 1.47</td>
<td>(2)</td>
<td>&lt; .05</td>
</tr>
<tr>
<td>7</td>
<td>11.30 ± 2.05</td>
<td>(2)</td>
<td>&gt; .05</td>
</tr>
<tr>
<td></td>
<td>AVERAGE</td>
<td>10.60 ± 5.93e</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PARENT</td>
<td>13.65 ± 4.20</td>
<td>(8)</td>
</tr>
</tbody>
</table>

a Cells in logarithmic growth were incubated in medium (37°C) containing 10 nM TCDD for 30 hours and AHH activity was assayed as described under "Materials and Methods". The values given for specific AHH activity were calculated by subtracting the activity for solvent treated cells from that of cells treated with solvent and inducing agent.

b Represents the number of measurements.

c Values from "t" test to estimate the value of each subclone in terms of the possibilities of belonging to the parent population.

d Mean ± Standard Deviation.

e Deviation of the Means.
Table 8. Analysis Of TCDD-Induced AH Activity In HepG2 cl-5 subclones

<table>
<thead>
<tr>
<th>SUBCLONE</th>
<th>AH Activity&lt;sup&gt;a&lt;/sup&gt; (units/mg prot.)</th>
<th>N&lt;sup&gt;b&lt;/sup&gt;</th>
<th>p&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.21 ± 0.86&lt;sup&gt;d&lt;/sup&gt;</td>
<td>(2)</td>
<td>&gt; .05</td>
</tr>
<tr>
<td>2</td>
<td>6.92 ± 2.05</td>
<td>(2)</td>
<td>&gt; .05</td>
</tr>
<tr>
<td>3</td>
<td>3.85 ± 1.02</td>
<td>(2)</td>
<td>&lt; .05</td>
</tr>
<tr>
<td>4</td>
<td>2.40 ± 0.90</td>
<td>(2)</td>
<td>&lt; .005</td>
</tr>
<tr>
<td>5</td>
<td>9.91 ± 1.52</td>
<td>(2)</td>
<td>&lt; .005</td>
</tr>
<tr>
<td>6</td>
<td>5.72 ± 1.92</td>
<td>(2)</td>
<td>&gt; .05</td>
</tr>
<tr>
<td>7</td>
<td>4.05 ± 1.05</td>
<td>(2)</td>
<td>&lt; .05</td>
</tr>
</tbody>
</table>

|          | AVERAGE 5.44 ± 2.45<sup>e</sup>        |             |             |
|          | PARENT 6.28 ± 2.20                      | (8)         |             |

<sup>a</sup> Cells in logarithmic growth were incubated in medium (37°C) containing 10 nM TCDD for 30 hours and AH activity was assayed as described under "Materials and Methods". The values presented for AH activity were calculated by subtracting the activity for solvent treated cells from that for cells treated with solvent and inducing agent.<br>
<sup>b</sup> Represents the number of measurements.<br>
<sup>c</sup> Values from "t" test to estimate value of each subclone in terms of the possibilities of belonging to the parent population.<br>
<sup>d</sup> Mean ± Standard Deviation.<br>
<sup>e</sup> Deviation of the Means.
subclones as described below.

Of the seven subclones of HepG2 cl-2, six were significantly
different from the parent in terms of TCDD-induced AHH
activity (Table 7). Of these HepG2 cl-2-1 (p < .005) and
HepG2 cl-2-5 (p < .001) demonstrated higher; and HepG2 cl-2-2
(p < .001), HepG2 cl-2-3 (p < .001), HepG2 cl-2-4 (p < .01) and
HepG2 cl2-6 (p < .05) demonstrating lower AHH activity relative
to the first generation parent. Although not as evident,
significant variation was also observed in HepG2 cl-5 subclones
with one of the seven subclones, HepG2 cl-5-5 (p < .005) demon-
strating higher and three others, HepG2 cl-5-3 (p < .05), HepG2
cl-5-4 (p < .005) and HepG2 cl-5-7 (p < .05) demonstrating lower
TCDD-induced AHH activity compared to the first generation parent.
The relative level of AHH activity in both first and second
generation clones remained stable during the course of study.

