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HUMAN MICROSOMAL EPOXIDE HYDROLASE (EPHX1): GENETIC POLYMORPHISM AND REGULATION BY CHEMOPREVENTIVE AGENTS

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ABSTRACT

Microsomal epoxide hydrolase (EPHX1) is a critical catalytic determinant in the formation of the highly reactive electrophilic, and ultimately carcinogenic, epoxide metabolites of the polyaromatic hydrocarbons. A central hypothesis of our research is that genetic variability and differential regulation of EPHX1 in human tissue are likely important determinants of interindividual responsiveness, resulting toxicities, and carcinogenicity outcomes related to chemical exposures. Our laboratory has demonstrated that the expression of the human EPHX1 gene is driven by the use of alternative promoters. An alternative promoter region, termed the E1-b promoter, is localized ~ 18.5 kb 5'-upstream from the structural region of the EPHX1 gene. The E1-b promoter is used exclusively to drive expression of EPHX1 mRNA transcripts in most tissues, along with a more proximal and highly liver-specific E1 promoter. Results of quantitative Real Time-PCR analyses demonstrated that the E1-b variant transcript is preferentially and broadly expressed in most tissues, such that it accounts for the majority of total EPHX1 transcript *in vivo*. In the studies conducted within this thesis research, detailed analysis of the E1-b promoter region demonstrated that this upstream EPHX1 promoter is replete with transposable elements. Further, we identified that two specific Alu elements are polymorphic (i.e. some chromosomes carry the two Alu insertions, whereas other do not) in the genome structure of different individuals. Results of luciferase gene reporter assays conducted in several human cell lines with E1-b promoter constructs demonstrated that the inclusion of the Alu (+/+) insertion significantly decreases basal transcriptional activities. Although we identified a putative aryl hydrocarbon receptor (AhR) binding motif within the Alu element structure, expression of human EPHX1 in an *in vitro* system was only modestly responsive to AhR ligands and we conclude that AhR regulation does not associate with the presence/absence of Alu elements. Two non-synonomous genetic polymorphisms that alter the EPHX1 amino acid structure were identified in

our laboratory's previous research efforts and several epidemiologic investigations have now implicated these EPHX1 coding region polymorphisms as a risk factor for lung cancer. In this study, using haplotype block analyses, we determined that the E1-b polymorphic promoter region was not in linkage disequilibrium with two previously identified non-synonomous SNPs in the coding region or with functional SNPs previously identified in the proximal promoter region of the gene.

Modulation of xenobiotic-metabolizing enzymes by chemopreventive agents is a promising strategy offering protection against toxicity mediated by certain chemical carcinogens. In this research, we discovered that in human cells, EPHX1 mRNA and protein expression were regulated tissue-specificaly by the potent chemopreventive agent, sulforaphane. Subsequent mechanistic studies revealed that Nrf2/ARE pathway plays a central role in sulforaphane's modulation of EPHX1. In the HepG2 cell line, co-expression of Nrf2 was found to activate EPHX1 promoter activity. However, in BEAS-2B cells, the presence of Nrf2 suppresses the EPHX1 transcriptional expression. A novel E1-b' transcript was further identified in this research and our results suggest that upstream open reading frames existing in the EPHX1 gene transcripts may function to regulate translational efficiency of EPHX1 expression. In summary, the investigations contributed by this research have substantially expanded our knowledge of the genetics and complex transcriptional regulation of EPHX1; and, given the significant association between EPHX1 genetic polymorphisms and lung cancer risk, highlight the chemopreventive potential of targeting EPHX1 as a defense against carcinogens.

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Chapter 1

Introduction

Xenobiotic metabolism in man is subject to large interindividual differences (Agundez, 2004;Wilke et al., 2005). A variety of parameters are known to contribute to this phenomenon, and genetic constitution is clearly of major importance (Evans and Relling, 1999). In addition to the inherited differences in chemical metabolism capacity and specificity, additional contributers to interindividual differences in risk of toxicity resulting from xenobiotic exposure include age, gender, disease states, tissue differences, and environmental influences, such as diet, smoking habits, occupational exposure to chemicals, and intake of drugs (Wilke et al., 2005;Clapper, 2000). Achieving a detailed comprehension of the interplay of such variables with man's inherent genetics is a formidable challenge for the foreseeable future. The ability to predict potential hazards resulting from human exposure to chemicals requires that we elucidate the genetic and regulatory factors that contribute importantly to the risk scenarios underlying human variability in biotransformation and xenobiotic disposition.

Xenobiotic metabolism results principally in detoxification. However, in certain instances, bioactivated and highly toxic intermediates are generated. Frequently reactive and unstable, epoxide metabolites, formed via the action of the cytochrome P450 monooxygenases, have been identified as ultimate carcinogenic and cytotoxic reaction products (Guengerich, 1982;Sayer et al., 1985;Thakker et al., 1986). Ultimately, the overall balance between bioactivation and detoxification pathways will determine the kinetics and fate of reactive intermediates within target cells. It appears likely that interindividual differences in susceptibility to toxic sequelae, including cancer incidence, may be associated with an altered genetic predisposition to detoxify

epoxides. The cell has developed the capacity to metabolize epoxides through several pathways. Prominent among these are the epoxide hydrolases.

1.1 Epoxide Hydrolases

Epoxide hydrolases are metabolic enzymes that catalyze the hydrolysis of epoxides to their corresponding diols. Several types of mammalian epoxide hydrolase have been characterized. These include: 1) a microsomal enzyme specific for cholesterol epoxide metabolism widely distributed in all tissues; 2) a membrane-associated hepoxilin A3 hydrolase participating in arachidonic acid metabolism in CNS and vascular cells; 3) a cytosolic leukotriene A4 hydrolase that mediates inflammatory and allergic responses; 4) a soluble epoxide hydrolase (EPHX2) that is active in the metabolism of arachidonic acid-derived epoxides and other endogenous epoxides; and, 5) a microsomal epoxide hydrolase (EPHX1, mEH) that is active in the metabolism of a broad range of xenobiotic compounds, such as the mutagenic polyaromatic hydrocarbon-derived epoxides (Fretland and Omiecinski, 2000). Recently, two new EHs, suggested as EPHX3 and EPHX4, were cloned from *Caenorhabditis elegans* that appear structurally closely related to EPHX2 and shown to metabolize epoxyeicosatrienoic acids, as well as epoxide metabolites of linoleic acid as well as epoxide metabolites of linoleic acids (Harris et al., 2008).

EPHX1 and EPHX2 are the two most investigated mammalian epoxide hydrolases and are members of the broader α/β hydrolase-fold family of proteins. EPHX1 and EPHX2 are not only distinguished on the subcellular level, but they also possess highly distinctive substrate specificities and physiological roles. It is likely that two independent forces drove the evolution of EPHX1 and EPHX2, cytoprotection and cellular signaling, respectively (Newman et al., 2005). In this review, we focus on the function and genetic regulation of EPHX1 and EPHX2.

1.2 Micosomal Epoxide Hydrolase (EPHX1)

1.2.1 Introduction to Micosomal Epoxide Hydrolase

As the earliest known epoxide hydrolase, EPHX1 plays a critical role in the metabolism of xenobiotic compounds. Studies in the 1970s by Brooks first characterized the microsomal enzyme, focusing on its role in the metabolism of pestcides (Brooks et al., 1970;Morisseau and Hammock, 2008). This enzyme catalyzes the *trans*- addition of water to epoxide, resulting in the formation of a dihydrodiol metabolite, a metabolite that is more water soluble and typically more amenable to phase II conjugation and excretion. In these respects, EPHX1 is generally considered to catalzye a detoxification function. On the other hand, the dihydrodiol derivative of certain substrates may undergo further oxidization by the cytochrome P450s to form diol epoxides, reaction products that are potentially highly electrophilic, biologically reactive and potentially carcinogenic (Miyata et al., 1999). For example, benzo[α]pryrene, a prevalent polyaromatic hydrocarbon present in tobacco smoke can be converted to highly mutagenic diol-epoxide intermediates, binding covalently to protein and DNA (Sayer et al., 1985;Sims et al., 1974). Genetic variation in EPHX1 activity and expression therefore may potentially lead to differences in pre-carcinogen bioactivation; thus, interindividual differences in cancer susceptibility may result from inherited EPHX1 genetic polymorphisms.

The complete cDNA of EPHX1 was previously isolated and sequenced in human and other mammalian species by our laboratory (Hassett et al., 1994b;Fretland and Omiecinski, 2000). The EPHX1 gene exists as a single copy and is localized in the long arm of human chromosome 1 at 1q42.1. The EPHX1 gene locus spans approximately 20kb. The structural features of the gene and resulting mRNA transcript have been more recently elucidated by our laboratory and include alternative non-coding exon1 (E1 and E1-b) and eight coding exons translated from exon 2 to

exon 9. The human EPHX1 protein contains 455 amino acids, resulting in the production of a ~50kDa protein. EPHX1 coding sequences are highly conserved (>75%) between human, rat and rabbit (Fretland and Omiecinski, 2000), as well as other vertebrate species (Yang et al., 2009). Human EPHX1 is expressed in all tissues thus far examined with highest levels in the liver, lower yet comparable levels in kidney and ovary, and lower levels in testis, lung, adrenal glands and lymphocytes (Liang et al., 2005;Newman et al., 2005;Yang et al., 2009). Intracellularly, EPHX1 has been primarily isolated from hepatic smooth endoplasmic reticulum membrane (Fretland and Omiecinski, 2000), but also been identified at the plasma membrane where it is reported as bile acid transporter by one laboratory (Alves et al., 1993). Large interindividual variations in EPHX1 activities have been described in human tissues. For example, EPHX1 activity was reported to vary from 8-fold up to 63-fold in panels of human liver samples (Hassett et al., 1997;Omiecinski et al., 1993;Mertes et al., 1985). Developmentally, EPHX1 expression in human fetal tissues is relatively low during early gestation, but increases as gestation progress (Omiecinski et al., 1994).

The microsomal epoxide hydrolase EPHX1 and soluble epoxide hydrolase EPHX2 enzymes belongs to the α/β hydrolase-fold family that includes bacterial haloalkane dehalogenase. The catalytic mechanism of EPHX1 involves two steps, and is similar to that described for EPHX2 (Fretland and Omiecinski, 2000). In this mechanism, first there is attack of a nucleophilic aspartic acid on the oxirane ring to yield an alkyl-enzyme intermediate, then subsequent hydrolysis of the intermediate by water. Based on the sequence alignment analysis, the catalytic triad of the EPHX1 was identified as consisting of Asp226, His431, and Glu404 (Laughlin et al., 1998;Arand et al., 1999). Subsequently, site-directed mutagenesis experiments in a rodent model confirmed these catalytic amino acids are important for EPHX1 enzymatic activity(Morisseau and Hammock, 2005).

The substrates of EPHX1 broadly range from aliphatic epoxides to polyaromatic oxides. EPHX1 has been recognized as an important contributor to the metabolism of xenobiotic

epoxides, including numerous environmental contaminates, such as polyaromatic hydrocarbons (PAHs), and clinically-used anticonvulsant drugs such as phenytoin (Hartsfield, Jr. et al., 1995;Riley et al., 1988;Van Dyke et al., 1991) and carbamazepine (Bellucci et al., 1987;Eugster et al., 1991). The common environmental toxins that generate epoxide intermidiates metabolized by EPHX1 include benzo[α]pyrene, 1,3-butadiene (Krause and Elfarra, 1997;Krause et al., 1997), benzene (Lindstrom et al., 1997), aflatoxin B_1 (Guengerich et al., 1998;Guengerich and Johnson, 1999;Walters and Combes, 1986), chrysene (Glatt et al., 1993), naphthalene, anthracene (van Bladeren et al., 1985;Hall et al., 1988) and nitropyrene (Heflich et al., 1990). In addition , a few studies reported estroxide and androstene oxide as mEH endogenous substrates (Vogelbindel et al., 1982). Styrene oxide (Carlson, 1998;Gadberry et al., 1996;Herrero et al., 1997), benzo[α]pyrene-4,5-oxide (Hassett et al., 1994a;Hassett et al., 1997;Laurenzana et al., 1998) and cis-stilbene oxide (Bellucci et al., 1994;Kitteringham et al., 1996;Moody and Hammock, 1987) are commonly used probe EPHX1 substrates. EPHX1 prefers mono- and *cis*-disubstituted epoxides, and these substrates are generally highly specific to this hydrolase. Differential substrate specificity is widely used to discirminate between microsomal EPHX1 and soluble EPHX2 activity.

In early studies, several epoxide-containing compounds were discovered as EPHX1 inhibitors. For example, 1,1,1-trichloropropene-2,3-oxide (TCPO) (Papadopoulos et al., 1985;Prestwich et al., 1985) and cyclohexene oxide (Ariyoshi et al., 1994;Magdalou and Hammock, 1988) have been widely used EPHX1 inhibitors. Some recent reports described primary ureas, amides and amines, and heavy metals such as divalent mercury and zinc as potent EPHX1 inhibitors (Draper and Hammock, 1999).

1.2.2 Biological Functions of EPHX1

Role in xenobiotic metabolism: In the last four decades, EPHX1 has been established as an enzyme involved in both detoxification of xenobiotic compounds and bioactivation of carcinogens. In addition to its role in xenobiotic metabolism, several additional studies suggested endogenous roles of EPHX1, including an involvement in steroid metabolism, bile acid transport and as a component member of the vitamin K reductase complex (Guenthner et al., 1998). EPHX1 exhibits broad substrate specificity, and most epoxides intermediates are generated *in situ* by phase I oxidation reactions (Gasser, 1996). As discussed previously, EPHX1 enzymatic activity can serve to detoxify compounds, but in some cases, it is responsible for the generation of highly reactive metabolites. A case study published in 1988 suggested the protective role of EPHX1 in anticonvulsant hypersensitive syndrome, indicating that patients susceptible to anticonvulsant toxicity had decreased epoxide metabolic activity (Shear and Spielberg, 1988). In a similar respect, bioactivation of aflatoxin B1 by cytochrome P450 enzymes generates a highly reactive epoxide metabolite, and incubation with the EPHX1 enzyme is reported to reduce the aflatoxin carcinogenic effect (Kelly et al., 1997;Kelly et al., 2002). However, in the case of PAHs, which are incomplete combustion products found in automobile exhaust and cigarette smoke, the bioactivation of PAH carcinogen is largely dependent on EPHX1 activity. In fact, EPHX1 null mice are completely resistant to the tumorigenic effects of dimethylbenz[a]anthracene (DMBA) in a complete carcinogenesis assay (Miyata et al., 1999). Furthermore, many epidemiologic investigations have now implicated human EPHX1 gene coding region polymorphism as a risk factor for lung cancer (Kiyohara et al., 2006).

EPHX1 contributes to the formation of highly reactive and mutagenic bay region (planar PAHs) and fjord region (non-planar PAHs) diol-epoxide intermediates. Enzymatic activation to sterically hindered bay and fjord region dihydrodiol epoxides (DEs) (Conney, 1982;Conney et al.,

1994;Cheng et al., 1989;Baird and Ralston, 1997;Dipple et al., 1984;Geacintov et al., 1997;Harvey and Geacintov, 1988;Thakker et al., 1985) is a three step process (see Figure 1-1): initial epoxidation by the cytochrome P-450 monooxygenases, subsequent EPHX1-mediated hydrolysis to the *trans* dihydrodiol, followed by a second epoxidation at the adjacent double bond. The diol epoxides thus generated react readily with mutational hot spots in DNA to form stable adducts both *in vitro* and *in vivo* (Hruszkewycz et al., 1992;Vousden et al., 1986).

Physiologic Roles of EPHX1: While EPHX1 has been recognized for its role in xenobiotic metabolism, more recent studies suggest that EPHX1 also plays a role in physiologic homeostatis. Several lines of evidence suggest EPHX1 plays a role in steroid synthesis and/or metabolism (Newman et al., 2005). For example, estroxide and androstene oxide are good EPHX1 substrates (Vogel et al., 1982;Fandrich et al., 1995), EPHX1 has been identified as a subunit of the anti-estrogen binding site (Mesange et al., 1998), and EPHX1 is well expressed in ovaries(Vogelbindel et al., 1982;Lee et al., 2002a), especially in follicle cells (Cannady et al., 2002). It is hypothesized that EPHX1 may be important for cellular protection against reactive metabolites of endogenous compounds, such as epoxy steroids. Human EPHX1 was reported to be expressed in fetus tissues as early as gestational day 53 (Omiecinski et al., 1994), suggesting it may be important for protection from toxic epoxide intermediates during embryonic and fetal development. Further, Cheong et al. have shown that oviductal EPHX1expression is up-regulated during the process of mouse embryogenesis, and that increased oviductal EPHX1 may be help to reduce reactive oxygen species (ROS), thereby potentially enhancing mouse embryo development (Cheong AW et al., 2009).

A role for EPHX1 in bile acid homeostasis is also emerging: EPHX1 appears to be part of a multi-protein transport system that is responsible for sodium-dependent bile acid uptake in liver (Ananthanarayanan et al., 1988;von Dippe et al., 1993;von Dippe et al., 1996;von Dippe et al., 2003). In this respect, several reports have indicated that EPHX1 is expressed at the hepatocyte

plasma membrane and in the endoplasmic reticulum where it can exist in two topological orientations. The bile acid uptake appears dependent on EPHX1 expression at the plasma membrane (von Dippe et al., 2003). Another study suggested that EPHX1 is a functional component of the vitamin K1 oxide reductase complex in rat liver microsomes (Guenthner et al., 1998). Possibly, potent EPHX1 inhibitors can help to address the mechanism by which EPHX1 participates in bile acid absorption as well as vitamin K reductase. However, the specific role and relative importance of EPHX1 in these physiologic functions remains to be more clearly elucidated.

1.2.3 Polymorphisms

After the full-length human EPHX1 DNA sequence was cloned and compared, two non-synonymous amino acid changes were identified in the EPHX1 coding region. The polymorphism in exon3 corresponds to amino acid position 113 and results in Tyr (Y) to His (H) substitution (rs1051740). An exon 4 polymorphism at position 139 (rs2234922), codes for a His (H) to Arg (R) substitution. For the polymorphism at 113, 7.6% individuals were observed with homozygous His, and the frequency of 139 Arg/Arg is 4.6%. The distribution of the two alleles follows Hardy-Weinberg equilibrium (Hassett et al., 1994a;Fretland and Omiecinski, 2000). There are other non-synonymous polymorphisms reported or listed in NCBI dbSNP database, but either they have not been validated or else have only been identified at very low frequency. Consequently, the 113Y/H and 139H/R polymorphisms are recognized currently as the most common human EPHX1 amino acid variants in the human population.

When each of the EPHX1 polymorphic variants was expressed in an *in vitro* system, the mRNA expression levels achieved was largely similar. Using benzo[α]pyrene-4,5-oxide as substrate, *in vitro* experiments indicated that the substitution of His for Tyr at 113 was associated

with approximately 40% decrease in enzyme activity, while His139Arg substitution increased EPHX1 activity ~25% (Hassett et al., 1994a;Hassett et al., 1997;Laurenzana et al., 1998). Based on these results, many epidemiology studies have since classified EPHX1 activity as low, intermediate and high according to the variant amino acids at position 113 and 139 (Gresner et al., 2007) . However, it is important to indicate that the previous studies actually concluded that there were only minimal differences in specific activities of the variants enzymes, i.e., activities measured following normalization to the corresponding EPHX1 immunoreactive protein levels (Hassett et al., 1994a).

After the initial discovery of the four allelic variants, several similar investigations have evaluated the respective functional activities in different conditions, but were still unable to provide strong evidence to support any correlative relationship between the polymorphic amino acids and enzyme activity (Laurenzana et al., 1998;Hassett et al., 1997). For example, a study published in 2004 evaluated the enzymatic profile with the EPHX1 substrate *cis*-stilbene oxide (cSO) and benzo[a]pyrene-4,5-oxide (BaPo) using purified baculovirus-expressed EPHX1 variants or human liver microsomes (Hosagrahara et al., 2004). The Y113/H139 polymorphism in the purified protein increased the hydrolysis activity about 2-fold relative to wild type; however, these differences were not apparent using human liver microsomes representing the different EPHX1 variants. So, even though many epidemiology studies use descriptors such as “low activity allele,” or “high activity allele,” in an attempt to explain the functional basis of their results, the underlying basis for the EPHX1 activity claim is not yet well-substantiated.

Some studies of the EPHX1 variants suggested that these coding region polymorphisms may affect protein stability. For example, in a panel of 40 human livers, EPHX1 enzyme activity demonstrated strong correlation with the protein level. However, neither the EPHX1 protein nor activity was associated with the EPHX1 mRNA levels, which suggested some post-transcriptional mechanism regulated mEH protein expression. Further, in both fetal and adult samples, the

EPHX1 activity in the liver or lung is strongly correlated with EPHX1 protein contents, whereas no correlation between activity and corresponding mRNA exists. Following on this finding, the translational efficiency, mRNA half-life, and protein half-life of mEH allelic variants were determined by in vitro transcription and translation using constructs encoding four EPHX1 alleles. The coding region polymorphisms do not appear to affect translational efficiency or mRNA decay rate. Although the calculated EPHX1 variant protein half-lives suggested that polymorphic amino acid substitution may result in altered protein stability (Laurenzana et al., 1998), these differences in protein half-lives were minimal and likely to have little impact on overall protein levels.

Since the initial identification of these two EPHX1 coding polymorphisms, a large number of epidemiologic investigations have analyzed the potential association of disease incidence with EPHX1 genetic polymorphisms. Two meta-analysis studies of selected molecular epidemiological investigations examining associations of EPHX1 genotype with lung cancer susceptibility have been published. Interestingly, EPHX1 coding region polymorphisms were consistently reported as a risk factor associated with lung cancer in a number of studies (Lee et al., 2002b;Kiyohara et al., 2006). For example, Kiyohara et al. reported a protective effect of lung cancer in white population associated with the “low-activity” exon 3 113His/113His genotype, after applying an appropriate correction to eliminate the heterogeneity of groups. Moreover, the interaction of EPHX1 with other metabolizing enzymes, such as the glutathione S-transferases and N-acetyltransferases, may additionally contribute to modulate the association with lung cancer (Gresner et al., 2007). Given the apparent epidemiological association of genetically-encoded differences in EPHX1 protein with the incidence of certain cancers, the functional impact of EPHX1 coding region polymorphisms needs rigorous characterization.

As introduced above, EPHX1 is largely responsible for the formation of PAH reactive metabolites. The mutagenic and carcinogenic potency of the PAHs appears largely dependent on

their structural features (see Figure 1-2). In particular, fjord region diol epoxides exhibit substantially more potent carcinogenic activities than those derived from bay regions, and fjord region-modified DNA adducts are more difficult to repair than the bay region adducts (Buterin et al., 2000;Dreij et al., 2005;Lloyd and Hanawalt, 2000;Lloyd and Hanawalt, 2002). The role of the EPHX1 polymorphic variants in the formation of these carcinogenic PAH metabolites have not been assessed. It is interesting to hypothesize that the EPHX1 protein variants have differential catalytic activities toward these substrates, and that this could in turn, render some individuals more susceptible to PAH-induced cancers.

1.2.4 Regulation of EPHX1

It is well established that RNA diversity in mammals is expanded markedly through the use of alternative promoters and differential RNA splicing mechanisms, and it has been shown that these mechanisms play a role in modulating the EPHX1 gene. For example, Gaedigk reported complex splicing processes at the exon 1/2 boundary generated eight putative alternative exon 1 variant sequences in addition to the well known exon 1, E1, previously defined from liver (Skoda et al., 1988;Gaedigk et al., 1997). Liang et al. later determined that many of the previously characterized alternative exon 1 sequences were mis-identified, and rather, shared identity with sequences derived from the signal recognition particle 9-kDa structural gene (SRP9, NM_003133), a gene that exists upstream of the EPHX1 coding region (Liang et al., 2005). In the more comprehensive study of Liang et al, a stringent 5'-RACE technique was used to identify two bone fide unique first exons from human liver-drived mRNA samples, termed E1 and exon 1b (E1-b). The E1 transcript initiated from the initially characterized promoter, positioned immediately proximal to the exon 2 of EPHX1 coding region, while the E1-b variant exon 1 was localized to a genomic region ~18.5 kb upstream of exon 2. Northern hybridizations

demonstrated that the E1-b variant was expressed ubiquitously in human fetal and adult tissues. In contrast, the E1 transcript was found almost exclusively in liver. A recent study using a highly sensitive and quantitive real-time PCR method, suggested the E1-b promoter functions as the primary driver of EPHX1 expression in human tissues including liver (Yang et al., 2009). These results confirmed that the tissue specific expression of the human EPHX1 gene is driven by the use of alternative promoters.

To examine the basis for liver-specific usage of the E1 promoter, a recent study identified several potential *cis*-regulatory elements that include GATA (-110/-105) and HNF3 (-96/-88) motifs (Liang et al., 2005). Mutation of the GATA site resulted in the largest (70%) decrease of basal transcription activity. GATA-4 was the principal GATA family member interacting with its respective motif, whereas both HNF3α and HNF3β were capable of interacting with the HNF3 element. Site-directed mutagenesis and transactivation analyses of the E1 promoter revealed that GATA-4 is likely a principle factor that regulates liver-specific expression of the E1 variant, with HNF3α and HNF3β acting to negatively regulate GATA-4 function in hepatic cells. GATA4 has been described to regulate tissue-specific expression with assistance from other tissue-restricted transcription factors (Zhu et al., 2004b). The cotransfection analysis with HNF3α or HNF3β inhibited GATA4 activation of the E1 promoter. The CCAAT/Enhancer-binding proteinα (C/EBPα) and nuclear factor Y (NF-Y) have also been shown to form a complex and bind directly to CCAAT box in E1 promoter, activating EPHX1 E1 transcription (Zhu et al., 2004a). Currently, little is known about the factors that bind to E1-b promoter that regulate the basal and inducible expression in most tissues.

In addition to the coding region polymorphisms, non-coding polymorphisms also were identified in the 5'-region of the EPHX1 gene (Raaka et al., 1998). The seven polymorphic sites in the E1 upstream region, exist in two linkages, the –200 linkage (–200C/T, –259C/T, –290T/G) and the –600 linkage (–362A/G, –613T/C, –699T/C). *In vitro* studies suggest these sites play a

role in regulating EPHX1 transcriptional activity. Interestingly, an epidemiological investigation concluded that the -600 linkage was significantly associated with toxic effects resulting from occupational exposures to 1,3-butadiene (BD), suggesting that the sensitivity to the carcinogenic effects of BD is inversely correlated with predicted EPHX1 activity (Abdel-Rahman et al., 2001;Abdel-Rahman et al., 2005). Therefore, when considering the association of genetic polymorphisms with the risk of human disease, structural region EPHX1polymorphisms alone likely do not account for the complete spectrum of variation influencing EPHX1 activity; polymorphisms occurring within the 5'-regulatory regions of the gene may be additional, important genetic determinants to consider.

In rodents, EPHX1 expression can be highly inducible by a variety of compounds, including phenobarbital, methylcholanthrene, polychlorinated biphenyls, *trans*-stilbene oxide (Schilter et al., 2000), peroxisome proliferators (Newman et al., 2005), radiation (Nam et al., 1998), heavy metals (Fretland and Omiecinski, 2000), and certain steroids (Fandrich et al., 1995). While EPHX1 induction has been well studied in rodent species, human EPHX1 expression levels appear to be only modestly affected by common prototypic chemical inducers in primary human hepatocytes. Sequence comparison of EPHX1 5'-flanking regions among several vertebrate species reveals few similarities outside primate, suggesting that the regulation of EPHX1 expression may involve distinct promoter regulatory mechanisms. In this regard, the EPHX1 induction results in rodents cannot be easily extrapolated to human.

1.3 Soluble Epoxide Hydrolase (EPHX2)

1.3.1 Introduction to Soluble Epoxide Hydrolase

As indicted by the name, microsomal epoxide hydrolase (EPHX1) and soluble epoxide hydrolase (EPHX2) were first distinguished by their subcellular localizations, and these two enzymes were found to have distinct and complementary substrates. EPHX2 was discovered a few years after EPHX1, and it was first thought to participate in xenobiotic metabolism. However, experimental evidence has now established that the major role of EPHX2 is metabolism of endogenous epoxy fatty acids (Newman et al., 2005), with the epoxyeicosatrienoic acids (EETs) as the best studied EPHX2 substrates. Numerous investigations have demonstrated the importance of EPHX2 in the regulation of high blood pressure (Sinal et al., 2000;Yu et al., 2000) and inflamination (Slim et al., 2001;Node et al., 1999). Recently, with the development of EPHX2 inhibitors, the generation of EPHX2 null mice and the analysis of EPHX2 polymorphisms, the biological role of mammalian EPHX2 is better understood.

