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CHARACTERIZATION OF THE GLUTATHIONE TRANSFERASE ALPHA GENES: ROLES IN DRUG RESISTANCE AND CHEMOPROTECTION

by

Nasser Fotouhi-Ardakani

A thesis submitted to the Faculty of Graduate Studies and research, McGill university, in partial requirements for the degree of Doctor of Philosophy

Department of Medicine
Division of Experimental Medicine
McGill University, Montréal, Canada
Submitted during November 2000

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ABSTRACT

The glutathione-S-transferases (GSTs) are a multigene family of enzymes that catalyze the conjugation of glutathione (GSH) with toxic endogenous and xenobiotic compounds including anticancer drugs, as part of detoxification pathways. Our laboratory previously isolated a melphalan resistant rat mammary carcinoma cell-line (MLNr MatB). The in vitro and in vivo studies demonstrated increased GST activity, especially elevated GST alpha class in MLNr cells. At the outset of my work, I examined the nature of the increased GST form in MLNr cells. I cloned the GSTA3 cDNA which is overexpressed in MLNr cells and found that it is identical to the previously described GSTA3 mRNA, suggesting that there is no additional GST alpha gene induced by melphalan. To study the role of the rat GSTA3 gene in chemotherapy resistance, and understand the mechanism of its overexpression in drug resistant tumors, I isolated the entire rGSTA3 subunit gene, including its regulatory regions, from a genomic library and characterized it. The rGSTA3 gene is approximately 15 kb in length and consists of seven exons interrupted by introns of different lengths. The functional activity of its promoter was shown by its ability to drive the expression of a CAT reporter gene in MatB cells, and its activity was greater in melphalan resistant cells. I also presented evidence that rGSTA3 subunit evolved as a duplication of the rGSTA5 subunit gene, increasing the diversification and functional benefits. In addition to drug resistance, I have been examining the implications of GST to carcinogenesis. Epidemiological studies have shown an association between Hepatitis B Virus (HBV) infection and exposure to chemical carcinogens in the incidence of hepatocellular carcinoma (HCC). The exact mechanism of these interactions is not known, but reduced GST activity could increase cellular sensitivity to chemical carcinogens. Semiquantitative RT-PCR revealed that HBV infected cells contain a significant decrease in the GST alpha level. Transient
transfection experiments using both rat and human GST alpha (rGSTA5 and hGSTA1) promoters in HepG2 cells show a reduced CAT activity upon HBx expression, suggesting a transcriptional regulation of both genes by HBx. Treatment with oltipraz, an inducer of GST alpha, partially overcome the effect of HBx on both promoters. Promoter deletion studies indicate that oltipraz acts through responsive elements distinct from AP-1 and NF-κB transcription factors. Thus, HBV infection alters phase II detoxifying enzymes via different mechanisms than those modulated by treatment with oltipraz.
Les glutathione-S-transférases (GSTs) font partie d’une famille de plusieurs gènes qui expriment des enzymes catalysant la conjugaison du glutathione (GSH) avec des composés toxiques endogènes et des xénobiotiques, incluant les médicaments anticancéreux. Ces réactions font partie du système de détoxification de la cellule. Notre laboratoire a précédemment identifié une lignée cellulaire de carcinome mammaire de rat (MLNrMatB) qui est résistante au melphalan. Les études in vitro et in vivo ont démontré une augmentation de l’activité de la GST, spécialement pour la classe GST alpha chez les cellules MLNr. Au début de cette étude, j’ai examiné la nature de l’augmentation de l’activité de cette classe de GST chez les cellules MLNr. J’ai cloné l’ADNe de GSTA3, lequel est surexprimé chez les cellules MLNr, et j’ai constaté qu’il est identique à l’ARNm de GSTA3 précédemment décrit, ce qui suggère qu’il n’existe pas un autre gène GST alpha qui est induit par le melphalan. Pour étudier le rôle du gène GSTA3 (rat) dans la résistance à la chimiothérapie, et pour mieux comprendre le mécanisme de sa surexpression dans les tumeurs résistantes, j’ai isolé le gène GSTA3 dans sa totalité, incluant ses régions régulatrices, et cela, à partir d’une librairie génomique de façon à le caractériser. Le gène rGSTA3 mesure environ 15 kb et est constitué de sept exons interrompus par des introns de différentes longueurs. L’activité fonctionnelle de son promoteur a été démontrée par sa capacité à réguler l’expression d’un gène rapporteur CAT dans les cellules MatB, et son activité était plus grande chez les cellules résistantes au melphalan. J’ai aussi démontré que la sous-unité rGSTA3 a évolué comme une duplication du gène rGSTA5, de façon à augmenter la diversification et les bénéfices fonctionnels. En plus de la résistance à la drogue, j’ai étudié les implications de la GST en rapport avec la carcinogène. Des études épidémiologiques ont montré une association entre l’infection du virus de l’hépatitte B (HBV) et l’exposition à des
cancérogènes chimiques dans l’incidence du carcinome hépatocellulaire (HCC). Le mécanisme exact de cette interaction n’est pas connu, mais une activité GST réduite pourrait augmenter la susceptibilité cellulaire aux cancérogènes chimiques. Des RT-PCR semi-quantitatifs ont révélé que des cellules infectées avec HBV montrent une diminution importante de GST alpha. Des essais de transfection transitoire utilisant à la fois des promoteurs GST alpha de rat et d’humain (rGSTA5 et hGSTA1) dans des cellules HepG2, ont montré une diminution de l’activité CAT lors de l’expression de HBx, ce qui suggère une régulation transcriptionnelle des deux gènes par HBx. Le traitement avec l’oltipraz, un inducuteur de GST alpha, a partiellement annulé l’effet de HBx sur les deux promoteurs. Des études de délétion du promoteur démontrent que l’oltipraz agit à travers des éléments de réponse distincts des facteurs de transcription AP-1 et NF-κB. Par conséquent, l’infection avec HBV modifie les enzymes de détoxicification de phase II, par des mécanismes différents que ceux modulés par le traitement avec l’oltipraz.
This Ph.D. thesis is written following the guidelines for thesis preparation from the faculty of Graduate Studies and Research at McGill University. I have chosen the option of writing the thesis as a manuscript-based thesis. The thesis guidelines states: "The candidate has the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted for publication, or the duplicated published text (not the reprints) of one or more original papers. The texts of the thesis must still conform to all other requirements of the thesis preparation with respect to font size, line spacing and margin sizes, and must be bound together as an integral part of the thesis. The thesis must be more than collection of manuscripts. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between different manuscripts are usually desirable in the interest of cohesion. The thesis must include an abstract in English and French, an introduction and literature review (in addition to that covered in the introduction of each paper), and a final conclusion. Additional material, where appropriate, must be provided in sufficient detail (e.g., in appendices) to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled Contributions of Authors as a preface to the thesis". As a result, I have included four original manuscripts as chapters of this thesis, which I have published. Each of these papers (chapters 2 to 5) contains an abstract, introduction, materials and methods, results, discussion, and references sections. In addition, certain figures are included as annexes to complement the chapters. In order to provide continuity, a preface
is added at the beginning of each chapter. In accordance with the thesis guidelines of McGill University, a general introduction and literature review is presented in chapter 1, and a final general discussion is given in chapter 6. The thesis also includes a general abstract in English and French.

Papers included in this thesis are as follows:


(*These authors have contributed equally to this work and are jointly considered as first authors).

Contributions of Authors:

I have performed most of the research experiments included in this thesis. The contributions of the other authors to this project are stated below:
In chapter 2, the isolation of the fragment A from a cDNA library described in figure 1, was performed by Marlena Lewandowska. The experiments to determine the GSH levels in Mat B WT and Mat B MLNr cells, as well as their responsiveness to the drug melphalan shown in Table 1, was done by Annie Woo. Robyn Schecter contributed in critical reading and English correction of the manuscript. In chapter 4, Robyn L. Schecter contributed in helpful discussions, and also in reviewing the manuscript. In chapter 5, Iris Jaitovitch-Groisman performed subcloning and preparation of retroviral producers containing the HBx gene and cell transduction experiments using AP2 and AP2-HBx particles. CAT assays using hGSTA1 promoter and CAT assays on Drosophila SL2 cells using both human and rat GST reporter plasmids. Annie Woo and Robyn Schecter performed Northern blot analysis (Fig. 1) and cytotoxicity assays shown in Tables 1 and 2.

All the studies were performed under the supervision of Dr. Gerald Batist.
ACKNOWLEDGMENTS

I would like to express great gratitude to my supervisor, Dr. Gerald Batist, his support encouragement, and guidance during my Ph.D. study. Dr. Batist gave me the opportunity to fulfill my dream. His enthusiasm, freedom of thoughts and trust in me as well as his optimistic outlook towards life and research made this experience a true success.

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<th>Full Form</th>
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<td>AFB&lt;sub&gt;1&lt;/sub&gt;</td>
<td>aflatoxin B&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>AP-1</td>
<td>activation protein-1</td>
</tr>
<tr>
<td>ARE</td>
<td>antioxidant-responsive element</td>
</tr>
<tr>
<td>Arnt</td>
<td>aryl hydrocarbon (Ah) receptor nuclear transporter</td>
</tr>
<tr>
<td>BCNU</td>
<td>1,3-bis(2-chloroethyl)-1-nitrosourea</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>CAT</td>
<td>chloramphenicol acetyl transferase</td>
</tr>
<tr>
<td>CDNB</td>
<td>1-chloro-2,4-dinitrobenzene</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>ERE</td>
<td>estrogen response element</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>5'-RACE</td>
<td>rapid amplification of cDNA 5' ends</td>
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<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>GSH</td>
<td>reduced glutathione</td>
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<td>GST</td>
<td>glutathione transferase</td>
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<td>HBV</td>
<td>hepatitis B virus</td>
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<td>HCC</td>
<td>hepatocellular carcinoma</td>
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<tr>
<td>HNF-5</td>
<td>hepatocyte nuclear factor-5</td>
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<tr>
<td>kb</td>
<td>kilobase pair(s)</td>
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<tr>
<td>kD</td>
<td>kilodalton</td>
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<td>MatB</td>
<td>rat mammary carcinoma cell line</td>
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<td>MLNr</td>
<td>melphalan resistant</td>
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<td>MYA</td>
<td>million years ago</td>
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<td>NF-(\kappa)B</td>
<td>nuclear factor-kappa B</td>
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<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>r</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RRE</td>
<td>ras-response element</td>
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<tr>
<td>RT</td>
<td>reverse transcription</td>
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<td>untranslated region</td>
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<td>wild type</td>
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CHAPTER 1

LITERATURE REVIEW

1. GENERAL INTRODUCTION

Cancer kills approximately six million people annually worldwide. A high percentage of North Americans will eventually be diagnosed with cancer, killing one in five. An alarming high percent of fatal cancer is caused by extrinsic factors such as lifestyle, especially dietary practices, smoking, alcohol consumption, lack of exercise. In addition, environmental stress such as exposure to natural and synthetic carcinogens, ionizing radiation, and infectious agents are known to play important role in the etiology of cancer. Formation of reactive oxygen species (ROS; free radicals) within the body such as hydroxyl radical, superoxide and hydrogen peroxide as a result of aerobic metabolism causes cellular damage through interaction with macromolecules such as DNA, protein, and membrane lipids. All organisms, including humans, are constantly exposed to carcinogenic compounds present in their surroundings. Many of the toxic agents are found naturally in the environment. For instance, one group of natural toxins, mold aflatoxin, is among the most potent cancer causing agents. Aflatoxin, particularly aflatoxin B₁, induces mutations in genes involved in controlling cell proliferation and differentiation.

To ensure protection against a wide spectrum of toxic compounds, nature has provided various defense mechanisms including chemical metabolism (Jakoby, 1980), repair of target sites (Fox et al. 1987), sequestration (Satoh et al. 1993), and chemical efflux by membrane pumps (Gottesman et al. 1993). In particular, chemical metabolism provides a versatile protective mechanism against a broad range of noxious compounds. The metabolism of xenobiotic compounds usually takes place in two distinct phases...
using two families of enzymes: phase I and phase II. In phase I metabolism, cytochrome P450 (CYP) monooxygenases activate foreign compounds through an initial oxidation forming highly reactive carcinogens. In the second phase of chemical metabolism, which is primarily a detoxification stage, phase II enzymes such as glutathione transferases (GSTs), sulfotransferases, and UDP-glucuronosyltransferases catalyse the conjugation of reactive metabolites with endogenous substrates (e.g. glutathione, sulfate, glucuronic acid). The balance between the two phases of metabolism, (phase I activation and phase II detoxification) is critical in carcinogenesis. Xenobiotic compounds that induce only phase II detoxifying enzymes such as GSTs are known as monofunctional inducers, while those that induce both phase I (CYP450) and phase II enzymes are identified as bifunctional inducers (Hayes et al. 1995).

Among the cellular deactivation and detoxification mechanisms, GSTs have been shown to play important roles in protecting cells from endogenous and xenobiotic electrophilic compounds. GSTs are a multi-gene family of detoxifying enzymes. They are widely present in nature, being found in some bacteria (Nishida et al. 1994) and all eukaryotes such as fungi (Sheehan et al. 1993), insects (Toung et al. 1993), plants (Holt et al. 1995), fish (Leaver et al. 1993), birds (Liu et al. 1991), and mammals (Mannervik et al. 1985). GSTs have evolved to protect organisms from toxic chemicals. They are a family of detoxifying enzymes that catalyze the inactivation of a broad range of hydrophobic toxic chemicals by conjugation with tripeptide glutathione (GSH, γ glutamyl-cysteiny1-glycine). GSTs have been shown to be involved in the metabolism of anticancer drugs, such as alkylating agents, resulting in cancer chemoresistance. They play an important role in xenobiotic detoxification with regards to cellular resistance to therapeutic drugs as well as chemical carcinogenesis. GSTs have also been implicated in hepatocarcinogenesis in several studies (Zhou et al. 1997; Wang et al. 1999). This section is to provide an overview of these mechanisms.
2. OVERVIEW OF GLUTATHIONE TRANSFERASES

The first publications on GST activity in rat liver date back to 1961 (Booth et al. 1961; Combes et al. 1961). Since then a large number of reports have been published on the subject. GSTs are a multi-gene family of detoxifying enzymes that catalyse the addition of the thiol group of reduced glutathione (GSH) to a wide variety of electrophilic chemicals containing various functional groups such as chloronitrobenzenes (Booth et al. 1961), hydroxyalkenals (Hubatsch et al. 1998), o-quinones (Baez et al. 1997) and α, β-unsaturated aldehydes (Berhane et al. 1994). GSTs also bind to hydrophobic compounds such as bilirubin, dexamethasone, heme, and polycyclic aromatic hydrocarbons (Mannervik 1985) resulting in less toxic and more easily excretable metabolites.

GST isoenzymes have been purified from various tissues, and subsequently their genes have been cloned and characterized. The availability of various affinity chromatography gels to which the GST enzymes bind have facilitated the purification and characterization of these enzymes (Habig et al. 1974; Simons et al. 1977; Guthenberg et al. 1979).

Kamiska et al. (1975) for the first time reported the cationic (basic) form of GST isoenzymes whose PI values (isoelectric points) ranged from 7.8 to 9.9. They were purified from human liver cytosol and designated by Greek letters as GSTα, β, γ, σ and ε, in order of ascending isoelectric points. Later, the anionic (acidic) form of GST isoenzymes (PI values of 4.5-4.9) were identified in various human tissues, designated GST ρ or GST π (Marcus et al. 1978; Guthenberg et al. 1981). The third type of GST isoenzymes, having the near neutral isoelectric point (PI values of 5.1-6.8) were demonstrated in several tissues (Warholm et al. 1981, 1983). These isoenzymes were later grouped in the class Mu of the GSTs by Mannervik et al. (1985). Board (1981) suggested that the near neutral, cationic, and anionic forms of the GSTs were the
products of distinct genetic loci termed as GST1, GST2, and GST3, respectively. The near neutral type of GST (GST1, GSTμ) has been reported to be absent in liver of 50% adults due to a gene deletion (Seidegard et al. 1988). Several reviews have recently described the GSTs in great detail (Hayes et al. 1995; Shen et al. 1997; Salinas et al. 1999).

3. CLASSIFICATION OF GSTs

So far three groups of GST enzymes, which have arisen through both convergent and divergent pathways of evolution, have been identified (Table 1). The mammalian soluble or cytosolic GSTs were discovered in 1961 (Booth et al. 1961; Combes et al. 1961) and are the best studied group. They have been classified into at least seven classes, namely alpha (α), kappa (κ), mu (μ), pi (π), sigma (σ), theta (τ), and zeta (ζ) based on substrate specificity and sequence similarity (Mannervik et al. 1985). It should be mentioned that there are no established rules concerning the percentage of sequence similarity needed to include a GST in a specific class. Usually GSTs that share greater than 40%-50% sequence similarity are placed in the same class. Additionally, emphasis is given to the N-terminal region of the primary structure of the GSTs for classification since this region tends to be more conserved within the classes. In general, the sequence similarities for the GST members within a class is greater than 70%, and it is usually less than 30% between the members of different classes. The GST members in each class appear to have distinct gene structures and chromosomal localizations. Each GST class consists of several subunits. The active GST enzymes form homo- or hetero-dimeric combinations of the subunits within each class evolving distinct substrate specificities.
Table 1. Three distinct groups of glutathione transferases.

<table>
<thead>
<tr>
<th>Group</th>
<th>Divergent Classes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Cytosolic</td>
<td>Alpha, Kappa, Mu, Pi, Sigma, Theta, Zeta</td>
</tr>
<tr>
<td>2 Membrane-bound</td>
<td>Microsomal, leukotriene C4 synthetase</td>
</tr>
<tr>
<td>3 Metallo-</td>
<td>Fosfomycin proteins (FosA, FosB)</td>
</tr>
</tbody>
</table>
The second group of GSTs is the membrane-bound form that includes microsomal GST and leukotriene C4 synthase (Morgenstern et al. 1982; Jakobsson et al. 1996). Neither of them share sequence identity with the cytosolic GST enzymes (DeJong et al. 1988, Lam et al. 1994). It is not known yet how widely the membrane bound GSTs are distributed in nature. The microsomal GST, originally isolated from the microsome fraction of rat liver, is found in rodents, humans, and cows. It is involved in the detoxification of xenobiotic compounds, while leukotriene C4 synthetase conjugates leukotriene A4 with GSH. Human microsomal GST has a subunit size of 17 kD and, unlike cytosolic GSTs, the active isoenzyme is a trimer. Ahmad et al. (1990) have suggested that various GST isoenzymes have possibly evolved from microsomal GST via gene duplication and divergence.

The third group of GSTs is the metalloglutathione transferases which are represented by a bacterial GST (FosA) involve in resistance to the antibiotic fosfomycin (Arca et al. 1990). The FosA was shown to catalyse the conjugation of the GSH with the antibiotic fosfomycin, leading to its inactivation (Arca et al. 1988). FosA is a 16 kD dimeric enzyme, and was shown to have higher catalytic activity in the presence of divalent metal ions such as Fe$^{2+}$, Mn$^{2+}$, Co$^{2+}$. Metalloglutathione transferase superfamily has diverse catalytic members including FosA, FosB, glyoxalase I, and estradiol dioxygenases (Table 1). The possible roles of metal ions in the catalytic activity of FosA include stabilization of the protein folding, GSH thiol activation for nucleophilic attack of fosfomycin (Laughlin et al. 1998).

### 3.1. Nomenclature of GSTs

The cytosolic GSTs are encoded by a number of genes grouped in seven distinct classes. Over a period of years, several nomenclatures have been suggested for GST subunits, which has caused concern in the researchers in this area.
Boyland et al. (1969) classified various forms of GSTs based on their substrate specificities. As a result, they introduced terms such as epoxide transferase, aryltransferase, and alkyltransferase. At the time, the available knowledge about GSTs would recommend substrate-based nomenclature. However, identification of new members in this family of enzymes revealed that they displayed overlapping substrate specificities resulting in replacement of the original nomenclature system by a system based on the structural or physical properties of the GST enzymes (Askelof et al. 1975).

Later, the Y-based nomenclature system for rat GST subunits was proposed by Bass et al. (1977). This system of nomenclature was based on the relative mobilities of the GSTs in SDS-polyacrylamide gel electrophoresis leading to identification of Ya, Yb, and Yc subunits, in order of their decreasing mobility. The main drawback of this system is that it does not allow interspecies subunit comparison.

Subsequently, Jakoby et al. (1984) suggested the Arabic numeral nomenclature for GSTs. According to this system of classification, a GST enzyme was named based on its subunit combinations. In other words, this system would allow subunit constituents to be displayed (e.g. GST1-1). Mannervik et al. (1982) had shown earlier that homo- and heterodimeric combinations of different subunits possess distinct substrate specificities. The disadvantage of this system is that it does not clearly show to which gene family each subunit belongs.

Recently, Mannervik et al. (1992) have proposed a species-independent nomenclature for GSTs, grouping the subunits into recognized classes and numbering them sequentially within each class on the basis of the order of identification. In this new system of classification, a subunit is designated by a single letter followed by an Arabic numeral. This shows their assignments to one of the classes according to the order they have been described. For instance, class alpha subunits of the GST are designated as GSTA1, GSTA2, etc. The dimeric isoenzymes are designated by a capital letter (signifying the class), two Arabic numerals separated by a hyphen (signifying two
subunits). Thus, the class alpha heterodimer (Yc1Yc2, GST2-10) originally described in rat liver, is now represented as rGSTA3-5 (rat GST alpha 3-5).

4. EVOLUTION OF THE GSTs

In nature, evolution of macromolecules such as DNA and proteins take place constantly from the ancestral precursors over a long period of time. At the DNA level, events such as gene duplication and recombination of gene fragments of the existing genes occur that leads to new members with novel and divergent function. Information on structures of the various proteins including GSTs have shown that most proteins have arisen from the predecessors by limited modifications such as amino acid substitutions, deletion or insertion of peptide fragments, or fusion of different domains of the protein, acquiring diversified, new functions.

GST enzymes have evolved to protect cells from toxic endogenous and xenobiotic electrophilic compounds. As mentioned earlier, GSTs are extremely diversified and are classified into seven classes, on the basis of their sequence similarities and subcellular distribution. The relatedness of GST subunits within each class is determined primarily on the basis of sequence comparisons. However, there are no established criteria for the extent of sequence similarity necessary to be considered as a member of one class over another. Although the evolutionary relationships of the various isoenzyme classes are not entirely certain, analysis of both the sequences and exon-intron boundaries of several GST genes provides some insight. Comparison of GST classes show that class theta (τ) is remotely related to all other classes. Specifically, class theta has 5-15% identity with other classes, while all others share more than 20% identity among themselves. According to these sequence comparisons, Pemble et al. (1992) has proposed that class theta was possibly the evolutionary precursor for genes encoding alpha, mu, and pi proteins. Class theta is considered as the most ancient gene among the GSTs. In other
words, all other GST classes evolved from a theta-gene duplication. The analysis of their sequences have also suggested that class sigma (δ) GST was probably diverged from the ancestral precursor first, followed by class mu divergence from common alpha/mu/π proteins (Fig. 1). This is possibly followed by alpha/π divergence (Dirr et al. 1994).

FIGURE 1. The proposed evolutionary pathway of GSTs.

