Biochemical and genetic studies on the expression of aryl hydrocarbon hydroxylase in murine and human cell lines by somatic cell hybridization and DNA mediated gene transfer.

Gubbi Subbaraya. Manjunath
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BIOCHEMICAL AND GENETIC STUDIES ON THE EXPRESSION OF ARYL HYDROCARBON HYDROXYLASE IN MURINE AND HUMAN CELL LINES BY SOMATIC CELL HYBRIDIZATION AND DNA MEDIATED GENE TRANSFER

by

Gubbi Subbaraya Manjunath

A thesis submitted to the Faculty of Graduate Studies and Research through the Department of Biological Sciences in Partial Fulfillment of the Requirements for the Degree of Master of Science at the University of Windsor

Windsor, Ontario, Canada

1986
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by

Gubbi Subbaraya Manjunath

ABSTRACT

Cell cultures of mouse hepatoma cell line, Hepa c1-9, the mouse L-cell line, LMTk- c1-1, and the rat hepatoma cell line, HTC c1-1, have been used to demonstrate that the lack of aryl hydrocarbon hydroxylase (benzo[a]pyrene) hydroxylase (EC 1.14.14.1) induction by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in HTC c1-1 is not caused by the lack of nuclear Aromatic hydrocarbon receptor or by a deficiency in the activity of NADPH cytochrome c (P-450) reductase (EC 1.6.4). Treatment of HTC c1-1 with 1.0 nM TCDD for 18 h resulted in a 5-6 fold increase in reductase activity without concomitant increase in aryl hydrocarbon hydroxylase activity. This is the first report of induction by TCDD of NADPH cytochrome c
reductase.

Somatic cell hybrids were formed between AHH inducible mouse hepatoma (Hepa cl-9) cells resistant to 8-azaguanine (hypoxanthine guanine phosphoribosyl transferase negative) and AHH noninducible human primary lung carcinoma (R1 cl-6) cells resistant to 5-bromodeoxyuridine (thymidine kinase negative). TCDD inducible AHH activity, a phenotype specific to the mouse Hepa cl-9 was not expressed in the hybrids.

A thymidine kinase negative variant of human primary lung carcinoma cell line, obtained at a frequency of $3.75 \times 10^{-6}$, was transfected with the marker thymidine kinase gene using two recombinant plasmids containing this gene. A transfection frequency of $5.5 \times 10^{-5}$ was obtained. The transfected variants were phenotypically very stable, and noninducible for aryl hydrocarbon hydroxylase by TCDD.
To my Parents

X
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<tr>
<td>Ah</td>
<td>Aromatic Hydrocarbon</td>
</tr>
<tr>
<td>AHH</td>
<td>Aryl Hydrocarbon Hydroxylase</td>
</tr>
<tr>
<td>8-AG</td>
<td>8-Azaguanine</td>
</tr>
<tr>
<td>BA</td>
<td>Benz[a]anthracene</td>
</tr>
<tr>
<td>BP</td>
<td>Benzo[a]pyrene</td>
</tr>
<tr>
<td>BCDGP</td>
<td>Benzo[ghi]perylene</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-Bromodeoxyuridine</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>HGPRT</td>
<td>Hypoxanthine Guanine</td>
</tr>
<tr>
<td>HAT</td>
<td>Phosphoribosyl Transferase</td>
</tr>
<tr>
<td>MEM</td>
<td>Alpha Minimal Essential Medium</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic Aromatic Hydrocarbon</td>
</tr>
<tr>
<td>PB</td>
<td>Phenobarbital</td>
</tr>
<tr>
<td>TK</td>
<td>Thymidine Kinase</td>
</tr>
<tr>
<td>TCDD</td>
<td>2,3,7,8-Tetrachlorodibenzo-p-dioxin</td>
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INTRODUCTION

The endoplasmic reticulum of most cells contains flavoproteins and cytochromes organized in enzyme systems that function in electron transport reactions requiring oxygen and reduced pyridine nucleotides. One of these, the microsomal cytochrome P-450 dependent mixed function oxidases constitute a family of 9, 7 or 4 isozymes in rat, mouse and rabbit respectively (Funae and Imaoka, 1985). They metabolize numerous drugs, environmental pollutants, chemical carcinogens, other foreign compounds like pesticides, and endogenous substrates such as steroids (Nebert et al; 1975, Nebert and Felton, 1976). The mixed function oxidases, or monoxygenases, transfer one atom of oxygen to the substrate and the other atom of oxygen is reduced to water. This basic pattern of metabolism of the multistep substrate enzymes results in an increased hydrophilicity of the product.

The three functional components of the mixed function oxidase system (MFO) are: (1) a phospholipid fraction which plays an essential role in electron transfer from NADPH to cytochrome P-450; (2) a flavoprotein called NADPH cytochrome c(P-450) reductase which serves as electron carrier shuttling electrons from NADPH to cytochrome P-450;
(3) a heme protein which is the substrate and oxygen binding site of the MFO system. The role of cytochrome P-450 in the oxidative metabolism of a broad spectrum of organic compounds is summarized in Figure 1. Reductase and P-450 are membrane bound and occur in a mole ratio of approximately 1/20 (reductase/P-450) depending on the state of induction in the microsomal membranes (Miwa and Lu, 1984).

In the mouse, cytochrome P1-450 is associated with aryl hydrocarbon hydroxylase. This activity can be induced 8 to 50 fold by polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene (BP), benz[a]anthracene (BA), 3-methylcholanthrene (3-MC) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Lang and Nebert, 1981, Negishi and Nebert, 1979, Okey et al; 1980, Dufresne and Dosescu, 1985). The TCDD inducible P-450 gene family represents a small subset of the overall number of P-450 genes (Nebert and Gonzalez, 1985). At present five P-450 gene families (with less than 50% similarity of their aminoacid sequences) are known to exist each with two to more than twenty genes and pseudogenes (reviewed by Nebert and Gonzalez, 1985). The mouse TCDD inducible and rat phenobarbital inducible gene families probably diverged from a common ancestor more than 200 million years ago (Gonzalez, et al; 1985).
Scheme for the Mechanism of Cytochrome P-450 Dependent Hydroxylation Reactions. RH represents a substrate and ROH the corresponding product. The arrows and numbers depict the sequence and steps respectively. (Adapted from White and Coon, 1980).

1. The interaction of oxidized ferric P-450 with the substrate to form a complex.
2. The first reduction of this complex to the ferrous state by an electron donated by NADPH transferred via the action of NADPH cytochrome c reductase.
3. The interaction of oxygen with the reduced P-450 to form an oxy-heme P-450 complex still tightly bound by the substrate.
4. Further reduction of the ternary complex of oxygen, substrate, and P-450 by a second electron donated by NADPH cytochrome c reductase and converting the bound oxygen to the oxygen radical.
5. Activation of oxygen for interaction with the organic substrate. One atom of oxygen is lost as water.
6. The other oxygen atom ('activated oxygen') is inserted into a carbon-hydrogen bond of the substrate.
7. The hydroxylated substrate is formed.
8. Dissociation of the hydroxylated substrate with the regeneration of ferric and oxidized form of P-450.
Role of the AHH Enzyme System.

Induced P450, inserts one atom of oxygen to the hydrophobic PAH substrate, BP. The first oxygenated compound i.e. epoxide undergoes a hydration reaction catalyzed by the enzyme epoxide hydrolase. The resulting transdihydriodiol of BP may be conjugated with glutathione resulting in the formation of water soluble excretable products leading to detoxification. Alternatively, the dihydriodiol may undergo a second oxygenation resulting in highly electrophilic BP-diol epoxide. This compound may bind to cellular macromolecules such as DNA, RNA, and protein and cause cell transformation and cancer. (Adapted from Nebert and Gonzalez, 1985).
Figure 2
In mouse, P₁-450 and P₃-450 represent those forms of TCDD inducible cytochrome P-450 enzymes having the highest metabolism for induced aryl hydrocarbon (benzo(a)pyrene) hydroxylase and acetanilide 4-hydroxylase respectively (Negishii and Nebert, 1979, and Nebert et al; 1984). Induction of aryl hydrocarbon hydroxylase results in increased metabolism of hydrophobic substrates.

Benzo(a)pyrene is the most studied substrate for AHH (Dipere and Ernst, 1978, Fig 2). Products of BP metabolism such as dihydrodiol exhibit increased polarities which facilitates rapid elimination of these products from the organism. However, before these products are formed, reactive intermediates such as 7,8-diol-9,10-epoxide are formed. These have been proved to be toxic, mutagenic and carcinogenic (Conney, 1982). This latter activity, known as "activation", is also associated with the aryl hydrocarbon hydroxylase (AHH) enzyme system. This is an important process which determines the carcinogenic potential of any PAH.

The relationship between the capacity for moderate to high levels of AHH induction by PAH and tumor incidence in mice is well established (Kouri et al; 1974, Kouri et al; 1980), and is significant to the problem of human tumorigenesis for many reasons.
First, AHH activity has been detected in virtually every human tissue and cell system examined (Merrill and Campbell, 1974, Okuda, et al; 1977). Second, humans are unavoidably exposed to PAH inducers of AHH activity which may be carcinogenic and/or toxic. The relevance of AHH induction does not extend only to carcinogenesis. It is precisely related to TCDD toxicity in the environment. Finally the genetically mediated inducibility of AHH has been shown to be associated with lung cancer in humans (Kellerman, et al; 1978, McLemore, et al; 1981, Nebert, 1981).

In murine systems, induction of aryl hydrocarbon hydroxylase activity is regulated by the Ah gene complex. This gene complex is viewed as a combination of regulatory, structural and possibly temporal genes. Regulation is mediated by the product of a regulatory gene, called the Ah receptor (Poland, et al; 1976). The molecular events leading to the induction of AHH activity by PAH have been extensively studied (Okey, et al; 1980). The various steps are as follows:

(1) A PAH passively diffuses into a cell where it binds specifically to the Ah receptor.

(2) Once formed, the PAH-receptor complex is translocated from the cytosol to the nucleus.
(3) The PAH-receptor complex interacts with relevant structural genes of the \textit{Ah} gene complex. These events result in stimulation of transcription and translation of AHH associated structural genes such as cytochrome \textit{P}$_1$-450 and ultimately an increase in metabolism of polycyclic aromatic hydrocarbons (Israel and Whitlock, 1984).

Significant progress has been made towards elucidating the genetic regulation of AHH induction using whole animal and cell culture systems. In whole animal systems for example, TCDD inducible mouse cytochrome P-450 isozymes, \textit{P}$_1$-450 and \textit{P}$_3$-450 are believed to be members of the same gene family (Kimura, \textit{et al.}; 1984). This conclusion is based on the following observations:

(1) Both proteins are most closely associated with AHH activity;

(2) Both isozymes are located on mouse chromosome 9;

(3) Both are regulated by the \textit{Ah} receptor;

(4) Both proteins have similar cDNA and aminoacid sequences;

(5) They have similar nucleotide and protein homologies of 68\% and 73\% respectively;

(6) The genes for both proteins have 7 exons and similar intron-exon patterns (Kimura, \textit{et al.}; 1984).

Very recent work reported the complete full length cDNA nucleotide sequence of cytochrome \textit{P}$_1$-450 and \textit{P}$_3$-450 (Gonzalez \textit{et al.}; 1985).
In cell culture systems the technique of somatic cell hybridization has provided a suitable approach to the study of expression of AHH induction. This approach which led to the identification of three different genes that affect the functioning of the Ah receptor raises the most valuable question whether the receptor may be composed of more than one subunit (Hankinson, et al; 1985). For this reason, it is worthwhile to pursue the same for genetic studies on the expression of aryl hydrocarbon hydroxylase.

Hankinson has isolated variants of inducible mouse hepatoma cells Hepa-1 resistant to benzo[a]pyrene. These mutants are AHH noninducible or very poorly inducible (Hankinson, 1983). In situ somatic cell hybridization between the noninducible mutant and the inducible wild type demonstrated that the mutations were recessive. Three classes of induction variants were classified into three complementation groups, i.e. genes A, B, and C (Hankinson, 1983). Class B and C mutants were defective in the functioning of the Ah receptor. Cytochrome P_{1-450} mRNA in these mutants was synthesized at the same level as in AHH responsive wild type Hepa-1 cells. The AHH nonresponsive mutants of class A lacked cytochrome P_{1-450} mRNA. The results of complementation analysis and molecular characterization of these mutants suggested that gene A may be the structural gene for cytochrome P_{1-450}. 
Further evidence for this conclusion was obtained by treatment of recipient class A mutants with calcium phosphate precipitates of genomic DNA obtained from rat, mouse, and human cells using DNA mediated gene transfer. (Montisano and Hankinson, 1985).

Another research group (Miller and Whitlock, 1981, Miller et al; 1983) isolated and characterized two groups of mutants. Group 1 variants of mouse hepatoma Hepa-1cl cells showed low levels of basal and induced AHH activity; these cells also possessed an Ah receptor with altered binding characteristics for the ligand (TCDD). However, in these mutants the nuclear binding of the TCDD-receptor complex was normal. Group 2 variants displayed normal ligand binding to the Ah receptor, but were altered with respect to the accumulation of the TCDD-receptor in the nucleus. This group had no capacity for basal or induced AHH activity. Cell to cell fusion followed by complementation analysis showed that both mutants were recessive with respect to wild type, and that different genes harboured these defects.

Biochemical and genetic analysis of another class of variants of Hepa-1cl yielded mutants which overtranscribe the P₁-450 gene upon TCDD treatment (Jones et al; 1984). The mutant is codominant to the wild type. These authors postulated that the variants contained an altered cis-acting genomic element regulating the expression of P₁-450 gene.
Based on these results, it has been postulated that group one variants contain a lesion in the ligand binding domain and group two contain a lesion in the chromatin binding domain (Whitlock and Galeazzi, 1984).

The studies described thus far have utilized intraspecific (i.e. same cell type) somatic cell hybridization to examine the linkage groups by complementation analysis. The hybrids produced in these studies are important only for this purpose. Therefore, the application of these studies via this experimental approach becomes limited for further analysis, for example in the area of phenotypic expression of AHH activity and the regulatory gene product, the Ah receptor. For these reasons interspecific hybridization as an approach is more informative. The regulation of expression of inducible AHH activity has also been examined using interspecific (i.e. different cell type) somatic cell hybridization. Studies recently published from our laboratory (Dufresne and Dosevscu, 1985) have examined the mechanism of AHH induction in TCDD-noninducible mouse L-cells (LMtk^-) x TCDD-inducible mouse hepatoma (Hepa cl-9) cell hybrids. In brief, it was shown that the TCDD inducible AHH activity expressed in Hepa cl-9 was also expressed in hybrids. Furthermore, the molecular characteristics of the Ah receptor were qualitatively and quantitatively similar in Hepa cl-9 cells and hybrids.
On the basis of these results, the authors conclude that the induction of the Ah gene complex at the genome level, appears to involve the expression only of the Hepa cl-9 regulatory and structural genes. In other words these results suggest that the expression of inducible AHH activity is "dominant" in hybrids.

These results from cell culture systems used raise several questions:

(1) Is the dominance in the expression of aryl hydrocarbon hydroxylase observed in murine cell hybrids be observed in the more relevant human cell systems?

(2) What does this dominance in the expression of Ah regulatory and structural genes reflect at the molecular level?

(3) What is the contribution of specific genes to the induction of AHH activity?

To answer question 1 outlined above, pattern of expression of TCDD inducible aryl hydrocarbon hydroxylase must be examined in a somatic cell hybrid formed between AHH inducible mouse x AHH noninducible human cells. Initial studies on the feasibility of plasmid DNA mediated marker thymidine kinase gene transfer, into AHH negative murine and human cell recipients represents a first step in the direction of plasmid DNA mediated restoration of aryl hydrocarbon hydroxylase activity to the same recipients by transfection.
This is because, the transfectibility and sensitivity of the recipient cell to selection are two important prerequisites for the successful transfer of cytochrome P₄₅₀ inducibility and enzymatic activity.

The ability to introduce isolated DNA containing specific sequences into mammalian cells has proven to be one of the most powerful tools for the study of mammalian gene expression. The technique is currently being used to identify and analyze transcriptional control signals in eucaryotic genomes, to investigate RNA splicing, to analyze mechanisms of gene modulation by regulators such as hormones and chemicals during differentiation, and to identify and analyze cellular oncogenes implicated in carcinogenesis (Shih and Weinberg, 1982).

Various methods are available to introduce genes into mammalian cells. These methods can be divided into direct or indirect methods of introducing DNA. Microinjection of purified DNA into the nuclei of somatic cells can be considered a direct method. Some workers have microinjected somatic cells with purified donor DNA with or without a selection system, clonally derived populations of cells were then analysed for the molecular presence and expression of the genes included on that exogenously added DNA (Anderson, et al; 1980). Indirect methods of introducing DNA would involve packaging DNA in a variety of forms and presenting it to the cultured cells.
The most widely used indirect method is the calcium phosphate coprecipitation of the DNA. Early workers showed that the infectivity of the adenoviral DNA is enhanced if the DNA is mixed with calcium and phosphate (Graham and van der Eb, 1973). This technique was later used to show that specific restriction fragments of Herpes Simplex Viral (HSV) DNA are capable of conferring the wild type thymidine kinase (TK+) phenotype to mouse cells deficient in this gene (Wigler, et al; 1977, Maitland and McDougall, 1977).

Since then several variations of this protocol have been used to introduce exogenous genes into mammalian cells (Alleem, 1983). The genes transferred are prokaryotic, viral, and eucaryotic (Pellicer, et al; 1978, Wigler, et al; 1979, Wold, et al; 1980, Hassel, et al; 1980, Mulligan and Berg 1980, Jolly et al; 1982). The basic protocol involves mixing DNA with calcium chloride and a phosphate buffer solution to form a fine precipitate. This precipitate is presented to cultured cells. The work cited above have further shown that the donor DNA is stably integrated into the host cell genome and gene coded products expressed. Among the sources of transfecting donor DNA introduced in this manner are genomic (Jolly, et al; 1982), viral (Pavalalis and Hamer, 1983, Milrani-Rosenbaum et al; 1983, Murray, et al; 1983), or phage vectors (Ishiura, et al; 1982).
Most of the early transfection experiments were conducted with microgram quantities of selectable DNA often mixed with a large excess of Salmon sperm, calf thymus or homologous cell DNA as carrier and are believed to be essential for gene transfer. Results obtained from subsequent studies clearly demonstrated that there is no absolute need for carrier DNA in DNA mediated gene transfer experiments (Shih and Weinberg, 1982).

Other experiments involving the transfer of the TK+ phenotype to mouse cells showed that linearized plasmid is five times more efficient than its circular counterpart (Colbere-Garapin et al; 1979). A number of studies have been conducted to examine the uptake and the fate of the exogenously added DNA in the recipient cell. It has been shown that the expression of foreign DNA and hence the success of gene transfer depends upon the formation of DNA-calcium phosphate complex, at the time of presentation to cultured cells in medium containing serum. Naked DNA added to cultured cells was rapidly degraded. On the basis of these results, it was concluded that the DNA-CaPO₄ complex is resistant to degradation (Loyter, et al; 1982). The exact mechanism by means of which the DNA present in the cytoplasm moves into the nucleus is not understood. The DNA that enters the nucleus may remain unintegrated.
Alternatively, it may become covalently integrated into the host cell DNA. An approach to check these possibilities involves the measurement of the stability of the phenotype in the absence of selection. If the DNA is unintegrated, it may not segregate equally to the progeny cells after each cell division. Such an improper segregation results in the loss of phenotype among a certain percentage of cells in the population.