F. Summary Statement of General Results

1) Assay conditions for Hep3B and HepG2, two human hepatoma-
cell lines differ from those for the mouse Hepa cl-9 cell line.

2) At conditions which produce maximum AHH activity in human
cells, first generation clones show significant variation
with respect to expression of induced AHH activity relative
to the Hep3B and HepG2 parent cell lines.

3) When first generation clones with high and low levels of
induced AHH activity relative to the parent are cloned, their
subclones show significant variation with respect to the
expression of induced AHH activity.
CHAPTER IV
DISCUSSION

It has been established that next to heart disease, cancer is the major cause of disease-related deaths in humans (Nebert, D.W., 1981). Of these, approximately 80% of the environmentally-induced cancers are caused by chemicals such as polycyclic aromatic hydrocarbons (Gelboin, H.V. and Ts'O, F.O., 1961). Although advances have been made in the treatment of cancers and in legislation to control the levels of pollutants entering the environment from automobile exhaust and industrial waste, mortality from chemically-induced cancers continues to increase. In view of this increase, much research is focusing on the prevention and early detection of cancer by unique biochemical and/or immunological markers which might signal the presence of cancer in its early, treatable stages and/or identify those individuals at greater risk for development of this disease. Such markers for example, might be used to counsel high-risk individuals against exposure to certain drugs, habits or work environments. One marker that has aroused considerable interest in this regard is the aryl hydrocarbon hydroxylase (AHH) enzyme system (Okey, A.B. and Nebert, D.W., 1982; Kourl, R.E., et al., 1982; Ishimura, Y., et al., 1978; Gelboin, H.V.,
and Ts'0, P.O., 1982; Tscharley, F.H., 1986).

It is well established that there is a direct (positive) correlation between AHH induction levels and the susceptibility to polycyclic aromatic hydrocarbon-induced tumors in mice (reviewed in Okey, A.B. and Nebert, D.W., 1982). However, a similar relationship has been more difficult to establish in humans. This is in part due to the use of lymphocytes which require mitogen activation (Kouri, R.E., et al., 1980, 1981, 1982) and also the large degree of interindividual and intraindividual variability in levels of AHH activity (Levine, A.S. et al., 1984; Kouri, R.E. et al., 1962; Okuda, T., et al., 1977).

Over the years considerable research has been devoted toward standardizing the assay of AHH activity and thereby achieving reproducible, significant levels of mitogen activation and subsequent AHH induction in human peripheral blood lymphocytes and other cell systems. Kouri and his colleagues (Kouri, R.E., et al., 1979) suggested the use of human AB serum rather than fetal calf serum in the cell culture medium. According to this early report, fetal calf serum affects both the level of AHH activity in control and induced lymphocytes and the time at which peak activities are observed. This effect, which varies with the lot of serum used, is probably related to the variable levels of chemical constituents, some of which are capable of activating
human lymphocytes (Zielinski, J.V. and Golub, S.H., 1976). This does not appear to be a major source of variation in cells established from liver which do not require mitogen activation (Tukey, R.H., et al., 1984; Whitlock, J.P. Jr., et al., 1984). The cells used in the studies described in this thesis were all established in fetal calf serum and the same lot of fetal calf serum was used throughout.

Another factor examined with respect to standardization of the AHH assay, was the concentration of cells initially seeded. In the lymphocyte system it was found that if cells were initially seed at a concentration of $10^6$/ml, a more reproducible measurement of AHH activity was obtained. It was further demonstrated that monitoring at least two time points improved detection of AHH activity and improved the basis for comparison of levels among different individuals (Kouri, R.E., et al., 1980, 1981, 1982). In the studies in human liver cells described in this thesis the more critical parameter appears to be the state of the cells at the time of exposure to inducer. This is indirectly related to initial seeding concentration in that cells seeded at a low initial concentration form packed colonies before reaching confluency and cells seeded at high initial concentrations reach confluency before exposure to inducer. In both cases, levels of AHH induction are reduced as a result of cell death and low
protein yields. Therefore, in these studies cells were always seeded at initial concentrations that permitted exposure of inducer to cells just prior to confluency.

