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Sexual Dimorphism of Hepatic Genes Expressed in Hexachlorobenzene-Treated Rats

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ABSTRACT

Hexachlorobenzene (HCB) is an epigenetic carcinogen that is persistent in the environment and has a tendency to bio-accumulate. HCB is stored in fatty tissues of animals and humans. When rats are exposed to HCB, females are more susceptible to develop tumors as compared to males. The genes responsible for such an expression are poorly understood. The objective of this study was to determine the gender-specific hepatic gene expression profiles observed in rats exposed to HCB.

Four experimental groups were used; control males; control females; HCB-treated males and HCB-treated females. Treated rats were exposed to HCB (100 mg/kg) for five consecutive days and sampled on day 50 of the experiment. The *in vivo* exposure model used in the present study in which rats are sampled at day 50 represents a "steady state" situation following from HCB exposure. Total RNA was isolated from the liver and reverse transcribed and used to screen an Atlas™ 1.2 II Rat Array containing 1176 genes. Data were analyzed using four individual rats per experimental group. Genes expressed in at least 3 out of 4 trials were selected for analysis. In addition, the genes must have had signal intensities at least 2X background level.

Our data indicates a sexual dimorphism in the expression of hepatic genes due to HCB-treatment. The female control group displayed comparable gene expression as the male control group (185 versus 184 genes). Seven genes were exclusively found in the male control group; ERp29, synaptogyrin 1, synaptojanin, DPPIII, kinesin-related protein, atrophin 1 and glycoprotein 55. Two genes were exclusively expressed in females; Mammalian achaete scute homolog 2 (MASH-2) and carboxypeptidase Z.

In HCB-treated males a total of 189 genes were detected by the cDNA microarray. Four of those genes had significant differential expressions; EGR2, dihydropyrimidinase and a liver-specific transport protein were down-regulated while epoxide hydrolase, an enzyme involved in the biotransformation of xenobiotics and epoxides was up-regulated.

No genes were exclusively expressed in HCB-treated females. However, HCB caused an 18% decrease (31 genes) in the total number of genes detected by the microarray in livers of HCB-treated females compared to control females. Twenty-nine percent of the 31 genes decreased in HCB-treated females have already been reported to be down-regulated in liver cancer. Furthermore we noticed a sexual dimorphism at the genetic level since in HCB-treated females surprisingly only 151 were expressed among which five genes were differentially expressed in HCB-treated females only; phosphatidylethanolamine N-methyltransferase, phosphatidylethanolamine binding-protein, ribosomal protein L18 and testis-specific farnesyl pyrophosphate were up-regulated while protectin/CD59 was repressed.

Thirty-six genes were altered by HCB treatment and may be implicated in the epigenetic mechanism of HCB-induced hepatocarcinogenesis. Of those genes 31 were found to be putative tumor suppressor genes whose mRNA levels were undetectable in HCB-treated females and 5 that showed sexual dimorphism in terms of genetic expression. The functional genomic approach used in this study has enabled us to target and identify trends in the expressions of clusters/families of genes that have been linked to HCB-induced hepatocarcinogenesis in HCB-treated females only. The results of this study expose additional insights into the mechanisms underlying HCB carcinogenesis. We propose a primary mechanism by which HCB alters the DNA methylation machinery of the cell, a known factor implicated in epigenetic carcinogens and reported to contribute to the early steps of carcinogenesis.

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LIST OF ABBREVIATIONS

- **Ah-R** Aryl hydrocarbon receptor
- **ALCL** Anaplastic large-cell lymphoma
- **ARNT** Ah-R nuclear translocater protein
- **BAC** Bacterial artificial chromosome
- **BRAC1 & 2** Breast cancer genes
- **cDNA** Complementary deoxyribonucleic acid
- **CXC** Chemokine (Cysteine-X-Cysteine) motif
- **DCPA** Dimethyl tetrachloroterephthalate
- **DEN** Diethylnitrosamine
- **DES** Diethylstilbestrol
- **DNA** Deoxyribonucleic acid
- **DNMT** DNA methyltransferase
- **DRE** DNA response elements
- **EGF** Epidermal growth factor
- **EGR** Epidermal growth receptor
- **EPA** Environmental protection agency
- **ESTs** Expressed sequence tags
- **FDA** Food and drug administration
- **GDP** Guanosine diphosphate
- **GGT** Gamma-glutamyltransferase
- **GSH** Glutathione
- **GSSG** Glutathione disulfide
- **GSSH** Glutathione persulfide
- **GSTs** Glutathione S-transferase
- **GTP** Guanosine diphosphate
- **HAH'S** Halogenated aromatic hydrocarbons

- **HBV** Hepatitis B Virus infected patients
- **HCB** Hexachlorobenzene
- **HCC** Hepatocellular carcinoma
- **HCE** Hexachloroethane
- **HGP** Human Genome Project
- **HSP** Heat shock proteins
- **IARC** International Agency of Cancer Research
- **KCLR** Killer cell lectin-like receptor
- **LCT** Liver-cell tumors
- **m^5C** Cytosine 5'-methylated
- **MAC** Membrane attack complex
- **MASH** Mammalian achaete scute homolog
- **MHC** Major histocompatibility complex
- **MMLV** Mouse Moloney murine leukemia virus
- **mRNA** Messenger ribonucleic acid
- **Na+/K+ATPase** Sodium/potassium adenotriphosphate pump
- **NF** Nuclear factor
- **NIOHS** National occupation hazard survey
- **P450IIIA1/2** Cytochrome P450 isoenzyme complex
- **PBB** Polybrominated Biphenyl
- **PCB** Polychlorinated Biphenyl
- **Pc-G/trx** Polycomb-trithorax group protein
- **PCNB** Pentachloronitrobenzene
- **PCP** Pentachlorophenol
- **PCT** *Porphyria cutanea tarda*
- **PEBT** Phosphatidylethanolamine binding-protein
- **PEMT** Phosphatidylethanolamine methyltransferase
- **PTK** Protein tyrosine kinase
- **RB** Retinoblastoma gene
- **RNA** Ribonucleic acid

- **RT-PCR** Reverse transcriptase-Polymerase Chain Reaction
- **SRI** Stanford Research Institute
- **T4** Thyroxine
- **TAO** Troleandomycin
- **TBA** Tumor bearing-animals
- **TCDD** 2,3,7,8-tetrachlorodibenzo-*p*-dioxin
- **TCHQ** Tetrachlorohydroquinone
- **TDI** Tolerable daily intake
- **TSH** Thyroid stimulating hormone
- **TTR** Transthyretin
- **URO-D** Uroporphyrinogen decarboxylase
- **WHO** World Health Organization
- **WT** Wilms tumor gene
- **XRE** Xenobiotic response elements
- **YAC** Yeast artificial chromosome

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INTRODUCTION SECTION

Hexachlorobenzene (HCB) is a pollutant widespread in the environment. It was used principally in the 1960's and 1970's as a fungicide for cereal crops (WHO 1998). Once the toxic potential of HCB had been recognized the Government of Canada, in accordance with other industrialized countries, decided to ban HCB production in 1973. However, even if it is no longer used as a fungicide, HCB is still being synthesized as a by-product during the manufacture of chlorinated solvents, pesticides, and graphite anodes. Other applications of HCB include; as a wood-preserving agent; in the production of munitions; and uses in the rubber industry. HCB therefore accumulates in fatty tissues and maternal milk of people living in industrialized countries (Sala et al., 1999; Mes et al., 1982; Williams et al., 1984). The average total daily intake of HCB in the general population varies between 0.4 and 3 ng HCB/kg body weight/day (WHO, 1998).

HCB is a potent chemical carcinogen that can induce hepatomas, hepatocellular carcinomas, bile duct adenomas, thyroid neoplasms and parathyroid adenomas in both laboratory animals and humans (WHO, 1998; Cabral et al., 1979).

A group of studies have shown that HCB induces tumours at a greater incidence in adult female rats as compared to males. Legault et al. (1997) showed that ovariectomy caused female rats to become insensitive to the porphyric effects of HCB. However, when these same female rats were given estradiol they were again responsive to HCB, suggesting that there is an estrogenic component in the sensitivity to HCB-treatment for the induction of porphyria. Plante et al. (2002) reported that HCB decreases the expression of genes that code for proteins (connexins) involved in intercellular

communication in female while male rats were unresponsive. HCB has also been shown to disrupt phospholipid metabolism in female rats resulting in increased phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol hepatic levels following 1-4 weeks of exposure (Cochon et al., 2001). HCB is incorporated between the fatty acid chains of membrane lipids leading to increased membrane fluidity in hepatocytes (Koszo et al., 1982).

The mechanism by which HCB induces tumor formation in females is not understood. HCB is reportedly unable to either induce chromosomal damage or alter DNA repair and it exhibits weak mutagenic activity (WHO, 1998; Khera, 1974; Simon et al., 1979). Mitotic indices in human peripheral blood lymphocytes exposed to HCB suggest that it acts as an epigenetic carcinogen (Siekel et al., 1991).

The development of microarray technology may provide novel information for identifying either specific mechanisms of action of environmental toxicants but it may also provide new information on macroscopic effects of chemicals on the expression of the genome and its relation to specific pathologies. HCB is an interesting chemical to study not only to determine its effects on tumor promotion but also to identify pathways that are involved in gender-specific tumor promotion. **In this study our objective was to outline the mechanisms of HCB-induced hepatic tumor promotion by examining gender-specific expression in clusters of genes in the livers of rats treated with HCB.** To address this objective a genomic approach was used to identify a cluster of candidate genes that play a role in HCB-induced hepatocarcinogenesis. The experimental model used is excellent as it offers the possibility of looking at gene expression in a context of a

gender-specific response and 'steady-state' of expression since HCB liver concentration decreases from day 5 to 50.

The first part of this thesis comprises a literature review focusing on HCB, carcinogenesis and toxicogenomics. The first section entitled 'hexachlorobenzene' discusses in detail HCB; product usage and production, toxicological effects, effects at the molecular level and biotransformation and metabolism of HCB. The second chapter entitled 'HCB as a Co-Carcinogen or Promoter of cancer' discusses the implications of HCB in carcinogenesis and in promotion of cancer. This section introduces and integrates several critical elements involving HCB and cancer, namely: epigenetic properties of HCB, DNA methylation, hepatocellular carcinoma (HCC) in animal studies and chemical-induced sexual dimorphism of HCB in rats. The final section of the literature review entitled 'Toxicogenomics' discusses the impact of toxicogenomics as a tool in profiling gene expression in HCB-treated rats. This section searches for patterns and trends among clusters or families of genes by identifying critical genes that are differentially expressed between males and females as a result of HCB-treatment. The implications and impact of microarrays in liver cancer research is discussed as well as microarray use in identification of tumor-specific markers. The section toxicogenomics serves as a link between the first two sections in that it shows the crucial associations between HCB and cancer at the genetic level.

These three sections display the significance of this master's research project towards greater understanding of the mechanisms implicated in carcinogenesis. These sections will provide the background for the formulation of hypotheses and the specific objectives of our research, which are represented in the final sections of this thesis.

The second part of the thesis represents the various techniques and methods used in the experimental research including the results which contribute to aid in the understanding of mechanisms of hepatocarcinogenesis. Results of this research have been compiled in an article submitted to *Carcinogenesis.*

The final part of this thesis presents a general discussion including several conclusions based on the results. This section highlights the importance of this study in relation to the scientific literature and hypotheses on HCB and carcinogenesis. Furthermore attached to this manuscript is a synthesis prepared in French discussing all the key topics of this research project.

FIRST PART : LITERATURE REVIEW

SECTION 1 : HEXACHLOROBENZENE

1. HEXACHLOROBENZENE (HCB)

1.1 Product Identity, Usage and Production

Hexachlorobenzene (HCB) is comprised of six chlorine molecules attached to a parent benzene molecule (Figure 1). It is insoluble in water but is soluble in ether, benzene, chloroform and hot ethanol. HCB has a high octanol/water partition coefficient (log K_{ow} = 5.5) allowing it to pass freely through the plasma membrane (WHO 1998).

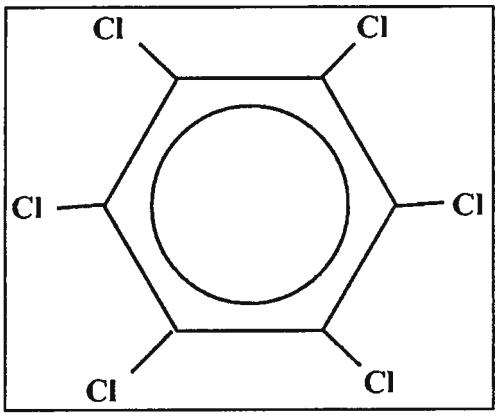

Figure 1 : Chemical composition of hexachlorobenzene. The molecule is comprised of an aromatic benzene ring surrounded by six chlorine atoms attached to each carbon atom (C_6Cl_6).

1.2 Product Uses

HCB was introduced as a fungicide in the 1940's to control for smut infestation in cereal crops such as wheat, barley, oats and rye (Government of Canada, 1993). Between

1948 and 1972, 17 different fungicides registered under the Canadian *Pest Control Products Act* contained HCB in the amounts of up to 80% (Tuttle, 1979). HCB was also produced as an impurity in the manufacture of chlorinated solvents (e.g., tetrachloroethylene, trichloroethylene, carbon tetrachloride), chlorinated compounds (e.g. vinyl chloride) and chlorinated pesticides (e.g. PCNB, DCPA, PCP) until the 1970`s (Hamdi, 1988). Other common uses of HCB include: a fluxing agent in the manufacture of aluminum, in the manufacture of graphite anodes, as a wood-preserving agent; in the production of munitions; and uses in the rubber industry (WHO 1998). HCB has been detected in the emissions from several industries, including; paint manufacturers, coal and steel producers, pulp and paper mills, textile mills, pyrotechnics producers and soap producers (Quinlivan et al., 1975; Alves and Chevalier, 1980). HCB has not been produced commercially in Canada since 1980, and as such, most of the studies reporting HCB production are referenced prior to 1980.

1.3 Production of HCB Worldwide:

Between 1978-1981 production of HCB exceeded 10,000 tons/year world-wide. The European Community produced 8000 tons/year of HCB in 1978 (WHO, 1998). Commercial production of HCB in the United States first began in 1933 (IARC V.20, 1979). Since 1982, HCB has not been produced commercially in the United States, but imports in 1982 totaled 38,000 lb. In 1990 there were two manufacturers that produced HCB for on-site use and processing and five other manufacturers who produced HCB as a by-product or impurity (SRI, 1990).

Although the Canadian government banned further use of HCB in fungicides in 1972, the levels of HCB present in the environment still remain a concern, partly due to the 30 tons of HCB that were imported into Canada between 1980 and 1983. Despite the ban on production of HCB in numerous countries, HCB remains persistent in the environment since it has a very slow half-life of degradation, ranging between 4 and 5 years (US EPA, 2002). HCB has been classified among the most persistent environmental pollutants because of its stability, resistance to degradation, and potential for bioaccumulation in the environment, animals and humans (Sala et al., 1999).

1.4 Toxicological Effects of HCB in Animals:

Animal studies have shown that HCB causes cancer and affects a wide range of organ systems including; the liver, lungs, kidneys, thyroid, reproductive tissues and the nervous and immune systems (WHO, 1998). Although the effects of HCB are widespread, those of short-term repeated exposure to HCB are primarily neurotoxic and hepatotoxic. Within the liver, the pathway involving the synthesis of heme appears to be the major target of HCB toxicity.

1.4.1 Porphyria

Porphyria is a disorder that has been characterized by a hepatic accumulation and increased urinary excretion of highly carboxylated porphyrins i.e. mainly uroporphyrin and heptacarboxyporphyrin (San Martin de Viale 1970; Elder et al., 1978). Various experimental protocols have been designed in order to assess the dose and time-response of HCB in inducing porphyria in animal models. Porphyria has been reported in rats after

short and long-term exposures between 2.5 and 15 mg HCB/kg body weight per day (Krishnan et al., 1991).

Although the mechanism of HCB-induced porphyria has not yet been fully characterized, the favored hypothesis points to a disturbance in the heme enzyme, uroporphyrinogen decarboxylase (URO-D) (San Martin de Viale et al., 1976). An unstable, reactive intermediate or metabolite of HCB is suspected to react with the catalytic SH-portion of the URO-D enzyme in the liver cytosol (Debets et al., 1980). URO-D catalyses the conversion of uroporphyrinogen to coproporphyrinogen by sequential decarboxylation of uroporphyrinogen forming decarboxylated intermediates (Tomio et al., 1970). Pentachlorophenol (PCP), the major metabolite of HCB, has been shown to accumulate excessively in the membranes of smooth endoplasmic reticulum and destroy the cytochrome P-450 complex. This can indirectly stimulate heme synthesis and result in marked porphyria caused by inhibition of URO-D (Debets et al., 1980). Mylchreest and Charbonneau (1997) observed a 4-week delay between the exposure to HCB and decreased URO-D activity accompanied by a 100 to 300-fold accumulation of uroporphyrin and heptacarboxyporphyrin in the liver and increased urinary excretion of uroporphyrin.

In Sprague-Dawley rats, females but not males have been found to be susceptible to the porphyrinogenic effects of HCB (Krishnan et al., 1991). Regardless of the dose used, similar porphyrinogenic responses were obtained 8 to 10 weeks after repeated exposure. In vivo, HCB treatment caused modification at the active center of rat liver URO-D (Billi de Catabbi, 1994). Since a decrease in URO-D activity was not observed before porphyria developed, inhibition of URO-D coincided with the onset of porphyria

in HCB-treated females. Additionally, URO-D activity was not decreased in male or female rats that did not develop porphyria (Legault et al., 1997).

1.4.2 Effects on the thyroid:

The liver is a major organ for thyroid hormone metabolism (Pisarev et al., 1995). HCB is known to be hepatotoxic and highly active in the liver by triggering changes in liver enzyme concentrations which creates disturbances in thyroid hormone homeostasis. Pisarev et al., (1995) searched for the effects of HCB on thyroid hormones and found that the HCB molecule interacts with the liver 5'-deiodinase enzyme complex by binding to a site on the enzyme which triggers a reaction in the blood stream responsible for displacing thyroxine (T4) from its carrier proteins. Pisarev et al. (1995) confirmed results from previous research (Van Den Berg, 1990) that total and free T4 levels decreased, and TSH levels increased. Furthermore, Van Den Berg (1990) has shown that HCB enhances glucuronidation of T4 through induction of UDP-glucuronyltransferases, leading to a decrease in the excretion of T4.

Some studies have examined not only the effects of HCB on thyroid hormone levels but also the effects of its major metabolite, PCP. PCP competes strongly with T4 for serum carrier proteins, which could result in an increase in free T4 entering the liver (Van Raaij et al., 1991). PCP interacts with binding sites on serum binding proteins, and specially transthyretin (TTR) (Van Raaij, 1993). Collectively, these studies demonstrate that HCB and its metabolites exert a significant toxic effect on thyroid hormones, and can alter thyroid hormone homeostasis.

1.5 Exposure levels of HCB in Humans:

The primary routes of exposure to HCB are ingestion, inhalation and dermal contact. The production of HCB as a fungicide and pesticide and as a by-product in the manufacturing of chemicals and solvents indicates that the exposure to HCB may be occupational or non-occupational in that people can be exposed to HCB in the environment and/or in the workplace. The National Occupational Hazard Survey, conducted by NIOSH from 1972 to 1974, estimated that 4400 workers were possibly exposed to hexachlorobenzene in the workplace.

It is estimated that the total average daily intake (TDI) in the general population varies between 0.4 and 3 ng/kg body weight whereas nursing infants are exposed to HCB through breast milk at a range of 0.018 to 5.1 μg/kg body weight (WHO, 1998). HCB has been detected in food. In the Food and Drug Administration's (FDA) Total Diet Program conducted in 30 cities, levels ranged from 0.006 to 0.040 mg/kg, and the average daily intake by humans in 1973 and 1974 was 0.04 μg/day and 0.07 μg/day, respectively. Moreover it was reported by the EPA's monitoring of human adipose tissue samples across the United States in 1986 that approximately 76% of the population had traceable amounts of HCB in their tissues. The recent daily intake of HCB in air, drinking water, soil and food for the average Canadian (70 kg) has been estimated at 6.2 ng/kg/day (Newhook and Meeks, 1994).