A typical experiment to distinguish between these possibilities is to use the principle of selection against or for selective genetic markers.

Selectable markers are genes whose expression in cells can be positively selected for using simple chemical additions to the growth medium and are therefore of great importance in the selection of transformants in gene transfer experiments. They can be classified into recessive or dominant acting. Recessive genes are those that can be introduced only into cells which are mutant and which lack the normal expression of the wild type gene. The genes coding for thymidine kinase (TK), adenine phosphoribosyltransferase (APRT) and hypoxanthine phosphoribosyltransferase (HPRT) are examples of this category. Dominant acting genes are those that can be introduced to any cells, the methotrexate resistant dihydrofolate reductase gene belongs to the latter type.
The transfer of recessive genes depends entirely on the availability of mutant cells lacking the specific genes. Recently genes are available which can be introduced to any type of cells. Two of the widely used genes are the bacterial neomycin resistance gene and the bacterial xanthine phosphoribosyl transferase genes (Mulligan and Berg, 1980).

Thymidine kinase (TK) genes are expressed in most eukaryotic cells. The enzyme is a part of the salvage pathway for the synthesis of thymine nucleotides and converts thymidine into thymidine monophosphate. HAT medium selects for TK+ cells because it contains aminopterin in addition to hypoxanthine and thymidine. Aminopterin inhibits one carbon metabolism and thus inhibits de novo synthesis of TTP from dUMP as well as de novo synthesis of dATP and dGTP. Hypoxanthine is a substrate for the salvage pathway for dATP and dGTP and therefore allows these nucleotides to be synthesized. However, with HAT medium the syntheses of TTP is totally dependent on an exogenous source of thymidine and an active TK gene.
Thymidine kinase genes can be introduced into cells deficient in this enzyme and HAT medium used to select for TK+ transformants. The mouse L-cells are noninducible for AHH activity, yet are the best known thymidine kinase gene recipients, because they have a negligible rate of spontaneous reversion and a high efficiency of transfection. A clone of this line (LMtk− cl-1) has been used for somatic cell hybridization in our laboratory.

RESEARCH OBJECTIVES

The majority of studies on the genetics of aryl hydrocarbon hydroxylase induction have utilized rodent tissue and cell culture systems. But little is known about biochemical and genetic mechanisms of expression of AHH activity in human cell culture systems. To gain an insight into this area the following objectives have been addressed:

(1) Characterization of the expression of inducible AHH activity by PAH's in mouse, rat and human cell lines. The possible involvement of the Ah receptor and NADPH cytochrome c reductase levels as limiting factors for the expression of inducible AHH activity in noninducible mouse and rat cell lines were examined.
Ah receptor has been shown to be required for the expression of inducible AHH activity. The reductase levels were examined because, expression of AHH activity might be limited by the supply of electrons via the action of the reductase (Masters et al; 1971).

(2) Examination of the genetic basis of expression of AHH activity in human cells in culture, using the technique of somatic cell hybridization. These studies were performed because (a) the technique has not been used to examine the involvement of activation or repression mechanisms in expression of AHH activity in AHH inducible mouse x AHH noninducible human cell hybrids; (b) the pattern of expression (i.e. dominance, suppression) is not known; (c) Not much is known about the genetic nature of AHH induction in human cells.

(3) Examination of the feasibility of plasmid DNA mediated marker thymidine kinase gene transfer to murine and human cells, as an initial step towards the DNA mediated restoration of AHH activity. This goal was approached by conducting transfection experiments using thymidine kinase negative mouse (LMtk- cl-1) and human (RLcl-6) recipient cells. Lack of data on transfection of human cells involved in AHH induction formed the basis for human transfection.
MATERIALS AND METHODS

Cell Lines

Hepa cl-9 used in this study, was a clone of the mouse hepatoma cell line Hepa-1c1, which was derived from a transplanted hepatoma BW 7756 originally produced in the C57 L/J mouse (Okey, et al; 1980). Mouse L cells, generally regarded as fibroblasts (i.e. produce collagen), which were 5-bromodeoxyuridine (5-BrdU) resistant and lacked thymidine kinase, originated as described earlier (Dufresne, et al; 1976), were cloned and used in the studies reported in this dissertation. HTC cl-1 is a line derived from an ascites tumor which in turn had been derived from a rat hepatoma 7288c (Thompson, et al; 1966). R1 cl-6 is a line derived from human primary lung carcinoma (HPL-R1) established in our laboratory (Dufresne and Dosecsu, unpublished results). Bromodeoxyuridine resistant and thymidine kinase negative variants of human R1 cl-6 cells originated as described later (page # 37 under "Materials and Methods). Mouse hepatoma (Hepa cl-9) cell variant deficient in the synthesis of hypoxanthine phosphoribosyl transferase (i.e. HGPRT−, and 8-azaguanine) was established in our laboratory.
All the cell lines were grown in alpha minimal essential medium (alpha MEM, Gibco Laboratories, Okey et al.; 1980) supplemented with either 5% or 10% fetal calf serum and 50\(\mu\)g Gentamicin per ml, and maintained at 37\(^{\circ}\)C in an atmosphere of 5% CO\(_2\) and 95% air.

**Cell Culture Techniques**

i) Subcultures:
Cells were maintained in continuous culture by plating one 25 cm\(^2\) tissue culture flask (Nunclon) per cell line with an initial density of approximately 4-5 x 10\(^5\) cells. Cell transfers were routinely made every 3-4 days. When the cells reached near confluency, the medium on the surface of cells was decanted. Cells were then washed with 1-2 ml of citrate saline (0.015 M citrate saline, pH 7.8 prewarmed at 37\(^{\circ}\)C), and incubated with 1-2 ml of trypsin (0.125\% [w/v]) at 37\(^{\circ}\)C for 3-5 minutes, to dissociate the cells attached to the culture dish. Trypsinized cells were collected with a pasteur pipette into growth medium in a 100 x 17 mm polypropylene tube (Falcon) at a dilution ratio of 1:4 (v/v). An aliquot of the single cell suspension was added to both chambers of a hemocytometer to determine the cell count. Cells were pelleted by centrifugation at 1500 rpm for six minutes in an IEC HNS centrifuge. The pellet was resuspended in growth medium to give the appropriate cell concentration. Cells were then plated into the required plasticware.
ii) Efficiency of Plating (E.O.P.):
Monolayer cultures of cells were used to set up four well
Linbro tissue culture multiwell plate (6.0 x 1.5 cm approx,
Flow Laboratories) at densities of $10^2$ to $10^3$ or $5 \times$
$10^2$ to $5 \times 10^3$ cells. Two wells of each plate were seeded
with each of the above mentioned concentrations of cells in
3 ml of medium per well. When most colonies contained
twenty or more cells, i.e. approximately one week after
plating, the medium was decanted, cells were washed with
citrate saline and stained with methylene blue ($7 \times 10^{-2}$
M in 50% (v/v) methanol). The wells were scored for the
number of colonies (i.e. clones) and the efficiency of
plating was estimated as the number of colonies formed,
divided by the number of cells plated, multiplied by 100.

iii) Growth Curves:
Cells were initially plated at a density of $10^5$ cells per
60 mm dish at 0 time in duplicate. Then the total number of
cells per plate was calculated approximately every twelve
hours by detachment with trypsin. The protocol was
repeated, until the majority of cells in culture detached
from the surface of the plate. The cell counts were plotted
on a semilogarithmic graph paper against the appropriate
time point. Two points lying within the log phase were
chosen, the difference between these two points on the
abscissa gives generation time, and the region where the
curve plateaus gives saturation density.
Cloning

The medium was decanted from the cells during the logarithmic phase of their growth. The cell surface was washed with warm citrate saline and the cells were treated with trypsin for 3-5 minutes, as described under "subcultures". Trypsinized cells were diluted into fresh medium and counted using a hemocytometer. Cells were pelleted by centrifugation and resuspended in growth medium at a concentration of $10^6$ cells per ml. Then a serial dilution was set up such that ten ml each of 100, 10, and 5 cells per ml of growth medium was obtained. In order to isolate homogeneous populations of cells, Linbro microtest plates (96 wells, Falcon) were then plated with four drops per well of the 5 or 10 cells per ml concentrations. Growth controls involved 3 wells of each Linbro plated with four drops of 100 cells per ml concentration. The plates were incubated at $37^\circ$C (5% CO$_2$, 95% air). Within twenty four hours of incubation all the wells were screened microscopically to identify wells containing one cell (i.e. clones). The cells were then carefully observed for colonial growth. When the growth of the clones was well established (> 50 cells/culture/well), the cells were washed with few drops of citrate saline and dissociated with trypsin. Colonies were grown into plasticware of increasing area until they could be transferred to 25 cm$^2$ Falcon tissue culture flasks, where they were allowed to grow.
These clones were characterized and used directly in experiments and / or stored frozen at \(-80^\circ\text{C}\) until further use.

**Karyotype**

Cells, growing as monolayer cultures during the logarithmic phase of growth were treated with 0.8\(\mu\text{g}\) of colcemid per ml of fresh medium for 2.5-3 hours at 37\(^\circ\text{C}\) to enrich the population for metaphase cells. At that time, the medium was decanted; cells were washed with warm citrate saline, trypsinized, pelleted in fresh medium and exposed to 0.015-0.075 M KCl for 15-20 minutes at 37\(^\circ\text{C}\). Cells were then pelleted, fixed in cold methanol : acetic acid (3:1, v/v) for twenty minutes at room temperature. Aliquots of the cell suspension were dropped onto cold slides at a distance of approximately a meter using pasteur pipettes, flamed dry, and stained for fifteen minutes with a freshly prepared solution of 4\% Giemsa (v/v in distilled water). At least ten metaphase spreads were counted per cell population.

**Cell Storage**

Long term storage of cells was necessary to minimize possible problems of loss of cells in culture due to factors such as senescence, contamination, and also alteration in growth characteristics, genotype and phenotype, which may occur during prolonged maintenance in culture.
Semilogarithmic growth phase cell cultures were trypsinized and counted as described under "subculture". Cells were then resuspended in growth medium so as to obtain a concentration of 1-2 x 10^6 cells per milliliter in a medium consisting of 75% alpha MEM + 10% (v/v) dimethyl sulfoxide + 15% fetal calf serum. The dimethyl sulfoxide (DMSO) serves as a cryoprotective agent. One milliliter aliquots of cell samples in 2-ml capacity Nunc cryotubes (Gibco Laboratories), were either directly frozen at -80°C in a ultralow Revco freezer or first frozen at -20°C for 24 hours and then subsequently transferred to the -80°C Revco freezer. The latter method proved to be more efficient with regard to cell viability as inferred from determination of concentration of total cells before and after freezing (data not shown). Approximately one week later, one of the frozen vials was quickly thawed in a 37°C water bath, diluted into growth medium and centrifuged. The pellet was resuspended, and the cell suspension inoculated into 25 cm^2 flasks. The cells were monitored for viability and contamination. This represents viability test.

Aryl Hydrocarbon Hydroxylase Assay
(Modified from Nebert and Gielen, 1972).
(See Fig 3 for the current concept of AHH activity).
Cells grown in 75 cm^2 flasks as subconfluent monolayer cultures were utilized to seed 100 mm culture dishes (Falcon) at a density of 10^6 cells /dish.
When cells were in the logarithmic phase of growth as inferred from growth curves (see "Growth Curves"), the medium was decanted from each dish. The cells were washed with citrate saline and fresh medium containing either the appropriate concentration of the PAH inducer reconstituted in solvent (dimethyl sulfoxide); solvent only (control #1); or no solvent or inducing agent (control #2) were added. The dimethyl sulfoxide concentration on the surface of the cells did not exceed 0.1% (v/v). All handling of PAH chemicals and cells during induction was carried out in the dark. The cells were incubated in the above medium at 37°C for 18 hours in an atmosphere of 5% CO₂ and 95% air. The induction period of 18 hours was chosen for all the cells used, because results from our and other laboratories have indicated that this is the time up to which the AHH activity is linear (Dufresne and Dosescu, 1985). Unless otherwise stated, all operations described below were carried out at 0⁰-4⁰C. After eighteen hour induction the medium was decanted, cells were washed with 2-ml of cold PBS buffer solution and collected by scraping with a rubber policeman into the same volume of the buffer. The composition of the PBS buffer was: 274 mM KCl; 1.5 mM KH₂PO₄, 0.15 M Na₂HPO₄·7H₂O, 0.14 M NaCl (pH adjusted to 7.6 with 1.0 N NaOH). The cells were washed three times with PBS buffer by centrifugation at 1,000 x g for ten minutes.
The final total cellular pellet was resuspended in three hundred μl of glycerol phosphate buffer. The GPO₄ buffer contained 0.25 M K₂HPO₄ and 0.25 M KH₂PO₄ in 30% (v/v) glycerol.

The possible effect of PBS buffer for washing step, at first and the resuspension of the cellular pellet in glycerol phosphate on the AHH activity was not determined. Glycerol phosphate was used as a physical resuspension medium.

One hundred μl of the whole cell suspension (in GPO₄) containing approximately one to two mgs of cellular protein per ml was added to 900 μl of a freshly prepared reaction mixture in a final volume of 1.0 ml consisting of 0.1 M MgCl₂, 0.36 mM NADPH, 0.42 mM NADH, 0.7 mg bovine serum albumin per ml in 0.2 M Tris-HCl buffer (pH 7.5). The enzymatic conversion of BP to hydroxylated BP was initiated by the addition of 50 μl of a 2 mM benzo[a]pyrene to each reaction flask at every fifteen second intervals. The mixture was incubated for 20 minutes at 37°C in a shaking water bath in open air. At that time the reaction was stopped by the addition of 3 ml of cold hexane : acetone (3.25 : 1.0 v/v). In order to ensure the maximum extraction of benzo[a]pyrene into the organic layer after the addition of hexane : acetone solvent the mixture was incubated with shaking at 37°C for ten more minutes. The flasks were covered to prevent any evaporation.
A 1.0 ml sample of the organic phase was added to 3.0 ml of 1.0 N NaOH, to extract the phenolic and other polar metabolites of BP. The concentration of the extracted hydroxylated BP in the alkali phase was immediately determined using a Turner Model 430 spectrofluorometer, with an excitation at 396 nm and emission at 522 nm using 12 x 75 mm borosilicate glass tubes. Cells from each of the control and/or treatment plates were added to two separate reaction flasks, for the determination of enzyme activities, and compared to a blank (all reaction components except the cellular protein), to assess the background fluorescence. One unit of AHH activity is defined as the amount of enzyme catalyzing per minute at 37°C the formation of hydroxylated product causing the fluorescence equivalent to one picomole of 3-hydroxybenz(a)pyrene. The specific AHH activities were expressed as picomoles of 3-OH BP formed per milligram protein per minute.
The modified version of the AH assay included

(1) the use of DMSO rather than dioxane as solvent, in order to avoid induction of cells by exposure to the solvent alone.

(2) Elimination of the step of homogenization of cells in favour of simple vortexing to minimize the time required for the assay.

(3) Increasing the pH of the reaction mixture buffer (0.2 M Tris) from 7.25-7.5. This value was chosen, because it has been established that a pH of 7.5 is optimal for induced aryl hydrocarbon hydroxylase in the Tris-chloride buffer system (Nebert and Gelboin, 1968). A pH value of 8.0 might be optimal for mixed function oxidases in general, but when these authors used a pH of 8.0 they found inactivation of induced aryl hydrocarbon hydroxylase.

Magnesium ion concentration of 0.1 M was used for all experiments, while other laboratories use low concentrations (2-3 mM). Intact cells were used for all the aryl hydrocarbon hydroxylase assays as a source of protein. The assay conditions used raises the possibility that they might have played a role in the observed variation in AH activity. This question is addressed under "Results".

With benzo[a]pyrene as a substrate in vitro, AH activity is equated with the rate of formation of 3-hydroxybenzo[a]pyrene. The 3-OH BP is measured by its strong fluorescence at an excitation wavelength of 396 nm and emission wavelength of 522 nm. The cytochrome P-450 dependent enzymatic catalysis of BP to hydroxylated metabolite 3-OH BP requires NADPH, NADH, Mg++, and oxygen. (Adapted from Nebert, 1978).
ARYL HYDROCARBON HYDROXYLASE "ACTIVITY"

BENZO[a]PYRENE

NADPH  O₂  MICROSOMES
NADH
Mg ++

DIHYDRODIOLS  QUINONES  POLYHYDROXY  CONJUGATED  COVALENTLY BOUND

PHENOLIC BENZO[a]PYRENE (3-HYDROXYBENZO[a]PYRENE)

PRODUCTS

Fig. 3
NADPH Cytochrome c Reductase Assay
(Modified from the methods of Phillips and Langdon, 1962
and Masters et al; 1967).
i) Preparation of microsomes from cultured cells:
Monolayer cultures were established by plating cells at
10^6 cells per 100 mm tissue culture dishes in ten ml of
minimal essential medium (alpha) supplemented with 5% fetal
bovine serum (Flow laboratories), and 50 μg/ml
Gentamicin (Gibco). At subconfluence, the medium was
decanted, and cell surface washed with 2 ml of cold PBS
buffer. The cells were harvested by scraping with a rubber
policeman into 2 ml of cold PBS. Usually the cells from ten
plates were collected into a single 30 ml Corex tube
(Dupont). The cells were washed three times with PBS and
centrifuged at 1,000 x g in a Sorvall RC-2B refrigerated
centrifuge at 4°C for ten minutes. The final total
cellular pellet was resuspended in 2 ml of 150 mM KCl
containing 50 mM Tris-HCl, pH 7.4 by vortexing. The
suspension was homogenized with a Polytron PT-10 (Brinkmann
Instruments) at setting 4 for twenty seconds (x6). The
cellular homogenate was centrifuged at 700 x g for ten
minutes at 4°C, to remove the nuclei and cell debris. The
supernatant referred to as the whole homogenate was
centrifuged at 10,000 x g for twenty minutes at 4°C. The
mitochondrial pellet was discarded.
The microsomal fraction was sedimented from the original mitochondrial supernatant by centrifugation at 105,000 x g for one hour in a Beckman 60 Ti fixed angle rotor using a Beckman Model L5-65, class "H" preparative ultracentrifuge. The resultant pinkish pellet constituted the microsomal fraction. The pellet was either assayed immediately or stored at -80°C for later assay. When stored at -80°C the pellet was usually assayed within a week.

ii) Enzyme Assay

All the initial rate measurements with regard to the reductase assay were performed at 25°C in a Perkin Elmer Spectrophotometer equipped with a recorder. The reaction mixture for the assay of NADPH cytochrome c reductase activity contained 0.33 M potassium phosphate buffer pH 7.6, 5 x 10⁻⁵ M NADPH, 10⁻³ M KCN, 47 x 10⁻⁶ M cytochrome c, and the enzyme in a total volume of 3 ml. The concentration of phosphate buffer used here was different from other literature values (i.e. 0.033 M). This was done to maximize the detection of basal and induced levels of reductase in cell lines. Control cuvettes contained all components except NADPH. The enzymatic reaction was initiated by the addition of NADPH to the sample cuvettes. The increase in absorbance at 550 mM due to the appearance of reduced cytochrome c, was recorded for six minutes. The specific reductase activity was estimated using an extinction coefficient of 19.6 cm⁻¹ x mM⁻¹ at 550 nm. This specific activity determined from cyt.c reduction
was expressed as nanomoles of cytochrome c reduced per milligram protein per minute. One unit of NADPH cytochrome c reductase activity is defined as the amount of enzyme which will catalyze the reduction of cytochrome c at an initial rate of one micromole per minute. Typically, two values of reductase activity for each variable were determined for each experiment. For every experiment, a substrate blank rate was always subtracted from the substrate dependent rate.