An important breakthrough with respect to standardization of assay conditions for the measurement of AHH activity was the use of cryopreserved tissue samples (Kouri, R.B. et al., 1982). It was reported that lymphocytes, and other human cells and tissues, could be stored at \(-196^\circ C\) for some time without loss of AHH activity. As a result, tissue and cell samples could be collected from many individuals in various geographical areas and at different times. These tissues and cells could then be cultured and assayed at the same time thus minimizing interexperiment variation. This same technique was used in this study in that cell pellets collected after exposure to inducer were routinely frozen at \(-80^\circ C\) prior to assay. Cells frozen in this manner retained control levels of AHH activity for up to two weeks.

Despite the progress made in establishing the human lymphocyte system, low, variable levels of AHH activity continue to make interpretation of AHH levels difficult. Analyses in other human cell systems has been difficult since inducible human cells have not been available for research. In this study, two human hepatoma cell lines, Hep3B and HepG2, were used to
study the expression of AHH activity. These two cell lines provide an advantage over previous analyses in that they do not require mitogen activation; they are more highly inducible than lymphocyte cells; and they can be compared to the established murine hepatoma cells (Levine, A.S., et al., 1984; Legraverend, C., et al., 1984; Greenlee, W.F. and Neal, R.A., 1985). With respect to this latter advantage, the mouse hepatoma cell line Hepa cl-9, previously established with respect to the induction of AHH activity was used as an internal control (Okey, A.B., et al., 1980, Okey, A.B., et al., 1983; Feeley, M.M. and Dufresne, M.J., 1982; Dufresne, M.J. and Dosescu, J., 1985; Forster-Gibson, C.J. and Dufresne, M.J., 1986; Isreal, D.I. and Whitlock, J.P.Jr., 1983; Isreal, D.I., et al., 1985; Tukey, R.H., et al., 1984).

Although the level of induced AHH activity varied as a function of type of PAH inducer in Hep3B and HepG2 (i.e. TCDD MCA DBA), the general pattern of inducer potency was the same as that reported for the mouse hepatoma cells with TCDD always inducing the highest levels of AHH activity (Okey, A.B., et al., 1980; Dufresne, M.J. and Dosescu, J., 1985). However, a 10-fold higher concentration of each inducer was required to induce maximum AHH activities in the human cells relative to the mouse Hepa cl-9 cells. There are—several reasons for this. First,
it is possible that it is more difficult for the PAH to cross
the membrane in these human cells such that a lower concentration
of inducer diffuses into the cytoplasm. Measurements to determine
the intracellular concentration of radiolabelled PAH, however,
have not been accomplished. However, in a recent report, it
has been demonstrated that incubation of cell-free, cytosolic
preparations from human lung tissue and HepG2 cells with 4 nM
and 10 nM $^3$H-TCDD result in levels of PAH:receptor complex
which are 10 to 20-fold lower than those detected in Hepa cl-9
cells incubated with 1 nM $^3$H-TCDD (Roberts, E. and Okey, A.B.,
1986). Since these preparations are cell free, diffusion across
a membrane is not necessary; therefore, the requirement for
higher concentrations of TCDD might reflect a difference at
the level of receptor, for example receptor content or affinity
of binding sites. Furthermore, if the mechanism of AH induction
in human cells is similar to that reported for murine cells,
the difference in sensitivity to PAH could be explained on
the basis of how much receptor:PAH complex translocates into
the nucleus and becomes available for interaction with the Ah
gene complex. It is well established that the level of induction
in murine cells is a direct function of nuclear receptor (Tukay,
R.H., et al., 1982). Thus in Hep3B and HepG2, a higher concentration
of intracellular TCDD may be required to induce similar or lower
levels of AHH activity than that observed in Hepa cl-9. These possibilities however cannot be presently distinguished since measurement of receptor levels in human cells has not yet been established. Two factors which however, should be examined are the levels of cytochrome c(P-450) reductase and the level of cytochrome P,450 in the human cells compared to Hepa cl-9. These measurements, while possible, present a problem in cell culture systems since large amounts of sample are required and for this reason were beyond the scope of the present study.