1.5.1 Occupational exposure:

There are certain occupations in which the risk of human exposure to HCB is a concern. Human exposure can occur during industrial processes such as organic

chemical synthesis, the manufacturing of chlorinated solvents and during other thermal processes (Michielsen et al. 1999). Exposure to HCB may possibly occur as a result of its presence in wastes from pesticide manufacture; where the waste is transported, HCB contaminates the ground and exposes grazing cattle around depositaries along roads (Government of Canada, 1993).

1.5.2 Toxic effects of HCB on humans:

1.5.2.1 Effects of HCB in PCT and on the thyroid

Available data on the effects of HCB are limited principally to an accidental exposure that occurred in Turkey between 1955 and 1959. Fungicides were imported from Western Europe into Turkey to combat the wheat fungus, *Tilletia tritici*. The fungicides were named Cholorable and Surmesan, and contained 10 % HCB (Peters et al., 1987). Two kilograms of fungicides were added to 1000 kg of seed, and because the treated wheat seed had arrived after planting time, it replaced edible stores of wheat. There were reports of at least 3000 people affected by HCB, the majority of which developed porphyria cutanea tarda (PCT) which contributed to a mortality rate of 10% (Peters et al., 1987). Infants of exposed mothers developed cutaneous lesions and had a high mortality rate (Peters et al., 1987). Breast-fed infants of exposed mothers developed a disorder called "pembe yara" ("pink sore") and most died within a year, experiencing weakness, convulsions and localized cutaneous annular erythema. The interval between HCB ingestion and the apparition of symptoms was approximately 6 months. Initial symptoms included weakness and the inability to handle eating utensils, rise from a squat or climb stairs and a loss of appetite. Enlargement of the thyroid gland, Parkinsonism

and other neurological effects were observed as well. Victims were exposed to a concentration of 50-200 mg HCB/day for an unknown period of time. HCB caused disturbances in porphyrin metabolism, dermatological lesions, hyperpigmentation, enlarged liver, enlargement of the thyroid gland and lymph nodes, osteoporosis and arthritis (primarily in children).

Peters et al. (1987) conducted a 30-year study on the Turkish epidemic in which they studied the health status of 225 patients in eastern Turkey that had a history of PCT between 1958-1963. The authors initially concluded from their findings that in fact the PCT had been HCB-induced since it becomes clinically evident at a very early age unlike genetic hepatic porphyria which manifests itself at a later period in life during maturity (Peters et al., 1987). Secondly, ingestion of HCB by mothers resulted in rapid transmission of the chemical in large amounts in maternal milk, killing the infant within 2 years of age. The authors concluded that even 25-30 years after initially intoxicated with HCB, many patients still showed dermatological, neurological and orthopedic symptoms (Gocmen et al., 1989).

Another event in which human health was compromised through exposure to HCB occurred in the rural village of Flix, Spain. The village comprised of roughly 5000 inhabitants and was located in proximity to an electrochemical factory (Sala et al., 1999). The factory produced volatile chlorinated solvents during several decades. Sala et al. (1999) sampled 1800 inhabitants from the region and noted that serum HCB levels (54.6 ng/ml) were 50 to 100 times higher than reported in a general population (1.5ng/ml) (Needham et al., 1990). They also found that men who had worked in the plant had a

higher prevalence of hypothyroidism, goiter, Parkinson's disease and cancer prevalence than nonworkers.

In addition several reports have shown that in North-East Spain there are areas polluted by organochlorine residues, in which the human population has accumulated high levels of HCB in adipose tissue (To-Figueras 1997). To-Figueras (1995) performed a study to determine if the baseline levels in the general population of Barcelona are cause for concern. Results indicated that HCB was detected in all (n=100) of the serum samples analyzed and 91 % of the volunteers in the experiment showed serum HCB baseline levels of > 1 ng/ml. Moreover, Grimalt et al. (1994) identified a possible link between a high exposure to HCB in the area of Flix, Spain (small population) and an excess risk of soft-tissue sarcoma, thyroid cancer and brain cancer. The serum HCB levels of the Flix inhabitants showed only a 5-6 fold increase in comparison with the baseline levels shown here in the general population of Barcelona (>1 ng/ml). Given that the serum HCB baseline levels (> 1ng/ml) are a representative sample of a very large human population the toxic risk does not seem negligible (To-Figueras et al., 1995).

Recently a cross-sectional study conducted by Sala et al. (2001) on the general population of Flix, Spain displayed specific associations between serum HCB levels and markers of thyroid and liver function. They found HCB to be present in the serum of patients at a concentration of 36.7 ng/ml. HCB had no effect on TSH or free T4 concentrations, however a significant reduction in total T4 hormone levels was reported which is consistent with effects seen in animal models. The authors also reported a positive correlation between HCB levels and γ-glutamyltransferase (GGT) a biological marker of liver function. They believe HCB caused an induction of enzymatic activity

thereby causing an increase in GGT levels. The results of this study suggest that the present dose of HCB in this population has a metabolic effect on thyroid and liver function as well as the enzymatic induction of HCB.

Selden et al. (1989) reported an incident in which a 65 year old man had been occupationally exposed to high concentrations of HCB for 50 years, and as a result, developed hepatocellular carcinoma. The patient devoted 25% of his time to the procedure of aluminum smelting, in which a degassing agent containing 30% hexachloroethane (HCE) was added to each melting pot. As a result of the HCE additions, the melting pot emitted strong chlorine smelling smoke and heavy gas emissions. Four years after retirement, the patient was diagnosed with a large malignant tumor in the right liver lobe, showing typical signs of highly differentiated hepatocellular carcinoma (Selden et al., 1989). The authors strongly believe that given the carcinogenic potential already exemplified in numerous animal studies (Cabral et al., 1979; Arnold et al., 1985) this human case study shows a strong correlation between exposure to HCB and developing liver cancer.

1.6 Effects of HCB at the Genetic and Molecular level:

1.6.1 Effects of HCB on the plasma membrane and phospholipid metabolism:

Due to the lipophilic character of HCB, lipid-rich membranes (i.e. plasma membrane) are important targets for its interactions with living organisms. The data from spin-label studies suggest that HCB is able to incorporate itself between the fatty acid chains of individual phospholipids (Koszo et al., 1982). This leads to increased fluidity of the plasma membrane and disturbs the general homogeneity of the membrane.

Membranes that have been treated with HCB show an increased phospholipid content and a decrease in free fatty acids (Cantoni et al., 1987). In addition, disrupted phospholipid metabolism has been documented in female rats treated with HCB (Cantoni et al., 1987; Billi de Catabbi et al., 1997; Cochon et al., 1999). Recently a study demonstrated that HCB increased phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol hepatic levels during the first 4 weeks of HCB treatment and subsequently decreased after 8 weeks of exposure. The early increase of these phospholipids occurred as a result of proliferation in hepatic microsomal membranes (Billi de Catabbi et al., 2000). At the microsomal level, HCB is metabolized by specific cytochrome P-450 enzyme complexes.

In order to better understand the effects of HCB on lipid-rich membranes Suwalsky et al. (1999) incubated human erythrocyte (red blood cells) membranes with HCB to examine if HCB had the ability to deform human lipid-rich plasma membranes. Their results indicated that HCB induced a gross alteration in the morphology of the membrane from a normal discoid biconcave shape to a more invaginated shape "stomatocytic form".

Considering the importance of membrane surface proteins and enzymes in maintaining normal cellular function, Randi et al. (1998) analyzed the effects of HCB in altering the activities of membrane-bound enzymes and protein expression. HCB (100 mg/100 g b.w.) was administered daily to female Wistar rats for 2, 5, 10, 20 and 30 days. Two plasma membrane-bound enzymes: 5'nucleotidase and Na^+/K^+ ATPase activity were significantly reduced by HCB treatment. Additionally, the authors noted significant alterations in the binding efficiency of EGF to the hepatic membrane (134 fmol/mg in

control versus 468 fmol/mg in HCB-treated animals) after 10 days of HCB treatment. The authors also noticed time-dependent effects of HCB treatment on the phosphorylation activity of the rat liver microsomal membrane protein tyrosine kinase (PTK) (Randi et al. 1998). Significant increases in PTK activity were observed after 2 and 5 days of treatment. However, by day 10 of treatment, PTK activity significantly decreased and subsequently returned to control levels after 20 days. It has been shown that the binding of dioxin-like chemicals (i.e. hexachlorobenzene) to the aryl-hydrocarbon receptor complex (Ah-R) could activate the phosphorylation of important proteins in the cytosol and plasma membrane eliciting functional changes (Matsumura, 1995).

1.6.2 The AhR receptor:

Most of the toxic effects of linear halogenated aromatic hydrocarbons (HAH's), such as TCDD (dioxin), are mediated through the Ah-R. The Ah locus controls the induction of the cytochrome P-450-mediated mono-oxygenase activities by the polycyclic aromatic hydrocarbons (Hahn 1988). The Ah locus includes structural, regulatory and temporal genes (Eisen et al., 1983). Temporal genes control the time and place of action of other genes and the differentiation of cells and tissues. HCB is classified as a dioxin-like compound since it binds to the Ah-R resulting in dioxin-like effects (Hahn, 1988). The Ah-R is a ligand-dependent transcription factor that belongs to the basic-helix-loop-helix superfamily of DNA-binding proteins.

HCB, being highly lipophilic, enters directly through the plasma membrane and binds to the Ah receptor complex. The complex is comprised of the heat shock proteins:

HSP 90, HSP 70, HSP 50, the Src-protein Kinase and the Ah receptor (Gonzales et al., 1993). After the ligand binds to the receptor complex, the Ah receptor dissociates itself from the heat shock proteins, leaves the cytosol and *trans*-locates into the nucleus. Once in the nucleus the Ah-R/HCB complex forms a heterodimer with the Ah-R nuclear translocater protein, and it is then able to bind to *cis*-elements on the DNA referred to as xenobiotic responsive elements (XRE) (Loaiza-Pérez et al. 1999).

1.7 Kinetics and Metabolism of Hexachlorobenzene

Organisms are constantly exposed to a large number of xenobiotics, such as drugs, pesticides, food additives and industrial chemicals. Unlike bacteria, animals have evolved ways in which to eliminate foreign substances. Most xenobiotics are highly lipophilic (i.e. HCB) and would remain quite persistent in the human body if they were not biotransformed and made more hydrophilic. The process of biotransformation is regulated by several enzyme systems referred to as Phase I and Phase II enzyme complexes. In phase I enzyme systems the main purpose is to transform the highly lipophilic substance into a water-soluble complex that can be eliminated from the body through excretion (Sipes and Gandolfi, 1986).

Biotransformation of xenobiotic substances predominately occurs in the liver although extrahepatic metabolism have been observed in other organs such as; lung, kidney, skin and gastrointestinal mucosa (Kaminski and Fasco, 1992; Dahl and Hadley, 1991). The activities of biotransforming enzymes can be activated or repressed following treatment with specific chemicals. Activation of the phase I or Phase II enzyme complexes can alter the pharmacological or toxicological response of xenobiotics and even increase their toxicity.

1.7.1 Cytochrome P450 complex: enzyme activation and/or repression

It is well documented that the cytochrome P450 mono-oxygenase system plays a crucial role in the metabolism of exogenous (foreign agents) and endogenous substances (steroids and fatty acids) (Wislocki et al. 1980). In addition, halogenated aromatic hydrocarbons such as TCDD, PCB's and PBB's have been shown to induce specific isoenzymes of hepatic cytochrome P-450 (Goldstein et al. 1986). In rats, humans, rabbits and mice, HCB are inducers of the Cytochrome P450IA1/2 complexes (den Besten, 1992). HCB is classified as a mixed-type inducer in that it induces not only P-450c and P-450d complexes but also P-450b and P-450e types. Furthermore, HCB has been documented to increase the translatable mRNA coding for these cytochrome proteins (Hardwick et al., 1985).

The cytochrome P450 complex is incorporated in the endoplasmic reticulum in combination with the electron transport chain at the cell membrane. The catalytic site of the complex contains a heme prosthetic group that has been shown to be a crucial element in the manifestation of several key physiological and toxicological responses of HCB during its biotransformation to its metabolites.

1.7.2 Metabolism of chlorinated benzenes:

Several investigators have carried out experiments designed to decipher the mechanism of action responsible for the biotransformation of HCB (Figure 2). When female Wistar rats were administered 2 to 3 injections of 130mg/kg ^{14}C-HCB, only 7 % and 27 % of HCB was excreted in the urine and faeces respectively. One month after

treatment more than 90% of labeled HCB was excreted through the urine as metabolites. However, only 25 % of the labeled HCB excreted by the faeces was in the form of metabolites, the majority (75 %) of the ^{14}C labeled HCB remained unchanged. Cytochrome P450-mediated oxidation of HCB results in the formation of its major metabolites: pentachlorophenol (PCP), tetrachlorohydroquinone (TCHQ), pentachlorothiophenol and a minor metabolite; tetrachlorothiophenol (Koss et al., 1976). To estimate the extent of HCB biodegradation, the authors examined the amount of the label present in HCB in comparison with the amount contained in its metabolites. They determined that about 2/3 of the recovered label was still retained within the animals body as unchanged HCB. Whereas only 1/3 was excreted from the body among which only half was converted into metabolites, concluding that only 16 % of the xenobiotic was being metabolized whereas the majority is stored in adipose tissue and is quite stable. In a follow–up experiment conducted over a 53-week period, the authors investigated the effects of HCB on long-term administration in the female Wistar rat. HCB was administered at a concentration of 50mg/kg every other day for a period of 15 weeks (Koss et al., 1978). One observation was that 53 weeks after initial treatment the only substance that could be found in the blood, liver, urine or feces besides HCB was PCP. In addition a rat excretes 60 % of the xenobiotic unchanged and 40 % in the form of metabolites. Since the elimination phase of HCB decreases over time, 9 to 12 weeks after HCB administration the level of HCB in the bloodstream is maintained at an equilibrium state. Furthermore the half-life of HCB degradation was calculated to be between 4-5 months.

The major isoenzyme involved in the hydroxylation of HCB is the P450IIIA1/2 (Van Ommen et al. 1989). Selective inhibition of the isoenzyme P450IIIA1/2 was confirmed by pre-treatment of HCB with TAO a selective inhibitor of the cytochrome complex (den Besten, 1992). It was shown that co-treatment of TAO with HCB reduces the formation of PCP and TCHQ (den Besten, 1992). In a long-term study Koss et al. (1978) treated female Wistar rats via oral gavage of 50mg/kg for 15 weeks. They noticed that the concentration of HCB in adipose tissue was about 30-60 times higher than originally. Moreover other researchers noticed a rapid accumulation of HCB in adipose tissue after exposure to a level 200-500-fold higher than reported in serum.

1.7.3 Glutathione Conjugation of HCB

The gluthathione S-transferases (GSTs) are a class of dimeric proteins involved in cellular detoxification of electrophilic substrates (Thomas et al., 1998; Mannervik and Jensson, 1982; Whalen and Boyer, 1998). They are among the class of Phase II biotransformation enzymes induced by a variety of xenobiotics. Conjugation of the thiol group of glutathione to an electrophilic center increases the solubility of that substrate and in its excretion from the cell. HCB is a broad inducer of the GSTs (Thomas et al., 1998). The major functions of cellular GSH include; detoxification of reactive electrophiles and peroxides, maintaining vitamins C and E in functional forms and supplying extracellular GSH. The ratio of cellular GSH/GSSG is a dynamic indicator of oxidative stress. When glutathione is predominantly in its reduced form, its primary role is the elimination of reactive oxygen species. A higher GSH/GSSH ratio induces thiol-

disulfide balance of proteins, enzymes receptors and transcription factors. These proteins allow xenobiotics to be easily eliminated from the liver (den Besten, 1992).

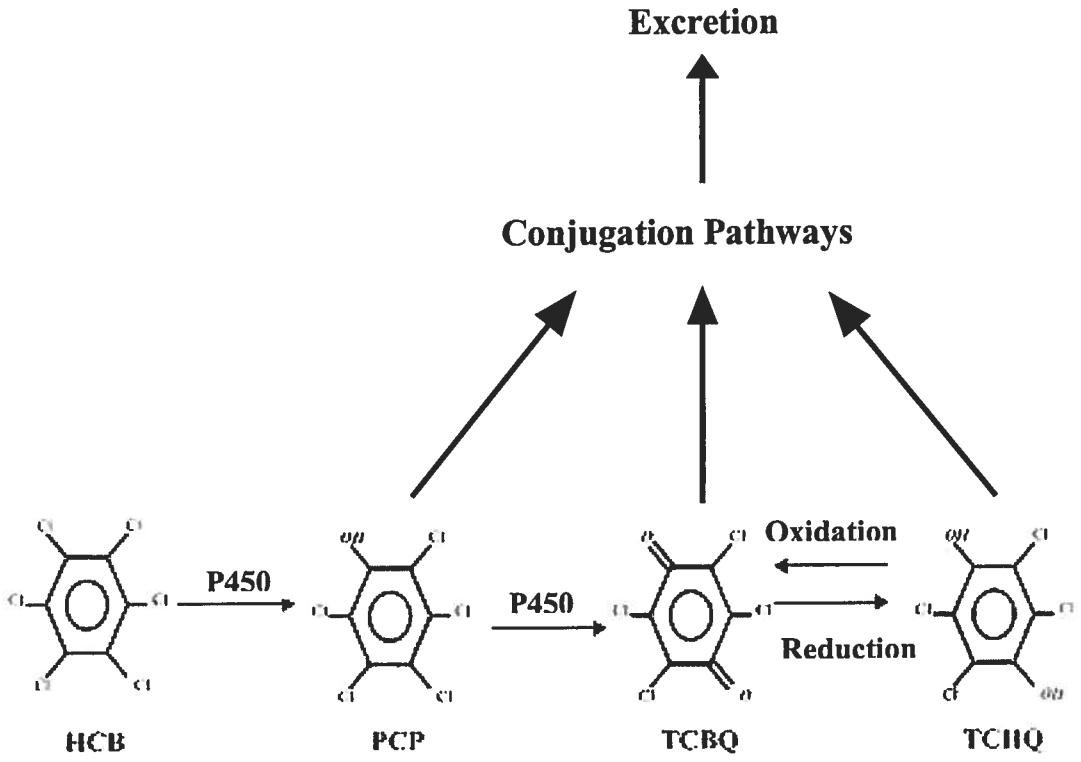

Figure 2 : Metabolic pathways and biotransformation of hexachlorobenzene. Production of different metabolites by the cytochrome P450 enzyme complex, oxidation and glutathione conjugation mechanisms. Cytochrome P450-mediated oxidation of HCB results in the formation of the major metabolites of HCB; pentachlorophenol (PCP) and tetrachlorobenzoquinone (TCBQ) and then reduced into tetrachlorohydroquinone (TCHQ) (den Besteen, 1992).

SECTION 2 : HCB AS A Co-CARCINOGEN OR PROMOTER OF CANCER

2. Liver Cancer:

Liver cancer is the fifth most common cancer worldwide with almost a half million cases reported in 1990 (Parkin et al., 1999) and hepatocellular carcinomas (HCC) are accountable for 85% of the liver cancer cases (Graveel et al., 2001). Liver cancer is highest in the areas of Asia and Africa where the population shows a high predisposition to hepatitis B and C infection (Buendia, 2000). Unlike other cancers such as skin and colon, a clear mutational model for liver cancer has not yet been elucidated that could help clarify the invasive, progressive and metastatic potential of liver tumors.

2.1 Tumors:

The liver is susceptible to induction of cancer by a large number of carcinogens. These may be of synthetic or natural origin. *Adenomas* consist almost entirely of hepatocytes and don't display a lobular or portal structure. Focal *hepatocellular changes* may result from administration of xenobiotics (Vandenberghe, 1996). If the focal changes cover a surface area larger than a liver lobule and causes compression of the surrounding liver parenchyma, they are referred to as hyperplastic nodules. They are similar to adenomas in man.

True hepatocellular tumors or *carcinomas* rarely occur spontaneously. They are characterized by altered mitotic activity and/or invasion and/or metastasis (Vandenberghe, 1996). Prominent occurrence of these tumors as readily observed in

HCB-treated animals, arises from induction through initiators and promoters. In humans, some cases of hepatocellular carcinoma have been associated with the use of steroids, contraceptives and estrogens (Mays and Christopherson, 1984; Marino et al., 1998). *Hemangiomas* and *hemangiosarcomas* (benign and malignant tumors of the blood vessels) have been reported in rats and mice.