The validity of the enzyme assay under these conditions was determined. Simple experiments were performed to study the effect of concentration of the microsomal protein on the kinetics of reduction of cytochrome c. The enzyme activity was proportional to the microsomal protein concentration.

Protein determination:

The protein concentration values for AHH and NADPH cytochrome c reductase activities were determined in duplicate by the method of Bradford (1976) using a Bio-rad protein assay kit and gamma globulin as standard. This microassay measures protein concentration by the differential colour change that occurs on binding of protein to Coomassie Brilliant Blue G-250. When the protein binds to the dye, the absorption maximum shifts from 465 nm to 595 nm for the dye. The dye reagent (supplied by Biorad) contains Coomassie Brilliant Blue G-250 in methanol and phosphoric acid.
The final concentrations according to Bradford (1976) are: 0.01% (w/v) dye, 4.7% (w/v) methanol, and 8.5% (w/v) phosphoric acid. The dye was filtered before use. For every assay, 100 μl of protein sample was added to 5 ml of dye reagent, vortexed and the absorbance at 595 nm determined.

**Formation of Somatic Cell Hybrids**

i) Selection of thymidine kinase negative human R1 clone-6 cells by 5-bromo-2-deoxyuridine (5-BrdU):

(See Fig. 4 for the principle of this procedure)

Wild type human primary lung carcinoma (R1 cl-6) cells were first tested for the thymidine kinase (TK) phenotype according to the following protocol:

Stock cell cultures of mouse hepatoma (Hepa cl-9), L-cell (LMtk- cl-1), and human R1 cl-6 cell lines from a stock 25 cm² flask were used to set up cells in four well Linbro dishes (6.0 x 1.5 cm approx) at predetermined concentrations of 1 x 10³ and 5 x 10³ cells per well in duplicate for each Linbro plate. Cells were plated both in nonselective medium (alpha MEM + 5% fetal calf serum and 50 μg per ml Gentamicin) and HAT selective medium consisting of 10⁻⁴ M hypoxanthine, 4 x 10⁻⁷ M aminopterin and 1.7 x 10⁻⁵ M thymidine in the above medium. The HAT medium was prepared immediately prior to use. The cells were incubated for seven days at 37°C (5% CO₂ and 95% air) to allow for colonies to arise.
At that time, the medium was decanted, from the colonies in the wells. The wells were washed with 2 ml of citrate saline, and the colonies in the wells were fixed with 2 ml per well of 95% (v/v) ethanol and air dried. Then the colonies were stained with Giemsa. The TK⁻ mouse L-cells (LMtk⁻ cl-1) were killed in HAT (i.e. HAT⁵), because aminopterin blocks the normal de novo dCDP-dTDP step. In this case the only source of dTTP for DNA biosynthesis is through thymidine kinase. The mouse hepatoma (Hepa cl-9) and human primary lung carcinoma (R1 cl-6) cells retained their ability to grow in HAT medium, thus suggesting the HAT⁺ and TK⁺ phenotype. These R1 cl-6 cells were selected for the isolation of TK⁻ variants by treatment with 5-bromodeoxyuridine.

For the isolation of thymidine kinase negative human R1 cl-6 variants, replicate cultures were established in forty 100 mm tissue culture dishes seeded at a concentration of 8 x 10⁵ cells per plate. Initially the cells were exposed to 5-bromodeoxyuridine at a concentration of 500 μg/ml. Within thirtysix hours after plating, the plates were monitored for cell death and the concentration of the thymidine analogue was decreased to 50 μg/ml. The cells were then maintained in continuous culture in growth medium containing 50 μg/ml BrdU.
When three or four colonies resistant to this drug arose, the plates were washed with citrate saline, and the cells were detached with trypsin, and grown under continued selection pressure on plasticware of increasing area until they could be transferred into 25 cm$^2$ flasks. These clones were characterized with respect to HAT$^+$ phenotype, AHH induction and used directly in experiments, and simultaneously stored frozen for later use.

In this selection procedure thymidine kinase positive population of the wild type cells and TK$^{-}$ mutant cells used as controls were treated with BrdU. This nucleoside analog when incorporated into DNA, is lethal to the cell. BrdU however, is incorporated into DNA only if it is first phosphorylated by the action of thymidine kinase to bromodeoxyuridine monophosphate (BrUMP). Cells with functional TK activity are thus killed by BrdU (BrdU$^+$), whereas the rare TK$^{-}$ mutants will survive (BrdU$^-$).
Principle of the selection procedure used to obtain TK$^-$ mutants.

Wild type cells with functional thymidine kinase enzyme phosphorylates bromodeoxyuridine (BrdU) to bromodeoxyuridine monophosphate (BrUMP), which is subsequently incorporated into the cells DNA. Brominated DNA is extremely sensitive to visible light and the cells die. The rare and deficient (TK$^-$) mutant survives. (Modified from Watson, 1983).
BROMODEOXYURIDINE

THYMIDINE KINASE

Br UMP

Br UTP

INCORPORATED INTO DNA

THYMIDINE-KINASE-DEFICIENT CELLS SURVIVE

DNA FRAGMENTED

CELLS DIE

Fig. 4
ii) Cell Hybridization:
(Modified from Vincent and Buttin, 1979).

The parental cells of mouse Hepa c1-9 (HGPRT\(^-\), Azaguanine \(r\)) at 1.5 \(\times\) 10\(^6\) cells/dish and human R1 c1-6 (TK\(^-\), BrdU\(^r\)) at 1.5 \(\times\) 10\(^5\) cells/dish were cocultivated for 24 hours in 60 mm petri dishes in 5 ml of alpha MEM supplemented with 5% fetal calf serum and 50 \(\mu\)g/ml Gentamicin, so as to form a mixed suspension. Cells derived from the same parent cell lines were mixed in a mock cross. After incubation for 24 hours the medium was removed by aspiration, and 2.5 ml of Polyethylene glycol 1000 (50% w/v in serum-free medium) was added. After one minute the PEG was aspirated and the petri dishes were rinsed three times with 5 ml of serum-free medium. Five ml of complete medium was added, and cells were grown for a further 24 hours. They were then trypsinized, and plated at a density of 4.2 \(\times\) 10\(^4\) cells in six well Linbros culture plates containing 3 ml per well of the standard medium supplemented with 1 \(\times\) 10\(^{-4}\) M hypoxanthine, 4 \(\times\) 10\(^{-7}\) M aminopterin and 1.7 \(\times\) 10\(^{-5}\) M thymidine (HAT). Two days later, the HAT medium was changed. After one week of incubation at 37\(^\circ\)C, the colonies surviving hybrid selection were trypsinized and transferred to 25 cm\(^2\) flasks, cloned in selection medium and analyzed for chromosomal composition. Once identified, true hybrid (i.e. synkaryon) clones were frozen and/or used directly in characterization and other experiments.
The selection strategy employed involved the fusion of parental cells that are each deficient for each enzyme, followed by culture in a medium that supported the growth of cells that produce both the enzymes. The murine parent used in this hybridization is a hypoxanthine guanine phosphoribosyl transferase deficient (HGPRT⁻) mutant of Hepa cl-9 cells. The human parent is a thymidine kinase deficient mutant of R1 cl-6 cells. The HGPRT⁻ Hepa cl-9 cells were resistant to 8-azaguanine, whereas the TK⁻ R1 cl-6 cells were resistant to 5-bromodeoxyuridine. HGPRT catalyzes reactions of the bases hypoxanthine and guanine with 5-phosphoribosyl-1-pyrophosphate to form the corresponding nucleotides IMP (inosine-5-PO₄, a precursor of AMP and GMP), and GMP respectively. TK catalyzes the conversion of thymidine to TMP. AMP, GMP, and TMP can be converted to the corresponding deoxynucleoside triphosphates and incorporated into DNA. The enzymes HGPRT and TK are not required by cells under normal culture conditions, since neither enzyme is involved in the major pathway of de novo nucleotide biosynthesis.
However, they are only involved in the salvage pathway for nucleotide synthesis. In the HAT medium, the \textit{de novo} synthesis of GMP and TMP are blocked by the folic acid analogue aminopterin. Mutant cells that lack either HGPRT or TK die because of their inability to synthesize nucleotides from hypoxanthine (HGPRT\textsuperscript{−}), or from thymidine (TK\textsuperscript{−}) supplied exogenously in the medium. The hybrid cells which contain both the enzymes (HGPRT\textsuperscript{+}, TK\textsuperscript{+}), can bypass the aminopterin block and survive in HAT since it supplies exogenous hypoxanthine and thymidine. In the hybrid, one parental chromosome set which is HGPRT\textsuperscript{−} but TK\textsuperscript{+} (Hepa cl-9 cells), and the other which is HGPRT\textsuperscript{+} but TK\textsuperscript{−} (R1 cl-6 cells), complement each other, since the parental genomes are combined in one cell. Fig. 5 represents the role of HAT medium in major and minor pathways for nucleotide biosynthesis.
The major and salvage pathways for nucleotide synthesis.

On the HAT medium the major pathway of nucleotide is blocked by aminopterin. An alternative (salvage) depends on preformed bases and nucleosides and the enzymes TK and HGPRT.

Cells lacking HGPRT or TK enzymes die in the HAT selective medium.
Molecular Genetics Techniques

Preparation of Genomic DNA:
(Modified from Shih and Weinberg, 1982)

Confluent monolayer cultures of cells established in 100 x
20 mm tissue culture dishes were rinsed twice with cold PBS
(27 \( \mu \)M KCl, 1.5 mM KH\(_2\)PO\(_4\), 0.15 M Na\(_2\)HPO\(_4\) \( \cdot \)7H\(_2\)O
and 0.14 M NaCl [pH 7.5]) buffer solution. Cells were
harvested by scraping with a rubber policeman using 2 ml of
PBS into a 30 ml capacity Dupont Corex tube. Cells were
washed three times by centrifugation at 1000 x g for ten
minutes at 4\( ^{\circ} \)C. Four ml of lysis buffer (0.5% SDS, 0.1 M
NaCl, 40 mM Tris-HCl, 20 mM EDTA [pH 7.0]) containing 0.2
mg/ml Proteinase K (Boehringer Mannheim) was then directly
applied onto the cells. Cells were lysed by incubation at
37\( ^{\circ} \)C with gentle shaking for at least two hours. At that
time the viscous lysate was extracted twice with an equal
volume of phenol saturated with 0.1 M Tris-HCl, pH 7.0. The
phases were separated by centrifugation at 1,000 x g for
ten minutes at 4\( ^{\circ} \)C. The aqueous phase was extracted twice
with an equal volume of chloroform and isoamyl alcohol
(24:1, v/v). The DNA solution was made 0.2 M with respect
to the final concentration of NaCl, before precipitation
with two volumes of cold 95% ethanol at -20\( ^{\circ} \)C overnight.
Clumps of precipitated genomic DNA were then removed with a
pasteur pipette and transferred to 10 mM Tris-HCl, 1 mM
EDTA (pH 8.0). The contaminating RNA was removed by
treatment with ribonucleases A and T\(_1\) (Sigma).
Both enzymes were heated for fifteen minutes at 100 degrees centigrade immediately prior to use to inactivate contaminating deoxyribonucleases if present. They were added to final concentrations of 10 μg and 5 units respectively. After incubation for sixty minutes at 37°C, the DNA was reprecipitated with two volumes of cold 95% ethanol in the presence of 0.2 M NaCl overnight at -20°C. The precipitated DNA was finally dissolved in sterile 10 mM Tris-HCl, 1 mM EDTA (pH 8.0). The concentration of the DNA was determined by measuring the absorbance at 260 nm in a Beckman Model 25 Spectrophotometer, using the relationship (A) at A260 nm = 1, corresponds to 50 μg/ml. The purity of the isolated DNA was estimated by determining the absorbance (A), 260 nm/280 nm ratio, since the absorption maxima for DNA and protein (a common contaminant in DNA preparations) is 260 nm and 280 nm respectively. A A260/A280 ratio of 2.0 was taken to indicate absolute purity of the sample. The total cellular genomic DNA prepared by this method was used directly in experiments and/or stored at 4°C for later use.

**Isolation of Plasmid DNA by a Rapid Alkaline Extraction Procedure**

(Method of Birnboim, 1983 with minor modifications).

1. Cell Growth:

Twenty ml of LB medium (Peptone, 5g/L; Tryptone, 10g/L; NaCl, 5g/L; Glucose, 2g/L) containing 100μg of ampicillin per ml was inoculated with a single pAR-tk plasmid
bearing E. Coli strain HB 101 (F\(^-\), hsd\(_{20}\) \(r_b \, m_b^-\), rec A13, ara-14, pro A2, lac Y1) and/or pX1-tk plasmid contained in strain LE 392 (F\(^-\), hsd R 514 \(r_k^-\), \(m_k^-\), sup E 44, lac Y1, gal k\(_2\), gal T 22) previously tested for the presence of the plasmid and grown to saturation in a 250 ml flask at 37°C with vigorous shaking overnight. The following morning, 500 ml of LB medium in 2, two liter flasks containing 100\(\mu\)g per ml of ampicillin and/or 12.5\(\mu\)g per ml of tetracycline were inoculated with ten ml of the overnight culture. Both the flasks were incubated at 37°C with vigorous shaking. When the O.D. at 600 nm reached a value of 0.8, the plasmids were selectively amplified by the addition of chloramphenicol to a final concentration of 170\(\mu\)g per ml, and incubation was continued for a further 18 hour, to obtain a ten to twenty fold increase in the yield of plasmids in the LB medium. One liter culture of amplified cells were used to extract plasmid DNA by the following steps:

2. Harvesting:
The cells were harvested by centrifugation at 6000 x g in 250 ml bottles for ten minutes at 0°C in a Sorvall RC-2B refrigerated centrifuge. The supernatant was discarded, the pellet was washed with 50 ml of water and recentrifuged. This step renders the cells free of culture media and other soluble components (Mg\(^++\), salts, buffers) that might inhibit later steps.
3. Lysis:

The cells were suspended in 1 ml of 50 mM glucose-10 mM cyclohexanediaminetetraacetic acid (CDTA)-25 mM Tris-HCl, pH 8.0 (containing 10 mgs of lysozyme added immediately prior to use) and the cell suspension was kept at 0°C for thirty minutes. At that time, twenty ml of alkaline SDS (1% SDS, 0.2 N NaOH) was added at room temperature. The mixture was stirred gently with a sterile glass rod until clear and homogeneous. It was kept at 0°C for ten minutes, then 15 ml of high salt solution (3 M potassium acetate, 1.8 M formic acid) was added. The mixture was stirred more vigorously until a coarse white precipitate was formed. After standing at 0°C for thirty minutes, the precipitate was removed by centrifugation at 12,000 x g for ten minutes at 0°C.

The CDTA is used here to remove Ca++ from the cell wall thereby making the action of lysozyme more accessible. Lysozyme was used fresh because it is unstable at alkaline pH. Routine use of this technique indicated that the pH 8.0 of the lysis buffer is critical. This is so because the enzyme loses its efficiency below a pH of 8.0. Lysozyme digests the peptidoglycan layer of the cell wall. Alkaline SDS was used to cause the lysis of lysozyme treated cells, and denaturation of the chromosomal DNA. The high salt served to neutralize the alkali used earlier, and helped to precipitate chromosomal DNA, RNA, and protein.

The supernatant obtained by 12,000 x g centrifugation was
transferred to a sterile Corex tube, and two volumes of cold 95% ethanol was added. The nucleic acids were precipitated overnight at -20°C. The precipitate was recovered by centrifugation and dissolved in 5 ml of acetate-MOPS (0.1 M sodium acetate, 0.05 M morpholinopropanesulfonic acid adjusted to pH 8.0 with NaOH). The nucleic acids were precipitated a second time with two volumes of cold 95% ethanol at -20°C for one hour. The precipitate was collected by centrifugation, and dissolved in 2 ml of sterile water.

4. Lithium Chloride Treatment:
An equal volume (2 ml) of 5 M LiCl, 0.05 M MOPS (pH adjusted to 8.0 with NaOH) was added. The sample was left on ice for fifteen minutes at 0°C. The heavy precipitate formed was removed by centrifugation at 12,000 x g for ten minutes at 0°C. The very clear supernatant was heated at 60°C for ten minutes, and ethanol precipitated at -20°C for 45 minutes. The recovered precipitate was redissolved in 2.5 ml of acetate-MOPS solution. After another ethanol precipitation, the plasmid DNA was dissolved in 2 ml of 1.0 mM CDTA, 10 mM Tris-HCl (pH 7.5) buffer.

5. Removal of RNA:
The RNA was removed by treatment with ribonucleases A and T₁ to final concentrations of 10 g and 5 units respectively. The samples were incubated at 37°C for fifteen minutes. At that time 0.04 ml of 10% SDS solution and 2 ml of acetate-MOPS were added.
Precipitation of the plasmid DNA was started with the dropwise addition of cold isopropanol. The sample was left at room temperature for fifteen minutes.

6. Ethanol Precipitation:
The precipitate was collected by centrifugation at 20°C, 2 ml of acetate-MOPS solution was added, and the plasmid DNA was reprecipitated with two volumes of cold 95% (v/v) ethanol overnight at -20°C. The precipitated plasmid DNA was dissolved in 2 ml of 1.0 mM CDTA, 10 mM Tris-HCl (pH 7.5) buffer, dispensed into 100 μl aliquots in sterile 1.5 ml eppendorf tubes and stored either frozen at -20°C or at 4°C over a drop of chloroform to keep it sterile. The plasmid DNA was used directly in characterization and other experiments.

**Analysis of the Plasmid DNA by Agarose Gel Electrophoresis**
The alkali extracted plasmid DNA was characterized by agarose gel electrophoresis as follows:

i) Agarose Gel Preparation:
A 0.7 g quantity of agarose (ultrapure DNA grade purchased from Biorad) was added to 100 ml of 1 x Tris-borate (0.089 M boric acid, 0.002 M EDTA) electrophoresis buffer. The slurry was heated until the agarose dissolved. The solution was cooled to 55°C, and poured into a clean plexi glass base plate with sealed edges, and a clamped comb, the teeth of which would form sample wells. The gel was allowed to set for 30-45 minutes at room temperature. At that time, a gentle back and forth rocking motion was used to remove the
comb, taking care not to tear the bottom of the gel.
ii) Electrophoresis Conditions:
The samples were applied to a 0.7% horizontal agarose gel, electrophoresed for 4 hours at 70 V at a gradient of 4.57 V/cm, at room temperature. The DNA was visualized by staining with 0.5 μg/ml ethidium bromide for fifteen minutes. The gel was photographed using UV light with a polaroid Type 665 positive/negative land pack film. The molecular size of the DNA was estimated from the electrophoretic migration pattern of commercially available molecular weight ladders.