A common feature of the GSTs is their ability to bind glutathione. Another property is their ability to recognize and detoxify compounds with diverse chemical structures. The evolution of the GSTs in which relatively low binding affinities are offset by broad substrate specificities constitutes an energy efficient response to toxin exposure. Although substrate specificities are somewhat overlapping, the enzymes are critical to eukaryotic organisms in that they display unique activities toward a variety of toxic compounds (Board et al. 1997).
5. TISSUE DISTRIBUTION OF GSTs

Various GST isoenzymes are expressed in a tissue-specific fashion. GSTs represent approximately 1% of total cellular protein, and about 5% of the cytosolic protein in the liver, suggesting that they play an important role in maintaining cellular homeostasis (Mannervik 1985). Normal tissues exhibit quantitative and qualitative differences in GST content (Zimniak et al. 1994). The various GST isoenzymes have different patterns of tissue distribution and catalytic activities, suggesting that they may be a contributing factor in tissue-specific susceptibility to the carcinogenic process. More than 90% of GSTs present in all tissues belong to the alpha, pi and mu classes. In humans and rodents, GST alpha is the predominant form expressed in liver. GST pi is only minimally present in hepatocytes, and GST mu is present in the liver of approximately 50% of the general population as a result of frequent mutation (Warholm et al. 1981).

GST alpha is also substantially expressed in other tissues such as kidney, fetal fibroblasts, adrenal gland, lens, and testis. In kidney, besides class alpha, class mu and pi GSTs are present in substantial amounts. In brain and lung, over 90% of GST activity towards CDNB (1-chloro-2,4-dinitrobenzene) has been shown to be due to class pi isoenzymes (Campbell et al. 1990; Singhal et al. 1992). In muscle, GST alpha subunits are not expressed, but pi, mu, and zeta GST classes are predominantly present (Singh et al. 1988).

In both humans and mice, GST pi is highly expressed in most tissues except liver, kidney, fetal fibroblasts, testis, adrenal gland, and retina. However, in rats, this class of GST is not present in most tissues. Human erythrocytes contain class mu as well as sigma GSTs in substantial amounts (Peter et al. 1989; Hallier et al. 1990).
Tissue-specific regulatory elements have been identified in the promoter of GST subunit genes (Paulson et al. 1990; Pulford et al. 1996). Rat GSTA1, A2, and A5 subunits have been shown to be expressed specifically in hepatocytes. These genes possess enhancer elements, termed hepatocyte nuclear factor (HNF). Elimination of these elements has revealed reduction in basal expression of GST in HepG2 (human hepatoma) cell lines (Paulson et al. 1990).

Rat GSTM3 subunit gene (class mu) was shown to be expressed primarily in the brain and testis (Abramovitz et al. 1995). Sequence analysis of the regulatory region of this gene revealed the presence of two octamer sequences (ATTTGCAT) separated by a 6-bp spacer. Functional analysis have demonstrated that the two octamer repeats were necessary for expression of the rGSTM3 gene in C6 glioma cells. In addition, electrophoretic mobility shift assays confirmed that the nuclear extracts from C6 glioma cells bind specifically to the synthetic octamer sequences (Abramovitz et al. 1995), suggesting their importance in tissue-specific expression of the rGSTM3 gene in glioma cells, and possibly in brain and testis.

6. STRUCTURE OF THE GSTs

A knowledge of structure of the GSTs is a prerequisite in understanding their properties. Over the last couple of years, various laboratories have determined the three dimensional structure of different GST isoenzymes by x-ray crystallography (Sinning et al. 1993; Dirr et al. 1994; Wilce et al. 1994). The information provided by GST structure analysis gives an insight into their catalytic mechanisms. All GSTs have a molecular weight of approximately 26 kD and the biologically active forms are either homo- or heterodimeric proteins. The x-ray crystallography has shown that each monomer contains two distinct ligand binding pockets, the G-site and the H-site. The G-site binds GSH in a highly specific manner, while the H-site interacts with a variety of
hydrophobic substrates with low specificity. The three-dimensional structure of the GST classes has revealed that each subunit contains two domains (Rossjohan et al. 2000). The smaller N-terminal domain or domain I comprises the amino acids that form the G-site (GSH-binding site). The larger C-terminal domain or domain II contains the amino acids that form the H-site (second substrate-binding site). A general features of GST catalysis seems to be initial activation of the thiol of GSH to the thiolate anion to enhance its nucleophilicity, followed by stabilization of the deprotonated thiolate anion (Rossjohan et al. 2000). It is thought that due to the considerably higher cellular concentration (1-10 mM) of GSH, it binds first to the GST enzymes. Analysis of the three-dimensional structure of the GSTs has identified the residues involved in the subunit dimerization. Ji et al. (1992, 1995) have demonstrated that an arginine-arginine interaction located in domain I, as well as several salt bridges and hydrogen bonds formed in the middle of the dimer interface are responsible for subunit dimerization of the GSTs.

The three-dimensional structures of GSTs have a conserved chain fold of the glutathione-binding domain (Wilce et al. 1994). The second domain is more variable and is principally involved with the electrophilic substrate. The structural differences contribute to the differential substrate selectivity seen between isozymes (Mannervik 1985). For example, it was proposed that in rat GSTA5 subunit gene, Y108 and D208 residues could be involved in the high activity of this isoenzyme for hepatocarcinogen aflatoxin B1 exo-8, 9-epoxide (Hayes et al. 1994). The generation of isozymes with novel substrate specificities has been attributed to gene conversion and exon shuffling (Mannervik 1985).

Various site-directed mutagenesis and x-ray crystallography studies have identified important GSH binding residues within the active sites of GSTs. The protein conformation was shown to be similar for class Alpha, Mu, and Pi GSTs with active sites located in similar positions. For example, in hGSTP1 and hGSTA1, hexyl and benzyl
moieties of the inhibitors bind in a similar location corresponding to the H-site of the class Mu protein.

Crystal structure of the cytosolic GSTs have revealed that their overall chain fold is similar, but each class possesses some unique features. The structural differences of the GST enzymes appears to be due to modifications in the active site as well as the C-terminal region (Dirr et al. 1994; Wilce et al. 1994; Armstrong 1997). For instance, both class Mu and Pi GSTs are shorter than human GSTA1 by 4 to 8 residues. The H-site of both Mu and Pi is larger and more hydrophilic while in hGSTA1 the H-site is more hydrophobic, binding to nonpolar substrates. Sinning et al. (1993) have resolved the structure of the human GSTA1-1 (hGSTA1-1) by X-ray crystallography.

The sequence alignment of all mammalian GSTs has revealed the existence of an active, conserved Tyr site (Sinning et al. 1993), and a single replacement of the conserved Tyr with Phe was shown to result in loss of the specific activity for the mutated GST (Rushmore et al. 1993).

Wilce et al. (1995) have characterized the crystal structure of a GST theta from Blowfly insect. The theta class is distinguished by a conserved Ser9 site located at the N-terminus.

It is of great value to identify and compare the active sites of various GSTs, and to determine how their respective inhibitors bind to these sites. These results could be used in designing more class-specific inhibitors for GST enzymes in order for chemotherapeutical agents to become more effective in cancer treatment. In fact, several studies have shown elevated levels of specific GSTs in cells that exhibit resistance to anticancer drugs (Linsenmeyer et al. 1992).
7. BIOCHEMICAL CHARACTERISTICS OF GSTs

The major function of GST isoenzymes is to detoxify both endogenous and exogenous xenobiotic compounds, and to protect against their adverse effects such as oxidative stress and cancer. The basic GST chemistry involves the generation of the thiolate anion (GS⋅) of the tri-peptide glutathione (GSH, \( \gamma \)-glutamyl cysteinyl glycine), increasing its nucleophilic attack at the electrophilic center of hydrophobic substrates (Figure 2A). The resulting conjugated products become less reactive, more soluble and hence more easily excreted from cells. Thus, the catalytic function of the GSTs takes place both through the generation of GS⋅ and the formation of a thioether bond between GSH and hydrophobic electrophilic substrates. This is the first stage in the biosynthesis of excretory metabolites such as mercapturic acids (Mannervik 1985). The subsequent stages involve sequential reactions represented by enzymes including \( \gamma \)-glutamyl transpeptidase, cysteinyl glycinease and N-acetyl transferase (Figure 2A; Boyland et al. 1969).

GSTs also function as glutathione peroxidase, catalysing the reduction of organic hydroperoxides to their respective alcohol (Figure 2B). These reactions involve nucleophilic attack by GSH on hydrophobic chemicals at electrophilic oxygen centers. Organic compounds such as phospholipids, fatty acids, and DNA hydroperoxides generated in the cells by lipid peroxidation and oxidative DNA damage are the GST substrates in reduction reactions resulting in their detoxification.

In addition to their enzymatic functions, GSTs have been reported to bind both covalently and noncovalently to a variety of compounds including carcinogens (e.g. dimethylaminoazobenzene and 3-methylcholanthrene) (Ketterer et al. 1967), steroids, bilirubin, bile acids and thyroid hormones. (Hayes et al. 1986; Danger et al. 1992) thus acting as regulatory proteins.
FIGURE 2. GST chemical reactions. A) Conjugation reaction leading to mercapturic acid pathway  B) Peroxidase reaction. GSH (γ-Glu-CysH-Gly), glutathione; R, aryl or alkyl group; X, electrophilic leaving groups; GSSG, oxidized glutathione.
Most GST substrates are either xenobiotic compounds or products of endogenous oxidative stress. GST isoenzymes have broad substrate specificity, ensuring effective detoxification of the majority of chemical compounds. For instance, GST pi specifically conjugates base propenals (the major low molecular weight products of DNA degradation produced by many toxic chemicals), and GST alpha and mu have shown specific activity against 4-hydroxyalkenals (the product of free radical initiated lipid peroxidation process). Many anticancer alkylating drugs and their metabolites, such as melphalan, cyclophosphamide, chlorambucil, mechlorethamine, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and aldophosphamide are potent electrophilic agents which have been shown to be GST substrates. These and some other important substrates of the GSTs are listed in Table 2. Alkylating agents have the capacity to modulate replication of DNA by causing single- or double-stranded DNA cross links and/or breaks. The detoxification of alkylating agents in GST catalyzed reactions has been well described. For example, GSTs have been demonstrated to catalyse the conjugation of GSH to 1,3-bis(2-chloroethyl)-1-nitrosourea (Smith et al. 1989), cyclophosphamide (Yuan et al. 1990), chlorambucil (Ciaccio et al. 1990), and melphalan (Bolton et al. 1991).

Several chemical carcinogens and their activated metabolites such as AFB₁-8,9-oxide, the potent hepatocarcinogenic metabolite of aflatoxin B₁ (AFB₁), is detoxified by its conjugation with GSH. Numerous studies attributed detoxification of AFB₁-8,9-oxide to specific subunits of the class alpha GST (Buetler et al. 1992; Kelly et al. 2000). For example, rGSTA5 subunit has been shown to have substantial activity towards AFB₁-8,9-oxide (Hayes et al. 1994). Introduction of the murine GSTA3 (Yc) subunit gene into a hamster V79 cells was reported to cause a fivefold increase in resistance to AFB₁ cytotoxicity, and 3.3-fold decrease in DNA adduct formation following AFB₁ exposure (Townsend et al. 1998). Another group of carcinogens, polycyclic hydrocarbons such as benzo[a]pyrene, are also conjugated with GSH by GST isoenzymes. Benzo[a]pyrene is
activated by CYP450 to a more potent carcinogen, (+)-anti-benzo[a]pyrene-7,8-diol-9,10-oxide (anti-BPDE). This chemical compound has been reported to be a substrate for human mu and pi GST classes.

Recently, Adler et al. (1999) characterized GST pi as an endogenous inhibitor of Jun N-terminal kinase (JNK), providing a novel function for this complex, multifunctional family of enzymes. One of the end results of the JNK activity was earlier reported to be induction of apoptosis (Kasibhatla et al. 1998). Thus, based on JNK inhibition by GST pi, cancer cells overexpressing this enzyme may inhibit intrinsic JNK activity which leads to resistance to apoptosis.
Table 2. Examples of biologically important GST substrates

1) Carcinogens
   Aflatoxin B1-8,9-oxide
   Anti-benzo[a]pyrene-7,8-diol-9,10-epoxide

2) Antineoplastic agents (Alkylating agents)
   A) Nitrogen mustards
      Melphalan
      Chlorambucil
      Cyclophosphamide
      Mechlorethamine
      Ifosfamide
   B) Nitrosoureas
      1,3-bis(2-chloroethyl)-1-nitrosourea
      Dacarbazine
      Procarbazine
   C) Aziridines and epoxides
      Mitomycin C
      Thiotepa

3) Oxidation products of membrane and DNA
   Fatty acid hydroperoxides
   DNA hydroperoxides
   4-hydroxy alkenals
8. ANTICANCER DRUG RESISTANCE

8.1. Introduction

The ability of cells to combat chemical insult is either a de novo property of the cells (intrinsic resistance) or a result of emerged resistance to chemotherapy (acquired resistance). Although very little is known about intrinsic resistance, there has been a great deal of progress in understanding acquired resistance. Intrinsic resistance is a refractory characteristic of tumor cells which exists even in the absence of earlier drug exposure. Acquired drug resistance is developed as a result of cellular changes occurring due to drug exposure which gives cells a selective growth advantage. The development of anticancer drug resistance is a major problem in cancer treatment with chemotherapy. A number of potential mechanisms have been reported to account for tumor cell drug resistance (Figure 3); some of these include alterations in drug transport through the multi-drug resistance P-glycoprotein (MDR) and multidrug resistance-associated protein (MRP) efflux pump (Ames 1986; Fardel et al. 1996), changes in DNA repair pathway (Brown et al. 1997; Barret et al. 1998) especially DNA topoisomerases (Withoff et al. 1996), resistance to apoptosis (Hichman 1992; Anthoney et al. 1996), and alteration of GSH and GSH-related enzymes (Batist et al. 1986; Tew 1994). Several studies have demonstrated the involvement of GST isoforms (Tew 1994; Shen et al. 1997) in chemotherapy resistance to various drugs, particularly to alkylating agents (Ban et al. 1996).
FIGURE 3. Some potential mechanisms of drug resistance.
8.2. Role of GSTs in anticancer drug resistance

Numerous publications have reported that the levels of GST expression in various organisms contribute significantly to their resistance to toxic chemicals. Evidence from various studies suggest that when GSTs have been associated with resistance to toxic xenobiotic chemicals, the level of GST expression was highly elevated. For instance, bacterial resistance to antibiotics (Arca et al. 1990), insect resistance to pesticides (Ranson et al. 2000), plant resistance to herbicides (Edward et al. 2000) and resistance of some rodent species to hepatocarcinogen aflatoxin B₁ (Buetler et al. 1992).

A large body of literature has implicated GST isoenzymes in drug resistance. GSTs catalyse the conjugation of GSH with highly toxic chemicals, leading to less toxic, more water-soluble products which are easily eliminated from cells. However, this invaluable service of GSTs becomes disadvantageous during chemotherapy where tumor cells develop drug resistance.

The cellular glutathione and GSH-related enzymes including GSTs have been shown to be a critical cellular detoxification system. As defined earlier, GSTs are phase II detoxification enzymes which catalyze the binding of GSH via its thiol (-SH group) with the reactive group of the cytotoxic agents. The resulting conjugated products become less reactive, more water-soluble, and are excreted from the cell. The elimination of the inactivated metabolites takes place with the participation of the transporter proteins termed GS-X.

GSH is the most abundant intracellular non-protein thiol (0.3-10 mM). It has many important functions such as cellular defense and metabolism, and cell protection from oxidative stress and toxic electrophilic compounds. Enhanced levels of intracellular GSH were reported in cell lines resistant to alkylating agents such as melphalan, chlorambucil, BCNU and cyclophosphamide. (Tew 1994). Depletion of GSH with agents including buthionine sulfoximine (BSO), an inhibitor of γ-glutamyl-cysteine synthetase (γ-GCS
enzyme), have been reported to increase the cytotoxicity of drugs such as melphalan (Kramer et al. 1987) and cyclophosphamide (Tomashesky et al. 1985). GST enzymes catalyse the addition of GSH with alkylating drugs, resulting in drug inactivation and detoxification. Therefore, increased expression and activation of GST enzymes could lead to drug resistance (Tew 1994). In fact, numerous studies have reported that overexpression of GSTs is associated with the development of cell-line resistance to a broad spectrum of compounds including alkylating agents (Table 3).

GST isoenzymes have a broad range of functions as well as substrate specificities. They could confer drug resistance through 1) addition of the thiol-containing GSH to the electrophilic chemical compounds, 2) covalent and noncovalent binding of hydrophobic chemicals and 3) inactivation of DNA as well as lipid hydroperoxides by de novo peroxidase activity (Jakoby et al. 1978; Hayes et al. 1995).

The majority of cell lines demonstrating multi-drug resistance have revealed increased level of GST pi, but several laboratories including ours, have shown increased expression of GST alpha (rGSTA1, rGSTA3) subunit genes in cell-lines selected for resistance to alkylating agents such as melphalan (Lewis et al. 1987; Schecter et al. 1993). Clapper et al. (1991) has also reported that alkylating agents can selectively induce some members of the GST alpha class. In addition, studies involving GST enzyme inhibition implicated class alpha GST in alkylator resistance. When resistant cells were treated with GST inhibitors like ethacrynic acid, treatment resulted in decreased drug resistance of the tumor cells (Tew et al. 1988). Furthermore, clinical specimens collected from patients with resistance to chemotherapy, were shown to have enhanced GST activity (Kuroda et al. 1991). In other studies, Ban et al. (1996) demonstrated that transfection of GST pi antisense cDNA could enhance the sensitivity of a colon cancer cell line to various antineoplastic drugs such as melphalan.
<table>
<thead>
<tr>
<th>Alkylating agent</th>
<th>Cell line</th>
<th>GST isozyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Melphalan</td>
<td>Rat mammary carcinoma</td>
<td>alpha</td>
</tr>
<tr>
<td></td>
<td>Human melanoma</td>
<td>Pi</td>
</tr>
<tr>
<td></td>
<td>Human myeloma</td>
<td>Pi</td>
</tr>
<tr>
<td>2) Chlorambucil</td>
<td>Chinese hamster ovary</td>
<td>alpha</td>
</tr>
<tr>
<td></td>
<td>Human ovarian adenocarcinoma</td>
<td>alpha</td>
</tr>
<tr>
<td></td>
<td>Walker rat cells</td>
<td>alpha</td>
</tr>
<tr>
<td>3) Cyclophosphamide</td>
<td>Yoshida rat sarcoma</td>
<td>alpha</td>
</tr>
<tr>
<td>4) CDNB</td>
<td>Human lung</td>
<td>alpha</td>
</tr>
<tr>
<td>5) BCNU</td>
<td>Human melanoma</td>
<td>Pi</td>
</tr>
<tr>
<td></td>
<td>Rat gliosarcoma</td>
<td>mu</td>
</tr>
</tbody>
</table>
Caffrey et al. (1999) reported that exposure of melphalan resistant human ovary tumor cells (A2780) to ethacrynic acid resulted in both decreased GST enzyme activity and increased drug sensitivity, indicating the involvement of GSTs in melphalan resistance. GSTs have been demonstrated to catalyse the addition of GSH to various alkylating agents such as nitrosourea (Smith et al. 1989), cyclophosphamide (Yuan et al. 1990), chlorambucil (Ciaccio et al. 1990), and melphalan (Bolton et al. 1991), resulting in their detoxification. The interaction of alkylating agents with DNA is thought to be an important event that leads to cell killing (Chasseaud et al. 1979). This interaction may be competed by GSH nonenzymatically or through GST mediated reactions, inhibiting DNA-crosslinking formation of the drugs. In addition, it has been proposed that both GSH and GST affect cellular DNA repair machinery (Ketterer et al. 1989).

To understand anticancer drug resistance, our laboratory has previously reported the isolation of a drug resistant rat mammary carcinoma cell-line (MatB) by exposing cells to increasing concentrations of the alkylating agent melphalan (MLNr MatB cell line). The earlier in vitro and in vivo studies in this laboratory showed enhanced GST activity, especially increased Yc subunit (now designated as rGSTA3) in MLNr cells (Schecter et al. 1991, Schecter et al. 1993). The introduction of the rat liver Yc cDNA (clone pGTB42) into WT MatB cells was reported to confer several-fold resistance to melphalan (Schecter et al. 1991). This newly emerged melphalan resistant cell line showed cross-resistant to mechlorethamine and chlorambucil.

The association between GST over-expression and increased antineoplastic drug resistance of tumors creates a rational to modulate GST expression and activity throughout chemotherapy by GST inhibitors such as ethacrynic acid or GSH analogues (Table 4). The approach is to reverse the drug resistance by GST inhibiting agents. Drug resistant cell-lines derived from mammalian tumors have been useful in this
investigation. Ethacrynic acid has been one of the best characterized inhibitor of the GSTs. It inhibits GSTs in both competitive and non competitive ways. Its inhibition of all GST classes was reported to be competitive towards CDNB (a GST substrate) and non competitive towards GSH (Ploemen et al. 1990). Moreover, the GSH-ethacrynic acid conjugate was found to be a GST inhibitor as well. Ciaccio et al. (1990) demonstrated that GST alpha mediated conjugation of chlorambucil and GSH is inhibited by ethacrynic acid, supporting the notion that GST inhibitors could modify the efficacy of alkylating agents through interruption of their GST-catalyzed conjugation with GSH. Ethacrynic acid has also been shown to restore the sensitivity of tumor cells to drugs such as adriamycin (Nagourney et al. 1990), melphalan (Hansson et al, 1991), and mitomycin C (Xu et al. 1992). It is interesting to mention that some tumor cells were shown to acquire resistance to ethacrynic acid as a result of increased GST expression and activity (Kuzmich et al. 1992). Since some of the existing GST inhibitors are not very specific for tumor cells, the development of GST-specific inhibitors with lesser side effects appears to be essential for use in chemotherapy of tumors. For example, Lyttle et al. (1994) designed an inhibitor termed TER 117 (γ-L-glutamyl-S-(benzyl)-L-cysteinyl-R-(-)-phenylglycine) that specifically inhibits human GST P1-1, reverting drug resistance of tumor cells. Thus, the modulation of GST activity during chemotherapy by inhibitors such as GSH analogues or by GST inhibitors including ethacrynic acid, ellagic acid, pipirprost, gossypol, tienilic acid, sulfasalazine, indomethacin, triphenyltin chloride, and other novel inhibitors including TER 117 could be a promising strategy against cancer.

Over-expression and increased activity of GSTs with subsequent protection against toxic chemical and carcinogenic compounds led to the identification of cis-acting regulatory elements in the promoters of the GST subunit genes, mediating their induction. Two of these regulatory elements are known as the xenobiotic responsive element (XRE), and the antioxidant responsive element (ARE). An XRE with core
sequence (GCGTG) was identified in the 5' flanking region of the Ya gene (now designated as rGSTA2) (Paulson et al. 1990) which is identical to the XRE found in the rat cytochrome P450 (CYP1A1) gene, being recognized by the liganded Ah receptor complex. The Ah receptor binds to some xenobiotic compounds such as polycyclic aromatic hydrocarbon (PAH), resulting in translocation of the ligand-Ah receptor complex to the nucleus and ultimately interacts with the XRE sequence, transactivating the XRE-containing genes (Reyes et al. 1992). Deletion analysis by Rushmore et al. (1991) led to the identification of another important cis-acting element (ARE) with the core sequence of TGACNNNGC, mediating the induction of the rGSTA2 gene by monofunctional inducers.