2.2 Cancer:

Cancer can be viewed as a complex group of diseases affecting a wide range of cells and tissues. A genetic link to cancer was proposed early in the twentieth century and has served as a foundation for cancer research (Klug and Cummings, 1997). Mutations that alter the genome or gene expression are now regarded as a common feature of all cancers. Genomic alterations associated with cancer can include small-scale and large-scale events. Small-scale changes include single nucleotide substitutions. Large-scale events include visible chromosome rearrangements (Klug and Cummings, 1997). Cancer cells have two properties in common: 1. uncontrolled growth and 2. the ability to spread or metastasize from their original site to other locations in the body. The metastasis of cancer cells is controlled by gene products that are localized to the cell surface. A metastatic cancer cell can spread from a primary tumor by entering the blood or lymphatic circulatory system. These cells are carried in circulation until they become fixed into the capillary bed (Klug and Cummings, 1997). Normally 99% of the cells die, but the surviving cells invade tissue adjacent to the capillary bed and begin dividing to form a secondary tumor. To reach a new target cell, the tumor cells pass through a layer of epithelial cells lining the wall of the capillary (or lymph vessel) to penetrate the

surrounding extracellular matrix. The surrounding matrix is a meshwork of proteins and carbohydrates that serves to inhibit the migration of cells. However tumor cells secrete enzymes that digest proteins on the outside membrane of the matrix creating "tunnels" or holes in which they pass through and invade subsequent cells. These cells will continue with this process since there is no control and regulation is lost (Klug and Cummings, 1997).

Genetic studies of several different cancers have targeted a set of genes that must be mutated in order to trigger the development of cancer or maintain the growth of malignant cells. In general, cell division can be regulated in two ways: 1) by **tumor suppressor genes** that normally suppress cell division; and 2) by **proto-oncogenes** that promote cell division.

2.2.1 Tumor suppressor genes:

Tumor suppressor genes inactivate or repress passage through the cell cycle and cell division. Both copies of these genes or their products must be absent or inactive for cell division to take place (Klug and Cummings, 1997). In this respect the genes are "recessive" since both copies must be mutated to have a tumor-promoting effect (Wolfe, 1995). If these genes become permanently inactive, control over cell division is lost, and the cell begins to proliferate in an uncontrollable fashion. Common examples of tumor suppressor genes include the retinoblastoma (RB) gene, the wilms tumor (WT) gene, p53, NF-1 and the breast cancer genes (BRCA1 & BRCA2).

2.2.2 Oncogenes:

Proto-oncogenes normally function to promote cell division. The mutated forms are known as oncogenes. These genes can be turned "on" or "off", but when they are activated they promote cell division. There are two mechanisms by which protooncogenes are transformed into oncogenes: 1. changes in the structure of the gene, resulting in the synthesis of an abnormal gene product having an irregular function and 2. changes in regulation of gene expression, resulting in enhanced production of the normal growth-promoting protein (Cotran et al., 1999; Klug and Cummings, 1997). If proto-oncogenes become permanently switched on, then uncontrollable cell division occurs leading to tumor formation (Klug and Cummings, 1997). Oncogenes are of the "dominant" form in that only a single mutated copy of the gene is needed to promote uncontrolled growth. A study searching for the origin and initiation of human tumors showed that when DNA is extracted from various human tumors and transfected into fibroblast cell lines, the recipient cells will exhibit neoplastic properties. The researchers concluded that the DNA of spontaneously arising cancers contains oncogenic sequences or oncogenes (Cotran et al., 1999). Common oncogenes include; *fos, jun, myc, p53, raf, ras, src.* The *myc* gene for instance can bind directly to DNA and regulate genes controlling the transition between G1-S (Wolfe, 1995).

2.3 Epigenetic or Genotoxic Carcinogen:

The carcinogenicity of HCB has been assessed in several bioassays in rats, mice and hamsters. Khera et al. (1974) administered HCB to male rats for 10 consecutive days

with a concentration of up to 60 mg/kg/day and reported the failure of HCB to induce dominant lethal mutations in rats. Another study conducted by Simon et al. (1979) set out to further identify whether HCB has the ability to directly act as a genotoxic agent. The authors administered up to 221 mg/kg HCB for 5 consecutive days to male rats and then mated them with 2 nulliparous (no pregnancies) females in order to determine lethal mutations and reproductive effects. As reported by Khera et al. (1974), HCB did not induce any classical dominant lethal effects. Most chemicals that cause dominant mutations manifest themselves within the first weeks (1-5) of mating (Simon et al. 1979). In this experiment, significant effects were only observed 10-14 weeks after initial administration of HCB, indicating that HCB may act as a non-genotoxic agent, i.e. epigenetic carcinogen. Additionally, an *in vitro* study conducted by the IARC reported the failure of HCB to induce chromosomal aberration on Chinese hamster cells.

Other studies have examined the mutagenic properties of HCB on auxotrophic mutants and bacterial strains. Reverse mutation tests were carried out on *Salmonella typhimurium* and *E.scherichia coli* strains. Siekel et al. (1991) reported seeing no significant increase in the mutagenic activity of either strain as a result of HCB. A subsequent test was set up to examine the genetic activity of HCB on bacterial strains. The authors created an assay that contained bacterial strains showing different levels of mutations in their DNA repair system and reported the effects on growth inhibition of the assays due to various mutagens. HCB had no genotoxic activity in the DNA repair system that has been used as a sensitive assay in indicating prokaryotic DNA damage (Siekel et al. 1991). The authors also examined the effects of HCB on proliferation of human peripheral blood lymphocytes. They looked at the occurrence of chromosome and

chromatid breaks and calculated the percentage of cells with aberrations. The cytotoxic effects of HCB were assessed by evaluating their mitotic indices. The occurrence of chromosomal aberration did not suggest any clastogenic or mutagenic activity of HCB and results showed only an insignificant cytotoxic effect in comparing the mitotic indices between experimental groups. The conclusions of these studies examining potential mutagenic properties of HCB on bacterial strains, auxotrophic mutants and a slew of animal models was that HCB does not act as a genotoxic agent, but rather as an epigenetic carcinogen.

Several common characteristics of non-genotoxic carcinogens include specificity, existence of a threshold and reversibility (Lima, 2000). HCB exhibits several of these properties. HCB has a tumorigenic potential more specific to one sex, i.e. females are more likely to develop hepatic tumors than males treated with HCB. In addition, the development of tumors only occurs after an administration of a minimum concentration of HCB i.e. HCB five consecutive daily doses of 100mg/kg were used to attain a minimal level of HCB in the blood of the animal to produce a prolonged interference with the normal physiological control of cellular proliferation patterns (Lima, 2000). These results help categorize HCB as an epigenetic carcinogen.

2.3.1 Epigenetic Mechanisms of Carcinogenesis: DNA Methylation

Epigenetics is defined as "The study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence" (Russo et al, 1996). There are two epigenetic systems that affect animal development and heritability; DNA methylation and polycomb-trithorax group (Pc-G/trx) protein

complexes (Bird, 2002). This review will focus on the important links between DNA methylation and carcinogenesis since the results of our research have displayed several common trends indicating significant modulations in methylation machinery.

Cytosine 5'-methylated (m^5C) DNA is the end-product of a chemical reaction between CpG rich DNA and S-adenosylmethionine, catalysed by DNA methyltransferse enzymes (DNMT) (Patra et al. 2001). DNA methylation is a highly dynamic system that responds well to endogenous and exogenous substances (Zingg et al. 1997). Methylation only occurs on 'CpG islands' which are short stretches of CpG rich regions associated with the promoter region of genes (Toyota and Issa, 1999). They are often 1kb long and often found in the 5' region of genes. There are variable patterns of methylation in different species. The insect *Drosophila melanogaster* for instance contains a low methylation (m^5C) level (Gowher et al, 2000). C. *elegans* has a high level of DNA methylation. Methylation is dispersed over much of the genome for vertebrates, a pattern referred to as global methylation. The diversity of DNA methylation patterns in various species can reflect the potential for the different functions of DNA methylation (Colot and Rossignol, 1999). In human somatic cells, m^5C accounts for ~1% of total DNA bases and therefore affects 70-80% of all CpG dinucleotides in the genome. These clusters are also 'hot spots' for mutations as with the P53 gene (Toyota and Issa, 1999). Studies indicated that ~60% of human genes are associated with CpG islands, of which the great majority are unmethylated at all stages of development and in all tissue types (Bird, 2002).

Croce et al. (2002) have shown that cancer cells induce both hypermethylation of regions of CpG clusters within the DNA as well as global hypomethylation of the

genome. They have shown that DNA methylation of tumor suppressor genes is a frequent mechanism of transcriptional silencing in cancer. Methylated genes become transcriptionally inert. The inactive state of silent genes is stabilized through cell proliferation and differentiation (McBurney, 1999). Aberrant methylation of CpG islands in tumors serves as an alternative pathway for complete inactivation of tumor suppressor genes (Ivanova et al. 2002). Berger et al. (2002) showed that methylation is the mechanism responsible for epigenetic silencing of nuclear receptor genes during carcinogenesis and tumor promotion. Counts and Goodman (1995) proposed that examining DNA methylation patterns has the potential for discovering alterations in gene expression, cell proliferation, mutation, chromatin aberrations and inactivation of tumor suppressor genes.

2.4 Carcinogenesis of HCB in liver of animals:

Experimental evidence on the carcinogenicity of HCB was first provided by Cabral et al. (1977). The authors reported a significant increase of "liver cell tumours (hepatomas)" in male and female Syrian Golden hamsters that were fed 50, 100, or 200 ppm HCB in their diets for life. The incidence of liver "haemangioendotheliomas" was increased in both sexes at 200 ppm and so was the occurrence of thyroid adenomas. Subsequent to the experiment on hampsters Cabral decided to demonstrate if HCB is carcinogenic to mice.

Outbred male and female Swiss mice were fed HCB at concentrations of 0, 50, 100 and 200 ppm for 120 weeks. At 90 weeks, 4% of the males and none of the females survived as compared to the survival rates for the controls 50 % for males and 48% for

females. In controls, the percentage of tumor-bearing animals (TBA) were 80% in females and 47 % in males. The tumors most frequently observed in both control and treated mice were lymphomas and lung adenomas while liver-cell tumors were only observed in HCB-treated animals (Cabral et. al, 1979). In HCB-treated females exposed to 200 ppm there was a significant increase in the incidence of liver-cell tumors (hepatomas) (34% for females, 16% for males).

Having witnessed an increased incidence of liver-cell tumors (LCT) and the carcinogenic potential of HCB, Smith and Cabral (1980) investigated the effects of HCB on female Agus rats that have been fed 100 ppm (5 mg/kg bw/day) for up to 90 weeks. Groups of female Agus rats (n=14) and female MRC Wistar rats (n=6) were sacrificed at 75 and 90 weeks after treatment and analyzed for incidences of liver-cell tumors. Results indicate that all HCB-treated female Agus rats showed multiple liver-cell tumors and none of the controls showed any signs of liver-cell tumors. In addition 4 of the 6 HCB–treated Wistar rats showed liver-cell tumors and 2 showed hepatocellular hypertrophy whereas none of the controls had any tumor development.

Arnold et al. (1985) performed an *in utero* exposure of HCB to Sprague-Dawley rats. Weaning male and female rats were fed diets containing 0.3, 1.6, 8.0 or 40 ppm HCB. Three months after treatment, the F_0 rats were bred and 50 F_1 pups from each sex were randomly selected. The F_1 pups were then continued on the same diet (from weaning) for up to 130 weeks. The findings of this study indicated that nodules in the livers of female rats constituted the only neoplastic liver lesion for which there was a dose response.

In a similar experiment Lambrecht et al. (1983) weanling male and female Sprague-Dawley rats were fed diets containing 0.75 or 150 ppm of HCB for up to 2 years. Significant increases in incidence of "hepatomas/hemangiomas" and of renal cell adenomas were observed in both sexes. Notable increases in HCC and bile duct adenomas/carcinomas were only observed in females at both doses. Furthermore, increases in adrenal cortical adenomas at 75 ppm and phaeochromocytomas at both doses were exclusive to females (U.S. EPA, 1985).

2.5 HCB as a cocarcinogen or promoter of cancer:

While there are numerous studies that illustrate the carcinogenic properties of HCB directly, there are few studies that address the mechanism by which HCB could act as a promoter of cancer or as a cocarcinogen. Simultaneous exposure to HCB in the diet of mice treated with polychlorinated terphenyl enhanced the induction of liver tumors (Shirai et al., 1978). Smith et al., (1989) showed that dietary exposure to HCB subsequent to iron administration will also lead to a promotion in the development of liver tumors in rats.

A study conducted by Pereira et al. (1982) set out to determine the effects of HCB in rats following pre-treatment with diethylnitrosamine (DEN; tumor initiator). One of the main objectives of the study was to determine whether HCB acted as an initiator or as a promoter of liver tumors leading to hepatocarcinogenesis. Results from a genetic interaction involved the covalent interaction of HCB with the DNA resulting in neoplastic progression (Pereira, 1982). Experimental evidence that HCB does not appear to be mutagenic itself (Khera et al. 1974; Simon et al. 1979; Siekel et al. 1991) indicated

that it is not an initiator but rather a promoter of carcinogenesis. Results of the study indicated that only rats pre-treated with DEN had a significant increase in GGTase-positive foci in the liver. GGTase-positive foci are putative precursor lesions and indicators of hepatocarcinogenic activity (Pereira 1981, Pitot 1980) and induction by HCB of the GGTase foci indicate that HCB is also a promoter of hepatocarcinogenesis. It would appear from this study that the hepatocarcinogenesis results from tumor promotion of already initiated hepatocytes. Furthermore, Pereira et al. (1982) observed a critical finding; females were much more susceptible to the development of these preneoplastic lesions, GGTase-positive foci, than males.

2.6 Chemical-induced Sexual Dimorphism of HCB in rats:

It has been demonstrated that female rats are more susceptible in the development and promotion of hepatic tumors than males (Pereira 1981, Pitot 1980). Rizzardini and Smith (1982) examined the sexual differences in the metabolism of HCB and the development of porphyria in Fisher rats. Male and female rats were administered 14 mg/kg bw/day for 103 days. The development of porphyria was much more pronounced in female rats in that the concentrations of porphyrins in the urine and livers were 40 and 310-fold higher, respectively than in HCB-treated males, which were similar to controls. Additionally, the levels of free hepatic pentachlorothiophenol, a metabolite of HCB (Koss 1976), was *3.6-fold* higher in females than in males. This result correlated well with the elimination of this metabolite in the urine, since urinary and total excretion of pentachlorothiophenol was significantly higher in females than males.

Once it had been identified that females were more susceptible to the hepatocarcinogenic and porphyrinogenic effects of HCB, Smith et al. (1985) wanted to determine whether a link existed between these toxicological effects. HCB was fed to male and female Fisher rats as 0.02% of their diet for up to 90 weeks. After 15 weeks of treatment, the investigators monitored the short-term toxicological effects of HCB. HCB-treated females showed a noticeable difference in the accumulation of porphyrins in the liver 548 nmol/g vs. 0.90 nmol/g. Morphological differences in the liver of treated females included: proliferation of smooth endoplasmic reticulum, reduction of glycogen content, large numbers of autophagic vacuoles, lipid droplets, abnormal mitochondria and increased peroxisomes.

The long-term toxicological effects were manifested after 90 weeks of treatment with HCB. The livers of all HCB-treated females (100%) that survived had several large dark red tumors (2-6 per liver), which stained positive for GGT (range 5-20mm). Only 2 males (16%) developed tumors; these were small and only one per liver occurred with no nodules or carcinomas.

In order to establish a link between the two toxicological effects of HCB, the authors proposed that the susceptibility of female rats to porphyria and tumor induction was related to their having a greater ability to store iron (Munro and Linder, 1978). However, in this study the authors preloaded males with iron and it showed no effect on their sensitivity to porphyria. It is difficult to establish a direct link between the susceptibility of females to the porphyrinogenic effects of HCB (short-term) and the development of hepatocarcinogenesis (long-term) since after 90 weeks, no URO-D activity was detected in treated livers of either sex (Smith et al. 1985). However, the

authors did notice a sexual dimorphism in that females had higher levels of porphyrins for a much longer period of time and it has been documented that patients with PCT have a higher risk of developing a primary hepatocellular carcinoma (Doss and Martini, 1978).

Other studies examined the role of sex hormones as the determining factor in the susceptibility of female rats to the development of porphyria. Grant et al. (1975) noticed that removal of the ovaries in females receiving a diet containing HCB decreased the level of porphyrins in the liver. Smith and Francis (1981) treated male rats for more than 100 days with HCB and observed only small changes in hepatic porphyrin and URO-D activity, a critical heme synthesis enzyme. However when the same rats were given the estrogenic drug, diethylstilbestrol (DES), there was a large increase in porphyria levels accompanied with a reduction in URO-D activity.

Legault et al. (1997) showed that the responsiveness of female rats, but not male rats, to the porphyrinogenic effect of HCB is dependent on the presence of estrogens rather than the absence of androgens. Groups of control females, ovariectomized females, castrated males and castrated males given 17β-estradiol (prior to HCB treatment) were given five consecutive daily doses of 100mg/kg HCB. This protocol has the advantage of giving a minimal amount of HCB to fully induce porphyria in a well-defined time frame (Legault 1997). The level of hepatic porphyria was measured through urinary uroporphyrin excretion level. The authors found that castrated males alone showed no signs of developing porphyria, whereas castrated males given 17β-estradiol developed significant levels of porphyria. Furthermore, ovariectomized females were unresponsive to the porphyrinogenic effects of HCB, but when these same ovariectomized females were given a subsequent round of HCB treatment concurrently with 17β-estradiol, there

were again responsive, clearly indicating a critical role for estrogens in HCB-induction of porphyria. Estrogen proved to be an independent risk factor for the development of PCT, but the mechanism responsible for the estrogen effect is unknown (Bulaj et al. 2000).

SECTION 3 : TOXICOGENOMICS

3. Toxicogenomics:

Genomics is a field that emerged as a result of the Human Genome Project (HGP). By understanding the sequence of the human genome we can now use this information as tools to assess how the expression of groups of genes is altered during the development of pathologies, such as cancer. Toxicogenomics is a rapidly developing discipline that promises to aid scientists in understanding the molecular and cellular effects of chemicals in biological systems (Hamadeh, 2002). Rather than using animal pathologies to identify illnesses it probes human or animal genetic material printed on plates called DNA arrays (Lovett, 2000). Using technologies such as DNA microarrays or high throughput NMR and protein expression analysis, this field displays a more global picture of biological mechanisms and pathways. The advantages of these tests are that they are fast, efficient and they reduce live-animal expenses which can range from $3000 a week, per animal (Lovett, 2000). Another benefit of using this new technology as it relates to environmental carcinogenesis, is that by using cDNA arrays, researchers are able to target metabolic precursors or inducers of slow-developing diseases without having to wait for tumors to develop which can take months or years. Cancer researchers have already been using these arrays to compare gene expression in healthy and diseased cells. Toxicologists are using the same technology to profile gene expression in cells exposed to foreign substances and xenobiotics.

3.1 The Human Genome Project:

The HGP is an international, collaborative research program whose goal is the complete mapping and understanding of all the genes of human beings. The HGP is the natural culmination of the history of genetics research. In 1911, Alfred Sturtevant, then an undergraduate researcher in the laboratory of Thomas Hunt Morgan, realized that he could- and had to, in order to manage his data – map the locations of the fruit fly *(Drosophila melanogaster)* genes whose mutations were tracked over several generations. HGP researchers are deciphering the human genome in three major ways; determining the 'sequence' of all the bases in our DNA; making maps that show the locations of genes for major sections of all our chromosomes; and producing linkage maps through which inherited traits can be tracked over generations.