Electroelution for Further Purification of the 6.3 Kilobase pAR-tk Plasmid DNA:
(Modified from Maniatis, et al; 1982.)
The alkali extracted pAR-tk plasmid DNA containing a heterogenous mixture of three different plasmids showing molecular sizes of 13 kb, 6.3 kb, and 4.2 kb were loaded onto a 1% agarose gel (15 cm), and electrophoresed at 70 V for four hours at room temperature as described under "Electrophoresis". After electrophoresis, the 6.3 kb plasmid DNA band was first localized using longwavelength ultraviolet light (300-360 nm). The slices of agarose containing the DNA band was cut using a scalpel equipped with a sterile surgical blade.

After excision the gel was examined to make sure that only the 6.3 kb band was taken out.
A narrow dialysis tubing was filled with 3 ml of sterile TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Gel slices were picked up with forceps and placed in fluid filled bags. Taking care to avoid air bubbles, the dialysis bag was tied just above the gel slice. The bag was immersed in 1 x TBE buffer and placed perpendicular to the original direction. The sample was electrophoresed for 2 hours at 70 V. The polarity of the current was switched to reverse for one minute to release the DNA from the wall of the dialysis tubing. Then the dialysis clip at the top end was opened, and all the buffer surrounding the gel slice was very carefully removed, and the volume measured. The bag was rinsed with a small volume of sterile TE. The gel slices were examined under UV to check that all the DNA was eluted. The electroeluted DNA was then treated with n-butanol saturated with NaCl and TE buffer to remove ethidium bromide. After n-butanol extraction, DNA was precipitated with 2 volumes of cold 95% ethanol at -20°C overnight. The pelleted DNA was recovered by centrifugation, and dissolved in sterile TE buffer. The DNA concentration and purity were determined by ultraviolet spectrophotometry. The repurified 6.3 kb pAR-tk plasmid DNA was immediately used as a source of donor DNA to assess its biological activity in bacterial transformation and later in DNA mediated gene transfer (DMGT) experiments.
Transformation of E. Coli with 6.3 kb pBRtk Plasmid DNA:

(Modified from Dagert and Erlich, 1979.)

i) Preparation of Competent Cells:

A 10 ml culture of E. Coli HB 101 (rec-) was grown overnight in L medium (Peptone, 5g/L; Tryptone, 10g/L; NaCl, 5g/L) without antibiotics at 37°C with vigorous shaking. This overnight culture was diluted 1:100 into fresh medium (50 ml in 250 ml flask). The culture was grown to an O.D. at 600 nm of 0.6 (approx. 6 x 10^7 cells/ml). This usually took two to three hours. The culture was divided in equal volumes and placed into sterile 100 x 17 mm polypropylene centrifuge tubes and sedimented at 2,800 rpm for ten minutes at 4°C. The supernatant was discarded. The cells were resuspended in half the original volume of an ice cold sterile solution of 50 mM CaCl_2.

The cell suspension was placed in ice for 30 minutes and then centrifuged at 2,800 rpm for 10 minutes at 4°C. The supernatant was decanted and the cells were resuspended in 1/10 th of the original volume of an ice cold sterile solution of 50 mM CaCl_2. The competent HB 101 cells were dispensed into sterile prechilled eppendorf tubes in 0.2 ml aliquots and stored at 4°C for 12-24 hours.

ii) Transformation Assay:

Five hundred nanograms of repurified 6.3 kb DNA, 3rd-band plasmid DNA, and pBR322 DNA (control) dissolved in TE buffer were added to 0.2 ml of cells.
The DNA was gently mixed with the cells and allowed to remain on ice for two hours. At that time, the samples were subjected to heat treatment at 40°C for 2 minutes, then brought to room temperature. Cells from each transformation reaction were layered on freshly prepared L agar plates containing 50 μg/ml ampicillin and 12.5 μg/ml tetracycline both alone and in combination. The control cells were plated on agar without any antibiotics. The plates were incubated in an inverted position at 37°C until the putative transformed clones arose. The colonies growing on selection media were picked with a sterile toothpick, arrayed using a patch master agar plate casted in L medium supplemented with 50 μg/ml ampicillin and 12.5 μg/ml tetracycline. The plates were stored inverted at 37°C. The 6.3 kb p<sup>A</sup>R-t<sup>k</sup> plasmid DNA transformed clones of E. coli HB 101 were used to extract the DNA by the rapid alkaline extraction procedure, and directly used in DNA transfection experiments.

**DNA Mediated Gene Transfer:**

(Modified from the method of Graham and van der Eb, 1973)
(See Figures 6 and 7 for a brief description of the plasmid DNA sources used in transfection experiments.)

Step 1. Preculture of Recipient Cells:

The transformation involved the use of mouse L<sup>Mtk</sup>~ cl-1 cells and a 5-bromodeoxyuridine resistant genetic variant of human primary lung carcinoma cells as recipients. On day 1, cells in the logarithmic phase of growth in stock 75 cm<sup>2</sup> flasks were dissociated with trypsin, and plated at a density
of 5 x 10^5 cells per 100 mm culture dishes (Nunc) containing 10 ml of alpha MEM supplemented with 5% fetal calf serum and 50 μg/ml of Gentamicin. The cells were incubated for 24 hours at 37°C under 5% CO2 and 95% air.

Step 2. Dilution of the Donor DNA and Calcium Phosphate Coprecipitation:
The transfecting recombinant plasmid DNAs (pAR-tk and pX1-tk) and ethanol precipitated calf thymus carrier DNA were first diluted in sterile transfection buffer (double strength (2x) HEPES-P04-buffered saline (HeBS) solution consisting of 280 mM NaCl, 50 mM HEPES (Sigma), 1.5 mM Na2PO4, pH 7.1), so as to yield a final concentration of 500 ng/ml. 2 M CaCl2 prepared immediately before use was added to a final concentration of 250 mM. To ensure gentle mixing, the tubes were flicked by hand 2-3 times. The DNA-calcium phosphate coprecipitate was allowed to form without any further agitation for thirty minutes at room temperature.

Step 4. Adsorption:
After the formation of the bluish fine calcium phosphate DNA precipitate, 1 ml of the suspension containing 20 μg of calf thymus carrier DNA and 500 ng of plasmid DNA was added directly to 100 mm dishes in 10 ml of the growth medium. The cells were maintained at 37°C for 24 hours.
Step 5. Expression:

After the adsorption period, the medium containing calcium phosphate DNA precipitate was removed from the transfected cultures, by aspiration. The cells were washed once with 2 ml of sterile 2 x HeBS buffer, fed again with 10 ml of fresh medium, and incubated for a further period of 24 hours at 37°C under 10% CO₂ and 90% air.

Step 6. Selection:

At 24 hour postexpression, the medium was replaced with HAT selective medium (alpha MEM supplemented with 5% fetal calf serum and containing 10⁻⁴ M hypoxanthine, 4 x 10⁻⁷ M aminopterin and 1.6 x 10⁻⁵ M thymidine) to select cells with thymidine kinase expression after DNA transfection. The cells were maintained under continued selection pressure for at least two weeks, at 37°C. The HAT medium was renewed every day for the first three days, and then once every 3 days. When TK+ transformant clones arose, they were scored, isolated and expanded for characterization studies.

Biohazards:

The transfection of mouse LMtk⁻ cl-1 and thymidine kinase negative human primary lung carcinoma cell lines to the TK+ phenotype with the DNA of cloned HSV TK gene contained in recombinant plasmids were carried out using a "Level B" containment facility and according to the guidelines established by the Medical Research Council of Canada.
Stability of the Transfected Thymidine Kinase Gene

(Method of Wigler et al., 1979.)

The stability of the HAT-resistant phenotype was monitored by removing the transfected cells from HAT selective medium and growing in nonselective medium. At various times of 10, 20, and 40 days after transfer to nonselective conditions, $10^3$ and $5 \times 10^3$ cells were plated in duplicate in both HAT selective and nonselective medium in 10 ml per well of a four well Linbro plate. Ten days later, the wells of each four well Linbro were stained with methylene blue and the colonies were counted. The plating efficiencies were measured under selective and nonselective conditions.

Phenotypic stability was calculated as the ratio of plating efficiency under selective conditions to that under nonselective conditions. This ratio (relative plating efficiency) was used as an indication of the percentage of the population which retained the HAT" phenotype. A transfectant was considered phenotypically stable, if at least 60% efficiency of plating was demonstrated when returned to HAT, after growth in the absence of selection pressure for a defined period of time.
Figure 6.

pΔR-tk plasmid

Size: approx. 6.3 kb in length
Selective markers: Amp\(^r\), Tet\(^r\)
Single sites: Hinc II, Hind III, Bam HI, Sal I, Eco RI
Insertional inactivation: Amp\(^r\)-Hinc II
Tet\(^r\)-Bam HI, Sal I, Hind III
Reference: Dr. Barry Barclay (personal communication)
Comments: p R-tk is a derivative of pBR322 in which the Herpes Simplex Virus thymidine kinase gene is inserted into the Pvu II site. It is a versatile cloning vector and a source of transfecting TK DNA.
pXI-tk Plasmid
Size: approx. 7.8 kb in length
Selection: Amp¹
Single Sites: Pvu I, Hind II
Insertional inactivation: Nil
Reference: Enquist et al; 1979
Comments: pXI-tk is a derivative of pBR322
in which the Herpes Simplex Virus
TK gene is cloned into the Bam HI
site. It is suitable for gene transfer
studies.
Fig. 7

BamHI

PvuII

EcoRI

PstI

BglII

PstI

SmaI

PvuII

BamHI

HSV-tk

3.5 kb

HindIII

ClaI

EcoRI

SphI

SalI

XmaI

NruI

HincII

RnuI

PvuI

PstI

AmpR

3.0

pX1-tk

= 7.8 kb

1.0

ori

2.0

PvuII
RESULTS

(1) Aryl Hydrocarbon Hydroxylase Induction in Four Cell Lines

The standard spectrofluorometric AHH assay which measures the formation of hydroxylated benzo[a]pyrene product was employed with some minor modifications (described in "Materials and Methods"), to determine AHH activity in four cell lines. These studies utilized 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and other PAH inducers were applied to the cells for 18 hours. The cell lines studied were:

(1) Hepa cl-9, a clone derived from the mouse hepatoma cell line, Hepa-1c1;

(2) LMtk- cl-1, a clone of mouse L-cells derived from normal skeletal muscle;

(3) HTC cl-1, a line derived from a rat hepatoma;

(4) R1 cl-6, a clone of human primary lung carcinoma cells;

Table 1 shows the induced specific activities for each cell line. Hepa cl-9 cells demonstrated a 35 fold induction of AHH activity over basal levels when pretreated with 1 nM TCDD. With BA as an inducer at 1 μM concentration, 16 fold induction of AHH activity was observed. Treatment of preinduced Hepa cl-9 cells with another PAH,
benzo[ghi]perylene, for 2 hours resulted in the decrease of AHH activity to approximately one half of the value observed in the case of cells not treated with B[ghi]P. It is possible that benzoperylene competes with TCDD for binding sites on the Ah receptor. Both TCDD (1 nM) and BA (1 μM) did not produce an inducible level of AHH activity in LMTK− cl-1, HTC cl-1, and R1 cl-6 cells. The human R1 cl-6 cells used here were established much later, and when available AHH induction was studied and included here for comparison. The human cells showed no aryl hydrocarbon hydroxylase induction with any PAH tested (Table 1). The AHH activity in these cell lines appear to be noninducible.

(2) Effect of Increasing Concentrations of 2,3,7,8-tetrachlorodibenzo-p-dioxin on the Inducibility of Aryl Hydrocarbon Hydroxylase Activity in Four Cell Lines

The possibility that the noninducible cell lines LMTK− cl-1, HTC cl-1, and R1 cl-6 might require greater inducer concentrations to express AHH activity has to be considered. In order to determine whether this is correct, cells were treated with 1, 10, and 100 nM TCDD concentrations for 18 hours and assayed for AHH activity. Exposure of cells to these varying concentrations of TCDD produced differences in their induction response (Table 2). In the case of mouse Hepa cl-9 cells, increasing the TCDD concentration above the optimal 1.0 nM level to 10 or 100 nM resulted in considerable decrease of induced specific activity. It is possible that this observed decrease might
reflect toxicity especially at higher concentrations of TCDD. However, this is not true since all cell populations demonstrated control (i.e. cells not treated) levels of survival at these concentrations of TCDD (light microscopic observations of cells after induction and just prior to the assay). These data are consistent with the hypothesis that 1.0 nM TCDD is optimal and that at higher concentrations (i.e. 10 and 100 nM) the ligand binding sites on the Ah receptor are no longer of high affinity and low capacity, but of low affinity and high capacity. The rat HTC cl-1 and the human R1 cl-6 cells showed essentially no induction at 10 or 100 nM concentrations of TCDD. However, the mouse LMtk− cl-1 cells showed a 2-fold induction at 100 nM TCDD. If a mere increase in induced specific activity, be interpreted as induction regardless of factor of induction obtained (i.e. ratio of treated to untreated solvent cultures) and for the absolute values observed, then in a pharmacological sense, these cells show AhR-induction. In the studies reported here, a cell line is interpreted to be capable of supporting aryl hydrocarbon hydroxylase induction only if it demonstrates > 5 fold inducibility compared to the control cells treated with solvent alone. The standard chosen is based on convenience, since highly inducible cells used throughout this thesis, as the results demonstrate consistently show this level of AhH activity.
The activity of mouse L-cells showed some variation but was well within the standard 3-5 fold inducibility level. Innumerable reasons may be attributed to the lack of inducibility of AHH in the cell lines, which are addressed under "Discussion". For example, it is possible that the concentration of Mg$^{2+}$ (0.1 M) used in AHH assay might contribute to the observed lack of induction, because this level of Mg$^{2+}$ ions might inhibit reductase and glucose-6-phosphate dehydrogenase both of which are required for the AHH reaction. Furthermore, intact cells used as a source of protein, might prevent the transport of exogenous reducing equivalents (i.e. NADPH, NADH), added as components of the reaction mixture, thereby making the reaction to be dependent on endogenous supply of reducing equivalents. First, all cell lines used in the studies were analyzed for AHH induction, under absolutely identical set of conditions (i.e. 0.1 M Mg$^{2+}$). If Mg$^{2+}$ caused inhibition of the two key enzymes, resulted in noninducibility of AHH activity, then regardless of whether a cell line is inducible or not, even under same set of reaction conditions identical lack of detection in inducible AHH activity should occur. Results obtained throughout the thesis, show a greater than or equal to 5 fold inducibility of AHH for inducible cell lines. For this reason, lack of AHH induction of AHH activity in tissue culture cell lines examined may not have resulted due to the use of higher Mg$^{2+}$ ion concentrations.
Second, with regard to the possibility of the dependency of exogenous source of NADPH, and NADH on the AHH reaction with the use of intact cells. It has for long been established (Nebert, 1978) that whole cell suspension and not homogenized or sonicated cells used as a source of protein in cell culture systems have consistently resulted in the detection of greater than or equal to 0.1 p mol of the product per milligram protein. Even though, the exact mechanism of regeneration of endogenous NADPH in noninducible cancer cells is not known, detection of even very low levels of inducibility in some noninducible cells and high levels in mouse Hepa cl-9 cells (See Tables 1 and 2) argue against this possibility. Furthermore, simple vortexing of cells may be sufficient for providing channels on the plasma membrane for the transport of reducing equivalents. The variations seen in levels of AHH induction in mouse Hepa cl-9 cells in results presented in Tables 1 and 2 are most probably due to the senescence of cells in culture. Results obtained on the effect of senescence on inducible AHH in mouse Hepa cl-9 cells (data not shown) have unequivocally demonstrated a 20-30% decrease in TCDD induced AHH activity with time in culture.
Table 1. *Arul Hydrocarbon Hydroxylase Activity in Four Cell Lines.*

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>INDUCER</th>
<th>CONCENTRATION1</th>
<th>SPECIFIC ACTIVITY2</th>
</tr>
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<tbody>
<tr>
<td>Hepa cl-9</td>
<td>None</td>
<td>-</td>
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<tr>
<td></td>
<td>DMSO</td>
<td>0.1% (v/v)</td>
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<tr>
<td></td>
<td>TCDD</td>
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<td></td>
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<td>B[ghi]P</td>
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<tr>
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<td></td>
<td>TCDD</td>
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</tr>
<tr>
<td></td>
<td>BA</td>
<td>1 uM</td>
<td>0.00</td>
</tr>
<tr>
<td>R1 cl-6</td>
<td>None</td>
<td>-</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>0.1% (v/v)</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>TCDD</td>
<td>1 nM</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>BA</td>
<td>1 uM</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>B[ghi]P</td>
<td>0.5 uM</td>
<td>0.00</td>
</tr>
</tbody>
</table>

1. Cells in the logarithmic phase of growth were treated with various concentrations of PAH inducing agents for 18 hours. At post-induction time, the cells were harvested and assayed for AHH activity as described in "Materials and Methods".

The abbreviations used are as follows:
BA = Benz[a]anthracene
B[ghi]P = Benzo[ghi]perylene
TCDD = 2,3,7,8-Tetrachlorodibenzo-p-dioxin
DMSO = Dimethyl sulfoxide.

2. Expressed as picomoles 3-hydroxybenzo[a]pyrene formed per milligram protein per minute.

The data represent the mean of triplicate determinations, where N=1.

The individual values differed < 10% from the mean,
Table 2. The Effect of Increasing TCDD Concentrations on AHH activity in Four Cell Lines.

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>INDUCER</th>
<th>CONCENTRATION</th>
<th>SPECIFIC ACTIVITY²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepa c1-9</td>
<td>None</td>
<td>-</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>0.1% (v/v)</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>TCDD</td>
<td>1 nM</td>
<td>38.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 nM</td>
<td>16.93</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 nM</td>
<td>8.35</td>
</tr>
<tr>
<td>L(tk) c1-1</td>
<td>None</td>
<td>-</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>0.1% (v/v)</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td></td>
<td>TCDD</td>
<td>1 nM</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 nM</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 nM</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>HTC c1-1</td>
<td>None</td>
<td>-</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>0.1% (v/v)</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>TCDD</td>
<td>1 nM</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 nM</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 nM</td>
<td>0.00</td>
</tr>
<tr>
<td>R1 c1-6</td>
<td>None</td>
<td>-</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>0.1% (v/v)</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>TCDD</td>
<td>1 nM</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 nM</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 nM</td>
<td>0.00</td>
</tr>
</tbody>
</table>

1. Cells in the logarithmic phase of growth were induced for 18 hours with different concentrations of TCDD. At post-induction time, the AHH activity was assayed as described in "Materials and Methods".
2. Expressed as induced specific activity which is picomoles of 3-hydroxybenzo(a)pyrene formed per milligram protein per minute. One unit equals that amount of the enzyme catalyzing per minute, at 37°C, the formation of hydroxylated product causing fluorescence equivalent to that of 1 pmol of recrystallized 3-hydroxybenzo(a)pyrene (Gielen and Nebert, 1971). The data represents the mean of triplicate determinations, where N=1. These differed < 10% from the mean.
(3) **Characteristics of the Ah Receptor in Three Cell Lines**

In murine systems, the expression of inducible AHH activity by polycyclic aromatic hydrocarbons (PAH) is mediated by specific binding to the Ah receptor which is considered to be a major regulatory gene product of the Ah gene complex. The presence of Ah receptor in three cell lines were detected by:

1. exposing cell lines in culture to medium containing \[^{3}H\] TCDD for one hour at 37°C;
2. treating the cytosol or nuclear extracts of these cells with dextran coated charcoal to eliminate unbound \[^{3}H\] TCDD; and
3. assessing binding by sucrose density gradient analysis. Centrifugation of samples on a sucrose density gradient separates specific \[^{3}H\] TCDD binding from non-specifically bound \[^{3}H\] TCDD. The specifically bound \[^{3}H\] TCDD is represented by a binding peak that is eliminated in cells treated with both 1 nM \[^{3}H\] TCDD and an excess (100 nM) of unlabeled TCDD.