Table 4. Examples of GST inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>Drug Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Clofibrate</td>
<td>Antihyperlipidemic agent</td>
</tr>
<tr>
<td>2) Ethacrynic acid</td>
<td>Diuretic</td>
</tr>
<tr>
<td>3) GSH analogues</td>
<td>GST inhibitors</td>
</tr>
<tr>
<td>4) Gossypol</td>
<td>Antifertility drug</td>
</tr>
<tr>
<td>5) Indomethacin</td>
<td>NSAID</td>
</tr>
<tr>
<td>6) Misonidazole</td>
<td>Antifungal drug</td>
</tr>
<tr>
<td>7) Piriprost</td>
<td>NSAID</td>
</tr>
<tr>
<td>8) Sulfasalazine</td>
<td>Antibiotic</td>
</tr>
<tr>
<td>9) Ellagic acid</td>
<td>Phenol analogues</td>
</tr>
</tbody>
</table>
9. HEPATOCELLULAR CARCINOGENESIS:

9.1. Introduction

Hepatocellular carcinoma (HCC) is among the five most common cancers in the world. It is a major cause of cancer morbidity and mortality worldwide. Approximately half a million new cases are estimated annually, and almost an equal number of patients die as a result of this disease. The frequency of occurrence of HCC is subject to regional variations. Approximately eighty percent of cases occur in developing countries such as sub-Saharan Africa and eastern Asia (Parkin 1988). HCC is a serious disease in these areas. For example, it accounts for almost 10% of all adult male mortality in Western Africa. As well, HCC is increasingly becoming an important health issue in other parts of the world such as North America. For example, a recent study showed that the incidence of HCC has increased over the last two decades in the United States (El-Serag et al. 1999). Significant variations in HCC incidence have been observed among different ethnic groups living in the same geographic region and among the same ethnic groups who have migrated to different parts of the world (Bosch 1999). These variations in HCC development are probably a reflection of multi-causative factors such as exposure to chemical carcinogens, infection with hepatotropic viruses and life styles.

Based on epidemiological and experimental studies using animal models, chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) and exposure to aflatoxin B1 contaminated food are considered major risk factors for HCC (Hall et al., 1994). Life styles that include heavy cigarette smoking and alcohol consumption appear to be other risk factors for HCC development.

Exposure to aflatoxin is prevalent in developing countries where food storage facilities are poor and it is found that the regions of the world with highest exposure to aflatoxin are often those with high rates of HBV infection (Montesano et al. 1997). The risk of HCC associated with aflatoxin exposure has been shown to be greater in HBV...
infected individuals (Qian et al. 1994). The mechanism of interaction between these risk factors is not yet well understood. However, the research work in our laboratory in this field may contribute to the overall understanding of hepatocellular carcinogenesis and its prevention. Several lines of evidence have suggested the importance of glutathione transferases in hepatocellular carcinogenesis (Zhou et al. 1997; Wang et al. 1999) which will be described in the following section.

9.2. Implication of GSTs in HCC

The fact that the most potent human liver carcinogens such as aflatoxin B1 (AFB1) are important substrates for GSTs, and that these enzymes represent as much as 5% of the cytosolic protein in the liver, suggests that they may play an important role in protecting hepatocytes from the toxic effects of these chemicals. AFB1 is a naturally occurring liver carcinogen produced by the mold Aspergillus flavus (Eaton et al. 1994). In humans, it is activated to the ultimate carcinogen AFB1 exo-8,9-epoxide by phase I isoenzymes such as CYP1A2 and CYP3A4 (Iyer et al. 1994). The exo-8,9-epoxide has been shown to be detoxified by class alpha GSTs including rGSTA5, mGSTA3 and hGSTA1 by catalyzing conjugation with GSH (Buetler et al. 1992). The fact that mice with intrinsic resistance to AFB1 express high levels of mGSTA3 in the liver and that rats with high sensitivity to this toxin express substantially lower levels of rGSTA5, support the importance of GSH and GST mediated detoxification (Hayes et al. 1994).

It has been shown in many studies using animal models that treatment with compounds that induce GSTs including ethoxyquin, benzyl isothiocyanate (BITC), butylated hydroxy-anisole (BHA), oltipraz and coumarin (Kelly et al. 2000) protects animals from the toxicities of carcinogens such as AFB1. Oltipraz and the antioxidants ethoxyquin and BHA have been suggested to block AFB1 hepatocarcinogenesis. Several GST isoenzymes were shown to be induced following treatment with or consumption of the above compounds. GST induction was demonstrated to correlate with both reduced
formation of AFB\textsubscript{1}-DNA adduct and enhanced catalytic activity of GSTs toward AFB\textsubscript{1}-GSH conjugation. Some agents such as ethoxyquin and oltipraz proved to be potent inducers of rGSTA5, while others including coumarin induce GSTP1 subunit of the class pi (Kelly et al. 2000).

The antischistosomal agent oltipraz, which was demonstrated in experimental animals to induce GST isoenzymes, is currently being considered for human clinical trials as a protectant against aflatoxin B\textsubscript{1}-induced carcinogenesis. Oltipraz was shown in animal models to decrease both aflatoxin-albumin adducts as well as the risk of liver cancer (Kensler et al. 1997).

In addition to AFB\textsubscript{1}, hepatitis B virus (HBV) represents another major risk factor for the development of hepatocellular carcinoma (HCC). Human liver specimens obtained from HCC patients showed a drastic reduction in GST expression and activity (Zhou et al. 1997), suggesting decreased cellular protection from chemical carcinogen exposure and viral infection.

9.3. Role of GST in chemoprotection

The carcinogenesis process is a prolonged accumulation of injuries at several different biological levels and includes both genetic and biochemical cellular changes. Chemoprevention is a promising strategy to use natural and synthetic compounds to intervene in the early precancerous stages of carcinogenesis, before invasive disease begins. Several potential chemoprevention agents have been identified through animal research and cancer epidemiology (Table 5). In contrast to chemotherapeutic agents which have the ability to kill cells through their cytotoxic effect, chemopreventive agents must be non-toxic and relatively free of side effects, as they are meant for long term use in healthy people.
Fruits and vegetables are a good source of most chemopreventive compounds. Many phytochemicals protect against cancer in laboratory studies. These chemoprotective phytochemicals include vitamins, as well as compounds without nutritional value such as indoles, isothiocyanates, dithiolthiones and organosulfur compounds. They are thought to work by activating detoxifying enzymes in the liver (Kelly et al. 2000). For example, dithiolthiones found in cruciferous vegetables such as broccoli, cauliflower and cabbage are potential chemopreventive agents. They provide protection against carcinogens by inducing detoxification enzymes such as GSTs (Clapper et al. 1997). A synthetic dithiolthione called oltipraz has been shown to inhibit the development of tumors of the lung, colon, mammary glands and bladder in animal models. Like other preventive agents, oltipraz interferes with carcinogenesis in more than one way. Possible mechanisms of the protective effects of oltipraz include: (1) inhibition of the phase I enzymes to retard metabolic activation, (2) induction of phase II xenobiotic metabolising enzymes to enhance detoxification and elimination of the carcinogens, (3) nucleophilic trapping of the reactive intermediates, and (4) enhancement of the DNA repair processes. In addition, oltipraz has been shown to inhibit human immunodeficiency virus (HIV) replication and also blocks HBV transcription through p53 protein elevation.

Chemopreventive agents are substances that can block the onset of cancer. Some chemopreventive agents are intended to prevent initiation, while some arrest progression, either before or after genetic mutations cause a cell to become precancerous. Other chemopreventive agents divert cancer progression, leading to a benign outcome. For instance, while coumarin pretreatment of rats before AFB₁ exposure prevented hepatocyte foci formation completely, it did not show much effect when given after AFB₁ exposure (Kelly et al. 2000). This suggested that coumarin is effective at preventing the initiation of AFB₁ carcinogenesis.
Table 5. Some GST-inducing chemopreventive agents

<table>
<thead>
<tr>
<th>Compound</th>
<th>Source and type of agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Benzyl isothiocyanate</td>
<td>Garden cress</td>
</tr>
<tr>
<td>2) Coumarin</td>
<td>Leguminosae sp.</td>
</tr>
<tr>
<td>3) Indol-3-carbinol</td>
<td>Cruciferous vegetables</td>
</tr>
<tr>
<td>4) Sulforaphane</td>
<td>Broccoli</td>
</tr>
<tr>
<td>5) Oltipraz</td>
<td>Antischistosomal drug</td>
</tr>
<tr>
<td>6) Ethoxyquin</td>
<td>Synthetic antioxidant</td>
</tr>
<tr>
<td>7) Butylated hydroxyanisol</td>
<td>Synthetic antioxidant</td>
</tr>
<tr>
<td>8) Diethyl maleate</td>
<td>Synthetic drug</td>
</tr>
<tr>
<td>9) t-SO, trans-stilbene oxide</td>
<td>Synthetic model inducer</td>
</tr>
<tr>
<td>10) β-naphthoflavone</td>
<td>Synthetic flavonoid</td>
</tr>
</tbody>
</table>
9.4. Risk factors associated with HCC development

A large body of epidemiological and experimental studies has shown the involvement of various factors in the development of human liver cancer. The worldwide geographical differences in the distribution of HCC has been crucial in identification of the major risk factors. The main established risk factors in the development of HCC are exposure to hepatocarcinogen AFB₁, chronic infection with hepatitis B and/or C virus, heavy alcohol consumption, and cigarette smoking.

9.4.1. Exposure to aflatoxins

The incidence of HCC is greatest in areas where there is concomitant HBV infection and exposure to liver carcinogens such as aflatoxins (Wild et al. 2000). Food contamination by various undesirable fungi cause human exposure to a broad range of mycotoxins (fungal metabolites). Aflatoxins are one group of potent mycotoxins which consist of aflatoxin B₁ (AFB₁), B₂, G₁ and G₂. They are produced by fungal species such as Aspergillus flavus and A. parasiticus. Among them, AFB₁ is the most potent hepatocarcinogen. Food contamination with aflatoxin takes place in areas with hot, humid climates that have poor facilities for storage of the oilseed and cereal crops. Aflatoxins, like many chemical carcinogens, need to be activated by cytochrome P450 (CYP) enzymes to exert its carcinogenic effects. The secondary metabolite of AFB₁, the exo-8,9-epoxide forms adducts with DNA (Eaton et al. 1994). Studies have shown that in rats, HCC caused by AFB₁ exposure is associated with mutations in codons 12 and 13 of ras oncogenes (McMahon, Davis et al. 1990), while in humans it is associated with an AGG to AGT transversion at codon 249 of the p53 tumor suppressor gene (Aguilar et al. 1993; Hussain et al. 2000). The occurrence of human p53 mutation has been demonstrated to be common in HCC patients from Asia and Africa where AFB₁
exposure is prevalent. It is however, rare in other regions of the world where exposure to AFB₁ is low (Aguilar et al. 1993; Kirby et al. 1996; Hussain et al. 2000).

Even-though rats and humans are sensitive to aflatoxins, mice show a high level of resistance without developing liver disease (Hayes et al. 1992). The increased level of GST expression, which inactivates epoxidated AFB₁, has been suggested to play an important role in protection against carcinogenesis. In addition, liver cytosols from male mice was demonstrated to have approximately 50-times more GST activity towards AFB₁-epoxide than those from rats and humans (Monroe et al. 1987).

Aflatoxins have been found to be the most potent hepatocarcinogen known to induce cancer in animals and human (Hall et al. 1994). Epidemiological and laboratory studies have shown a synergistic interaction between aflatoxins and HBV in the incidence of HCC (Wang et al. 1996). The precise nature of the interaction between these factors is not yet known. One reason might be that aflatoxins are immunosuppressive, resulting in an increased susceptibility to viral infection and decreased response to vaccines (Nakamoto et al. 1998). Conversely, viral infection could diminish both immune system function and normal cellular detoxification potential, and therefore concomitant exposure to chemical carcinogens would be more likely to contribute to the development of HCC. The epoxidated aflatoxin binds DNA preferentially in GC rich regions at the N7 position of guanines, inducing G to T transversions (Aguilar et al. 1993). Several studies support the role of these mutations as the initiation step in the development of AFB₁ associated HCC.

The primary preventive measure to decrease aflatoxin exposure is to change the dietary habits of at risk individuals. However, because dietary changes are difficult to implement, an alternative measure would be the use of the natural or synthetic chemopreventive agents to decrease the toxic effects of the ingested aflatoxins.

It was demonstrated that the sensitivity to formation of aflatoxin-DNA and aflatoxin-protein adduct (Wild et al. 1996) as well as susceptibility to aflatoxin
carcinogenesis varies among animals species (Gorelick et al. 1990). Therefore, modulation of aflatoxin activation and detoxification in sensitive and susceptible groups is a promising strategy in carcinogenesis prevention. Induction of GSTs and aflatoxin aldehyde reductase was shown to decrease aflatoxin-DNA adduct formation and inhibit its carcinogenicity in rats (Roebuck et al. 1991; Judah et al. 1993; Egner et al. 1995).

Similarly, it was demonstrated that in humans exposed environmentally to aflatoxin, the administration of the synthetic drug oltipraz inhibited cytochrome P450 1A2 activity, which metabolises aflatoxin to the reactive epoxide, and, at the same time elevated the level of GST conjugation of aflatoxin 8,9-epoxide (Wang et al. 1996; Kensler et al. 1998).

9.4.2. Hepatitis B virus infection

An estimated 350 million people worldwide contract some form of hepatitis per year. It is well established that chronic infection with hepatotropic viruses such as HBV and HCV are major risk factors for development of HCC worldwide. The viruses seem to be responsible for the geographical pattern of HCC incidence, with HBV being prevalent in developing countries and HCV prevalent in developed countries with intermediate to high incidence (Kew 1998). The transmission of the HBV virus is usually related to domestic crowding of children and occurs through salivary exchange and open skin sores. Contaminated needles and multiple sexual partners are considered as the major means of HBV transmission. Vaccination is a good measure for control of the HBV infection, but sadly, it is currently not accessible to every one in the geographical areas at risk. For instance only 1% of African children have access to vaccine (Wild et al. 2000).

HBV viruses are hepadnaviruses that preferentially infect the liver of mammalian and avian species. The members of HBV family are human, woodchuck, squirrel, and
duck hepatitis B viruses. HBVs appear to be distantly related to retroviruses since, unlike other DNA viruses, they replicate through reverse transcription of pregenomic RNA.

The human HBV genome is a 3.2 kb circular DNA that consists of four open reading frames encoding surface, core, polymerase, and X proteins (HBx) as well as cis-elements that are involved in regulation of HBV gene expression. After HBV infection of hepatocytes, the partly double-stranded genome is converted to covalently closed circular DNA (cccDNA), which acts as the template that is transcribed by cellular RNA polymerase II. (Weiser et al. 1983; Schaller et al. 1991). Transcripts are differentiated on the basis of the transcription start sites located at their 5' ends. The synthesis of all viral transcripts is regulated at transcription level, and it precedes viral replication. In hepatocytes, viral gene expression is regulated by an enhancer element that binds several transcription activators. This enhancer consists of several functional elements termed as E, EP, GB, and R-S which are binding sites for various transcription factors. For example, the E element is a binding site for basic leucine zipper family of proteins such as AP-1 complex, C/EBP, and ATFs. C-Abl protein is known as an EP binding protein, and the R-S enhancer element of HBV is reported to be the binding site for p53.

Among the four proteins translated from the HBV genome, the X-gene product (HBx) has drawn much attention. The HBx encodes a basic protein of 154 amino acids with the molecular weight of about 17 kD. The pathogenesis of HBV induced HCC is not well understood, and different mechanisms have been suggested. Random integration of HBV DNA sequences are frequently detected in the genome of malignant hepatocytes (Robinson 1994). There is not much evidence to support the involvement of HBV DNA integration in hepatocarcinogenesis. However, many lines of evidence suggest a role for the X protein of HBV (HBx) in hepatocarcinogenesis both in humans and in animal models (Kim et al. 1991). The X open reading frame (ORF) is conserved in mammalian hepadnaviruses associated with HCC. This protein seems to be required for viral
infection of the woodchuck (Zoulim et al. 1994). Oncogenic effects of avian hepadnaviruses, which lack the X protein, have not yet been reported.

Many studies using a HBx transgenic mice model have reported the hepatocarcinogenic effects of this protein (Kim et al. 1991; Ullrich et al. 1994). However, not all HBx transgenic mice develop HCC, unless they are exposed to additional hepatocarcinogenic influences. For instance, Slagle et al. (1996) demonstrated increased tumor development in HBx transgenic mice when they are exposed to diethylnitrosamine.

Persistent infection with hepatitis B virus and incidence of the liver cancer in certain geographical areas of the world led to the association between HBV and HCC. Greenland is the only exception where HCC is a rare disease although chronic HBV infection is common. This observation may provide evidence that HCC, like other forms of cancer, is developed as a result of multi-factors such as viral infection, exposure to genotoxic environmental carcinogens, heavy alcohol consumption and cigarette smoking. For example, several epidemiological and experimental studies have demonstrated that viral infection or chemical exposure alone is not sufficient for HCC development (Hussain et al. 2000; Buendia 2000; Wogan 2000).
REFERENCES


BASS NM, KIRSCH RE, TUFF SA, MARKS I, SAUNDERS SJ. 1977. Ligandin heterogeneity: evidence that the two non-identical subunits are the monomers of two distinct proteins. Biochim. Biophys. Acta. 492: 163


BULLER AL, CLAPPER ML, TEW KD. Glutathione S-transferase in nitrogen mustard-resistant and -sensitive cell lines. Mol. Pharm. 31: 575-578.


KASIBHATLA S, BRUNNER T, GENESTIER L, ECHEVERRI F, MAHBOUBI A, GREEN M, DOUGLAS R. 1998. DNA damaging agents induce expression of Fas ligand

KELLY VP, ELLIS EM, MANSON MM, CHANAS SA, MOFFAT GJ, MCLEOD R, JUDAH DJ, NEAL GE, HAYES JD. 2000. Chemoprevention of aflatoxin B1 hepatocarcinogenesis by coumarin, a natural benzopyrone that is a potent inducer of aflatoxin B1-aldehyde reductase, the glutathione S-transferase A5 and p1 subunits, and NAD(P)H:Quinone oxidoreductase in rat liver. Cancer Res. 60: 957-969.


SMITH MT, EVANS CG, DOANE-SETZER P, CASTRO VM, TAHIR MK, MANNERVIK B. 1989. Denitrosation of 1,3-bis(chloroethyl)-1-nitrosourea by class mu


Chapter 2

Identification of the Yc₁ Glutathione S-transferase mRNA as the Overexpressed Species in a Nitrogen Mustard Resistant Rat Mammary Carcinoma Cell Line

PREFACE

GSTs have been shown to be overexpressed in several tumor cell-lines selected for resistance to anticancer drugs. A melphalan resistant rat mammary carcinoma cell line (MATB MLNr) was previously developed and used as a model to study the mechanisms of chemotherapeutic drug resistance. The in vitro and in vivo studies revealed enhanced GST activity, especially increased GST alpha in MLNr cells. In order to identify the enhanced GST form in the resistant cells, my objective was to characterize and clone the overexpressed cDNA species of the GST subunit in this study.

ABSTRACT

Glutathione transferases (GSTs) have been shown to be overexpressed in a number of tumor cell lines selected for resistance to chemotherapeutic drugs and have been implicated in some studies of clinical specimens. In tumor cell lines selected for resistance to chemicals that alkylate DNA the isoform most frequently overexpressed is GST-Yc₁, a member of the α class GSTs. To date two variations of the cDNA designated Yc₁ with subtle differences have been described, and Yc₂ is shown to be clearly distinct.
Transfection of a Yc1 cDNA constitutively expressed in rat liver into rat mammary cancer cells confers resistance to alkylators, however to a lesser extent than is observed in the cells selected for resistance. It has therefore been widely suggested that the GST that is overexpressed in selected resistant cells represents a distinct and novel isoform. We have previously described a rat mammary carcinoma cell line (MLNr) that is resistant to alkylating agents, and overexpresses a GST with characteristics similar to GST-Yc1 and not Yc2. It has many features common to the several other GST-Yc overexpressing alkylator resistant cell lines. We have cloned the specific Yc cDNA overexpressed in MLNr and analyzed it in detail and found that it is identical to one of the previously reported Yc1 cDNAs, suggesting that there is no additional Yc gene specifically induced by nitrogen mustards. Another hypothesis to explain the difference in the level of resistance in selected versus GST-Yc transfected cells is the lack of concurrent increased glutathione (GSH) in the transfectants, which is a common feature in the selected resistant cells. Experiments in which we modulated GSH levels suggest that this is not likely. These studies add to our speculation that other mechanisms may be involved in alkylator resistance.
INTRODUCTION

Cytosolic glutathione transferases play an important role in xenobiotic detoxification, both in the context of chemical carcinogenesis and in cellular resistance to therapeutic drugs in established tumors (1-4). Characterization of the members of this multi-gene family provides continued insights into the diversity of this enzyme system to detoxify environmental and therapeutic chemicals. Five separate classes of cytosolic GST have been described (α, μ, π, θ, and σ), and in general distinct predominant substrate specificities can be assigned to each. A feature common to all is the utilization of glutathione (GSH) either in conjugation reactions or as reducing equivalents. This is reflected in highly conserved amino acid sequences among all GSTs, representing a site of GSH binding. The resolution of crystal structures for all GST classes has provided important insights into the structure of these proteins and their microheterogeneity with regard to substrates other than GSH (5). Random mutagenesis of the hydrophobic binding site which accommodates electrophilic substrates in the GST-Yc, overexpressed in E. coli, followed by selection for nitrogen mustard resistance, yielded mutant transferases with higher catalytic activity toward the selecting agent, although not other alkylators or organic peroxides (6).

The α-class GSTs have significant organic peroxidase activity and also use known carcinogens and steroids as substrates (7, 8). In rats, five distinct α class GST subunit cDNAs have been identified that can be either homo- or heterodimers; Yα1, Yα2, Yc1, Yc2, Yk. The genes encoding these isoforms are strongly related on the basis of sequence, with overall sequence homology of 75%. The 5'-third of the coding region is the most homologous, consistent with the likely position of the GSH binding site in the protein. The first Yc cDNA isolated from a rat liver library was in the phage clone called pGTB42, which contains the entire coding sequence, a 5'-non-coding fragment of 44 bps, as well as a lengthy 3' non-coding sequence. A 178 base pair fragment of it, used
as a probe, effectively distinguishes the rat liver Yc from Ya mRNA. This contains 20 bp of 3' coding sequence, as well as 3' non-coding region (8). Since then, Hayes et al. independently cloned a Yc cDNA from a different rat liver cDNA library (9). It contains the identical 5'-non-coding sequence found in pGTB42, but it extends 5' by 105 additional bps. The coding region is identical, except for a C to T substitution at nucleotide +27, and it has a T to C substitution at nucleotide +788 in the 3' non-coding region. These substitutions have no effect on the primary sequence of the encoded proteins.

Recently, a distinct Yc protein, called Yc₂, has been described in ethoxyquin-treated rat liver, which has significantly higher reactivity with activated epoxide forms of aflatoxin (10). The peptide analysis has revealed a significant amino acid composition similarity but faster mobility in polyacrylamide gel. The two Yc subunits can be recognized as two distinct bands of Mr 25,800 (Yc₂) and 27,500 (Yc₁) on denaturing polyacrylamide gel respectively.