In the mid 1970's Frederick Sanger developed techniques to sequence DNA. The advent of automation of DNA sequencing led to the idea of sequencing the entire human genome. A committee appointed by the US National Research Council accepted the concept in 1988 but indicated the necessity of the creation of genetic, physical and sequence maps of the human genome along with parallel efforts in key model organisms such as bacteria, yeast, worms, flies and mice. In 1986 the Department of Energy announced its Human Genome Initiative which placed emphasis on gathering the proper resources and technologies for mapping, sequencing, computing and infrastructure support that would lead to a complete sequence of the human genome (National Research Council, 1988). Similar initiatives were established in different countries. Through 1995, work on the HGP progressed rapidly on two levels. First the construction of genetic and

physical maps of the human and mouse genomes provided key tools for identification of genes that were implicated in diseases. Second the sequencing of the yeast and worm genomes, as well as targeted regions of the mammalian genomes provided insight into position and clustering of genes (Int. Human Genome Sequencing Consortium, 2001). A large part of the early work on the HGP was devoted to improving molecular biological and genetic technologies. Several improved research techniques enabled rapid progress of the HGP techniques such as, use of restriction fragment-length polymorphisms, the polymerase chain reaction, BAC's, YAC's and pulsed-field gel electropheresis.

These pilot projects were instrumental in exemplifying that large-scale sequencing are feasible and they demonstrated that complete genomic sequences reveal key information on genes, regulatory regions and chromosome structure that was not readily available from cDNA's studies alone. Additional pilot projects were launched in 1999 to demonstrate large-scale sequencing of the human genome. These projects were successful in producing a finished sequence with 99.99% accuracy and no gaps (Int. Human Genome Sequencing Consortium, 2001). They also introduced a new large-insert cloning system, the bacterial artificial chromosome (BACs), which are more stable in DNA than cosmids or yeast artificial chromosome (YACs). The HGP has revealed that there are between 30,000 and 40,000 human genes. In February 2001, the International Human Genome Sequencing Consortium published the first draft of the human genome in the journal *Nature* (Feb. 15 2001) which included a 90% complete sequence of all three billion base pairs of the human genome.

3.2 Profiling Gene Expression:

The human genome project identified and characterized thousands of genes. Although many of these genes have been assigned to specific structural or functional classes, the roles they play in biological mechanisms and pathways are still being developed. Defining gene expression profiles is an effective way to discover the roles genes play in biological models and processes by comparing their patterns of expression in different tissues: normal versus diseased states. Alterations in gene expression are responsible for both morphological and phenotypic differences as well as monitoring the cellular responses to foreign substances (Lockhart and Winzeler, 2000). The expression profile or the 'transcriptome' is highly dynamic and changes rapidly in response to perturbations and during cellular events such as DNA replication and cell division. In addition, changes in multi-gene patterns of expression can provide clues about regulatory mechanisms and biochemical pathways (Lockhart and Winzeler, 2000).

There are other methods designed to examine differential gene expressions such as RT-PCR and northern blot analysis; however, these methods are rather time consuming, limited to only monitoring a few genes at a time and some require rather large volumes of animal tissues as starting material in which to extract sufficient total RNA for use and analysis (Zhumabayeva et al. 2001). Recently a more promising method has developed that is able to identify changes in multiple genes simultaneously and can use minute concentrations of tissue to construct probes. This method involves the hybridization of entire cDNA populations to nucleic acid arrays. This technique rean quires a μg aliquot of total RNA is reverse transcribed using MMLV reverse transcriptase and [α^{32}P] dATP with the Clontech Atlas™ Rat 1.2 II CDS primer mix (Clontech BD

Biosciences). The Atlas™ Labeling System uses streptavidin-coated magnetic beads and biotinylated oligo (dT) which allow for the simultaneous processing of poly A+ RNA enrichment and cDNA probe synthesis.

3.2.1 What is a Microarray?

DNA microarrays, also known as "DNA chips" are arrays of DNA embedded onto a membrane or surface area. The cDNAs on the membranes are comprised of PCR-amplified inserts from cDNA clones of known genes (BD Biosciences Clontech, Palo Alto, CA). The surface can be rigid and nonporous such as glass, or can be flexible and porous such as a nylon membrane (Figure 3). Arrays can also be composed of oligonucleotides synthesized by photolithography *in situ,* which form the basis of DNAchip technology (Affymetrix, Santa Clara, CA). cDNA arrays were originally developed by Brown and colleagues (Schena et al. 1995) and are another common form of microarray currently used in genetic research. These genes on the array bind to matching genetic material extracted from animals or cell cultures exposed to the xenobiotic being tested. The mRNA that binds to the array is representative of genes that are currently active, these genes are then labeled with a radioisotope to simplify detection (Lovett, 2000). Both treated and untreated samples are labeled and then tested onto a single array and the resulting spot shows the degree to which that gene has been modulated by a toxicant.

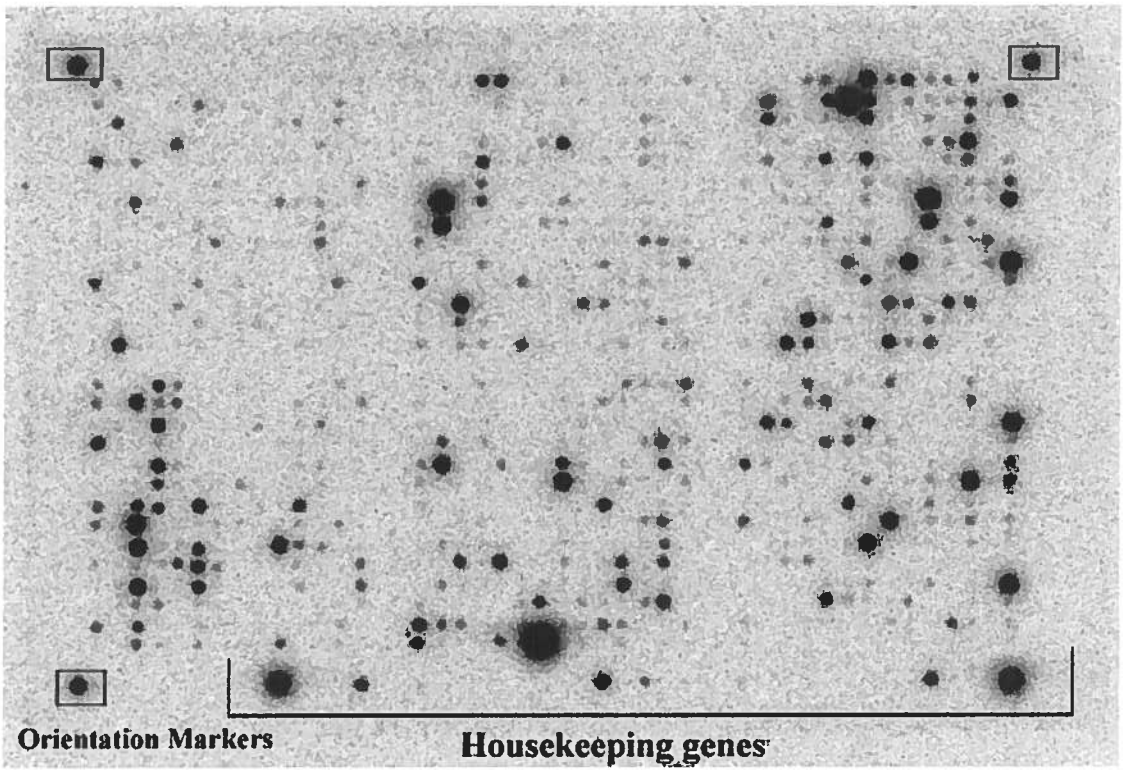

Figure 3: Representative cDNA Atlas™ Rat 1.2 Expression Array
This array represents 1,176 cDNAs immobilized on a nylon membrane. The bottom lane on the array represents the 9 housekeeping genes. The 4 spots at the corners represent orientation marks to align the membrane on a grid before quantification.

3.3 Significant application of microarrays in gene discovery and carcinogenesis:

The great promise of toxicogenomics is that it may be used to scan the entire human genome to see which genes are affected by specific toxicants. The immediate goal is to look at different classes of compounds and identify clusters of genes that are that are correlated to specific classes of toxicants (Lovett, 2000). One area of research that has clearly benefited recently from the integration and implementation of microarray technology in science is the elucidation of genes and mechanism promoting carcinogenesis in rat and human studies. Current notions of cancer progression infer that

accumulations of multiple mutations lead to unlimited cell proliferation observed in tumours. These mutations can lead to the up or down-regulation of several genes involved in the proliferative and metastatic potential of the tumor. Through the use of large-scale gene expression analysis the investigator should be able to monitor some of the distinct changes in gene expression that are related to the treatment. The investigator is able to survey a variety of transcriptional effects while focusing on a set of genes of interest.

3.3.1 Identification of genes/mechanisms in promotion of hepatocarcinogenesis:

Recently specific genes that are potentially important in the pathogenesis of liver carcinoma as well as targets for new strategies of cancer therapy have been identified using microarray technology. Tackels-Horne et al. (2002) compared gene expression from six primary hepatocellular carcinomas, five adenocarcinoma metastases to the liver with 8 normal liver tissues. Of the 20 most significantly up-regulated genes 7 were known, to be implicated with liver carcinoma or other types of cancer. Furthermore the authors identified 42 genes and 24 expressed sequence tags (EST's) that are expressed at a significant level in both HCC and metastastic tumors indicating a list of marker genes indicative of cancerous liver tissue. Another study looked at the effects of the hepatitis B viral X protein, a known transcription factor and a potential oncogene on promotion of carcinogenesis in human liver. Using the Atlas™ human cDNA array, Han et al. (2000) screened 588 cDNA's and targeted only 13 genes (2.2%) whose biological functions are known to be implicated in cellular proliferation. Identifiable genes pertained to classes such as oncogenes, apoptosis associated proteins, growth factors, cell cycle regulators

and stress response proteins. The overall analysis identified a cluster of genes which can be implicated when HBV-infected patients develop hepatocellular carcinoma.

cDNA application to the study of arsenic-induced liver carcinogenesis has revealed a set of genes whose expression has been significantly altered by treatment. Using the Atlas™ human cancer array (588 genes), Lu et al. (2001) identified 60 genes (10%) differentially expressed between treated and control samples. These genes were involved in cell-cycle regulation, DNA damage response and intermediate filaments. The results were consistent with an array analysis of chronic arsenic-transformed rat liver cells (Chen et al., 2001). Clearly a variety of gene expression changes may play a role in arsenic hepatotoxicity and carcinogenesis.

An intriguing study by Graveel et al. (2001) searched for a mutational model to describe liver cancer. The authors profiled normal and neoplastic livers of mice treated with diethylnitrosamine (DEN). Using oligonucleotide microarrays, the authors compared gene expression in liver tumors to three different states of normal liver: quiescent (dormant) adult, regenerating adult, and newborn. Only 22 genes were found to be deregulated in the tumors of all three comparisons. Furthermore, three of these genes were found to be up-regulated in human hepatocellular carcinoma. The results suggest that DEN-treated mice provide an excellent model for human hepatocellular carcinomas. Collectively these studies have displayed some of the key applications and findings using microarray technology to elucidate primary genes and mechanisms implicated in carcinogenesis. In addition, they have displayed the significant interrelationship and reproducibility across different species, i.e. human, mice and rats. Using microarrays, researchers have been able to rapidly yet efficiently target key genes, pathways and

markers of carcinogenesis and the data on the more critical sets of genes will be further highlighted through more detailed analysis and experimentation.

Microarray application has also given insight into the formation of hepatocellular carcinoma (HCC), the cancer that arises from hepatocytes, the major cell type of the liver. It is one of the most common malignant tumors worldwide, accounting for an estimated one million deaths annually (Shirota et al., 2001). HCC is the number one or number two cause of cancer death in parts of Asia and Africa (Bosch et al., 1999). The formation of this malignant tumor is complex and involves somatic mutations, the loss of tumor-suppressor genes and the possible activation of oncogenes (Cox, 2001). Tumor growth can be maintained by altering the expression of growth factors, oncogenes and the target receptors. Most of the growth factor receptors belong to the protein tyrosine-kinase family (PTK) of receptors implicated in maintaining normal cell growth (Hanks and Hunter, 1995).

Tsou et al. (1998) used cDNA arrays to determine whether a correlation existed between PTK gene expression profiles and tumorigenesis. The main objective of the experiment was to delineate expression profiles of normal, HCC liver and five different HCC cell lines. Starting with a high density replica array (768 clones), the authors identified three PTK genes that were up-regulated in HCC cell lines: the fibroblast growth factor receptor 4, tyk2 and MEKK-3. Furthermore, by using 11 pairs of PTK probes, Tsou et al. identified nearly 40% (310/768) of the clones from the cancerous part of the liver tissue. In comparison Graveel et al. (2001) also used a genomic approach to identify clusters of genes that are differentially expressed in hepatocellular carcinoma. Certain liver metabolic enzymes such as naphthalene hydroxylase were down-regulated

in HCC while others; (apolipoprotein A-IV and squalene epoxidase) were significantly up-regulated in HCC. Another cluster of genes differentially expressed in liver tumors are the cell adhesion and motility genes. Osteopontin is a gene that is up-regulated in gastric, lung and breast cancer (Tuck et al., 1998). Using cDNA arrays, Graveel et al. (2001) also observed an up-regulation of this transcript in three independent hepatocelluar carcinomas and in HepG2 cells.

Shirota et al., (2001) used Atlas™ glass cDNA expression arrays to further characterize genes that are differentially expressed in hepatocellular carcinoma. The array was uniquely designed to include 930 genes with various functions such as: apoptosis, cell cycle, cell-cell interaction, growth factors, oncogenes, transcription factors, DNA repair, stress-response and hematology-related genes. mRNA was extracted from cancerous and non-cancerous tissue of 10 patients with HCC. The authors identified 10 genes whose expression was up-regulated and 9 genes that were down-regulated in cancerous tissues of HCC patients. Shirota et al. identified a unique sequence of human DNA which contains a high proportion of genes that have already been shown to be up-regulated in HCC. The sequence contained a variety of genes such as ubiquitin-like protein gene, γ-aminobutyric acid B receptor 1a, 1b genes, Rhodopsin family gene, and 60S Ribosomal protein 13A gene. This human DNA sequence is likely to be an important gene for HCC (Shirota et al., 2001). This report further confirmed the differential expression of 5 other genes, already known to be perturbed in HCC i.e.; hepatoma-derived growth factor, glutathione S-transferase, fibronectin, c-myc transcription factor and major histocompatibility complex (MHC) class I.

One of the intriguing findings reported was that of the 9 genes down-regulated in HCC, 4 genes [GRO2 oncogene, interferon gamma-inducible protein 10 (IP-10), human intercrine-α (hIRH) and interleukin-8] belonged to the same CXC chemokine family. CXC chemokines such as IP-10 and IL-8 have been shown to activate lymphocytes and play primary roles in tumor immunity (Dias et al. 1998; Yoong et al. 1999). Therefore, reductions in the expression of this cluster of genes may repress the immune response and in effect enhance the progression or invasiveness of HCC tumors in the patient. It is evident that these studies illustrate well the potential use of microarrays in the field of cancer biology, in liver cancer in particular.

3.3.2 Identification of tumor-specific markers:

Recently, Wellmann et al. (2000) tested the utility of cDNA microarrays in identifying diagnostic markers of large-cell lymphomas. Using the Atlas Human Expression Array containing 588 well-defined cDNA transcripts of known sequence and function, the authors first set out to investigate the potential for the arrays to identify markers that are already known to be differentially expressed in various B-, T- and myeloid cell lines. The B and T cell lines were chosen to represent a broad variety of lymphoid neoplasms. cDNA arrays were capable of confirming known markers of lymphomas. For instance, CD19, a B-cell specific marker, was expressed only in B-cell lines whereas CD4, a T-cell-restricted marker within lymphoid neoplasms, was expressed only in T-cell lines. Once the authors were satisfied with the confirmation of their initial results they assessed the ability of this technique to identify novel markers of tumorigenesis. Among the genes that showed differential expression, only one gene,

clusterin, was strongly up-regulated in all anaplastic large-cell lymphoma (ALCL; form of T-cell lymphoma) cell lines but not in the other cell lines. This example illustrates the advantages of using microarrays in cancer biology, to identify diagnostic biomarkers.

SECOND PART : ARTICLE

Submitted for publication

Sexual Dimorphism of Hepatic Genes Expressed in Hexachlorobenzene-Treated Rats

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ABSTRACT

Hexachlorobenzene (HCB) is an epigenetic carcinogen that predisposes females to the formation of liver tumors. Short-term exposure (5 days) to HCB promotes DEN-induced liver tumors, predominantly in female rats, 95 days following the last HCB treatment. The objective of this study was to determine gender-specific differences in hepatic gene expression and their relationship to tumor promotion. To address this objective, four experimental groups of rats were used: vehicle-exposed males (control); vehicle-exposed females (control); HCB-treated males; HCB-treated females. Adult rats were administered corn oil (vehicle) or HCB (100 mg/kg) by gavage for 5 consecutive days and sampled on day 50 of the experiment. Cellular mRNA was isolated from the liver of each rat (n=4/group) and used to screen an Atlas™ Rat Array 1.2 II (BD Biosciences Clontech, Palo Alto, CA) containing 1176 genes. Results indicate that in control male rat liver, 185 genes were expressed using this array, and in the control female liver, 184 genes were expressed. In HCB-treated males, there were 189 genes expressed. Of these, 41 were found only in HCB-treated males: 9 genes (22%) were up-regulated while 32 genes (78%) were down-regulated at least two-fold. In HCB-treated females, only 151 genes were expressed. Of these, 35 genes (23%) were modulated by HCB treatment as compared to control females: 6 genes (17%) were up-regulated and 29 genes (83%) were down-regulated at least two-fold. These results suggest that in female rats there is a 15% decrease in the number of genes expressed in the HCB-treated rats as compared to control females. In summary, the functional genomic approach used in this study allowed us to identify among the 1176 genes examined a cluster of 36 genes that may be implicated in the epigenetic mechanisms of HCB-induced hepatocarcinogenesis in the female rat. A marked sexual dimorphism effect in the liver, attributed to HCB, has been seen at the level of gene expression, with a clear down-regulation in females as opposed to a slight up-regulation in males. We propose a primary mechanism in which HCB alters the methylation processes of the cell, known to be targeted by epigenetic carcinogens.

INTRODUCTION

Hexachlorobenzene (HCB), an organochlorine, was widely used as a fungicide for cereal crops and in the manufacture of chlorinated solvents and pesticides until the 1970`s (1). This has resulted in its widespread distribution in the environment. HCB, a persistent environmental contaminant, is found in the fatty tissues and maternal milk of people living in industrialized countries (2). The average total daily intake of HCB in the general population varies between 0.4 and 3 ng HCB/kg body weight/day (1).

HCB is a potent chemical carcinogen that can induce hepatomas, hepatocellular carcinomas, bile duct adenomas, thyroid neoplasms and parathyroid adenomas in both laboratory animals and humans (1,3). Several studies have indicated that HCB induces tumors at a greater incidence in adult female rats as compared to males. Legault *et al.* (4) showed that ovariectomy caused female rats to become insensitive to the porphyric effects of HCB. However, when these same female rats were given estradiol they were again responsive to HCB, suggesting that there is an estrogenic component in the sensitivity to HCB-treatment for the induction of porphyria. Plante *et al.* (5) reported that HCB decreased the expression of genes that code for proteins (connexins) involved in intercellular communication in females, while in male rats, no changes in gene expression were observed. HCB has also been shown to disrupt phospholipid metabolism in female rats resulting in increased hepatic levels of phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol following 1-4 weeks of exposure (6). HCB is incorporated between the fatty acid chains of membrane lipids, leading to increased membrane fluidity in hepatocytes (7). The mechanism by which HCB induces tumor formation in females is not understood. HCB does not induce chromosomal

damage nor alter DNA repair (1). Mitotic indices in human peripheral blood lymphocytes exposed to HCB suggest that it acts as an epigenetic carcinogen (8).

Microarray technology could provide novel information for identifying specific mechanisms of action of environmental toxicants, but it could also provide new information on macroscopic effects of chemicals on expression of the genome and its relationship to specific pathologies. HCB represents an interesting chemical for studying its effects on tumor promotion and also for elucidating pathways that may be involved in gender-specific tumor promotion.