Human EPHX2 is a single gene that consists of 19 exons, encoding a 62.5kDa protein (Sandberg and Meijer, 1996). The human soluble EH exists largely as homodimers of the monomeric subunit. Each monomer is two functional domains joined by a proline rich linker. The C-terminal domain contains the α/β-hydrolase structure homologous to haloalkane dehalogenase and is responsible for the epoxide hydrolase activity(Argiriadi et al., 1999;Newman et al., 2005). The N-terminal domain is similar to haloacid dehalogenase, and functions as a lipid phosphatase (Cronin et al., 2003;Newman et al., 2003;Newman et al., 2005). EPHX2 is widely distributed in numerous tissues, with highest activity in the liver (Schladt et al., 1986), followed by the kidney (Yu et al., 2004), where its distribution is concentrated within renal cortex. The primary isolation

of EPHX2 is from the cytosolic or soluble fraction, but in some cases EPHX2 activity is localized in the peroxisomes (Yu et al., 2000). In rodents, drugs such as the peroxisome proliferator-activated receptor alpha (PPARα) agonists are strong inducers of EPHX2 (Hammock and Ota, 1983;Pinot et al., 1995), although functional PPARα response elements have not been identified in the upstream of human EPHX2 gene.

1.3.2 Biological Functions of EPHX2

EPHX2 prefers *trans*- over *cis*- substituted epoxides of sterically hindered substrates, and *trans*-stilbene oxide is often used to distinguish EPHX2 activity from EPHX1 activity. In recent years, it has become clear that fatty acid epoxides are the major endogenous substrates for EPHX2, with the cytochrome P450 derived epoxides of arachidonate acids (epoxyeicosatrienoic acids, EETs) being the most well studied. Numerous investigations have demonstrated that EETs are chemical mediators producing important biological effects, such as antihypertensive and anti-inflammatory actions in the cardiovascular and renal systems. Over the past several years, selective pharmacological EPHX2 inhibitors have been designed to treat a variety of diseases.

It is well known that EETs have significant roles in the regulation of hypertension, inflammation, angiogenesis, and have mitogenic effects in the kidney (Spector and Norris, 2007). Generally, the hydrolysis catalyzed by EPHX2 eliminates the biological activity of the lipids. It is belived that EPHX2 is involved in blood pressure regulation because 14,15-EET is a potent vasodilator. Further, EPHX2 null mice have decreased blood pressure (Sinal et al., 2000), and selective EPHX2 inhibitor effectively decrease blood pressure in a hypertensive rat model. Therefore, inhibition of EPHX2 is considered as a new therapeutic approach for hypertension. In addition to vasodilation, EETs also display anti-inflammatory role in vascular endothelial cells by inhibiting cytokine-induced NF-κB transcription. It has been suggested that EPHX2 may play a

key role in the regulation of inflammation by metabolizing anti-inflammatory EETs and bioactivating toxic and pro-inflammatory leukotoxin. In cell culture, inhibiting EPHX2 results in EETs accumulation and enhances their anti-inflammation effect (Morisseau and Hammock, 2005).

1.3.3 Polymorphisms

A common polymorphism, which results in the substitution from arginine to glutamine at codon 287, has been identified in exon 8 of human EPHX2 (Sandberg et al., 2000;Fornage et al., 2004). *In vitro* assay and analysis of the crystal structure suggested the Arg287Gln mutation decreased EPHX2 enzyme activity as well as protein stability (Przybyla-Zawislak et al., 2003). In addition, the Arg287Gln polymorphism is strongly associated with a genetic cardiovascular disease and familial hypercholesterolemia (Sato et al., 2004). Another study report an association between the Arg287Gln polymorphism of the EPHX2 and coronary artery calcification in young African-American after adjusting for other risk factors (Fornage et al., 2004).

1.3.4 Regulation of EPHX2

Although the biological function of human EPHX2 has been extensively investigated, little is known about the molecular mechanisms of EPHX2 transcriptional regulation. Several observations in rodents have shown that EPHX2 can be regulated by endogenous chemical mediators and by some xenobiotics. In rodents, EPHX2 expression is induced by the administration of peroxisome proliferator activated receptor alpha (PPARα) agonists. Peroxisome proliferators are compounds that induce the size and number of hepatic peroxisomes. PPARα agonist include clinically used hypolipidemic drugs, endogenous compounds such as steroids, dietary fatty acids and commercial plastisizers (Peraza et al., 2006). The response to these

agonists suggests EPHX2 plays a possible role in peroxisome proliferators-induced liver toxicity in rodents. Additionally, male mice show higher EPHX2 activities than female mice in liver and kidney (Newman et al., 2005), which suggests that sex hormones may be involved in EPHX2 regulation. Furthermore, cigarette smoke and gamma radiation seem to have impact on human EPHX2 expressions (Petruzzelli et al., 1992;Park et al., 2002).

A recent report found that angiotensin-II (Ang II), a potent vessel constrictor that elevates blood pressure in animal models, appears to directly upregulate EPHX2 transcription expression mediated by the AP-1 motif in the human EPHX2 promoter (Ai et al., 2007). These studies further demonstrated that the transcription factor mediating EPHX2 induction by Ang II is a c-Jun/c-Fos binding to the AP-1 site at -446. In addition, overexpression of the mutant c-Jun lacking the transactivation domain significantly attenuated the EPHX2 induction after treatment of angiotensin-II. Interestingly, Monti et al. have recently identified EPHX2 as a heart failure susceptibility gene. Their studies show that the metabolism of cardioprotective EETs may be affected by EPHX2 allelic variants which result in altered transcript and protin levels (Monti et al., 2008). The core promoter of EPHX2 was recently characterized by Tanaka et al. (Tanaka et al., 2008). No PPARα responsive element was identified in this region; however, Sp1 regulatory elements located in the GC-rich promoter region are necessary for the EPHX2 transcriptional regulation.

Alternative splicing appears to play a role in EPHX2 expression. For example, in mouse ovary, a unique variant of EPHX2 is generated by alternative splicing and this variant lacks phosphatase activity (Hennebold et al., 2005). This short isoform of EPHX2 has an altered N-terminal sequence spliced from the second intron. However, to our knowledge, no such spliced EPHX2 variant has been found in human.

1.4 Transposable Elements

Mammalian genomes are very complex and dynamic, merely a small fraction is occupied by protein coding exons, while up to 50% is contributed by repetitive elements and the remaining 48% is called unique DNA (Makalowski, 2000). For example, nearly half of the human genomic sequences are derived from ancient transposable elements, which encompass both transposons and retrotransposons, including short and long interspersed elements (SINE & LINE), long terminal repeats (LTR), and DNA transposons (Lorenc and Makalowski, 2003). Transposable elements (TEs) are DNA sequences that are capable of integrating from one site in the genome to a new one via a "cut and paste" mechanism (Kazazian, 2004). First described as "junk" DNA, the importance and function of TEs have been increasingly appreciated recently. These transposable or mobile elements participate in genome formation and benefit the evolution of genes by affecting their functions. TEs have the ability to promote genetic diversification and regulatory variation by serving as recombination hot spots, altering protein coding content or by regulating gene transcription (Kamat et al., 2002;van de Lagemaat et al., 2003). TEs have been shown to serve as alternative promoters for many genes, including aromatase P450 (CYP19; (Carlton et al., 2003)), carbonic anhydrase 1 (CA1; (Mighell et al., 1997)), and bile acid CoA: amino acid N-transferase (BAAT; (Grover et al., 2004)), resulting in tissue-specific regulation of gene expression.

Alu elements are one important type of TEs that insert into the human genome and affect regulation of gene expression. Alu elements are the major components of SINEs; their 1.1 million copies occupy over 10% of the human genome and contribute to a significant portion of human genetic diseases. The typical Alu element is ~300 nucleotides in length and derived from a process in which the element is transcribed by RNA polymerase III and then reverse-transcribed

and inserted into the genome (Korenberg and Rykowski, 1988). The Alu repeats, which are unevenly spread throughout the entire genome, are positively correlated with gene density (Moyzis et al., 1989) and many studies have suggested that transcriptionally active regions of the genome are enriched with Alu elements (Deininger et al., 1992;Kapitonov and Jurka, 1996).

It is clear that Alu elements integrated early in primate evolution. The Alu elements are divided into several subfamilies according to their insertion age (Deininger and Batzer, 1999;Korenberg and Rykowski, 1988). In human genome, the majority of Alu elements belong to the old Alu subfamilies. Although present at relatively low copy numbers, the younger Alu elements are considered the most active with respect to biological function (Carroll et al., 2001). Almost all of the Alu insertions occur specifically in the human genome and belong to four closely related subfamilies, Alu Y, Ya5, Ya8, and Yb8 (Salem et al., 2003). These young or recent Alu insertions also produce genetic variation in human populations by generating polymorphic loci (Jasinska and Krzyzosiak, 2004;Miki et al., 1996). It has been suggested that Alu elements can integrate into an mRNA open reading frame by direct insertion or indirectly recruiting an intronic TE (Lorenc and Makalowski, 2003). Insertion/deletion of these Alu elements has been associated with many human diseases (Salem et al., 2003). The vast majority of the disease-associated Alu insertions occur at coding exon regions, resulting in disruption or alteration of protein structure, as noted for the BRCA2 gene for breast cancer, Factor IX for hemophilia, and CaR for hypocalciuric hypercalcemia (Vidaud et al., 1993;Rowe et al., 1995;Wallace et al., 1991). Another type of Alu insertion occurs within intronic regions and may result in altered gene splicing; such as in NF1 for neurofibromatosis, the progesterone receptor gene relating to ovarian carcinoma, and the glycerol kinase gene resulting in glycerol kinase deficiency (Bailey et al., 2003;Chen et al., 1989;Zhang et al., 2000).

It should be noted that many mutation detection strategies are designed to identify mutations selectively in coding regions of genes and thus may overlook Alu insertions occurring

within gene regulatory regions. It is likely that the contribution of transcriptional regulation by Alu insertions to the actual spectrum of human disease is underestimated. For example, some recent investigations found that human Alu RNA represses mRNA transcription by binding RNA polymerase II (Pol II) and preventing its proper interaction with promoter regions during closed complex formation (Yakovchuk et al., 2009). Currently, Alu-mediated recombination events have been linked to ~50 human diseases, including hypercholesterolemia, BRCA1-related breast cancer, and acute myelogenous leukemia (Deininger and Batzer, 1999). In addition to insertion-associated human diseases, Alu elements also promote genetic recombinations that may result in large-scale deletions, duplications, and translocations (Iafrate et al., 2004;McNeil, 2004).

Results presented in this thesis research demonstrate that the E1-b promoter region of human EPHX1 is replete with repetitive elements, including those of the Alu class, and that humans are genetically polymorphic for the inclusion of a specific double Alu repeat cluster.

1.5 Chemoprevention and Phase II Gene Induction.

1.5.1 Isothiocyanates

Chemoprevention has been well received as a great weapon in the anticancer arsenal. It can be defined as prevention of cancer by the use of chemical compounds to reverse, suppress, or prevent the development of cancer (Morse and Stoner, 1993). Some identified chemopreventive agents include synthetic drugs and biological constituents of the diet. Fruits and vegetables are rich sources of effective chemopreventive agents and numerous epidemiologic and animal studies suggest that increased ingestion of fruits and vegetables has the potential to prevent human cancers. Isothiocyanates (ITCs) are naturally occurring cancer chemopreventive compounds that are found in cruciferous vegetables including broccoli, cabbage, cauliflower, radish, brussels sprouts, and kale. High intake of ITCs can enhance excretion of reactive carcinogenic metabolites to decrease DNA damage. Preclinical and clinical studies have suggested an inverse relationship between consumption of cruciferous vegetables and tumorigenesis at a variety of sites, including liver (Kensler et al., 2003), lung, breast, pancreas, colon, intestine, and prostate (Juge et al., 2007)

Sulforaphane (SFN, figure 1-3), is the main isothiothiocyanate found in broccoli (Kensler et al., 2003;Juge et al., 2007) and it appears to have chemopreventive activity in different stages of cancer development. At the carcinogenesis initiation level, SFN exhibits dual mechanisms by blocking phase I gene expression resulting in decreased formation of reactive metabolites, and inducing Phase II gene expression to increase detoxification of carcinogens. Furthermore, SFN induce cell cycle arrest, apoptosis, angiogenesis inhibition in cancer cells (Clarke et al., 2008). In addition, SFN has anti-inflammatory effects. For example, SFN significantly decreased the DNA binding capability of nuclear factor κB (NF-κB), which is a transcription factor regulates the

expression of proinflammatory proteins including iNOS, Cox-2, and TNF-α. SFN probably inactivates NF-κB by direct, reversible and thiol-dependent modification of its subunit or interact to relevant co-factors (Heiss et al., 2001). Constitutive activation of NFκB is common in various human cancers, thus inhibition of NFκB activation by SFN is considered as another evidence for its chemoprevention property.

Selenium also plays a major role in the field of chemoprevention. Novel synthetic isoselenocyanates (ISCs), selenium analogs of naturally occurring ITCs (Figure 1-4), have been identified as potent anti-tumor agents (Sharma et al., 2008). ISCs appear as potentially more effective as compared to their corresponding ITCs with respect to inhibiting cell proliferation and inducing cell apoptosis in different cancer cell lines, as well as in the reduction of tumor size in mice (Sharma et al., 2008;Sharma et al., 2009). The results presented in this thesis research demonstrate that SFN and perhaps ISC modulators regulate the expression of EPHX1 expression in primary human hepatocytes and several cultured cell lines.

1.5.2 Nrf2/ARE pathway

As indicated above, SFN exerts some of its chemopreventive effects by inducing phase II metabolism. Transcription induction of these genes by SFN occurs via the antioxidant response element (ARE), a *cis*-acting binding sequence, located in the 5'-flanking regions. Nrf2 (nuclear factor erythroid 2-related factor 2) is the transcription factor known to bind the ARE motif. Normally, Kelch ECH associating protein 1 (Keap1) retains Nrf2 in the cytoplasm and upon exposure to phase II gene inducers the interaction with Keap1 is disrupted, allowing NRF2 to translocate to the nucleus and bind to AREs (see figure 1-5). Keap1 acts as the specific negative regulator of Nrf2, and several models have been suggested to explain how Keap1 regulates Nrf2. For example, Keap1 may retain Nrf2 in the cytoplasm through interaction with cytoskeletal

network, and Keap1-Nrf2 complex has been proposed to facilitate the degradation of Nrf2 (Kensler et al., 2007). The Keap1-Nrf2 complex appears to induce the degradation of Nrf2 via ubiquitin proteasome pathway. The multiple cysteines in Keap1 appear to be excellent sensors for inducers and modification of Keap1 cysteine content is important for Nrf2 nuclear accumulation. Thus it has been suggested that the electrophilic SFN can modify Keap1, resulting in release of Nrf2 and its translocation of Nrf2 to the nucleus, inducing the expression of phase II detoxification enzymes. Studies suggest that the Keap1-Nrf2-ARE signaling pathway is the major mechanism by which Nrf2 protects cells from endogenous and exogenous stresses. For example, Nrf2-disrupted mice have been shown to be more sensitive to a variety of carcinogens, including aflatoxin (Yates et al., 2006;Ramos-Gomez et al., 2003), and these mice are not protected by chemopreventive agents, such as oltipraze and sulforaphane (Ramos-Gomez et al., 2001;Fahey et al., 2002). In contrast, mice with hepatocyte-specific disruption of of Keap1 demonstrate striking resistance to high doses of hepatocarcinogens.

Increasing evidence from several investigations implies that cross-talk exists between Nrf2 and other transcription factors (Kensler et al., 2007). Nrf1 and Nrf2 both belong to cap-n-collar (CNC) basic-leucine-zipper (bZIP) family and activate gene transcription through binding to the ARE as a heterodimers with small-Maf protein. It was shown that the N-terminally truncated form (p65) of Nrf1 blocks the Nrf2-mediated activation. Overexpression of this Nrf1 isoform results in suppression of the endogenous ARE-mediated gene transcription. Immunoprecipitation studies have demonstrated that this Nrf1 isoform is a competitor of Nrf2 for heterodimer with small Maf and binding to ARE.

Small Maf proteins are a subgroup of bZIP transcription factors that emerged as crucial regulators of gene expression. Small Mafs do not contain any apparent transcriptional activation domain, and they function as heterodimers by interacting with CNC bZIP factors, like Nrf1, Nrf2, Nrf3, Bach1, Bach2 and Fos (Blank, 2008;Motohashi et al., 2000). The resulting CNC bZIP/small

Maf complex can recognize Maf recognition element (MARE), as well as ARE. As essential dimerization partners, tipping the balance of the levels or activity of small Maf leads to major changes in gene expression. In various cellular models, the minimal changes in small Mafs concentration is sufficient to reverse from activation of ARE-mediated gene transcription activity to repressor activity (Li et al., 2008;Nguyen et al., 2000). Recent studies suggested that one of the small Maf proteins is subject to post-translational modification in order to form an active repressor complex (Motohashi et al., 2006).

1.5.3 Roles of Keap1-Nrf2 pathway in regulation of EPHX1 expression

Microarray analysis of gene expression profiles using Nrf2-deficient mice identified several clusters of genes dependent on Nrf2. The microarray studies in rodent models suggested that EPHX1 is likely regulated by SFN via Keap1-Nrf2 pathway. For example, an investigation comparing small intestine of Nrf2-deficient mice and wild type with or without SFN treatment showed that EPHX1 expression was elevated in the wild type mice compared to mutant; and selectively induced in response to SFN in wild type only (Thimmulappa et al., 2002). Microarray analysis using wild-type and Nrf2 knockout mice confirmed that EPHX1 was induced in the liver following 6 hour treatment with another potent chemopreventive agent, and resulted in decreased aflatoxin-DNA adducts in the liver (Yates et al., 2006). These studies clearly suggested that in rodents, EPHX1 expression can be regulated by chemopreventive agents, such as SFN, and the Keap1-Nrf2 pathway may therefore play a central role in EPHX1 transcriptional regulation.

1.6 Upstream Open Reading Frames as Regulators of Gene Expression.

Transcriptional control is the major checkpoint in regulate gene expression; however, many events are required before the protein is synthesized from the RNA. These post-transcriptional regulations consist of mRNA processing, mRNA localization, mRNA stabilization and translational regulation. In cancer cells, an efficient and rapid way to alter gene expression is via modification of translational efficiency (Audic and Hartley, 2004). For example, MDM2 is an oncoprotein that is overexpressed in breast cancer cells. In normal cells MDM2 is poorly translated, as a result of two upstream AUG codons (see figure 1-6). Increased expression of MDM2 occurs through alternative splicing in the MDM2 RNA, which results in a short form mRNA that eliminates two uAUGs (Okumura et al., 2002;Audic and Hartley, 2004). So the Mdm2 oncogene overexpressed in human tumor cells correlated to enhanced translation of its mRNA in the absence of gene amplification

Protein synthesis is mainly regulated at the initiation phase, which is very complex and involves more than 25 regulatory factors (Kozak, 2005;Preiss and Hentze, 2003). The first step of translation initiation is assisted by some initiation factors (eIFs). The small (40S) ribosomal subunit migrates through the 5' untranslated region (UTR) until it encounters the first AUG. Following AUG recognition, a 60S ribosomal subunit enters to begin the elongation phase. In most cases, 5'-UTRs that enable efficient translation are short, low in G+C content, relatively unstructured and do not contain uAUG codons (Meijer and Thomas, 2002). The mechanism of ribosome scanning indicates the dominant role of position, such that most uAUGs are translated as they exist as the closest AUG to the 5' end of the mRNA transcript (Kozak, 2005). Therefore, the presence of AUG codons within the leader sequences will affect the translation efficiency of the main coding region. Some ribosome becomes stalled by the synthesized peptide from uAUG,

blocking the additional scanning to the further downstream. Frequently, ribosomes will reinitiate downstream, and the efficiency depends on many *trans*-acting factors and the mRNA structure. Inefficient translation is not always caused by the presence of uAUG in the 5'-UTR. In the case of human PDGF2 mRNA, which has extremely GC-rich leader sequences, adding or removing an uAUG has little effect (Kozak, 2005;Kozak, 2006).

In addition to control of ribosomal assembly and scanning, another regulatory function of uAUG is the selection of the start codon, which results in the synthesis of different proteins. For example, the CCAAT/enhancer binding proteins (C/EBP) are a family of transcription factors that are crucial for differentiation and cell proliferation. The various C/EBP binding proteins are derived from two unique C/EBPα and C/EBPβ mRNA by using alternative translation initiation sites. Thus, the genereated isoforms have different N-terminal domains, which allow for differential gene regulation, and identical C-terminal DNA-binding domain (Calkhoven et al., 2000). uAUG can also trigger the mRNA nonsense-mediated decay (NMD) pathway (Morris and Geballe, 2000) leading to mRNA degradation. mRNA stability is controlled through a complex network of mRNA-protein (mRNP) interactions.

In this thesis research, we discovered that human EPHX1 translation is likely regulated by use of upstream open reading frames. These pathways represent complex and not well understood modes of post-transcriptional regulation, but further studies may help better elucidate the impact of these regulatory events on EPXH1 functional expression.

1.7 Hypothesis of the Current Study

As an important xenobiotic metabolizing enzyme, human EPHX1 has been well characterized for its biological and biochemical properties, however, studies of EPHX1's complex genetics and transcriptional regulation are relatively lacking. Human EPHX1 expression has been detected in most tissues, with highest activities in liver. A recent report from our laboratory demonstrated that the expression of the EPHX1 gene in extrahepatic tissues is driven, likely exclusively, by the use of a novel alternative promoter (Liang et al., 2005). The alternative promoter region, the E1-b promoter, is localized ~18.5 kb 5'-upstream from the coding region of EPHX1. The previously identified promoter, E1, lies directly upstream of the EPHX1 coding region (Hassett et al., 1994b;Skoda et al., 1988), and is responsible for driving EPHX1 expression specifically in the liver. Our sequence analysis of the E1-b region has identified the insertions of transposable Alu elements. We hypothesize that Alu polymorphisms are differentially present among individuals and their presence or absence will differentially impact rates of EPHX1 basal transcription. The specific hypothesis being tested is that a far upstream gene promoter region drives EPHX1 expression preferentially/ exclusively in multiple human tissues and that the associated transcriptional activity of this promoter region is interindividually regulated by the presence of transposable genetic elements.

The outcome of human exposure to potential toxic agent is largely determined by the balance between the bioactivation of reactive intermediates and the the detoxification of these reactive species. Chemoprevention by isothiocyanates is an area of great interest because of the ability of these agents to inhibite the metabolic activation of carcinogens by phase I oxidation reaction and to simultaneously increase detoxification of reactive metabolites through induction of phase II conjugation enzymes. These actions of isothiocyanates are thought to be mediated via

the Nrf2 pathway. With the development of Nrf2-deficient mice, EPHX1 has been identified as a cytoprotective gene regulated by Nrf2 (Thimmulappa et al., 2002). Administration of isothiocyanate SFN in cells has been shown to regulate EPHX1 mRNA expression, as well as protein content. The mechanism underling these observations may therefore involve Nrf2 regulation. Consistent with this idea, *in silico* analysis of the EPHX1 E1-b promoter region was examined for possible transcription factor binding sites, and ARE-like motifs were located in the -300bp region. Together, these findings suggest that EPHX1 transcriptional expression can be regulated by exposure to chemopreventive isothiocyanates. We hypothesize that isothiocyanates modulate human EPHX1 gene expression largely through the Nrf2-regulated pathway, and that these inhibition/induction activities ultimately contribute to the protection against carcinogenesis and mutagenesis mediated by carcinogens.

Modifications of mRNA stability or translational efficiency are increasingly reported in regulatory proteins that are involved in tumorgenesis and cancer progression. The translational efficiency may be altered by the presence of multiple upstream reading frames and highly structured GC-rich leader sequences located in the 5' untranslated region (UTR). Further, our data demonstrate that the human EPHX1 E1-b' transcript contains two uAUGs in its 5'-UTR, and that this variant demonstrates lower translational activity when compared with either the E1 or E1-b transcript. We hypothesize that the uAUGs in the E1-b' 5'-UTR are functional and act to limit E1-b' translation efficiency, as well as overall EPHX1 enzyme activity levels. Alterations in the EPHX1 protein level and activity likely impact xenobiotic metabolism capacity, in turn leading to differences in tissue susceptibility to carcinogens.

In summary, it is clear that EPHX1 represents an important detoxification/ bioactivation pathway for xenobiotic metabolism. It follows that the genetics and regulation of EPHX1 in human tissues is likely an important determinant of differences in individual responsiveness, resulting toxicities, and carcinogenicity outcomes related to chemical exposure. Investigations

using EPHX1 knockout mice have demonstrated convincingly that this enzyme contributes to polyaromatic hydrocarbon induced carcinogenesis. Modulation of EPHX1 expression by chemopreventive isothiocyanates can facilitate carcinogen detoxification and excretion. Post-transcriptional events are important for EPHX1 expression after the gene has been transcribed, and our identification of E1-b' transcripts with upstream open reading frames suggeste that translation efficiency might be a specific mechanisms linked to EPHX1 regulation.

Therefore, the specific aims of this dissertation research project are as follows:

Aim 1: To characterize the impact of transposable genetic Alu elements within the novel far-upstream promoter region of EPHX1 as interindividual regulators of transcriptional expression in multiple human tissues.

Aim 2: To delineate the molecular mechanisms that determine the activities of EPHX1 associated with potent protection through sulforaphane, a natural chemoprevention agent rich in broccoli.

Aim 3: To assess the association of upstream open reading frames and structured leader sequence in the 5'-UTR with translation efficiency of the new EPHX1 variant E1-b' mRNA.

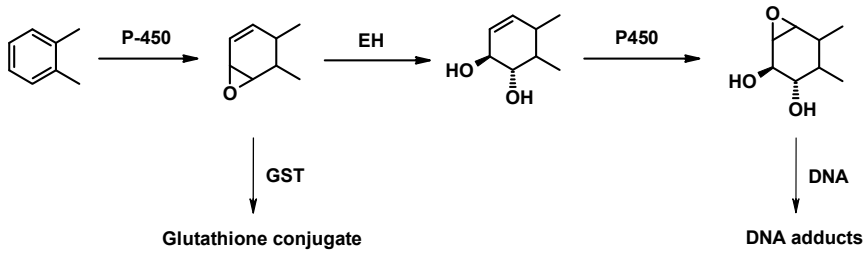


Figure 1-1 Metabolism of the PAH. Carcinogenic PAH can be bioactived by EPHX1, another pathway describe the detoxification mediated by phase II enzyme (GST).

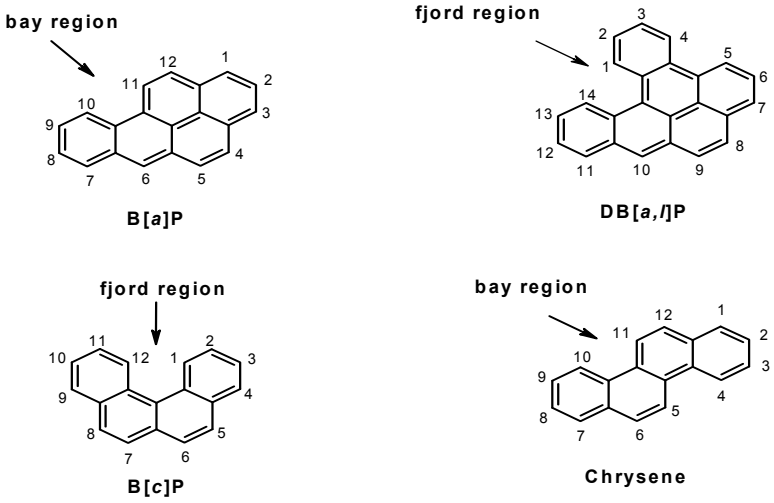


Figure 1-2 Structural characteristics of carcinogenic PAHs.

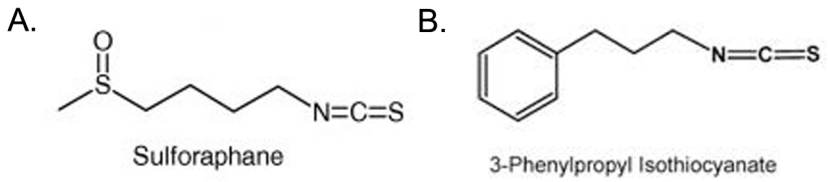


Figure 1-3 Structure of isothiocyanates. A. Sulforaphane B. phenylpropyl isothiocyanate

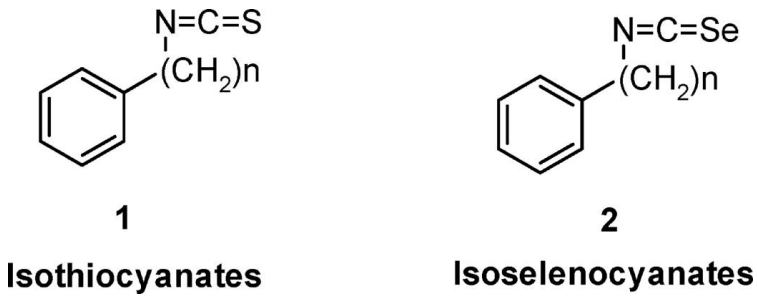


Figure 1-4 General structures of (1) phenylalkyl isothiocyanates (ITCs) and (2) phenylalkyl isoselenocyanates (ISCs).

Adapted from Sharma AK et al., *J. Med. Chem.* **2008,** 51, 7820-7826.