We and others have demonstrated significant overexpression of a GST-Yc in tumor cell lines selected for resistance to chemicals which alkylate DNA (1-3). In rat mammary tumor cells, the Yc gene is transcriptionally activated, and in another Chinese Hamster Ovary cell line selected for resistance to Chlorambucil, Yc gene amplification was also demonstrated (11,12). Two-fold increased cellular GSH concentration is commonly also present in the cells selected for alkylator resistance. By Western blotting, there is a single band consistent with Yc₁. We have demonstrated that expression of the rat liver GST-Yc coding region digested from pGTB42 can confer resistance to these drugs in sensitive cells, using both calcium phosphate transfection and retroviral transduction. However the level of resistance is lower when the Yc₁ protein level is normalized between transfectants and selected resistant cells (11,13). This has led to speculation that the absence of elevated GSH, or an altered GST protein in the selected cells are responsible. Recently, the Yc protein from a bifunctional alkylating
agent resistant cell line was purified and shown to catalyze the reaction of GSH with these chemicals. However, it was not directly compared to Yc purified from rat liver for this activity, and was not sequenced or otherwise characterized (14). In this paper we have examined the effect of GSH level on cellular resistance, and we have also characterized the specific GST-Yc cDNA that is overexpressed in alkylator resistant cells.
MATERIALS AND METHODS

Reagents- Cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum (Gibco). The GST cDNAs in plasmids pGTB30, 38, and 42 were the kind gift of Dr. Cecil Pickett (Merck), and they hybridize to Ya, Ya/Yc and Yc mRNAs respectively. Restriction enzymes were purchased from BRL (Burlington, Ontario), and radioisotopes from ICN (St. Laurent, Quebec) and from Amersham (Oakville, Ontario). Both melphalan and L-buthionine-S,R-sulfoximine (BSO) were obtained from Sigma Chemical Co. (St. Louis, MO). Reagents for GSH analysis were obtained from Boehringer Mannheim (Laval, Quebec). Oligonucleotides were synthesized using the Applied Biosystems model 392 DNA/RNA synthesizer.

Cell lines- MatB cells are rat mammary carcinoma cells that grow both in vitro and in vivo. MatB MLNr is a subline selected for resistance to melphalan, which has a 13-fold increase in GST-Yc expression at the protein and mRNA levels and 2-fold higher GSH concentration. There is an approximately 20% decrease in drug accumulation in these cells compared to the WT controls. Southern blot shows no gene rearrangement or amplification and nuclear run-on demonstrates transcriptional activation (2). A cDNA library was constructed from these cells.

To examine the role of GSH relative to GST in the resistance of the MLNr cells, cells in exponential growth were exposed to various doses of L-buthionine-S,R sulfoximine at various concentrations (1-100 μM) for 20 hours and then harvested for estimation of GSH according to a previously described method (15).

To measure cytotoxicity of melphalan in the WT and MLNr cells, we used the MTT assay as previously described (16). Cells were exposed to various concentrations of BSO for 20 hours and then divided for analysis of GSH concentration and for
subsequent exposure to melphalan. BSO itself was not cytotoxic in the dose range studied, but the addition of BSO resulted in sensitization of the cells to melphalan (17).

cDNA library construction and cloning-The cDNA was synthesized from polyA+ RNA (Pharmacia oligo dT) from MLNr cells. The probes used to screen the library included: I. 550 bp fragment from PstI-BglII restriction digest of pGTB 38 which hybridizes to both Ya and Yc, II. 300 bp fragment from PstI digestion of plasmid pGTB 38 which is specific for a Ya GST, III. 178 bp fragment which results from PstI digestion of pGTB42 and encodes 3' coding and non-coding sequences of pGTB 42 and is specific for both Yc subunits. Initially, the filters were screened with probe I to identify cDNAs in the α-GST class. Positive clones were then screened using probe II, to exclude Ya cDNAs, since Ya GST is expressed in the MatB MLNr cells. The remaining clones were then probed with the 178 bp fragment to determine the relationship of these to the known GST-Yc. All 5 of these in fact hybridized to probe III, i.e. were not Ya and were Yc, and they were subsequently subcloned into pBluescript SK- vector for sequencing. The longest sequence information was contained in a single cDNA clone including the 3' half of the coding region in addition to 3' non-coding sequences (fragment A).

Cloning Strategy- The full length cDNA was cloned in two steps. A cDNA library was constructed, and plaque hybridization of the library was performed using Ya-specific, and the Yc-specific cDNA sequences. Five independent clones, representing partial cDNA fragments that represented the 3' half of the coding region, including most or all of nucleotides 358-663 as well as non-coding sequence (nucleotides 664-865), were isolated. They were all identical in sequence. Using a specific primer near the 5' end of the largest cDNA fragment (nucleotide 390-372), and another primer derived from the
first 21 nucleotide of Yc, which are both highly conserved among α-GSTs, we PCR amplified the intervening fragment and directly sequenced it.

**PCR amplification and primers used**- In order to clone the rest of the GST-Yc in MLNṛ cells, total RNA was isolated according to recommended procedures using RNA Zol Premix solution (Tel-Test, Friendswood, Texas). Four μg of isolated RNA were reverse-transcribed using Superscript reverse transcriptase (GibcoBRL) with the primer synthesized from the 5' end of the partially cloned Yc cDNA (#2): 5’-GTAACGGTTCCCTGCTTTG-3’. PCR was performed with first strand cDNA for 35 cycles using 2.5 units of Pfu DNA polymerase using the methods recommended by Stratagene. The upstream primer was the same used in RT (#2) and the downstream primer (#1) was synthesized from the 5' end of the non-coding region of Yc¿. The PCR product was a single product that hybridized to a fragment of pGTB42 that encodes 5' non-coding as well as coding sequences, as a single band (data not shown). It was resolved in 1% agarose gel and transferred onto Hybond-N Nylon membrane (Amersham) for Southern-blot hybridization using fragments of pGTB42 3' to the primer.

**Sequencing**-The cDNAs isolated from the library were subcloned into pBluescript SK-vector, and the sequencing reactions were carried out according to the instructions provided with the Sequenase Version 2.0 DNA Sequencing Kit (U.S. Biochemical Corporation/Amersham). Reaction mixtures were heated to 95°C for 3 minutes prior to loading onto a 7M urea, 6% acrylamide gel. The gel was dried and exposed to XOMAT film at room temperature. Direct sequencing of the PCR amplified fragment was performed following the instructions suggested by USB/Amersham using 5 pmole of each primer. The sequencing primers used were the PCR primers (#1 and 2) described above in addition to three other internal primers (#3) :5’-CCTCTATGGGAAGGACATGAAGG-3’, (#4): 5’-GCCTGGCCAGGTCATCCCGAG-
3', and (#5): 5'-GTAAAAAGCTGCTCCCTCTAAG-3'. The result was the remaining 5' region, Fragment B.

**Primer extension**- Since two previously cloned Yc1 cDNAs differ in the length of the 5' non-coding sequences, we performed primer extension analysis to determine the transcription start site of the cloned cDNA. Primer extension was performed as described by Sambrook et al. (18). A 5' end-labelled oligonucleotide complementary to bases -47 to -25 (primer #5, Figure 1) of the GST-Yc1 cDNA sequence described by Hayes (9) was used and hybridized with 5 µg of total RNA from MLNr cell line at 45°C. The primer was extended using Superscript reverse transcriptase (Gibco BRL) and analysed on a 6% sequencing gel. An M13 sequencing ladder was used as a size marker.

**GST gene expression**- To confirm that the sequenced cDNA is indeed representative of the Yc1 gene overexpressed in the melphalan resistant cells, we performed a Northern blot using a highly specific probe. Total RNA was extracted from MatB WT and MLNr cell lines using the RNAzol B method (Tel Test, Friendswood, TX). Fifteen micrograms of RNA was electrophoresed in 1.2% agarose containing 2% formaldehyde and was blotted onto Hybond-N nylon membrane (Amersham) in 20x SSC (3M NaCl, 0.3M Trisodium citrate) buffer. A 125 bp PCR fragment specific to the 5' non-coding region of the Yc1 cDNA was used as the probe. The PCR primers used to synthesize this probe were: (#1), 5'-GCAGCGGGGACCTTATTGGAC-3', complementary to nucleotides -149 to -129; (#5), 5'-GTAAAAAGCTGCTCCCTCTAAG-3', complementary to nucleotides -47 to -25. We have previously reported on the expression of the GST-Ya in these cells, and found no difference in the resistant cells (2). In order to complete the characterization of α class GST expression, we also performed Northern blots with probes designed to identify the Yc2 and Yk GST subunit coding genes. The Yc2 probe
was a PCR generated fragment corresponding to nucleotides +66 to +192 from the transcription start, and the Yk probe was a synthesized oligonucleotide including +694 to +757, located in the 3' non-coding region of the cDNA which is specific for this subunit (9,19). Hybridization was carried out overnight at 42°C in a solution containing 5x SSPE (0.9M NaCl, 5 mM NaH2PO4, 1mM EDTA), 10% dextran sulfate, 1% SDS and 500 μg/ml of salmon sperm DNA. The filter was then washed 3 times in 2x SSC-0.1% SDS, 1x SSC-0.1% SDS, 0.5x SSC-0.1% SDS at 42°C for 15 mins. for each washing. β-actin was used to control for sample loading and transfer efficiency.
RESULTS

Mat B cells selected for MLN resistance

Table 1 shows the comparison of determinations of the GSH levels in the various cells, as well as the responsiveness to the cytotoxic drug melphalan. At a BSO concentration of 50 µM over 20 hours the GSH level in the MLNr cells was reduced to the same as that measured in the WT cells with no significant effect on GST activity. This is also identical to the level of GSH found in the GST-Yc transfectants we have previously described (12). This reduced GSH is accompanied by a decrease in the level of resistance in the MLNr cells from approximately 63-fold to approximately 40-fold (i.e. >30% increase in the sensitivity of the cells to melphalan), suggesting that GSH plays an independent but limited role in the resistance (2).

cDNA cloned and sequenced from MLNr cells

Our sequence is nearly identical to the previously reported Yc1 cDNA nucleotide sequence (8,9). Figure 1 is a schematic of the cDNA cloned from the MLNr cDNA library and by PCR amplification. It includes the entire coding region and the 5′ and 3′ non-coding regions, which is identical to that reported for Yc1 cDNA by Hayes et al. (9). Fragment A was cloned from the cDNA library, and provided strong evidence that the overexpressed Yc form is Yc1. In order to complete the sequencing of the overexpressed Yc subunit, fragment B was generated by PCR amplification. It differs from the pGTB42 sequence in the two base substitutions (C to T at +27, and T to C at +788). The primers used in this and the sequencing reactions are shown.
Table 1. This table shows the relationship between GSH concentration and the concentration required to inhibit 50% of Mat B cells ($IC_{50}$) for melphalan. Results showed that 20 hour exposure to 50 μM BSO lowered the GSH concentration in MLNr to that in WT cells, and this changes the $IC_{50}$ by 30%. These results were obtained from three separate experiments, in which samples were done in triplicate.

<table>
<thead>
<tr>
<th>Cells</th>
<th>GSH (nmol/10^6 cells)</th>
<th>IC$_{50}$</th>
</tr>
</thead>
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<tr>
<td>WT</td>
<td>12.39 ± 0.6</td>
<td>0.63 ± 0.2</td>
</tr>
<tr>
<td>MLNr</td>
<td>40.96 ± 5.5 *</td>
<td>39.49 ± 5.2 *</td>
</tr>
<tr>
<td>MLNr + BSO 50μM</td>
<td>12.49 ± 3.1 **</td>
<td>24.94 ± 6.1 * ^</td>
</tr>
</tbody>
</table>

* is $p<0.01$ compared to WT  
** is $p<0.01$ compared to MLNr  
^ is $p<0.05$ compared to MLNr
FIGURE 1. This figure is a schematic representation of the Yc cDNA analyzed in these studies. Fragment A was isolated from a cDNA library and includes both coding and non-coding sequences as shown. Fragment B was derived from PCR amplification, using primers #1 and 2. Additional internal primers were used to complete the sequencing of this fragment, #3, 4, and 5. The coding region and 3' non-coding region are identical to the previously reported pGTB42, except for 1 base in the 3' non-coding region. The 178 bp fragment used to identify Yc sequences includes the last 20 base pairs in the coding region and 158 bases in the non-coding 3' terminus.

Primers are:  
#1: 5'-GCAGCGGACCTATTGGGAC-3'  
#2: 5'-GTAACGGTTCCTTGCTTTG-3'  
#3: 5'-CCTCTATGGGAAGGACATGAAGG-3'  
#4: 5'-GCCTGGCCAGGTCATCCCGAG-3'  
#5: 5'-GTTAAAAAGCTGCTCCCTCTAAG-3'
Northern blotting with cloned sequence

The expression of the GST-Yc₁ gene specifically in the MatB MLNr cells compared to the WT cells is shown in Figure 2. The probe used was synthesized by PCR, and it was directly sequenced to confirm that it contains the desired Yc₁-specific sequences. This probe detects a 1.3 Kb mRNA species, consistent with the known Yc₁ mRNA, which is abundant in the MLNr cells, and virtually undetectable in the WT cells. Neither the Yc₂ nor the Yk subunit genes are expressed in either the WT or the MLNr cells (data not shown).

Primer extension analysis

Figure 3 shows the results of primer extension with an oligonucleotide corresponding to sequences in the 5' flanking region, which detected a predominant fragment of 219 bp. This demonstrates that the mRNA overexpressed in the MLNr cells is identical to the Yc₁ isolated by Hayes et al. (9).
FIGURE 2. The results of Northern blotting of RNA obtained from the WT and the MLNr subline of MATB rat mammary carcinoma cells. The probe is a PCR-synthesized 125-bp fragment specific to the Yc1 gene. β-actin was used to control for sample loading and transfer efficiency.

FIGURE 3. The result of primer extension analysis of the MLNr cell line, and a predominant fragment of 219 bp is indicated.
DISCUSSION

Our previous results obtained after transfection of a rat Yc, DNA in sensitive cells demonstrated that this GST can confer approximately 15-fold resistance quite selectively to alkylating agents, while the GST activity measured as GSH conjugation with CDNB is essentially the same as the MLNr cells which were selected and are 60-fold resistant to melphalan (2,12). Although the cross-resistance pattern of the transfectants is similar to that observed in the selected cells, the level of resistance conferred is significantly less than would be expected when the amount of Yc protein is normalized. While the higher resistance level in the selected cell line MLNr is also partly due to the drug accumulation defect in the selected resistance cells (<20%), we and others have hypothesized that the lack of concurrent increase in GSH concentration in the transfectants may account for the diminished resistance compared to the selected cells (3). Table 1 suggests that this is not the case.

An alternative hypothesis was that the rat liver-derived Yc cDNA encodes a protein with lower specific activity than a theoretical novel "nitrogen mustard-induced isoform" in the MLNr selected cells. The recently reported analysis of GST-Yc protein purified from a Chinese Hamster Ovary subline that is selected for alkylating agent resistant cell line did indeed show its ability to catalyze GSH conjugation with melphalan, but this was not shown to be greater than that observed for rat liver derived GST-Yc (15). This purified protein was not sequenced. Although it is possible that post-translational modification of the Yc protein is present, examination of phosphorylation of the Yc-GST in MLNr cells, using an in vivo cellular assay system did not demonstrate this modification (not shown). While this may require further study, to date there have been no demonstrations of a critical effect of post-translational modifications on GST activity.

In the present studies we have sequenced 5 independently isolated cDNAs that are identical and are overexpressed in the bifunctional alkylator resistant rat mammary
carcinoma cells and found that they encode the same Yc peptide that is constitutively expressed in liver, i.e. the Yc1 cDNA (8,9). The present study demonstrates that there is no novel Yc gene overexpressed in the MatB MLNr cells. There have actually been two independently cloned Yc1 cDNAs previously described, differing in the length of the 5' non-coding sequence, as well as one base substitution in the coding region and another in the 3'non-coding segment. Sequence and primer extension analyses demonstrate that the 5'-flanking sequence of the overexpressed gene in MLNr cells is identical to one previously reported (9). While it is possible that there are two truly different genes with very subtle differences, it is more likely that the other cDNA which is in pGTB42 is not full length. The base substitution in the coding region does not result in a change in the amino acid tyrosine which is translated. We conclude that there is likely only one Yc1 gene, and that it is both constitutively expressed in rat liver as well as overexpressed in the MLNr cell line.

The mechanism of overexpression of the GST-Yc in nitrogen mustard resistant cells remains unknown. While much has been learned about the regulation of the Ya gene, another a-class GST, GST Yc is significantly different in this regard. Its expression in various tissues is quite distinct, and previous data using in vivo treated rat liver RNA suggested that the Yc1 form does not respond to common inducers as does the GST-Ya (18). The mechanism of this induction awaits elucidation of the regulatory elements of the GST-Yc to determine if response elements with some specificity for alkylating agent chemotherapy are present, or whether the chronic exposure to these agents results in stable overexpression of transactivating factors that bind these regulatory sequences.

ACKNOWLEDGEMENTS

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REFERENCES


Chapter 3

Genomic Cloning and Characterization of the Rat Glutathione S-Transferase A3 Subunit Gene

PREFACE

In previous chapter, I showed that rGSTA3 mRNA is overexpressed in melphalan resistant cell-line and found that it is identical to the previously described GSTA3 mRNA with no additional GST alpha being induced by melphalan. To study the role of GSTA3 subunit gene in drug resistance, and to understand the mechanism of overexpression of this gene in drug resistant tumors, my goal in this study was to isolate and characterize the entire rGSTA3 gene, including its regulatory region.

ABSTRACT

The rat glutathione S-transferase A3 subunit gene (rGSTA3) is a member of class alpha GST which we have previously reported to be overexpressed in anticancer drug resistant cells. In this study, we report the isolation and characterization of the entire rat GSTA3 (rGST Yc₁) subunit gene. The rat GSTA3 gene is approximately 15 kb in length and consists of seven exons interrupted by introns of different lengths. Exon 1, with length of 219 bp contains only the 5' untranslated region of the gene. Each exon-intron splicing junction exhibited the consensus sequence for mammalian splice site. The transcription start site and exon 1 of the rGSTA3 were characterized by a combination of
primer extension and rapid amplification of the cDNA ends (5'-RACE). Position +1 was identified 219 bp upstream of the first exon-intron splicing junction. The proximal promoter region of the rGSTA3 gene do not contain typical TATA or CAAT boxes. A computer-based search for potential transcription factor binding sites revealed the existence of a number of motifs such as ARE, AP-1, NF-κB, CREB, Barbie box, RRE, and E box. The functional activity of the regulatory region of the rat GSTA3 gene was shown by its ability to drive the expression of a CAT reporter gene in rat mammary carcinoma cells, and its activity was greater in melphalan resistant cells known to have transcriptional activation of this gene by previous studies. The structure of the gene, with a large intron upstream of the translation initiation site, may explain why the isolation of this promoter has been so elusive. This information will provide the opportunity to examine the involvement of the rGSTA3 subunit gene in drug resistance and carcinogenesis.
INTRODUCTION

The glutathione-S-transferases (GSTs) are a family of enzymes that catalyze the conjugation of glutathione (GSH) with xenobiotics as part of detoxification and drug resistance pathways [1-3]. The GSTs are present in almost all eukaryotic species, and thus far, six classes of this enzyme have been described, viz., alpha, mu, pi, sigma, theta and zeta [3, 4]. In rats, each class consists of several subunits with various nomenclature in the literature [3]. The rat GSTA3 (rGST Yc,) subunit belongs to the alpha class, and is located on chromosome 9 [5]. Several reviews have recently described the GSTs in great detail [1-3].

The development of anticancer drug resistance is a major problem in treatment of cancer by chemotherapy. A number of pathways have been reported to be involved in drug resistance; among them are alterations in drug transport through P-glycoprotein and multidrug resistance protein [6, 7], increased DNA repair [8, 9], changes in DNA topoisomerases [10] resistance to apoptosis [11, 12], and alteration of GSH and related enzymes [2, 13, 14]. Several studies have demonstrated the involvement of GST isoforms [1, 2, 15] in chemotherapy resistance to various drugs, particularly to alkylating agents [16-18].

To study the mechanisms of anticancer drug resistance, we have previously reported the isolation of a drug resistant rat mammary carcinoma cell-line (MatB) by exposing cells to increasing concentrations of the alkylating agent melphalan (MLNr MatB). The in vitro and in vivo studies in our laboratory demonstrated increased GST activity, especially elevated GSTA3 subunit in MLNr cells [19, 20]. The introduction of the rat liver GSTA3 cDNA (clone pGTB42) into WT MatB cells were reported to confer several fold resistance to melphalan [19]. Nuclear run-on experiments showed that the induced GSTA3 mRNA in MLNr cells is due to transcriptional activation [21]. We have recently isolated the GSTA3 cDNA overexpressed in these cells and confirmed that in
fact it is virtually identical to the previously described GSTA3 subunit of GST mRNA [22] which has been designated GST Yc1 in the past.

Although at least three different laboratories [22-24] have isolated rGSTA3 cDNAs, the role of this gene in chemotherapy resistance has not been amenable to study since isolating its promoter has been extremely elusive. We here report the first isolation and characterization of the rGSTA3 gene including its regulatory regions. This information will permit detailed studies of the mechanism of overexpression of this gene in drug resistant tumours.
MATERIALS AND METHODS

Isolation of Genomic Clone- We have isolated three overlapping clones from λDASH rat liver genomic library (Stratagene) named λBF7, λBF11 and λBF59, respectively. The probe for screening the library was initially a PCR generated fragment of 125 bp located at the 5'-end of rat GST GSTA3 cDNA corresponding to nucleotides -149 to -25 (translation start site as +1) [24]. Using this probe, we obtained clones λBF7 and λBF11, which contain the entire 5'-flanking region as well as the first 4 introns and exons. The rat genomic library was screened following the standard protocols and conditions suggested by the manufacturer (Stratagene). To isolate the remaining 3' regions of the rGSTA3 gene, we further screened the library with a probe made from a fragment located at the 3' end of the λBF7 clone. This resulted in the isolation of the third clone, λBF59. The clones were subjected to restriction digestion with EcoRI and XbaI enzymes and the fragments were subcloned into pBluescriptIIKS plasmid (Stratagene) for analysis. A 4.2 kb EcoRI fragment of clone λBF11, that hybridized with the 5'-noncoding cDNA probe, was also subcloned into plasmid pBluescriptIIKS for sequencing (named as prA3/4.2E1). This construct is comprised of about 3 kb of the promoter plus exon 1, as well as 1.2 kb of the first intron. The first intron is located 22 bp upstream of the translation start site.

Southern-blot Hybridization of λ phage DNA- Small-scale phage DNAs were extracted from the rGSTA3 positive clones using Wizard lambda preps kit (Promega). The λ DNAs were digested with EcoRI and XbaI restriction enzymes and Southern-blot hybridization were performed for analysis. In brief, the fractionated λ DNA from each clone were separated on a 0.8% agarose gel, and transferred onto a Hybond-N nylon membrane (Amersham) in 0.4 N NaOH. Hybridization of the membrane with the [α-32P]dCTP labeled probe was carried out at 42 °C for overnight in a solution containing 5
× SSPE (0.9 M NaCl, 5 mM NaH₂PO₄, 1 mM EDTA), 10% dextran sulfate, 1% SDS (sodium dodecyl sulfate), and 500 µg/mL of salmon sperm DNA. The membrane was washed sequentially in 2 × SSC (3 M NaCl, 0.3 M trisodium citrate) and 0.1% SDS, 1 × SSC-0.1% SDS, 0.5 × SSC-0.1% SDS at 42 °C for 15 min each washing. The EcoRI and XbaI fragments were subcloned into pBluescriptIIKS plasmid for sequencing and mapping of the exon-intron splicing junctions.