Specific genes that are potentially important in the pathogenesis of liver carcinoma as well as targets for new strategies of cancer therapy have been identified recently using microarray technology. Tackels-Horne *et al.* (9) compared gene expression from six primary hepatocellular carcinomas and five adenocarcinoma metastases to the liver, with 8 normal liver tissues. Of the 20 most significantly up-regulated genes, 7 were known to be associated with liver carcinoma or other types of cancer. Furthermore, the authors identified 42 genes and 24 expressed sequence tags (EST's) expressed at a significant level in both HCC and metastastic tumors, establishing a list of marker genes indicative of cancerous liver tissue. Another study examined the effects of the hepatitis B viral X protein, a known transcription factor and a potential oncogene, on promotion of carcinogenesis in human liver. Using the Atlas™ human cDNA array, Han *et al.* (10) screened 588 cDNA's and targeted only 13 genes (2.2%) whose biological functions are known to be implicated in cellular proliferation: identifiable genes pertained to classes such as oncogenes, apoptosis proteins, growth factors, cell cycle regulators and stress response proteins. Using microarray technology, these studies have targeted a select

group of candidate genes that could be involved in the mechanism in which HBV-infected patients develop hepatocellular carcinoma.

In the search for a mutation model to describe liver cancer, Graveel *et al.* (11) profiled normal and neoplastic livers of mice treated with diethylnitrosamine (DEN). Using oligonucleotide microarrays the authors compared gene expression in liver tumors to three different states of normal liver: quiescent (dormant) adult, regenerating adult, and newborn. Changes in expression of only 22 genes were found in the tumors of all three groups. Three of these genes were found to be up-regulated in human hepatocellular carcinoma. The results suggest that DEN-treated mice provide an excellent model for human hepatocellular carcinomas. Collectively, these studies have demonstrated some of the key applications and findings using microarray technology to elucidate the primary genes and mechanisms involved in carcinogenesis. In addition, they have displayed significant interrelationship and reproducibility across different species, i.e. humans, mice and rats. Using microarrays, researchers have been able to rapidly yet efficiently target key genes, develop pathways and identify markers of carcinogenesis. Genes that have been identified as critical will be further examined through more detailed analysis and experimentation.

In this study, our objective was to investigate the mechanisms of HCB-induced hepatic tumor promotion by examining gender-specific expression of genes in the livers of rats treated with HCB. A genomic approach was used to identify a cluster of candidate genes involved in HCB-induced hepatocarcinogenesis. In this experimental model, gender-specific gene expression can be analyzed in the context of 'steady-state' of

expression, since HCB liver concentration decreases from day 5 to 50, at which point it is almost undetectable or remains constant, 45 days after administration of HCB.

MATERIALS AND METHODS

Animals

Adult male (225-275 g) and female (175-200 g) Sprague-Dawley rats were purchased from Charles River Canada Inc. (St-Constant, QC). Rats were acclimated for 7 days prior to the start of the experiment and received water and food *ad libitum.* Room temperature was maintained at 21°C with a 12-hr light/dark cycle. Animals were segregated by gender and housed three per cage. All animal manipulations were approved by the University animal care committee.

Four experimental groups were used; control males (n=4), control females (n=4), HCB-treated males (n=4) and HCB-treated females (n=4).

Treatment

Rats were administered HCB (100 mg/kg) or vehicle (corn oil, controls) by gavage for 5 consecutive days. The control groups received corn oil only (10 ml/kg/day) while the treated animals received 5 consecutive daily doses of HCB (Sigma-Aldrich Chemical Company Inc., Mississauga, ON) dissolved in corn oil (100 mg in 10 ml/kg) by gavage. Forty-five days after HCB treatment the animals were anaesthetized with isofluorane gas and sacrificed by exsanguination from blood withdrawn from the abdominal aorta. Previous studies from our laboratory have shown that at this point in time proteins involved in intercellular communication were significantly lower in treated female rats (5). At the time of sampling, the liver from each rat was surgically excised, immediately frozen in liquid nitrogen and stored at -86°C.

RNA Isolation

Total RNA was extracted from the livers of control or HCB-treated rats using the Atlas™ Pure Total RNA Labeling System (BD Biosciences Clontech, Palo Alto, CA). Frozen liver samples (100 mg) were placed in liquid nitrogen, ground to a powder, and homogenized with 1 ml of denaturing solution (BD Biosciences Clontech). RNA was then extracted with three rounds of phenol:chloroform extraction and precipitated with 2 ml of isopropanol. The RNA was precipitated for 10 min on ice and recovered by centrifugation at 12 000 rpm for 15 min at 4°C. The RNA pellet was then washed in 80 % ethanol and suspended in RNAase-free H_2O to ensure an RNA concentration of 1-2 $\mu g/\mu l$. The RNA was then treated with 1 unit/μl of RNAase-free DNAase I (Clontech BD Biosciences) to eliminate genomic DNA contamination. Finally, the quality of DNAase-treated RNA samples was confirmed by optical density at 260/280nm and by electrophoresis in a denaturing agarose gel.

Synthesis of cDNA Probes

A 50-μg aliquot of total RNA was reverse transcribed using MMLV reverse transcriptase and [α^{32}P] dATP (3 000 Ci/mmol; Amersham Pharmacia Biotech; Baie d'Urfé, QC) with the Clontech Atlas™ Rat 1.2 II CDS primer mix (Clontech BD Biosciences). The Atlas™ Labeling System uses streptavidin-coated magnetic beads and biotinylated oligo (dT) which allow for the simultaneous processing of poly A+ RNA enrichment and probe synthesis. The ^{32}P-labelled cDNA probes were then purified by column chromatography (CHROMA SPIN™-200 Columns; Clontech BD Biosciences).

cDNA Hybridization

The cDNA Atlas™ Rat 1.2 Expression Array used in this study represents 1,176 cDNAs immobilized on a nylon membrane. The cDNAs on the membranes are

comprised of PCR-amplified inserts from cDNA clones of known genes (BD Biosciences Clontech, Palo Alto, CA). The bottom lane on the array represents the 9 housekeeping genes and the 4 spots at the corners of the array represent orientation marks to align the membrane on a grid before quantification. The membranes containing the microarrays were prehybridized for 30 min with 5 ml ExpressHyb™ hybridization solution (Clontech BD Biosciences) warmed to 68°C and mixed with 0.5mg of heat-denatured sheared salmon testes DNA. A 5 μl aliquot of C_0t-1 DNA was added to the labelled probe. The sample was denatured by boiling for 2 min and rapidly cooled for 2 min. A 2-3 μl aliquot of ExpressHyb™ hybridization solution was then added to the probe and the arrays were hybridized overnight with continuous agitation at 68°C. Following the hybridization, the membranes were washed four times for 30 min at 68°C with wash solution 1 (2X 0.3M sodium citrate (SSC), pH 7.0, 1% SDS) and once for 30 min with wash solution 2 (0.1X 0.015M SSC, pH 7.0, 0.5% SDS). The membranes were then washed at room temperature for 5 min with 2X SSC, and the membranes were sealed in plastic wrap and exposed on a phosphorus screen.

Quantification of Gene Expression

Unsaturated exposed phosphorus screens were visualized with a PhosphorImager SI (Molecular Dynamics Inc., Amersham Pharmacia Biotech Inc., Piscataway, NJ) using the ImageQuant software (Version 5,1, Molecular Dynamics). The membranes were exposed for different lengths of time, ranging from 1 hour to 2 weeks, in order to estimate optimal exposure time. Quantification of gene expression was performed using AtlasImage™ 1.5 software (Clontech BD Biosciences). The intensity of hybridization signals amongst pairs of membranes was normalized using a global method technique,

which identifies the level of expression of one gene with respect to the sum of all gene intensities on the microarray. Analyses were done using four individual rats per experimental group.

Determination of Expressed Genes

In order to analyze individual changes in gene expression and because of the low variation between individual rats, only genes that were expressed in at least three out of four trials in any one subset and had signal intensities corresponding to at least 2X the background level were selected for data analysis. When the array is hybridized with a 32P-labeled cDNA probe, the background level is sufficiently low to permit detection of an mRNA that is present at only about 10–20 copies per cell: an abundance level of approximately 0.0025%, permitting the detection of RNAs that may be expressed at very low levels in the cell.

Data Analysis

Statistical significance between groups was determined using a 2-way ANOVA followed a posteriori by a Bonferroni or Student-Neuman-Keuls test (STATISTICA 6.0; StatSoft, Inc., Tulsa, OK). All analyses were two-tailed and significance was established at $p<0.05$.

RESULTS

The Clontech Rat 1.2 II cDNA Expression Array microarray contains 1176 genes whose genetic sequences and biological functions are known. Figure 1 shows a phosphorimage of a representative microarray hybridized with a ^{32}P-labeled cDNA probe made with liver cells from an HCB-treated female rat. In control male rat liver, 185 expressed genes were detected using this array (Fig. 2). While there was some variation between individuals, 83% of the expressed hepatic genes were common to at least 3 of the 4 individuals. In the female livers, 184 genes were expressed and 86% of these were common to at least 3 of the 4 individuals. In HCB-treated males, there were 189 genes expressed with 76% of these genes in common to 3 individuals. Surprisingly, in HCB-treated females, there were only 151 expressed genes detected with 84% in common to 3 individuals (Fig. 2).

Genes expressed in Controls

In comparison to the female rat control group, control male rat livers showed similar numbers with regards to the total number of genes detected on the Rat 1.2 II Atlas array membrane, i.e. 185 versus 184 genes. Within the controls 7 genes were expressed exclusively in males; ERp29, synaptogyrin 1, synaptojanin, DPPIII, kinesin-related protein, atrophin 1 and glycoprotein 55.

A total of 184 genes were expressed in the livers of the control females and 145 of those genes overlapped all 4 experimental groups (Fig. 2). Only 2 genes were uniquely expressed in control females: Mammalian achaete scute homolog 2 (MASH-2) and carboxypeptidase Z. MASH-2 is a basic helix-loop-helix transcription factor previously

shown to be involved in placenta development and is also expressed by Schwann cells of adult peripheral nerves. Carboxypeptidase Z is a 120 Kd lysosomal membrane glycoprotein. Additionally, 3 genes were detected in all experimental groups except male controls: i.e. (Sprague-Dawley) H-rev 107, B61 and the myosin heavy chain 3.

Genes expressed in HCB-treated males

A single gene was expressed exclusively in HCB-treated males, i.e. phosphoglucomutase, an enzyme involved in carbohydrate metabolism. Since these animals do not develop cancer, one could speculate that this gene may confer protection against tumor development. However, no reports regarding such an effect have been found in the literature. Unlike HCB-treated females, but similar to control male and female rats there was little individual variation in the number of genes expressed in HCB-treated male rats. Statistical comparison of the genes expressed in control and HCB-treated male rats indicated that the levels of expression of 4 genes were significantly altered in the male; 3 of these were down-regulated (Fig. 3) and 1 was up-regulated (Table 2).

The EGR2/KROX-20 is highly down-regulated in the males, but not females treated with HCB. Furthermore, the expression level of this gene is similar in male and female controls (Fig 3). Dihydropyrimidinase, an enzyme implicated in nucleotide metabolism, was significantly down-regulated only in HCB-treated males although there was a tendency for down-regulation in the HCB-treated females as well (Fig 3). Epoxide hydrolase, an enzyme involved in the biotransformation of xenobiotics and epoxides, was regulated differently in male and female rats treated with HCB (Fig 3). This gene was

up-regulated in HCB-treated males but very low in both control and HCB-treated females.

Genes expressed in HCB-treated females

There were no genes exclusively expressed in HCB-treated females. On the contrary, HCB caused an 18% decrease in the total number of expressed genes detected in the livers of female rats treated with HCB as compared to control females or to both control and HCB-treated males. Expression of 31 genes that was decreased in HCB-treated females, which have been classified in 3 functional groups listed in Table 1. Interestingly, 29% of these genes have been reported to be down-regulated in liver cancer. Statistical analyses using two-way analysis of variance on the entire subset of genes from all 4 experimental groups allowed us to identify 9 other genes that were significantly modulated by the HCB treatment (Table 2); the expression of 5 of these genes was significantly modulated in the female rats (Fig. 4), with 4 being up-regulated and 1 down-regulated (Table 2).

DISCUSSION

HCB is an epigenetic carcinogen (1,8,12,13). Previous studies have shown that HCB decreases the expression of the connexin 32 and 26 genes in female but not in male rats, resulting in reduced intercellular communication (5). It was proposed that this is an important component in the mechanism of HCB-induced liver carcinogenesis (14). These observations suggest that HCB may repress the expression of hepatic genes. In the present study, 1176 genes representing approximately 2 % of the entire rat genome were screened to determine if HCB altered the expression of specific gene clusters, or if there were general effects on the expression of the genome in the liver. In females, the number of detected expressed genes in HCB-treated rats was 18 % lower than in controls, whereas male HCB-treated rats showed a negligible increase (2 %) when compared to controls. Thus, it would appear that HCB can repress the expression of the genome in the liver of female rats.

The *in vivo* exposure model used in the present study in which rats are treated and sampled at day 50 represents a "steady state" situation following from HCB exposure. This, as well as the sexual dimorphism in liver tumor promotion, allows us to "dissect" gene expression effects in order to identify the mechanism of liver tumor formation. The expression analysis data identified 36 genes whose expression was altered by HCB treatment and could contribute in the epigenetic mechanism of HCB-induced hepatocarcinogenesis. This group is comprosed of 31 putative tumor suppressor genes that are undetectable in HCB-treated females and 5 genes which show predisposition to HCB-induced hepatic tumors that were significantly modulated by HCB in the female.

No genes were expressed exclusively in either HCB-treated females only, or in HCB-treated males and females as compared to controls.

In order to target candidate genes relevant to the promotion of hepatocarcinogenesis in females, two groups of genes (labeled with an asterisk in figure 2) were analysed in greater depth. One group of 31 genes (Table 1) is present in all experimental groups except HCB-treated females, suggesting that these genes may provide protection to male rats, which exhibit low tumor induction as compared to females. More detailed analyses of these 31 genes indicate that 11 of these are down regulated during carcinogenesis. Of these, 3 are related to amino acid metabolism (malate dehydrogenase, L-glutamine amidohydrolase and histidase) while 4 others are either cell surface antigens (killer cell lectin-like receptor and metastasis suppressor homolog) or extracellular matrix proteins (alpha albumin gene and fibronectin). Interestingly two other genes involved with amino acid metabolism, but for which we did not find a published link with carcinogenesis, are also repressed by HCB in female rats. The bulk of the genes expressed in HCB-treated females (145 out of 151 genes) were also expressed in the other three experimental groups (figure 2).

The modulation of gene expression by HCB may involve the aryl-hydrocarbon receptor (Ah-R), since HCB is a weak agonist for this receptor. The Ah-R is a ligand-dependent transcription factor that belongs to the basic-helix-loop-helix superfamily of DNA-binding proteins (15). Once bound to the Ah-R complex (composed of HSP 90, HSP 70, HSP 50 and the Src-protein Kinase), the HCB-Ah-R complex leaves the cytosol and moves into the nucleus. Once in the nucleus the Ah-R-HCB complex forms a heterodimer with the Ah-R nuclear translocater protein (ARNT), and is then able to

modulate the expression of genes that have specific xenobiotic or dioxin-responsive elements (XRE or DRE) in their promoter regions (15). It can be suggested that the HCB-activated Ah-R complex targets promoters of tumor-suppressor genes such as the metastasis suppressor homolog KAI1/CD82 and the killer cell lectin-like receptor. The transcriptional regulators AhR and Arnt modulate the transcription of genes involved in cellular differentiation and proliferation (16). Recent studies suggest that the AhR directly regulates cell growth (17).

It is well documented that increased methylation of the 5'-promoter region of a given gene is a mechanism for silencing the expression of the gene. This has been reported to contribute to the early steps of carcinogenesis (18,19). Increased DNA methylation may represent a mechanism by which HCB silences the expression of 30% of hepatic genes. The fact that phosphatidylethanolamine N-methyltransferase (PEMT) and phosphatidylethanolamine binding-protein (PEBP), both of which are implicated in DNA methylation, are increased in HCB-treated female rats (Table 2) supports the potential of HCB acting via an increase in DNA methylation. In HCB-treated females, PEMT mRNA levels are up-regulated, while in HCB-treated males there is no effect. Newly methylated CpGs become docking sites for methyl binding proteins such as PEBP, which binds to phosphotidylethanolamine as well as to a variety of nucleotides associated with signalling mechanisms, including GTP and GDP (20). Banfield *et al.* (21) reported that PEBP plays a key role in signal transduction from the interior membrane surface. If the initial recruitment step is not prevented, it may lead to hypermethylation of the neighbouring regions of the promoter, locking the genes into a stable silenced chromatin state. One could speculate that PEMT increases methylation of specific CpG

dinucleotide clusters within the promoter region of the KAI1 gene. The 5' promoter region of the KAI1 gene is G + C rich (22) implying that there is a high potential for hypermethylation in the promoter region and hence a silencing of this metastasis-suppressor gene in HCB-treated female but not male rats. Down regulation of KAI1 expression has been shown to be associated with formation of metastases in prostate and pancreatic cancer (23) and in common solid epithelial tumors found in lung, hepatic, colorectal, ovarian, esophageal, pancreatic and prostatic cancers (24). Reduced KAI1 mRNA expression in HCC cells seems to influence their metastatic ability and enhance the malignant potential of HCC (23).

In the last decade, increased attention has been directed towards epigenetic mechanisms of gene regulation such as DNA methylation (25,26). The PEMT pathway in the liver is an important process of cell proliferation and carcinogenesis (27). PEMT expression has been shown in human liver, heart and testis, with the highest level of expression in the liver (28). Moreover, choline deficiency has been reported to cause sexually dimorphic activation of liver and brain PEMT, i.e. brain PEMT was increased in females by 49% with no increase in males, whereas hepatic PEMT activity was unaffected in females while increased by 34% in males (29).

Two other pathways potentially disrupted in HCB-treated females but not in males could also support a mechanistic explanation for the sex-specific liver tumor promotion. One involves the up-regulation of ribosomal protein L18 expression (Table 2), a novel inhibitor of double-stranded RNA-activated protein kinase (PKR). When activated, PKR inhibits cell growth and induces apoptosis. L18 inhibits PKR phosphorylation. An up-regulation of the ribosomal protein L18 was found in HCB-

treated females only, inhibiting the activation of PKR and resulting in unregulated cell growth (30). Ribosomal protein L18 is overexpressed in other cancers such as colorectal cancer (30).

The other pathway that merits mention relates to protectin/CD59 (Table 2). Activation of the plasma complement (C) system leads to formation of a cytolytic membrane attack complex (MAC) which results in a large pore that spans the membrane of the cell being attacked, allowing ions to flow freely outside of the cell. Spiller *et al.* (31) have shown that cytokines caused increased secretion by hepatoma cells of complement regulatory proteins, providing protection from complement attack. The CD59 antigen (MACIF, MIRL, HRF-20 or protectin) is one such complement C regulator that acts by inhibiting the formation of the MAC present on human cell membranes (32). Up-regulation of membrane C regulators has been implicated as a survival strategy in numerous human tumors (33). The HCB-induced down-regulation of CD59 activity observed in females may predispose them to hepatic tumor development as compared to males.

In summary, the functional genomic approach used in this study allowed us to identify, among the 1176 genes examined, a cluster of 36 genes that may be implicated in the epigenetic mechanism of HCB-induced hepatocarcinogenesis in the female rat. A marked sexual dimorphism in the liver effect by HCB has been seen at the gene expression level, with a clear down-regulation in females as opposed to a slight up-regulation in males. We propose a primary mechanism in which HCB alters the methylation machinery of the cell, a known factor for epigenetic carcinogens.

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Table 1 Genes whose level of expression is silenced in HCB-treated female but not male rats.
Genes and their functions are presented in three groups according to their known implications in liver or other types of cancer. Sprague-Dawley Rats were administered HCB (100mg/kg) by gavage every day for 5 days and sampled on day 50 of the experiment. Total RNA was isolated using the Atlas™ Pure Total RNA Labeling System. A α^{33}P-labelled cDNA probe was hybridized onto an Atlas™ Rat cDNA Expression Array. Unsaturated exposed phosphorus screens were visualized with a PhosphorImager SI. Gene expression was quantified using the AtlasImage™ 1.5 software.