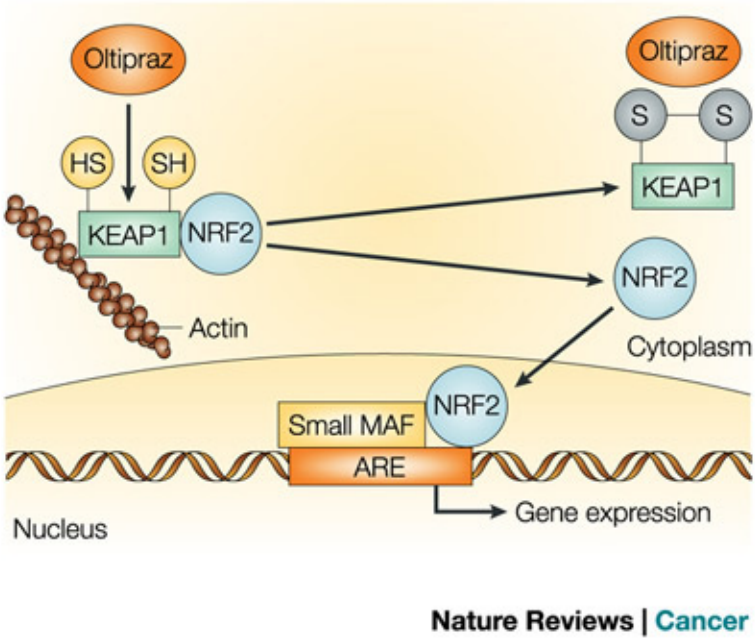


Figure 1-5: Mechanism of Keap1-Nrf2 pathway: A common cis-acting sequence, the antioxidant response element (ARE), is found in the promoter regions of phase II genes. Several transcription factors are known to bind this motif, such as members of the basic leucine zipper NF-E2 (nuclear factor erythroid-derived 2) family (for example, NRF2). An actin-binding protein, KEAP1 (Kelch-like ECH-associated protein 1), sequesters NRF2 in the cytoplasm by binding to its amino-terminal regulatory domain. KEAP1 is a sulphydryl (S)-rich protein, and several cysteine residues mediate the KEAP1–inducer interaction. Treatment with oltipraz disrupts the interaction between KEAP1 and NRF2, allowing NRF2 to translocate to the nucleus. In the nucleus, it forms heterodimers with small MAF-family proteins to activate the expression of GSTs and other genes.

Adapted from Kensler T.W. et al., Nature Reviews 3, 321 -329 (2003)

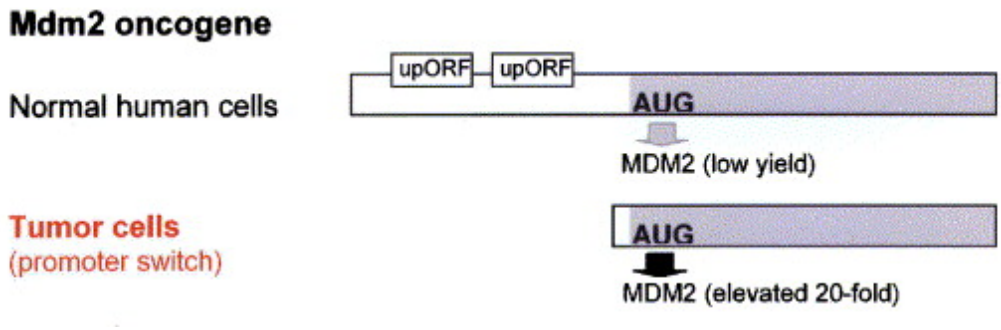


Figure 1-6: **Small upstream ORFs down-regulate translation by imposing an inefficient reinitiation mechanism.** Overexpression of the mdm2 oncogen in human tumor cells is caused by a switch in the transcriptional start site which eliminates two small uORFs, thereby elevating translation 20-fold.

Adapted from Kozak M., Gene 361, p13-37 (2005)

Chapter 2

The Expression of Human Microsomal Epoxide Hydrolase is Predominantly Driven by a Genetically Polymorphic Far Upstream Promoter

Statement of collaboration

Chapter 2 contains most of the data used in a paper that was published in J Pharmacol Exp Ther. (2009).

Xi Yang, the author of thesis, performed most of the analysis described in this chapter. Shun-Hsin Liang contributed to the identification of Alu-insertion and promoter activities analysis. Denise Weyant contributed to genotyping in human population. Philip Lazarus and Carla Gallagher contributed to the analysis of HapMap data. Curt Omiecinski helped with suggestions and critical comments throughout the experimental work.

2.1 Abstract

Microsomal epoxide hydrolase, EPHX1, biotransforms epoxide derivatives of pharmaceuticals, including metabolites of certain antiepileptic medications such as phenytoin and carbamazepine, and many environmental epoxides, such as those derived from butadiene, benzene and carcinogenic polyaromatic hydrocarbons. We previously identified a far upstream promoter region, designated E1-b, in the EPHX1 gene that directs expression of an alternatively spliced EPHX1 mRNA transcript in human tissues. In this investigation, we characterized the structural features and expression character of the E1-b promoter region. Results of quantitative Real Time-PCR analyses demonstrated that the E1-b variant transcript is preferentially and broadly expressed in most tissues, such that it accounts for the majority of total EPHX1 transcript *in vivo*. Comparative genomic sequence comparisons indicated that the human EPHX1 E1-b gene regulatory region is primate-specific. Direct sequencing and genotyping approaches in 450 individuals demonstrated that the E1-b promoter region harbors a series of transposable element cassettes, including a polymorphic double Alu insertion. Results of reporter assays conducted in several human cell lines demonstrated that the inclusion of the Alu (+/+) insertion significantly decreases basal transcriptional activities. Further, using haplotype block analyses, we determined that the E1-b polymorphic promoter region was not in linkage disequilibrium with two previously identified non-synonomous SNPs in the coding region or with functional SNPs previously identified in the proximal promoter region of the gene. These results demonstrate that the upstream E1-b promoter is the major regulator of EPHX1 expression in human tissues and that polymorphism in this region may contribute an interindividual risk determinant to xenobiotic-induced toxicities.

2.2 Introduction

Although xenobiotic metabolism often results in detoxification, in certain instances, both in cases of pharmaceutical and environmental chemical metabolism, bioactivated and highly toxic intermediates are generated. In particular, cellular levels of epoxide moieties resulting from chemical metabolism appear to be critical initiators of toxic damage, including genetic mutation. Frequently reactive and unstable, epoxide metabolites, formed via the action of the cytochrome P450 monooxygenases, have been identified as ultimate carcinogenic and cytotoxic reaction products (Sayer et al., 1985;Thakker et al., 1986;Fretland and Omiecinski, 2000). Ultimately, the overall balance between bioactivation and detoxication pathways will determine the kinetics and fate of reactive intermediates within target cells. It appears likely that interindividual differences in susceptibility to toxic sequelae, including cancer incidence, may be associated with an altered genetic predisposition to detoxify epoxides. Tissues have developed the capacity to metabolize xenobiotic epoxides through several pathways. Prominent among these is the microsomal epoxide hydrolase (EPHX1) pathway.

A wide variation of interindividual EPHX1 activities have been reported across human tissues (Hassett et al., 1997;Omiecinski et al., 1993;Mertes et al., 1985). Previously, we characterized two structural polymorphisms, an exon 3 polymorphism corresponding to amino acid position 113, with a resulting Tyr (Y) to His (H) substitution (rs1051740), and an exon 4 polymorphism at position 139, coding for a His(H) to Arg (R) substitution (rs2234922) (Hassett et al., 1994a). Tyr is the predominant amino acid at the 113 position in Caucasian populations, with His most common at 139. A large number of investigations have now been published examining the potential association of these coding region polymorphisms with altered xenobiotic disposition and disease incidence. Reports include potential associations with EPXH1 genetics and warfarin dose requirements (Loebstein et al., 2005), estrogen production (Hattori et al., 2000)

and anticonvulsant disposition (Robbins et al., 1990). Epidemiological studies focusing on cancer susceptibility have reported associations with the H113H genotype of EPHX1 and a decreased risk of lung cancer (Kiyohara et al., 2006). Other reports have indicated an increased risk of chronic obstructive pulmonary disease (Park et al., 2005;Brogger et al., 2006) and emphysema (Smith and Harrison, 1997) with the H113H genotype, as well as a protective association of the Y113H genotype with COPD (Brogger et al., 2006). Certain of these results may be contributed by differential activities of the various EPHX1 allelic proteins on specific substrates involved in diverse disease etiologies.

It is well-established that the RNA transcriptome in mammals is expanded markedly through the use of alternative promoters and differential RNA splicing mechanisms and that the use of alternative promoters is an important source of generating protein and regulatory multiplicity (Kimura et al., 2006;Davuluri et al., 2008). Recently, we reported that expression of the human EPHX1 gene is driven by an alternative promoter region, termed the E1-b promoter, that is localized ~18.5 kb 5'-upstream from the structural region of the EPHX1 gene, and is responsible for driving expression of EPHX1 mRNA transcripts in many tissues from both adult and fetal sources (Liang et al., 2005). Prior to this discovery, only the proximal E1 promoter was recognized, which lies directly upstream of the EPHX1 coding region (Skoda et al., 1988;Hassett et al., 1994b). However, the E1 promoter appeared to drive EPHX1 expression selectively in the liver (Liang et al., 2005).

Given the importance of the upstream EPHX1 promoter as a regulator of its functional expression in most human tissues, in this study we conducted comparative genomic analyses on this region and identified the variable presence of polymorphic repetitive elements of the Alu class among humans within a ~3-kb region of the E1-b promoter. In vitro promoter activity assays conducted in several human cell lines demonstrated that these polymorphisms were associated with reduced transcriptional activities and therefore may represent important

contributors to interindividual differences in EPHX1 activity within tissues targeted by xenobiotic compounds.

2.3 Materials and Methods

2.3.1 Quantitative Real-Time PCR.

Human tissue RNA was obtained from the FirstChoice Human total RNA survey panel (Ambion, Austin, TX). The total RNA was converted to cDNA using the High Capacity cDNA Archive Kit according to the manufacturers' instructions. Real-time PCR was performed using Custom Taqman Gene Expression Products (Applied Biosystems, Foster City, CA) following the manufacturer's standard protocol. The E1 variant was detected using the forward primer (5'-CTCCACAGCTCTCTTTCCCAA -3'), reverse primer (5'CCACCAGGCTCCACGTT -3') and probe (5'- TCACCCTCTGATTACTCC -3'). Similarly, the E1-b variant was detected using the forward primer (5'- GATCGCGCGCCTGC -3'), reverse primer (5'-GTGAGGAGGATTTCTAGCCACATG -3') and probe (5'- CTCGCAGGCTCCGGC -3'). Real-time RT-PCR data were analyzed using methods previously described (Page et al., 2007;Olsavsky et al., 2007). Plasmids containing cloned E1 or E1-b full length sequences were diluted to create standard curves, ranging from 30 copies to 3x 107 copies. DNA samples of known target sequence were used to verify the specificity of the assays.

2.3.2 Cell Culture

The hepatoma HepG2 and lung carcinoma A549 cell lines were grown in minimal essential medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum, 0.1 mM

nonessential amino acids, and 1.5 g/L sodium bicarbonate. The transformed human embryonic kidney lines 293A and 293T, and breast adenocarcinoma MCF7 cell line were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. All cells were maintained at 37˚C with 5% CO_2.

2.3.3 Plasmid Construction and PCR Genotyping.

A ~2.8-Kb DNA fragment upstream of E1-b was obtained by first PCR amplification of genomic DNA by forward (5'GGGTAGTAAACTGATTGGCCTC-3') and reverse (5'-CGGGCGCTTACGGTCTCG-3') primers followed by a second PCR with nested primers (5'-GACTGGTACCGGAATTGATCTACAATTTTTATCC-3') and (5'-GAAGATCTCTCTCCGGCTCCCTGGCTCTCCTC-3') using UniPOL DNA polymerase (GeneChoice, San Diego, CA). This 2.8-Kb fragment was inserted into the KpnI and XhoI sites of the luciferase-reporter vector, pGL3-basic. The insert sequences were sequenced with forward (5'CTAGCAAAATAGGCTGTCCC -3') and reverse (5'-CTTTATGTTTTTGGCGTCTTCCA-3') primers using a CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc., Fullerton, CA). For the PCR genotyping of the Alu insertion, the primers (5'- ACCAAGTAGGAGGGTATTAG-3') and (5'- ACCTGCAGTCTGGG-AGAGTTCTTT-3') were used for PCR of 10 to 30 ng of genomic DNA [DNA Polymorphism Discovery Resource (PDR), Coriell Institute, Camden NJ] with Taq DNA polymerase (GeneChoice, San Diego, CA).

2.3.4 Western Immunoblotting.

Equal amounts (10μg) of total cell lysate obtained from HepG2, A549 and 293A cells were loaded on a 10% SDS-polyacrylamide gel. After separation, proteins were transferred to a

polyvinylidene fluoride membrane. Detection of EPHX1 proteins was performed after membranes were incubated with EPHX1 peptide-directed polyclonal antibody, mouse anti rabbit secondary antibodies as described previously(Laurenzana et al., 1998). Signals were visualized using the Lumi-Light Western blotting substrate (Roche Diagnostics, Inc., Indianapolis, IL). Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) antibody (Sigma-Aldrich, Inc., St. Louis, MO) was used as a loading control.

2.3.5 Cell Transfection and Luciferase Activity Assays.

A549, HepG2 and MCF7 cells were transfected with Lipofectamine 2000 (Invitrogen). The 293 cells were transfected with FuGENE 6 (Roche Diagnostics). For the AhR ligand study, the transfection mixture contained AhR and ARNT expression vectors (a gift from Dr. Gary Perdew), luciferase reporter containing E1-b Alu-insertion promoter and pRL-CMV control. The transfection procedure and luciferase assay were performed as previously described.

2.3.6 Haplotype analysis.

The EPHX1 haplotype block structure was determined by the Haploview algorithm based on data provided from the Hapmap Project (Barrett et al., 2005).

2.3.7 Chemical Treatment in Hepatocytes

AhR ligands, beta-Naphtoflavone (β-NF), indirubin (IR) and dioxin-like 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126), are provided by Dr. Gary Perdew (Penn State University, PA). Approximately 72 hours post Matrigel™ addition, hepatocytes were exposed to chemopreventive

agents for 24 hours. Prior cells underwent treatment, media was replaced in the day three (24 h post matrigel) and hepatocytes adapted to the new culture conditions for approximately 4 days. Human hepatocytes were treated with indirubin for 6 hours. Chemical inducers were dissolved in dimthyl sulfoxide (DMSO), and DMSO was maintained at 0.1% for all treatment and control.

2.3.8 Statistical analysis.

All statistical analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software Inc., San Diego, CA). To assess the impact of the Alu repeat polymorphism on transcriptional activities in A549, HepG2, MCF7, and 293A cells, a two-tailed Student's t test was used to examine differences between each pair of haplotypes. In figure 2-5, one-way analysis of variance (ANOVA) in combination with Tukey's post hoc test was used to determine significance of the difference in the E1-b EPHX1 transcript levels between 293T and either the A549 or HepG2 cell lines. Significant differences were designated in instances where p was < 0.05.

2.4 Results

2.4.1 The E1-b Variant Is the Primary Transcript in Human Tissues.

We designed specific, quantitative real time PCR (qRT-PCR) assays to assess the expression of the E1 and E1-b alternative EPHX1 transcripts across a panel of 20 human tissues. Each tissue sample is a pooled sample, obtained from at least 3 different adult individuals. As shown in Figure 2-1, both the E1 and the E1-b EPHX1 transcripts were detected at high levels in the liver, although E1-b was the major hepatic transcript, accounting for ~ 70% of the total

EPHX1 RNA expressed in this tissue (Figure 2-1). Although the expression of the E1 transcript was largely liver-selective, very low levels of E1 were also detected in ovary, small intestine and testes. In contrast, the E1-b transcript was detected as the predominant EPHX1 transcript in all tissues examined.

2.4.2 Identification of Alu Insertion Polymorphisms in the Proximal Upstream Region of the E1-b EPHX1 Variant.

In efforts to further characterize the structure of the E1-b promoter region, we cloned and analyzed a 2.8-kb DNA fragment from various individuals within the Polymorphism Discovery Resource genomic panel (DNA Polymorphism Discovery Resource (PDR), Coriell Institute). During these studies, we observed different genomic patterns occurring in different individuals. Overall, three types of PCR profiles were encountered: 1) a homozygous, wild-type fragment profile, 2) a profile consisting of a wild-type fragment plus a larger fragment, and 3) a profile containing a homozygous, larger molecular weight fragment (Figure 2-2A). All of the genomic fragments were subjected to DNA sequence analysis. The larger genomic fragment was characterized as containing two Alu insertions (2xAlu) at nucleotide positions -2214 and -1392 (Figure 2-2B). It is interesting these two Alu insertions that occurred at separate positions were always linked together, i.e., in no instance have we detected only single Alu polymorphic insertions within a given haplotype. By searching the RepeatMasker database (http://www.repeatmasker.org), we determined that both the -2214 and -1392 Alu insertional elements belong to the Ya5 Alu family, evolutionarily a relatively young Alu insertion. Although largely conserved in their sequence, the Alu insertions did possess several single base differences within their nucleotide content (data not shown).

A PCR genotyping protocol was used to determine the frequency of these Alu insertions in the human population. In this analysis, the E1-b upstream DNA region (-2428 to -1305) was analyzed across 450 individuals of the Coriell Institute's human genomic DNA collection, containing samples from 450 U.S. residents with ancestry from all the major regions of the world(Collins et al., 1998). The results revealed that ~60% of the population had no Alu insertion [Alu(-/-)]; whereas ~36% of the individuals were heterozygous for the 2xAlu insertion elements [Alu(+/-)], and 4% of the population were homozygous for the 2xAlu insertion (Table 1).

2.4.3 Multi-species Sequence Alignments of the E1-b Upstream Promoter Region.

We used the UCSC Genome Browser (www.genome.ucsc.edu) to assess evolutionary conservation within the distal 3Kb 5'-flanking sequence of the EPHX1 E1-b promoter region among 44 vertebrates, including mammalian, amphibian, bird, and fish species. The alignment analysis was based on the genomic sequences from 44 species, and we chose to display several representative species that have pretty good sequence quality (Figure 2-3A). To reduce potential errors in the browser alignment algorithms, segments of EPHX1 sequences were also submitted to the BLASTN program to search directly for sequence conservation against all genomic NCBI databases. The results of the analysis revealed that the E1-b 5'-flanking region is highly conserved among human, chimp and rhesus, but is not identified in any species outside the primate clade. The region comprising the immediate 300bp 5'of the human E1b transcriptional start site was conserved in both chimp and rhesus; however, the Alu-insertion polymorphic elements identified in the human EPXH1 gene locus (Figure 2-2B; chromosome 1: 224062300 - 224063000), as well as the repetitive element structure surrounding this insertion region, were present only in the human and chimp genomes. In contrast, within the structural regions of the gene in the human and other vertebrate species, the protein coding sequences of EPHX1 are well

conserved with relatively high conservation score. Interestingly, in the Human and Chimp genomes, EPHX1 was localized to the Chromosome 1 'plus' strand; whereas for Rhesus, EPHX1 sequences were localized in the Chromosome 1 'reverse' strand (Figure 2-3B).

2.4.4 Analyses of promoter activities of polymorphic E1-b 5'-flanking region.

We reasoned that the existence of multiple polymorphisms within the proximal upstream region of the E1-b promoter region may affect the transcriptional activity of the gene, perhaps leading to interindividual differences in EPHX1 expression. To assess the functional significance of these sequence variations, two polymorphic haplotypes were cloned by PCR amplification and ligated into the luciferase reporter vector, pGL3-basic. The promoter activities of the resulting 2xAlu and wild type constructs were analyzed in vitro using transfection assays conducted in four different human cell lines, 293A, A549, HepG2, and MCF7. The presence of the 2xAlu double insertion resulted in lower associated transcriptional activities, although these activities varied among the cell types tested. A statistically significant reduction in promoter activity was associated with the insertion assayed in A549, HepG2, and 293A cells (Figure 2-4), with the maximal difference (40% reduction) occurring in A549 cells. Also trending lower was the Alu associated transcriptional activity level assessed in the MCF7 breast cancer cell line; however the lower trend observed in the MCF7 cells was not statistically significant. Overall, these data suggest that the presence of the genetically polymorphic Alu repeat elements within the 5'flanking region of the EPHX1 E1-b promoter down-regulates resultant EPHX1 gene transcriptional activity.

We further analyzed the Alu insertion region within selected cell lines by the specific PCR amplification of a DNA fragment between -2428 and -1305 of E1-b. Of interest, 293A (data not shown) and 293T kidney cells possessed the homozygous 2xAlu insertion genotype, whereas

the 2xAlu elements were not present in A549 lung cells and HepG2 hepatoma cells (Figure 2-5 A). We used our quantitative RT-PCR assays to measure the E1-b transcript levels in these respective cell lines and determined that the 293T cells expressed significantly less transcript than the either of the other cell lines (Figure 2-5B). We further assessed the EPHX1 protein levels in these cells using immunoblotting methods and observed that the 293T cells were similarly reduced in their corresponding EPHX1 protein content (Figure 2-5C).

2.4.5 HapMap Structure of EPHX1 Coding Region and 5' Upstream Region.

The EPHX1 gene plays an important role in the activation of carcinogenic compounds such as PAHs in cigarette smoke. Results from several epidemiological investigations have now suggested that EPHX1 coding region polymorphisms may alter the risk of developing lung cancer, as summarized by Kiyohara and co-workers(Kiyohara et al., 2006) (Kiyohara et al., 2006), as well as other human diseases, such as COPD and emphysema. Thus, it was of interest to examine the genetic linkage of the coding polymorphisms with that of the E1-b promoter region Alu-insertion polymorphism.

We used Haploview software, the algorithm derived for analysis of HapMap project data, to determine the EPHX1 gene locus linkage disequilibrium (LD) structure. LD describes the situation where combinations of alleles or genetic markers occur more or less frequently in a population than would otherwise be predicted as a random combination of allelic haplotypes based on their known frequencies of occurrence. Non-random associations between genetic polymorphisms at different loci are thus measured by the degree of LD. The map of these projections is presented in Figure 2-7. The structural region of the gene together with its extended 5' sequence, including the distal E1-b promoter region (human chromosome 1: 222304957 to 222301977), is indicated in the upper portion of the figure, with the exon 3 and exon 4

polymorphism positions marked with red ovals. Across this genetic region, 3 distinct haplotype blocks are apparent, delineated by the black triangular borders that are filled in with predominantly red squares. What is clear from the HapMap is that the distal E1-b promoter region is not in linkage disequilibrium with the coding region of the gene, which itself is divided into 2 haplotype blocks, with one block harboring the exon 3 113 SNP and another harboring the exon 4 139 SNP. Therefore, in the EPHX1 coding-region, the exon 3 and exon 4 nonsynonomous SNPs were not in linkage disequilibrium with each other.

2.4.6 Effects of AhR Ligands on the E1-b Promoter +/- Double Alu Insertion

In silico analysis of the Alu insertions in EPHX1 E1-b promoter was performed to identify possible transcription factor binding sites. The computer scans using TESS and TRANSFAC algorithms with high stringency parameters identified one AhR/ARNT transcription factor motif in the inserted Alu element (data not shown). Two human epithelial lung cell lines, the 293A cell line, that had been characterized as possessing the Alu (+/+) genotype, and the BEAS-2B cell line, with an Alu -/- genotype, were used to assess potential AhR-mediated transcriptional activities. However, no differences in EPHX1 mRNA expression level were ascertained subsequent to treatment with β-NF for 6 h (Figure 2-7 A). For an internal positive control, levels of CYP1A1 mRNA expression were similarly assessed and the marked induction of CYP1A1 confirmed that the cell culture conditions used were indeed permissive for gene responsiveness following AhR ligand treatment; (Figure 2-7B). Additional potent and highly selective AhR ligands were also tested in tested in the 293A cell assasys, including indirubin (IR) and dioxin-like 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126).. Only modest EPHX1 transcript increases were observed for IR (Figure 2-7 C). Further, we conducted similar experiments using cultured human hepatocytes, following analysis of the Alu polymorphism genotype, to determine

EPHX1 expression pattern following IR exposure.However, no homozygous 2xAlu insertion genotypes could be identified among the five available donors. For the Alu-/- genotypes, exposure to IR resulted in varied EPHX1 expression induction, ranging from no significant change to approximately 6.8- fold increase (Figure 2-7 D). Additionally, luciferase assays were used to evaluate the potential transcriptional regulation. In these assays, we transfected 293A cells with EPHX1 E1-b promoter constructs, either wild type (Alu -/-) or containing the Alu +/+ insertion, in the presence of AhR and ARNT expressing plasmids. Following AhR ligand treatments, using either PCB 126 or IR, no differences in luciferase reporter activities could be detected between the Alu (+/+) and Alu (-/-) promoter constructs (Figure 2-8A). Silimar results were also obtrained in the HepG2 cell line (Figure 2-8B).

2.5 Discussion

The primary coding sequences of the human, rodent and rabbit EPHX1 genes are highly conserved, with alignments possessing >75% similarity (Fretland and Omiecinski, 2000). These observations alone may suggest that the EPHX1 gene product has important biological function. Indeed, EPHX1 is an important contributor to the metabolism of many xenobiotic epoxides, including those derived from the metabolism of certain pharmaceuticals as well as a host of environmental toxins such as polyaromatic hydrocarbons, butadiene and acrylamide(Morisseau and Hammock, 2005;Fretland and Omiecinski, 2000). The data presented in this study demonstrate conclusively that EPHX1 expression in most human tissues is directed predominantly through the use of a far upstream alternative gene promoter, termed the E1-b promoter. Our analyses of the E1-b 5' flanking promoter region further identified a large concentration of transposable elements (TEs) (Figure 2-2B). Nearly half of the human genome is derived from ancient TEs, including short and long interspersed elements (SINEs; LINEs), long

terminal repeats, and DNA transposons (Jasinska and Krzyzosiak, 2004). TEs can promote genetic diversification and regulatory variation by serving as recombination hot spots or acquiring specific cellular functions such as adopting portions of protein coding regions or by affecting gene transcription. Further, TEs have been identified to function as alternative promoters of many genes, including aromatase P450 (CYP19;(Kamat et al., 2002)), carbonic anhydrase 1(Brady et al., 1989), and bile acid CoA: amino acid N-transferase(Carlton et al., 2003), resulting in tissue-specific regulation of gene expression.

We used comparative sequence analysis to discover that the EPHX1 E1-b promoter region is polymorphic among individuals, specifically with respect to the presence of dual TEs of the Alu-class. As one important component of TEs, Alu elements are primate-specific and represent the most abundant of the SINE group; their 1.1 million copies occupy over 10% of the human genome(Mighell et al., 1997). Alu repeats are unevenly distributed, with transcriptionally active regions of the genome especially densely populated, and likely play roles in human gene regulation (Grover et al., 2004;Korenberg and Rykowski, 1988;Moyzis et al., 1989). Alu elements are divided into several subfamilies according to their insertion age (Mighell et al., 1997). Almost all of the insertions occurring specifically in the human genome belong to four closely related subfamilies, Alu Y, Ya5, Ya8, and Yb8 (Deininger and Batzer, 1999). The majority of human Alu elements belong to old or intermediate subfamilies. Although present in relatively low copy numbers, the younger Alus are considered the most active and those most likely to possess biological function (Deininger et al., 1992). These more recent Alu insertions also result in genetic variation in human populations by generating polymorphic loci (Carroll et al., 2001;Salem et al., 2003). In this study we report the new finding of Alu insertion polymorphisms of the youngest Alu class, the Ya5 class, in the E1-b 5'-flanking region of the EPHX1 gene. These elements may serve as geneticists as markers for the study of human population genetics, disease associations, and genomic diversity and evolution.

Frequency analysis in a panel of 450 individuals demonstrated that the combination of Alu insertion/deletion polymorphisms results in three different EPHX1 E1-b promoter haplotypes: Alu (-/-) genotype (59.9%) Alu (+/-) genotype (36.1%) and Alu (+/+) genotype (4.0%) (Table 2-1). Further, data obtained from in vitro promoter activity assays indicates that the presence of the 2X Alu insertion down-regulates the transcriptional level of the associated E1-b EPHX1 transcription in several cells types, suggesting that individuals with the Alu (+/+) genotype may possess lower EPXH1 activities in certain tissues. Comparative species assessments using the UCSC genome browser indicated that the EPHX1 E1-b promoter region context was a specific attribute of primate species (Figure 2-3).

These results, together with our previous findings that genetic polymorphisms exist both in the coding region and the proximal 5'-flanking region of the EPHX1 gene (Hassett et al., 1994a; Raaka et al., 1998), may constitute determinants of interindividual and tissue-specific differences in capacities to biotransform xenobiotic-derived epoxides. The upstream promoter region Alu element polymorphism identified in this study is of particular interest in the respect in that E1-b is the primary EPHX1 transcript expressed in human tissues. EPHX1 transcript level analyses within a panel of human tissues demonstrated the largely liver-specific expression pattern of the E1 proximal promoter-derived transcript; in contrast, the far distal E1-b promoter ubiquitously and predominantly drives EPHX1 expression in all tissues examined. Human/primate genetic evolution has apparently selected the alternative E1-b promoter as the primary regulator of EPHX1 expression.