**DNA sequence analysis** - The sequencing of the isolated genomic DNAs of rat GSTA3 subunit gene was performed using Sequenase (Amersham) and synthetic oligonucleotide primers (Life Technologies Inc.) corresponding to internal sequences (Table 1). Both manual and automated sequencing methods were used to determine the sequence of the 5'-flanking region in both orientations. The BLAST program of the GenBank database [25] was employed to search for sequence homology. For identification of the putative consensus elements and potential transcription factor binding sites in the 5'-regulatory region of the rGSTA3, the TRANSFAC program from the Internet database (http://transfac.gbf.de/TRANSFAC) was used [26]. The nucleotide sequences reported in this paper has been submitted to the DDBJ/EMBL/GenBank with accession number AF067442 and AF111160.

**PCR amplification** - For generation of the cDNA probe corresponding to nucleotides -149 to -25 (ATG reference as +1) [24], PCR amplification was performed as described previously [27] using Y1-9 and Y1-10 primers (Table 1). For determination of the sizes of introns 1 and 2, PCR amplification was performed using the Expand™ Long Template PCR system (Boehringer Mannheim, Germany), the λBF7 clone as template, and 50 pM of each primer (Life Technologies Inc., Table 1). Reaction mixtures were amplified using a programmable thermal cycle (MJ Research). The amplification parameters were: denaturation at 94 °C for 3 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 1 min.
and 68 °C for 3 min. The amplified products were subjected to agarose gel electrophoresis and sequencing for verification.

**Mapping of the transcription start site** - To identify the transcription start site of the rGSTA3 subunit gene, primer extension was performed following the standard methods [28]. Briefly, primer Y1-10 (Table 1) complementary to the rGSTA3 mRNA was end-labeled with \( \gamma^{32}\)-pATP and then it was annealed to 5 μg of total RNA from MLNr MatB cells at 60 °C. Yeast tRNA was used as control for extension specificity. To prevent the premature termination due to the secondary structure of mRNA, the reverse transcription was performed using Murine Leukaemia Virus reverse transcriptase (Gibco BRL) at 52 °C for 30 min. The extended product was run on a 6% sequencing gel along with sequencing reaction of prA3/4.2E1, using Y1-10 as the primer (Figure 2A). This experiment was repeated several times to ensure the reproducibility of the results.

To confirm the transcription start site of the rGSTA3 gene, rapid amplification of cDNA 5'-ends (5'-RACE) method was performed following the protocols described by the manufacturer (Clontech, USA). In brief, 10 μg of total RNA from MatB cells was annealed with a gene specific oligonucleotide Y1-10 corresponding to the 3' end of the first exon (Figure 2B) and the 5'-end of the cDNA was generated. The synthesized cDNA was ligated to an oligonucleotide adaptor provided. A fraction of this reaction product was used as a template for PCR amplification using the same gene specific primer (Y1-10) and an anchored primer (primer A). The PCR amplification was carried out as described in the previous section. As positive control, two gene specific primers Y1-91 and Y1-10 were included in PCR amplification. A single oligonucleotide (primer A or Y1-10) was used in PCR reactions as negative control. The PCR products were separated on a 1% agarose gel for analysis.
FIGURE 1. Structure of rGSTA3 (Yc1) subunit gene and alignment of isolated genomic clones. Three phage clones, λBF7, λBF11 and λBF59 are represented as horizontal lines. The seven exons (1-7), six introns and 5'-flanking region of the rGSTA3 gene are shown to scale. The closed boxes represent coding exons and the open boxes 5' and 3' untranslated regions. The initiation (ATG) and stop (TAA) codons are depicted by bars. The positions of restriction sites are shown for B, BamHI; E, EcoRI; Ev, EcoRV; X, XbaI; Xh, Xhol; P, PstI; H, HindIII; Xm, XmnI.
Table 1 The oligonucleotide primers used in this study

<table>
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<th>Primer</th>
<th>Sequence</th>
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<th>Assay*</th>
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</table>

* Ex-Int, Primers for Exon-Intron splicing junction; PE, primer extension; Seq., Sequencing of rGSTA3 subclones; PR, primers used to generate cDNA probe for the genomic library screening; 5UT and 3UT, Primers involved in the sequencing of 5'UTR and 3'UTR; RC, 5'-RACE experiment.
Construction of plasmids- A 4.2 kb EcoRI (-2797 to +1413) fragment from clone λBF11 that contains the 5' regulatory region of the rGSTA3 was subcloned into plasmid pBluescript IIKS for sequencing. We designate this plasmid as prA3/4.2E1. The genomic fragment was excised from plasmid prA3/4.2E1 using KpnI and XbaI restriction sites and inserted into the pCAT-Basic (Promega) reporter plasmid, for analysis of its transcriptional activity in both WT and MLNr MatB cells. The above construct was designated prA3/4.2-CAT. A 5' deletion mutant of the rGSTA3 regulatory region was constructed by deletion of the 1.7 kb EcoRV fragment from plasmid prA3/4.2-CAT. The resulting plasmid was named prA3/2.5-CAT. Similarly, the 3' deletion mutant of the rGSTA3 gene was obtained by deletion of the 1.4 kb XmnI and SmaI fragment from plasmid prA3/4.2-CAT. This resulted in plasmid prA3/2.7-CAT (Figure 5A).

Cell culture and transient transfection- Rat mammary carcinoma cells (WT MatB) and the melphalan resistant subline (MLNr MatB) were grown in minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (Gibco-BRL) at 37 °C with 5% CO₂. Transfection of cells with recombinant plasmids were performed using lipofectamine (Gibco-BRL). The plasmids used for transfection were purified using QIAGEN maxi-prep kit (QIAGEN Inc.). WT and MLNr MatB cells were seeded into 6-welled plates at a density of 2.5 × 10⁵ cells per 35 mm well and maintained overnight. The next day the cells were rinsed twice and complete medium was replaced with serum free α-MEM. Three μg of DNA was incubated with 6 μl of lipofectamine at room temperature for 30 min prior to being added to each respective well. The cells were transferred to a 37 °C incubator for 5 h, then the complete medium was substituted for 24 h prior to harvest. For control of transfection efficiency, the cells were cotransfected with 1 μg of plasmid containing β-galactosidase gene (pSV-βgal ). The cells were harvested and prepared for protein assays (Bradford method) [29], β-galactosidase activity, and CAT-assays [30].

Chloramphenicol Acetyltransferase (CAT) assays- CAT assays were performed as
described by Rushmore et al. [30]. In brief, 50 μg of protein was incubated with acetyl coenzyme A and [14C] chloramphenicol for 4 h at 37 °C. They were then extracted with 1 ml of ethyl acetate and dried by speed vacuum. The chloramphenicol acetylated products were separated by TLC plates and visualized by exposure to X-ray films, and then were quantitated by phosphorimage analysis.
FIGURE 2. Mapping of exon one and determination of transcription initiation site. A) The transcription start site was mapped by using primer extension analysis. The primer Y1-10 was end-labeled, annealed with 5 µg of total RNA from MLNr cells, and reversed transcribed. The extended product was run on a 6% sequencing gel. The sequencing reaction of rGSTA3 genomic template with Y1-10 primer was used as marker. lane 1, primer extension with yeast tRNA as control; lane 2, with total RNA from MLNr MatB cells. The position of the transcription start site (+1) is indicated with the arrow. B) Determination of rGSTA3 transcription initiation site by 5'-RACE method. The position of the three primers and the PCR fragments obtained in this experiment are shown. Primer Y1-10 was used in reverse transcription reaction and all three primers were included in PCR amplification. M, molecular size marker in bp; lane 1, 5'-RACE product; lane 2, positive control using two gene specific primers Y1-91 and Y1-10; lane 3 and 4, negative controls using only primer A or Y1-10, respectively.
RESULTS AND DISCUSSION

Isolation of rGSTA3 (rGST-Yc,) genomic clones- A rat liver genomic library (Stratagene) was screened with a PCR fragment located in the 5'-end of rGSTA3 cDNA (previously designated GST Yc,) and resulted in the isolation of two independent genomic clones. Restriction enzyme mapping and sequencing of these clones revealed that they overlap, and contain a long 5'-flanking region and the first 4 introns. To obtain the remaining 3' end of the gene, further screening of the same library was performed using the most 3' fragment of clone λBF7 as a probe. Screening approximately $6 \times 10^3$ plaques resulted in the isolation of clone λBF59. These three phage clones together contain the entire rGSTA3 subunit gene (Figure 1).

Structural Organization of the rGSTA3 gene- The structural map of the rGSTA3 subunit gene was determined by a combination of restriction enzyme digestion, Southern-blot hybridization, PCR analysis, and DNA sequencing. The rGSTA3 gene is approximately 15 kb in length and consists of seven exons interrupted by six introns of different lengths. The first exon with the length of 219 bp contains only 5'-untranslated region of the mRNA. The ATG start codon is found 22 bp downstream from the intron I-exon 2 boundary (Figure 1 and 3), therefore, exons 2-7 provide the coding information for this gene. Interestingly, in rat GSTA2 and mouse GSTA2, intron 1 is also located 22 bp upstream from the ATG start codon [31, 32]. The class alpha GST genes characterized thus far, with the exception of rGSTA5 have an intron in the 5' non-coding region [31-34]. The lengths of the coding exons 3 to 6 of rGSTA3 are identical to that of other class alpha GSTs (Table 2). This could have arisen as a result of the ancestral duplication of the GST alpha gene.

The sequencing analysis of our phage genomic clones (λBF7 and λBF11) revealed that their codon 9 is similar to that of the rGSTA3 cDNA clone previously reported by
Telakowski-Hopkins et al. [23] and is TAC. This contrasts with another rGSTA3 cDNA clone (λJH24) reported by Hayes et al. [24] which contains TAT at codon 9. Moreover, λBF7 and λBF11 differ from λJH24 in four nucleotides located between 120 bp and 123 bp upstream of the first exon-intron splicing junction (Figure 4). Both of our phage clones contain TTTT while λJH24 has GGGG. These differences might be due to the presence of polymorphisms in different rats.

Introns 1 and 2 of the rGSTA3 are the longest of the six introns that are present, with the estimated lengths of approximately 5 kb and 5.1 kb, respectively (Figure 1). The sequences of the exon-intron boundaries of this gene are consistent with the consensus splice site sequences of mammalian genes, containing "gt-at" (Figure 3). In general, the organization of the rGSTA3 subunit gene is similar to that of other mammalian alpha class GST genes, with the exception of rGSTA5, in terms of number of exons and introns (Table 2). However, the rGSTA3 with a size of about 15 kb, appears to be larger than other members of the class alpha GSTs.

Mapping of the transcription start site- The transcription initiation site of rGSTA3 gene was determined by both primer extension and 5'-RACE studies using total RNA from MLNr MatB cells, in which the gene is highly expressed [22]. Primer extension analysis was performed with antisense primer Y1-10 (Table 1). The elongated products were analyzed on a 6% sequencing gel (Figure 2A). The end points of the primer extension were determined with the help of a sequencing ladder derived from prA3/4.2EI as the template and Y1-10 as the primer. The end point of the primer extension which is the start site for the transcription of the rGSTA3 gene was determined to be an adenine (+1), corresponding to 219 nucleotides upstream of the first intron-exon splicing junction (Figure 2A). We obtained the same results when we used the total RNA from rat liver tissue.
Figure 3. Analysis of Exon-Intron splice junctions of the rGSTA3 gene, including 3'UTR. Exon sequences including 3'UTR are shown in capital letters and intron sequences in lowercase letters, as determined by sequence analysis. The deduced amino acid sequence of the rGSTA3 cDNA are displayed below the nucleotide sequence. The numbers on the upper lines indicate the nucleotide positions of the junctions in reference to the transcription start site, while the positions of amino acids are shown on the lower lines and starts at the initiation codon methionine. Intron 1 interrupts 5'-UTR of the gene at 22 bp upstream of the ATG start codon. Introns 3 and 4 interrupt codon triplets of the rGSTA3 gene. Intron sizes were determined by a combination of sequencing (intron 3 and 4), PCR amplification of genomic clones using cDNA primers, or restriction digest analysis of genomic clones. The complete coding sequences of rGSTA3 cDNA have been previously published [23, 24].
The results from primer extension were confirmed by 5'-RACE studies using the gene specific primer Y1-10 and the anchor primer A (Figure 2B). Thus the combination of results obtained from 5'-RACE and primer extension analysis were in agreement with each other, and the transcription start site was identified to be located 219 bp upstream of the first exon-intron boundary and shown as +1 in Figure 2A. In this experiment two gene specific primers Y1-91 and Y1-10 were used as positive control and the expected 125 bp fragment was obtained by PCR amplification of the generated cDNA 5'-ends (Figure 2B).

Analysis of the 5'-flanking region of rGSTA3- A 4.2 kb EcoRI fragment of rGSTA3 genomic DNA that spans sequence -2797 to +1413, was subcloned into pBluescriptIKS plasmid and sequenced (Figure 4). Analysis of the sequence immediately upstream of the potential transcription start site revealed no canonical TATA or CAAT boxes, characteristic of genes that are widely expressed in different cell types [35]. The computer-based analysis of the 5'-flanking region of the rGSTA3 gene identified several potential transcription factor binding sites. Such motifs included binding sites for the so-called antioxidant responsive elements (ARE), Barbie box, Sp-1 (GC box), and a half site element for ERE. These sequences have been previously found in the promoter of rGSTA5, the other member of the same subfamily of the GST alpha class [33]. In addition, the 5' upstream region of the rGSTA3 contains consensus sequences for AP-1, NF-κB, E box, CREB, and RRE.

Recently, it has been demonstrated that in extracts of livers from male rats, the GSTA3 subunit is induced by a number of antioxidant and metabolizable xenobiotic agents such as coumarin, ethoxyquin, β-naphthoflavone, trans-stilbene oxide, indole-3-carbinol, oltipraz, phenobarbital, benzyl isothiocyanate, butylated hydroxyanisole and diethylmaleate [36]. The putative enhancer elements that are identified in the 5' flanking
Table 2  Comparative analysis of the rGSTA3 gene with other class-alpha GST genes.

Data for other GST alpha genes were obtained from previous studies (31-34). r, rat; h, Human; m, mouse; NA, not applicable.

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region of the rGSTA3 subunit gene (Fig. 4) may be involved in its inducibility by the above agents.

The sequence analysis of the rGSTA3 promoter revealed the existence of three potential AREs, G/ATGAC/GNNNGCA/G [37, 38], of which one is located in 5'-UTR, 30 bp downstream of the transcription start site, and the other two in the distal portion of the 5'-flanking region at -1941 and -2220. Whether either or all AREs are involved in the regulation of the rGSTA3 by the antioxidants that induce this gene [36] is a subject of further analysis. The promoter of the human γ-glutamylcysteine synthetase (γ-GCS) heavy subunit gene contains four AREs, of which only a distal ARE sequence was found to be involved in the constitutive and β-naphthoflavone-induced expression of this gene [38]. The second element which was reported to be present in the regulatory region of most GSTs, is the Barbie box (at -606) with the core sequence of "AAAG" being common in all of them. This element might be responsible for the induction of the GST genes by phenobarbital [33, 39]. Seven putative AP-1 consensus sites were found throughout the promoter region of rGSTA3 gene at nucleotides +19, -450, -768, -931, -1924, -1998 and -2644 (Figure 4). The Fos-Jun family of transcription factors and related proteins like Nrl and Maf have been demonstrated to bind to the AP-1 sites of many promoters, resulting in their activation [40-42]. Another regulatory element in the promoter region was the presence of two sites for an NF-κB-like element at -714 and -1104. NF-κB activation, in contrast to AP-1 [43], has been shown to be inhibited by antioxidants [44]. The possibility of interactions between the transcription factors that bind to both AP-1 as well as NF-κB sites has been previously demonstrated in human GST P1-1 gene [42] and will be explored in rat GSTA3 gene. The next potential element is an E box motif CACGTG (Arnt) which is located at nucleotide -1327. It has been shown that this motif is recognized by a number of basic helix-loop-helix (bHLH) transcription factors such as Max, USF and Arnt leading to transcriptional activation of
the target genes [45-47]. Two sites for CREB (cAMP response element-binding protein) which trans-activates cAMP-responsive genes [48] were also seen in the promoter of the rGSTA3 gene at -591 and -816. Another element found in the 5' upstream region of this gene was RRE which is situated at nucleotide -337 and may function as a silencer element influencing the basal expression of this gene. This element was previously hypothesized to function as a repressor in a mu class glutathione S-transferase, hGSTYBX [49]. The nucleotide sequence analysis of the 5' upstream region of the rGSTA3 also showed the presence of eleven copies of the tetranucleotides "GATA" within the distal region of the promoter between nucleotides -2226 and -2769. The function of this motif with regard to transcriptional expression of rGSTA3 gene is unknown at present. The consensus sequence HNF-5 (TGTITGC) which is present in the promoter of the rGSTA5 and might be a reason for its tissue-specific expression in the rat livers, is interestingly not seen in the 5'-flanking region of the rGSTA3 we studied here [33].

An interesting motif found within the first intron (between nucleotides +254 and +308) was a 56-bp "CA" pyrimidine-purine microsatellite repeat (Figure 4). This tandem repeat could contain polymorphism information that provides an excellent genetic marker for the rGSTA3 gene to be used in linkage analysis of physiologically important traits, perhaps relating to detoxification of relevant contaminants.
FIGURE 4. Sequence analysis of the proximal promoter region, the first exon (5'UTR), and a portion of the first intron of the rat GSTA3 gene. The transcription start site is shown and denoted as +1. The putative cis-acting elements like ARE, AP-1, Sp-1, NF-kB, Barbie box, ERE (half-site), E box, CREB and RRE are indicated. The nucleotide sequence of the first exon (5'UTR) and some nucleotide sequence of the first intron are presented as well. The "GATA" tetranucleotide repeats located in the distal portion of the promoter, and a "CA" pyrimidine-purine repeat within the intron 1 are underlined.
**Functional activity of the rGSTA3 regulatory region** - A 4.2 kb EcoRI fragment from the rGSTA3 subunit gene that includes 2.8 kb of the promoter region and 1.2 kb of intron 1 was inserted 5' of the CAT reporter gene (prA3/4.2-CAT). This construct along with its two deletion mutants, prA3/2.5-CAT and prA3/2.7-CAT (Figure SA) were transiently transfected into either WT or into MLNr MatB cells which are known to have significantly higher expression of the rGSTA3 mRNA [22]. The transfection efficiency was normalized by cotransfection of a β-galactosidase plasmid driven by the SV40 promoter. The rGSTA3 construct (prA3/4.2-CAT) exhibited higher promoter activity in MLNr cells compared to the WT cells (Figure SB). These results demonstrate that this region of the rGSTA3 gene could act as a functional regulatory region that contains the necessary cis-acting elements for driving the expression of this gene in MatB cells. At the same time, these results clearly indicate that this region contains enhancer element(s) that appear to be important for its elevated expression in melphalan resistant cells. The identification of cis-acting element(s) that are involved in overexpression of the rGSTA3 gene in melphalan resistant cells is currently underway.

The 5' deletion mutant of rGSTA3 gene (construct prA3/2.5-CAT) revealed significantly higher promoter activity in MLNr cells as compared with plasmid prA3/4.2-CAT (Figure SB). This difference was not significant in WT cells. Thus, this data indicate the existence of the regulatory element(s) within the distal region of the rGSTA3 promoter that affect the expression of this gene in MLNr cells. Further analysis is needed for identification of the sequences in this region of the promoter that might influence the expression of the rGSTA3 gene in MLNr cells.

Deletion of the region between nucleotides -46 and +1413 (construct prA3/2.7-CAT) that removes the important region surrounding the transcription start site, resulted in virtually complete elimination of the basal CAT activity (Figure SB). This is consistent with previous studies demonstrating that the promoters that lack TATA box
require initiator or elements downstream of the transcription initiation site for binding of
general transcription factors such as TFIID complex [50].
FIGURE 5. Functional activity of the rGSTA3 regulatory region. A) Schematic representation of the rGSTA3 5'-flanking region/CAT constructs. The restriction enzymes used for generation of the mutant constructs are shown; +1, transcription start site; large open box, mRNA untranslated region. The putative response elements are indicated. B) Relative CAT activity of the rat GSTA3 upstream sequence. The CAT expression plasmids indicated in panel A were used to transfet WT and MLNr MatB cells. The CAT activity was measured as described in Experimental procedures. The co-transfected β-gal activity was used to normalize the transfection efficiency. This result is the average of at least three experiments.
CONCLUSIONS

In this study, we reported the molecular cloning and characterization of the entire rGSTA3 subunit gene including its 5'-flanking sequences. We also presented evidence for its enhanced promoter activity in drug resistant cancer cells. Several potential binding sites for transcription factors such as ARE, AP-1, NF-kB, Barbie box, CREB, RRE, E box were found in the 5'-flanking region of the rGSTA3 gene. Their possible functions remain to be tested. The availability of the rGSTA3 genomic clones and the complete sequences of its regulatory regions make it now feasible to initiate detailed investigation of the molecular mechanisms of anticancer drug resistance in GSH and GST dependent pathway. We are presently analyzing the involvement of cis- and trans-acting element(s) in transcriptional regulation of rGSTA3 gene in anticancer drug resistant tumor cells. This study also provides new insights into the structural and functional relationships among human and rat GST alpha class members.

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REFERENCES

1 Tew, K. D. (1994) Cancer Res. 54, 4313-4320
43 Bergelson, S., Pinkus, R., and Daniel, V. (1994) Oncogene. 9, 565-571


Chapter 4

Evidence for Genomic Duplication of the Glutathione Transferase A3 Gene in Genus *Rattus*

**PREFACE**

During the course of analysis of the rGSTA3 subunit gene, I identified the presence of some nucleotide sequences identical to the 5'-flanking region of another GST alpha subunit gene (rGSTA5). In order to confirm this observation and to elucidate the molecular evolution of the GST multigene family, the study in this chapter was undertaken.

**ABSTRACT**

Glutathione transferases are a family of proteins that are multifunctional in drug biotransformation and in xenobiotic metabolism. The sequencing analysis of two independently isolated genomic phage clones of the rat glutathione transferase A3 (rGSTA3) subunit gene has revealed that the 3' region of intron 1 contains sequences identical to a portion of the 5'-flanking region of the rGSTA5 subunit gene. Polymerase chain reaction analysis of the genomic DNAs from a rat mammary carcinoma cell line and rat liver tissue showed that high sequence identity between the respective regions of the two subunit genes actually exists in cells and tissues and therefore, confirmed this observation. The transient transfection experiments showed that the duplicated region of
the rGSTA3 gene are nonfunctional under normal conditions. Our findings support the notion that these two subunits of the alpha class gene are diversified as a result of ancestral duplication.
INTRODUCTION

The glutathione transferases (GSTs) are a multigene family of detoxifying enzymes that are present in all eukaryotes and some bacteria. This group of enzymes has evolved to protect cells from toxic endogenous and xenobiotic electrophilic compounds. There are three distinct superfamilies of enzymes with glutathione transferase activity namely; cytosolic, membrane-bound and metallo forms (Arca, Hardisson, and Suarez 1990). The natural evolution of GSTs has occurred by both convergent and divergent pathways (Pemble and Taylor 1992; Ji et al. 1995). The evolutionary pressure driving the diversification of GSTs are the reactive oxygen species which are generated in cells exposed to an aerobic environment. The cytosolic enzymes are the best characterized examples of divergent evolution.