Group 1 : Genes known to be down-regulated in liver cancer	**Function**	**Reference**
1. Killer cell lectin-like receptor subfamily D member 1	Cell surface antigen	**34**
2. Metastasis suppressor homolog KAI1	Cell surface antigen	**23,24**
3. Alpha albumin gene	Extracellular transporter	**35**
4. Fibronectin	Extracellular matrix protein	**36**
5. Malate dehydrogenase	Amino acid metabolism	**37**
6. L-glutamine amidohydrolase	Amino acid metabolism	**38**
7. Histidase	Amino acid metabolism	**39**
8. Uroporphyrinogen decarboxylase	Metabolism of cofactors	**40**
9. Kupffer cell receptor	Endocytosis protein	**41**
Group 2 : Genes known to be down-regulated in other types of cancer	**Function**	
10. Thyroid peroxidase	Metabolism of enzymes	**42**
11. 40S Ribosomal protein S5	Ribosomal protein	**43**
Group 3 : Genes not proven to be related to cancer or whose expression is silenced but rather increased in cancer	**Function**	
12. Vascular ATP diphosphohydrolase (ATPDase); lymphoid cell activation antigen; CD39 antigen	Nucleotide metabolism	**44**
13. Transcription factor IIIC alpha subunit	Transcription factor	**45**
14. Y box-binding protein	Transcription activator & repressor	**46**
15. Sperm membrane protein	Cell-Cell adhesion receptor	**47**
16. Neuronal pentraxin precursor	Extracellular transporter	**48**
17. Alpha-soluble NSF attachment protein	Targeting protein	**49**
18. 7-dehydrocholesterol reductase	Simple lipid metabolism	**50**
19. Glutamate oxaloacetic transaminase 1	Amino Acid metabolism	**51**
20. Kan-1 protein	Amino Acid metabolism	**52**
21. Heme oxygenase-3	Metabolism of cofactors	**53**
22. Hydroxyindole-O-methyltransferase	Metabolism of enzymes	**54**
23. N-acetylglucosaminyltransferase I	Protein modification enzymes	**55**
24. Ribophorin I	Protein involved in translation	**56**
25. 25-Dx	Receptor	**57**
26. Clathrin-associated protein medium 1	Intracellular adaptor	**58**
27. GTPase Rab8b	Exocytosis G-protein	**59**
28. Pepsinogen precursor	Aspartic protease	**60**
29. Alpha-1-macroglobulin	Protease inhibitor	**61**
30. Estrogen-responsive uterine gene	Unclassified protein	**62**
31. Leucine rich protein	Unclassified protein	**63**

Table 2 Genes that are significantly differentially expressed by HCB-treatment in male and female HCB-treated rats. A α^{32}P-labelled cDNA probe was hybridized onto an Atlas™ Rat cDNA Expression Array. Unsaturated exposed phosphorus screens were visualized with a PhosphorImager SI. Gene expression was quantified using the AtlasImage™ 1.5 software. Data was standardized via the global method, using the housekeeping genes on the array.

Differential Expression	♂ MALE (N=4)	♀ FEMALE (N=4)
Up-Regulated	**Epoxide Hydrolase**	**Phosphotidylethanolamine N-methyltransferase**
		Phosphatidylethanolamine Binding-protein
		Ribosomal Protein L18
		Testis-specific Farnesyl Pyrophosphate
Down-Regulated	**EGR2/KROX-20**	**Protectin/CD59**
	Dihydropyrimidinase	
	Liver-specific transport protein	

FIGURE LEGENDS

Figure 1 Phosphorimage of a representative Atlas™ Rat 1.2 II Array hybridized with a ^{32}P-labeled cDNA probe made from RNA isolated from liver cells of an HCB-treated female Sprague-Dawley rat. Sprague-Dawley Rats were administered HCB (100mg/kg) by gavage every day for 5 days and sampled on day 50 of the experiment. Total RNA was isolated using the Atlas™ Pure Total RNA Labeling System. The genes that are identified are among those that have levels of genetic expression significantly altered by HCB-treatment. This array represents 1,176 cDNAs immobilized on a nylon membrane. The bottom lane on the array represents the 9 housekeeping genes. The 4 spots at the corners represent orientation marks to align the membrane on a grid before quantification.

Figure 2 Venn diagram displaying total number of genes expressed in male and female rats control and HCB-treated. Sprague-Dawley Rats were administered HCB (100mg/kg) by gavage every day for 5 days and sampled on day 50 of the experiment. Total RNA was isolated using the Atlas™ Pure Total RNA Labeling System. A α^{32}P-labelled cDNA probe was hybridized onto an Atlas™ Rat cDNA Expression Array. Unsaturated exposed phosphorus screens were visualized with a PhosphorImager SI. Gene expression was quantified using the AtlasImage™ 1.5 software. Data was standardized via the global method, using the housekeeping genes on the array. The genes that are identified are among those that have levels of genetic expression altered by HCB-treatment.

Figure 3 Expression of genes altered by HCB in male but not female rat liver: krox-20 (A), dihydropyrimidinase (B) and epoxide hydrolase (C). Sprague-Dawley Rats were administered HCB (100mg/kg) by gavage every day for 5 days and sampled on day 50 of the experiment. Total RNA was isolated using the Atlas™ Pure Total RNA Labeling System. A α^{32}P-labelled cDNA probe was hybridized onto an Atlas™ Rat cDNA Expression Array. Unsaturated exposed phosphorus screens were visualized with a PhosphorImager SI. Gene expression was quantified using the AtlasImage™ 1.5 software. Data were standardized via the global method using the housekeeping genes on the array.Data are means ± SEM (n=4) for control (open bars) and treated (filled bars) rats. * Significantly different from control of the same sex ($p < 0.05$). ‡ Significantly different from HCB-treated female rat liver tissues ($p < 0.05$).

Figure 4 Expression of genes up-regulated in HCB-treated female but not male rat liver: phosphatidylethanolamine N-methyltransferase (A), phosphatidylethanolamine binding-protein (B) and ribosomal protein L18 (C). Sprague-Dawley Rats were administered HCB (100mg/kg) by gavage every day for 5 days and sampled on day 50 of the experiment. Total RNA was isolated using the Atlas™ Pure Total RNA Labeling System. A α^{32}P-labelled cDNA probe was hybridized onto an Atlas™ Rat cDNA Expression Array. Unsaturated exposed phosphorus screens were visualized with a PhosphorImager SI. Gene expression was quantified using the AtlasImage™ 1.5 software. Data were standardized via the global method using the housekeeping genes on the array. Data are means ± SEM (n=4) for control (open bars) and treated (filled bars) rats. * Significantly different from control of the same sex ($p < 0.05$). ‡ Significantly different from HCB-treated male rat liver tissues ($p < 0.05$).

Figure 1

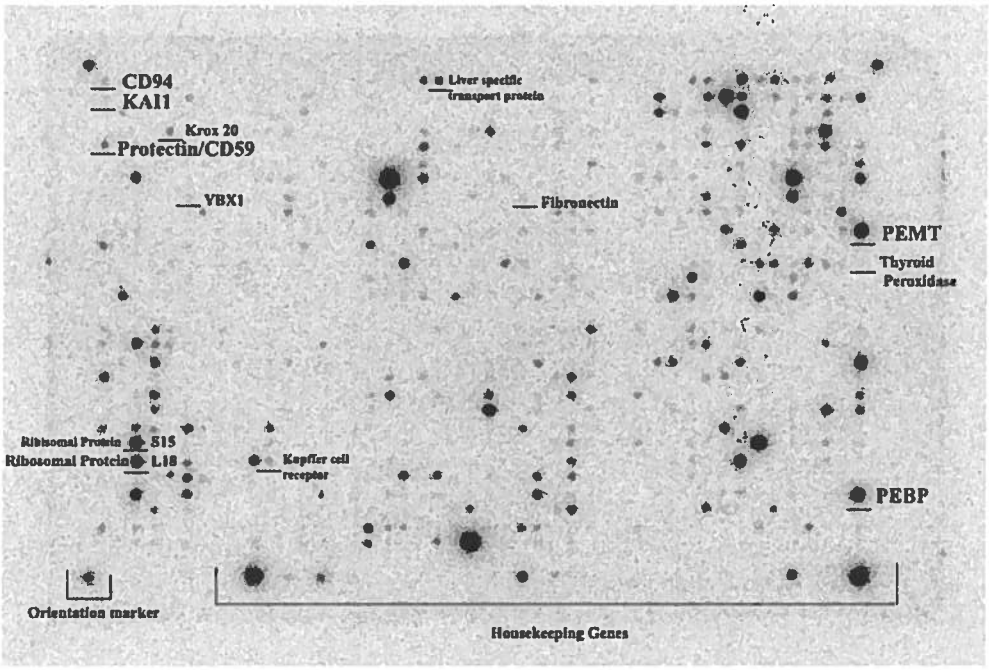


Figure 2

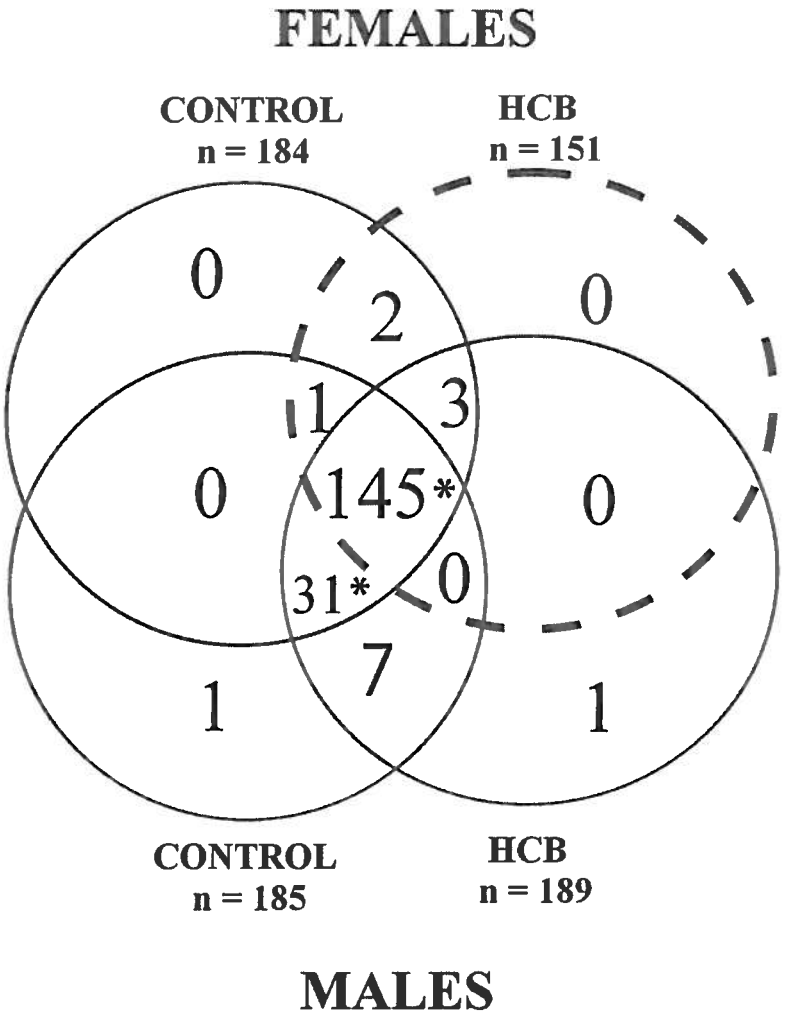
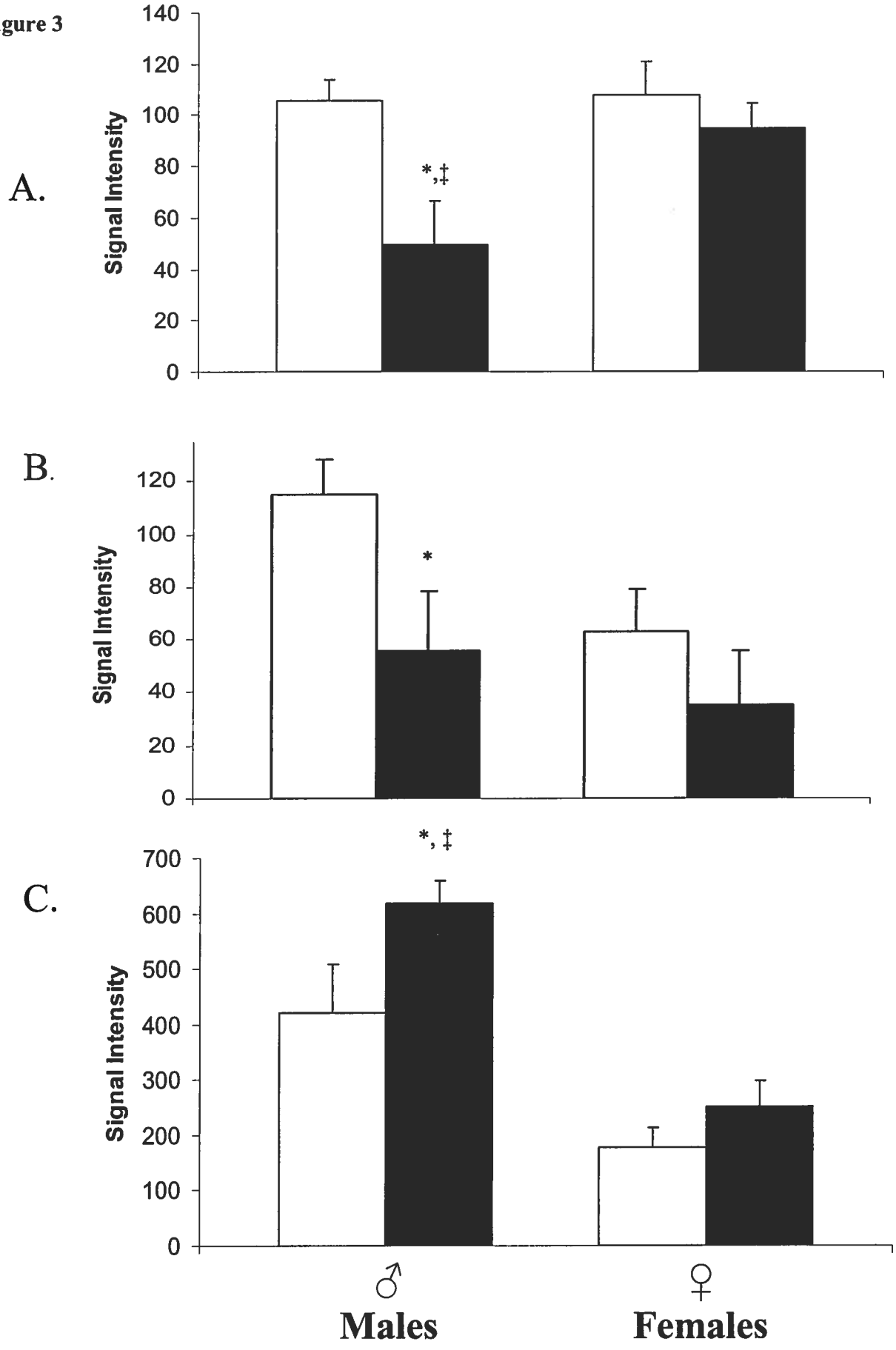


Figure 3


A.
Signal Intensity
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120
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*,‡
B.
Signal Intensity
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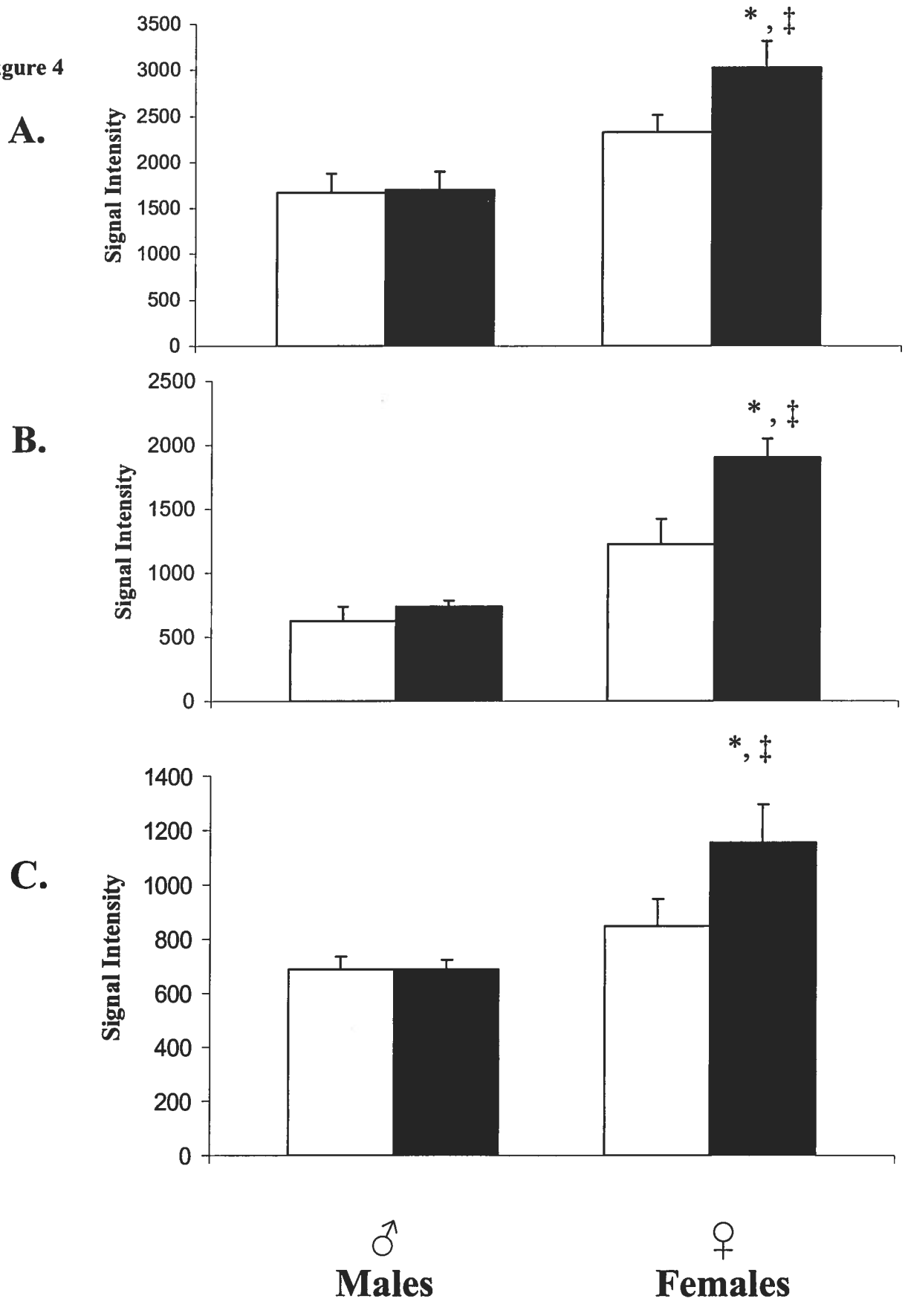
Males
Females

Figure 4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Signal Intensity
3500
3000
2500
2000
1500
1000
500
0
*, ‡
B.
Signal Intensity
2500
2000
1500
1000
500
0
*, ‡
C.
Signal Intensity
1400
1200
1000
800
600
400
200
0
*, ‡
♂
Males
♀
Females

THIRD PART

DISCUSSION AND CONCLUSIONS

Several environmental pollutants have been shown to act as tumor promoters, manifesting themselves through epigenetic mechanisms of carcinogenesis. Certain mechanisms have been identified and implicated in the promotion phase of carcinogenesis. HCB is known to act as an epigenetic carcinogen (WHO, 1998; Siekel et al., 1991). These aforementioned observations suggested that HCB could act to repress the expression of hepatic genes. Given the significant bioaccumulation and persistence of HCB in the environment and especially its stable presence in human adipose tissues, it is imperative to identify the effects of HCB in carcinogenesis. **The research hypothesis of this study was that HCB causes a marked sexual dimorphism through the differential expression of certain candidate genes, thereby promoting HCB-induced liver carcinogenesis in females but not males.**

Building on earlier studies which showed the sexual dimorphism is a well known phenomenon, the purpose of this study was to determine whether it was also apparent at the level of gene expression. In the present study, expressions of 1176 genes were screened, representing approximately 2 % of the entire rat genome. We searched for significant trends in expression with respect to similar clusters of genes. By identifying similar patterns of expression among families of genes, we can propose and outline candidate genes that are mechanistically relevant for HCB-induced liver carcinogenesis.