Figure 8 presents the results of this study. Based on the criteria described above, Ah receptor was detectable in the nucleus of Hepa cl-9 and HTC cl-1 cells, and in the cytosol of Hepa cl-9 and HTC cl-1 cells. Incubation of mouse LMTk\(^{-}\) cl-1 cells with 1 nM \[^{3}H\] TCDD,
did not result in either cytosolic or nuclear peaks suggesting the absence of a detectable Ah receptor. R1 cl-6 cells derived from freshly resectioned human primary lung carcinoma, demonstrate a specific cytosolic receptor for TCDD in culture (Dufresne, personal communication 1985). These studies from our laboratory also showed that the translocation of TCDD : receptor complex into the nucleus was not detectable in this cell line.

(4) Characterization of the NADPH Cytochrome c (P-450) Reductase in Three Cell Lines

Since NADPH specific cytochrome c reductase (EC 1.6.4) is an integral component of the microsomal mixed function oxidase system, and differences in the kinetics of microsomal cytochrome c reductase have been shown to be associated with the Ah locus (Blumer and mieyal, 1978), the expression of inducible AHH activity might be limited by the supply of electrons via the action of reductase. This possibility was examined in mouse Hepa cl-9 cells, mouse LMTK- cl-1, and rat HTC cl-1 cells.

The data presented in this section were obtained through the spectrophotometric facilities kindly provided by Dr. Bruce Virgo of the Department of Biological Sciences. Table 3 presents the basal NADPH cytochrome c reductase activities for each cell line. The results showed that AHH inducible mouse hepatoma cell line Hepa cl-9 demonstrated a basal level of the
enzyme similar to the AHH noninducible LMTk cl-1 and HTC cl-1 cell lines, even though the rat HTC cells showed lower basal levels than either of the other two cell lines. The validity of the enzyme assay was determined by examining the effect of the concentration of the microsomal protein on the kinetics of reduction of cytochrome c. Figure 9 shows the relationship between the rate of reductase catalyzed reduction of cytochrome c and the concentration of the cellular microsomal protein. Under the reaction conditions used, the initial velocity of cytochrome c reduced was proportional to the enzyme concentration. Therefore, these conditions were routinely used in other experiments.

In order to characterize more closely the reductase in these cell lines, the in vitro effect of phenobarbital and TCDD on reductase activities were examined to determine induction. Table 4 shows the effect of phenobarbital (PB) on reductase activity in Hepa cl-9 and LMTK^- cl-1 cells. Incubation of mouse Hepa cl-9 cells with 1/4 M PB for 72 hours produced a 3 fold increase in the activity of reductase compared to basal levels. Incubation of mouse LMTK^- cl-1 cells, on the other hand did result in a 1.4 fold induction of reductase. These results suggest that PB treatment results in an increased level of reductase in Hepa cl-9 cells which express inducible AHH activity (Table 1). PB
showed a lower level of reductase activity in mouse LMtk cl-1 cells which do not express inducible AHH activity (Table 1).

Table 5 presents the effect of TCDD on NADPH Cytochrome c reductase activity in these cell lines. In the absence of the inducer, both mouse Hepa cl-9 and mouse LMtk− cl-1 cells have similar basal levels of the enzyme. Treatment of these cell lines with 1 nM TCDD, under conditions identical to those used for the induction of AHH activity, did result in a slight increase in reductase level in Hepa cl-9 cells. However, the rat HTC cl-1 cells demonstrated a significant 5-6 fold increase in reductase activity. The mouse LMtk− cl-1 cells were not induced for reductase.

These results were reproducible.

The human cells established much later in our laboratory were made available for later studies, as also the hybrids and transfectants, as the work progressed. For this reason, they were not characterized with regard to reductase.

To further characterize the nature of TCDD induced reductase in HTC cl-1 cells, the kinetics of induction of the enzyme were examined. The results of this study are presented in Fig 9. It is seen that the induction of NADPH Cytochrome c reductase activity is a function of the induction time in rat HTC cl-1 cells. The reductase is induced after 18 h of exposure to 1 nM TCDD. These results prove reductase induction by TCDD.
Sucrose density gradient detection of specific high affinity $[^3H] \text{TCDD}$ binding in cytosol and nuclear extracts from: (A) Hepa cl-9, (B) LMtk$^{-}$ cl-1, (C) HTC cl-1 cells. Cells in logarithmic growth were incubated with nonradiolabeled TCDD (O) 1.0 nM $[^3H] \text{TCDD}$ plus 100 nM nonradiolabeled TCDD (●) for 1 h at 37°C. Preparation of cytosolic and nuclear extracts and determination of specific binding was done according to Dufresne and Dosescu, 1985.
Table 3. Basal NADPH Cytochrome c Reductase Activities in Three Cell Lines

<table>
<thead>
<tr>
<th>CELL LINE²</th>
<th>NADPH CYTOCHROME c REDUCTASE ACTIVITY³</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMTK⁻ cl-1</td>
<td>25.46</td>
</tr>
<tr>
<td>Hepa cl-9</td>
<td>27.10</td>
</tr>
<tr>
<td>HTC cl-1</td>
<td>10.00</td>
</tr>
</tbody>
</table>

1. Mid-logarithmic phase cell cultures established in 100 mm dishes were used to prepare the microsomal fraction and to assay for NADPH cytochrome c reductase activities, as described under *Materials and Methods*.
2. LMTK⁻ cl-1 (mouse L-cell clone 1), Hepa cl-9 (mouse hepatoma cell clone 9), HTC cl-1 (rat hepatoma cell clone 1).
3. Enzyme activity is expressed as nanomoles of cytochrome c reduced per milligram microsomal protein per minute. The cytochrome c was quantitated by the reduction of oxidized cytochrome c with an $E = 19.6 \text{ mM}^{-1} \text{ cm}^{-1}$ for reduced cytochrome c.
   A substrate blank rate was subtracted from the substrate dependent rate.
   The values are based on the mean activity from duplicate determinations. These differed < 10% from the mean. N=2.
Fig. 9.
Initial velocity of the reductase catalyzed reaction as a function of the concentration of microsomal protein in mouse Hepa cl-9 cells. The 105,000 x g pellet fraction was used in this figure and in all subsequent experiments depicting measurements of NADPH cytochrome c reductase activity. Each data point is the mean of two separate determinations. Results of duplicate determinations were always within 10% of each other, N=2. The assay conditions were as described under "Materials and Methods".
Fig. 9

INITIAL VELOCITY
NANOMOLES CYTOCHROME C REDUCED MIN⁻¹

MILLIGRAM MICROSONAL PROTEIN
Table 4. The Effect of Phenobarbital on Cytochrome c Reductase Activity in Hepa cl-9 and LMtk cl-1 cells

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>Cytochrome c reductase activity²</th>
<th>n mol/min/mg protein</th>
<th>UNTREATED PB-TREATED FACTOR OF INDUCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepa cl-9</td>
<td>9.00</td>
<td>23.71</td>
<td>2.63</td>
</tr>
<tr>
<td>LMtk cl-1</td>
<td>11.85</td>
<td>17.09</td>
<td>1.44</td>
</tr>
</tbody>
</table>

1. Mid-logarithmic phase cell cultures established in 100 mm dishes were exposed to 1 μM PB for 72 h. At the end of induction period cells were harvested and assayed for NADPH cytochrome c reductase activity.
2. Mean value of duplicate determinations; these differed by < 10% from the mean, N=1.
Factor of induction is defined as the ratio of PB treated to untreated control cultures.
Table 5. The Effect of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) on NADPH Cytochrome c Reductase Activity in Three Cell Lines

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>Cytochrome c reductase activity&lt;sup&gt;2&lt;/sup&gt;</th>
<th>n mol / min / mg protein</th>
<th>UNTREATED</th>
<th>TCDD</th>
<th>FACTOR OF TREATED INDUCTION&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepa cl-9</td>
<td>25.46</td>
<td>38.00</td>
<td>1.49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LMtk- cl-1</td>
<td>27.10</td>
<td>27.10</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HTC cl-1</td>
<td>10.00</td>
<td>56.10</td>
<td>5.61</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Mid-logarithmic phase growth cultures established in 100 mm dishes were exposed to 1.0 nM TCDD for 18 hours. At the end of the induction period, microsomal fractions were prepared from the three cell lines and used for the assay of reductase activity according to procedures described under "Materials and Methods".
2. The values represent the arithmetic mean of duplicate determinations. These differed < 10% from the mean, N=3.
3. Defined as a ratio of TCDD treated to untreated control cultures.
Figure 10.
Kinetics of induction by TCDD of NADPH cytochrome c Reductase Activity in Rat HTC Cells
Monolayer cultures established in 100 mm dishes were exposed to 1 nM TCDD for various indicated time periods. At each time point, cells were harvested and used to prepare the microsomal pellet for the determination of reductase activity. Each data point represents the arithmetic mean of two separate determinations. Each value was always within 10% of the other.
SPECIFIC REDUCTASE ACTIVITY (NMOL/MIN/MG PROTEIN)

TIME OF INDUCTION (HOURS)

Fig. 10
(1) **Selection of a Thymidine Kinase Negative Variant of Human R1 Clone 6 Cells**

(See Figure 11 for the morphology of the 5-bromodeoxyuridine resistant TK⁻ variant).

A thymidine kinase deficient (TK⁻) variant of the human R1 cl-6 cells, was derived from the parental wild type cells by growth in the permanent presence of 5-bromodeoxyuridine (BrdU). This selective agent is cytotoxic to the wild type cells. The growth of the wild type cells by continuous culture in BrdU was done to ensure the elimination of possible revertants. The results of this study are presented in Table 6. It is seen that the thymidine kinase deficient variants appeared with a frequency of \(3.75 \times 10^{-6}\). Five clones were independently isolated by this procedure and were analyzed for the phenotype after mass cultivation. All five clones retained their ability to grow in selective medium containing 50 g/ml BrdU when subcultured continuously for 40 or more generations.
(2) Characterization of Bromodeoxyuridine Resistant Variants of Human RI Clone 6 Cells

The BrdU-resistant clones (1-5) were chosen for phenotypic characterization. For a precise determination of the TK phenotype, the isolated clones were tested for their sensitivity to HAT (hypoxanthine, aminopterin, thymidine) selective medium. After 7 days of initial plating the growth and the viability of the clones were measured in the presence of both selective and nonselective medium. The results are presented in Table 7. The growth of wild type human RI cl-6 cells was inhibited in the presence of bromodeoxyuridine, but not in the presence of HAT medium. The growth of mouse L-cells (LMTk- cl-1) was inhibited in the presence of HAT medium, but showed normal growth when plated in medium containing BrdU. Three out of five BrdU-resistant clones of wild type RI cl-6 cells demonstrated inhibition of growth in HAT selective medium, but not in medium containing BrdU. These clones were selected for hybridization and for the DNA-mediated transfer of the Herpes Simplex Virus thymidine kinase gene.
Table 6. The Selection of a Thymidine Kinase Deficient Variant of the Human Primary Lung Carcinoma Cells Using 5-Bromodeoxyuridine

<table>
<thead>
<tr>
<th>CELL LINE&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MEAN NUMBER OF RESISTANT COLONIES&lt;sup&gt;c&lt;/sup&gt;</th>
<th>NUMBER OF CELLS PLATED</th>
<th>FREQUENCY OF APPEARANCE OF RESISTANT CLONES</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1 clone 6</td>
<td>3.0</td>
<td>8 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>3.75 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>: R1 clone 6 resistant to 5-bromodeoxyuridine was established by continuous culture in growth medium containing 50 μg/ml BrdU. Ten replicate monolayer cultures were seeded at a density of 8 x 10<sup>5</sup> cells per 100 mm dish at the time of exposure.

The clones were isolated, and expanded into mass cultures for phenotypic characterization and other experiments.

<sup>b</sup>: R1 clone 6 cells represent a clone of the human primary lung carcinoma cells.

<sup>c</sup>: The value is based on the mean number of 10 separate determinations. These differed < 10% from the mean.

<sup>d</sup>: Defined as the ratio of mean number of resistant clones to the number of cells at the time of BrdU exposure.
Table 7. **Phenotypic Characterization of 5-Bromodeoxyuridine Resistant Clones of Human R1 Clone 6 Cells**

<table>
<thead>
<tr>
<th>CELL LINE&lt;sup&gt;b&lt;/sup&gt;</th>
<th>GROWTH MEDIUM&lt;sup&gt;c&lt;/sup&gt;</th>
<th>NONSELECTIVE</th>
<th>HAT</th>
<th>BrdU</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1 clone 6 (control)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LMTK&lt;sup&gt;-&lt;/sup&gt; clone 1 (control)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Clone 1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Clone 2</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Clone 3</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Clone 4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Clone 5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Approximately 2 x 10⁵ cells of various cell lines were plated in nonselective (drug-free), HAT (10⁻⁴ M hypoxanthine, 4 x 10⁻⁷ M aminopterin, 1.6 x 10⁻⁵ M thymidine) and BrdU (50 μg/ml) selective medium respectively. The cells were incubated at 37°C for 7 days to allow viable colonies to arise. At that time, the cells were stained with Giemsa and scored for the presence of growth.

<sup>b</sup> LMTK<sup>-</sup> clone 1 (mouse L-cell clone 1), R1 clone 6 (human wild type cells), Clones 1-5 (BrdU-resistant clones).

<sup>c</sup> The symbol (+) indicates normal growth and viability whereas (-) indicates no detectable growth and viability. Normal growth and viability is detected by a healthy monolayer of cells. The table represents one of the three experiments which gave similar results, N=3.
Fig. 11.
Morphology of the Thymidine Kinase Negative (TK−) variants of human R1 clone 6 Cells Using Light Microscopy (mag x 200):
a) Growth in nonselective medium,
b) Growth in HAT selective medium.
The Effect of 8-Azaguanine Treatment on Arul Hydrocarbon Hydrxylase in Mouse Hepatoma (Hepa cl-9) Cells

(See Fig. 12 for the morphology of 8-azaguanine resistant Hepa cl-9 cells).

The 8-azaguanine resistant and hypoxanthine guanine phosphoribosyl transferase deficient (HGPRT-) mouse Hepa cl-9 cells was assayed for AHH activity after selection for resistance to 8-azaguanine, in order to assess the utility of this cell line for the formation of somatic cell hybrids. The results of this analysis are presented in Table 6. It is seen that the 8-azaguanine resistant mouse hepatoma (Hepa cl-9) cells demonstrated an inducible level of AHH activity similar to the control wild type Hepa cl-9 cells from which the former had been derived. However, the levels of wild type Hepa cl-9 cells with regard to AHH activity were 20% that present in results presented in tables 1 and 2. Wild type Hepa cl-9 cells with higher passage numbers have shown a decrease in AHH activity of 20-30% (data not shown). The human R1 cl-6 cells did not show inducible AHH activity as expected. These results suggested that the murine parent (8-azaguanine resistant HGPRT-, Hepa cl-9) and the human parent (5-bromodeoxyuridine resistant TK- R1 cl-6) could be used for hybrid construction in which each parent carries one recessive marker, with HAT selection.
(4) Formation of Hybrid Cells

(See Figure 13 for the morphology of the RH [R1 cl-6 x Hepa cl-9] hybrid cells).

Table 9 shows the results of a cross between a HGPRT- variant line isolated from Hepa cl-9 cells and a TK- variant line isolated from human primary lung carcinoma (R1 cl-6) cells. The results demonstrate that putative hybrid colonies resistant to HAT were obtained every time the two parents were used. After 3-4 days in HAT medium, during selection for the hybrid and against the parental cells, it was observed that the unfused mouse Hepa cl-9 mutant cells and human R1 cl-6 mutant cells had rounded up and begun to detach from the substratum. This was followed by the appearance of colonies of cells that were larger than either parent (Fig. 13 demonstrates this point, and represents the morphology after isolation). When cells derived from the same cell line were mixed in a mock cross, no HAT resistant clones were obtained. The frequency of hybrid colonies obtained by this procedure was around \(10^{-5}\) of the number of minority parental cells. Two of the five hybrid clones independently isolated in this cross were immediately used for further characterization. Cells were determined in the presence of both nonselective and selective medium containing the indicated concentrations of various drugs.
(5) Phenotypic Characterization of RH Hybrid Cells

The two putative RH hybrids were characterized further with regard to the phenotype (HAT, 8-azaguanine, and BrdU). The results of this study are presented in Table 10. After 7 days of initial plating, the growth and viability of parental cells and the putative hybrid.

The growth of the murine parent (Hepa cl-9) was inhibited in HAT (i.e. HAT$^S$), and BrdU (i.e. BrdU$^S$) but not in medium supplemented with 8-azaguanine (i.e. 8-AG$^r$). The growth of the human parent (TK$^{-}$, R1 cl-6) was inhibited both in HAT and 8-azaguanine (i.e. HAT$^S$, 8-AG$^S$), but not in medium containing BrdU (i.e. BrdU$^r$). The growth of the two putative RH hybrids was inhibited both in 8-azaguanine (i.e. 8-AG$^S$) and 5-bromodeoxyuridine (BrdU$^S$), but not in the presence of HAT medium (i.e. HAT$^r$). These results confirmed the dominance of the HAT resistance phenotype in the RH hybrid cell lines.

In the hybrid selection system used in this work, the hybrids were produced after fusion of 8-azaguanine resistant mouse Hepa cl-9 cells (lacking the enzyme HGPRT$^{-}$) with human R1 cl-6 cells resistant to 5-bromodeoxyuridine (lacking the enzyme TK). Both the parental cells die in HAT, because cells are unable to synthesize nucleotides from hypoxanthine (HGPRT$^{-}$ cells) or from thymidine (TK$^{-}$ cells). The sensitivity data prove the presence of allelic genes from both the parents.
(6) Chromosomal Analysis of RH Hybrid Cells

(See Fig. 14 for the determination of chromosome numbers of parental cells and the hybrids).

A comparison of chromosome numbers of parental cells and hybrid cells are presented in Table 11. The results demonstrate that the RH hybrids contain approximately, the number of chromosomes carried by each parent. Preferential loss of human chromosomes from a mouse x human cell hybrid would explain why the hybrids do not contain absolute chromosome complement from both the parental lines. These data did provide evidence for the presence of a true hybrid.

(7) Identification of Hepa cl-9 x R1 cl-6 Sunkaryons on the basis of Electrophoresis in Single Dimension

Electrophoresis in single dimension was carried out on sodium dodecyl sulfate polyacrylamide gels according to the procedure of Laemmli, 1970. The two independently derived hybrids expressed the protein bands specific to both the parental cell lines Hepa cl-9 and R1 cl-6. These RH hybrids are designated by RH-1 and RH-2 in the studies reported in this thesis. The results of the migration pattern of cytosolic proteins from the parental cells and the hybrids is given by Fig 15.