GSTs are extremely diversified and are grouped into a number of classes designated as alpha, mu, pi, sigma, theta, zeta, etc. (Board et al. 1997) which originate from an ancient group of proteins. Each class is comprised of several subunits, where the biologically active forms are either homo- or heterodimeric proteins. A common feature of the GSTs is their ability to bind glutathione, another property is their ability to recognize and detoxify compounds with diverse chemical structures. The evolution of the GSTs in which relatively low binding affinities are offset by broad substrate specificities constitutes an energy efficient response to toxin exposure. Although substrate specificities are somewhat overlapping, the enzymes are critical to eukaryotic organisms in that they display unique activities toward a variety of harmful compounds (Board et al. 1997).

The three-dimensional structures of GSTs have a conserved chain fold of the glutathione-binding domain (Wilce and Parker 1994). The second domain is more variable and is principally involved with the electrophilic substrate. The structural differences contribute to the differential substrate selectivity seen between isozymes.
The generation of isozymes with novel substrate specificities has been attributed to gene conversion and exon shuffling (Mannervik 1985).

Although the evolutionary relationships of the various isoenzyme classes are not entirely certain, analysis of both the sequences and exon-intron boundaries of several GST genes provides some insight. For example, the theta class protein is thought to be the evolutionary precursor for genes encoding alpha, mu, and pi proteins (Pemble and Taylor 1992) and the sigma class enzymes diverged from an ancestral precursor prior to the divergence of the alpha / mu / pi precursor (Ji et al. 1995). The relatedness of one subunit to another is determined primarily on the basis of sequence comparisons. However, there are no established criteria for the extent of sequence similarity necessary to be considered as a member of one class over another.

The isolation and structural organization of two overlapped phage clones of the glutathione transferase A3 gene of the Rattus sp. (rGSTA3 gene) was recently reported by our laboratory (Fotouhi-Ardakani and Batist 1999). During the course of analysis of the exon/intron splicing junctions of the rGSTA3 gene, we detected the presence of some nucleotide sequences identical to the 5'-flanking region of the rGSTA5 (Pulford and Hayes 1996). In order to confirm and extend this result and to elucidate the molecular evolution of the GST multigene family, the sequencing of the entire intron 1 through intron 2 of the rGSTA3 subunit gene was performed in this study.
MATERIALS AND METHODS

Nucleotide sequence analysis- DNA sequencing of the rGSTA3 clones (λBF7 and λBF11) were performed by the dideoxy chain termination method (9) using [α-35S]dATP and T7 Sequenase version 2.0 (Amersham). All DNA sequences were confirmed by sequencing both strands. The direct sequencing of the PCR products were performed using [γ-32P]ATP end-labeled primer and ds DNA cycle sequencing system (Gibco-BRL). The BLAST program of the GenBank database (1) was employed for construction of the sequence alignments.

Polymerase chain reaction- PCR amplification was performed as described previously (4) using pfu DNA polymerase (Stratagene). The oligonucleotide primers used in the PCR analysis were primer 1: 5'-GAGGGGCACCTGAGT AGTTCTT AGC-3' (forward), primer 2: 5'-CCAGTTTAGTGTATCCATCGG-3' (reverse) and primer 3: 5'-GGCTTCCCCGGCATGGCAGCA-3' (reverse). The amplification parameters were: denaturation at 94 °C for 3 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 1 min and 70 °C for 1 min. The amplified products were subjected to agarose gel electrophoresis and sequencing for verification.
FIGURE 1. (A) The schematic diagram of the 5' region of the rGSTA5 and A3 gene. The regions of the rGSTA3 clone (analyzed in this study) that contain sequences identical to the segments of the rGSTA5 are located between two dotted lines. Open boxes, untranslated exons; Closed boxes, coding exons; +1, transcription start sites; ATG, translation start sites; The positions of the primers used in PCR analysis are marked with arrow heads. The modified structure of the rGSTA5 was obtained from Pulford and Hayes 1996.

(B) Nucleotide sequence of the rGSTA3 subunit gene spanning the 3' region of intron 1 to a portion of intron 2. The nucleotide sequences of the identical regions in rGSTA3 and A5 subunits are aligned and dashed lines in A3 show nucleotides which are identical to those in rGSTA5. The gaps (*) were made to generate maximum alignments. The respective coding exons are shown in capital letters. The nucleotide sequences of the rGSTA5 were obtained from Pulford and Hayes 1996. +1, transcription start site of the rGSTA5; ATG, start codons; Introne/exon splicing junctions are indicated by curved arrows. The positions of the primers used in PCR analysis are underlined. Dideoxy sequencing reactions (Sanger, Nicklen, and Coulson 1977) were performed using T7 Sequenase version 2.0 (Amersham). The BLAST (basic local alignment search tool) program of the GenBank database (Altschul et al. 1997) was employed for construction of the sequence alignments.
FIGURE 1 (Continued)
RESULTS AND DISCUSSION

We have identified a nucleotide fragment of 690 bp in the 3' region of intron 1 of the rGSTA3 subunit gene which is similar to a segment of the 5'-flanking region (nucleotides -483 to +207) of the rGSTA5 gene. (fig. 1A). This region of the rGSTA5 with high degree of nucleotide sequence identity to the 3'-end of intron 1 of the rGSTA3 consists of a transcription start site, a TATA box and two putative enhancer elements namely hepatocyte nuclear factor-5 (HNF-5 at -392 bp), a liver specific consensus binding site; and an antioxidant responsive element (ARE) located at -421 bp (fig. 1A). The sequence analysis revealed that HNF-5 and ARE consensus elements in intron 1 of the rGSTA3 clones were modified by one and two bases, respectively (fig. 1B).

Similarly, the 5'-end of intron 2 of the rGSTA3 clone has a stretch of 102 bp identical to the nucleotide sequences located in the 5'-end of intron 1 of the rGSTA5 clone (fig. 1A and B), forming the identical regions of 900 bp in length.

To verify that our observation was a true finding and not the result of cloning artifact, polymerase chain reaction (PCR) analysis was employed using the genomic DNAs from a rat mammary carcinoma cell line (MatB) and rat liver tissue, respectively. PCR reactions were performed as described previously (Fotouhi-Ardakani and Batist 1999) using primers 1, 2 and 3 (fig. 1) where primer 1 was designed to be specific only to the rGSTA3. The PCR products were analyzed on a 1% agarose gel (fig. 2A) and then sequenced directly following the ds DNA cycle sequencing protocol (Gibco-BRL). PCR analysis (fig. 2A) and sequencing of the products showed that the 3' region of intron 1 of the rGSTA3 with high identity to 5'-flanking region of the rGSTA5 is actually present in cells and tissues.

In order to determine whether this region of the rGSTA3 clone with 95% identity to the regulatory region of the rGSTA5 clone has been altered to nonfunctionality or evolved a new function, we tested the rGSTA3 intronic sequence (fig. 1B) and its homologous rGSTA5 promoter region in the same transient transfection experiments.
The rGSTA5 sequence was included in the experiment as a control. The transcriptional activity of the rGSTA5 promoter was previously demonstrated by Pulford and Hayes (1996). The transfection and CAT (chloramphenicol acetyltransferase) assay conditions were as described elsewhere (Fotouhi-Ardakani and Batist 1999). Briefly, the PCR generated DNA fragments of the respective regions of both subunit genes were inserted into the pCAT-Basic reporter plasmids. These constructs were then transiently transfected into the HepG2 (human hepatoma) cell line. The proteins were extracted 36 h after the transfection, followed by the CAT assay analysis (fig. 2B). The transfection of HepG2 cell line with this region of the rGSTA3 gene showed that it lacks the capability of modulating transcriptional activity and indeed these intron sequences are non-functional under normal conditions (fig. 2B).

An important force in evolution of genome is gene duplication. Recently in an empirical study, it was suggested that approximately half of all duplicated genes become functionally divergent and novel, while the remaining half become pseudogenes (Nadeau and Sankoff 1997). The ongoing large scale sequencing of eukaryotic genome is an excellent tool for providing, among others, direct information about gene duplication events. In this communication, we report a remarkable sequence identity between rGSTA3 and A5 subunit genes which extends beyond their coding regions. Genomic sequences of the 711 bp regions upstream of their start codons contain a total of 38 individual nucleotide modifications that accounts for a 5% difference between the two subunits. In addition, there are a total of 48 bp differences in the coding region of the rGSTA3 and rGSTA5 subunit genes (Pulford and Hayes 1996; Fotouhi-Ardakani and Batist 1999). These differences in sequence between rGSTA3 and A5 will translate into structural differences and explain the differences in their substrate specificity. For example, the rGSTA5 homodimer was reported to exhibit 120- to 150-fold higher aflatoxin B1 exo-8, 9-epoxide conjugating activity as compared to the rGSTA3 homodimer (Buetler et al. 1996). It is well known that the rGSTA3 enzyme has very
high specific activity towards alkylating agents such as melphalan (Bolton, Colvin and Hilton 1991). However, there is no report in literature for such activity for the rGSTA5. The relationship between the coding exons and a high conservation of nucleotide sequence in the intervening sequences reflects a gene duplication event during the evolution of the rat genome. This duplication event presumably lead to mutations with ultimate diversification and functional benefits. The striking 95% identity between the promoter region of the rGSTA5, and the intronic region of the rGSTA3 gene suggests that this duplication event occurred relatively recently. Assuming the spontaneous mutation rate of $10^{-9} / \text{bp} / \text{yr}$ for vertebrates (Ohno 1985), we estimate that the duplication event of this region of the rGSTA3 took place approximately 53 MYA.

In humans, it has been well documented that the GST genes occur in class-specific clusters on different chromosomes. Although there is less data available for other species, there is probably a similar arrangement in other mammals. For example, mouse GST (mGST) alpha and mGST pi genes are clustered on chromosomes 9 and 1, respectively. In rats, GST Yc genes (GSTA3 and GSTA5) are clustered on chromosome 9 (Yamada et. al, 1992). This strongly supports the observation that the evolution of the GSTs has involved multiple gene duplication events.

In summary, we report evidence for a gene duplication within the alpha class of the GSTs in rats. These data suggest that the rGSTA3 subunit evolved as a duplication of the rGSTA5 subunit gene increasing the range of catalytic activity within this class and affording further protection to deleterious agents.
FIGURE 2. (A) PCR analysis of 3' region of intron 1 of the rGSTA3 subunit gene. Agarose gel electrophoresis shows the PCR products from MatB cell line and rat liver tissue, in addition to a cloned phage. The PCR fragments were isolated from the gel and sequenced directly. M, molecular size marker in kb (λ DNA cleaved with HindIII); Lanes 1 & 4, a rGSTA3 phage clone, λBF7 (Fotouhi-Ardakani and Batist 1999); Lanes 2 & 5, MatB cells; Lanes 3 & 6, rat liver tissue. Oligonucleotide primers used in the PCR analysis were: primer 1 (5'-gaggggcacctgagtagttcttagc-3'), primer 2 (5'-ccagtttagtgtatccatcgg-3'), primer 3 (5'-ggcttccccggcatggcagca-3'). The positions of these primers are indicated in fig. 1.
FIGURE 2. (B) Lack of functional activity of the rGSTA3 intronic sequences. The CAT expression plasmids, carrying the 3' region of intron 1 of the rGSTA3 gene (fig. 2A) or its homologous rGSTA5 promoter region (-647+192, see fig. 1B), were used to transfect HepG2 cells. The CAT assays were performed as described previously (Fotouhi-Ardakani and Batist 1999). pRSV-CAT and pCAT-Basic plasmids were used as positive and negative controls, respectively. The transfection efficiency was normalized by co-transfection with the plasmid containing the β-galactosidase gene. These experiments were repeated at least three times in triplicate.
ACKNOWLEDGMENTS

This work is supported by a grant from the National Cancer Institute of Canada. We wish to thank Dr. Marco Di Fruscio for critical reading of this manuscript. The nucleotide sequence reported in this study is deposited in the DDBJ/EMBL/GenBank with the accession number AF146746.
LITERATURE CITED


CHAPTER 5

Modulation of Glutathione S-Transferase Alpha by Hepatitis B Virus and the Chemopreventive Drug Oltipraz

PREFACE

During the course of isolation of the rGSTA3 promoter, which has been very elusive, I also isolated the regulatory region of the rGSTA5 subunit gene. Many experimental data have previously shown a chemopreventive role of the rGSTA5 gene against aflatoxin B1-induced hepatocarcinogenesis. This prompted me to use the rGSTA5 promoter in the following study to examine further the implications of GST in carcinogenesis. Several studies demonstrated the association of HBV infection and exposure to chemical carcinogens with the development of hepatocellular carcinoma (HCC). The exact mechanism of these interactions is not known. Reduced GST activity could enhance sensitivity of cells to chemical carcinogens, and previous studies have demonstrated the modulation of GST expression in HBV infected human specimens. To study the mechanism of GST implication in HCC, GST regulation was examined in both HepG2 and in a HBV-transfected subline, using both rat and human GST alpha promoters.

ABSTRACT

Persistent infection by hepatitis B virus (HBV) and exposure to chemical carcinogens correlates with the prevalence of hepatocellular carcinoma in endemic areas. The precise nature of the interaction between these factors is not known.
Glutathione transferases (GST) are responsible for the cellular metabolism and detoxification of a variety of cytotoxic and carcinogenic compounds by catalysis of their conjugation with glutathione. Diminished GST activity could enhance cellular sensitivity to chemical carcinogens. We have investigated GST isozyme expression in hepatocellular HepG2 cells and in an HBV-transfected subline. Total GST activity and Se-independent glutathione peroxidase activity are significantly decreased in HBV transfected cells. On immunoblotting, HBV transfected cells demonstrate a significant decrease in the level of GST alpha class. Cytotoxicity assays reveal that the HBV transfected cells are more sensitive to a wide range of compounds known to be detoxified by GST alpha conjugation. While no significant difference in protein half-life between the two cell lines was found, semi-quantitative RT-PCR shows a reduced amount of GST alpha mRNA in the transfected cells. Since the HBV x protein (HBx) seems to play a role in HBV transfection, we also demonstrated that expression of the HBx gene into HepG2 cells decreased the amount of GST alpha protein. Transient transfection experiments using both rat and human GST alpha (rGSTA5 and hGSTA1) promoters in HepG2 cells show a decreased CAT activity upon HBx expression, supporting a transcriptional regulation of both genes by HBx. This effect is independent of HBx interaction with Sp1. Treatment with oltipraz, an inducer of GST alpha, partially overcomes the effect of HBx on both promoters. Promoter deletion studies indicate that oltipraz works through responsive elements distinct from AP1 or NF-κB transcription factors. Thus, HBV infection alters phase II metabolizing enzymes via different mechanisms than those modulated by treatment with oltipraz.
INTRODUCTION

An estimated 350 million people worldwide contract some form of hepatitis per year. Epidemiological and experimental data have demonstrated that individuals chronically infected with HBV have a high incidence of developing HCC (1-4) and HBV DNA sequences have been shown to be integrated into cellular DNA of human HCC (5). Strikingly, the incidence of HCC is even greater in areas where there is also exposure to liver carcinogens such as aflatoxins (6-8).

Different mechanisms have been suggested as significant in the development of HCC following HBV infection. An activated host immune response and increased production of reactive oxygen species have been shown to be important in triggering abnormal liver cell growth (9). In addition, HBV encodes a small protein x (HBx) which seems to play a critical role in hepatocarcinogenesis both in humans and in animal models (10, 11). HBx has been found to be expressed in chronic hepatitis, cirrhotic liver and HCC from individuals infected with HBV (11-15). The HBx protein demonstrates trans-activating ability for viral and cellular genes through protein-protein interaction with several components of the transcription machinery and signaling cascades (16-20). HBx has been shown to bind and inactivate the p53 tumor suppressor protein, alter the cell cycle, interfere with apoptosis and DNA repair mechanisms (21-26). The processes that associate development of HCC with viral infection, HBx biological properties, as well as the role and action of concomitant exposure to liver carcinogens are still not clear.

The glutathione transferases are members of a family of detoxification enzymes which metabolize a variety of carcinogens by conjugating lipophilic electrophiles to glutathione. They also bind non-substrate ligands including bile acids and bilirubin (27, 28). The mammalian cytosolic GST isozymes are dimeric and have been divided into seven classes: Alpha, Kappa, Mu, Pi, Sigma, Theta and Zeta. Alpha GSTs in all species are designated as GSTA1 through A5. The human GSTA1 shows a high degree of
homology with rat GSTA5. In human and rodent liver, GST alpha is the predominant form expressed and GST pi protein is not found in normal adult hepatocytes. GST mu is absent in approximately 50% of the general population as a result of a frequent mutation (29). The fact that GSTs represent as much as 5% of the cytosolic protein in the liver, suggests that they may play an important role in maintaining cellular homeostasis. The various GST isozymes have different catalytic activities and patterns of tissue distribution, suggesting that they may be a contributing factor in tissue specific susceptibility to the carcinogenic process. Some highly reactive chemicals are able to conjugate glutathione directly, however, in most cases the biotransformation of these compounds via phase I activation results in a more electrophilic molecule. For instance, AFB1-8,9-epoxide, the hepatocarcinogenic derivative of aflatoxin B1 (AFB1), is detoxified by the formation of a glutathionyl-AFB1 conjugate. Studies with purified rat isozymes demonstrate that this reaction is catalyzed by enzymes containing alpha class subunits of GST (30, 31).

The GST alpha isozyme can be induced by agents such as phenobarbital and oltipraz (31, 32). Oltipraz (4-methyl-5-pyrazinyl-3H-1,2-dithiole-3-thione) has been found to have cancer chemoprotective properties (33, 34). Inhibition of phase I enzymes, induction of phase II xenobiotic metabolizing enzymes, regulation of oxygen reactive metabolites and enhancement of DNA repair processes are known properties of oltipraz (35-41). Since GST alpha from human liver is inducible by oltipraz, alpha GSTs may also reduce the susceptibility to hepatocarcinogenesis by enhancing carcinogen detoxification and elimination (36, 38).

While much is known about the structure and function of GSTs, little work has focused on the changes that occur in the different GSTs following liver disease. In malignant hepatocytes from clinical specimens, alpha subunit expression is almost always dramatically decreased. The reduction in expression appears to parallel the cellular transformation process, since adenomas demonstrate intermediary levels of
GST alpha (42-46). This loss of GST protection could increase the susceptibility of preneoplastic populations of hepatocytes to further contribute to genotoxic injury by chemicals during malignant progression.

In these studies, we reasoned that HBV transfection could diminish normal cellular detoxification potential and thus, concomitant exposure to chemical carcinogens would be more likely to contribute to HCC development. We have identified changes that occur specifically in GST alpha following the expression of HBV gene products in hepatocytes. Furthermore, we have determined the potential of oltipraz to overcome this effect.
EXPERIMENTAL PROCEDURES

Cell Lines—The human hepatoblastoma cell line, HepG2 and its HBV transfected counterpart, HepG2/HBV (kindly provided by Dr. Wands, Massachusetts General Hospital, Boston, Mass.) (47) were grown in α-MEM with non-essential amino acids, sodium pyruvate and Earle's BSS 90% and 10% fetal bovine serum (FBS). The rate of growth of both cell lines was shown to be similar (unpublished data). CCL13 cells are human liver epithelial cells. These cells were maintained in D-MEM, 10% FBS, and 50 μg/ml of gentamicin. The 293GPG packaging cell line (48) was kept in D-MEM medium containing 10% FBS, 1 μg/ml tetracycline, 2 μg/ml puromycin and 0.3 μg/ml geneticin. All cells were maintained at 37 °C in an atmosphere of 5% CO₂. Drosophila SL2 cells were cultured in Grace's insect cell culture medium (Life Technologies, Burlington, Ontario), supplemented with 10% FBS and kept at room temperature.

Northern Blot Analysis—RNA extractions and Northern blot analyses were performed according to standard protocols (49). Fifteen micrograms of each RNA sample was electrophoresed in 2% agarose/formaldehyde gel at 100 V for 3 h, followed by transfer onto Zeta-Probe membrane (Bio-Rad, Hercules, CA). An EcoR I fragment of pGEM-adw2 containing the entire HBV viral genome was radiolabeled with [α-32P]dCTP by random primer extension (Oligo Labeling Kit; Pharmacia, Montreal, Canada) and used as probe. Hybridization was carried out at 42 °C using 50% formamide, 4× SSC, 4× Denhardt’s reagent, 1.2% SDS, and 0.2 mg/ml salmon sperm DNA. Final washes of blots were done with 2× SSC and 0.1% SDS at 65°C. The same membrane was reprobed with β-actin cDNA to control for sample loading and transfer efficiency.
Protein Turnover Studies—The turnover of GST alpha was measured in exponentially growing HepG2 and HepG2/HBV cultures. Cells were incubated in complete medium supplemented with 150 μg/ml cycloheximide (ICN Biomedicals Inc., Mississauga, Ontario) for 2 h, 4 h, 8 h and 10 h. Following these time periods the cells were washed twice with PBS and lysed by resuspending them in 1 ml of lysis buffer (10 mM Tris-HCl pH 8, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 0.5% NP-40, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 0.5 μg/ml aprotinin per 4 x 10^7 cells).

Enzymatic Assays—Cytosolic extracts from the various cell lines were prepared by cell lysis and centrifugation of debris at 12,000 x g for 1 h. Protein concentrations were determined according to the method of Lowry, using bovine serum albumin as the standard. Total GST activity was assayed using 2-chloro-1,3-dinitrobenzene as the substrate (43). Selenium-independent glutathione peroxidase activity was assayed using cumene hydroperoxide (44). Student's t test was used for statistical analysis.

Cytotoxicity Assays—Cells were plated in 100 μl of medium at a concentration of 2-10 x 10^3 cells/flat-bottomed well in 96-well microtiter plates which were incubated for 24 h at 37 °C in an atmosphere of 5% CO_2. One hundred μl of medium containing drug dissolved in appropriate solvent were added to triplicate wells and incubated for a further 72 h. Medium (180 μl) was then removed from each well and replaced by 150 μl of medium containing 10 mM 1,4-piperazinediethane sulfonic acid (pH 7.4) and 50 ml MTT (Sigma Chemical Co., St. Louis, MO) at 2 mg/ml in PBS. Plates were then wrapped in aluminum foil and incubated for 4 h at 37 °C. The formazan crystals were dissolved in 180 μl dimethyl sulfoxide (Fisher Scientific, Montreal, Canada) and 25 μl glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5) by mixing on a shaker for 5 min. The formazan product formed by viable cells was then quantitated by measuring the
absorbance at a wavelength of 570 nm on a microtiter plate reader. IC50 was determined by plotting the percentage of surviving cells versus the log of drug concentration. Statistical analysis was performed using Student's t test for paired samples.