Previous experiments demonstrated that HCB decreases the expression of the connexin 32 and 26 genes in female but not male rats (Plante et al., 2002). Our results were consistent with that finding in that the number of expressed genes in HCB-treated female rats was 18 % lower than in controls, whereas in males, HCB-treated rats show a negligible increase (2 %) when compared to controls. Thus, it would appear that HCB can repress the expression of the genome in the liver of female rats. In addition we targeted a cluster of 31 genes present in all experimental groups except HCB-treated females, suggesting that these candidate genes may be involved in providing a protective role for non-responsive male rats. Upon subsequent analyses of these 31 genes, we noticed that 11 of these genes (35%) were shown to be down- regulated during carcinogenesis. These results are consistent with the fact that females are more susceptible to the development of hepatic tumors than males, who develop very few, if any hepatic tumors.

Among possible hypotheses for HCB-induced liver carcinogenesis this study points out a disturbance of HCB at the level of transcription. HCB is a weak agonist for the aryl-hydrocarbon receptor (Ah-R), a transcription factor belonging to the basic-helix-loop-helix superfamily of DNA-binding proteins. Once bound to the Ah-R complex, the HCB-Ah-R complex leaves the cytosol and moves into the nucleus forming a heterodimer with the AhR nuclear translocater protein (ARNT). In this configuration, the heterodimer is then able to modulate the expression of genes containing specific xenobiotic or dioxin-responsive elements in their promoter regions (Loaiza-Pérez et al., 1999).

After screening the array we noticed that HCB treatment caused a down-regulation in the expression of the metastasis suppressor homolog KAI1/CD82 and the killer cell lectin-like receptor, both candidate tumor-suppressor genes. Reduced KAI1 mRNA expression in HCC cells influences their metastatic ability and enhances the malignant potential of HCC (Guo et al., 1998). Furthermore, down regulation of KAI1 expression has been shown to be associated with formation of metastases in prostate and pancreatic cancer (Guo et al., 1998), and in common solid epithelial tumors found in lung, hepatic, colorectal, ovarian, esophageal, pancreatic and prostatic cancers (Liu et al., 2001). It could be suggested that the HCB-activated AhR complex targets promoters of tumor-suppressor genes, such as the KAI1 and the KCLR. Moreover, the association between these candidate genes and the transcriptional regulators, AhR and Arnt, should not be overlooked, since they have been involved in cellular differentiation and proliferation (Khorram et al., 2002).

Our favored hypothesis of HCB-induced carcinogenesis points to a disturbance in the DNA methylation machinery. It is well documented that increased methylation of the 5'-promoter region of a given gene is a mechanism for silencing the expression of the gene. This has been reported to contribute to the early steps of carcinogenesis (Ivanova et al., 2002). DNA methylation is a highly dynamic system that responds well to endogenous and exogenous substances (Zingg et al. 1997). Methylation only occurs on 'CpG islands', the short stretches of CpG rich regions associated with the promoter region of genes (Toyota and Issa, 1999). As is the case with the P53 gene, these regions are also 'hot spots' for mutations (Toyota and Issa, 1999). Several studies have indicated the significance of DNA methylation as an epigenetic mechanism promoting

carcinogenesis. Croce et al. (2002) have shown that cancer cells induce both hypermethylation of regions of CpG clusters within the DNA, as well as global hypomethlation of the genome. They have shown that DNA methylation of tumor suppressor genes is a frequent mechanism of transcriptional silencing in cancer. As genes become transcriptionally inert, the inactive state of these now silent genes is stabilized through cell proliferation and differentiation (McBurney, 1999). Aberrant methylation of CpG islands in tumors serves as an alternative pathway for complete inactivation of tumor suppressor genes (Ivanova et al. 2002). Berger et al. (2002) showed that many nuclear receptor genes can be silenced through methylation in tumors; epigenetic silencing is the mechanism that modifies expression of key genes during carcinogenesis. Counts and Goodman (1995) also proposed that examining DNA methylation patterns leads to the potential for discovering alterations in gene expression, cell proliferation, mutation, chromatin aberrations and inactivation of tumor suppressor genes.

Our results have indicated key patterns and trends in the expression of families/clusters of genes implicated in DNA methylation mechanisms. Increased DNA methylation may represent the mechanism by which HCB silences the expression of 30% of hepatic genes in females. We noticed that both the phosphatidylethanolamine N-methyltransferase (PEMT) and phosphatidylethanolamine binding-protein (PEBP) which are both implicated in DNA methylation were increased in HCB-treated female rats only. This realization is significant because it displays consistent patterns and trends among genes within the same cluster and/or family. In addition, it had been shown by Bucquoy et al. (1994) that newly methylated CpGs sites become docking sites for methyl binding proteins such as PEBP. These, in turn, bind to phosphotidylethanolamine as well as a

variety nucleotides associated with signaling mechanisms, including GTP and GDP. Banfield et al. (1998) reported that PEBP plays a key role in signal transduction from the interior of membrane surface. If the initial recruitment step is not prevented, it may lead to the spreading of hypermethylation to the neighboring regions of the promoter region, locking the genes into a stable silenced chromatin state.

The PEMT pathway in the liver is an important process of cell proliferation and carcinogenesis (Vance et al., 1998). PEMT is expressed in human liver, heart and the testis, with the highest level of expression in the liver (Shields et al., 2001). Moreover, choline deficiency has been reported to cause sexually dimorphic activation of liver and brain PEMT, i.e. brain PEMT was increased in females by 49% with no effect in males, whereas in contrast hepatic PEMT activity was unaffected in females while increased by 34% in males (Johnson et al., 1998).

There are two other possible pathways that should be acknowledged with respect to their implications in HCB-induced liver carcinogenesis in females. We noticed a significant up-regulation of ribosomal protein L18 expression. This protein is an inhibitor of dsRNA-activated protein kinase (PKR) activity. PKR activity is a necessary control step in the regulation of protein synthesis. When activated, PKR inhibits cell growth and induces apoptosis. However, if L18 expression is inhibited, as was noticed in our study, it can lead to unregulated cell growth (Kumar et al., 1999).

The other pathway that merits recognition relates to protectin/CD59. Activation of the plasma complement (C) system leads to formation of a cytolytic membrane attack complex (MAC). This results in a large pore that spans the membrane of the cell being attacked, allowing ions to flow freely outside of the cell, and leading to osmotic burst.

HCB has been shown to disrupt phospholipid metabolism in female rats (Cohon et al., 2001) and to incorporate itself between the fatty acid chains of membrane lipids. This suggests a key role for HCB at the plasma membrane leading to increased membrane fluidity in hepatocytes (Koszo et al., 1982). Spiller et al. (2000) have shown that cytokines caused increased secretion by hepatoma cells of complement regulatory proteins, providing protection from complement attack. The protectin/CD59 antigen acts by inhibiting the formation of the MAC present on human cell membranes (Okada et al., 1989). Up-regulation of membrane C regulators has been implicated as a survival strategy in numerous human tumors (Varsano et al., 1998). In this study, however, we noticed that HCB down-regulated CD59 activity in females only, and this regulation should be recognized in HCB-induced liver carcinogenesis.

The *in vivo* exposure model used in the present study in which rats are sampled at day 50, represents true "steady state" perturbations resulting from HCB exposure. This, as well as the sexual dimorphism in liver tumor promotion, allows us to "dissect" gene expression effects in order to identify the mechanism of liver tumor formation. The expression analysis data indicate that 36 genes whose expression was altered by HCB treatment may be implicated in the epigenetic mechanism of HCB-induced hepatocarcinogenesis. This group is made of 31 putative tumor suppressor genes that are undetectable in HCB-treated females, and 5 genes that were significantly modulated by HCB in the female which show a predisposition to HCB-induced hepatic tumors. No genes were expressed exclusively in either HCB-treated females alone or in HCB-treated males and females simultaneously. We propose a primary mechanism in which HCB alters the methylation machinery of the cell, a known factor for epigenetic carcinogens.

In summary, the functional genomic approach used in this study allowed us to identify among the 1176 genes examined, a cluster of 36 genes that may be implicated in the epigenetic mechanisms of HCB-induced promotion hepatocarcinogenesis in the female rat. A marked sexual dimorphism in the liver effect by HCB has been seen at expression of genes with a clear down-regulation in females, as opposed to a slight up-regulation in males. Our results are essential in understanding hepatocarcinogenesis in the rat. They unveil a mechanistic pathway crucial in the development of hepatic tumors. This pathway is critical in evaluating the risks of exposure to HCB in hepatocarcinogenesis. Furthermore, they demonstrate that the predisposition of females to liver tumors arise, partly, from a sexual dimorphism exhibited at the genetic level. The understanding of the mechanisms in which environmental pollutants cause the development of hepatic tumors is vital, in that it allows us to search for therapies that can alter the invasive and metastatic abilities of cancer. Finally, understanding the mechanisms will allow for the proper judgment for extrapolation of animal carcinogenicity data to humans and appropriate assessment of an acceptable environmental level within the population, and for the institution of necessary risks prior to using these xenobiotic substances.

SYNTHÈSE DE LA MÉMOIRE REDIGE EN FRANÇAIS

L'hexachlorobenzène (HCB), un organochloré, était notamment utilisé jusqu'en 1970 comme fongicide pour la culture des céréales et pour la fabrication de solvants chlorés et de pesticides (WHO, 1998). En conséquence ce produit s'est largement répandu dans l'environnement. L'HCB est un contaminant environnemental que l'on retrouve dans les tissus gras et le lait maternel des personnes vivant dans les pays industrialisés (Sala, 1999). La moyenne totale de la consommation journalière d'HCB dans la population varie entre 0.4 et 3 ng HCB/kg poids/d (WHO, 1998).

Plusieurs études ont démontré que l'HCB crée des tumeurs chez les rats femelles, mais peu chez les rats mâles. Legault *et al.* (1997) ont démontré qu'une ovariectomie provoque chez le rat femelle une insensibilité aux effets porphyriques de l'HCB. Toutefois, lorsque ces femelles ont reçu un traitement d'estradiol, elles ont développé une porphyrie suggérant qu'il y a une composante estrogénique dans la sensibilité au traitement à l'HCB pour le déclenchement de la porphyrie. Plante *et al.* (2002) ont rapporté que chez le rat femelle, mais pas chez le rat mâle, l'HCB réduit l'expression des gènes qui codent pour les protéines, les connexines, engagées dans la communication intercellulaire. L'HCB cause aussi une perturbation du métabolisme phospholipidique chez les rats femelles résultant en une augmentation de phophatidylcholine, de phosphatidylsérine, de phosphatidyléthanolamine et de phosphatidylinositol hépatique suite à une exposition de une à quatre semaines (Cochon *et al.*, 2001). L'HCB est

incorporé entre les chaînes d'acides gras des membranes lipides entraînant une augmentation de la fluidité membranaire des hépatocytes (Koszo *et al.* 1982).

La porphyrie est un désordre caractérisé par une accumulation hépatique massive et une excrétion urinaire importante de porphyrines grandement carboxylées, à savoir l'uroporphyrine et l'heptacarboporphyrine (San Martin de Viale, 1970 ; Elder, 1978). Chez le rat, la porphyrie induite par l'HCB présente un dimorphisme sexuel, la femelle étant susceptible mais non le mâle. Même si le mécanisme d'induction de la porphyrie par l'HCB n'a pas encore été complètement élucidé, l'hypothèse la plus courante met en cause l'inhibition d'une enzyme de la synthèse de l'hème, l'uroporphyrinogène décarboxylase (URO-D) (San Martin de Viale *et al.*, 1976). L'URO-D catalyse la conversion de l'uroporphyrinogène en coproporphyrinogène par des décarboxylations séquentielle de l'uroporphyrinogène formant successivement quatre intermédiaires décarboxylés. On suspecte qu'un intermédiaire réactif instable réagit avec la portion-SH catalytique de l'enzyme URO-D dans le cytosol du foie (Debets *et al.*, 1980). Legault *et al.* (1997) ont observé un délai de 4 semaines entre l'exposition à l'HCB et la diminution de l'activité de l'URO-D accompagnée d'une accumulation de 100 à 300 fois d'uroporphyrine et d'heptacarboxyporphyrine dans le foie et d'une augmentation de l'excrétion urinaire d'uroporphyrine. L'inhibition de l'URO-D coïncide avec le début de la porphyrie chez les femelles traitées à l'HCB, puisqu'une diminution de l'activité de l'URO-D n'a pas été observée avant que la porphyrie ne se développe. De plus, l'activité

de l'URO-D n'etait pas diminuée ni chez les mâles non chez les femelles qui nont pas développé de porphyrie (Legault *et al.* 1997).

Randi *et al.* (1998) ont observé des changements significatifs dans l'efficacité de la liaison de l'EGF (« Epidermal Growth Factor ») à la membrane plasmatique (134 fmol/mg chez les témoins versus 468 fmol/mg chez les animaux traités) après 10 jours de traitement à l'HCB. Ils ont aussi noté des effets dépendant du temps de traitement sur le niveau de phosphorylation de la protéine tyrosine kinase (PTK) dans le foie du rat ; des augmentations significatives de l'activité dans la PTK ont été observées après deux et cinq jours, puis au dixième jour du traitement l'activité de la PTK était significativement diminuée et elle était subséquemment revenue au niveau témoin après 20 jours.

Le récepteur Ah est un facteur de transcription « ligand-dépendent » qui appartient à la superfamille *basic-helix-loop-helix* des protéines liantes de l'ADN (Loaiza-Pérez *et al.*, 1999). Il fut démontré que la liaison des produits chimiques de type dioxine au récepteur Ah peut activer la phosphorylation de protéines importantes dans le cytosol et la membrane plasmatique, provoquant ainsi des changements fonctionnels (Matsumura, 1995). Le locus Ah contrôle l'induction de l'activité des cytochromes P-450 par les hydrocarbures aromatiques polycycliques (Hahn *et al.* 1988). Le locus Ah comprend des gènes structuraux, régulateurs et temporels (Eisen *et al.* 1983). L'HCB peut être considéré comme un composé de type dioxine parce qu'il se lie, bien que faiblement, au récepteur Ah-R et qu'il en résulte des effets semblables à ceux induits par la dioxine (Hahn *et al.* 1989).

L'HCB est un inducteur de type mixte du cytochrome P-450 induisant en autres les cytochromes P450 $1A_1$ et $1A_2$ qui sont les plus importants. L'oxydation de l'HCB par les cytochromes P-450 résulte en la production des métabolites pentachlorophénol (PCP), tétrachlorohyodroquinone (TCHQ), pentachlorothiophénol et le tétrachlorothiophénol (Koss *et al.*, 1976). L'HCB possède une demie-vie d'environ 4 à 5 ans et donc sa concentration est très stable dans l'environnement (EPA, 2002). De plus l'HCB a été classé parmi les polluants les plus persistants a cause de sa stabilité, sa résistance à la dégradation, et son potentiel de bioaccumulation dans l'environnement, les animaux et les humains (Sala *et al.* 1999; Mes *et al.*, 1982).

L'HCB est un carcinogène chimique puissant qui peut induire des hépatomes, des carcinomes hépatocellulaires et des adénomes biliaires chez les animaux de laboratoire (WHO, 1998 ; Cabral *et al.*, 1979). Le mécanisme par lequel HCB entraîne la formation de tumeurs chez le rat femelle demeure inconnu. De nombreuses études ont démontré des propriétés carcinogènes complètes de l'HCB, alors que d'autres ont mis en lumière son potentiel pour promouvoir le cancer à titre de co-carcinogène ou agent promoteur du cancer. Une exposition simultanée à l'HCB dans la diète de la souris traitée avec du terphénylène polychloré facilite l'induction de tumeurs de foie (Shirai *et al.,* 1978). Une étude menée par Pereira *et al.* (1982) étudiant les effets de l'HCB chez le rat suivant un prétraitement de diéthylnitrosamine (DEN - initiateur de tumeur) ont déterminé que l'HCB agissait comme promoteur de tumeurs de foie.

La promotion des tumeurs implique l'expansion clonale des cellules initiées via un mécanisme épigénétique réduisant le temps nécessaire pour la progression de la tumeur. L'HCB n'apparaît pas être mutagène (Khera *et al.* 1974; Simon *et al.* 1979; Siekel *et al.* 1991), ce qui suggère qu'il n'est pas initiateur mais plutôt promoteur de l'hépatocarcinogenèse. Les études ont montré que seuls les rats pré-traités avec la DEN avaient une augmentation significative des foyers de cellules hépatiques positives en enzyme gamma-glutamyl transférase. Ces foyers sont des précurseurs de lésions cancéreuses et donc des indicateurs d'une activité hépatocarcinogène (Pereira *et al.* 1981). L'induction de ces foyers par l'HCB indique que celui-ci est un promoteur de l'hépatocarcinogenèse. Pereira *et al.* (1981) ont fait aussi une découverte critique, à savoir que les femelles sont beaucoup plus susceptibles de développer ces lésions pré-néoplasiques que les mâles.

L'HCB a été rapporté incapable d'induire des dommages au chromosome (WHO, 1998). Les indices mitotiques dans les lymphocytes périphériques exposés à l'HCB suggèrent qu'il agit comme un carcinogène epigénétique. Le potentiel cancérogène de l'HCB a été démontré par plusieurs analyses biologiques chez les rats, les souris et hamsters. Khera *et al.* (1974) ont administré de l'HCB à des rats mâles pendant 10 jours consécutifs à une concentration allant jusqu'à 60 mg/kg/j et ils ont observé que l'HCB n'a pas réussi à induire des mutations létales dominantes. Une autre étude conduite par Simon *et al.* (1979) cherchait à savoir si l'HCB pouvait agir directement comme agent

génotoxique. Ils ont administré jusqu'à 221 mg/kg d'HCB pendant cinq jours consécutifs à des rats mâles et ils les ont ensuite accouplés avec deux femelles nullipares naïves pour enregistrer des mutations létales et des effets reproductifs. L'HCB n'a induit aucun des effets létaux dominants classiques. La plupart des produits chimiques qui causent des mutations dominantes le manifestent dans les premières semaines (1-5) de l'accouplement. Dans cette expérience, des effets significatifs ne furent observés que 10-14 semaines après la première administration d'HCB, indiquant alors que l'HCB est un agent non-génotoxique, c'est-à-dire carcinogène épigénétique. De plus, une étude *in vitro* conduite par IARC ont rapporté l'échec de l'HCB à induire une aberration chromosomique sur des cellules de hamsters chinois.

Les puces à ADN

La technologie de puces à ADN *(microarray)* peut fournir de nouvelles informations pour identifier des mécanismes d'action spécifiques pour les toxiques environnementaux et elle peut également fournir de nouvelles informations sur les effets macroscopiques des produits chimiques sur l'expression du génome et sa relation avec des pathologies spécifiques. L'avantage de ces tests est qu'ils sont rapides, efficaces et que la grande quantité de mesures simultanées permet de réduire l'utilisation d'animaux de laboratoire (Lovett, 2000). Un autre bénéfice d'utilisation de cette nouvelle technologie s'applique spécifiquement à la toxicologie des cancérogènes. En utilisant des déploiements d'ADNc, il est possible de cibler les précurseurs et inducteurs des maladies se développant lentement, tel le cancer, sans avoir à attendre que des lésions complètes se

développent au terme de mois, voire des années. Les chercheurs en cancérologie utilisent déjà ces déploiements depuis plusieurs années pour comparer l'expression génique dans les cellules saines à celle des cellules cancéreuses. Les toxicologues utilisent cette technologie pour établir le profil de l'expression génique dans les cellules exposées à des xénobiotiques. L'HCB représente un produit chimique intéressant non seulement pour déterminer ses effets sur la promotion des tumeurs, mais également pour identifier des voies responsables de la progression des tumeurs présentant un dimorphisme sexuel.