These data provide evidence for the presence of true hybrids.
Table 8. The Effect of 8-Azaquanine Treatment on AHH activity in Mouse Hepatoma (Hepa c1-9) Cells

| CELL LINE | CONDITION | AH Activity | INDUCTION RATIO
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(nM) TCDD</td>
<td>(B/A)</td>
</tr>
<tr>
<td></td>
<td>UNTREATED</td>
<td>(A)</td>
<td>(B)</td>
</tr>
</tbody>
</table>

|       |          | 1.00        | 8.39            | 8.39            |
| Hepa c1-9 | wild type | 1.00        | 9.23            | 9.23            |
| RTK^-     | variant   | 0.00        | 0.00            | -               |

1. Cells in logarithmic phase of growth were exposed to 1.0 nM TCDD in growth medium for 18 hours. At the end of the induction period the cells were harvested and assayed for AHH activity according to the procedure described under "Materials and Methods".
2. Hepa c1-9 (mouse hepatoma cell clone 9), RTK^- (thymidine kinase negative variant of human R1 clone 6 cells).
3. The specific AHH activities are expressed as picomoles of 3-hydroxybenzo[a]pyrene formed per milligram protein per minute. The values represent the mean of 3 separate determinations. These differed < 10% from the mean. N = 3.
4. Defined as the ratio of TCDD treated to untreated control cultures.
Table 9. The Dominance of the Hypoxanthine, Aminopterin, and Thymidine Resistance Phenotype in R1 cl-6 x Hepa cl-9 Hybrid Cells

<table>
<thead>
<tr>
<th>CROSS</th>
<th>HAT SELECTIVE MEDIUM²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepa cl-9 x Hepa cl-9 (HGPRT⁻)</td>
<td>0</td>
</tr>
<tr>
<td>RTK⁻ x RTK⁻ (TK⁻)</td>
<td>0</td>
</tr>
<tr>
<td>Hepa cl-9 x RTK⁻ (HGPRT⁻) (TK⁻)</td>
<td>2</td>
</tr>
</tbody>
</table>

1. 1.5 x 10⁶ HGPRT⁻ Hepa cl-9 cells (murine parent) were seeded together with 1.5 x 10⁵ TK⁻ R1 cl-6 cells (human parent) in petri dishes containing 5 ml of alpha MEM medium. After 24 h incubation at 37°C, the cells were fused in situ with PEG 1000 and processed for the isolation of hybrid, as described under "Materials and Methods".

2. The results are presented as the number of colonies per plate (mean value of 6 plates). The hybrid clones were identified by their growth on HAT selective medium.
Table 10. Phenotypic Characterization of RH Hybrid Cells

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>GROWTH MEDIUM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NONSELECTIVE</td>
</tr>
<tr>
<td>Hepa cl-9</td>
<td>+</td>
</tr>
<tr>
<td>RTK^-</td>
<td>+</td>
</tr>
<tr>
<td>RH hybrid-1</td>
<td>+</td>
</tr>
<tr>
<td>RH hybrid-2</td>
<td>+</td>
</tr>
</tbody>
</table>

1. Approximately $1 \times 10^4$ and $1 \times 10^5$ cells of various cell lines were plated in nonselective (drug-free), HAT ($10^{-4}$ M hypoxanthine, $4 \times 10^{-7}$ M aminopterin, $1.6 \times 10^{-5}$ M thymidine), BrdU (50 ug/ml), B-AG (8 ug/ml) selective medium respectively. The cells were incubated at 37°C for seven days to allow for colonies to arise. The resulting cells were scored for the presence of growth.

2. Hepa cl-9 (8-azaguanine resistant variant of mouse hepatoma cell clone 9), RTK^- (thymidine kinase negative variant of human R1 cl-6 cells), RH (RTK^- x Hepa cl-9 hybrid).

3. The symbol (+) indicates normal growth whereas (-) indicates no detectable growth.

The table represents one of the three experiments which gave similar results.
Table 11. Chromosomal Analysis of RH Hybrid Cells

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>CHROMOSOME NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepa cl-9</td>
<td>57</td>
</tr>
<tr>
<td>RTK⁻</td>
<td>49</td>
</tr>
<tr>
<td>RH hybrid-1</td>
<td>90</td>
</tr>
<tr>
<td>RH hybrid-2</td>
<td>96</td>
</tr>
</tbody>
</table>

1. Logarithmic phase growth cultures of cell lines were treated with 0.8 μg of colcemid per ml, exposed to 0.05-0.075 M KCl for 15 minutes, fixed in cold methanol : acetic acid (3:1, v/v), for metaphase spreads as described under "Materials and Methods".
2. Hepa cl-9 (8-azaquanine resistant mouse hepatoma cell clone 9), RTK⁻ (thymidine kinase negative variant of human R1 cl-6 cells), RH (RTK⁻ x Hepa cl-9 hybrids).
3. The number presented represents mean chromosome number where N = 10.
Fig. 12
Morphology of the 8-Azaguanine Resistant Variant of mouse Hepatoma (Hepa cl-9) Cells using Light Microscopy (mag x 200):

a) Growth in culture in nonselective medium,
b) Growth in culture in HAT selective medium.
**Fig. 13.**

Morphology of the RH Hybrid Cells using Light Microscopy (mag x 200):

a) Growth in culture in nonselective medium,
b) Growth in culture in HAT selective medium.
Fig. 14.
Karyotype of Parental Cells and RH Hybrids using Light Microscopy (mag x 200):
a) Hepa c1-9,
b) RTK-,
c) RH hybrids
Figure 15.
Identification of Hepa cl-9 and R1 cl-6 Cell Phenotypes by Single Dimension Gel Electrophoresis
Cytosolic proteins were separated in a 7-10% gradient sodium dodecyl sulfate polyacrylamide gel. Channel 1, 8-azaguanine resistant mouse Hepa cl-9 cell line, channel 2, 5-bromodeoxyuridine resistant human R1 cl-6 cell line, channel 3-4, RH hybrid-1 and RH hybrid-2. The arrow designates the regions of comigration of protein bands expressed by the hybrids derived from both parents.
Determination of Induced AHH Activity in Hepa cl-9, RTK- and the RH Hybrids

The expression of inducible AHH activity, a phenotype specific to the 8-azaguanine resistant mouse Hepa cl-9 parent in this hybridization was examined in RH hybrid-1, and RH hybrid-2 when the cells were pretreated with 1.0 nM TCDD. The results of this study are presented in Table 12. It is seen that TCDD inducible AHH activity observed in Hepa cl-9 was not expressed in the RH hybrids. However, the observed low value of induced specific activity (< 1.0) is not considered significant when compared to the value with control Hepa cl-9 (18.0). Thus the positive control cells demonstrated 18 fold induction of AHH activity. The absolute specific activity was around 50% of the values presented in Tables 1 and 2. Senescence of cells in culture possibly contributed to this variation.
Table 12. Determination of Induced AHH activity in Hepa cl-9, RTK and in RH hybrid-1 and RH hybrid-2

<table>
<thead>
<tr>
<th>CELL LINES</th>
<th>INDUCER</th>
<th>CONCENTRATION</th>
<th>SPECIFIC ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepa cl-9</td>
<td>None</td>
<td>-</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>0.1% (v/v)</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>TCDD</td>
<td>1 nM</td>
<td>18.00</td>
</tr>
<tr>
<td>RH-1</td>
<td>None</td>
<td>-</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>0.1% (v/v)</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>TCDD</td>
<td>1 nM</td>
<td>0.00</td>
</tr>
<tr>
<td>RH-2</td>
<td>None</td>
<td>-</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>0.1% (v/v)</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>TCDD</td>
<td>1 nM</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

1. Cells in the logarithmic phase of growth were exposed to 1 nM TCDD for 18 hours. After induction, the cells were harvested and assayed for AHH activity as described under "Materials and Methods".
2. Hepa cl-9 (8-azaguanine resistant mouse hepatoma cell clone-9 cells), RTK (5-bromodeoxyuridine resistant variant of human R1 cl-6 cells), RH-1 (RTK x Hepa cl-9 hybrid-1), RH-2 (RTK x Hepa cl-9 hybrid-2).
3. The specific AHH activities are expressed as picomoles of 3-hydroxybenz(a)pyrene formed per milligram protein per minute. The values represent the mean of triplicate determinations, where N=2. These differed < 10% from the mean.
1) **Purification of Genomic and Plasmid Sources of DNA for Transfection**

Both genomic and plasmid DNAs were purified in order to assess their biological activity in DNA mediated gene transfer to aryl hydrocarbon hydroxylase negative recipient mouse and human cells. Table 10 provides a brief summary of results obtained from these experiments. Reproducibly, from experiment to experiment, the genomic and plasmid DNAs yielded an A 260/A 280 ratio of 1.8-2.0. The purity of the plasmid DNA was further examined by agarose gel electrophoresis.

2) **Characterization of Plasmid DNAs by Agarose Gel Electrophoresis**

The alkali extracted plasmid DNAs were characterized by agarose gel electrophoresis. Fig. 16 shows the analysis of the p\(\beta\)-R-\(tk\) plasmid DNA. Bands with size estimates of 13 kb, 6.3 kb, and 4.2 kb were observed. The 3 band pattern of purified uncut p\(\beta\)-R-\(tk\) DNA was found to be reproducible. These results suggested the necessity of further purification of the thymidine kinase gene bearing 6.3 kb p\(\beta\)-R-\(tk\) plasmid. Fig. 17 gives the pXI-\(tk\) plasmid DNA analysis and Bam HI restriction
enzyme digestion by agarose gel electrophoresis. The 6.3 kb plasmid exactly matched the expected size estimate. The 13.0 kb band might represent another plasmid, possibly a doublet of the 6.3 species. The 4.2 kb band corresponds approximately to a size of 4.3 kb representing the plasmid pBR322. These results suggested the possibility that the two different plasmids of 13.0 kb and 4.2 kb may represent vector DNA molecules that have recircularized during the cloning of the HSV-tk gene into pBR322. In order to minimize the effort required to distinguish between the transfecants, produced in DNA mediated gene transfer experiments, resulting from either the 13.0 kb plasmid or the 6.3 kb species, the relevant 6.3 kb plasmid was extensively purified and analyzed for its biological activity.

3) Transformation of E.Coli Strain HB101 with Repurified 6.3 kb pA R-tk Plasmid DNA

The 6.3 kb plasmid DNA obtained by a combination of agarose gel electrophoresis and electroelution techniques was immediately used to determine its biological activity in bacterial transformation assays. The results of this study are given in Table 14. The transformation frequency was expressed as the number of transformants per nanogram of the transforming DNA, were 0.80, 1.31, and 0.80 for pBR322, 3-band plasmid, 6.3 kb plasmid DNAs respectively. These results confirmed active 6.3 kb DNA.
Table 13. Summary of Genomic and Plasmid DNA Purifications

<table>
<thead>
<tr>
<th>SOURCE OF DNA*</th>
<th>A 260 : A 280</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMtk&lt;sup&gt;-&lt;/sup&gt; cl-1 cell genomic</td>
<td>2.00</td>
</tr>
<tr>
<td>p&lt;sup&gt;AR-tk&lt;/sup&gt; plasmid</td>
<td>1.80</td>
</tr>
<tr>
<td>px1&lt;sup&gt;-tk&lt;/sup&gt; plasmid</td>
<td>1.90</td>
</tr>
</tbody>
</table>

* Unfractionated total cellular genomic DNA was prepared from cultured cells as described under "Materials and Methods".

p<sup>AR-tk</sup> and px1<sup>-tk</sup> are recombinant plasmids containing the Herpes Simplex Virus thymidine kinase gene. Their DNA was purified by a rapid alkaline extraction procedure described under "Materials and Methods".

Both sources of plasmid DNA and genomic DNA were used in DNA transfection experiments. The data represents one of the several experiments which gave similar results.
Fig. 16.
Characterization of the p①R–tk plasmid DNA by Agarose Gel Electrophoresis.
The alkali extracted plasmid DNA in 1 mM cyclohexanediamine tetraacetic acid (CDTA), 10 mM Tris-HCl (pH 7.5) was applied to a horizontal 0.7% agarose gel. The electrophoresis buffer contained 0.08 M Tris base, 0.08 M boric acid, and 0.002 M EDTA (pH 8.0). Electrophoresis was carried out at room temperature with an applied voltage of 70 V for 4 h. Gels were stained for 15 minutes with ethidium bromide (0.1 μg/ml in 1 x TBE buffer) and photographed through a 6 x 14" filter with 302 middle wave (UV-B) illumination (Spectronics Corporation) using polaroid type 665 positive /negative film.
The figure represents one of the three experiments which gave similar results.
Figure 17.
Characterization of pX1-tk Plasmid DNA and its Bam HI
Restriction Enzyme Digestibility.
The alkali extracted plasmid DNA in TE buffer was applied
to a 0.7% agarose gel. Electrophoresis was carried out as
described in the legend to Fig 18. Restriction endonuclease
cleavage (Bam HI) was performed in buffer containing 100 mM
NaCl, 50 mM Tris-HCl, 10 mM MgCl₂, 1 mM dithiothreitol
(pH 7.5). The enzyme to DNA ratio was at least two units/ug
of DNA, and the reaction mixtures were incubated for
atleast 2 h (one unit is the amount of enzyme that digests
1 ug of DNA in 1 h).
Samples in slots A, B, and C correspond to reference
marker, undigested DNA, and Bam HI digested DNA
respectively.
Fig. 17
4) **Plasmid DNA Mediated Transfer of the Herpes Simplex Virus Thymidine Kinase Gene to AHH Negative Mouse LMtk cl-1 Cells**

(See Fig. 18 for the morphology of the recipient and transfected cells).

Murine hybridization studies from our laboratory (reviewed under "Introduction") indicated that the expression of AHH activity is dominant in the PAH inducible mouse Hepa cl-9 × PAH noninducible mouse LMtk− cl-1 cell hybrids. A phenotype also expressed in the hybrids was resistance to HAT (hypoxanthine/aminopterin/thymidine) suggesting the transfer of the thymidine kinase activity. In order to examine the molecular basis of the dominant thymidine kinase gene expression in somatic cell hybrids, and more importantly, to determine the feasibility of the application of the DNA mediated gene transfer to AHH negative mouse LMtk− cl-1 cells, transfection was carried out using purified pAR-tk plasmid DNA.

The more relevant human recipient could be transfected with a marker gene, only if a transfection system, using a recipient well characterized for genetics of AHH expression by somatic cell approach, such as LMtk− cl-1 cell line, can be established. Readily available HAT selection system was used. This technology was made available later to transfect a human cell, when the cell line was made available. Therefore, transfection of a mouse cell was significant on its own merits.
Noninducible mouse L-cells deficient in the enzyme thymidine kinase were cloned and tested for their AHH induction. These clonally derived cells were then used for DNA transfection experiments. The mouse LMtk^- cl-1 cells were chosen because they serve as AHH negative recipients that are both competent for transfection and sensitive for selection. In addition, the frequency at which the recipient cells spontaneously revert to selection resistance is lower than the frequency of transfection (<10^-9). Table 12 presents the results of these studies. As shown, the LMtk^- cl-1 cells (deficient for TK) undergo transformation to the TK+ phenotype at a rate of 8 transfectant colonies per 5 x 10^5 cells per microgram donor DNA. HAT resistant colonies were not observed on control plates. When DNA from LMtk^- cl-1 cells was used as donor, it was unable to transfer the TK gene as expected. Treatment of recipient cells with calf thymus carrier DNA alone did not result in any HAT resistant colonies. These results therefore strongly suggest that LMtk^- cl-1 cells which contain the Herpes Simplex Virus thymidine kinase gene gave rise to transformed colonies. The term transformation in this and subsequent studies is defined as the change in the genotype of a recipient cell mediated by the introduction of DNA. The results also show that transfection occurs with a low frequency (10^-5) and is detectable by the ability of the rare transformed cell to grow under appropriate selection conditions.
Table 14. **Transformation of E. coli Strain HB 101 with Further Purified 6.3 kb Plasmid DNA**

<table>
<thead>
<tr>
<th>SOURCE OF DNA</th>
<th>COLONIES / 500 ng</th>
<th>NUMBER OF TRANSFORMANTS/ng DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR322 (control)</td>
<td>400</td>
<td>0.80</td>
</tr>
<tr>
<td>3-band plasmid2</td>
<td>656</td>
<td>1.31</td>
</tr>
<tr>
<td>6.3 kb plasmid3</td>
<td>408</td>
<td>0.80</td>
</tr>
</tbody>
</table>

1. Transformations were carried out using two hundred microliters of competent cells. Selection plates were cast in agar containing L-medium supplemented with 50 μg/ml ampicillin and 12.5 μg/ml tetracycline added immediately before use. Control plates contained no antibiotics; i.e., neither ampicillin nor tetracycline. Putative bacterial clones arising from the transformation reaction were further analyzed by arraying a patch master agar plate containing Lamp and Ltet.

2. 3-band plasmid represents the heterogeneous plasmid DNA containing 13.0, 6.3, and 4.2 kb plasmids.

3. The 6.3 kb plasmid DNA containing the selectable marker thymidine kinase gene was purified from initial heterogeneous plasmid as described under "Materials and Methods".
Table 15. Transfection of Mouse LMTk cl-1 Cells with p^R-tk Plasmid DNA\(^1,2\)

<table>
<thead>
<tr>
<th>CARRIER DNA (20 μg)</th>
<th>AMOUNT OF DONOR DNA ADDED (μg/dish)</th>
<th>MEAN NUMBER OF HAT(^+) COLONIES(^3)</th>
<th>NUMBER OF TRANSFECTANTS PER MICROGRAM DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf thymus</td>
<td>0</td>
<td>0(3)(^4)</td>
<td>0</td>
</tr>
<tr>
<td>Calf thymus</td>
<td>LMTk(^-) cl-1 20(^5)</td>
<td>0(3)</td>
<td>0</td>
</tr>
<tr>
<td>Calf thymus</td>
<td>p^R-tk 0.5</td>
<td>4(3)</td>
<td>8</td>
</tr>
</tbody>
</table>

1. DNA transfections were carried out according to the method of Graham and van der Eb (see "Materials and Methods"). Five hundred nanograms of plasmid DNA and 20 μg of calf thymus carrier DNA as calcium phosphate coprecipitate was added to each dish containing 5 x 10\(^5\) cells.
2. Mock\(^0\) transfection of LMTk\(^-\) was carried out using CaPO\(_4\) alone in place of transforming DNA. Control plates of recipient cells under the transformation conditions did not receive CaPO\(_4\) or DNA.
3. The HAT selective medium consisted of 10\(^{-4}\) M hypoxanthine, 4 x 10\(^{-7}\) M aminopterin, and 1.6 x 10\(^{-5}\) M thymidine respectively.
4. The number in parenthesis represents the number of separate transfection experiments carried out with different DNA preparations.
5. Represents homologous cell genomic DNA used as a source of transforming DNA.
Fig. 18.
Morphology of untransformed and pA R-tk Plasmid Transformed Cells of Mouse LMtk^- cl-1 cells using Light Microscopy (mag x 200):
a) Growth in culture in nonselective medium of recipient,
b) Growth in culture in HAT selective medium of transfected cells
5) **Phenotypic Stability of the Transfected Thymidine Kinase Gene in Mouse L- cells**

The appearance of surviving colonies following transfection assays with the plasmid donor DNA (Table 15) could result either from the reactivation of the murine TK gene or from the integration of a new wild type thymidine kinase gene into the recipients DNA. The DNA may or may not be covalently integrated into LMTk^- cl-1 DNA. In order to distinguish between these possibilities it was asked if the expression of TK was stable in the absence of selective pressure. In these experiments, the recipient LMTk^- cl-1 cells and the transfected cells were first grown into mass culture under selective pressure. They were subsequently subcultured for various times in the absence of selective pressure. The fraction of cells that retained the TK+ phenotype was determined by measuring plating efficiencies in selective and nonselective medium. The results of this study are given in Table 16. It is seen as expected that the recipient LMTk^- cl-1 cells were unable to grow in selective medium (HAT) after passage under nonselective conditions. The plasmid transfected cell line retained the ability to grow in HAT when cultured in the absence of selective pressure for 10, 20, and 40 days respectively. These results unequivocally demonstrate the stability of the TK+ transformed phenotype, and therefore suggest that the transfected cells express the TK gene.
Many transformants of mouse L-cells, transfected with DNA for different genes such as adenine phosphoribosyltransferase (Wigler et al.; 1979), thymidine kinase (Scangos et al.; 1981) and methotrexate genes (Lewis et al.; 1980) have been characterized as generally unstable in that they lose their ability to grow in selective medium after being grown for a period of time in nonselective medium. Because this seemed a frequent attribute of transfected cells, the stability of HAT\(^{+}\) colonies were examined. The transfectants demonstrated 80-100% efficiency of plating (% of control) when returned to HAT, after switch to nonselective medium. The results unequivocally demonstrate a high level of stability of their HAT\(^{+}\) phenotype. Thus the studies suggest that mouse AHH negative L\(\text{Mtk}^{-}\) cl-1 cells transfected with plasmid DNA for a marker thymidine kinase gene is stable for the selectable phenotype.