The association between genetic polymorphisms of the biotransformation enzymes and the risk of cancer has been of particular interest for the study of carcinogenesis. One major group of strong carcinogenic materials in tobacco smoking is the polycyclic aromatic hydrocarbon with bay or fjord regions (Hecht et al., 1994). The ultimate carcinogenic PAH metabolites are generated through the concerted action of multiple enzymes, such as the cytochrome P450s,

glutathione-S-transferases and EPHX1. EPHX1-null mice are completely resistant to the tumorigenic effects of dimethylbenz[a]anthracene in a complete carcinogenesis assay (Miyata et al., 1999), and results of several human epidemiologic studies suggest a reduced risk of lung cancer in individuals with a His113 genotype, as summarized by Kiyohara et al (Kiyohara et al., 2006). The pair of ~300 bp Alu insertion elements described in the current investigation provide several potential binding sites for transcription factors and hormone receptors (Shankar et al., 2004). Additional epidemiological studies are required to investigate any potential associations of the polymorphic Alu insertions within the upstream promoter region of the EPHX1 gene with human cancers or with the incidence of other disease states previously implicated with EPHX1 genetics (e.g., (Smith and Harrison, 1997;Brogger et al., 2006;Fretland and Omiecinski, 2000).

An AhR/ARNT motif was predicted in the Alu insertion sequence using computer scans with high stringency matrix scoring parameters. Given the ability of the AhR inducible CYP1A1 to generate epoxide derivatives of various PAH species, we reasoned that certain individuals, those possessing Alu +/+ insertions in theirE1-b promoter regions, may be of an especially high risk class, as induction of both pathways within an individual would likely lead to a synergistic enhancement in the generation of reactive diol-epoxid-containing chemical species. However, only modest modulatory effects on EPHX1 expression were detected following direct treatment of cell lines or primary hepatocytes with potent AhR ligands (β-NF, IR, PCB 126). Luciferase assays and real-time PCR were used to assess transcription activity in 293 lung and HepG2 hepatoma cells, which have been characterized with Alu+/+ and Alu-/- genotype, respectively. Cultures of primary human hepatocytes isolated from five donors were also included for real-time PCR analysis and Alu polymorphisms were analyzed by PCR method. Using Lipofectomine-based transfection schemes, luciferase reporter assays demonstrated surprisingly no response to AhR ligand exposure. Cytochrome P4501A1 mRNA levels were measured following exposure to AhR ligands to permit independent evaluation of inducer responsiveness. Marked induction in

CYP1A1 mRNA expression verified that the human cell lines and hepatocytes models used had preserved robust responsiveness to AhR ligand treatment. These results indicate that the expression of human EPHX1 in an *in vitro* system is only modestly responsive to AhR ligands and the AhR regulation does not associate with the presence/absence of transposable EPHX1 Alu elements interindividually.

When considering the association of genetic polymorphisms with the risk of human disease, to better predict complex phenotypes or disease processes one clearly needs to consider pairwise LD or haplotype blocks (several linked polymorphic alleles), rather than reliance on a single polymorphic site (Crawford and Nickerson, 2005). The HapMap structure of the EPHX1 gene (Figure 2-6) indicated that the E1-b Alu polymorphisms in the regulatory region were not in the same haplotype block with either the Tyr113/His or His139/Arg coding region polymorphisms previously identified by our laboratory, and that the respective coding region polymorphisms also are predicted to reside in separate haplotype blocks, indicating that there is not a strong linkage association among these three polymorphisms and that variations in each polymorphism can affect the phenotype independently.

In summary, the results from our current study serve to further characterize the tissue-selective expression context of human EPHX1, and have identified the polymorphic nature of the far upstream E1-b promoter region of EPHX1, the promoter that serves as the predominant transcriptional driver of EPHX1 in human cells. These studies have also elucidated the haplotype structure of the extended EPHX1 genomic locus, thereby contributing a context for further investigations examining the associations of EPHX1 genetic polymorphisms with human diseases arising from environmental or pharmaceutical exposures that are subject to metabolism through the EPHX1 enzymati**c** pathway.

Table 2-1 The Alu insertion polymorphisms at the 5'- flanking region of E1-b in human population.

Genotype	Number of Samples	Percentage (%)
Alu (-/-)	269	59.9
Alu (+/-)	162	36.1
Alu(+/+)	18	4.0

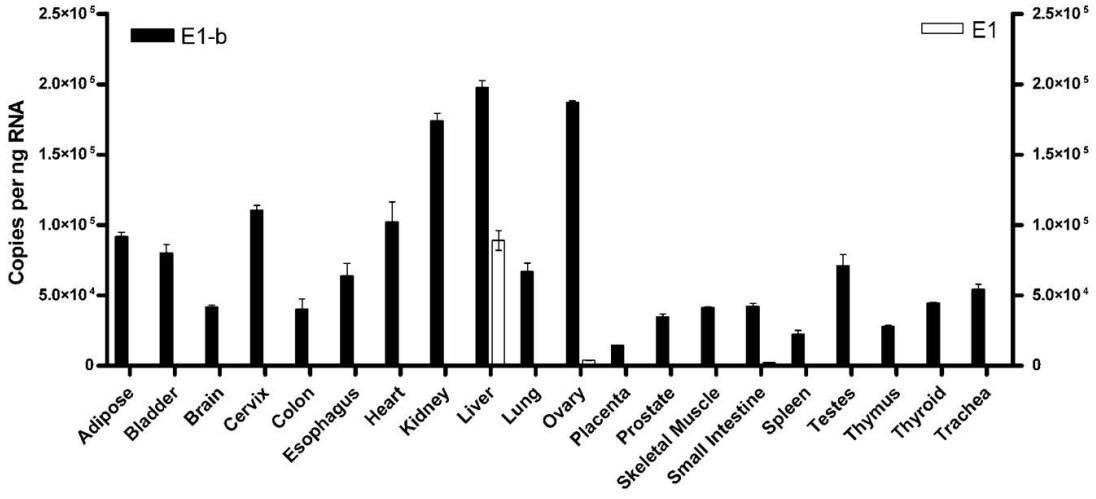


Figure 2-1. Quantification of the EPHX1 E1 and E1-b transcripts expression in different human tissues. Real-Time PCR was performed with RNA obtained from 20 human tissues; each sample represents a pool of at least three individuals. E1-b (black bar) and E1 (white bar) transcript copy numbers were quantified by absolute quantification based on standard curves determined using plasmid DNA templates.

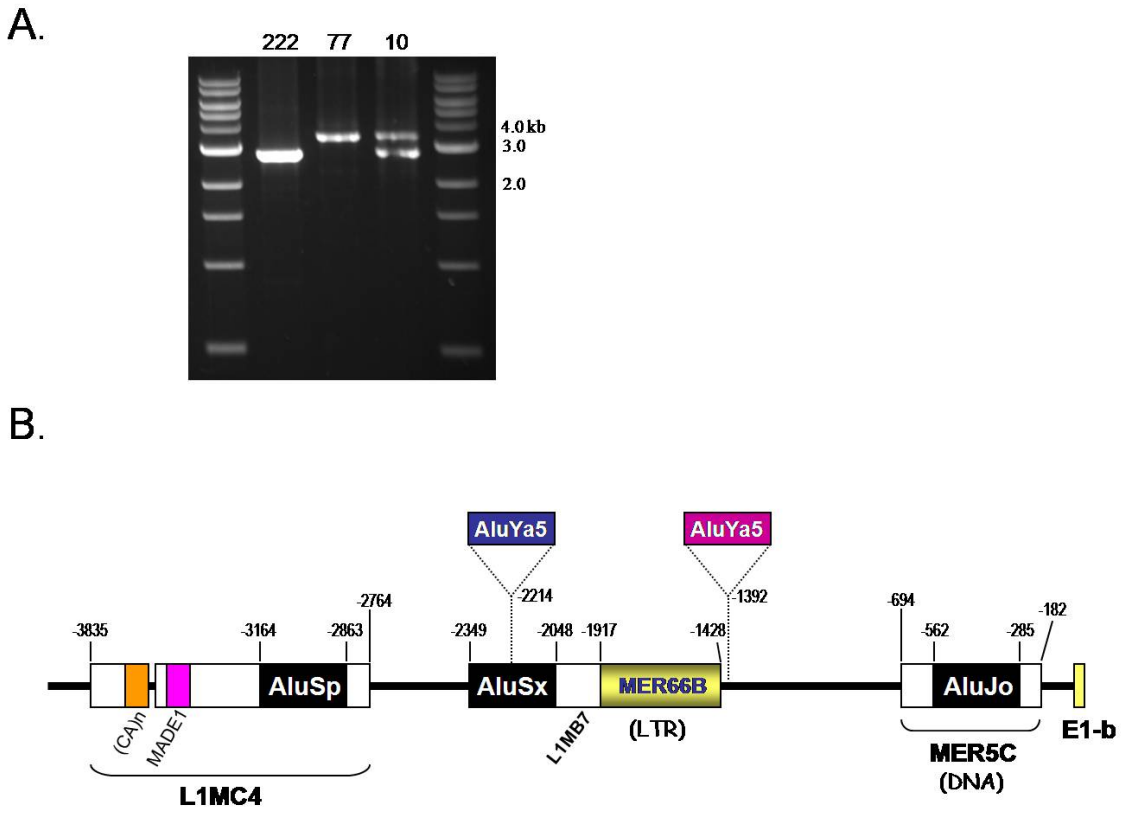
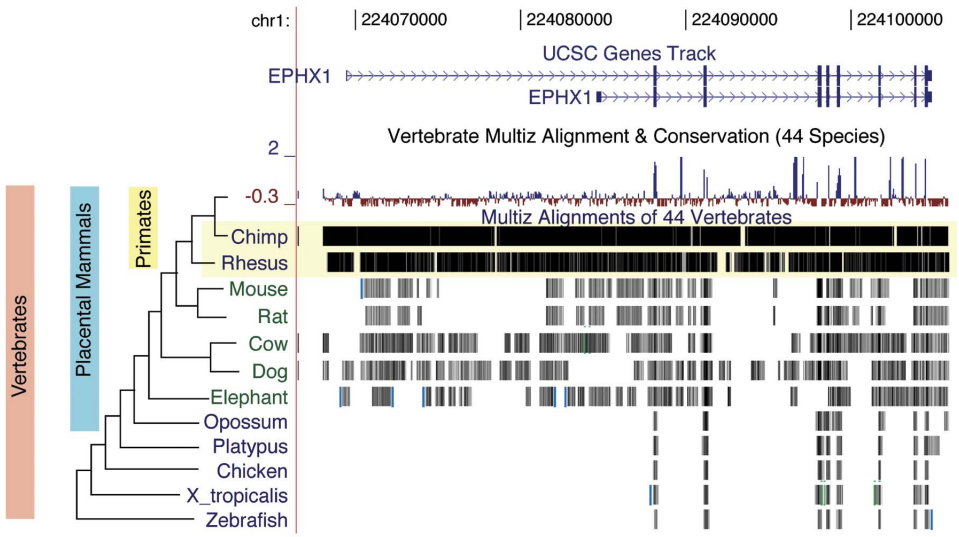


Figure 2-2. Identification of Alu-insertion polymorphisms in the 5'-flanking region of E1-b. (A) PCR analysis of polymorphic DNA fragments ~2.8-kb upstream of E1-b. The genomic DNA (20 ng) of three individuals (#10, 77, 222 of DNA Polymorphism Discovery Resource, Coriell Institute) were subsequently amplified using two primer sets as described in Materials and Methods. (B) Schematic representation of the polymorphic Alu (Ya5) insertions upstream of E1-b. Also indicated are the transposable element (TE) cassettes formed through combinations of different types of TEs that belong to SINE (AluSp, AluSx, AluJo), LINE (L1MC4, L1MB7), LTR (MER66B), tandem repeats (CA)n, or DNA transposons (MADE1, MER5C) classifications.

A.



B.

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Human  -GGACATACATCATGCAAGTTTGGATTTTTGTTTTTAGATTCAACTGACGAAGTTACGGGATCAAACTGCTGTAGGAGCTGCCAA
Chimp  AGGACATACATCAAGCAAGTTTGGATTTTTGTTTTTAGATTCAACTGACGAAGTTACGGGATCAAACTGCTGTACGAGCTGCCAA
Rhesus AAGACGTACATCATGCAAGTTTGGATTTTTGTTTTTAGATTCA-CTGACAAAGTTACGGGATCAAACTGCTGTACAAGCTGCTAA
         **** ******* ***************************** ***** ************************ ****** ***

Human  ACGCTTCTCTCCATTTCTGGCCGCGGGGCCGCGGACCGCCCTTTAAGTAGCCCGTTTTATCCCTGGCAGAGGTGGAGCCTTAGGC
Chimp  ACGCTTCTCTCCATTTCTGGCCGCGGGGCCGCGGACCGCCCTTTAAGTAGCCCGTTTTATCCCCGGCAGAGGTGGAGCCTTAGGC
Rhesus ACGCTTCTCTCCATTTCTGTCATCGGGGCCGCGGACCGCCCTTTGAGTAGCCTGTTTTATCCCCTGCGGAGGTGGAGCCCTGAGG
       ******************** *  ********************** ******* **********  ** *********** *  *

Human  AGGCCTAGAGACTTTCCCGGGTCCTCCAGG----------------------------------------CCGGGGAACGCCCCGCT
Chimp  AGGCCTAGAGACTTTCCCGGGTCCTCCAGG----------------------------------------CCGGGGAACGCCCCGCT
Rhesus AGGTGTAGAGACATTCCCGGATCGCTCAGGTAACGGGCGGGGAAGTGCGGAACCCAGGTTCTCCGGGGCCGGGGAACGCCCCGCT
       ***  ******* ******* **   ****                                          **************
                                                                               ↓
Human  CGGAGGCCGGGCTTGGGCGGAGACTGCGCCGGGGCTGCTGAAAACTAGCCGAGGAGAGCCAGGGAGCCGGAGAGATCGCGCGCCT
Chimp  CGGAGGCCGGGCTTGGGCGGAGACTGCGCCGGGGCTGCTGAAAACTAGCCGAGGACAGCCAGGGAGCCGGAGAGATCGCGCGCCT
Rhesus CGGAGGCCGGGCTTGGGCGGAGACTGCGCCGGGGCGGCTGGAGACTAGCCGAGGGGAGCCGGGGAGTCGGAGAGACCGCGCGCCT
       ************************************ **** * ***********  **** ***** ******** *********

Human  GCCGCCGCCGGAGCCTGCGAGCCGAGACC
Chimp  GCCGCCGCCGGAGCCTGCGAGCCGAGACC
Rhesus GCAGCCGCCGGAGCCTGCGAGCCGAGACC
       ** *************************
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Figure 2-3 E1-b 5'-flanking sequence comparisons. Multiple alignments of E1-b upstream region, showing E1-b promoter is conserved only in primates. (A) The 44 vertebrate species alignments of the E1-b 5'-flanking region were generated by the UCSC Genome Browser, representative species with good sequence quality were chosen to display. For each species, conserved sequences were displayed with grayscale density plot that indicates alignment quality, the darker the better. The E1-b promoter is conserved among Human/Chimp/Rhesus, but only Human and Chimp have the Alu insertion sites. The coding exons share similar sequences and exhibit high conservation score (range from - 0.3 to 2, the higher the better). (B) EPHX1 E1-b promoter region (E1-b transcription start site is indicted by arrow) compared between Human/Chimp/Rhesus by Clustal W2. In Human and Chimp genomes (98% match), EPHX1 was located in the Chromosome 1 plus strand; whereas for Rhesus (91% match to Human), sequences were found in Chromosome 1 reverse strand.

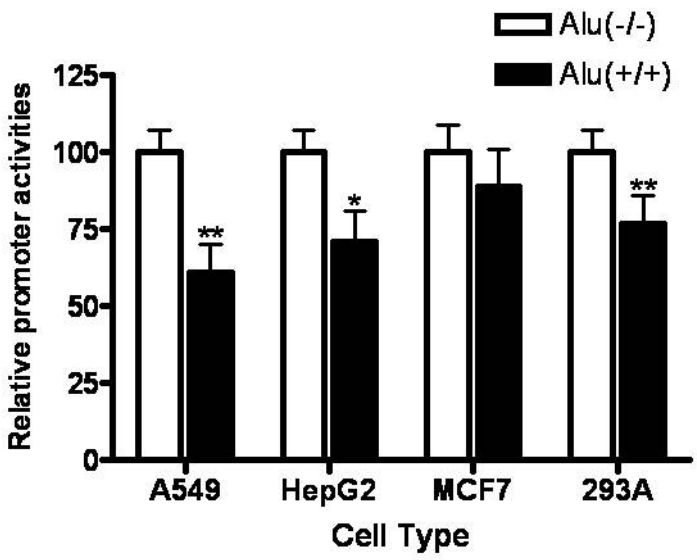


Figure 2-4. Analysis of promoter activities of the polymorphic E1-b 5'-flanking region. Luciferase-based promoter activities were analyzed by cloning the E1-b 5'-flanking DNA region (-2763/+6) for different haplotypes into the pGL3-basic vector. The data shown depict means and S.D. values derived from five separate experiments, each performed in duplicate. Inclusion of the 2xAlu element results in a statistically significant decreased promoter activity in A549, HepG2 and 293A cells (*, $p<0.05$; **, $p< 0.01$).

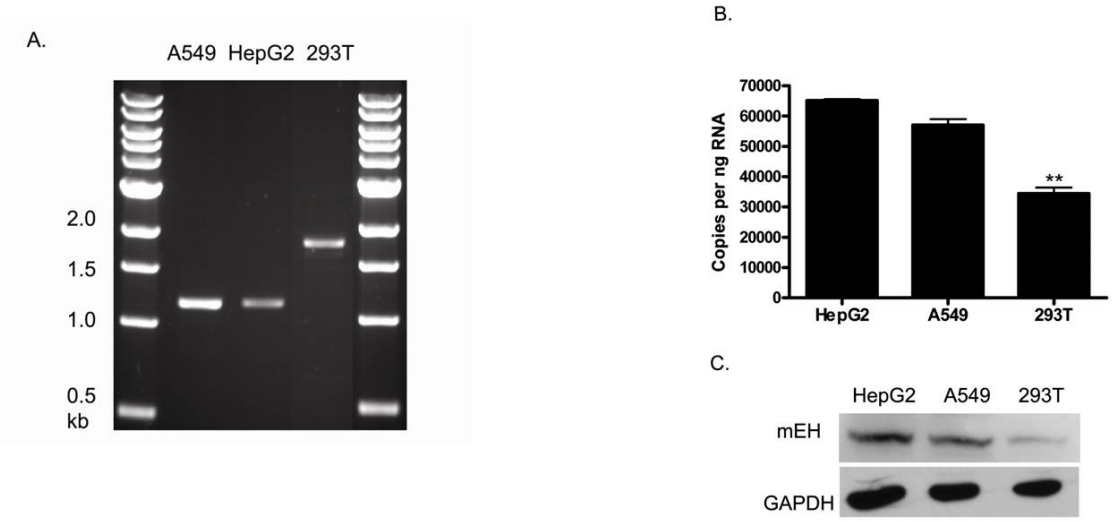


Figure 2-5 Characterization of the E1-b promoter region and EPHX1 expression in human cell lines. (A) PCR genotyping of Alu insertion polymorphism in A549, HepG2, and 293T cells. The DNA fragment between _2428 and _1305 of E1-b was specifically amplified from genomic DNA demonstrating the presence of the 2xAlu insertion in 293T cells but not A549 or HepG2 cells. (B) Real-time PCR was performed with RNA obtained from the respective cell lines. The data shown depict means and S.D. values derived from two separate experiments, each performed in triplicate. The 293T cells exhibited a significantly lower level of EPHX1 E1-b transcript expression compared either with HepG2 or A549 cells (**, $p < 0.01$). (C) Western immunoblot blot analysis assessing corresponding EPHX1 protein levels.

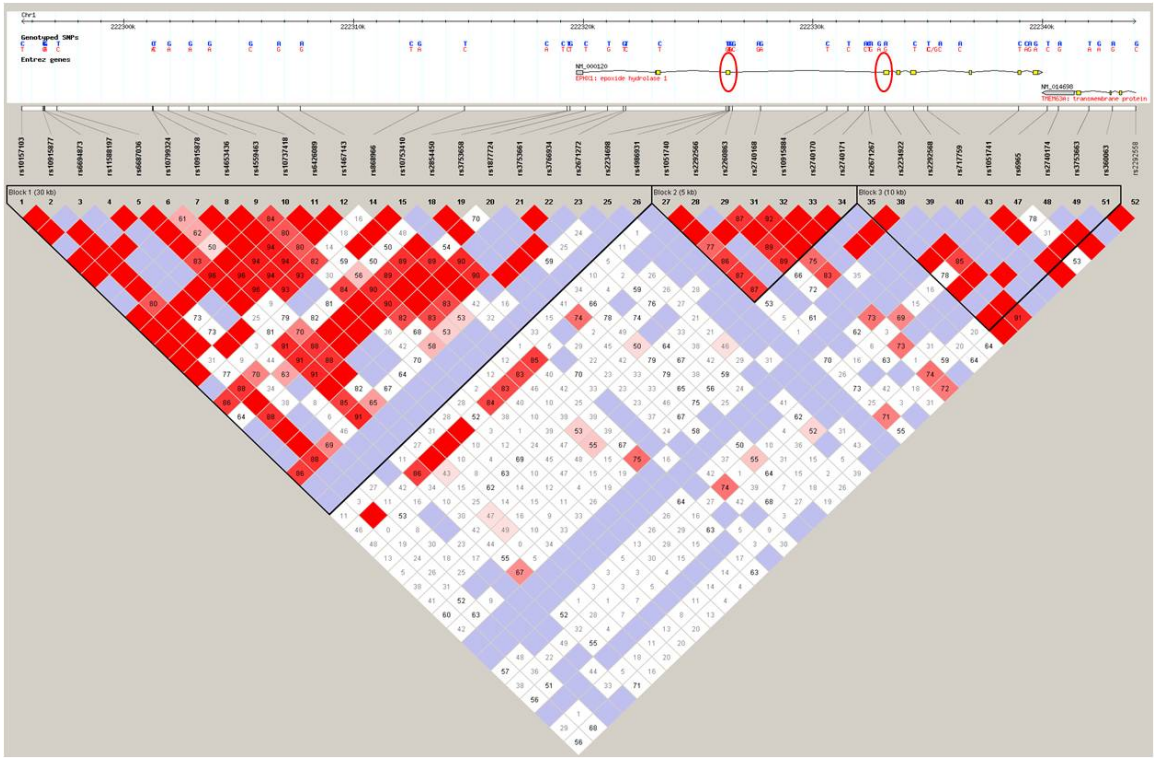


Figure 2-6. Linkage disequilibrium (LD) structure of a 52 kb region spanning EPHX1 and extended promoter in European Americans. EPHX1 genotypes from HapMap (http://www.hapmap.org/) were downloaded and the LD was determined using Haploview software. D' values are displayed in the squares (empty squares have a pairwise D'=1.00). Red squares show high pairwise LD, gradually coloring down to white squares of low pairwise LD. The black triangles indicate the haplotype blocks. There are 3 haplotype blocks in this region (one block in the large promoter region that spans into the first 2 exons of the gene, one block that spans exon 3 and nearby intronic regions, and one block that spans exons 4-9). At the upper portion gene structure region, ovals indicate the coding polymorphisms in exon 3 (rs1051740) and exon 4 (rs2234922) which are not in LD with each other, nor with the promoter region.

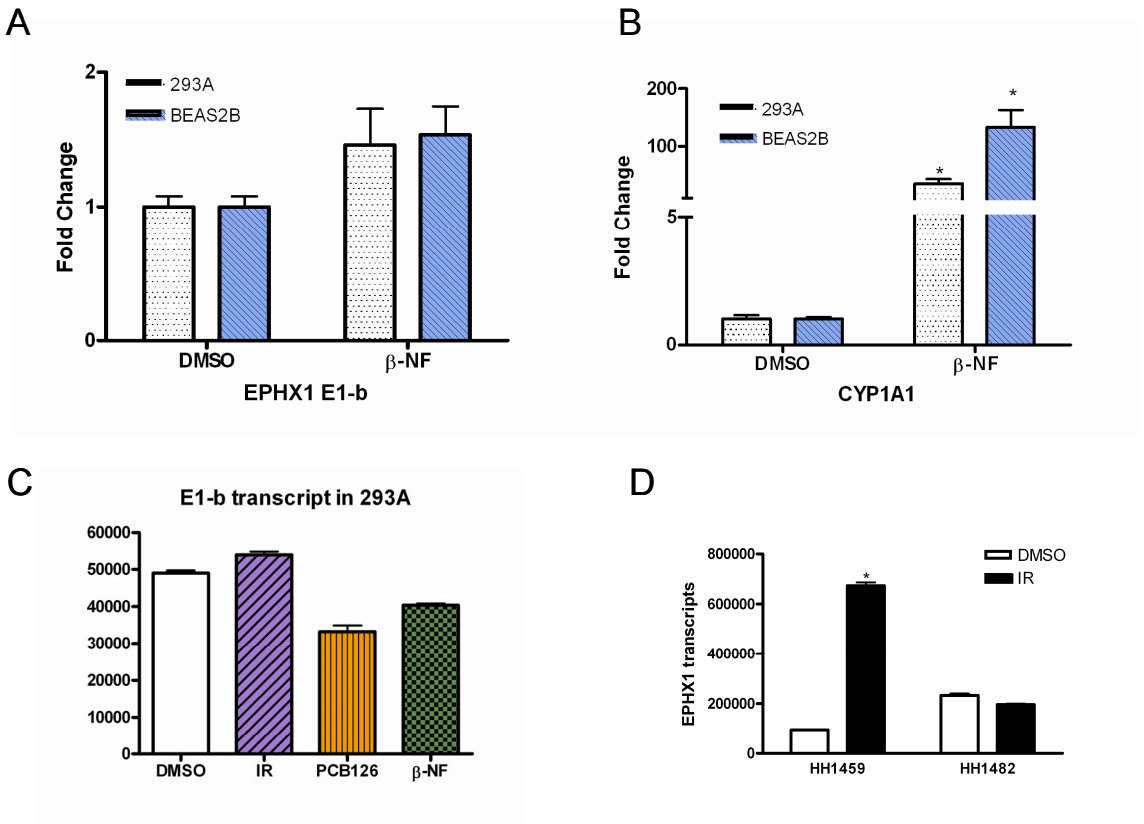


Figure 2-7. Effects of AhR ligands on EPHX1 expression in cell lines and human hepatocytes. (A) EPHX1 E1-b transcript expression in 293A (Alu(+/+) genotype) and BEAS-2B (Alu(-/-) genotype) was quantified by TaqMan real-time PCR assay after β-NF (1 μM) treatment for 6 h. (B) CYP1A1 mRNA expression was analyzed as positive control following β-NF (1 μM) treatment (C) AhR ligands, IR, PCB 126 and β-NF, were tested for their ability to regulate EPHX1 expression in 293A cells. (D) EPHX1 mRNA expression in hepatocytes (Alu(-/-) genotype) was quantified by TaqMan real-time PCR assay after β-NF (1 μM) treatment for 6 h. (*, $p<0.05$, paired t-test)

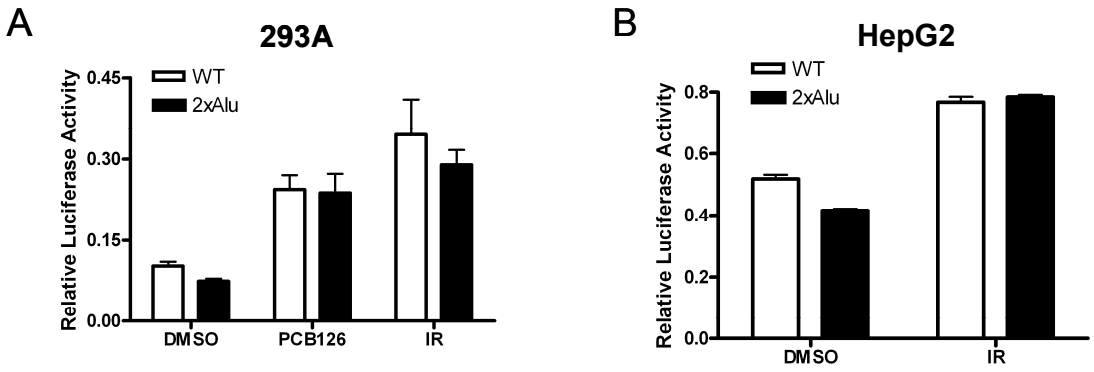


Figure 2-8. Analysis of promoter activities by AhR ligands. (A) 293A cells were co-transfected with the wild type (WT) E1-b promoter or Alu insertion (2xAlu) construct and AhR/ARNT expressing vectors and subsequently treated with PCB 126, IR or DMSO. At 6 hr post-treatment, cells were lysed and assayed for luciferase activity. (B) HepG2 cells were transfected with the wild type (WT) E1-b promoter or Alu insertion (2xAlu) construct and subsequently treated with IR or DMSO. At 6 hr post-treatment, cells were lysed and assayed for luciferase activity. The data shown depict means and S.D. values derived from three separate experiments, each performed in duplicate.