The measurement of AHH activity involves measuring the amount of hydroxylated product (3-OH-BP) formed from benzo(a)pyrene fluorometrically. The fluorescence units are then routinely converted to units/mg protein as described in Chapter II. Thus, two factors which might affect the level of AHH activity are the amount of benzo(a)pyrene (i.e. substrate) in the reaction and the concentration of sample protein (i.e. enzyme). Both factors affected the level of AHH activity in Hep3B and HepG2. However, concentrations of BP which resulted in maximum levels of TCDD-induced AHH activity in the human lines were similar to that reported for Hepa cl-9. Nevertheless, the concentration of BP in the reaction mix should be examined for each human cell system.

The effect of sample protein concentration on levels of TCDD-induced activity was different for each cell line examined in
that Hepa cl-9, Hep3B and HepG2 all displayed different protein optima. In this study, HepG2 required the highest protein concentration (in mg/ml) followed by Hep3B and finally Hepa cl-9. Since the levels of induced AHH activity are considerably lower on both sides of the optimum level, it is apparent that assaying at sub-optimum protein concentrations could lead to misinterpretation with respect to relative levels of AHH activity among and between cell lines. In this study, at optimum concentrations of sample protein, induced AHH activity expressed in units/mg protein, was always highest in Hepa cl-9 and lowest in Hep3B. These results support the conclusion that Hepa cl-9 is more inducible than HepG2 which, in turn, is more inducible than Hep3B. However, the difference in size and saturation density of these three cell lines permits a reassessment of this conclusion.

Although there has been variation in the absolute levels of AHH activity from experiment to experiment, the relative levels of AHH activity in Hepa cl-9, HepG2 and Hep3B have been consistent (Table 9). When expressed in units/mg protein, the level of induced AHH activity in Hepa cl-9 is routinely at least twice that of HepG2 and four times that of Hep3B. Levels of basal AHH activity, on the other hand are similar in all three and always less than 1 unit/mg protein. Interestingly, when the number of cells per mg of protein is determined, Hepa cl-9 has
### Table 9. A Comparison Of TCDD-Induced AHH Activity In Hepa cl-9, Hep3B And HepG2

<table>
<thead>
<tr>
<th>CELL LINES</th>
<th>AHH ACTIVITY&lt;sup&gt;a&lt;/sup&gt; (units/mg prot.)</th>
<th>CELLS/MG PROT.</th>
<th>AHH ACTIVITY&lt;sup&gt;b&lt;/sup&gt; (units/10&lt;sup&gt;6&lt;/sup&gt;cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepa-cl9</td>
<td>27.33 ± 4.81&lt;sup&gt;c&lt;/sup&gt;</td>
<td>17.3 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.58</td>
</tr>
<tr>
<td>HepG2</td>
<td>11.70 ± 2.08</td>
<td>9.1 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.29</td>
</tr>
<tr>
<td>Hep3B</td>
<td>6.73 ± 1.26</td>
<td>5.3 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.27</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cell lines were induced with 1.0 nM TCDD for Hepa cl-9, 10.0 nM TCDD for both Hep3B and HepG2, other conditions were the optimum conditions to induce the maximum AH3H activity in each cell line. The values given for specific AH3H activity were calculated by subtracting the activity for solvent treated cells from the activity for cells treated with solvent and TCDD.

<sup>b</sup> AH3H activity of units/10<sup>6</sup>cells was calculated by dividing AH3H activity in AH3H units/mg protein by the number of cells /mg protein as listed in this table.

<sup>c</sup> Mean ± Standard Deviation. (N = 3 ).
approximately twice that of HepG2 and four times that of Hep3B. If AHH activity is then reexpressed in terms of units/10^6 cells, the difference in levels of induced AHH activity in all three cell lines is relatively similar with less than a one fold difference between Hepa c1-9 and the human cells. It has been reported that the level of AHH activity is proportional, among other things, to P1-450 gene dosage in the cell (Jones, P.B.C., et al., 1984). A larger content of non-AHH associated proteins in the cell would tend to dilute the AHH relevant proteins in the assay mix. Thus on a per mg protein basis, the larger cell would require a greater protein concentration than a cell having the same level of AHH structural proteins but lower total protein. Referring back to the findings in this study, the two human cell lines show different protein content per cell but similar levels of AHH activity per cell. Thus the initial conclusion that HepG2 was more inducible than Hep3B was incorrect since on a per cell basis they are equally inducible. Therefore in cell culture systems, it is important to express results in units which represent the cell (i.e. per cell of per ug DNA) when comparing levels of AHH activity. If the question of gene dosage is to be considered than the latter representation, units/ug DNA, is most suitable.