While the molecular presence of the transfected thymidine kinase gene in these transfectants by southern blot analysis has not been done, these results strongly suggest stable integration of the TK gene into the recipient cell.

Furthermore, these results may be considered significant in the light of transformation of AHH negative hepatoma cell mutants with total genomic DNA from an AHH positive donor (Montisano and Hankinson, 1985). While these authors observed transfection of AHH activity, the transfectants were unstable, rapidly losing AHH activity with time in culture.
This occurs even after prolonged passaging under nonselective conditions.

6) **Comparison of Growth Parameters of LMTk-1 cl-1 Recipient and Transfected Cells**

Growth characteristics of the thymidine kinase gene transfer recipient and its plasmid DNA transfected counterpart were examined. The results of this study are presented in Table 17. It is seen that both cell lines, have the same generation times. With respect to the efficiency of plating, the transfected cells showed a higher saturation density as compared to the recipient mouse LMTk-1 cl-1 cells.

7) **Comparison of Aryl Hydrocarbon Hydroxylase Expression in LMTk-1 cl-1 and Transfected Cells**

To assess the effect of transfection of the TK gene (to LMTk-1 cl-1 cells) on the AHH phenotype, the levels of AHH activity were determined in both the cell lines by the standard fluorometric assay (see "Materials and Methods"). Table 18 gives the results of this analysis. Mouse Hepa cl-9 cells used as a control, demonstrated a 16 fold induction of AHH activity over basal levels when pretreated with 1 nM TCDD. BA (1 μM) produced a 6 fold induction of AHH activity. However, the treatment of LMTk-1 cl-1 and transfected cell lines with 1 nM TCDD or 1 μM BA did not result in the induction of AHH activity.
It is understood that these results were rather predictable. The reason being, a viral marker gene carried by a recombinant plasmid transfected into an AHH negative cell, cannot be expected to result in the expression of the AHH structural gene (i.e. cytochrome P<sub>450</sub> gene), because expression of AHH activity is the result of interactions between the components of a complex membrane bound multicomponent system. It was considered worthwhile to pursue the answer to this question because of several reasons. First, Hankinson's research group (Montisano and Hankinson, 1985) reported the transfection of cytochrome P<sub>450</sub> inducibility and enzymatic activity to a hepatoma cell mutant. In their system they used a free and unligated plasmid with the AHH+ genomic DNA. Transient expression of AHH activity in the recipient cell raises the question whether genomic DNA was stably integrated. The loss of AHH activity in these transfectants show that the cytochrome P<sub>450</sub> gene was perhaps not stably integrated. What is the possible involvement if any of the marker plasmid? Second, when a selectable marker plasmid gene is ligated to AHH gene, it can be transfected to a AHH negative cell. This approach will not be feasible if the phenotype of the noninducible cell is altered by the integration of the plasmid. These results demonstrate the utility of using a recombinant plasmid marker gene for the study of expression of AHH activity.
Table 16. Phenotypic Stability of the Transfected Thymidine Kinase Gene in a Transfectant clone of Mouse L\(\text{Mtk}^-\) cl-1 Cells

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>TIME OF PASSAGE UNDER NONSELECTIVE CONDITIONS (days)</th>
<th>PLATING EFFICIENCY IN SELECTIVE MEDIUM(^2) (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L(\text{Mtk}^-) cl-1</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>Transfected clone</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>88</td>
</tr>
</tbody>
</table>

1. L\(\text{Mtk}^-\) cl-1 (mouse L-cell clone 1), Transfected clone (p\(\Delta R\)-tk plasmid DNA transformant).
2. The transfectant clone was initially grown in selective medium, cells were detached by trypsinization and grown in nonselective medium for the indicated times prior to measuring the plating efficiencies in selective and nonselective medium. The results are expressed as efficiency of plating as a percent of control. The values represent the mean of 4 different determinations. These differed < 10% from the mean.
Table 17. Growth Parameters of the LMTk- cl-1 cells and their Plasmid DNA Transformed Counterparts

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>EOP (%)</th>
<th>TD (h)</th>
<th>SD (cells $\times 10^4$/cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMTk- cl-1 Transfected clone</td>
<td>30</td>
<td>16.00</td>
<td>3.74</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>15.60</td>
<td>6.36</td>
</tr>
</tbody>
</table>

1. Efficiency of plating is defined as the number of cells that grow into colonies as compared to the number of cells initially plated.
2. TD (time of doubling) is defined as the time it takes for a culture to double in number during exponential growth.
3. Saturation density is defined as the maximum number of cells/cm$^2$ to which a given cell culture is capable of packing.
2,3. Determined from growth curves.
Table 18. The Effect of Plasmid DNA Mediated Thymidine Kinase Gene Transfer on the AHH Phenotype of Mouse LMTk cl-1 Cells

<table>
<thead>
<tr>
<th>CELL LINES1</th>
<th>INDUCER</th>
<th>CONCENTRATION</th>
<th>SPECIFIC ACTIVITY2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepa cl-9</td>
<td>None</td>
<td>-</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>0.1% (v/v)</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td>TCDD</td>
<td>1 nM</td>
<td>8.35</td>
</tr>
<tr>
<td></td>
<td>BA</td>
<td>1 uM</td>
<td>6.26</td>
</tr>
<tr>
<td>LMTk- cl-1</td>
<td>None</td>
<td>-</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>0.1% (v/v)</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>TCDD</td>
<td>1 nM</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>BA</td>
<td>1 uM</td>
<td>0.20</td>
</tr>
<tr>
<td>Transfected clone</td>
<td>None</td>
<td>-</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>0.1% (v/v)</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>TCDD</td>
<td>1 nM</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>BA</td>
<td>1 uM</td>
<td>0.25</td>
</tr>
</tbody>
</table>

1. Hepa cl-9 (mouse hepatoma cell clone 9), LMTk- cl-1 (mouse L-cell clone 1), Transfected clone (p\textsuperscript{AR-tk} transformed clone)
2. Expressed as picomoles 3-hydroxybenzo[a]pyrene formed per milligram protein per minute.
The values represent the mean of triplicate determinations. These differed < 10% from the mean.
Transfection of Human Cells

1) DNA Mediated Transfer of the Herpes Simplex Virus

Thymidine Kinase Gene to a Bromodeoxyuridine

Resistant Variant of Human Primary Lung Carcinoma Cells

(See Fig 20 for the morphology of RTK⁻ variant, and the two transfectants)

In the light of successful transfection of noninducible (AHH negative) mouse L-cells for a marker thymidine kinase gene, genetic analysis of the expression of AHH activity using this approach was extended to human cells. Results from this laboratory have demonstrated the presence of a detectable cytosolic Ah receptor for this cell line incapable of nuclear translocation (M.J. Dufresne, Personal Communications). DNA mediated transfer of the Ah gene complex, and hence the restoration of inducible AHH activity to a human AHH negative cell can be realized only if the recipient cell does not prove refractory to gene transfer. Therefore, it was considered significant to determine if the transfection of a marker thymidine kinase gene to a noninducible human cell could be achieved. Furthermore for continued genetic and molecular analysis of transfected cell lines, the transformed phenotype should be stable. Thus it is appropriate to determine if the transfected thymidine kinase gene was as stable in human cells as in mouse cells (See Table 16).
The experimental protocol in brief involved the treatment of thymidine kinase negative variants of human R1 cl-6 cells with calcium phosphate precipitated DNA which was purified from the recombinant plasmids bearing the thymidine kinase gene. The bonafide transfectants were selected using HAT medium. Table 15 gives the results of these studies. It is clearly seen that the human thymidine kinase negative R1 cl-6 cells undergo transfection to the TK+ phenotype at a frequency of $5.5 \times 10^{-5}$ with either p\textsuperscript{tk} R-tk or pX1-tk donor DNA. HAT resistant colonies were not observed on control plates. When homologous RTK\textsuperscript{-} cell genomic DNA was used as donor, it was unable to transfer the TK gene to RTK\textsuperscript{-} recipient cells. Treatment of recipient cells with calf thymus carrier DNA alone did not result in HAT resistant colonies. pBR322 DNA used as an additional control did not produce HAT resistant colonies. These results therefore suggested that RTK\textsuperscript{-} cells which contain the Herpes Simplex Virus thymidine kinase gene gave rise to transformed colonies.
2) **Phenotypic Stability of the Transfected Thymidine Kinase Gene in a Bromodeoxyuridine Resistant Variant of R1 cl-6 Cells**

Since the ultimate goal of our genetic studies, is the analysis in a human system of the expression of AHH induction, the transfectants of RTK- human cells were subjected to phenotypic stability analysis. In these experiments, the recipient thymidine kinase negative R1 cl-6 cells (control) and the transfectants were first grown under selective pressure and were subsequently subcultured for various times in the absence of selection. The fraction of cells that retained the TK+ phenotype was determined by measuring the plating efficiencies in selective and nonselective medium. The results of this study are given in Table 20. It is observed that the recipient cells were unable to grow in HAT selective medium after passaging under nonselective conditions. Both plasmid mediated transfectants retained their ability to grow in HAT (hypoxanthine/aminopterin/thymidine) medium when subcultured in the absence of selective pressure for 10, 20, and 40 days respectively. These results demonstrate the stability of the TK+ transfected phenotype.
3) Comparison of Growth Parameters of RTK− Recipient cells and the Transfectants

Growth characteristics of the recipient RTK− cells and the two plasmid DNA mediated transfectants were examined. The results are presented in Table 17. The time of doubling (TD) or generation time in both transfectants resembled the recipient cells. However, the saturation densities were lower compared to the TK− recipient cells and were similar to saturation densities of wild type cells.

4) Comparison of Aryl Hydrocarbon Hydroxylase Activity in RTK− cells and the Transfectants

To determine the effect of plasmid DNA mediated thymidine kinase gene transfer on the inducibility of AHH activity in recipient human RTK− cells, the levels of TCDD induced AHH activity were determined in transfectants and compared to the recipient RTK− cells and wild type R1 cl-6 cells (see Materials and Methods for the assay procedure). The results of this study are presented in Table 18. The control mouse Hepa cl-9 cells demonstrated a 28-29 fold induction at 1.0 nM TCDD concentration.
The results (Table 18) demonstrate no detectable AHH activity in wild type R1 cl-6, RTK^- cells, and the two plasmid transfected cell lines. These results are in absolute agreement with those of murine transfection studies (see Table 14) suggesting that the plasmid DNA mediated thymidine kinase gene transfer to AHH negative murine (LMtk^- cl-1 cells) or human (TK^- variant of R1 cl-6 cells) recipients does not interfere with their AHH negative phenotypes. This suggests that AHH structural genes from an AHH positive donor could be ligated to the plasmid carrying a marker thymidine kinase gene, and the chimeric plasmid DNA be presented by transfection to a mouse (LMtk^- cl-1) or human (TK^- variant of R1 cl-6) recipient.
Table 19.  Thymidine Kinase Gene Transfer into a Bromodeoxyuridine Resistant Variant of Human Primary Lung Carcinoma Cells

<table>
<thead>
<tr>
<th>DONOR DNA²</th>
<th>TOTAL TK+ COLONIES / TOTAL PLATES</th>
<th>AVERAGE PLATE</th>
<th>TRANSFECTION FREQUENCY ¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBR322</td>
<td>0/3 (3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>0/3 (3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Calf thymus</td>
<td>0/3 (3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RTK⁻</td>
<td>0/3 (3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pA²R-tk</td>
<td>200/9 (3)</td>
<td>22.2</td>
<td>5.5 x 10⁻⁵</td>
</tr>
<tr>
<td>pX1-tk</td>
<td>200/9 (3)</td>
<td>22.2</td>
<td>5.5 x 10⁻⁵</td>
</tr>
</tbody>
</table>

1. Plasmid and genomic DNAs were purified and coprecipitated with calcium phosphate in the presence of 33 ug calf thymus carrier DNA. 1.0 μg of precipitated plasmid DNA (in 1.0 ml) and 20.0 μg of homologous cell DNA (without carrier) (in 1.0 ml) was directly added to approximately 4 x 10⁵ TK⁻ cells in each dish. Transfectants were scored for TK⁺ phenotype after two weeks of HAT selection.

2. pA²R-tk and pX1-tk are recombinant plasmids bearing the thymidine kinase gene from Herpes Simplex Virus. Mock transfections of RTK⁻ was carried out using CaP₀₄ alone in place of transforming DNA.

3. The number in parenthesis represents the number of separate transfection experiments carried out using different DNA preparations.

4. Defined as the ratio of mean number of TK⁺ colonies to the number of recipient RTK⁻ cells at the time of DNA exposure.
Table 20. Phenotypic Stability of the Transfected Thymidine Kinase Gene in Transfectants of Human Bromodeoxyuridine Resistant R1 Cl-6 Cells

<table>
<thead>
<tr>
<th>CELL LINE(^1)</th>
<th>TIME OF PASSAGE UNDER NONSELECTIVE CONDITIONS (days)</th>
<th>PLATING EFFICIENCY IN SELECTIVE MEDIUM(^2) (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTK(^-)</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>p(\uparrow R-tk/RTK(^-)</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>76</td>
</tr>
<tr>
<td>pX1-tk/RTK(^-)</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>81</td>
</tr>
</tbody>
</table>

1. RTK\(^-\) (thymidine kinase negative variant of human R1 cl-6 cells), p\(\uparrow R-tk/RTK\(^-\) (p\(\uparrow R-tk\) plasmid DNA transfec tant of RTK\(^-\) cells), pX1-tk (pX1-tk plasmid DNA transfec tant of RTK\(^-\) cells).

2. The transfected cells were grown initially in selective medium; cells were then detached by trypsinization and grown in selective medium for the indicated times prior to measuring the plating efficiency in selective and nonselective medium.

The results are expressed as the plating efficiency; i.e., a percent of control. The values represent the mean of 4 different determinations. These differed < 10% from the mean.
Table 21. Growth parameters of wild type R1 cl-6, RTK− cells, and the two Transfectants

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>GENERATION TIME (h)</th>
<th>SATURATION DENSITY cells (x 10^5)/cm^3</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1 cl-6</td>
<td>11.0</td>
<td>1.35</td>
</tr>
<tr>
<td>RTK−</td>
<td>12.0</td>
<td>1.70</td>
</tr>
<tr>
<td>RTK−/p▲R-tk</td>
<td>10.0</td>
<td>1.00</td>
</tr>
<tr>
<td>RTK−/pX1-tk</td>
<td>12.0</td>
<td>1.20</td>
</tr>
</tbody>
</table>

1. R1 cl-6 (human primary lung carcinoma cell clone 6), RTK− (thymidine kinase negative variant), RTK−/p▲R-tk (p▲R-tk plasmid DNA transfected cell line), RTK−/pX1-tk (pX1-tk plasmid DNA transfected cell line).
2. Determined from the logarithmic phase of growth and defined as the time it takes for the cells in a culture to double in number.
3. Saturation density is defined as the maximum number of cells/cm^2 to which a given cell culture is capable of packing.
Table 22. The Effect of Plasmid DNA Mediated Gene Transfer on the AHH activity in Human RTK Cells

<table>
<thead>
<tr>
<th>CELL LINES</th>
<th>INDUCER</th>
<th>CONCENTRATION</th>
<th>SPECIFIC AHH ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepa c1-9</td>
<td>None</td>
<td>-</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>0.1% (v/v)</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>TCDD</td>
<td>1 nM</td>
<td>28.76</td>
</tr>
<tr>
<td>R1 c1-6</td>
<td>None</td>
<td>-</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>0.1% (v/v)</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>TCDD</td>
<td>1 nM</td>
<td>0.00</td>
</tr>
<tr>
<td>RTK⁻</td>
<td>None</td>
<td>-</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>0.1% (v/v)</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>TCDD</td>
<td>1 nM</td>
<td>0.00</td>
</tr>
<tr>
<td>RTK⁻/pΔR-tk</td>
<td>None</td>
<td>-</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>0.1% (v/v)</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>TCDD</td>
<td>1 nM</td>
<td>0.00</td>
</tr>
<tr>
<td>RTK⁻/pX1-tk</td>
<td>None</td>
<td>-</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>0.1% (v/v)</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>TCDD</td>
<td>1 nM</td>
<td>0.00</td>
</tr>
</tbody>
</table>

1. Cells in logarithmic phase of growth were exposed to different concentrations of TCDD in growth medium for 18 hours. At that time, the cells were harvested and assayed for AHH activity as described under "Materials and Methods".

2. TCDD = 2,3,7,8-tetrachlorodibenzo-p-dioxin
3. Specific AHH activities are expressed as picomoles of 3-hydroxybenzofuraprene formed per milligram protein per minute.
The values represent the mean of triplicate determinations. These differed < 10% from the mean.
ND = Not detectable.
Morphology of the RTK variant, RTK/p\text{\textdagger}R-tk Transfectant, and RTK/pX1-tk Transfectant using Light microscopy (mag x 200).

Growth of the two plasmid mediated transfectants in HAT selective medium.

(a) p\text{\textdagger}R-tk transfectant

(b) pX1-tk transfectant
DISCUSSION

1) **Aryl Hydrocarbon Hydroxylase Induction**

The assessment of inducible AHH activity in Hepa cl-9, LMTk⁻ cl-1, HTC cl-1, and RI cl-6 cells revealed differences between these cell lines with respect to their ability to respond to aryl hydrocarbon hydroxylase induction after exposure to inducing agents. The mouse hepatoma (Hepa cl-9) cells demonstrated a higher level of expression of inducible AHH activity after pretreatment with 1.0 nM TCDD, rather than 1.0 μM BA in DMSO. These results are consistent with the fact that TCDD is one of the most potent inducers of AHH activity (Kimura et al.; 1984). Inducible AHH activity was not expressed in mouse LMTk⁻ cl-1, rat HTC cl-1, or human RI cl-6 cells. AHH induction could not be restored to LMTk⁻ cl-1, HTC cl-1, or RI cl-6 cells by altering either the concentration of TCDD or by using another type of PAH inducer. These results on the noninducibility of AHH activity are consistent with those previously published (Dufresne, and Dosescu, 1985).