Chapter 3

Human Microsomal Epoxide Hydrolase Expressions is Regulated by the Chemopreventive Agent, Isothiocyanate

3.1 Abstract

Sulforaphane (SFN) has been identified as a highly potent chemopreventive agent and this naturally occurring isothiocyanate (ITC) is found in broccoli and broccoli sprouts at high level. Recent research suggests sulforaphane acts through many pathways, such as inhibition of Phase I enzymes involved in carcinogen activation and induction of the Phase II enzymes involved in detoxification of reactive metabolites. Here we evaluated the ability of the isothiocyanates, sulforaphane and phenylpropyl isothiocyanate (PPITC), as well as a related group of isoselenocyantes, to regulate EPHX1 transcription and protein expression. In the BEAS-2B human lung cell line, both SFN and PPITC reduced the EPHX1 mRNA in a dose-dependent manner. However, in HepG2 human hepatocarcinoma cells and primary human hepatocytes, EPHX1 mRNA expression was significantly induced after the SFN treatment. EPHX1 protein levels were quantified by immunoblotting for all treated and non-treated groups. In general, there was a strong concordance with corresponding EPHX1 mRNA levels. SFN has been shown to regulate cytoprotective genes at the transcriptional level through the nuclear factor-erythroid2-related factor (Nrf2) pathway. Luciferase reporter assays of the E1-b promoter revealed that Nrf2 is likely involved in the tissue-specific regulation of EPHX1 transcription. In HepG2 cell lines, co-expression of Nrf2 activates EPHX1 promoter activity more potently than SFN treatment alone. Similarly, in BEAS-2B cells, the presence of Nrf2 enhances the efficacy of SFN treatment. Therefore, our investigations indicate that the chemopreventive agent, SFN, and the synthetic

isothiocyanate, PPITC, differentially modulate EPHX1 expression in a tissue-specific manner and that these effects are likely mediated though an interplay of the Nrf-2 regulatory pathway.

3.2 Introduction

Epidemiological studies highlight the inverse relationship between high intake of fruits and vegetables and cancer risk, including liver, lung, breast, prostate and colon cancers (Juge et al., 2007;Kensler et al., 2003;de Kok et al., 2008). Isothiocyanantes (ITCs) are naturally occurring chemopreventive compounds found in cruciferous vegetables, which have gained much attention because of their potential cancer inhibitory effects in culture models as well as animal models. Broccoli, an anti-cancer cruciferous vegetable widely consumed by Western societies, is a rich source of glucosinolates, which are precursors of ITCs. The biological active isothiocyanates, including sulforaphane (SFN), phenethyl isothiocyanate (PEITC) and phenylpropyl isothiocyanate (PPITC), are identified as potent chemopreventive agents inducing Phase II detoxification enzymes, such as quinine reductase, glutathione S-transferases and glutamate cysteine ligase (McWalter et al., 2004). For example, SFN has been shown to prevent carcinogen-induced tumorigenesis in rat mammary glands (Zhang et al., 1994;Osburn and Kensler, 2008). These chemopreventive effects induced by SFN were initially attributed to the increase of Phase II enzymes and glutathione (GSH) level, as well as inhibition of cytochromes P-450 and other enzymes involved in carcinogen activation. Recently, SFN has been found to augment cellular defense systems against carcinogenesis through additional pathways, including of cell cycle inhibition and the induction of apoptosis (Juge et al., 2007;Myzak and Dashwood, 2006).

Several key studies identified the Keap1-Nrf2 pathway as an important modulator of toxicological insult. For example, Nrf2 knockout mice are more sensitive to a variety of acute and chronic chemical and biological stresses in multiple target organs (Kensler et al., 2007).The

protective effects of SFN against carcinogens appear to by mediated through Keap1-Nrf2 pathway. SFN has been shown to be very potent in inducing translocation of Nrf2 to the nucleus, where it functions as a transcription activator of genes containing the antioxidant response element (ARE) in the upstream region. Nrf2 knockout mice are more sensitive to a variety of acute and chronic chemical and biological stresses in multiple target organs (Kensler et al., 2007). Microarray studies in mice identified several classes of genes as targets of SFN regulation, including cellular NAPDH regenerating enzymes, xenobiotic metabolizing enzymes, antioxidant enzymes, and biosynthetic enzymes of glutathione and glucuronidation conjugation pathways (Thimmulappa et al., 2002). Many of these genes are modulated by the Keap1-Nrf2 pathway.

Human microsomal epoxide hydrolase (EPHX1; EC 3.3.2.3) catalyzes the conversion of a broad range of epoxide substrates to more polar *trans*-dihydrodiol metabolites, thus the active epoxide intermediates generated by Phase I oxidation reactions can be detoxified. However, in certain instances, the hydrolysis of the PAH-epoxides can lead to the generation of highly reactive and mutagenic products known as diol epoxides. For example, the environmental carcinogen, benzo[a]pyrene, which exists in tobacco smoke, requires region-selective hydrolysis by EPHX1 and epoxidation by P450 enzymes to form covalent adducts with DNA (Shou et al., 1996). Bioactivation of carcinogens by EPHX1 was further demonstrated in EPHX1-null mice, which are completely resistant to the tumorigenic effects of 7,12-dimethylbenz[a]anthracene, as compared to wild type mice. Association between EPHX1 polymorphisms and lung cancer risk has been observed in many case-controlled epidemiological studies. Beyond xenobiotic metabolism, human EPHX1 may also play a role in endogenous processes such as steroid metabolism, bile acid transport and in the vitamin K reductase complex (Newman et al., 2005).

Human EPHX1 activity is generally the highest in liver, with lower levels in kidney, ovary, lung, and heart (Liang et al., 2005;Newman et al., 2005). There is a wide variation of interindividual EPHX1 enzyme activities reported in human livers, which generally correlates

well with EPHX1 protein levels (Mertes et al., 1985;Guengerich and Turvy, 1991). A previous report identified two EPHX1 transcripts, E1 and E1-b, driven by the use of alternative promoters. E1, directly proximal to EPHX1 coding region, is expressed only in the liver. The E1-b promoter is used preferentially to drive expression of EPHX1 mRNA transcripts in most human adult and fetal tissues (Liang et al., 2005). Interestingly, the 5'-flanking region of the EPHX1 gene reveals few similarities across species even though the primary coding sequences of the vertebrate EPHX1 genes are highly conserved. Furthermore, in rodents, EPHX1 gene transcription is increased by several xenobiotic chemicals (Schilter et al., 2000), while the effects of chemical exposures on human EPHX1 gene expression are quite moderate (Hassett et al., 1998).

The present study aims to determine whether human EPHX1 can be regulated by chemopreventive agents, SFN and PPITC, using cell lines and primary hepatocytes. To examine this issue, we exposed the cells to the test chemopreventive chemicals for 24 hours, and the expression of EPHX1 mRNA transcripts and protein were analyzed by real-time PCR and by western blotting. In general, we found that human EPHX1 responded to chemopreventive agents in a tissue-specific manner. SFN and PPITC inhibited the EPHX1 expression in BEAS-2B cells, while treatment of hepatic cells resulted in a dose-dependent induction of EPHX1 mRNA and protein levels. Effects of synthetic isoselenocyanate derivatives were also assessed in these studies, although in general, these agents appeared less efficacious than the naturally-derived isothiocyanate, SFN.

3.3 Materials and Methods

3.3.1 Materials

Sulforaphane and dimethylsulfoxide (DMSO) were purchased from Biomol (Plymouth Meeting, PA), and Sigma (St Louis, MO.), respectively. Primers for PCR were purchased from Integrated DNA Technologies (Coralville, IO). Phenylpropyl isothiocyanate (PPITC) was a gift from Dr. Jeff Peters (Penn State University). Isoselenocyanates were synthesized in the laboratory of Dr. Shantu Amin (Penn State University, Hershey, PA). All materials and equipment for gel electrophoresis were from Bio-Rad. Unless indicated otherwise, all other chemicals were purchased from Sigma (St Louis, MO).

3.3.2 Cell Culture

The human bronchial lung epithelial cell line, BEAS-2B, and the human embryonic kidney, HEK293, were maintained and transfected in Dulbecco's modified Eagle medium with 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 0.15% sodium bicarbonate, 50 units/ml penicillin G and 50 μg/ml streptomycin (all purchased from Invitrogen Life Technologies, Carlsbad, CA.). The human hepatoma cells, HepG2 and human lung carcinoma A549 cell lines were grown in minimal essential medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum, 0.1 mM nonessential amino acids, and 1.5 g/L sodium bicarbonate.

Human hepatocytes were isolated in the laboratory of Dr. Stephen Strom (University of Pittsburgh, Pittsburgh, PA) with a procedure that has been described previously (Hassett et al., 1998;Olsavsky et al., 2007). When received in our laboratory the flasks were vented and incubated at 37˚C with 5% CO_2 for 1 hour. Then the media was changed to serum-free hepatocyte maintenance media supplemented with 25 nM dexamethasone. Where indicated, Matrigel™

(Collaborative Research) was added on the second day, swirled gently into solution as an overlay immediately following media change (final concentration, 233 µg/ml). Hepatocytes were maintained for a minimum of 3 days in serum-free media prior to treatment with test chemicals.

3.3.3 Plasmid Construction

A ~2.8-Kb DNA fragment upstream of E1-b was inserted into the KpnI and XhoI sites of the luciferase-reporter vector, pGL3-basic (Liang et al., 2005). Based on this template, the -1.5kb E1-b promoter fragment was amplified with forward (5'-TTGGTGGGTACCGCTAGTCCTCAATTTGGTCCTCAA -3') and reverse (5'-GAAGATCTCTCTCCGGCTCCCTGGCTCTCCTC-3') primers using UniPOL™ DNA polymerase (GeneChoice, San Diego, CA). The 300bp E1-b promoter was amplified with forward (5'-GATCTGGGTACTGATAGAGTGAGACTCTGT -3') and reverse (5'-GAAGATCTCTCTCCGGCTCCCTGGCTCTCCTC-3') primers. Nrf2 gene was amplified with forward (5'- GATCGCGGCCGCAATGGACTTGGAGCTGCCGCCG -3') and reverse (5'-GATCTCTAGACTAGTTTTTCTTAACATCTGG -3') primers. PCR amplifications were performed in a 50 µl reaction volume containing 3.0 mM MgCl2, 25 pmol of each primer and 0.5 µl of template. Cycling was conducted at 95º C for 30 sec, 60ºC for 30 sec, and 72ºC for 30 sec. The 1.5-Kb and 300bp fragment were inserted into the KpnI and XhoI sites of the luciferase-reporter vector pGL3-basic (Promega, Madison, WI), pGL3-basic. Nrf2 gene was inserted into 3XFLAG vector (Sigma, St Louis, MO) by using the NotI and XbaI sites. The constructs were sequenced using a CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc., Fullerton, CA).

3.3.4 Cell Transfection and Luciferase Activity Assays

All transfections for luciferase reporter assays were performed in a 48-well format. On the afternoon of day one, BEAS-2B and HepG2 cells were plated at a density of 50,000 cells/well, allowing cellular attachment overnight. On the morning of day two, DNA transfection mixtures were assembled using the Fugene6 transfection reagent (Roche Applied Science, Indianapolis, IN). In general, for assays involving standard reporters, a combination of 25 ng of CMV driven Nrf2 expression plasmid, 100 ng luciferase reporter containing E1-b -300bp promoter region and 10 ng of Renilla reniformis luciferase expression plasmid (pRL-CMV, added for transfection normalization; Promega, Madison, WI.) was used to transfect cells in each well. In all transfections the Fugene6 reagent was used at a ratio of 1:3 (micrograms of DNA: microliters of Fugene6 reagent), as recommended in the manufacturer's protocol. The DNA/reagent mixture was incubated at room temperature for up to 45 minutes. Within a given experiment all transfections contained the same total amount of DNA. At the time of transfection (~12 hrs post-plating), cells were approximately 80% confluent and had initiated cell division. The following day (24 h after transfection), cells were treated with chemicals as indicated in the figures. If chemical treatment was not performed, cells were lysed and gene-expression assay was performed 24 h post-transfection. In all treatments media levels of DMSO levels never exceeded 0.1% [vol/vol]. On the last day (24 h after chemical treatment), cells were washed with PBS and luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) and a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA). Previous studies in our laboratory found that dilution of luciferase reagent had no effect on normalized luciferase values, therefore the luciferase assay and stop & glow reagent were diluted with 1XTBS (Tris Buffer Saline, pH 8.0) to 0.5X concentration. All other aspects of the assay were performed in accordance to the manufacturer's protocol.

3.3.5 Chemical Treatment

Following the hepatocytes arrival, Matrigel™ was added (day two post harvest), then the media was replaced on day three. With these conditions the hepatocytes adapted to the new culture conditions for ~ 4 days before exposeure to test agents.

Prior to the cell lines undergoing treatment; BEAS-2B and HepG2 cells were plated at a density of 50,000 cells/well and incubated overnight. Chemical inducers were dissolved in dimthyl sulfoxide (DMSO), and DMSO was maintained at 0.1% for all treatment and control (24 hrs).

3.3.6 RNA isolation and quantitative Real-Time PCR

For human cell lines and primary hepatocytes, the media were aspirated and 1 ml of TRIzol® Reagent (Invitrogen, Carlsbad, CA) was added directly to each well of 6-well plate. RNA was isolated and processed according to the manufacturer's instructions. Extracted RNA was treated with DNA-free™ DNase Treatment and Removal Reagents (Ambion, Inc., Austin, TX) according to manufacturer's instructions to remove contaminating DNA. RNA concentrations and qualities were assessed with UV absorbance at 260 nm, using a SmartSpec 3000 spectrophotometer (BioRad, Hercules, CA).

Total RNA was converted to cDNA using the High Capacity cDNA Archive Kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Real-time PCR was performed using Custom Taqman Gene Expression Products following the manufacturer's standard protocol (Applied Biosystems). The E1 variant was detected using the forward primer (5'- CTCCACAGCTCTCTTTCCCAA -3'), reverse primer (5'CCACCAGGCTCCACGTT -3') and probe (5'- TCACCCTCTGATTACTCC -3'). Similarly,

the E1-b variant was detected using the forward primer (5'- GATCGCGCGCCTGC -3'), reverse primer (5'- GTGAGGAGGATTTCTAGCCACATG -3') and probe (5'- CTCGCAGGCTCCGGC -3'). Real-time RT-PCR data were analyzed using methods previously described (Page et al., 2007;Olsavsky et al., 2007). Assays were prepared according to the manufacturer's recommendations for a 50-µl reaction volume (5 µl cDNA, 1x TaqMan® Universal PCR Master Mix, and 1x Assays-on-Demand™ Gene Expression Assay Mix containing unlabeled PCR primers and TaqMan® FAM™ dye-labeled MGB probe), divided into duplicate 25 µl reactions in a 96-well plate, and conducted using an Applied Biosystems 7300 Real-Time PCR System. Thermal cycling consisted of a UNG activation step for 2 min at 50°C and an iniial denaturation step for 10 min at 95°C followed by 40 cycles of denaturation (15 s at 95°C) and annealing/extension (1 min at 60°C). Plasmids containing cloned E1 or E1-b full length sequences were diluted to create standard curves, ranging from 30 copies to 3x 10^7 copies. DNA samples of known target sequence were used to verify the specificity of the assays.

3.3.7 Western Immunoblotting.

Equal amounts (10µg) of total cell lysate were loaded on a 10% SDS-polyacrylamide gel. After separation, proteins were transferred to a polyvinylidene fluoride membrane. Detection of EPHX1 proteins was performed after membranes were incubated with EPHX1 peptide-directed polyclonal antibody, and mouse anti rabbit secondary antibody as described previously (Laurenzana et al., 1998). Signals were visualized using the Lumi-Light Western blotting substrate (Roche Diagnostics, Inc., Indianapolis, IL). Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) antibody (Sigma-Aldrich, Inc., St. Louis, MO) was used as a loading control.

3.3.8 Statistical analysis.

All statistical analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software Inc., San Diego, CA). When error bars are shown, data are reported as mean ± standard deviation, which were obtained from three independent experiments. Significant differences were designated in instances where p was < 0.05. One-way analysis of variance in combination with Tukey's post hoc test was used to determine significance of the difference in the EPHX1 transcript levels after chemical treatments and changes of luciferase activity after Nrf2 co-expression and/or treatment.

3.4 Results

3.4.1 Sulforaphane and PPITC inhibit EPHX1 expression in human lung cell line.

Using quantitative real-time RT-PCR, the EPHX1 E1-b transcript level in BEAS-2B was determined following SFN and PPITC treatment in the concentration range of 0-20 μM. Below 10 μM, there was no observable effect on cell viability by either compound. At higher concentrations (20μM), cell counts in BEAS-2B cells confirmed that SFN caused a significant decrease in cell viability (data not shown). This is in agreement with previous investigations showing that SFN reduced relative viability to 50% at 20 μM in A549 cells (Harris and Jeffery, 2007). However, at 20 μM PPITC had no observable effect on the viability of BEAS-2B cells. SFN caused a significant decrease in EPHX1 mRNA level at concentration as low as 5 μM (Figure 3-1A). EPHX1 protein, detected by the anti-EPHX1 antibody from the total cell extract, showed a decrease at 5 and 10 μM SFN compared to DMSO treated control cells (Figure 3-1B). PPITC was less potent compared to SFN, the significant decrease in E1-b transcript level was not

observed until a concentration of 10 μM had been reached (Figure 3-1A). However, SFN caused a significant change in EPHX1 protein level at 5 μM (Figure 3-1B).

3.4.2 Sulforaphane increase EPHX1 expression in liver cell lines and hepatocytes

The effect of SFN on EPHX1 expression in HepG2 cells and primary human hepatocytes was opposite of the BEAS-2B lung cell line. Following the treatment in the concentration range of 0-10μM, the E1 and E1-b transcripts of the HepG2 cell line exhibited a significant increase (Figure 3-2A&B), and the treatments had no observable effect on the cell viability. While E1 and E1-b transcripts are two separate EPHX1 isoforms, produced by alternative promoter usage, SFN treatment induced a dose-dependent increase in both the E1 and E1-b mRNA transcript level. The western blot results clearly confirmed these results at the protein level, as the EPHX1 protein levels also markedly increased subsequent to the SFN treatment in HepG2 cells (Figure 3-2C). In constrast, PPITC treatments did not produce a significant change in E1 or E1-b EPHX1 mRNA atranscript level (Figure 3-2A&B) at any of the doses studied.

Human hepatocytes maintained under well-defined experimental conditions have been used as a valuable *in vitro* method to model biological processes in the human liver. To examine the effects of SFN on human EPHX1 gene expression in liver, we exposed cultures of primary human hepatocytes isolated from several donors to various doses of SFN. There was no obvious cellular toxicity following the SFN treatment during the 24-hours duration. A representative induction of EPHX1 mRNA expression, obtained from HH1428, is presented in Figure 3-3. At 10 μM SFN, E1 and E1-b transcripts expressed approximately 2.6-fold and 3.5-fold induction, respectively, compared with the corresponding control cells. The western blot result indicated the significant increase in EPHX1 immunoreactive protein levels after the SFN treatment in HH1428

(Figure 3-3C). In all hepatocytes tested, 10 μM SFN treatment resulted in increased of EPHX1 mRNA and protein levels (data not shown).

The selenium analog of sulforaphane (SFN-isoSe) and other isoselenocyanates (ISCs), which are more effective at certain endpoint measures than their corresponding ITCs (Sharma et al., 2008), were evaluated in human hepatocytes for their ability to induce EPHX1 expression (Table 3-1). Our results suggested that SFN-isoSe induces EPHX1 mRNA level, and that protein expression is also upregulated (data not shown). However, we detected no effect by ISC2, and ISC6, which likely resulted from the substantial decrease in cell viability noted by these respective treatments. ISC4, which also appeared to produce cellular apoptosis at the doses tested, shows some induction in one donor, but no effect was seen in other samples. Oltipraz and chlorophyllin, which are potent chemopreventive agents, were also tested in human hepatocytes; however, no significant results were observed with these agents (Table 3-1).

3.4.3 E1-b -300bp promoter region is responsive to treatment

The finding that E1-b EPHX1 transcripts exhibit unique expression patterns after SFN treatment led us to hypothesize that the regulation is mediated through the E1-b 5'-promoter context and that Nrf2 may be a key player in these respects. Sequence analysis of the 5' promoter region revealed several potential ARE-like motifs (Figure 3-4A). We further examined which segment of the 5'-flanking region was responsive to SFN treatment. Both the characterized -300 bp promoter region and the more encompassing 1.5 kb upstream region of E1-b were subcloned into luciferase reporter constructs, and analyzed by transient transfection in the BEAS-2B cell line. In agreement with published data, the -300 region of E1-b contributed the highest basal transcriptional activity for the E1-b promoter (Liang et al., 2005). As shown in Figure 3-4B, the SFN treatment of cells transfected with the -300bp construct resulted in the reduction of

transcriptional activity, indicating that the -300 E1-b promoter region may contain *cis*-regulatory elements.

3.4.4 Nrf2 contributes to the transcriptional regulation

Because sequence analysis of the E1-b promoter region revealed the potential presence of several ARE motifs, we examined the ability of Nrf2 to modulate expression of the transcript. For these studies, we transfected BEAS-2B or HepG2 cells with the EPHX1 E1-b promoter construct with or without Nrf2-expressing plasmid (Figure 3-5). When E1-b promoter alone was transfected into BEAS-2B or HepG2 cells, the results were similar to our previous studies, i.e., SFN decreased luciferase activation in BEAS-2B cells, whereas it increased transcriptional activity in HepG2 cells. Co-transfection of BEAS-2B cells with the Nrf2-expression plasmid and the E1-b promoter construct produced a similar decrease in transcriptional activity without SFN treatment. Furthermore, these effects were enhanced when cells were co-transfected with the E1-b promoter and Nrf2 and exposed to SFN treatment for 24 hours.

3.5 Discussion

Human EPHX1 is a xenobiotic metabolizing enzyme that exhibits high protein sequence conservation between several vertebrate species (Hassett et al., 1994b). Additionally, EPHX1 is expressed widely across different tissues, and its activity is present early in the developing fetus (Omiecinski et al., 1994). These observations imply that the human EPHX1 gene product has important biological functions as a xenobiotic metabolizing enzyme. Furthermore, investigations using EPHX1 knockout mice have demonstrated that this enzyme plays a key role in the bioactivation of carcinogenic polyaromatic hydrocarbons (Miyata et al., 1999).

Chemopreventive compounds have gained considerable attention that act as primary protective agents by preventing, delaying or reversing carcinogenesis (Clarke et al., 2008;Yates and Kensler, 2007). Isothiocyanates, found as daily-consumed dietary compounds, are of particular interest because of their easy accessibility to the general population and many ITCs have been used in cancer chemoprevention clinical trials (Kensler et al., 2003). In this study, we have shown for the first time that human EPHX1 gene is responsive to chemopreventive agents. The effects of several cancer inhibitory ITCs on human EPHX1 expression patterns were evaluated in BEAS-2B, HepG2 cell lines and human primary hepatocytes. After SFN treatment, the EPHX1 mRNA and proteins levels were decreased in human BEAS-2B lung carcinoma cells but were increased in the human HepG2 hepatoma cell line and in human hepatocytes. EPHX1 expression was decreased after exposure to PPITC in BEAS-2B lung carcinoma cells but no obvious changes were detected in hepatic cells.

Accumulating evidence suggests that SFN is a potent preventive agent, due to its ability to confer chemoprotection through multiple pathways (Myzak and Dashwood, 2006;Juge et al., 2007). Early studies have shown that SFN is a potent inducer of phase II detoxification genes such as glutathione S-transferase, through the activation of the Nrf2 transcription factor. Under quiescent conditions, Nrf2 is sequestered in the cytoplasm by repressor protein Keap1. Upon the treatment with SFN, the interaction between Keap1 and Nrf2 is disrupted, and activated Nrf2 translocates to the nucleus, where it binds to antioxidant responsive elements (AREs) in the promoter region of target genes (Kensler et al., 2007;Kensler et al., 2003). Recently, microarray analysis of knockout mice treated with SFN identified a number of cytoprotective genes, which are directly or indirectly dependent on Nrf2 for transcriptional activation in response to SFN (Thimmulappa et al., 2002). Epoxide hydrolase was reported as one of the xenobiotic metabolizing enzymes dependent on Nrf2 for basal as well as inducible expression. The identification of novel downstream mediators for SFN chemoprevention facilitates the

understanding of cellular defenses against carcinogen and other toxins.Consistent with these findings in the rodent model, we have shown that the expressions of human EPHX1 was changed after exposure to SFN in cell lines and hepatocytes. Our results also suggest that SFN-induced changes in EPHX1 transcription are modulated by the Keap1-Nrf2 pathway. AREs have been detected in the promoters of of phase II genes like GST, mediating chemoprotective response through Nrf2 binding. We show that the -300bp region of E1-b promoter is regulated by SFN treatment, and that it harbors several ARE-like sequences. Overexpression of Nrf2 transcription factors repressed the E1-b promoter activity in BEAS-2B cells and increased E1-b promoter activity in HepG2 cells.

The results presented here define two distinct effects of SFN in cell lines derived from different human tissues and therefore raise the question as to the biological importance of the tissue-specific responses. Because the liver is an essential organ in detoxifying toxic compounds, high levels of biotransformation enzymes are presumed beneficial to provide an enhanced threshold of protection. In human liver, where extremely reactive epoxides intermediates generated via cytochrome P450 activation, detoxication activities tend to be high via usage of two alternative promoters driving additional hepatic expression of EPHX1. In a recent study using a rat model of aflatoxin-induced hepatic tumorigenesis, genes contributing to aflatoxin detoxication, namely EPHX1, were up-regulated in liver upon low dose of chemopreventive agent treatment (Yates et al., 2006).

Lung cancer is the most common malignancy in the world and most lung cancer cases might be associated with the environmental carcinogens, such as tobacco smoking. PAHs are pollutant occurring from incomplete combustion of organic compound (Harvey, 1991;Sims et al., 1974). They are of concern because they are identified as carcinogens and are widespread in the environment, such as in automobile exhaust, cigarette smoke, ambient air, water and soil (1973;1983;1984). EPHX1 bioactivates PAHs to exert their carcinogenic and genotoxic effects.

Previously, our laboratory characterized two non- synonymous amino acid changes in human EPHX1 coding region, an exon 3 polymorphism corresponding to amino acid position 113, with a Tyr to His substitution, and an exon 4 polymorphism correspondin to amino acid position 113, with a resulting His to Arg substitution (Hassett et al., 1994a). Available data from epidemiology studies indicates that there is a strong association between EPHX1 coding region polymorphisms and individual susceptibility to lung cancer. For example, using a 13-study meta analysis approach, it was reported that 'low activity' phenotypes seem to be protective against lung cancer (Kiyohara et al., 2006).

It should be noted that after exposure to SFN, the induction of both E1 and E1-b expression in hepatic cells was significantly correlated. The expression profile of E1-b transcript is apparently ubiquitous, while E1 appears subjected to tissue-specific regulation, with liver expressing the highest levels of E1 and E1-b transcripts. Evidence supporting the common liver-specific regulation of E1 and E1-b is derived from the observation that transcription factors GATA-4 and HNF3 participate in the regulation of both E1 and E1-b promoter activities (Liang et al., 2005).

Although additional studies are required to assess the regulation by Nrf2, it is clear that human EPHX1 expression is regulated by chemopreventive agents, SFN and PPITC, and these results provide insight into how EPHX1 is involved as providing cellular defense against carcinogens, and may offer an exciting new target for achieving cancer chemoprevention. Future analysis of the Nrf2/ARE regulatory mechanisms underlying the control of EPHX1 expression in different cell types should facilitate the necessary molecular understanding for potential development of EPHX1 as a target for chemopreventive agents, in particular for the lung.

Table 3-1 Effects of chemopreventive chemicals on EPHX1 mRNA expression in different cells. Real-Time PCR was performed with total RNA obtained from chemically treated (24h) cells and DMSO controls. For hepatocytes, results were reported as maximal change from one representative donor. ISC2, ISC4 and ISC6 treatments resulted in 50% ~ 95% reductions in cell viability estimated by cell morphologic change. ITCs, isothiocyanates. SFN, sulforaphane. PPITC, phenylpropyl isothiocyanate. ISCs, isoselenocyanates. SFN-isoSe, sulforaphane isoselenocyanate. ISC2, phenethyl isoselenocyanate. ISC4, phenylbutyl isoselenocyanate. ISC6, phenylhexyl isoselenocyanate. ND, not determined.

Compounds		Fold Change			
		Liver Hepatocytes	Liver HepG2	Lung A549	Lung BEAS-2B
ITCs	SFN 10μM	6.69 ± 0.03 ↑	2.59 ± 0.47 ↑	0.48 ± 0.02 ↓	0.04 ± 0.02 ↓
	Ergothionine 10μM	2.27 ± 0.11	ND	ND	1.10 ± 0.01
	PPITC 20μM	1.26 ± 0.10	0.89 ± 0.36	0.30 ± 0.02 ↓	0.49 ± 0.02 ↓
ISCs	SFN-isoSe 10μM	4.18 ± 0.68 ↑	ND	ND	0.55 ± 0.02 ↓
	ISC2 10μM	1.02 ± 0.02	ND	ND	0.16 ± 0.01
	ISC4 10μM	4.53 ± 0.07	ND	ND	ND
	ISC6 10μM	1.87 ± 0.86	ND	ND	0.24 ± 0.01
Drug	Oltipraz 25μM	0.92 ±0.06	1.09 ± 0.02	0.94 ± 0.17	ND
In diet	Chlorophyllin 50μM	1.13 ±0.05	1.06 ± 0.10	0.88 ± 0.01	ND

Values are mean ± S.D.