**Plasmid Constructs**—The expression vectors for the HBx protein used in this study were: pCMV-HBx, consisting of the HBx gene from the HBV ayw subtype (nt 1241 to nt 1991) ligated into the HindIII site of the pRc-CMV vector (Invitrogen, La Jolla, CA). The HBx gene was kindly provided by Dr. J. Cromlish (21). The pRc-CMV plasmid was used as a negative control and referred to as pRc-CMV. pAP2 is a retroviral expression vector encoding for a bicistronic non-splicing retrovector which incorporates a multiple cloning sites, allowing insertion of sequences, linked by an IRES to the enhanced green fluorescent protein (GFP) reporter. The HBx gene from the HBV subtype ayw (nt 1241 to nt 1991) (21) was subcloned into the XhoI-BamHI sites of the pAP2 vector, and referred to as pAP2-HBx. The pAP2 vector was used as a negative control. Retroviral producer cell lines were generated for both pAP2 and pAP2-HBx by stably transfecting the 293GPG packaging cell line (48). VSVG pseudotyped retroparticles were collected as described (48) and utilized to transduce target cells. The hGST A1-CAT reporter plasmid was a generous gift from Dr. Board (50). The 5' deletion constructs of the rGSTA5 promoter [accession number S82821, (51)] were created by PCR amplification followed by subcloning into the pCAT-Basic reporter plasmid. The primers were RP1 (+192): 5'-gcacagctgtttctaacagttgtc-3'; FP1 (-1836): 5'-ctgatgaggtcatcctgtcggca-3'; FP2 (-928): 5'-gataacaagacggctggcagagg-3'; FP3 (-761): 5'-gatagggaggccacagctggctgg-3'; FP4 (-460): 5'-aagggcgcacagttgtcggcc-3'; FP5 (-353): 5'-ccctctcgttcttcctgcatcc-3'. The position of each deletion site is indicated in Fig. 4A. The deletion clones produced by PCR were sequenced using T7 Sequenase v2.0 (Amersham Life Science Inc., Cleveland, OH). The chloramphenicol reporter construct pERE3-CAT containing AP1 responsive elements (52), and the reporter construct 5'tb
containing NF-κB sites were kindly provided by Dr. Mader (Université de Montréal, Montreal, Canada) and Dr. J. Hiscott (McGill University, Montreal, Canada).

Retroviral Production—293GPG cells were plated at a concentration of $4 \times 10^6$ per 60 mm dish the night before transfection in the appropriate medium. Five $\mu$g of linear DNA containing either pAP2 or pAP2-HBx were co-transfected at a ratio of 50:1 with the Zeocin-resistance plasmid pJ6Ω2bleo (48) using LipofectAmine according to the recommendations of the manufacturer (Life Technologies, Burlington, Ontario). Selection for cells stably transfected with pAP2 or pAP2-HBx was performed in three weeks in 293 cell medium containing 100 $\mu$g/ml of Zeocin (Invitrogen). FACS analysis was performed to determine the percentage of producer cells that expressed the GFP reporter protein. Cells expressing GFP were selected using a FACS STAR PLUS TURBO (530-30; FL-1) cell sorter, and immediately frozen or kept in culture. When cells reached 60% confluence, the selection medium was removed and replaced with D-MEM medium, 10% FBS, penicillin, and streptomycin (50 U/ml and 50 $\mu$g/ml, respectively). Three days after tetracyclcin withdrawal, the supernatant was collected, filtered and frozen daily for 6 to 7 days. Supernatants were thawed, pooled and retroviral particles AP2 and AP2-HBx were concentrated 20 to 30 times (v/v) by ultracentrifugation as described (48).

Immunoblot Analysis for GST Alpha Expression in HepG2 and HBV Cells—Polyacrylamide gel electrophoresis was performed according to the method of Laemmli using a 4% polyacrylamide stacking gel layered over a 12% resolving gel. Twenty five $\mu$g of cytosolic protein from HepG2 and HepG2/HBV, treated and untreated with 45 $\mu$M oltipraz for 36 h, were run at 100 V and transferred onto nitrocellulose membrane by electroblotting at 15 V for 16 h. The membranes were blocked with 10% BSA/PBS and
incubated overnight in 0.5% BSA containing a 1:3000 dilution of polyclonal rabbit antisera directed against human GSTA1 (kindly provided by Dr. A.J. Townsend, Bowman Gray School of Medicine, Winston-Salem, N.C.). The membranes were blocked with 10% low fat milk in PBS and incubated overnight with the corresponding antibody. Enhanced chemiluminescence detection was performed using ECL detection reagents (Amersham Pharmacia Biotech, Baie d’Urfé, Que.). Blots were subsequently stripped in 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7, 50 °C for 30 min, then immunoblotted with monoclonal anti-GAPDH (clone 6C5, Cedarlane Laboratories, Ontario). Densitometric analysis was performed using a Hoefer, scanning densitometer, model GS300 (Hoefer Scientific, Minnesota).

*Semi-quantitation of GST Alpha mRNA by Polymerase Chain Reaction*—Total RNA was isolated from HepG2 and HepG2/HBV treated and untreated with 45 µM oltipraz for 36 h, using the RNeasy Total RNA Kit (Qiagen Inc., Chastworth, CA). The RNA was treated with RNase-free DNase I and further cleaned (RNeasy Total RNA Kit). Each RNA sample (1 µg) was reverse transcribed using murine leukemia virus reverse transcriptase (Gibco/BRL, Burlington, Ontario) and oligo dT primer for 1 h at 37 °C. PCR amplification of hGSTA1 and GAPDH cDNAs were performed using 50 pmole of each primer, 5 µCi of [α-32P]dCTP, and 2.5 U of Taq polymerase (Amersham Pharmacia Biotech, Baie d’Urfé, Que.) for 30 cycles at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. The appropriate cDNA volumes were 1/100 for hGSTA1 and 1/5000 for GAPDH. These dilutions were determined to be in a linear range of each standard curve. Samples were run on a 10% acrylamide gel dried and exposed. The quantification was performed using the Bio-Rad Gelscan Phosphoimager and the Molecular Analyst (Bio-Rad, Hercules, CA) software program. The primer sequences for GAPDH are 5’-ccatggagaaggrggg-3’ (forward), 5’-ccaaagttgtcatggatgacc-3’ (reverse);
and for hGSTA1 are 5'-cgtgatggactccggtgacggg-3' (forward), 5'-
gatggagttgaaggtagtttcgtg-3' (reverse).

Transfection and CAT Assay—Cells were seeded at $2.7 \times 10^5$ cells per 35 mm-
diameter dish, using 6-well plates, and grown overnight in the appropriate media. The
following day, the cells were transiently transfected, using LipofectAMINE (Gibco/BRL,
Burlington, Ontario), with hGSTA1-CAT or rGSTA5-CAT together with pCMV-HBx
or pRc-CMV. As controls, pERE3-CAT was co-transfected with pCMV-HBx or pRc-
CMV. LipofectAMINE was used at a concentration of 3 $\mu$g per 1 $\mu$g of DNA. pRc-CMV
was added to equalize the amount of DNA transfected in each well when necessary.
Cells were incubated with DNA-LipofectAMINE complexes for 6 h, after which cells
were washed gently and cultured in fresh serum-supplemented medium. When treatment
with oltipraz was performed, 16 h after transfection oltipraz was added at a final
concentration of 45 $\mu$M/well. Cells were harvested 48 h after transfection and protein
extracts were used to determine CAT activity as described (53). The quantification of the
reaction products in the CAT assay was performed using the Bio-Rad Gelscan
Phosphoimager and the Molecular Analyst (Bio-Rad, Mississauga, Ontario) software
program. The percentage of chloramphenicol conversion to its acetylated metabolites
was determined for each sample in at least three independent experiments.

Cell Transduction for CAT Assay, RT-PCR and Immunoblot Analysis—for CAT
assay, $8 \times 10^5$ cells/35 mm diameter dish were plated and grown overnight in appropriate
medium. The following day, cells were transiently transfected with pERE3-CAT reporter
plasmid (1 $\mu$g/well) as described above. After 6 h incubation with Lipofectamine-DNA
complexes, medium was removed and cells were transduced with 20x concentrated
retroviral supernatant obtained from pAP2 or pAP2-HBx 293GPG producer cells and
1 \mu g/\mu l of Polybrene (Sigma, Oakville, Ontario). Samples were collected 24 h later and CAT assays were performed as described above.

For immunoblot analysis and RT-PCR, cells were plated at a concentration of $1 \times 10^6$ cells/35mm plate and transduced the following day with concentrated viral supernatant. A final concentration of 1 \mu g/\mu l of Polybrene (Sigma, Oakville, Ontario) was added to each well. Cells were harvested 24 h later and $5 \times 10^5$ cells were resuspended in the appropriate medium to quantify GFP expression by FACS analysis. The remaining cells were used either to isolate total RNA for RT-PCR or whole cell extract for immunoblot analysis. Total cell extracts from cells transduced with AP2 and AP2-HBx retroviral particles were used to examine the expression of the endogenous hGSTA1 protein by Western blot analysis as described above. The expression of hGSTA1 mRNA was evaluated by semiquantitative RT-PCR. Isolation of total RNA and reverse transcription was performed as described above.
FIGURE 1. Northern blot analysis for the expression of HBV mRNA. Twenty μg of total RNA from HepG2 cells (lane 1) and HBV transfected HepG2 cells (lane 2) were hybridized with an HBV cDNA which recognizes the viral genome. Transcripts of expected sizes were visualized by radioautography. The membrane was reprobed with β-actin cDNA for verification of sample loading and transfer efficiency.
TABLE I

Total GST activity and selenium-independent glutathione peroxidase activity of HepG2 and HBV transfected HepG2 cells. Total GST activity was assayed using 2-chloro-1,3-dinitrobenzene (CDNB) and selenium-independent glutathione peroxidase activity was assayed using cumene hydroperoxide.

<table>
<thead>
<tr>
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<th>Total GST ± SD (nmol/min/mg protein)</th>
<th>GSHPx ± SD (nmol/min/mg protein)</th>
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<tbody>
<tr>
<td>HepG2</td>
<td>9.8 ± 0.03</td>
<td>2.2 ± 0.4</td>
</tr>
<tr>
<td>HepG2/HBV</td>
<td>3.1 ± 0.2a</td>
<td>0.3 ± 0.3b</td>
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a p<0.01; b p<0.001

TABLE II

Cytotoxic effect of chemicals on HepG2 and HepG2/HBV cells. Drug sensitivity assays were performed using the MTT colorimetric assay. Results represent the mean of 3 independent assays, each cell line being tested in quadruplicate in every individual assay.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>IC50 mM (mean ± SD) HepG2</th>
<th>IC50 mM (mean ± SD) HepG2/HBV</th>
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<tbody>
<tr>
<td>Melphalan</td>
<td>7.91 ± 0.28</td>
<td>4.36 ± 1.26a</td>
</tr>
<tr>
<td>CDDP</td>
<td>1.46 ± 0.36</td>
<td>0.66 ± 0.11a</td>
</tr>
<tr>
<td>BCNU</td>
<td>271.0 ± 27.50</td>
<td>87.96 ± 8.12a</td>
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a p<0.01. IC50 is the concentration which inhibits 50% of cell growth.
RESULTS

Expression of HBV in HepG2 Cells—The integrity of the hepatitis B virus DNA sequence in HepG2 cells was verified by Northern blot analysis. Fig. 1 shows the results where total RNA from HepG2 and HepG2/HBV cells were hybridized with an HBV cDNA. Only infected cells carry the message for this gene. Our result showing more than one HBV transcripts, is in agreement with the earlier reports indicating that different viral transcripts are produced prior to viral replication (5). The same membrane was reprobed with β-actin cDNA to verify for sample loading and transfer efficiency.

Biochemical Characterization—The activities of GST and Se-independent glutathione peroxidase in HepG2 and HepG2/HBV are shown in Table I. There was, however, a significant difference in total GST activity toward CDNB between the two cell lines. GST activity in HepG2 was 3.2-fold higher than in HBV transfected HepG2 cells. Using cumene hydroperoxide to measure GST alpha (glutathione peroxidase) activity specifically, transfected cells demonstrated an 86% decrease in GSHPx activity relative to control cells.

Cytotoxicity Assays—The drug sensitivity of HBV transfected cells was examined in cytotoxicity assays. The results of these experiments are presented in Table II. The chemicals studied are known substrates for GST alpha detoxification. HBV transfected cells were hypersensitive to the antiproliferative effects of melphalan, cisplatin and BCNU. The increase in sensitivity ranged from 1.8 to 3-fold relative to uninfected cells.
FIGURE 2. GST alpha Protein and mRNA expression in HepG2 and HepG2/HBV cells. A) Twenty five μg of protein from whole cell extract from HepG2 and HepG2/HBV cells were separated by polyacrylamide gel electrophoresis and transferred into nitrocellulose. The membrane was probed with a polyclonal antibody for hGSTA, stripped and reprobed with a monoclonal antibody for GAPDH as described in Experimental Procedures. B) GST alpha turnover was measured after incubation with cycloheximide at different time points. Cells were lysed in protease inhibitors and cell extracts were subjected to SDS-PAGE. C) mRNA from HepG2 and HepG2 HBV cells treated and non-treated with oltipraz was reverse transcribed, PCR amplified with specific primers that amplify a fragment of 500 bp from hGSTA1 cDNA and semiquantitated as described in Experimental Procedures.
<table>
<thead>
<tr>
<th></th>
<th>HepG2</th>
<th>HepG2/HBV</th>
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<tbody>
<tr>
<td>0h</td>
<td></td>
<td></td>
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<td>2h</td>
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**FIGURE 2 (Continued)**
**GST Protein Expression**—The cytosolic protein from HepG2 and HepG2/HBV cells were analyzed by immunoblotting to examine the pattern of GST isozyme expression. Significant differences in protein expression were seen (Fig. 2A). As in normal liver, the predominant GST subtype in HepG2 cells is class alpha. GST alpha protein expression is strongly decreased in HBV transfected cells. GST mu isoenzyme protein is slightly decreased in HepG2/HBV cells and the absence of GST pi was a consistent feature in both the parental and HBV transformed cells (data not shown). The turnover time of GST alpha in HBV transfected cells was compared to that in untransfected cells. Both cell lines were exposed to 150 μg/µl of cycloheximide for up to 10 h. After consecutive time points, extracts were prepared and subjected to immunoblotting using anti hGSTA1 antibody (Fig. 2B). The stability of the protein did not appear to be any different between the two lines even up to 12 h in the presence of cycloheximide (not shown). The effect of cycloheximide on protein synthesis was confirmed by reacting the same samples with antibody to detect expression of NF-κB and demonstration of the anticipated protein half life (data not shown). While the levels of the hGSTA1 protein are decreased in HepG2/HBV cells, treatment with oltipraz increased the protein level, although not to the same level observed in the parental cell line (Fig. 2A).

**HBV and the Regulation of GST Alpha mRNA**—We investigated whether the decreased GST alpha protein and enzyme activity occurred at the transcriptional level. Total RNA was isolated and used to examine hGSTA1 mRNA expression by semiquantitative RT-PCR in HepG2 and HepG2/HBV cells treated and untreated with oltipraz. The concentrations of hGSTA1 and GAPDH cDNAs used here, were determined to be in the linear range based on standard curves. HBV transfected cells
showed a significantly reduced amount of hGSTA1 mRNA (Fig. 2C). As previously described, oltipraz treatment lead to a transcriptional activation of the hGSTA1 gene.

Regulation of GST Alpha Gene Expression—Since both GST alpha protein and mRNA were decreased, it appears that following HBV expression, the mechanism involved is related to a transcriptional down-regulation. Due to the interaction of the HBx protein of the HBV with the cellular transcription machinery, we speculate that the observed results could be a consequence of HBx activity. For this reason, we performed co-transfection experiments using the pCMV-HBx expression vector and a reporter plasmid, where CAT gene expression is regulated by the hGSTA1 promoter (50) or the equivalent rGSTA5 promoter. Strikingly, we found a dramatic transcriptional inhibition in both GST alpha promoters. A dose response curve shows a decrease in CAT activity from hGSTA1-CAT that correlates with the amount of pCMV-HBx plasmid transfected (Fig. 3A). Expression of HBx produced the same extent of decreased CAT activity when the rGSTA5 promoter was used (Fig. 3B). When HepG2 cells transiently expressing HBx were treated with oltipraz, the induction of the hGSTA1 and rGSTA5 promoters was observed, overcoming to a great extent the down-regulation induced by the viral protein (Fig. 3C and 3D). HBx inhibitory activity on hGSTA1 and rGSTA5 promoters was observed while HBx maintained its transactivation capacity on AP1 and NF-κB responsive elements (data not shown).
FIGURE 3. Effect of HBx on hGSTA1-CAT and rGSTA5-CAT activities. HepG2 cells were transfected with increasing amounts of pCMV-HBx (A) or with 3 μg/well of pCMV-HBx (B) or the negative control (C). Cell extracts were used to determine CAT activity as described in Experimental Procedures. The results, percentage of acetylated chloramphenicol to non-acetylated metabolites, are expressed as percentage of CAT Activity for each HBx transfected sample compared to the control. Each bar corresponds to the average ± SD for at least 3 independent experiments. For A) hGSTA1 driven CAT expression was used, whereas in B) we show the activity of both promoters upon HBx expression. C) and D) show CAT activity with and without oltipraz treatment following HBx expression or transfection with the negative control.
FIGURE 3 (Continued)
The mechanism of induction of phase II detoxifying enzymes by oltipraz is disputable, and therefore, not yet defined. Oltipraz clearly transactivates these genes through an effect on their promoters, however, some data suggest that this effect depends on an ARE (AP1-like element) sequence, while others support a rate limiting role for the NF-κB motif (54). To examine their involvement in GST alpha induction by oltipraz, we prepared a series of 5'-deletion constructs for the rGSTA5 promoter (Fig. 4A). Elimination of both NF-κB and ARE consensus elements do not affect the induction of the rGSTA5 promoter (Fig. 4B), suggesting that oltipraz does not require the presence of either NF-κB or AP1 transcription factors.

**Effect of HBx on the Endogenous Expression of GST Alpha Proteins**—Due to a low transfection efficiency using lipid complexes and the abundance of GST alpha in liver cells, a decrease in the endogenous levels of GST alpha was difficult to prove in the whole cell population. To overcome this limitation, we subcloned the HBx gene into a bicistronic retroviral vector expressing the Green Fluorescent Protein (GFP), which serves as a marker for retroviral expression. Following HepG2 cell transduction, 81% and 77% of the cells were positive for GFP in cells transduced with AP2 and AP2-HBx retroviral particles, respectively. As a positive control, to demonstrate that the HBx protein produced following retroviral expression was functional, we evaluated its transactivation capacity on regulatory elements known to be transactivated by HBx such as AP1 or NF-κB. For this purpose, HepG2 cells transduced with AP2 or AP2-HBx retroviral particles were transfected with two different reporter plasmids, containing either AP1 or NF-κB regulatory elements. CAT activity was fourfold higher in cells transduced with AP2-HBx than in cells transduced with the negative control
(unpublished data). These results confirmed that the HBx protein produced was functional.

FIGURE 4. Effect of HBx and oltipraz on the rGSTA5 promoter. A) Schematic representation of the rGSTA5 promoter constructs. Known responsive elements are shown. B) Promoter activity of the rGSTA5 deletion constructs. HepG2 cells were transfected with 3 μg of each rGSTA5 promoter construct (control), or co-transfected with 3 μg of pCMV-HBx expression vector (HBx). Twenty four hours later, cells were treated with 45 mM of oltipraz (Olz and HBx + Olz, respectively). The transfection efficiency was normalized by cotransfection with the plasmid containing β-galactosidase gene. C) ( Annex) Promoter activity of the rGSTA5/-175.CAT construct. The experimental condition is similar to that in panel B. This experiment demonstrates that basal expression, oltipraz induction, and HBx inhibition of rGSTA5 depends on the promoter sequences within 175 bp upstream of transcription start site. Each bar corresponds to the average ± SD for at least 3 independent experiments.
FIGURE 4 (Continued)

B

rGSTA5 deletion constructs

C

Treatment

FIGURE 4 (Continued)
In order to determine the effect of HBx on endogenous GST alpha protein, we examined its expression on HepG2 following cell transduction with AP2 or AP2-HBx retroviral particles. Compared to control cells, expression of HBx down-regulates the endogenous expression of hGSTA1 (Fig. 5A), while no significant differences were observed between HepG2-AP2 transduced and HepG2 non-transduced cells (data not shown). To confirm that this effect is not cell restricted, CCL13 (Chang) cells were transduced following the same protocol. The levels of hGSTA1 were also reduced in CCL13 cells (Fig. 5B).

The transcriptional regulation of endogenous GST alpha protein by HBx was confirmed by semiquantitative RT-PCR on HepG2 transduced cells. Cells expressing HBx show lower level of hGSTA1 mRNA compared to cells transduced with the negative control (Fig. 5C). When HepG2 cells were treated with oltipraz, the hGSTA1 mRNA levels increased even upon expression of HBx (Fig. 5C).

Involvement of Sp1 Transcription Factor and p53 on the Regulation of GST Alpha by HBx—Although no previous report exists on the involvement of Sp1 transcription factor and the tumor suppressor protein p53 on the regulation of the hGSTA1 and rGSTA5 promoter activity, HBx has been shown to interact with both proteins. Conversely, since both promoters contain Sp1 responsive elements (Fig. 6A), we further investigated if the observed transcriptional down-regulation was linked to HBx interaction with Sp1.

Transient transfection experiments on HepG2 cells using the rGSTA5 deletion constructs suggest that Sp1 is not involved in the down-regulation of GST alpha by HBx protein (Fig. 4B). To confirm our findings, we used the Drosophila SL2 cells in the next transfection experiments. The Drosophila SL2 cells provide an Sp1 deficient background
to assess the activity of both promoters in the absence of Sp1. Both hGSTA1 and rGSTA5 promoters are active in this cell line. Furthermore, HBx decreased CAT activity of both hGSTA1 and rGSTA5 promoters in these cells, suggesting that the previously described HBx-Sp1 interaction is not the mechanism involved in GST alpha transcriptional down-regulation (Fig. 6B). Since these cells do not express human wt p53, we can also infer that hGSTA1 and rGSTA5 promoter down-regulation by HBx does not depend on p53 status in the cells. The absence of p53 consensus sequence on both promoters provide another support for p53 independent regulation of the GST alpha by HBx.
FIGURE 5. GST alpha expression in cells expressing HBx. Panel A and B are Western blots for hGSTA1 expression of whole cell extracts from HepG2 and CCL13 cells, respectively, that were transduced with AP2-HBx or AP2 retroviral particles as described in “Experimental Procedures”. Panel C shows the results of a semiquantitative RT-PCR for hGSTA1 mRNA with and without oltipraz treatment.
FIGURE 6. GST promoter activity in SL2 cells. A) Several regulatory elements important for GST expression in both human and rat promoters are depicted. B) Two reporter plasmids containing either hGSTA1- or rGSTA5-driven CAT expression were used. The expression vector for HBx (pCMV-HBx) or the empty vector (pRc-CMV) was cotransfected with either hGSTA1-CAT or rGSTA5-CAT. CAT activity for either promoter is relative to pRc-CMV. 100% CAT activity represents the percentage of acetylated to non-acetylated metabolite obtained after transfection with pRc-CMV. Each bar corresponds to the average ± SD for at least three independent experiments.
Analysis of the proteins binding to the proximal promoter region of the rGSTA5 gene—As demonstrated above through the use of deletion constructs, transactivation of rGSTA5 gene by oltipraz does not require either ARE or NF-κB transcription factors. Interestingly, this data reveals that basal expression, oltipraz induction, as well as HBx repression of rGSTA5 depends on the promoter sequences within 175 bp upstream of transcription start site (Fig. 4C). Electrophoretic mobility shift assay was then performed using the promoter fragment (-175 to +192) which is shown by CAT assays to be both responsive to the oltipraz transactivation as well as HBx inhibition. The binding assay was performed using 10 μg of whole cell extracts, and 0.2 ng of the indicated 32P-labeled probe (nucleotides -175 to +192). Competition analysis revealed that addition of unlabeled excess DNA from fragment 1 (-175 to +192), fragment 3 (-82 to +192), and fragment 4 (-82 to +7) compete with the retarded band (see arrow in Fig. 7A).