Récemment la technologie des puces à l'ADN a permis d'identifier les gènes spécifiques potentiellement importants dans la pathogenèse du cancer du foie. En effet, Tackels-Horne *et al.* (2002) ont réalisé une étude comparant l'expression des gènes de six hépatocarcinomes primaires, cinq adénocarcinomes métastatiques du foie et huit foies normaux. Les auteurs ont ciblé 20 gènes significativement surexprimés ("up-regulated ") et parmi eux sept étaient déjà reconnus être impliqué dans le cancer du foie et les autres types de cancer. De plus, les auteurs ont identifié 42 gènes et 24 séquences (EST's) qui sont exprimées à un taux élevé dans les carcinomes hépatocellulaires. En utilisant la technologie des puces à l'ADN les auteurs ont réussi à identifier, parmi plusieurs gènes potentiels, un groupe de gènes marqueurs de tissus hépatique cancéreux. Une autre étude a rapporté les effets physiologiques d'un oncogène reconnu dans la littérature, la protéine du virus l'hépatite B (HBV), en utilisant l'Atlas^{TM} humain cDNA (*microarray)* ; Han *et al.* (2000) ont analysé 588 cDNA et réussi à cibler 13 gènes (2.2%) dont les fonctions biologiques sont impliquées dans la prolifération cellulaire et dans ce cas

significativement surexprimés dans le mécanisme des carcinomes hépatocellulaire chez l'humain. Les gènes identifiables appartiennent aux classes suivantes : oncogènes, protéines de l'apoptose, facteurs de croissance, régulateurs de cycle cellulaire et protéines de stress. L'analyse a fourni une nouvelle démarche pour comprendre le mécanisme par lequel des patients affectés par le virus HBV, peuvent développer des carcinomes hépatocellulaires.

Une étude intéressante de Graveel *et al.* (2001) a présenté un modèle mutationnel pour décrire le cancer du foie chez la souris. En utilisant une puce d'oligonucleotides, les auteurs ont comparé l'expression génique des tumeurs dans le foie à trois différents états du foie, soit l'état normal chez l'adulte, l'état de régénération, et l'état en développement chez le nourrisson. Ils ont observé qu'en induisant des tumeurs avec la diéthylnitrosamine seulement 22 gènes étaient dérégulés dans les tumeurs induites chez les trois groupes. En outre, trois de ces gènes sont *"up-regulated"* dans les hépatomes humains. Les résultats suggèrent que les souris traitées au DEN fournissent un excellent modèle pour l'hépatome humain.

En résumé, les études précédentes avec les puces d'ADN ont démontré le potentiel solide de cette technique pour expliquer les gènes primaires et les mécanismes impliqués dans la cancérogenèse. De plus, cette technique permet l'étude des relations à travers les différentes espèces, soit l'humain, la souris et le rat.

Travaux expérimentaux

Dans cette étude notre objectif était de déterminer les mécanismes d'induction des tumeurs chez le rat traités à l'HCB en observant l'expression génique dans le foie en fonction du genre. Pour aborder cet objectif, une approche génomique a été utilisée pour identifier un groupe de gènes candidats qui jouent un rôle dans l'hépatocarcinogenèse induite par l'HCB. Le modèle expérimental utilisé est excellent puisqu'il offre la possibilité d'observer l'expression génique dans un contexte d'équilibre dynamique d'expression (« steady state »).

Notre modèle expérimental comprend quatre groupes de rats Sprague-Dawley : les mâles témoins (n=4), les femelles témoins (n=4), les mâles traitées à l'HCB (n=4) et les femelles traitées à l'HCB (n=4). L'HCB a été administré (100 mg/kg/jour dans 10 ml/kg/jour d'huile de maïs) par gavage pendant 5 jours consécutifs ; les témoins ont reçu l'huile de maïs seulement (10ml/kg/jour). Quarante-cinq jours après cette étape, les rats ont été anesthésiés avec le gaz isofluorane, puis sacrifiés par exsanguination via un prélèvement de sang retiré via l'aorte abdominale. Le foie du rat était chirurgicalement prélevé, puis immédiatement gelé dans l'azote liquide et préservé à 86°C.

Pour la fabrication des sondes, l'ARN total a été extrait du foie des rats témoins et des rats traitées a l'HCB en utilisant «l'Atlas™ Pure Total Labeling System ». De plus, l'ARN a également été traité avec une DNAase libre en RNAase pour éliminer la possibilité de contamination par de l'ADN génomique. L'ARN total est ensuite inversement transcrit en ADNc en utilisant le MMLV, puis marqué avec du [α-^{32}P]-dATP. La sonde est hybridée à la membrane durant environ 12 heures. Après

l'hybridation, les membranes sont scellées dans une enveloppe en plastique et exposées sur un écran de phosphore. Les membranes sont exposées pendant des durées différentes, variant entre 1 heure à 2 semaines, pour obtenir un temps d'exposition optimal pour chacune. Une puce à ADN a été préparée pour chacun des quatre rats par groupe expérimental.

La membrane « *Clontech* Rat 1.2 II cDNA Atlas™ expression Array » (*microarray*) contient 1176 gènes dont les séquences génétiques et les fonctions biologiques sont connues. Le schéma 1 (p. 57) montre un autoradiogramme d'une puce représentative hybridée avec une sonde d'ADN marquée au ^{32}P préparée à partir du foie d'un rat femelle traitée à l'HCB. Les résultats indiquent que chez les rat mâles témoins environ 185 gènes sont exprimés sur la membrane (p. 57). Bien qu'il y avait une différence entre les individus, 83% des gènes hépatiques exprimés étaient communs chez au moins trois des quatre essais individuels. Dans le foie des femelles, 184 gènes étaient exprimés et 86% de ceux-ci étaient communs chez trois des quatre femelles. Chez les mâles traités à l'HCB il y avait 189 gènes exprimés et 76 % de ces gènes étaient communs chez trois mâles individuels. Chez les femelles traitées à l'HCB il y avait seulement 151 gènes exprimés et 84 % étaient communs entre trois des quatre essais individuels (p.57).

La comparaison des résultats du groupe des femelles témoins avec le groupe des mâles témoins démontre des nombres similaires en ce qui concerne le numéro total de gènes exprimés sur la membrane, soit 185 et 184 gènes respectivement. Dans le groupe

des mâles témoins il y a sept gènes qui y étaient exclusivement exprimés ; ERp29, synaptogyrin 1, synaptojanin, DPPIII, la protéine kinesin-related, atrophin 1 et la glycoprotéine 55.

Un total de 184 gènes était exprimé dans le foies des femelles témoins et 145 de ces gènes chevauchent les quatre groupes expérimentaux. Il y avait seulement 2 gènes qui étaient uniquement exprimés dans les femelles, soit « l'achaete mammifère scute homolog 2 (MASH- 2 » et le carboxypeptidase Z (p.57). Le MASH 2 est un facteur de transcription de base "hélice-boucle-hélice" précédemment montré pour être impliqué dans le développement du placenta et être aussi exprimé par les cellules de Schwann des nerfs périphériques adultes. La carboxypeptidase Z est une glycoprotéine de la membrane des lysosomes dont la taille est de 120 Kd. Il y avait de plus 3 gènes qui étaient présents dans tous les groupes expérimentaux sauf chez les mâles témoins : le H-rev 107, le B61 et la chaîne lourde myosin-3.

Un seul gène étaient exprimé exclusivement chez les mâles traitées à l'HCB, soit celui de la phosphoglucomutase, une enzyme impliquée dans le métabolisme des hydrates de carbone. Comme ces rats ne développent pas de tumeurs ils est raisonnable de penser que ce gène protège contre le développement de tumeurs ; cependant il n'y a aucune évidence de ceci dans la littérature. À l'opposé de ce qui est observé chez les femelles traitées à l'HCB il y avait beaucoup moins de variation individuelle dans le nombre de gènes exprimés chez les rats mâles traités à l'HCB. À cet égard ceux-ci sont semblables

aux rats de témoins mâles et femelles. Les analyses statistiques des gènes exprimés chez les mâles témoins et mâles traités à l'HCB indiquent que les niveaux d'expression de quatre gènes étaient significativement changés (p. 58). De ces 4 gènes, 3 étaient régulés à la baisse "down-regulated" et un était surexprimé ("up-regulated" - Table 2, p.61).

Le gène EGR2/KROX-20 est fortement "down-regulated" chez les mâles (p. 58). Cependant, les niveaux de ce gène sont inchangés chez les femelles traitées à l'HCB. En outre, le niveau d'expression de ce gène chez les mâles était semblable à celui des femelles, cependant chez les rats traitées à l'HCB il était fortement diminué ("down-regulated") mais seulement chez les mâles. La dihydropyrimidinase, une enzyme impliquée dans le métabolisme des nucléotides, étaient significativement "down-regulated" mais seulement chez les mâles traitées à l'HCB bien qu'il y avait une tendance à la diminution chez les femelles (p. 58). L'époxide hydrolase, une enzyme impliquée dans la biotransformation des xénobiotiques et des époxides était régulée différemment chez les rats mâles et femelles traités à l'HCB (p. 58). Ce gène est surexprimé chez les mâles traités à l'HCB, mais très diminué chez les rats femelles témoins et femelles traitées à l'HCB.

Il n'y avait aucun gène exclusivement exprimé chez les femelles traitées à l'HCB. De plus, l'HCB a causé une diminution de 18 % dans le nombre de gènes exprimés dans le foie de rats femelles traitées à l'HCB en comparaison des témoins femelles, des mâles témoins, et des mâles traités à l'HCB. Il y avait 31 gènes qui étaient diminués chez les

femelles traitées à l'HCB. Ceux-ci ont été classés en 3 groupes fonctionnels répertoriés au Tableau 1 (p.60). De façon intéressante, 29 % de ces gènes avaient une expression réduite ("down-regulated") dans le cancer de foie. Des analyses statistiques utilisant l'analyse des variations bilatérales dans le sous-ensemble entier des gènes des quatre groupes expérimentaux ont permis d'identifier 9 gènes significativement modulés par le traitement (Tableau 2, p.61). L'expression de cinq de ces gènes était significativement modulée chez les rats femelles (p. 59). De ces 5 gènes, 4 étaient surexprimés et un était réduit ("down-regulated") (Tableau 2, p. 61).

DISCUSSION ET CONCLUSIONS

Plusieurs études ont démontré que divers polluants environnementaux agissent comme des promoteurs de tumeur via des mécanismes épigénétiques de cancérogenèse. Certains mécanismes ont été identifiés et peuvent être impliqués dans la phase d'expansion clonale des cellules initiées. Des études précédentes dans notre laboratoire ont montré que l'HCB diminue l'expression des gènes des connexins 32 et 26 chez le rat femelle, mais pas chez le rat mâle, réduisant ainsi la communication intracellulaire selon un patron empreint d'un dimorphisme sexuel (Plante *et al.* 2002). Nos résultats sont compatibles avec cette découverte. En effet, le nombre de gènes exprimés chez les rats femelles traités à l'HCB est 18 % plus bas que chez celui des témoins des deux sexes ou des mâles traités. Ainsi, il apparait que l'HCB peut réprimer l'expression du génome dans le foie de rats femelles.

De plus, nous avons identifié un groupe de 31 gènes qui sont exprimés chez tous les groupes expérimentaux sauf chez les femelles traitées à l'HCB suggérant que ces gènes candidats peuvent être impliqués dans la protection de la cancérogenèse. L'analyse de ces 31 gènes nous a permis de découvrir que 11 de ces gènes (35 %) sont déjà reconnus être "down-regulated" pendant la carcinogenèse. Ces résultats supportent le fait que les rats femelles sont plus susceptibles au développement de tumeurs hépatiques que les mâles, qui eux développent très peu, voire aucune tumeur hépatique.

Étant donné la bioaccumulation significative et la persistance de HCB dans l'environnement, et particulièrement sa grande stabilité dans les tissus adipeux humains, il est impératif d'identifier les effets de l`HCB sur la carcinogenèse chez l'humain. L`HCB agit comme un cancérigène épigénétique (WHO, 1998; Simon *et al.* 1979). L'hypothèse de recherche de cette étude était que le dimorphisme sexuel dans la carcinogenèse du foie induite par l`HCB reposait sur un dimorphisme sexuel au niveau de l'expression différentielle de certains gènes avec un nombre de gènes protecteurs plus faiblement exprimés, ou encore de gènes activateurs plus fortement exprimés, chez les rats femelles traitées a l'HCB que chez les mâles.

Le premier objectif de cette étude était de déterminer si le dimorphisme sexuel clairement manifesté au niveau morphologique, était aussi apparent au niveau génétique et moléculaire. Dans l'étude présente, 1176 gènes représentant approxi ativement 2 % du génome de rat entier ont été examinés. Nous avons cherché des différences significatives dans l'expression en ce qui concerne les groupes semblables de gènes. En identifiant les niveaux d'expression parmi les familles de gènes il est possible de proposer et peu décrire des gènes-candidats potentiellement impliqués dans le mécanisme de la carcinogenèse du foie.

Une hypothèse quant au mécanisme de la cancérogenèse du foie induite par l`HCB suggère une modulation du niveau de la transcription des gènes. L`HCB a été démontré capable de changer les niveaux d'expression de certains facteurs de

transcription (Loaiza-Pérez *et al.,* 1999). L`HCB réagit comme un agoniste faible du récepteur Ah. Le récepteur Ah est un facteur de transcription qui appartient à la superfamille "hélice-boucle-hélice" des protéines liantes à l'ADN (Loaiza-Pérez *et al,* 1999). Une fois le ligand attache au récepteur Ah, le complèxe HCB-Ah-R quitte le cytosol et se déplace vers le noyau de la cellule où il forme un hétérodimère avec la protéine translocatrice nucléaire Ah-R (ARNT) qui est capable de moduler l'expression des gènes qui ont une séquence spécifique répondant aux xénobiotiques ou à la dioxine (XRE ou DRE) dans la région de leur promoteur.

Le modèle d'exposition *in vivo* utilisé dans la présente étude dans laquelle les rats étaient échantillonnés au 50^{ieme} jour permet d'obtenir une évaluation pour un "'état stable" de perturbations résultant de l'exposition l`HCB. Ceci, de même que le dimorphisme sexuel dans la promotion de la tumeur du foie, nous permet "de disséquer" spécifiquement les effets d'expression génique afin d'identifier le mécanisme de la formation des tumeurs dans le foie. L'analyse des données de ces expériences de puces à ADN indique que 36 gènes dont l'expression a été changée par le traitement de l`HCB peuvent être impliqués dans le mécanisme épigénétique de l'hépatocarcinogenèse. Ce groupe est composé de 31 gènes, dont l'expression est absente chez les femelles traitées à l`HCB, potentiellement des marqueurs de tumeurs, et de cinq gènes qui sont significativement modulés par l`HCB chez la femelle. Aucun gène n'a été exprimé exclusivement chez les femelles traitées à l`HCB seul.

Afin de trouver des gènes candidats appropriés à la promotion de l'hépatocarcinogenèse chez les femelles, deux groupes de gènes (marqués d'un astérisque dans la figure 2, p.57) ont été analysés en profondeur. Ce groupe de 31 gènes est présent dans tous les groupes expérimentaux sauf chez les femelles traitées à l'HCB suggérant que ces gènes-candidats peuvent conférer un rôle protecteur chez les rats mâles qui ne développent pas de tumeurs. L'analyse plus détaillée de ces 31 gènes indique que l'expression de 11 de ces gènes est déjà reconnus comme diminuée pendant l'hépatocancérogenèse. La plus grande portion des gènes exprimés chez les rats femelles traitées à l'HCB (145 de 151 gènes) a été aussi exprimée chez les trois autres groupes expérimentaux (fig. 2, p.57).

Il est bien documenté que l'augmentation de la méthylation de la région du promoteur d'un gène donné est un mécanisme pour réprimer l'expression de ce gène. Ceci contribue aux premiers pas vers la carcinogenèse (Croce *et al.* 2002; Counts and Goodman 1995). L'augmentation de la méthylation d'ADN peut représenter un mécanisme par lequel HCB réduit l'expression de 30 % des gènes hépatiques. En appui à ceci on note le fait que la phosphatidyléthanolamine N-méthyltransférase (PEMT) et la protéine liée à la phosphatidyléthanolamine (PEBP) sont deux gènes impliqués dans la méthylation de l'ADN qui sont augmentés chez les rats femelles traitées à l'HCB. De plus, alors que chez les femelles traitées à l'HCB les niveaux d'ARNm de la PEMT et de la PEBP sont fortement surexprimés, chez les rats mâles traités à l'HCB il n'y a aucun changement d'expression. Les CpGs nouvellement méthylés deviennent des sites de

liaison pour des protéines sensibles aux méthyles comme le PEBP qui se lie à la phosphotidyléthanolamine aussi bien qu'une variété de nucléotides associés aux mécanismes de signalisation, y compris la GTP et la GDP (Bucquoy *et al.* 1994). Banfield *et al.* (1998) ont suggéré que la PEBP joue un rôle clé dans la transduction de signaux. Ce phénomène peut mener à l'hyperméthylation des régions voisines de la région promotrice, plaçant davantage le gène dans un état de chromatine stable. On pourrait spéculer que la PEMT augmente la méthylation des groupes spécifiques dinucleotides CpG dans la région de promoteur du gène KAI1 par exemple. La région 5 ' du promoteur de ce gène KAI1 est riche en G+C (Dong *et al.* 1997) impliquant qu'il y a un fort potentiel pour une hyperméthylation dans cette région promotrice, ce qui pourrait expliquer la réduction d'expression de ce gène chez les rats femelles traitées à l`HCB mais non chez les rats mâles. La "down-regulation" de l`expression de KAI1 est associée à la formation de métastases dans la prostate au cancer pancréatique et à des tumeurs épithéliales solides communes pulmonaires, de type hépatique, colo-rectale, ovarienne, oesophagienne, pancréatique et prostatique (Guo *et al.* 1998; Liu *et al.* 2001). Une réduction de l'expression de l'ARNm de la KAI1 dans des cellules d'hépatocarcinome semble influencer leur capacité métastatique et augmenter leur potentiel agressif (Guo *et al.* 1998).

Au cours des dernières années, un important effort de recherche a été dirigé vers la connaissance des mécanismes épigénétiques, en particulier relativement à la régulation des gènes via la méthylation de l'ADN (Ivanova *et al.* 2002; Berger *et al.* 2002). La voie

PEMT dans le foie est un processus important dans la prolifération des cellules et la carcinogenèse (Vance and Walkey, 1998). Le PEMT est exprimé dans le foie humain, le coeur et le testicule, avec le plus haut niveau d'expression dans le foie (Shields *et al.* 2001). De plus, à l'aide du modèle de déficience en choline un dimorphisme sexuel dans la PEMT dans le foie et le cerveau a été rapporté, à savoir que les nivaux dans le cerveau étaient augmentés chez les femelles de 49 %, sans effet chez les mâles, tandis que l'activité hépatique de la PEMT était inchangée dans les femelles mais augmentée de 34 % chez les mâles (Johnson and Blusztajn 1998).

Deux autres voies modulées chez les femelles traitées à l'HCB mais non chez les mâles, pourraient soutenir une explication mécanistique pour le dimorphisme sexuel dans la promotion de tumeurs du foie. Une première implique l'augmentation de l'expression de la protéine ribosomale L18, un nouvel inhibiteur des protéines PKR qui lorsqu'activée empêche la croissance cellulaire et induit l'apoptose (Kumar *et al.* 1999); une augmentation de l'expression de la protéine ribosomale L18 a été trouvée chez les femelles traitées à l'HCB seulement. La protéine Ribosomal L18 est surexprimée dans d'autres formations cancéreuses comme le cancer colorectal (Kumar *et al.* 1999).

Une seconde voie implique la protectine/CD59 qui est un régulateur du système du complément et qui a été impliquée comme une stratégie de survie dans de nombreuses tumeurs humaines (Varsano *et al.* 1998). L'HCB diminue l'activité de la CD59 chez les femelles uniquement les rendant plus prédisposées aux tumeurs hépatiques que les mâles.

En résumé, l'approche de génomique fonctionnelle utilisée dans ce mémoire nous a permis d'identifier parmi les 1176 gènes examinés, un groupe de 36 gènes qui peuvent être impliqués dans des mécanismes épigénétiques induis par l'HCB et responsables du développement de l'hépatocarcinogenèse chez le rat femelle. Les résultats ont montré un dimorphisme sexuel marqué à l'expression génique dans le foie des rats traités à l'HCB avec une régulation diminuée chez les femelles par opposition à une légère augmentation chez les mâles. L'analyse des résultats nous incite à proposer un mécanisme primaire dans lequel l'HCB module la machinerie de méthylation de la cellule, un mécanisme reconnu pour des hépatocancérogènes épigénétiques.

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