Up/Down arrow indicates significant ($p < 0.01$ vs. control; t-test) increase/decrease and no cellular cytotoxicity.

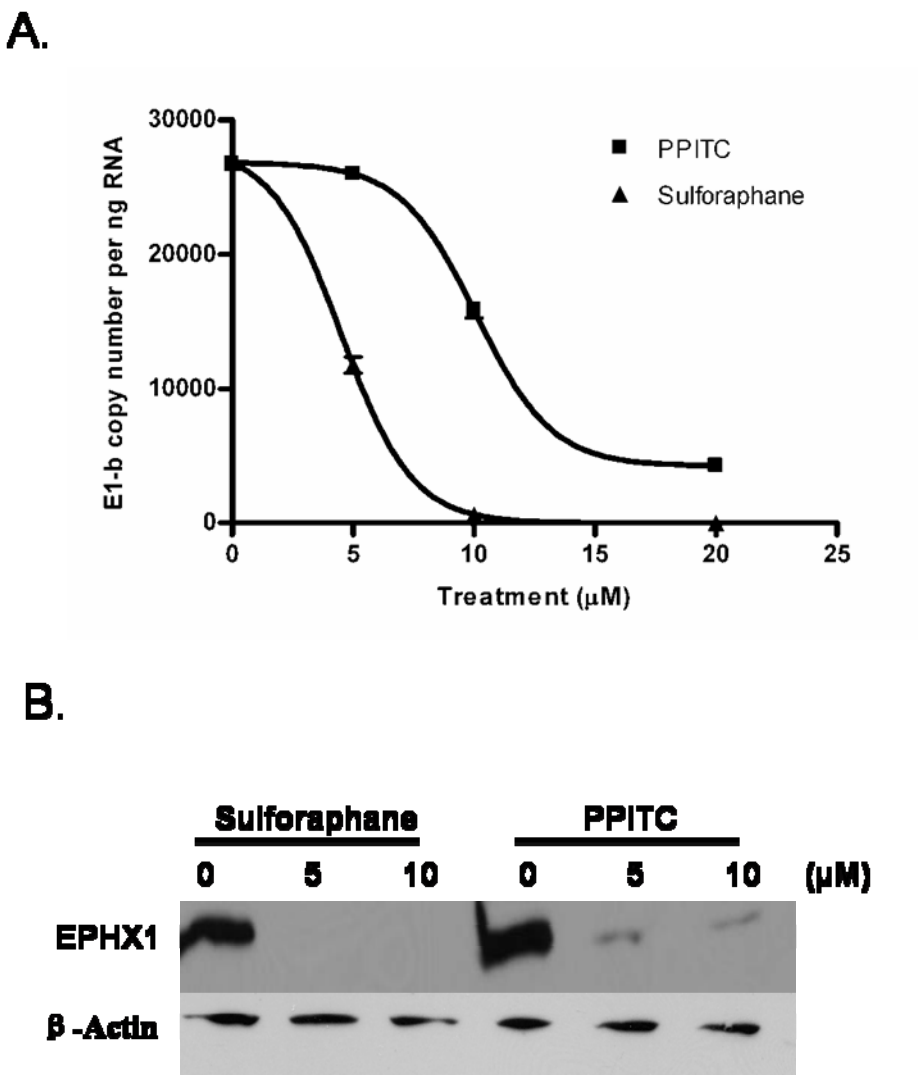


Figure 3-1. Inhibition of EPHX1 expression in BEAS-2B cell lines by Sulforaphane and PPITC. (A) EPHX1 E1-b transcript expression in BEAS-2B was quantified by TaqMan real-time PCR assay after Sulforaphane (0, 5, 10 or 25 μM) and PPITC (0, 5, 10 or 25 μM) treatment for 24 h. (B) Western immunoblot blot analysis assessing corresponding EPHX1 protein levels. The β-Actin blot is shown as a reference for the equal loading of total proteins.

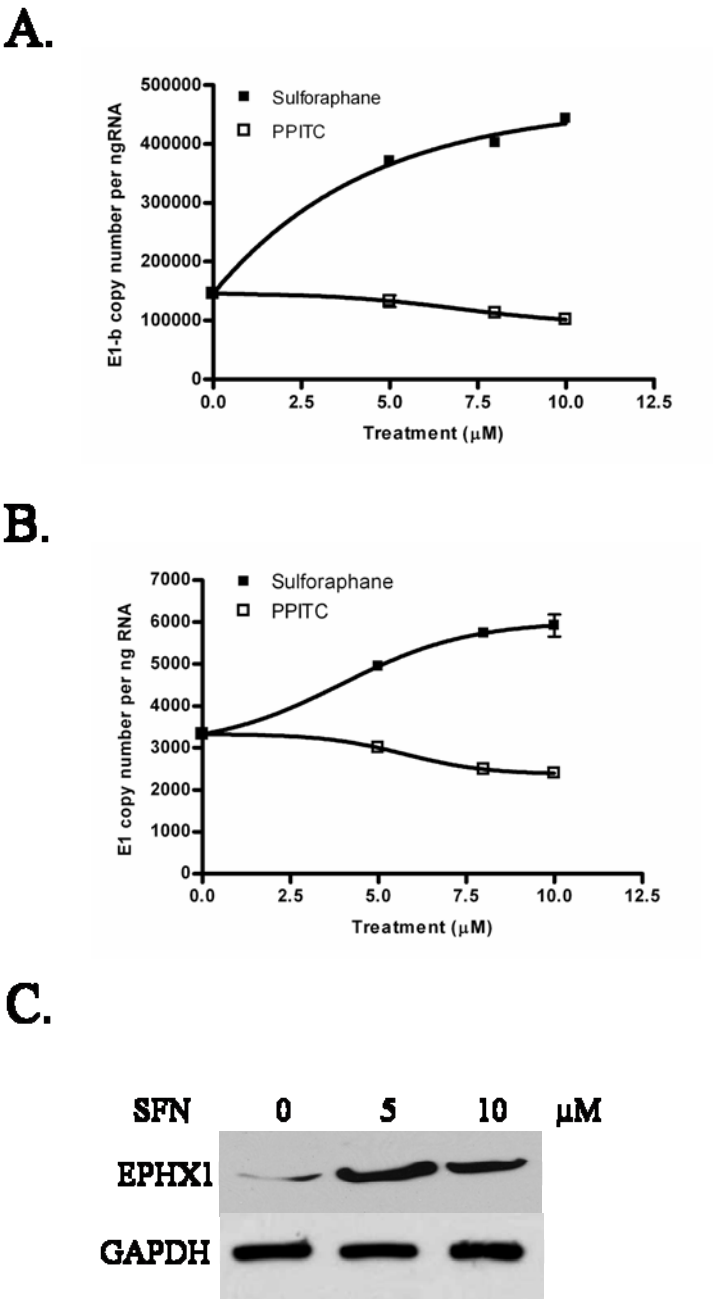
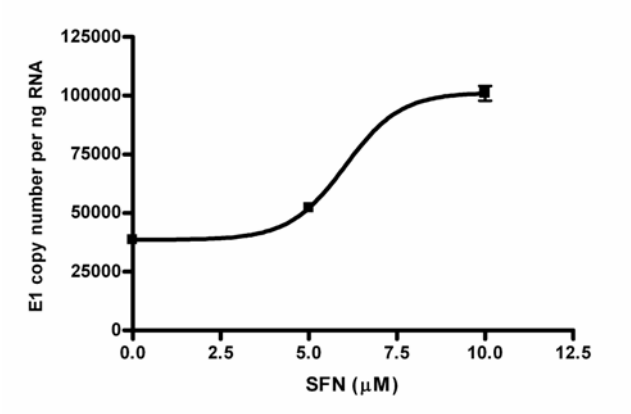
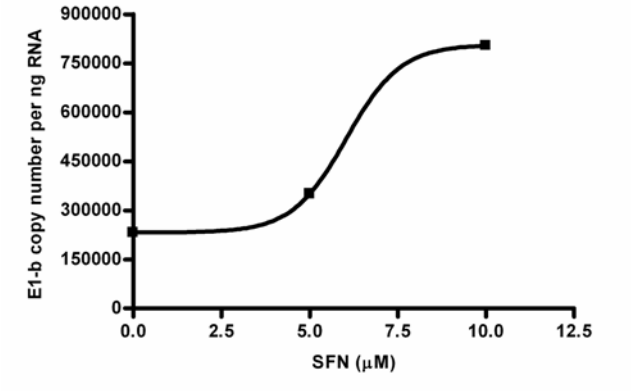


Figure 3-2: Induction of EPHX1 expressions in HepG2 cell lines by sulforaphane. (A–B) EPHX1 E1 (panel A) and E1-b (panel B) transcripts expression was quantified by TaqMan real-time PCR in cultured HepG2 treated with SFN (0, 5, 8 or 10 μM) and PPITC (0, 5, 8 or 10μM) for 24h. (C) Western immunoblot blot analysis assessing corresponding EPHX1 protein levels. GAPDH is shown as a reference for the equal loading of total proteins.

A.



B.



C.

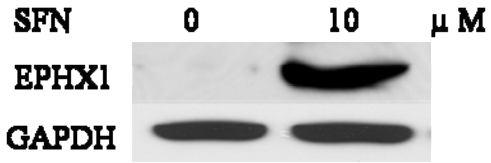


Figure 3-3. Induction of EPHX1 expressions in human hepatocytes by sulforaphane treatment. (A–B) EPHX1 E1 (panel A) and E1-b (panel B) transcripts were quantified by TaqMan real-time PCR using RNA extracted from hepatocytes after exposure to SFN (0, 5 or 10 μM) for 24h. (C) Western immunoblot blot analysis assessing corresponding EPHX1 protein levels. GAPDH is shown as a reference for the equal loading of total proteins.

A

GGTGATAGAGTGAGACTCTGTCTCCAAAAAAAAAAAGGACATACATCATGCAAGTTTG

GATTTTTGTTTTTAGATTCAACTGACGAAGTTACGGGATCAAACTGCTGTAGGAGCTG

ARE-1 ARE-2

CCAAACGCTTCTCTCCATTTCTGGCCGCGGGGCCGCGGACCGCCCTTTAAGTAGCCCG

ARE-3

TTTTATCCCTGGCAGAGGTGGAGCCTTAGGCAGGCCTAGAGACTTTCCCGGGTCCTCC

ARE-4

AGGCCGGGGAACGCCCCGCTCGGAGGCCGGGCTTGGGCGGAGACTGCGCCGGGGCTGC

TGAAAACTAGCCGAGGAGAGCCAGGGAGCCGGAGAGATCGCGCGCCTGCCGCCGCCGG

+1

AGCCTGCGAGCCGAGACC

B

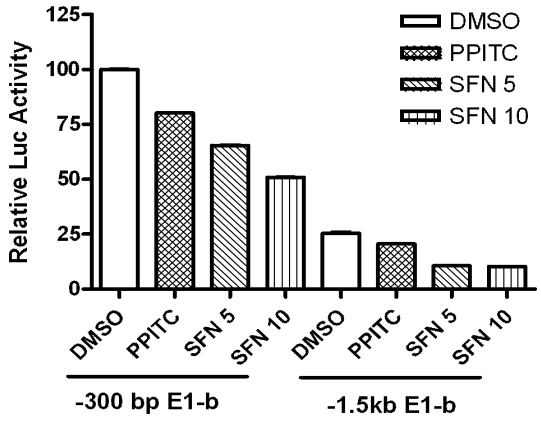


Figure 3-4. Effect of sulforaphane and PPITC treatment in E1-b upstream region promoter activity. (A) The E1-b -320bp 5'-upstream region was scanned for the currently accepted ARE consensus sequence. The transcription start site is indicated by +1. Four ARE-like motifs are indicted by underlined letters. (B) BEAS-2B cells were transfected with -300bp or -1.5Kb E1-b promoter sequence in pGL3 vector. Subsequently, transfected cells were treated with 5μM PPITC, 5μM or 10μM SFN or DMSO for 24h and cell lysates were analyzed for luciferase activity.

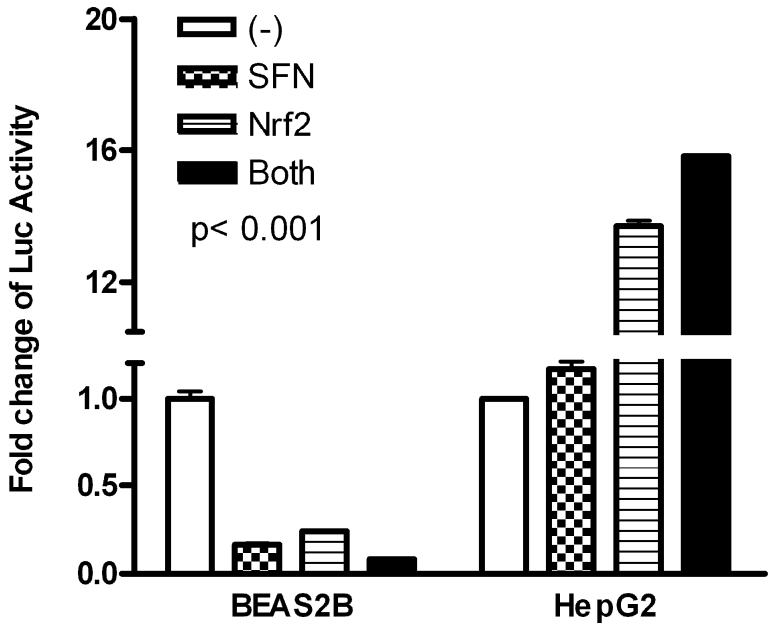


Figure 3-5. Modulation of E1-b promoter activities by Nrf2 protein. Cells were co-transfected with the E1-b -300bp promoter construct and Nrf2 or empty control vector and subsequently treated with SFN or DMSO. At 24 hr post-treatment, cells werelysed and assayed for luciferase activity. The data shown depict means and S.D. values derived from three separate experiments, each performed in duplicate. The co-expression or/and SFN treatment significantly changed the luciferase activity compared to control ($p < 0.001$, ANOVA).

Chapter 4

Translational Regulation of Human Microsomal Epoxide Hydrolase is Mediated by mRNA 5'-Untranslated Region Diversity

4.1 Abstract

Post-transcriptional regulation may participate in controlling EPHX1 activity in human tissues, as indicted in a previous study which showed that EPHX1 activity highly correlated with EPHX1 protein level but did not correlate with mRNA level. Rolling Circle Amplification - Rapid amplification of cDNA ends (RCA- RACE) analysis was carried out to characterize UTRs and to determine whether signals reside in the EPHX1 mRNA to account for translational regulation. These methods enabled us to identify a new EPHX1 E1-b' variant, in addition to the previously identified EPHX1 E1 and E1-b transcripts. E1-b' contains two upstream AUGs in its 5' GC-rich leader sequences that may inhibit the translation of the downstream open reading frame. Real-time -PCR was employed to verify and quantitate the E1-b' expression in numerous human tissues; in most tissues E1-b' was expressed with very low abundance among total EPHX1, but it exhibited relatively high expression levels in ovary (22%). Using an *in vitro* transcription/translation system and transfection expression in mammalian cell lines we demonstrated that, compared to the E1 and E1-b variants, the E1-b' 5'-UTR significantly inhibited translation activity. Mutation analysis suggested that the two uORFs acted synergistically to inhibit translation from the major ORF. Disruption of the uAUGs and sequential deletion of the 5'-leader sequence resulted in a corresponding increase in translation efficiency. Using a reporter assay system in which the different EPHX1 5'-UTRs were fused in front of the luciferase gene, we confirmed the effect of E1-b' 5'-UTR on translation. The first uORF of E1-b'

is in frame with the EPHX1 coding region, giving rise to an uORF containing 26 amino acids which terminates within the 5'-UTR region. In BEAS-2B cells, adding the E1-b' construct inhibited the translation of endogenous EPHX1. The frame-shift mutation of the first uORF, which encodes a similar length peptide but with entirely different amino acid content, lost the ability to decrease EPHX1 protein expression. E1-b and E1-b' mRNA may be driven by the same promoter as the luciferase assay results suggested the -300bp promoter region preceding the E1-b transcription start site also contributed to E1-b' transcription activity.

4.2 Introduction

Microsomal epoxide hydrolase (mEH, EPHX1) is an important enzyme that catalyzes the metabolism of numerous xenobiotic compounds, including epoxide derivatives of certain pharmaceuticals and epoxides of environmental toxins. In the case of polycyclic aromatic hydrocarbons, EPHX1 is involved in the bioactivation process, yielding highly mutagenic metabolites capable of forming DNA adducts (Lloyd and Hanawalt, 2000;Buterin et al., 2000). A study using the knockout mouse model illustrated EPHX1's procarcinogenic role; compared to their wild-type counterparts, mice deficient in EPHX1 were protected from carcinogenesis after exposure to DMBA (Miyata et al., 1999). EPHX1 also serves a cytoprotective function through its role in detoxification of many reactive epoxides to the less toxic dihydrodiols (Fretland and Omiecinski, 2000). In addition to xenobiotic metabolism, there are several lines of evidence suggesting an endogenous role for this enzyme, in estrogen metabolism, bile acid transport and in the vitamin K reductase complex (Guenthner et al., 1998). The expression of EPHX1 has been found in nearly all human tissues with maximum activity in liver, however activity levels between individuals are highly variable (Hassett et al., 1998;Newman et al., 2005). While a variety of parameters are known to contribute to the interindividual variability, genetic

polymorphism is clearly of major importance. Numerous studies have suggested that human EPHX1 gene is highly polymorphic; documented polymorphisms include two non-synonomous polymorphisms in the structural region and genetic polymorphisms in the promoter region and intron regions.

Alternative promoters are used to drive expression of human EPHX1 transcripts in a tissue-specific manner. A recent investigation by Liang et al. reported that the transcription of the human EPHX1 gene is initiated at two different promoters (Liang et al., 2005). E1, which is initiated from a proximal promoter close to the EPHX1 coding region, was expressed specifically in liver, whereas the E1-b promoter, localized ~18.5 kb upstream, was used ubiquitously in all tissues tested. The human EPHX1 coding region extends from exon 2 to exon 9, therefore despite distinct non-coding exon 1's, the E1 and E1-b transcripts generate the same EPHX1 protein.

As indicted in a previous study, post-transcriptional regulation likely also participates in controlling EPHX1 activity in human tissues (Laurenzana et al., 1998). In a study of EPHX1 protein content, enzyme activity and mRNA level in human liver and lung, Omiecinski et al.found that while EPHX1 enzyme activity was highly correlated with EPHX1 protein level, this correlation was not observed between activity and mRNA level (Omiecinski et al., 1994). Post-transcriptional control can happen at several steps before the protein is synthesized including mRNA processing, mRNA localization, mRNA stabilization and translational regulation (Audic and Hartley, 2004). One possible mechanism is manifested at the 5' untranslated regions (UTRs) of mRNA and involves upstream open reading frames (uORF) and RNA secondary structure (Morris and Geballe, 2000;Kozak, 2005). In the 5'-UTR, an upstream synthesized small peptide may affect ribosome reinitiation leading to lower translation efficiency, while the presence of complex secondary structure may cause the ribosome to pause during translation.

In this study, we identified the existence of a novel EPHX1 variant, termed E1-b', that contains two uORFs in the 5'-UTR. The presence of these uORFs suggests that this transcript can

be differentially regulated at the translational level. We investigated the differences in the translational potential contributed by 5'-UTR-containing EPHX1 constructs or 5'-UTR-containing-luciferase reporter constructs in cell-free system and transfected cell lines. We found that the uORFs in the 5' region of E1-b' impart an inhibitory effect on EPHX1 expression from the main downstream initiation site. We also found that the length of the 5'-leader sequence affects the translation efficiency. These data indicate that the uORFs can be recognized and translated by the ribosome, and that the synthesized peptide alters translation efficiency of the primary EPHX1 transcript.

4.3 Materials and Methods

4.3.1 Materials

Unless indicated otherwise, all chemicals were purchased from Sigma (St Louis, MO), and all cell culture consumables were purchased from VWR Scientific (West Chester, PA). Primers for PCR were purchased from Integrated DNA Technologies (Coralville, IO).

4.3.2 Cell Culture

The human lung epithelial cell line BEAS-2B and the human kidney cell lines 293A and 293T were maintained in Dulbecco's modified Eagle medium supplemented with 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 0.15% sodium bicarbonate, 50 units/ml penicillin G and 50 µg/ml streptomycin (all purchased from Invitrogen Life Technologies, Carlsbad, CA). The human hepatoma cell line HepG2 and the human lung carcinoma cell line A549 were grown in minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 0.1 mM

nonessential amino acids, and 1.5 g/L sodium bicarbonate. All cells were maintained at 37˚C with 5% CO_2.

One day prior to transfection, cells were trypsinized and plated onto 24-well plates (100,000 cells/well). Transfections were performed according to the manufacturer's recommendations.

For protein expression studies, 500 ng E1, E1-b or E1-b' were transfected into 1 well of a 6 well plate using the Fugene6 transfection reagent. Cells were incubated after the transfection mixture was added and harvested 24 h after transfection as detailed below.

4.3.3 Rolling Circle Amplification- Rapid Amplification of cDNA Ends (RCA-RACE)

A newly developed Rapid Amplification of cDNA Ends method (Polidoros et al., 2006) was used to simultaneously analyze the EPHX1 5' and 3'UTR. RNA (2ug) was reverse transcribed into first-strand cDNA in a reaction containing 0.5 μg oligo(dT)-adaptor primer [5'-GGCCACGCGTCGACTAGTAC$(T)_{18}$-3'] phosphorylated at the 5' end, 0.5 mM dNTPs, 10 mM dithiothreitol (DTT), 1× first strand buffer (Invitrogen, Paisley, UK), and 200 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen). The mixture was incubated at 37°C for 1 h followed by heat inactivation of reverse transcriptase at 70°C for 15 min. The RNA strand was then removed by the addition of 1 μl RNaseH (Invitrogen), incubated at 37°C for 20 min and purified using the QIAquick® PCR purification kit (Qiagen, USA). The purified cDNA (16 μL) was circularized using 1 μl CircLigase (Epicentre Biotechnologies) at 60°C for 1 h followed by inactivation of the enzyme at 80°C for 10 min. The circularized cDNA was then amplified in a RCA reaction using the Φ29 DNA polymerase and random primers.The 50 μl RCA mixture contained the following: 10-μL circularized cDNA, 1 mM dNTPs, 200 μg/mL bovine serum albumin (BSA), 1× Φ29 DNA polymerase reaction buffer (New England Biolabs), 1 μL Φ29

DNA polymerase, and 10 μM random hexamers modified by the addition of two phosphothioate linkages on the 3′ end to confer resistance to the Φ29 exonuclease activity. Utilizing the serially diluted RCA reaction as a template, PCR was carried out using AccuPOL DNA polymerase and EPHX1 forward (5'-TGGCAAAGTTTCCTCTTTGTCCCG -3') and reverse (5'-ATGGAGGCCTGGAAAGGAAGTTCT-3') primers. The PCR products were sequenced with specific E1, E1-b or E1-b' primers using a CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc., Fullerton, CA).

4.3.4 Plasmids

RNA was isolated from cultures of primary human hepatocytes using TRIzol® Reagent (Invitrogen, Carlsbad, CA) and total RNA was converted to cDNA using the High Capacity cDNA Archive Kit. DNA primers used for the PCR amplification were: EPHX1 E1 FP, (5'-GCCCTTAGAGCATCG - 3'), E1-b FP, (5'-GATCGCGCGCCTGC - 3'), and E1-b', (5'-AGGGAATTCCGCGTCCC -3'); and RP, (5'- GGTCATTGCCGCTCCAGCACCGAC-3'). Mutation of the uORFs in the E1-b' 5'-UT was performed using the indicated primers by a two-step PCR protocol (Table 4-1). Purified products were separated by agarose gel electrophoresis and DNA fragments were purified using a Qiagen gel purification kit (Qiagen, Chatsworth, CA). Purified fragments were ligated into the KpnI/BamHI sites of pcDNA3.1 and transformed into DH5α ultracompetent cells (Invitrogen, Carlsbad, CA). Colonies were screened by PCR to confirm the presence of the insert. Positive clones were grown overnight in 2 ml cultures, purified, and sequenced verified. Prior to transfection, plasmids were prepped using the Quantum Prep Plasmid Maxiprep Kit (Bio-Rad, Hercules, and CA).

4.3.5 Quantitative Real-Time PCR

Human tissue RNA was obtained from the FirstChoice Human total RNA survey panel (Ambion, Austin, TX). The total RNA was converted to cDNA using the High Capacity cDNA Archive Kit according to the manufacturer's instructions. Real-time PCR was performed using Custom Taqman Gene Expression Products (Applied Biosystems, Foster City, CA) following the manufacturer's standard protocol. The E1 variant was detected using the forward primer (5'-CTCCACAGCTCTCTTTCCCAA -3'), reverse primer (5'CCACCAGGCTCCACGTT -3') and probe (5'- TCACCCTCTGATTACTCC -3'). Similarly, the E1-b variant was detected using the forward primer (5'- GATCGCGCGCCTGC -3'), reverse primer (5'-GTGAGGAGGATTTCTAGCCACATG -3') and probe (5'- CTCGCAGGCTCCGGC -3'). E1-b' variant was detected using the forward primer (5'- CGGCGGTGAAATGCACTTAATT -3'), reverse primer (5'- CACGGACGCGCATGAAAAT -3') and probe (5'-CCAAGTCGGAACACTG -3'). Real-time RT-PCR data were analyzed using methods previously described (Page et al., 2007;Olsavsky et al., 2007). Plasmids containing cloned E1 or E1-b full length sequences were diluted to create standard curves ranging from 30 copies to 3x 10^7 copies. DNA samples of known target sequence were used to verify the specificity of the assays.

4.3.6 Protein Expression in Human Cell Lines

One day prior to transfection cells were plated in 6-well plates at a density of ~400,000 cells/well. Cells were transfected with 2 μg of EPHX1 expression plasmid using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's protocol. Whole cell lysates were generated 24 hr post-transfection by sonication in RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-

40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA). Following centrifugation, supernatant was stored at -20° C for subsequent analysis.

4.3.7 In vitro Transcription and Translation

PCR was employed to generate the full-length cDNA sequence of the EPHX1 variants (E1, E1-b and E1-b') or the mutated versions thereof, along with a 5' T7 promoter region directing 3' polyadenylation. The PCR products were then used as templates for transcription in the TnT® Quick-coupled reticulocyte lysate (Promega Corp., Madison, WI). Protein expression was carried out in the presence of [^{35}S]-methionine as described in the manufacturer's protocol. Protein was separated in a pre-cast 10% Tris-HCl gel (Biorad); the gel was subsequently dried and exposed to film.

4.3.8 Preparation of EPHX1-Luciferase Constructs

The chimeric EPHX1-Luciferase reporters were constructed such that the unique EPHX1 5'-UTRs were positioned directly upstream of the luciferase reporter gene. The three EPHX1 5'-UTRs were amplified using the forward primers HindIII E1, HindIII E1-b and HindIII E1-b', respectively, and the reverse primer EPHX1-LucR such that the amplicons contained a HindIII site on the 5' end and a segment of the luciferase gene with a NarI site at the 3' end (Table 4-1). The fragments were then digested with HindIII and NarI and cloned into pGL3 vector (Promega) so that the various EPHX1 5'-UTRs replaced the luciferase 5'-UTR. The constructs were sequenced using a CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc., Fullerton, CA).

4.3.9 Western Immunoblotting.

Equal amounts (10μg) of total cell lysate obtained from HepG2 cells were loaded on a 10% SDS-polyacrylamide gel. After separation, proteins were transferred to a polyvinylidene fluoride membrane. Detection of EPHX1 proteins was performed after membranes were incubated with EPHX1 peptide-directed polyclonal antibody, followed by incubation with HRP-conjugated goat anti-rabbit antibody (Biorad, Hercules, CA). Proteins were visualized using the Lumi-Light Western blotting substrate (Roche Diagnostics, Inc., Indianapolis, IL). Detection of Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) using a GAPDH antibody (Sigma-Aldrich, Inc., St. Louis, MO) served as a loading control.

4.3.10 Cell Transfection and Luciferase Activity Assays.

A549, HepG2 and BEAS-2B cells were transfected with Lipofectamine 2000 (Invitrogen). The 293 cells were transfected using FuGENE 6 (Roche Diagnostics). The transfection procedure and luciferase assay were performed as previously described (Liang et al., 2005).

4.3.11 Secondary Structure Modeling

The secondary structure of EPHX1 E1-b' mRNA 5'-UTRs were modeled using the GeneQuest module of Lasergene version 6 software (DNASTAR, Madison, WI).

4.3.12 Statistical analysis

All statistical analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software Inc., San Diego, CA). The student's t-test or ANOVA followed by an

appropriate post-hoc test was used where appropriate. Results were considered significant when $p < 0.05$.