This observation prompted us to use the fragment 4 (-82 to +7) as the radiolabeled probe in the following gel shift assay (Fig. 7B). In addition to HepG2, whole cell extracts from untreated and oltipraz-treated HepG2/HBV cells were also used in this experiment. The bands that were competed with unlabeled specific DNA are considered retarded bands. This experiment identified two retarded bands, designated as I and II, using fragment 4 as probe. (Fig. 7B). In the whole cell extracts from the oltipraz-treated HepG2 cells, the amount of bound protein (band II) appears to be higher than the untreated HepG2 cell. The upper DNA binding protein (termed as I) is clearly inhibited to bind to the rGSTA5 promoter fragment (-82 to +7) in the whole cell extracts from the HepG2/HBV cells. Similar result was obtained when the whole cell extracts from HBx-transfected HepG2 cells were used in the experiment (data not shown). As is clear in the Fig. 7B, oltipraz treatment of HepG2/HBV does not restore binding of the inhibited protein to the rGSTA5 promoter.
Taken together, these results suggest that there are two DNA-protein complexes formed via interactions between the transcription factors and the regulatory sites within the rGSTA5 core region (-82 to +7) that produce a repressive activity by the HBx protein, and transactivation by the chemopreventive drug oltipraz.
FIGURE 7 (Annex). Electrophoretic mobility shift assay. A) Whole cell extracts prepared from untreated and oltipraz (OLZ)-treated HepG2 cells were incubated with 32P-labeled PCR fragment of rGSTAS promoter encompassing nucleotides -175 to +192. Retarded bands were separated by polyacrylamide gel electrophoresis. Various PCR-produced fragments of this region were added as competitor DNA to the binding reactions. The retarded band inhibited by the fragments number 1, 3, and 4, respectively (see lower panel for their location).

B) Experiment similar to that in panel A, except that promoter region encompassing -82 to +7 (fragment 4) was used as radiolabeled probe. In addition to HepG2, whole cell extracts from untreated and OLZ-treated HepG2/HBV cells were also used in this experiment.
DISCUSSION

An emerging theme among DNA tumor viruses is that viral encoded oncoproteins interact specifically with critical cellular regulatory proteins, and that the oncogenic effects of these viruses are at least in part, a consequence of these specific interactions. Published data indicates that HBV itself is not a transforming virus, and thus it must act in some way to enhance the transforming capacity of other factors such as environmental toxins. Indeed, HCC in particular, occur more frequently in livers that have been damaged by both chemical toxins and HBV (6-8), but the mechanism of this synergism is not known. The recent observation that very high levels of DNA damage accumulate in hepatocytes of transgenic mice with HBV, suggests that antioxidant and DNA repair mechanisms are sub-optimal (9). In this regard, the organic peroxidase activity of GST alpha in liver is highly significant and critical. In most animal models of hepatocarcinogenesis there is the early appearance of pi class GST by neoplastic hepatocytes not present in normal adult liver cells. In humans, however, GST pi expression has not been found during hepatic neoplasia. On the other hand, much more variability exists in the expression of both alpha and mu class isozymes. The concentration of alpha class GST, the predominant isozyme found in normal liver, decreases dramatically in malignant hepatocytes (44, 45) and in HCC from HBV infected individuals (55). A reduction in alpha and mu class GST is seen in tumor as compared with normal tissues from kidney and breast (56,57).

HepG2 cells are moderately differentiated and produce a spectrum of normal hepatocyte proteins and the pattern of expression of GSTs is similar to normal adult liver (58). Although these cells express α-feto protein, they are not tumorigenic in nude mice. We recognize that the oncogenic potential of HBV cannot be determined in HepG2 cells which are derived from hepatoblastoma. However, the HBV transfected cells can be utilized to test the effects of various xenobiotics given the observation that many are detoxified by GST alpha enzymes (59, 60). We have shown that HBV transfected HepG2
cells have decreased GST alpha subunit levels concomitant with depressed GST and glutathione peroxidase activity and are hypersensitive to the effects of DNA alkylating agents such as melphalan, BCNU and cisplatin. We have found an increased sensitivity to AFB1 and B(a)P cytotoxicity using these assays, although not strong (unpublished data), probably because of the fact that these carcinogens require metabolic activation to a cytotoxic species by cytochrome P450 enzymes. Furthermore, the effects of decreased GST alpha may be more subtle regarding carcinogenicity than is obvious using a less sensitive cytotoxicity assay. Indeed, one study has provided evidence that expression of GST alpha can protect differentially against genotoxic and potentially mutagenic effects without necessarily affecting the cytotoxicity of electrophiles. The susceptibility of HBV infected cells to environmental toxins may be considerable, since it has been demonstrated that slight increases in alpha class isozymes are able to decrease DNA-adduct formation by 90% in AFB1 treated cells (61). Administration of AFB1 to woodchucks with viral hepatitis was shown to result in a significantly earlier appearance of hepatocellular neoplasm and a higher incidence of HCC compared to viral carriers not treated with AFB1 (62). In this model, the similarity of preneoplastic foci after both viral and chemical (AFB1) exposure suggest common underlying molecular mechanisms for carcinogenic development where GST alpha may be implicated. Finally, expression of HBx in the liver cell line CCLI3 sensitizes cells to carcinogens normally detoxified by GST enzymes (63). In addition, hepatocytes from adjacent areas of fibrosis and inflammation in sections of liver infected with HBV have shown a marked increase of cytochrome P450 (64), thus increasing the susceptibility to AFB1 genotoxicity.

The regulation of GST genes have been examined in several different models. There is evidence of altered GST pi half life and its mRNA in chemically treated tumor cells (65). Increased rates of transcription have also been demonstrated as a mechanism to regulate the GST alpha gene product (61). Hypermethylation of the GST pi promoter was shown as a mechanism to down-regulate pi in human prostate cancer (65). We have
provided evidence that GST expression in HBV transfected HepG2 cells is regulated transcriptionally.

The decrease in GST activity correlates with diminished levels of alpha class and to a lesser extent mu class, and suggests that propagation of HBV may be more favorable under these conditions. Interestingly, after SV40 infection of human fibroblasts, GST activity was found to decrease initially and then return to normal levels after passaging (66). We have shown here, that the presence of an HBV genome may functionally compromise liver cells by significantly reducing the detoxification potential offered by GST alpha. This change might favor the likelihood of increased susceptibility to carcinogenic development not only by exogenous chemicals but also by endogenous toxins normally metabolized by GST. Our data suggest that the interaction of HBV gene products with detoxifying enzymes may contribute to the synergistic effect of HBV and chemical carcinogens in the development of liver carcinogenesis.

Although we have not compared GST alpha protein structure from transfected and untransfected cells, amino acid sequencing of GST alpha from HepG2 and normal liver demonstrated that they were in fact identical (67). Cycloheximide inhibition of de-novo GST alpha synthesis, however, failed to show any difference between the rates of degradation in the two cell lines studied over the course of 12 hours. Exposure for greater than 12 hours was not performed since it has been previously demonstrated that cycloheximide degradation occurs after this time (68). If in fact a difference in turnover time exists between the protein in transfected versus untransfected cells, detection of this difference should have been apparent within 12 hours. Semiquantitative RT-PCR clearly shows that the reduced protein levels are due to a decreased transcription of the hGSTA1 gene. Moreover, treatment with oltipraz induces transcription of hGSTA1 in both HepG2 and HepG2/HBV cells, partially overcoming the viral effect on mRNA levels.

Three highly related hepadnaviruses, the human hepatitis B virus, the woodchuck hepatitis virus and the ground squirrel hepatitis virus cause liver cancer in their hosts (5).
Although there is still a poor understanding of the mechanisms that associate viral infection to carcinogenesis, it is striking that the three mammalian hepadnaviruses share the regulatory gene \( x \), while no counterpart of this gene is found in the non oncogenic duck hepatitis B virus. Many different biological properties have been ascribed to this \(~17\) kDa protein. It has been reported that HBx affects transcription, signal transduction, DNA repair, cell cycle control and apoptosis. One of the best documented activities of HBx is the transcriptional transactivation of a wide range of cellular promoters cellular genes such as interleukin 6, tumor necrosis factor alpha and transforming growth factor beta 1 (69). Whereas directly or indirectly, HBx associates with several transcription factors resulting in promoter transactivation, this activity may also interfere with the regular expression of other genes.

We report in this study that following HBx expression, a down-regulation of hGSTA1 and rGSTA5 promoter activities were observed. Both the human and rat homologue promoter are equally affected by this viral protein, strongly suggesting common conserved regulatory elements in their gene structure. Reduced levels of GST alpha protein were confirmed in HepG2 and CCL13 liver cell lines transduced with the AP2-HBx retroviral particles. The effect of HBx on hGSTA1 transcription was confirmed utilizing semiquantitative RT-PCR on HepG2 cells and upon HBx expression. Interestingly, when HepG2 cells expressing HBx were treated with oltipraz, the levels of hGSTA1 mRNA increased, overcoming in great proportion the effect of HBx. Several mechanisms may explain the protective effects attributable to oltipraz with respect to viral infection and chemical carcinogenesis. Inhibition of phase I enzymes, induction of phase II xenobiotic metabolizing enzymes, regulation of oxygen reactive metabolites and enhancement of DNA repair processes are known properties of oltipraz (33-41). Oltipraz was shown to have potent inhibitory activity against the reverse transcriptase of HIV and to inhibit HBV transcription through elevation of p53 protein (70). In this study we report that oltipraz overcomes the HBx transcriptional down-regulation of hGSTA1 thus,
adding strength to the case for testing oltipraz treatment in HBV infection. Adjacent areas of fibrosis and inflammation in sections of liver infected with HBV have shown a marked increase of cytochrome P450 (64). Interestingly, oltipraz decreases P450 expression (33). However, these experiments were performed independently.

The regulation of both hGSTA1 and rGSTA5 promoter activities has had limited study. Since decreased levels of the corresponding metabolizing enzymes have been shown to be decreased in neoplastic processes (65), we further investigated the involvement of two possible regulatory factors in down-regulation of GST alpha promoters by HBx. Promoter deletion studies as well as transfection in Drosophila SL2 cells clearly shows that this effect is independent of HBx indirect association with Sp1 (71). Although neither hGSTA1 nor rGSTA5 promoters contain any known p53 responsive element, because of the known interaction between HBx and p53 the contribution of this association on GST alpha transcriptional down-regulation by HBx could be also considered. The participation of the HBx/p53 association cannot be involved as mediating HBx transcriptional down-regulation of these enzymes, since the Drosophila SL2 cells do not express human wt p53. We did find that expression of Sp1 in these cells strongly stimulates CAT activity from both promoters while expression of human wt p53 protein does not modify it (unpublished data). Neither Sp1 nor p53 expression modifies the effect of HBx on GST alpha. Interestingly, the GSHPX enzyme which catalyses Se-dependent enzymatic reactions is strongly transactivated by the p53 tumor suppressor protein (72).

Since expression of the GST family of proteins is affected in HBV and HBx transfected cells, two different mechanisms seem to regulate the activity of phase II metabolizing enzymes during this process. One of them is probably relates to the oxidative damage induced by the surface/envelope viral protein, while the other is directly related to transcriptional modulation by HBx. We have shown that the use of oltipraz overcomes the effect of both HBV mechanisms in the regulation of GST
isozyms expression. The mechanism of action of oltipraz at the molecular level is controversial and still unidentified. In this study, we presented evidence that the transcriptional induction of the GST alpha genes by oltipraz is mediated through responsive elements distinct from NF-κB or AP1 transcription factors.

The fact that a variety of cell defense mechanisms are modified during HBV expression, and that oltipraz can at least partly overcome the effect on GST alpha, strengthens the case for studying the use of oltipraz as a chemopreventive agent in hepatocellular carcinoma.

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REFERENCES


Chapter 6

GENERAL DISCUSSION

The results presented in this thesis have already been extensively discussed in their respective chapters. Chapter 6 includes a resume of the discussions presented in chapters 2 to 5 including the latest developments in the field of GSTs in respect to drug resistance and chemoprotection. Some examples of future experiments that might be necessary for further characterization of the GST alpha are included in this chapter. A hypothetical model for transcriptional regulation of GST alpha by HBx and oltipraz is also proposed here.

1. ROLE OF GST IN ANTICANCER DRUG RESISTANCE

The emergence of antineoplastic drug resistance is a major cause for failure of cancer treatment by chemotherapy. In many instances, the biochemical and molecular basis for the development of resistance is still unclear. Alkylating agents are widely used for treatment of a variety of tumors, and resistance to these drugs is common. Thus, understanding the mechanism of resistance of the tumor cells to these agents is important for successful chemotherapy. GSTs, particularly GST alpha, are believed to play an important role in the cellular resistance to alkylating agents. There have been several studies reporting the overexpression and enhanced activity of the GST alpha in alkylator resistant tumor cells (Alaoui-Jamali et al. 1992; Ban et al. 1996).
To examine the potential involvement of GSTs in alkylating drug resistance of tumor cells, our laboratory previously reported the isolation of a drug resistant rat mammary carcinoma cell-line (MatB) by exposing cells to increasing concentrations of the alkylating agent melphalan (MLNr MatB). The in vitro and in vivo studies in our laboratory demonstrated increased GST activity, especially elevated GSTA3 subunit in MLNr cells (Schecter et al. 1991). The introduction of the rat liver GSTA3 cDNA (clone pGTB42) into WT MatB cells was reported to confer several fold resistance to melphalan (Schecter et al. 1991). Nuclear run-on experiments showed that the induced GSTA3 mRNA in MLNr cells was due to transcriptional activation (Schecter et al. 1991).

In present study, I demonstrated that only rGSTA3 mRNA is overexpressed in melphalan resistant cell-line and found that it is identical to the previously described GSTA3 mRNA (designated GST Yc, in the past) with no additional GST alpha being induced by melphalan. To further study the role of the rat GSTA3 gene in chemotherapy resistance, and understand the mechanism of its overexpression in drug resistant tumors, I isolated the entire rGSTA3 subunit gene, including its regulatory regions, from a genomic library and characterized it. The rGSTA3 gene is approximately 15 kb in length and consists of seven exons interrupted by 6 introns of different lengths. The functional activity of its promoter was shown by its ability to drive the expression of a CAT reporter gene in MatB cells, and its activity was greater in melphalan resistant cells. Several potential binding sites for transcription factors such as ARE, AP-1, NF-κB, Barbie box, CREB, RRE, E box were found in the 5'-flanking region of the rGSTA3 gene. Their possible functions remain to be tested. The availability of the rGSTA3 genomic clones and the complete sequences of its regulatory regions make it now feasible to initiate detailed investigation of the molecular mechanisms of anticancer drug resistance in GSH and GST dependent pathway. This study also provides new insights.
into the structural and functional relationships among human and rat GST alpha class members.

In chapter 2, I demonstrated, by Northern blot analysis, that the endogenous expression of the rGSTA3 mRNA was abundant in the melphalan resistant cells and virtually undetectable in the WT cells. However, this result did not complement the results from the next study presented in chapter 3, where the isolated promoter from rat liver library was used in transient transfection of both WT and MLNr cells. Although the promoter activity was greater in drug resistant cells as compared to WT cells, this activity did not appear to be as drastic as endogenous mRNA observed in the previous study. One reason might be because the promoter sequences used to drive CAT reporter gene in these experiments were cloned from rat liver tissue. There might be subtle differences in the promoter sequences of the WT and melphalan resistant cells that causes increased overexpression of the resistant cells. Thus, sequence analysis of the entire promoter region of the rGSTA3 gene from the resistant cell-line might clear the altered expression of the gene.

In chapter 4, I presented evidence for gene duplication within the alpha class of the GSTs in rats. I reported a remarkable sequence identity between rGSTA3 and rGSTA5 which extends beyond their coding regions. These data suggest that the rGSTA3 subunit evolved as a duplication of the rGSTA5 subunit gene increasing the range of catalytic activity within this class and affording further protection to deleterious agents.

2. ROLE OF GSTs IN HEPATOCELLULAR CARCINOGENESIS

In addition to drug resistance, I have been examining the role of GST in carcinogenesis. Several studies have demonstrated the association of HBV infection and exposure to chemical carcinogens with the development of HCC, but the mechanism of this synergism is not known. Published data indicates that HBV itself is not a
transforming virus, and thus it must act in some way to enhance the transforming capacity of other factors such as environmental toxins. Reduced GST activity could enhance sensitivity of cells to chemical carcinogens. Thus, we examined the status of GST family of detoxifying enzymes in hepatocellular HepG2 and in an HBV-transfected subline. As discussed in Chapter 2, we have found reduced enzyme activity that correlated with decreased expression levels of human GST alpha protein in HBV transfected cells. Since a reduced amount of GST alpha mRNA was detected in transfected cells, we reasoned that the regulation of the GST alpha genes by HBV might be at RNA level. Because the x protein of HBV appears to play a role in HBV transfection, we showed decreased CAT activity upon HBx expression, by transient transfection of HepG2 cells using both rat and human GST alpha (rGSTA5 and hGSTA1) promoters. This supports a transcriptional regulation of both genes by HBx. Treatment of HBV expressing cells with oltipraz, an inducer of GST alpha, was shown to partially overcome this effect, as was anticipated on the basis of reported data. The mechanism of induction of phase II detoxifying enzymes by oltipraz is still controversial. Oltipraz clearly transactivates these genes through an effect on their promoters, however, some data suggest that this effect depends on an ARE (AP1-like element) sequence, while others support a rate limiting role for the NF-κB motif. To study induction of GST alpha by oltipraz, we prepared a series of 5' -deletion constructs for the rGSTA5 promoter. Removal of both NF-κB and ARE consensus elements did not affect the induction of the rGSTA5 promoter, suggesting that oltipraz does not require the presence of either NF-κB or AP1 transcription factors.
3. A MODEL FOR TRANSCRIPTOINAL REGULATION OF rGSTA5 BY HBV AND OLTIPRAZ

Based on the data presented in chapter 5 of this thesis and some recent studies, both HBV transfection and oltipraz treatment modulate GST isoenzymes (Fig. 4). While cellular transfection by HBV, including its x protein (HBx) inhibit GST expression, the chemopreventive drug oltipraz partially overcomes the effect of HBx on GST expression.

The exact molecular mechanism of GST regulation by HBV and oltipraz remains to be determined. Evidence shows that both HBV and oltipraz exert their effects on the promoter of many genes (Mahe et al. 1991; Yao et al. 1995. The X gene product has been known to transactivate several viral and cellular genes (Spandau et al. 1998). However, our data suggest a novel function for HBx protein. We demonstrated that HBx is involved in transcriptional down-regulation of both human and rat GST alpha subunit genes. Promoter deletion studies indicate that HBx protein interferes with GST expression within 175 bp upstream of transcription start site (Fig. 4C). Electrophoretic mobility shift assays demonstrated that HBx had an inhibitory effect on the transcription of rGSTA5 and exerted its effect through interaction within the promoter region, 82 bp upstream from the initiation site of for rGSTA5 (Fig. 7B). Oltipraz, on the other hand, has been shown in this thesis and a number of other studies to induce phase II detoxifying enzymes including both rat and human GST alpha genes. The mechanism of induction of phase II genes by oltipraz appears to be controversial. Previous data suggested the involvement of NF-κB or ARE elements (Yao et al. 1995), but our results clearly show that these transcription factors are not involved in GST alpha induction by oltipraz. Both promoter deletion studies as well as gel shift assays show that oltipraz induction of rGSTA5 gene depends on promoter sequences within 82 bp upstream of the transcription start site (Fig. 4C and Fig. 7 of chapter 5). In addition, the absence of an
ARE consensus sequence in the promoter of human GSTA1 gene provide further support for ARE-independent regulation of the GST alpha by oltipraz.

Thus, based on the evidence presented in this thesis, a possible model is proposed to explain how HBx and oltipraz mediate transcriptional regulation of rGSTA5 (Fig. 1). We demonstrated that both HBx and oltipraz exert their antagonistic effect on promoter sequences 82 bp upstream of transcription initiation site of the rGSTA5 gene. This region consists of TATA box and therefore is the recognition site for RNA polymerase II (Pol II). A transcription initiation complex is formed which includes TFIID (TATA-binding protein [TBP] and associated factors such as TFIIB, TFII F, TFIIH, TFII E and TFII A (Pugh 1996). This controls the basal expression of the gene. The formation of complex is initiated by binding TFIID to the TATA box of the promoter, followed by interaction with other members of the complex and Pol II.

HBx seems to display its inhibitory action on GST expression through either displacing TFIID (by interrupting TBP and TAF) from the promoter, or by blocking the interactions between TFIID and the other members of the initiation complex. Our gel shift assays support the former scenario, since DNA binding proteins are drastically reduced in HBx-transfected HepG2 and in HepG2/HBV cells (Fig. 7B of chapter 5). Indeed, many inhibitors of gene transcription have been shown to act through the above two pathways (Auble et al. 1993). According to our hypothesis, oltipraz appears to act either directly or through a mediator protein (OMP), interacting with TFII A of the initiation complex. TFII A presumably acts through direct interaction with TBP (Kobayashi et al. 1995; Pugh 1996). Oltipraz induction of GST alpha genes does not seem to be so much due to an increased amount of protein(s) that binds to the promoter, but as a result of a conformation change in one of the basal factors of the initiation complex such as TFIIA.
FIGURE 1. A possible model for transcriptional regulation of GST alpha by HBx protein of HBV virus and the chemopreventive drug oltipraz. A) Basal expression, B) HBx-inhibition, C) Oltipraz (OLZ)-transactivation. Bold line represents the GST promoter; Bent arrow shows transcription start site; Star sign indicates protein-protein interruption; PolII, RNA polymerase II; TBP, TATA binding protein; TAF, TBP-associated factors; A, TFIIA; B, TFIIB; F, TFIIF.
4. CONCLUDING REMARKS

GSTs are an important family of phase II detoxifying enzymes that have been implicated in complex processes such as anticancer drug resistance as well as carcinogenesis. The isolation and characterization of the overexpressed GST mRNA species from alkylator resistant cells has opened the door for a better understanding of the molecular basis of antineoplastic drug resistance. In addition, more work needs to be performed to elucidate the regulatory elements that control modulation of GST isoenzymes by viral HBV infection and the chemopreventive drug oltipraz.
REFERENCES


CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

The work presented in this thesis has focused on molecular characterization of GST alpha subunit genes and their roles in drug resistance and chemoprotection. The major contributions to original knowledge are summarized below:

1. I showed that rGSTA3 mRNA is overexpressed in a melphalan resistant cell-line and found that it is identical to the previously described GSTA3 mRNA with no additional GST alpha being induced by melphalan.

2. To study the role of GSTA3 subunit gene in drug resistance, and to understand the mechanism of overexpression of this gene in drug resistant tumors, the entire rGSTA3 gene, including its regulatory region, was isolated from a genomic library and characterized for the first time.

3. I also presented evidence that the rGSTA3 subunit evolved as a duplication of the rGSTA5 subunit gene, increasing diversification and functional benefits.

4. I demonstrated that HBV/HBx down-regulates GST alpha expression and the chemopreventive drug oltipraz overcomes this effect. As well, I showed that novel regulatory elements, unlike those claimed previously, are involved in transcriptional regulation of GST alpha gene by HBV and oltipraz.