The lack of AHH inducibility in LMTk⁻ cl-1 cells can be readily explained on the basis of their lack of the Ah receptor.
DISCUSSION

1) Aryl_Hydrocarbon_Hydroxylase_Induction

The assessment of inducible AHH activity in Hepa cl-9, LMtk\textsuperscript{-} cl-1, HTC cl-1, and R1 cl-6 cells revealed differences between these cell lines with respect to their ability to respond to aryl hydrocarbon hydroxylase induction after exposure to inducing agents. The mouse hepatoma (Hepa cl-9) cells demonstrated a higher level of expression of inducible AHH activity after pretreatment with 1.0 nM TCDD, rather than 1.0 \textmu M BA in DMSO. These results are consistent with the fact that TCDD is one of the most potent inducers of AHH activity (Kimura et al.; 1984). Inducible AHH activity was not expressed in mouse LMtk\textsuperscript{-} cl-1, rat HTC cl-1, or human R1 cl-6 cells. AHH induction could not be restored to LMtk\textsuperscript{-} cl-1, HTC cl-1, or R1 cl-6 cells by altering either the concentration of TCDD or by using another type of PAH inducer. These results on the noninducibility of AHH activity are consistent with those previously published (Dufresne, and Dosecsu, 1985).

The lack of AHH inducibility in LMtk\textsuperscript{-} cl-1 cells can be readily explained on the basis of their lack of the Ah receptor.
However, the lack of AHH inducibility in rat HTC cl-1 cells cannot be explained on this basis, since it not only has Ah receptor, but translocates this receptor into the nucleus. These results of AHH inducibility with rat HTC cells are consistent with those of Benedict, et al; 1972, who found no detectable basal or induced levels of this enzyme in these cells. However, these authors used a higher concentration (13 uM) of benz[a]anthracene and not TCDD. The molecular basis for this nonexpression of AHH in HTC cells is not known. Israel and Whitlock, 1984 found that in variant mouse cells without a nuclear receptor, no basal or inducible cytochrome P{sub}1{-}450 mRNA was detectable. HTC cells present an even more interesting subject, because of a clearly demonstrable nuclear receptor (see "Results"). Thus it is worthwhile as also stated later, to examine detectable basal and/or inducible cytochrome P{sub}1{-}450 mRNA levels. These possibilities do not necessarily represent the only explanation for the lack of expression of inducible aryl hydrocarbon hydroxylase. The expression of AHH activity depends on correct functioning of a complex multicomponent membrane bound pathway. A block in any one step may prevent the expression of the end point (i.e. AHH)
2) **Ah Receptor Characteristics**

The receptor characteristics differed for each cell line. The AHH inducible Hepa cl-9 and the AHH deficient HTC cl-1 had clearly demonstrable cytosolic and nuclear binding peaks. These peaks are considered to be a result of the Ah receptor. The mouse L(tk- cl-1 cell line had no detectable Ah receptor. Once again these results are consistent with those previously published (Dufresne and Dosecu, 1985).

The noninducible rat HTC cl-1 cells had both nuclear and cytosolic receptor. The lack of AHH inducibility in this cell line could be due to a block in the induction mechanism before and after the translocation of the PAH-receptor complex into the nucleus. For example, the PAH may bind to a faulty receptor, which cannot be recognized by the DNA receptor sequences. In other words, the DNA binding domain of the PAH-Ah receptor complex might be defective. This might prevent the stimulation of transcription of the mRNA for cytochrome P450. The nonexpression of inducible AHH activity in HTC cells resembles Hankinson's Hepa-1 mutant cells (Hankinson, 1983). It would be of interest to determine if these cells have basal or inducible mRNA for P450 (Israel and Whitlock, 1984).
The induced cytochrome P$_1$-450 may not be functional due to an alteration in the enzyme structure. Some other possibilities include proteolysis of the reductase, post-translational modification, erroneous transcription or no transcription for cytochrome P$_1$-450 gene, or reducing equivalents might be limiting. Another possibility is that the lack of AHH inducibility might reflect a deficiency in the activity of the microsomal NADPH cytochrome c (P-450) reductase since differences in the activity of reductase have been shown to be associated with the Ah locus (Blumer and Mieyal, 1978). The receptor data for HTC cl-1, Hepa cl-9, and LMtK$^-$ cl-1 cells demonstrate that the binding of the PAH to the cytosolic Ah receptor and translocation of the resultant complex into the nucleus are necessary but not sufficient for the induction of AHH activity. The requirement of the Ah receptor for the induction of AHH activity has recently been questioned in a human cell line, MCF-7 (Jaiswal et al; 1985). The receptor may be present in small quantities so as to escape detection by the method used, or the seemingly undetectable receptor present in minute quantities might be very potent. It is also possible that the receptor mediated mechanism of AHH induction may not be operative in this cell line. The Cytochrome P$_1$-450 gene may be transcribed by a positive modulator produced in response to the PAH.
3) Analysis of NADPH Cytochrome c Reductase

The possibility that lack of inducible AHH activity in rat HTC cl-1 cells might reflect a deficiency in the reductase activity was examined in the three cell lines. The inducible mouse hepatoma (Hepa cl-9) cells and the noninducible LMt- cl-1 cells had similar basal levels of reductase. These results are consistent with those of Wiebel, et al; (1981) who found that the noninducible mouse RAG cells, showed levels of reductase similar to other cell lines expressing AHH activity. Pretreatment of mouse LMt- cl-1 cells with 1.0 nM TCDD did not result in reductase induction, whereas in the mouse hepatoma (Hepa cl-9) cells there appeared to be a slight (50%) increase in this enzyme. Wiebel et al; (1984) have reported a similar low level of increase in reductase activity (i.e. 30%) in 4-day old cultures of dedifferentiated H5 rat hepatoma cells treated with 1.6 mM phenobarbital for 72 hours. This increase occurs in the absence of inducibility of AHH activity but is not considered to be significant. Although rat HTC cl-1 cells have a lower level of basal reductase activity than either mouse cell lines, treatment of rat HTC cl-1 cells with 1.0 nM TCDD resulted in a reproducible increase (i.e. 500-600%) in reductase activity (see Results). This activity moreover, is greater than that found in AHH inducible Hepa cl-9 cells treated with TCDD.
These data suggest that the deficiency in inducible AHH activity does not seem to reflect a deficiency in measurable reductase activity. The results are consistent with those of Wiebel et al; (1981) where mouse RAG cells exhibited a considerable amount of reductase, but with no detectable inducible AHH activity. Whether induction of reductase in HTC cl-1 cells can occur with other inducers of cytochrome P450 or with phenobarbital remains to be determined. However the data for mouse LMtk− cl-1 and Hepa cl-9 cells demonstrate that treatment of both cell lines with 1.0 μM Phenobarbital results in a reproducible 40-50% and 200-300% increase in reductase activity respectively. Thus while PB marginally induces reductase activity in the AHH noninducible LMtk− cl-1 cells, it increases AHH inducibility to a significant extent in mouse Hepa cl-9 cells inducible for AHH. These findings suggest a possible relationship between the mechanism of AHH induction and the induction of cytochrome c reductase.

In reductase assays, a higher ionic strength of phosphate buffer (0.33 M) was used to maximize detectable levels of reductase consistently throughout the studies. Results have shown that the absolute reductase specific activity values were low but were comparable to the levels obtained when low ionic strength phosphate buffers (0.033 M) were used (See reductase data of Wiebel et al; 1981) in tissue culture cells.
The data from induction kinetics experiments provide additional evidence for the induction by TCDD of NADPH cytochrome c reductase. It is seen that reductase in rat HTC cl-1 cells is maximally induced after 16-18 hours of exposure to TCDD. Previous studies from our laboratory and others, have established that this is also the time required for 1.0 nM TCDD to maximally induce AHH activity in murine cells (Dufresne and Dosescu, 1985).

The results discussed in this section indicate that the rat HTC cl-1 cells have a functional NADPH cytochrome c reductase. They are consistent with the hypothesis that the inability to induce AHH activity in rat HTC cl-1 cells reflects a defective structural gene for cytochrome P450 induction. This does not necessarily mean the exclusion of other possibilities. Analysis of the P450 mRNA levels with the use of cDNA probes in this cell line, as compared to AHH inducible cell lines will be informative in this regard. Nondetectable levels of aryl hydrocarbon hydroxylase activity in response to TCDD has previously been shown to be associated with a similar nondetectable or reduced P450 mRNA. This could be due to reduced transcription of the P450 gene, or the defective structural gene might affect the lability of the mRNA (Hankinson et al; 1985). The rat HTC cl-1 cells would then resemble class A mutants of Hepa-1 (Hankinson, 1983). These mutants had translocatable cytosolic receptor.
These results demonstrate that the synthesis of cytochrome c reductase in rat HTC cl-1 cells is induced by TCDD.

Little is known about the molecular mechanism of induction of NADPH cytochrome c reductase. If TCDD can induce reductase activity, as the results suggest, the induction mechanism could be mediated in part by the Ah receptor or a different receptor. TCDD may exert its action by augmenting the transcriptional activity of the receptor gene.

Alternatively, physiological factors (cAMP or cAMP mediated factors) might modulate the expression of this gene. Given the fact that phenobarbital induces reductase in rat HTC cl-1 cells, it is possible that the induction mechanism may involve a broad spectrum of P-450 inducers of both phenobarbital and methylcholanthrene types. This assumption is reasonable given the central role of cytochrome c reductase in the overall metabolism of xenobiotics.
Expression of Inducible AHH activity in Human Cell Hybrids

Cell hybrids formed between AHH noninducible human primary lung carcinoma (R1 cl-6) cells and AHH inducible mouse hepatoma (Hepa cl-9) cells did not express inducible AHH activity. These results suggest a "recessive" phenotype of the inducible parent. The implications of these results can be considered by an examination of the data already reported in the literature. Dominance in the expression of TCDD inducible AHH activity characteristic of the inducible parent has been reported from our laboratory (Dufresne and Dosescu, 1985).

This hybridization involved the use of TCDD noninducible mouse L-cells and TCDD inducible mouse hepatoma cells as parents. Dominance in the expression of AHH activity has been reported in other somatic cell hybrid systems (Wiebel et al; 1981). However, the biochemical and genetic nature of expression of inducible AHH activity in human cell lines has not been clearly elucidated. The results reported here demonstrate that the inducible aryl hydrocarbon hydroxylase activity is not expressed in the hybrids. Studies from the literature on the dominance relationship for example has shown the suppression of melanin synthesis in hybrids between Syrian hamster melanoma cells and mouse fibroblasts (Davidson, 1972).
Using the same approach, but with a rat myoblast differentiation system, Dufresne et al. 1976, showed that in myoblast x fibroblast hybrids, the synthesis of muscle specific proteins (e.g. creatine phosphokinase, and heavy chain myosin) were not expressed.

The human hybrid cell data on the expression of AHH activity is consistent with the above findings. However, whether aryl hydrocarbon hydroxylase induction by 1.0 nM TCDD is reexpressed in at least one of the hybrids after the loss of some chromosomes remains to be determined. The present results suggest a "repressor-type" mechanism in the suppression of inducible AHH activity specific to the murine parent (8-azaguanine Hepa cl-9). Unless further molecular and genetic experimental evidence is obtained, it cannot be determined with certainty that the noninducible human R1 cl-6 cell genome is responsible for this observed suppression.

In the Hepa cl-9 x R1 cl-6 cell hybrids, what might cause the observed repression of aryl hydrocarbon hydroxylase induction, compared with AHH inducibility in the mouse Hepa cl-9 parent? One possibility is that the AHH noninducible human R1 cl-6 cell genome might code for a repressor which in some way blocks the Hepa cl-9 specific cytochrome P₄₅₀ mRNA, protein, and aryl hydrocarbon hydroxylase activity. Therefore it would be of significant interest to examine the cytochrome P₄₅₀ mRNA levels in the hybrids (RH) as compared to the Hepa cl-9 parent.
However, it must be made clear that the present data do not provide any direct or indirect experimental proof for the postulated "repressor-type" mechanism. Furthermore, if one assumes random chromosome loss, it is possible that the human chromosome carrying the repressor gene is not excluded or segregated. This model is only a possible explanation of the mechanism of repression of inducible AHH activity in cultured somatic cell fusions between an AHH inducible mouse cell and an AHH noninducible human cell. It is simplest, though not necessarily the only explanation for the suppression of AHH activity in the RH hybrids. It does not attempt to explain the molecular interactions accruing between the parental genomes during the production of this repression in inducible AHH activity. This point of view is supported by evidence from literature in which gene products unique to the hybrid cell could lead to the suppression of AHH activity described by Wiebel and coworkers for JEG-3/VA-2 human hybrids (Wiebel et al; 1981).

Other possibilities may contribute to the lack of AHH expression. For example a mouse chromosome bearing the AHH gene complex may not be present in the hybrid, a deficient heme biosynthetic pathway, or apocytochrome not synthesized and so on. The potential inherent in these hybrids if realized is a definite bonus, because for instance the human chromosome responsible for the synthesis of the repressor or repressor-like molecule could be identified.
DNA Mediated Transfer of the Herpes Simplex Virus Thymidine Kinase Gene to Aryl Hydrocarbon Hydroxylase Deficient Mouse LMTk-c1-1 Cells

i) Gene transfer to LMTk-c1-1 Cells

The results reported in this study demonstrated the transfection of TK- and AHH- mouse cells to TK+ using extensively purified 6.3 kb pAR-tk plasmid DNA as donor. A transfection frequency of 8 transfectants per microgram of donor DNA obtained in these experiments is consistent with previously published frequencies for this gene (Abraham and Gottesman, 1984). The rate of spontaneous reversion of LMTk-c1-1 to TK+ is < 10^-9 (Wigler et al., 1979).

Therefore, the appearance of even a single colony in cellular transformation experiments is significant, and for this reason strongly suggests that the expression of the thymidine kinase positive phenotype, (detected by resistance to HAT) in these experiments resulted from the introduction and expression of the Herpes Simplex Virus, thymidine kinase gene contained in the pAR-tk plasmid DNA.

However, the appearance of colonies surviving in HAT selective medium following transfection assays with plasmid DNA could result from the reactivation of the murine TK, or from the integration of a new wild type TK gene coded for by the donor DNA.
Plasmid DNA Mediated Transfer of the Herpes Simplex Virus Thymidine Kinase Gene to Bromodeoxyuridine Resistant Variant of Human Primary Lung Carcinoma Cells

i) Gene Transfer of Human Bromodeoxyuridine Resistant Variant of R1 cl-6 Cells

The results reported in this study demonstrate the transfection of thymidine kinase negative and AHH noninducible human R1 cl-6 cells to TK+ using both pAR-tk and pX-tk plasmid DNAs as donors. A transfection frequency of approximately 5.5 x 10^-5 was obtained when 1.0 µg of either plasmid DNA was added to each dish. Both recombinant plasmids used in these studies are derivatives of pBR322 bearing the Herpes Simplex Virus thymidine kinase gene. This frequency of transfection is much more efficient than other human transfections involving for example the transfer of xanthine phosphoribosyl transferase gene contained in the psv-gpt plasmid to hypoxanthine phosphoribosyl transferase negative human D98/AH-2 cells. In this transfection system a maximum attainable frequency of 2.0 x 10^-5 has been reported using 10 µg of plasmid donor (Srivatsan et al; 1984).

These results demonstrate that transfection is a feasible approach for the analysis of the expression of inducible aryl hydrocarbon hydroxylase in AHH deficient human cells.
The results suggest that human cells do not prove refractory to transfection. Furthermore, the results show that transfection in human cells can approach the levels observed with rodent cells as recipients (i.e. mouse LMtk- cl-1 cells). The human transfection data obtained in these studies is significant for several reasons. First, relatively few studies have examined the transfectability of human cells. Second, the results are an initial step towards a more definitive genetic assessment of the expression of AHH induction by PAH. Third, the studies describe conditions for a useful level of transfection of an AHH negative human cell with a selectable marker gene.

The efficiency of transfection for the genes contained in plasmids seem to depend on the particular cell line used as a recipient. In these studies, the frequency of gene transfer compares closely with that observed in murine transfection experiments. This conclusion is most probably due to the choice of a bromodeoxyuridine resistant variant as a recipient and the specific method of gene transfer used.

11) **Phenotypic Stability of Human TK+ Transfectants**

Since the major research objective of our laboratory is to analyze the expression of inducible AHH activity in a human cell culture system, it was important to examine whether the transfection of a human cell is as phenotypically
stable as that reported for a mouse transfecant. The results clearly suggest a high level of stability of the transfected thymidine kinase gene in transfecants of human TK⁻ R1 cl-6 cells. These results were obtained irrespective of the source of the donor gene. Thus the data on phenotypic stability analysis showed a high level of stable expression of the transfected phenotype, even though integration has not been shown using southern blot analysis. The transfecants demonstrated their ability to grow in HAT medium. These results argue strongly in favour of transfection rather than mutation to HAT resistance in TK⁻ human R1 cl-6 cells.

The human transfection results are consistent with those obtained for murine transfection.

iii) Effect of Thymidine Kinase Gene Transfer on the Aryl Hydrocarbon Hydroxylase Phenotype in Transfectants of Human R1 cl-6 Cells

The data presented on the effect of thymidine kinase gene transfection on inducible AHH activity in transfecants of thymidine kinase negative human R1 cl-6 cells demonstrate that the TK gene transfection does not interfere with the AHH negative phenotype of the recipient. At the same time, a gene carried by the plasmid is stably expressed in the recipient human cell. Once again these results are in agreement with those obtained for murine transfection studies.
Both the murine and human transfection studies, while
demonstrating the feasibility of DNA mediated gene transfer
in AHH induction studies, also suggest that the TK gene
will serve as a useful marker in future transfection
studies involving the transfection of AHH induction
associated genes.

This assumption is significant when a ligated
cotransfer recombinant DNA approach is used for the
transfection of AHH inducibility. In this approach the
nonselectable genomic DNA from an AHH positive cell could
be covalently ligated to a selectable marker gene (e.g.
thymidine kinase). The resulting chimeric plasmid could
then be presented to AHH negative recipient cells. Such an
approach provides a virtual certainty that the second gene
(i.e. AHH) can be introduced. Since the two genes would be
equimolar, it would be possible to select for cells which
contain a single copy of each of the genes.

However, a drawback of using the thymidine kinase gene is
that it is a recessive gene and only can be introduced into
mutant cells deficient in the expression of this gene.

Based on the results and discussion presented in this
thesis, Somatic cell genetics and DNA mediated gene
transfer constitute feasible approaches at the somatic cell
and molecular level to study the expression of aryl
hydrocarbon hydroxylase activity in murine and human cell
lines.
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Vita Auctoris

Gubbi Subbaraya Manjunath
Born May 1, 1953,
Bangalore City, India

Education:

1970-1973
Bangalore University,
Bangalore, India,
B.Sc.

1973-1974
Bangalore University,
Bangalore, India,
B.Sc. (Hons)

1974-1976
Bangalore University,
Bangalore, India,
M.Sc.

1982-1986
University of Windsor,
Windsor, Ontario
Candidate for the Degree
of Master of Science,
Biology, 1986.