4.4 Results

4.4.1 Identification of E1-b' transcript by RCA-RACE

By employing stringent 5'-RACE methodology on liver RNA, our laboratory previously identified two EPHX1 transcripts with unique first exons. A survey of the NCBI AceView cDNA-based transcript annotation, however, indicated the expression of a third mRNAwith a distinct exon 1 (designated E1-b') in select human tissues. A review of the CAGE database further verified the presence of a third transcription initiation site in the human EPHX1 gene. We used the rolling circle amplification - Rapid Amplification of cDNA Ends (RCA - RACE) technique, which utilizes gene-specific primers to eliminate the high background found in conventional RACE due to the use of a universal primer corresponding to the anchor sequence, to isolate rare transcripts based on a pool of amplified circular cDNA templates. The RACE results from total RNA isolated from A549 and HepG2 cell lines revealed the presence of the E1-b' transcript and sequence analysis of the clones confirmed this finding. The E1-b' transcript contains a 206-nt 5'-UTR with two upstream AUGs (uAUG1 and uAUG2; Figure 4-1B) in front of the main ORF in exon 2.

The uAUG1 is inframe with the EPHX1 coding region giving rise to a uORF comprised of 26 amino acids which terminates upstream of the main AUG. The uAUG2 has the least favorable Kozak context compared to the uAUG1 and the main AUG; the suboptimal translation initiation context of the uAUG2 gives rise to a 17 a.a. uORF that is not in frame with the main

AUG and terminates at the +10 position. The E1-b' 3'-UTR shares similar sequences with E1 and E1-b.

The E1-b' 5'-UTR has a stable secondary structure, as modeled by Lasergene6 software (data not shown). This region was predicted to fold into a complex stem-loop structure comprised of nine stable individual hairpin structures with an overall Gibbs free energy value of $\Delta G = -65.58$ kcal/mol . Secondary structures with a free energy value of less than -30 kcal/mol are considered to impair translation (Kozak, 1989). Therefore, it is likely that E1-b' 5'-UTR inhibits translation of the main ORF due to its uAUG and stable secondary structure.

4.4.2 Sequence Comparison

The alignment results suggested that 5'-UTR region of E1-b' transcript is highly conserved between human and chimp, while conservation in rhesus is considerably reduced (Figure 4-2). As expected, two upstream AUGs found in human EPHX1 E1-b' transcript were also present in the 5'-UTR region of both human and chimp. The reading frames of the uORFs were highly conserved as well, as was the entire sequence encoding the small peptide. However, the second uAUG was not present in rhesus. Interestingly, in the human and chimp genomes EPHX1 is localized to the Chromosome 1 'plus' strand whereas in the rhesus genome EPHX1 is localized in the Chromosome 1 'reverse' strand.

4.4.3 Real-time RT-PCR Analysis of the E1-b' mRNA

To evaluate E1-b' transcript expression levels in various human tissues, a series of assays were performed. Northern blots were carried out to assess transcripts levels in MCF7, 293A, A549 and HepG2 cell lines. Results indicated that while E1 and E1-b were expressed at moderate

levels, E1-b' was barely detectable by the E1-b' specific probe (data not shown). To better characterize and quantitate the respective expression patterns of these EPHX1 transcripts, real-time RT-PCR analysis was carried out on total RNA isolated from 20 human tissues. The data were analyzed by absolute quantification in which a standard curve is utilized to relate the PCR signal to the copy numbers of input genes. Standard curves based on recombinant DNA are highly reproducible and exhibit a large quantification range (6 orders of magnitude) with specific and sensitive data. Using the standard curve, the expression level of each transcript is presented as copy number per 1 ng total RNA. The analysis confirmed that, of the total EPHX1 mRNA, E1-b' was expressed at a low level (less than 1% in all tissues except ovary). Previous studies have indicated that the liver is the tissue where EPHX1 is expressed most highly, however we found that E1-b' transcript levels are highest in the ovary (Figure 4-3). Although the E1-b' variant was detected in other tissues, the expression levels were significantly lower than ovary.

4.4.4 In vitro Translational Efficiency of the 5'-UTR EPHX1 mRNAs

We then analyzed the possibility that E1-b' might be translated inefficiently, because sequence comparison of the E1, E1-b and E1-b' 5'UTR highlighted the following features of E1-b': the presence of two uORFs and the potential to form stable RNA secondary structures.

To examine the effects of the various 5'-UTR sequences on EPHX1 translation, we constructed full-length EPHX1 gene containing different 5'-UTR (E1, E1-b and E1-b') and inserted them into eukartotic expression vector pcDNA3.1+. A construct comprised of the EPHX1 main ORF from exon 2 to exon 9 was served as the control. All constructs were tested in *in vitro* transcription/translation assays conducted in the rabbit reticulocyte lysate system and the protein products were visualized by the incorporation of ^{35}S. SDS-PAGE analysis revealed that the full-length E1 and E1-b products were translated at least as well as the control sequence.

However, translation products of the E1-b' sequence indicated that this transcript was barely translatable (Figure 4-4 A). Comparable results were obtained when the constructs were expressed in transiently transfected HepG2 cells; the translation mediated by the E1-b' 5'-UTR was only 20% as efficient as that mediated by the E1-b (Figure 4-4 B). These differences suggested that E1-b' 5'-UTR had a pronounced negative effect on the EPHX1 translation efficiency in the cell-free lysate and in the transfected cells.

4.4.5 Mutational and Deletion Effects on EPHX1 mRNAs Translational Efficiency

Many experiments have demonstrated that translational repression of an mRNA can be assuaged when uAUG codons are mutated. Thus, mutational analysis of the uAUGs (both individually and in combination) was employed to evaluate their respective contributions to translational repression. As shown in figure 4-5, mutation of the first uAUG (Mut1), which is in frame with the main ORF and located within a favorable Kozak context to function as a translation start codon, resulted in a moderate increase in translation efficiency. We expected the uAUG2 (Mut2) mutation to increase translation to a lesser extent, as the second uAUG is located within a poor context for ribosome recognition. However, Mut2 also significantly increased EPHX1 protein translation, to levels comparable to the wild-type. The combined mutation eliminating both uORFs had the most dramatic effect on translation efficiency, increasing translation ~6.7-fold and thus restoring efficiency to the level of the control. These results suggest that both upstream AUGs can be efficiently recognized for initiation and thus the translation from the main cistron is decreased by ribosome diverting.

Deletion of the 5'-UTR may alter the length of the 5'-leader sequence, the distance between upstream AUGs and the mRNA 5' end, or the RNA secondary structure. Thus, the effects of sequential deletions from the 5'-end of the E1-b' 5'-UTR were analyzed in the TnT®

transcription/translation system. Deletion of the first 50 nt of the sequence resulted in a 2 -fold enhancement compared to E1-b' translation while the 110 nt deletion, which made the 5'-end just 5 nt ahead of the first uAUG, led to a 3.8-fold increase.

4.4.6 Effects of EPHX1 mRNA 5'-UTRs on Luciferase Expression in Transfection Studies

We next employed quantitative luciferase assay assessments to evaluate the effects of the various EPHX1 5'-UTRs on translation of the luciferase main ORF. The unique EPHX1 5'-UTRs were positioned upstream of a firefly luciferase reporter gene, and the constructs were then transiently co-transfected into HepG2 human hepatoma cells along with the renilla luciferase plasmid control. In addition, the E1-b' 5' UTR was mutated either by disruption of the uAUGs or serial deletions from the 5'/ GC-rich end region. The firefly luciferase values were corrected for transfection efficiency using the renilla luciferase activity measures. The results (Figure 4-6) were consistent with those obtained using the *in vitro* system. Compared to the pGL3 vector fused with E1-b 5' UTR, E1-b' expressed the lowest luciferase activity, only 37% of the E1-b control; E1 exhibited relatively strong luciferase activity. Both uAUG1 and uAUG2 contribute to the down-regulation of translation, as indicated in the mutation analysis. Mutation of the first uAUG or second uAUG resulted in the increase of protein translation relative to the wild type. Mutation of both uAUG1 and uAUG2 resulted in a restoration of the protein activity to the level of the E1-b control. As expected, deletions of the 5'-leader sequence resulted in increased levels of protein translation.

4.4.7 Functionality of the EPHX1 uAUG1

To demonstrate that the uORF1 is functional, the STOP1 mutation was created based on the full-length E1-b' construct. In the STOP1 construct, the stop codon of uORF1 was mutated to a lysine residue, thus extending the uORF1 translation product from the in-frame 26 a.a. peptide to a ~53 kDa protein (small peptide at the N-terminal of EPHX1 protein) that can be visualized on SDS-PAGE gel by ^{35}S labeling. The E1-b' and mutation constructs were expressed in the cell-free transcription/translation system with [^{35}S]-methionine incorporation and the translated products were analyzed by SDS-PAGE analysis (Figure 4-7). Translation of the E1-b'construct yielded a single band of EPHX1 protein. However, the STOP1 mutation resulted in two bands: the correct size EPHX1protein and a second, larger protein product. These results provide further evidence that ribosomal initiation can occur from the uORF1, thus the translation of the downstream main AUG is inhibited by the functional uORF1.

4.4.8 *Trans*-effect of E1-b' uORF1

To investigate possible trans effects of the E1-b' uORF, EPHX1 E1 or E1-b constructs were translated in the presence of the E1-b' construct *in vitro* (Figure 4-8A). The E1 construct without AUG on the 5'-UTR was used as a negative control. In the presence of E1-b', translation from both E1 and E1-b was suppressed. More protein products were translated from the E1 control constructs, suggesting that the E1-b' had a *trans*-suppressive effect rather than preventing the E1/E1-b translation by competing for translation resources. In order to determine the specificity of the *trans*-effect, the uORF1-fs construct was created with an insertion in the uORF1, such that the peptide product was the same length but was comprised of different amino acids. The E1-b' and uORF1-fs constructs were transfected into BEAS-2B cells and equal amounts of

total protein were analyzed by Western blot. Transfection of E1-b' resulted in a dose-dependent reduction in the endogenous EPHX1 protein level (Figure 4-8B). However, overexpression of the uORF1-fs construct had no effect on the endogenous EPHX1 expression.

4.4.9 Characterization of E1-b' Promoter Activity

E1-b and E1-b' EPHX1 transcripts are initiated from two different transcription start sites and their unique tissue expression patterns led us to hypothesize that a internal promoter located between E1-b and E1-b' was used independently of the main E1-b promoter region. A deletion analysis was performed to locate the minimal sequence responsible for the E1-b' promoter activity (Figure 4-9). First, we isolated the E1-b' 2 kb upstream region from human liver genomic DNA. Fragments yielded from sequential deletion were subcloned into a pGL3 luciferase reporter vector and their promoter activities were compared by transient transfection assays in HepG2, BEAS-2B and 293A cells. The results demonstrated that the region 600-bp upstream from the E1-b' transcription start site contributed the highest transcriptional activities. Similarly to the E1 and E1-b promoter, the presence of a longer upstream region resulted in a reduction in promoter activity. Deletion of the -600 to -300-bp region of E1-b', which was previously established as the E1-b 300-bp promoter region, significantly decreased the transcriptional activity. However, the transcriptional activity of E1-b' -600-bp upstream region was higher than the proximal 300-bp upstream region of E1-b.

4.5 Discussion

Microsomal epoxide hydrolase is an enzyme that plays an important role in the biotransformation system and catalyzes the hydrolysis of many carcinogenic or cytotoxic eletrophilic epoxides (Fretland and Omiecinski, 2000). In human liver and lung, EPHX1 enzymatic activity and protein levels were highly correlated; however, neither correlates well with respective messenger RNA levels (Omiecinski et al., 1994). These observations suggest that EPHX1 expression is modulated by one or more post-transcriptional mechanisms that determine the functional status of EPHX1 interindividually. In this chapter, we performed a detailed analysis of the EPHX1 5'UTR to investigate factors that affect translation efficiency from different mRNA variants.

Approximately 40% of mammalian mRNA sequences contain AUG trinucleotides upstream of the main open reading frame (Peri and Pandey, 2001;Crowe et al., 2006). In some cases; the upstream open reading frame encods a functional polypeptide that exerts regulatory effects on translation (Pendleton et al., 2005). In our study, the E1-b' transcript provides an opportunity to assess the role of uORFs in the translational control. Among the three EPHX1 mRNA isoforms producing the same protein, the newly identified E1-b' transcript differs in its expression profile and 5'UTR structure. First, the E1-b' transcript is expressed in most human tissues tested (with the ovary exhibiting ~100-fold higher expression than the other tissues), while the liver is the predominant site of EPHX1 activity. E1-b' mRNA represents the minor form of the total EPHX1 in most tissues while E1-b is the primary transcript in all tissues. Second, two upstream AUG codons were detected in the E1-b' 5'UTR region. Bioinformatics tools predicted that the E1-b' 206-nt 5'-leader sequence would form a complex base-paired secondary structure. The inhibitory effect of the uORF together with the stable secondary structure suggested that the

translational efficiency of the E1-b' main AUG would be reduced. Our *in vitro* system and transfection assay results confirmed that translation from E1-b' mRNA is indeed inhibited (Figure 4-4). The analysis of promoter activity suggested that the core promoter of E1-b' is located within the upstream -600bp region, which is the same as E1-b -300bp region, there could be other transcription factors which bind to the internal region between E1-b and E1-b' to regulate E1-b' expression (Figure 4-9).

The mRNA 5'UTR can modulate protein translation from the main ORF in part through upstream AUGs, uORFs, and secondary structures. Accumulated experimental evidence suggests that 5'UTR down-regulation is a commonly employed regulatory mechanism in situations where protein production needs to be tightly controlled (Audic and Hartley, 2004), such as in the translation of proto-oncogenes, transcription factors, and growth factors and their receptors (Kozak, 1991;Kozak, 2005). In general, regulation is exerted at the initiation phase of protein synthesis. In the ribosome scanning mechanism, ribosomes will initiate translation at the first AUG (e.g. upstream AUGs) encountered. Although leaky scanning and reinitiation of translation of the main ORF can occur, the translation efficiency is greatly reduced by the presence of uAUGs. Additionally, hairpins and stem-loop secondary structures serve to inhibit the scanning ribosome resulting in poor translation. Studies in both prokaryotes and eukaryotes have shown that translation initiation from uORFs contributes to translation inhibition of the downstream main ORF (Brown et al., 1999;Imataka et al., 1994;Kozak, 2005).

In the case of EPHX1, we mutated the upstream AUGs into GCAs either individually or in combination. According to the secondary structure prediction tools, these substitutions result in no significant changes to the hairpin structure (data not shown). The hypothesis that the upstream AUGs act to inhibit ribosome initiation at the main EPHX1 start codon was supported by the data. Indeed, the results of our mutation experiments show that the translation efficiency of the downstream main AUG is dependent on the two upstream ORFs (Figure 4-5). Rescue by

mutation of the second uAUG casts doubt on the possibility that the uAUG2 is skipped by leaky ribosome scanning as the absence of a Kozak sequence suggests. Interestingly, sequential deletions of the 5' GC-rich leader sequence resulted in elevated translation from the main coding region. This evidence supports the premise that the translation activity from the main AUG is inversely correlated with the length of the 5'-UTR leader sequence. It is possible that the 5'-leader sequence will create complex secondary structure such that ribosome scanning is difficult and initiates at the upstream AUG codon even it is in a weak context (Kozak, 2005). When the long 5'UTR is shortened, the scanning 40S complex has some ability to bypass the upstream AUG, leading to increased translation efficiency of the major ORF. Expression of luciferase from the chimeric EPHX1-Luc construct again demonstrated the functionality of E1-b' (Figure 4-6). Overexpression of E1-b' resulted in a dramatic decrease in EPHX1 expression (Figure 4-7), suggesting that the uORF1 can inhibit protein translation through *trans*-suppression in addition to its *cis* effects.

Many cancer studies suggest that the inefficient translation of uORFs is a regulatory mechanism used to limit expression of oncogene products which are required in small amounts but would be harmful if over-expressed. For example, the oncogene mdm2 is transcribed from two alternative promoters yielding L-mdm2, which contains two uORFs, and S-mdm2, which lacks these uORFs (Kozak, 2005;Brown et al., 1999;Jin et al., 2003). L-mdm2 is the dominant transcript in normal human cells where the oncoprotein MDM2 is inefficiently produced. In turmor cells, however, elevated MDM2 levels result from a switch in promoter usage which up-regulates the shorter mdm2, thereby increasing the translation of MDM2 ~ 20-fold (Landers et al., 1997). It is possible that under certain stress situations, modulation of EPHX1 enzyme activity is accomplished by a switch from the E1-b' promoter to the E1-b promoter which results in the generation of a shorter and simpler 5'-UTR.

In summary, there is remarkable diversity among the 5'-UTR EPHX1 mRNA variants. Here we demonstrate that the E1-b' transcript, which contains two uAUGs, has significantly lower translation efficiency. This regulation of translation may explain the reported discrepancies between EPHX1 mRNA and protein levels.

Table 4-1 Primer Sequences for PCR Amplification

Name	Primer Sequence	Name	Primer Sequence
E1 FP	GCCCTTAGAGCATCG	E1-b FP	GATCGCGCGCCTGC
E1-b' FP	AGGGAATTCCGCGTCCC	EPHX1 RP	GGTCATTGCCGCTCCAGCACCGAC
HindIII E1	GATCAAGCTTGCCCTTAGAGCATCGCC	HindIII E1-b	GATCAAGCTTGATCGCGCGCCTGC
HindIII E1-b'	GATCAAGCTTAGGGAATTCCGCGTCCC	EPHX1-LucR	ATAGAATGGCGCCGGGCCTTTCTTTATGTTTTTGGCGTCTTCCATGGCTC
FSa FP	CTCGGCGGTGAAATGGCACTTAATTTGTCA	FSa RP	GTGACAAATTAAGTGCCATTTCACCGCCGAG
FSb FP	CAAGTCGGAACACTGCTTTTCGCACGCGTCC	FSb RP	GGACGCGTGCGAAAAGCAGTGTTCCGACTTG
M1 FP	CGGCGGTGAAGCACACTTAATTTGTCAC	M1 RP	TAAGTGTGCTTCACCGCCGAGCGAGC
M2 FP	CGGAACACTGATTTTCGCACGCGTCCGTGTGAAGA	M2 RP	TTCACACGGACGCGTGCGAAAATCAGTGTTCCG
Del-50	CAAGCTCGCACCCCGAGAGCGCACCGCGTC	Del-110	GATCAAGCTTCTGCTTCCAGGGCCG
-2kb E1b'	TTGGTGGGTACCGCTAGTCCTCAATTTGGTCCTCAA	-600 E1b'	AGGACATACATCATGCA
-400 E1b'	GAGACTTTCCCGGGTCC	-300 E1b'	GGAGAGATCGCGCGCCTGC

Figure 4-1. Diverse 5'-leader sequence of the human EPHX1 gene. (A) Structural map of three human EPHX1 alternative exon1s. Both E1-b and E1-b' are 18.5kb upstream of the coding exon 2. Translation of the EPHX1 protein begins in exon2, so the three variants encode the same protein. (B) Exon 1 sequences of EPHX1. E1-b' was newly identified by 5'-RACE analysis. E1-b and E1-b' are separated by 238bp GC-rich sequence. The E1-b' uAUGs are marked by their position. The main AUG is in bold type. The first uAUG and uORF are underlined; the second uORF is underlined by a dashed line.

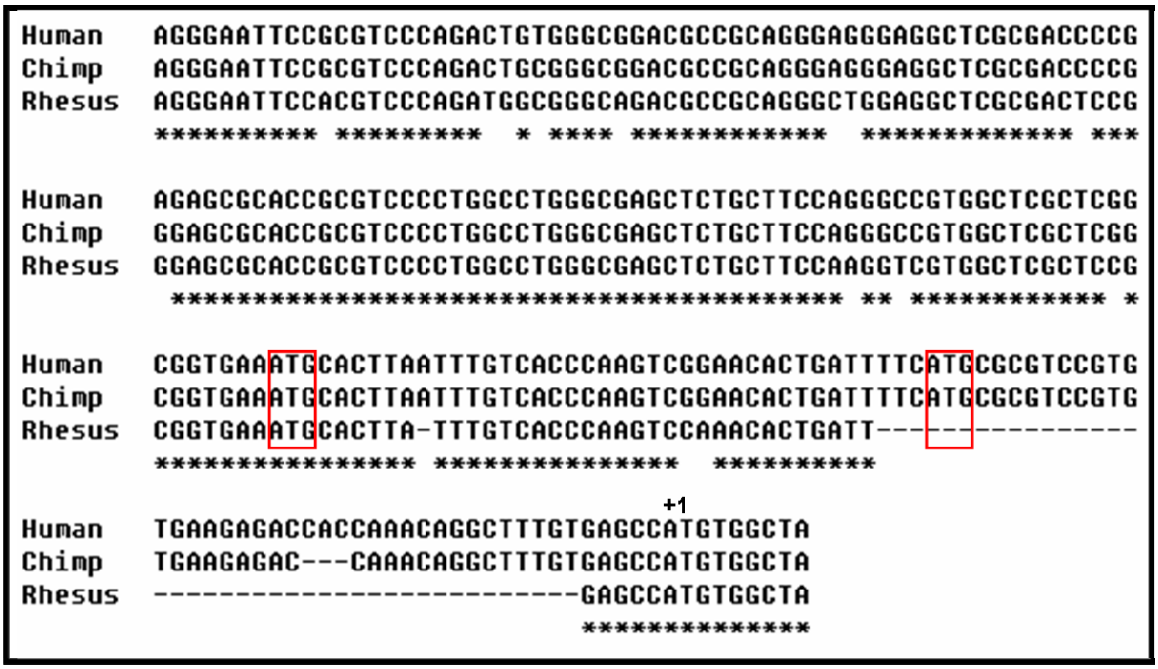


Figure 4-2. E1-b' 5'-UTR sequence comparisons. EPHX1 E1-b' 5'-UTR region was compared between human/chimp/rhesus by Clustal W2. The first upstream AUG is conserved in the three species, however the second uAUG is only identified in human and chimp. In human and chimp genomes (98% match), EPHX1 is located on the Chromosome 1 plus strand; in rhesus (70% match to human), sequences are located on the Chromosome 1 reverse strand.

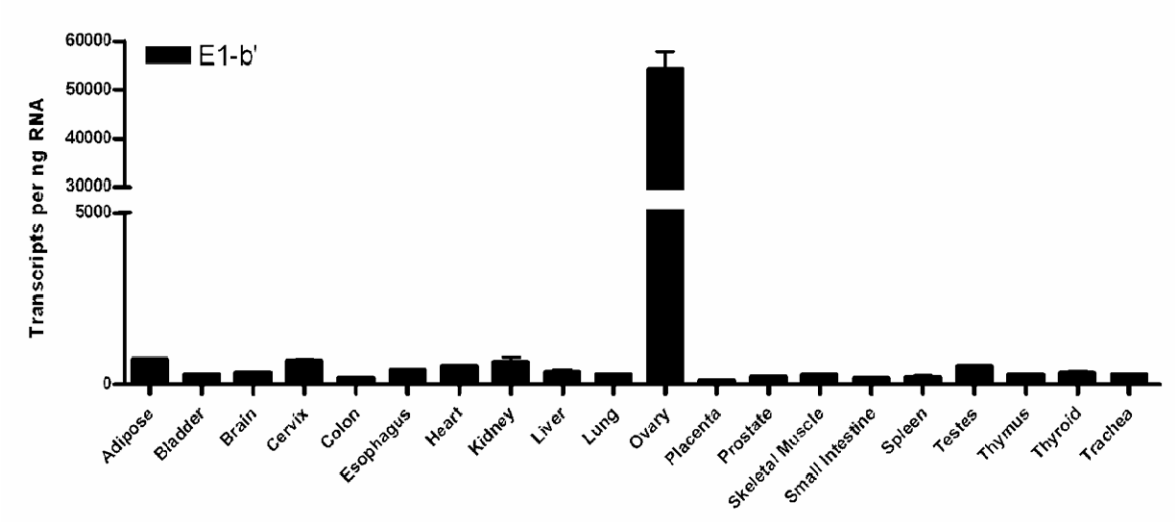


Figure 4-3. Quantification of the EPHX1 E1-b' transcript expression in different human tissues. Real-Time PCR was performed with RNA obtained from 20 human tissues; each sample represents a pool of at least three individuals. E1-b' transcript was quantified by absolute quantification based on standard curves determined using plasmid DNA templates. The E1-b' mRNA is significantly higher in ovary compared to other tissues ($p < 0.001$).

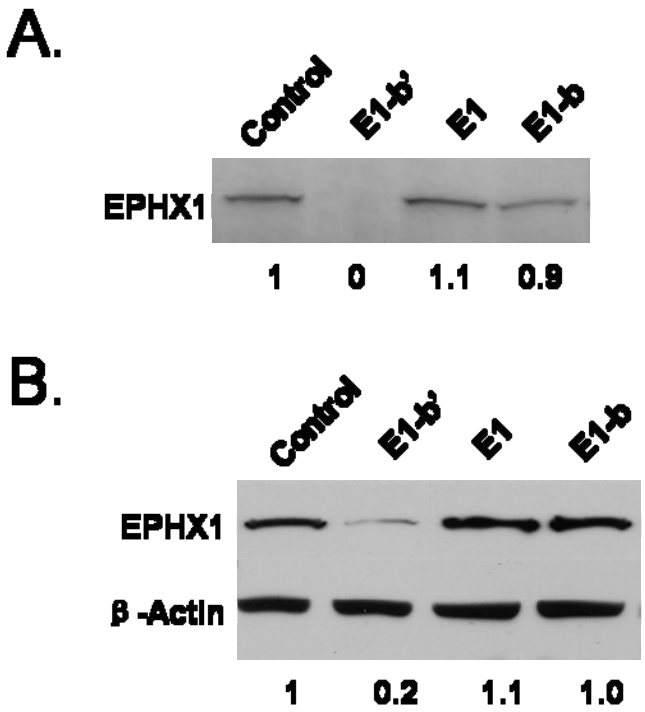


Figure 4-4. Translation efficiency of the EPHX1 variants. (A) Full length E1, E1-b and E1-b' cDNAs were subcloned into pGL3 vectors along with the control containing the main AUG starting from Exon 2. All constructs were expressed in an *in vitro* transcription/translation system. The [^{35}S]-methionine labeled protein products were separated by SDS-PAGE and the gels were dried and exposed to film. (B) All constructs were transfected into 293A cells. 24 h after transfection, equal amount of cell lysis were separated by SDS-PAGE, and EPHX1 proteins were detected by anti-EPHX1 antibody. Quantification of EPHX1 protein expression, normalized to β–Actin loading control, is indicated under the Western blot results.