The induction time differs in the two systems. Time for
for maximum induction of AHH activity in Hepa cl-9 was 18 h
compared to the 30 h induction period required for the two
human hepatoma cell lines. In the case of Hep3B, this increase
in induction time can be readily explained —perhaps incorrectly—
on the basis of its longer generation time of 30 h compared to
18.5 for Hepa cl-9. Since induction is known to involve numerous
steps in murine systems (Greenlee, W.P. and Neal, R.A., 1985) it
can be argued that it takes Hep3B longer to complete the numerous
steps necessary for expression of induction. Generation time,
however, cannot be used to explain HepG2's requirement for an
equivalent 30 h induction time since its generation time is
slightly less than Hepa cl-9 (16.5 and 18.5 h respectively).
A possible explanation for the need of a 30 h induction period
in HepG2 can be approached by addressing two questions: First,
how is the induction time distributed in the AHH induction process
both in human and mouse cells? Second, at which stage of the
AHH induction process is the induction period flexible in the
human and mouse cells?

The AHH activity induction period may comprise the following
events: transport of PAH into cytoplasm of cell; binding of PAH
to receptor; translocation of PAH:receptor into nucleus; activation
of transcription of AHH associated genes; translation of relevant
transcription products; and incorporation and expression of AHH
structural gene products. According to recent receptor measurements in Hep3B and HepG2 (Labruzzo, P., personal communication) it takes one hour for TCDD in the medium to appear as TCDD:receptor complex in the nucleus. This is consistent with the results published for mouse hepatoma cells (Okey, A.B., et al., 1980; Greenlee, W.F. and Neal, R.A., 1985). Thus the time required for binding of TCDD to receptor and the subsequent time- and temperature dependent translocation of the TCDD:receptor complex into the nucleus is not largely flexible and in fact takes up a small proportion of the required induction times. Therefore, at 37°C, subsequent activation of the cytochrome P1-450 gene, and other AHH related genes, and their translation is optimal after 17 h in the mouse and 29 h in human cells. Studies on the distribution of mouse cytochrome P1-450 mRNA (Yuan-Tsong, C. and Masahike, N., 1982) indicate that during the early phase of induction by MCA, 14% (at 3 hours) and 10% (at 6 hours) of P1-450 mRNAs are associated with free polysomes. At 24 hours this value decreases to 6%. It is interesting to note that during the first 6 hours of induction the specific content of P1-450 mRNA reaches 20% of its maximal induction level. However, the levels of the induced AHH activity and cytochrome P1-450 protein determined by immunological methods, are only 5% of their maximal levels at 6 hours following MCA treatment (Negishi, M., et al., 1981). In view of this, one
explanation for the difference in induction time between HepG2 and Hepa cl-9 could be that there is a time lag between the binding of $P_1-450$ mRNA containing polysomes to the endoplasmic reticulum and the accumulation of immunoprecipitable translated product (e.g., cytochrome $P_1-450$) in the endoplasmic reticulum. The time lag reported by Negishi and colleagues was not observed in the case of rat liver microsomal NADPH-cytochrome $P-450$ reductase or epoxide hydrase during their induction by phenobarbitol (Gonzalez, F.J., et al., 1984) and may thus apply to $P_1-450$ (and $P_3-450$?) genes.