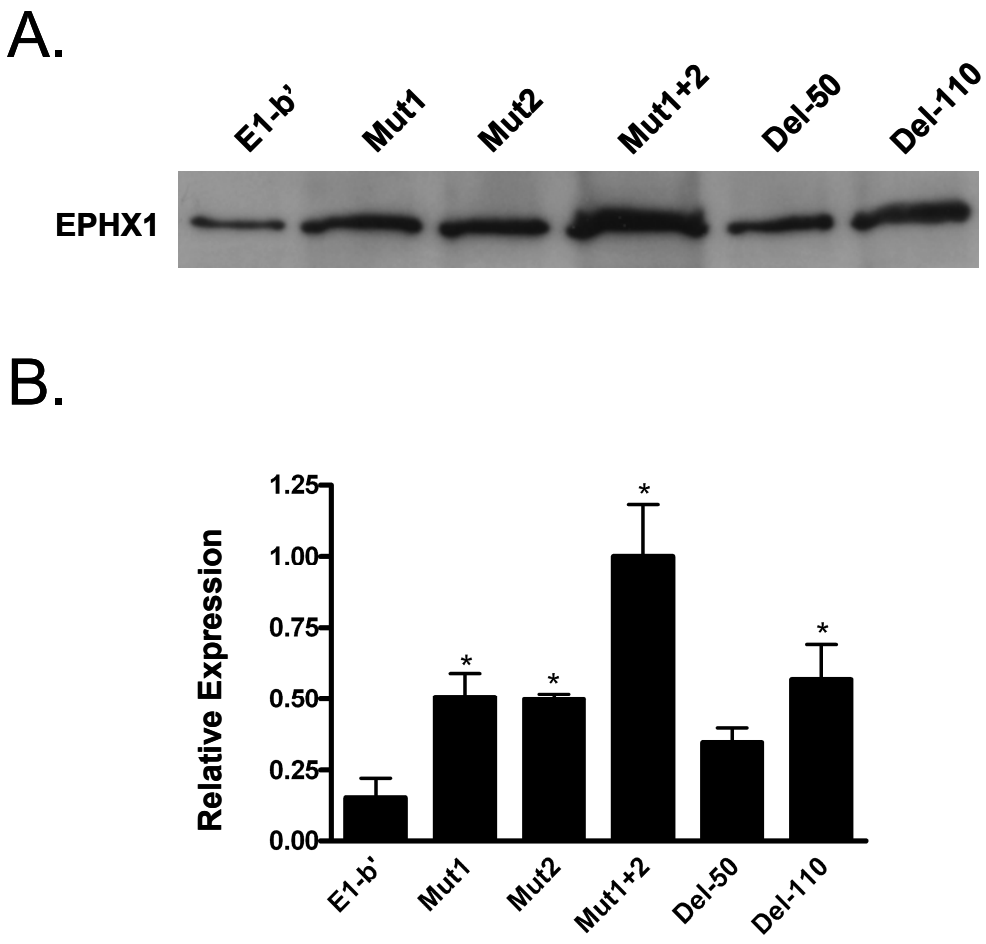


Figure 4-5. Deletion and mutational analysis of the E1-b' EPHX1. Translation of the main ORF depends on the uORFs and leader sequence. (A) Full length and mutant constructs were expressed *in vitro*. In uAUG1 or uAUG2, the uAUG was mutated to a nonininitiation codon. In uAUG1+2, both uAUGs were mutated. In the Del-50 or Del-110, the 5' leader sequence was deleted while the uAUGs were not affected. In vitro translated [^{35}S] methionine-labeled products were separated by SDS-PAGE, and gels were dried and exposed to film. (B) Quantification of EPHX1 protein expression from three separate experiments is indicated. (mutation/ deletion of E1-b' vs. E1-b'; *, $p < 0.05$, ANOVA)

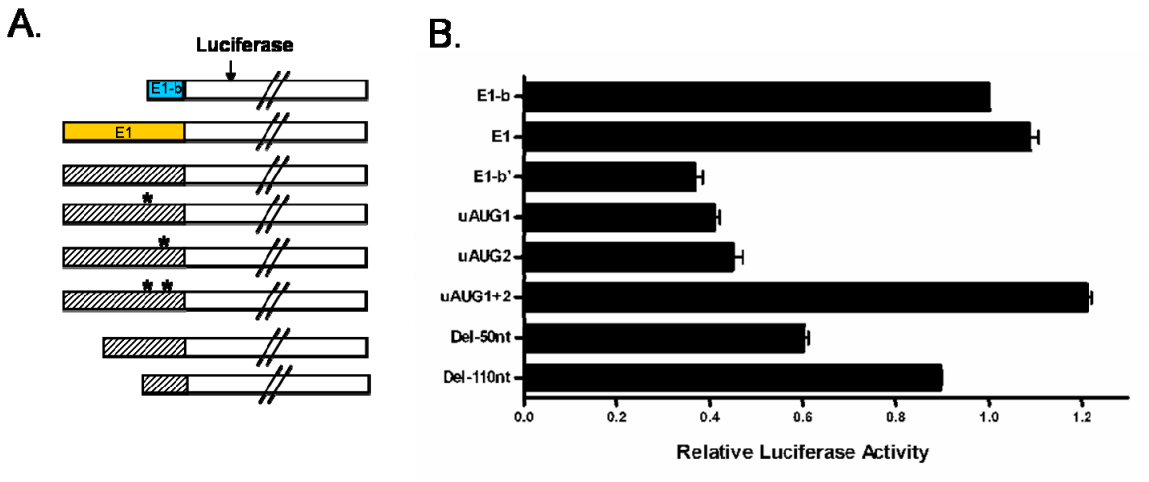


Figure 4-6. Functionality of E1-b' uORF when placed immediately upstream of a luciferase ORF. (A) EPHX1 variants E1, E1-b and E1-b' (WT, mutation or deletion) 5'-UTR sequences were cloned and replaced the luciferase 5'-leader sequence in the pGL3 control vector using the TK promoter. (B) Chimeric EPHX1-luciferase constructs were transfected into 293A cells. R. reniformis luciferase reporter (pRL-CMV) was used as an internal control. Luciferase assays were performed 24 h after transfection. The data shown depict means and S.D. values derived from three separate experiments, each performed in duplicate. (mutation/ deletion of E1-b' vs. E1-b'; $p<0.05$; ANOVA)

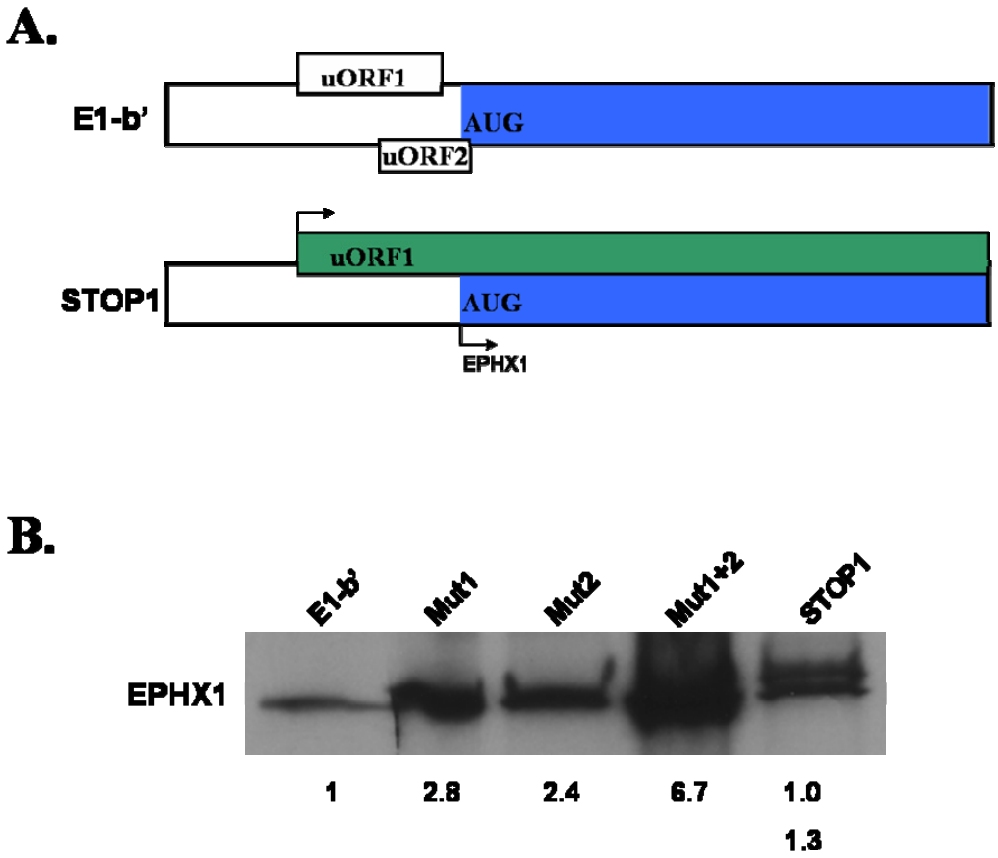


Figure 4-7. Visualization of E1-b' uORF1 expression by mutagenesis of stop codon. (A) Schematic showing the E1-b' and STOP1 construct. The stop codon of uORF1 was mutated to lysine and translation was extended to the EPHX1 coding region. (B) *In vitro* translated [^{35}S] methionine-labeled products were separated by SDS-PAGE, and gels were dried and exposed to film. After mutation, two translated products were generated from the STOP1 EPHX1 construct: one is the 50 kDa EPHX1 protein from the main AUG and the other is the upper band translated from the uORF1 with an extended N-terminal.

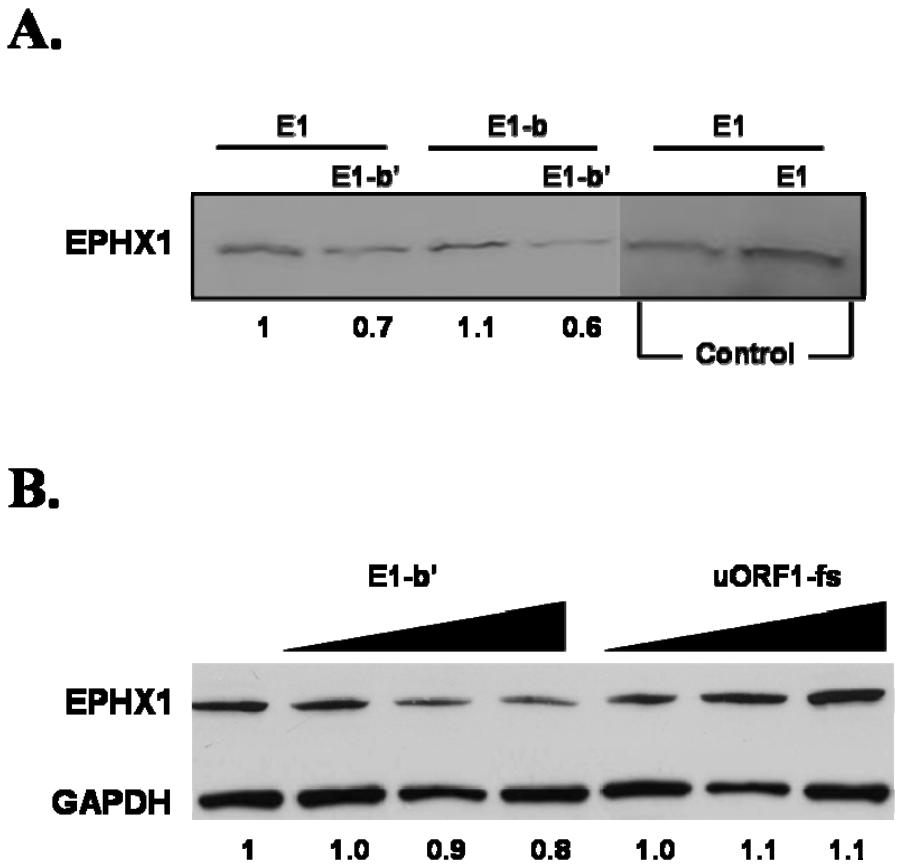


Figure 4-8. The effects of overexpression of E1-b' on EPHX1 expression. (A) Full-length E1 and E1-b transcripts were expressed in the present/absent of the E1-b' transcript. *In vitro* translated [^{35}S] methionine-labeled products were separated by SDS-PAGE, and gels were dried and exposed to film. E1 was used as a control to show that there is no competing for translation resources (B) Western blot analysis of EPHX1. E1-b' and the mutated constructs were transfected into HepG2 cells. The uORF1-fs construct, in which the entire uORF1 peptide is frame shifted but the length of 26 amino acids remains the same, was used as control. 24 h after transfection, equal amounts of cell lysate were separated by SDS-PAGE and EPHX1 proteins were detected by anti-EPHX1 antibody. Quantification of EPHX1 protein expression, normalized to loading control, is indicated under the Western blot results.

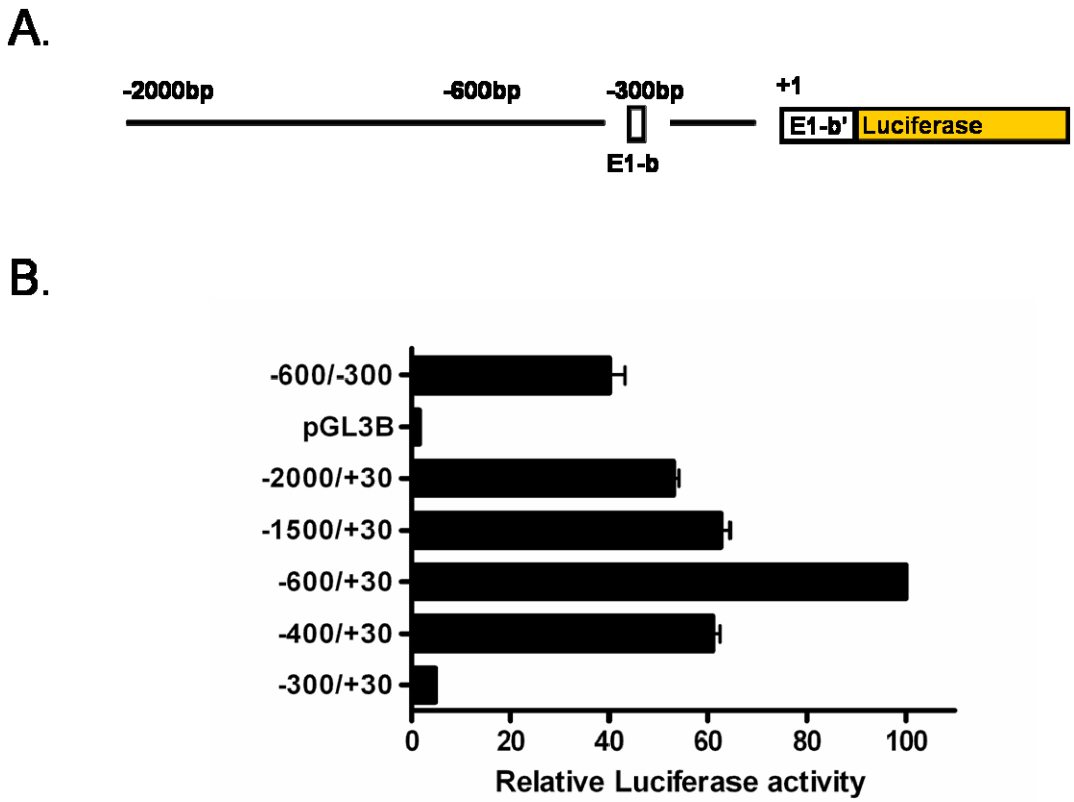


Figure 4-9. Identification of the potential promoter in the 5'-flanking region of E1-b'. (A) The upstream region of E1-b', including exon E1-b, is shown schematically. Various 5' truncated DNA fragments were linked to the luciferase reporter in pGL3-basic vector. The -600/-300 region has been identified as the E1-b promoter. (B) Constructs were co-transfected with the internal control vector pRL-CMV. E1-b promoter (-600/-300) was used as positive control. pGL3-basic (pGL3B) was used as a negative control. The data shown depict means and S.D. values derived from three separate experiments, each performed in duplicate. All constructs yielded similar results when tested in BEAS-2B and HepG2 cells. All the promoter constructs are significantly different than pGL3-basic control ($p < 0.01$, ANOVA).

Chapter 5

Conclusions and Discussions

Xenobiotic metabolizing enzymes play central roles in the biotransformation of foreign compounds, which are introduced to the human body through the diet, as pharmaceutical drugs, and through other routes, such as inhalation. In general, these enzymes protect the body against the potential harmful insults from the environmental toxins and drugs; however in some cases, xenobiotics may generate reactive intermediates that induce toxicity. To minimize the insults caused by these xenobiotics, the expression of xenobiotic metabolizing enzymes must be tightly regulated. The purpose of this investigation was to better understand the microsomal epoxide hydrolase (EPHX1) expression profile including the effects of promoter region polymorphisms and the underlying molecular mechanisms of EPHX1 translational regulation.

Since the identification of the microsomal epoxide hydrolase in the early 1970s, much has been learned with respect to the metabolic function of this enzyme. For example, EPHX1 is involved in bioactivation of carcinogenic PAH xenobiotics. In studies using EPHX1 null mice as a model system, the absence of EPHX1 protected against DMBA-induced toxicity as illustrated by resistance to carcinogenesis in EPHX1-null mice compared with wild-type mice (Miyata et al., 1999). Thus, regulation of EPHX1 enzymatic activities can alter the disposition and toxicologic fate of xenobiotics and differences in EPHX1 expression may result in altered response to chemical exposures. Human EPHX1 is genetically polymorphic, both in the coding exons and 5' promoter regions. A systematic review and meta-analysis of 13 case–control studies confirmed that the His113 allele appears protective for lung cancer incidence in humans (Kiyohara et al., 2006). Recent studies from our laboratory have shown that alternative promoters are used to drive EPHX1 expression according to tissue specific programs. A far upstream primate-specific

promoter, termed the E1-b promoter, predominantly drives EPHX1 expression in multiple human tissues, including liver.

The associated transcriptional activity of this E1-b promoter region is interindividually regulated by the presence of transposable genetic elements. We characterized the variable inclusion of Alu elements within this EPHX1promoter region as a potential regulator of transcriptional expression in human tissues. As described in detail in Chapter 2, our data demonstrate that the human EPHX1 E1-b promoter region contains a double insertion of Alu elements, and that these elements are variably present in the genome structure of different individuals. Our studies further demonstrate that the presence, or absence, of these Alu elements influences the basal transcriptional activities driving EPHX1 expression. When considering the association between genetic polymorphisms and human diseases, it should keep in mind that the haplotype blocks (several linked polymorphic alleles) need to be consided rather than reliance on a single polymorphic site (Crawford and Nickerson, 2005). The HapMap project is a catalog of commom human genetic variants that describes what the polymorphisms are, where they exist in the genomic DNA, and how they affect human disease and individual responses to xenobiotics. The HapMap structural determinations of the human EPHX1 indicated that the Alu insertion polymorphism in the E1-b promoter region is not linked with the coding region polymorphisms.

Several independent molecular epidemiological studies have identified an association between structural region EPHX1 polymorphisms and the incidence of lung cancer. However, the mechanistic basis for these apparent associations remains largely unknown. EPHX1 is a key biotransformation enzyme, active in the hydrolysis of a large number of epoxides, and a critical catalytic determinant in the formation of the highly reactive electrophilic, and ultimately carcinogenic, bay- and fjord-region diol-epoxide metabolites of the polyaromatic hydrocarbons (PAHs). Therefore, the genetic variability in EPHX1 is a key determinant of lung cancer risk in humans. In particular, we hypothesize that insertion/deletion of Alu elements in the E1-b EPHX1

promoter region may represent an important interindividual regulator of EPHX1 transcription and perhaps an associated risk factor in human lung cancer. To test this hypothesis, in future stdueis we plan to examine the association of EPHX1 E1-b promoter genotype and haplotype frequencies with lung cancer incidence in a lung cancer case: control study.

Based on computer prediction scans, we hypothesize that the Alu elements, identified as variably present in the EPHX1 far upstream gene promoter region, have potential interactions with active transcription factors, for example, with the aryl hydrocarbon receptor (AhR)/AhR nuclear tranlocator (ARNT) heterodimer. It is known that the expression of phase I and phase II genes can be induced via the AhR/ARNT pathway, in response polycyclic aromatic hydrocarbon (PAH) exposures (Marlowe and Puga, 2005). CYP1A1 is a CYP450 enzyme largely involved in the oxidation of various procarcinogens, including the PAHs, and that its induction is largly mediated by AhR. EPHX1 plays a dual role in biotransformation of epoxides generated by CYP1A1, because it is not only involved in detoxication of the epoxides but also generates certain *trans*-dihydrodiols that are converted to highly reactive toxic, mutagenic and carcinogenic diol-epoxide metabolites. Recalling the observation in our laboratory that the Alu element in the E1-b upstream region contains an AhR/ARNT binding site, we thought it likely that the presence of these Alus reduce the basal transcriptional activity through interactions with AhR, and that the EPHX1 gene may be transcriptionally up-regulated at this locus following exposure to AhR ligands. However, the results of our studies using both primary hepatocytes and cultured cell lines provided no evidence for a functional role of AhR in these respects (Figure 2-7). The potential limitations of *in vitro* systems as compared to *in vivo* studies always need to be considered. For example, studies of interactions between cell types may not be possible in cell culture systems, and loss of specific biotransformation functions may limit their predictive potential. Although these considerations are important to keep in mind, a previous study using primary human hepatocytes reported similar results as those obtained here in that only modest EPHX1 induction

was detected by the prototypic AhR ligand, beta-naphthoflavone (β-NF) (Hassett et al., 1998). The development of a knock-in humanized mouse model that would enable humanized control of EPHX1 gene expression in specific mouse tissues would likely provide a valuable biological model to assess such questions. The development of project is underway in our laboratory. In addition, future studies of mutation in the predicted binding motifs (i.e. AhR/ARNT, RAR) and protein/DNA interaction experiments, such as electromobility shift assays (EMSAs) and chromatin immunoprecipitation (ChIP) analyses, will allow us to investigate possible additional transcription factors that are involved in regulating EPHX1 gene expression.

In addition to transcriptional regulation, it is suggested that epigenetic modifications may play additional roles in controlling gene expression. The human genome has evolved epigenetic mechanisms to silence the expression and mobility of TEs, which are otherwise potentially active through their abilities to influence gene expression or contribute to genome rearrangement. Silencing of TEs is controlled by overlapping epigenetic mechanisms at transcriptional and post-transcriptional levels (Figure 5-1), including RNAi, chromation modifications, and RNAi mediated chromatin modifications (Slotkin and Martienssen, 2007). Recent studies have shown that modifications of histone tails, DNA methylation and alteration in chromatin packing and condensation are associated with transcriptionally repression. Alu elements are rich in CpG context, which can be targeted for methylation; and this methylation is inherited to daughter DNA strands upon DNA replication. In the human populations, specific Alu polymorphisms (i.e. some chromosomes carry the Alu insertion, wheres others do not) show interindividual epigenetic variability at the level of DNA methylation (Sandovici et al., 2005). Alu elements can also be regulated by histone methylation at Lys^9 on histone H3 (Kondo and Issa, 2003). In summary, DNA methylation and histone modification might each inactivate Alu elements and therefore potentially repress the EPHX1 expression in individuals harboring the Alu insertion genotype.

Isothiocyanates found in cruciferous vegetables, such sulforaphane (SFN) and 3-phenylpropyl isothiocyanate (PPITC), are known to reduce cancer risk in animal models through induction of phase II detoxification enzymes, as well as inhibition of phase I enzymes. In the studies presented in Chapter 3, we evaluated the ability of SFN and PPITC to modulate EPHX1 mRNA and protein expression. In BEAS-2B lung cancer cell line, SFN and PPITC caused a significant inhibition of EPHX1 expression in mRNA and protein levels. In contrast, SFN induced the EPHX1 expressions in HepG2 liver cells and primary human hepatocytes. The presence of antioxidant response element (ARE)-like motifs in the promoter region suggested the involvement of Nrf2 in EPHX1 regulation, as Nrf2 has been identified as a master regulator of antioxidant response pathways. Co-transfection with a Nrf2-expression plasmid and the E1-b promoter construct decreased the EPHX1 transcriptional activity in BEAS-2B (lung) cells, and increased the transcriptional activity in HepG2 (liver) cell lines. These effects were significantly enhanced when the co-transfected cells were exposed to SFN treatment. Therefore, these studies demonstrated that EPHX1 expression can be modulated by chemopreventive agents, leading to EPHX1 induction in liver and inhibition in lung cells, and that the Nrf2/ARE pathway likely functions as an important mechanistic contributor to this regulatory scheme.

EPHX1 plays a dual role in biotransformation and detoxication and this may contribute to the the tissue-specific regulation of EPHX1 by SFN. Because EPHX1 can activate carcinogens in the lung, our observations suggest that the SFN-induced decrease in EPHX1 expression in lung cells may be protective. On the other hand, in liver, since EPHX1 also detoxifies carcinogens and other xenobiotic-epoxides in this primary metabobolic organ, the observed SFN-induced increase in hepatic EPHX1 expression may be protective. Furthermore, tissue-specific regulation following carcinogen exposure may be the consequence of a range of biochemical factors, including those that affect absorption, metabolism, and DNA repair. For example, lung is a target tissue for PAH carcinogenesis associated with tobacco smoke. In several *in vivo* studies, the

tissue concentration of BaP, a model substrate for PAH toxicity study, is elevated in rat lung compared to the liver (Moir et al., 1998;Withey et al., 1993). Similarly, more BaP-DNA adducts are formed in lung than the liver when rat lung and liver slices are exposed to BaP *in vitro* (Harrigan et al., 2004). So the differences in carcinogen absorption could contribute to the tissue-specific regulation of EPHX1 expression.

At the transcriptional level, it is possible the tissue-specific mode of transcription regulation is controlled by cap-n-collar (CNC) subfamily members, including Nrf1 and Nrf2, through *cis*-acting motifs in the EPHX1 promoter region. Wang et al. demonstrated that the 65 kDa short form of Nrf1 functiond as a dominant inhibitor of Nrf2-mediated activation of ARE-dependent gene transcription (Wang et al., 2007). Additionally, this positive and negative regulation is likely further modulated through dynamic changes in activity of transcription factor heterodimer partners. In particular, the small Maf protein which interacts with NrF2, has been reported to reverse activation into repressor activity through minimal changes of concentration. Small Maf protein may either promote gene activation or repression by binding to different CNC transcription factors. Our future studies will test the regulatory roles of other related transcription factors, for example, the CNC family member Nrf1. In addition, the ARE-like motifs predicted in the E1-b EPHX1 promoter region will be examined for potential interactions with the CNC-bZIP factor/small Maf complex, such as Nrf2 and small Maf heterodimer complex. These studies should provide additional insight into the complex regulatory mechanisms controlling EPHX1 expression, including tissue-specific expression.

By using an improved 5'-RACE technique, an additional new EPHX1 transcript variant (E1-b') was discovered by our laboratory and further characterized in the studies reported here. Analysis of its expression in a human tissue panel found that the E1-b' transcript is detected at the highest levels in ovary. The 5'-UTR sequence of the E1-b' mRNA contains two upstream AUGs and substantial base-paired secondary structure, features that were shown to inhibit the translation

of the main open reading frame. Our studies also showed that production of the EPHX1 protein could be increased by mutation of the uAUGs, or the sequential deletion of the 5'-leader sequence. In other words, the inhibition of translation is relieved by removing the upstream ORF, or through truncation of the long, structured 5' UTR. Mutation of the uAUG1 stop codon, which serves to extend the translation product, demonstrated that the context of the uAUG is sufficient for the scanning ribosome to initiate translation. Further, two EPHX1 products were identified by *in vitro* transcription/translation analysis, showing the both the uAUG1 and downstream main AUG are recognized by the translational machinery. Not only does the uORF function to repress downstream EPHX1 translation due to its *cis* effect, the overexpression of the uORF also exhibited a *trans*-mediated effect, resulting in a dramatic decrease in total EPHX1 protein expression. We speculate that the small peptide produced through use of the uORF is required for *trans*-suppression of protein translation, as supported by the fact that EPHX1 expression was not suppressed when uORF was mutated by the loss of its start codon or after insertion of a frame-shift mutation that changed the entire peptide sequence. These observations suggest a novel post-transcriptional mechanism for the regulation of EPHX1 protein expression. In the future, we plan the use of a synthetic peptide identical to the uORF product as a probe to further elucidate the mechanism(s) that underly the inhibition of translation.

Together, the studies presented here have identified novel regulatory mechanisms for the human EPHX1 gene. Further, interindividual variations appear to exist in these regulatory elements. Because of the important role of EPHX1 in the activation and detoxification of xenobiotics, genetic variation in the EPHX1 far upstream promoter region, together with structural region functional polymorphisms, may represent critical risk modifiers of interindividual differences in human cancer susceptibility.

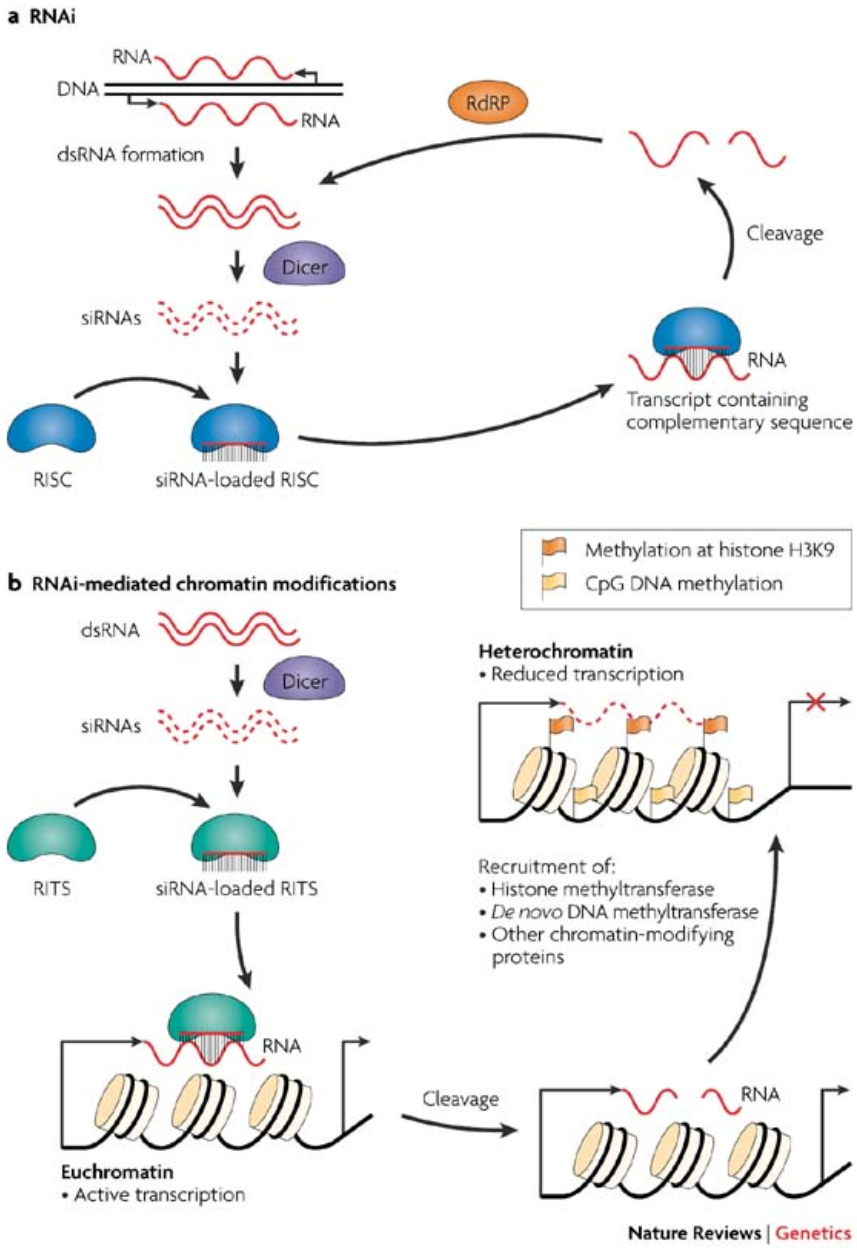


Figure 5-1 Mechanisms of transposable element silencing

Adapted from R. Keith Slotkin, Nature Reviews Genetics 8, 272-285 (2007)

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