The primary objective in examining factors in the expression of AH activity in human cells was not so much to establish all the necessary conditions but rather to establish conditions which would permit levels of activity comparable to those reported in mouse hepatoma cells, to be expressed. These levels would then permit an analysis of clonal variation in human cells to be addressed. In view of this, it is understood that other factors are important in the assay of human cells and should be examined in more detail in the future. These include the effect of $\text{MgCl}_2$ concentration and intra- and extracellular levels of NADPH as well as $pH$. Thus the levels of AH activity in human cells may in fact be higher than reported in this study, nevertheless, the reported values are sufficient for clonal analysis.
A high degree of spontaneous variation in the expression of induced AHH activity was found in clones of Hep3B and HepG2. Five of the seven Hep3B clones and four of the six HepG2 clones had significantly lower or higher induced levels of AHH activity compared to the parent cell lines. Two clones having the highest and lowest levels of AHH activity were selected for each parent cell line for further characterization. The AHH inducibility of the Hep3B cl-3 first generation clone was approximately 10-fold higher than that of Hep3B cl-6, while the AHH inducibility of HepG2 cl-2 was approximately 5-fold higher than that of HepG2 cl-5. The absolute levels of induced AHH activity in these first generation clones varied from experiment to experiment. However, the relative levels of AHH induction within experiments remained stable. This stability was seen between assays performed at different times of the year, using a variety of PAH inducers and before and after routine storage at -80°C.

Subclones of each of the four first generation clones also demonstrated a significant degree of spontaneous variation. Four of the six Hep3B cl-3 subclones, three of the six Hep3B cl-6 subclones, six of the seven HepG2 cl-2 subclones and four of the seven HepG2 cl-5 subclones had either significantly lower or higher induced levels of AHH activity than their first generation parents. Thus the potential for expression of variation
in AHH induction appears to be an inherited trait and not an artifact of the original Hep3B and HepG2 populations. These results are consistent with those obtained for clones and subclones of the mouse hepatoma cell line, Hepa lcl (Forster−Gibson, C.J. and Dufresne, M.J., 1986).

While the variation in expression of AHH activity in subclones is spontaneous, it is not entirely unpredictable. The average AHH activity of the subclones from the 'high activity' Hep3B cl-3 clone was approximately 2-fold higher than that of the average AHH activity of the subclones from the 'low activity' Hep3B cl-6 clone. A similar pattern was observed between HepG2 cl-2 and HepG2 cl-5 subclones. Thus the level of AHH activity in the parent clone approaches the mean of the activities in the subclones. Realistically this must have an upper and lower limit.

Having established that heritable variation in AHH induction does appear to occur in human cells maintained in culture, what is the basis, or mechanism of this variation? Studies with mouse hepatoma cells, Hepa lcl, suggest that both genetic and epigenetic mechanisms are responsible for the appearance of high levels of significant variation in induced AHH activity in clones (Tukey, R.H., et al., 1984: Forster−Gibson, C.J. and Dufresne, M.J. 1986). In this study of human cells, the combination of variable AHH activity and stable relative AHH activity between
clones and subclones indicates that the cause of variation lies in a factor(s) common to all cell populations. All cell populations, for a given parent, were always handled identically with respect to times of subculturing or plating for experiments, density of plating for AHH assay, time of harvest, freezing conditions and source of components for the growth and assay media. Therefore, it is unlikely that variation is a result of external factors. It is more probable that this apparently inherited variation reflects some common internal factor(s) such as random distribution or loss of chromosomes during cell growth in culture or tumor 'cell' heterogeneity.

Cell hybrids formed between TCDD-inducible mouse L-cells and TCDD-inducible mouse hepatoma, Hepa cl-9 cells express inducible AHH activity (Dufresne, M.J. and Dosescu, J., 1985). The apparent dominance of the responsive phenotype is also consistent with that reported for the expression of BP- and BP-induced AHH activity in other cell hybrid systems (Brown, S., et al., 1976; Wiebel, H., et al., 1981). In mouse, this dominant trait has been localized on chromosome 17 (Legravereend, C., et al., 1984) and is associated with the receptor which controls AHH induction. Further genetic studies, however, suggest the involvement of at least two non-linked loci operating in an unknown manner (Robinson, J.R. et al., 1980).
mouse these two loci have been localized to chromosome 9 (the P₄-450 gene) and chromosome 17 (regulatory gene controlling AHH induction) (Tukey, R.H., et al., 1984). In humans, AHH induction by benzantracene has been localized to human chromosome 2 (Wiebel, F.J., et al., 1981). However, a gene controlling the inducibility of P₄-450, as in the case of mouse chromosome 9, has not been reported. Determination of chromosome number in this study demonstrates that the majority of cells are aneuploid (Figure 15). Moreover, there is an uneven distribution of chromosome numbers in both Hep3B and HepG2. If a chromosome such as chromosome 2, which carries AHH associated gene(s), is lost or duplicated in a cell, the level of AHH activity would decrease (or increase) as a function of gene dosage (Isreal, D., et al., 1985). If AHH induction in human cells involves more than one chromosome, as observed with mouse cells, the possibility of a wider spectrum of variation becomes apparent. The extent of variation is limited only by the number of mechanisms controlling AHH induction and their distribution among chromosomes. A starting point for this possibility could involve chromosome banding studies for the identification of chromosome 2 in each clone and subsequent comparison of this karyotype with the level of TCDD-induced AHH activity. If the above assumption is true, then the cells with more than one chromosome
Figure 15. Chromosome Distribution of Hep3B and HepG2 showing the variation of the chromosome numbers in the cells of Hep3B and HepG2.
2, or other AHH associated chromosome(s), may have increased induction of activity. Such an analysis however requires some degree of stability in karyotype in clones and subclones once established and this may not be the case.

Similar patterns of non-specific, heritable variation have been reported in mouse hepatoma cell lines and provide the basis for alternative explanations of variation. The fluorescence-activated cell sorter isolated population of variant mouse hepatoma cells, when compared with wild-type cells, exhibit increased AHH activity and increased responsiveness of the AHH induction mechanism to TCDD. An increased rate of transcription of the cytochrome P1-450 genes accounts for the increased AHH activity (Jones; P.B.C., et al., 1984). Transcription of the cytochrome P1-450 gene(s) requires the accumulation of TCDD:receptor complex within the nucleus (Tukey, R.H., et al., 1982). Cells which fail to do so also fail to express the gene (Israel, D.I. and Whitlock, J.P. Jr., 1983). By analogy with other inducible systems, the TCDD:receptor complex presumably performs its regulatory function by interaction with a control element flanking the 5' -end of the cytochrome P1-450 structural gene (Karin, M., et al., 1984). The TCDD:receptor complex was not altered in the variant cells. Furthermore, the cytochrome P1-450 gene(s) was not amplified in the variant cells.
Thus the investigators conclude that it is likely that the variant cells contain an altered control element(s) which, in the presence of TCDD:receptor complexes, permits more effective transcription of the cytochrome P\textsubscript{450} gene(s).

The alteration of the control element can be explained in genetic and/or epigenetic terms. It is possible that a mutation in the relevant genetic material results in the alteration; the low frequency of variants is consistent with this observation. However, it is possible that there is no direct change in the genetic material per se. Studies in mouse by restriction analysis reveal that the cytochrome P\textsubscript{450} gene in the variants is relatively undermethylated with respect to the wild-type gene.

In view of the reported correlations in other systems between decreased gene methylation and increased gene expression (Razin, A. and Riggs, A.D., 1980; Ehrlich, M. and Wang, R.Y., 1981; Doerfler, W., 1983) the findings are consistent with an epigenetic mechanism, i.e. hypomethylation.

The variants obtained in the present study cannot be explained on the basis of mutation. The variants observed in Hep3B and HepG2 occur at rates much higher than those expected for spontaneous mutation in animal cells (Hankinson, O., 1981). Thus if an alteration in (a) controlling element(s) is contributing to variation in Hep3B and HepG2, it is more likely that it is
a result of epigenetic mechanism(s) and/or genetic drift resulting from a loss of relevant genetic material in some of the clones. This latter possibility is supported by the observation that in both Hep3B and HepG2, the mean value of induced AHH activity for all the clones was similar to the mean value for the parent cell line. Recall, for example, that when Hep3B cl-3 and Hep3B cl-6, with high and low levels of AHH activity respectively, were cloned, the mean value of activity for their subclones was similar to the parent values. A similar result was obtained in second generation clones of HepG2 (refer to Tables 5, 6, 7, and 8). This may indicate that the AHH induction gene copy is kept constant during cloning but that there is a random distribution of the relevant genes (chromosomes) in the initial cells of the clones.

Attempts have been made to determine the frequencies for spontaneous and induced mutation in two cloned human lymphblastoid lines which, over a period of several years in culture, showed phenotypic change in the expression of peptidase D (Povey, S., et al., 1977). The investigators did not find any spontaneous changes in gene expression in lines and sublines from 41 individuals assessed at 26 structural loci. However, treatment of the lines with chemical mutagens or ultraviolet radiation resulted in the identification of 3 changes in phenotype out of
5150 alleles tested. Of these, one appeared to be the result of a structural gene alteration, and two the result of changes at the regulatory loci. Therefore the investigators concluded that the high levels of spontaneous variation in peptidase D activity could not be the result of mutation. In the Chinese hamster ovary cell line, CHO, Siciliano and colleagues (Siciliano, M.J. et al., 1983) screened approximately 40 enzyme loci and found the frequency of spontaneous mutation in 383 clones to be $0.6 \times 10^{-4}$ per locus tested. This relatively high level of mutation was increased to $7.3 \times 10^{-4}$ after UV mutagenesis. However, even at these high rates, it is unlikely that spontaneous mutation can account for the high degree of variation seen in Hep3B and HepG2.

While rates of spontaneous mutation are relatively low, rates for epigenetic change have been estimated to be much higher. Epigenetic change has been described as a heritable alteration in phenotype that is not the result of a genotypic alteration (Lewin, B., 1980). The process of differentiation is commonly cited as an example of this kind of change. Peterson (Peterson, J.A., 1979), in analyzing the variation in albumin production by clones of H$\&$IIE-C3 rat hepatoma cells, calculated a rate of phenotypic variation of $0.5$ to $1.4 \times 10^{-2}$ per cell per generation for five hepatoma clones sequentially subcloned.
He suggest an epigenetic mechanism may be responsible for this high rate of variation. This has also been suggested as a contributing factor in the expression of the high frequency of spontaneous variation in the expression of AHH activity in clones of the mouse hepatoma cell line Hepa 1c1 (Forster-Gibson, C.J. and Dufresne, M.J., 1986). The occurrence of significant variation from the parent populations in their level of inducible AHH induction in clones and subclones of Hep3B and HepG2 is not inconsistent with this interpretation. However, the author of this thesis believes that another factor contributes to variation in Hep3B and HepG2, specifically drift based on the dividing characteristics of tumor cells in culture.

A review by Fidler and Hart (1982) of the biological diversity of metastatic tumors and tumor cell lines indicates that very high levels of variation exist in the metastatic potential of cloned populations. For example, a melanoma-K-1735 established in culture and cloned after the fifth passage in vitro produced 20 out of 22 clones that differed significantly from the parent tumor with respect to metastatic capacity. Similarly, 15 of 17 clones of the B16 melanoma line and 15 of the 21 clones from a recently induced fibrosarcoma differed significantly from the parent tumor in this regard. Thus the high rate of variation seen in AHH induction in Hep3B and HepG2 may, in fact, be
another example of inherited "drift" which is dependent on the origin of the tumor cells and their time in culture. Regardless of the mechanism of variation, i.e. genetic, epigenetic, chromosome distribution and/or tumor cell diversity, it is likely to interfere with the reliability and reproducibility of AHH measurements. On the basis of these results it would seem more appropriate to assay cells in culture as soon as possible after establishment in culture. Alternatively, a large number of measurements should be taken at various time points. The average value thus obtained might better reflect the level of AHH activity of the original sample.

In view of the high degree of inherited variation observed in Hep3B and HepG2 it is likely that variation will continue to be a problem in the measurement of AHH activity in any cell system. Therefore, the measurement of AHH activity may in fact not be appropriate as a clinical parameter. It has been suggested for example, that an alternate enzyme such as NADH-dependent cytochrome b₅ reductase (using cytochrome c as a substrate) be used as a basis for comparing AHH activities among different individuals. (Kouri, R.E., et al., 1982). This lymphocyte-associated enzyme is immunochemically similar to the NADH-cytochrome b₅-reductase and cytochrome b₅ found in liver microsomes, spleen microsomes, erythrocytes and liver mitochondrial membranes.
(Prough, R.A., et al., 1976) but distinct from NADH cytochrome P-450 reductase. Moreover, this microsomal enzyme is not influenced by mitogen activation of AHH induction and can be used as a marker for total cells. Alternatively, the detection of nuclear receptor in human cells may also be used as an indicator for AHH induction.
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