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Glutathione Transferases and Cancer

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ABSTRACT: The glutathione transferases, a family of multifunctional proteins, catalyze the glutathione conjugation reaction with electrophilic compounds biotransformed from xenobiotics, including carcinogens. In preneoplastic cells as well as neoplastic cells, specific molecular forms of glutathione tranferase are known to be expressed and have been known to participate in the mechanisms of their resistance to drugs. In this article, following a brief description of recently identified molecular forms, we review new findings regarding the respective molecular forms involved in carcinogenesis and anticancer drug resistance, with particular emphasis on Pi class forms in preneoplastic tissues. The rat Pi class form, GST-P (GST 7-7), is strongly expressed not only in hepatic foci and hepatomas, but also in initiated cells that occur at the very early stages of chemical hepatocarcinogenesis, and is regarded as one of the most reliable markers for preneoplastic lesions in the rat liver. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA)-responsive element-like sequences have been identified in upstream regions of the GST-P gene, and oncogene products *c*-jun and *c*-fos are suggested to activate the gene. The Pi-class forms possess unique enzymatic properties, including broad substrate specificity, glutathione peroxidase activity toward lipid hydroperoxides, low sensitivity to organic anion inhibitors, and high sensitivity to active oxygen species. The possible functions of Pi class glutathione transferases in neoplastic tissues and drug-resistant cells are discussed.

KEY WORDS: glutathione transferases, carcinogenesis, tumor markers, drug-metabolizing enzymes, induction, chemoprevention.

I. INTRODUCTION

Chemical carcinogenesis can be divided into several steps; the general consensus is that at least initiation, promotion, and progression stages can be distinguished.[1,2] The carcinogenic process is thought to be initiated by the covalent modification of DNA with electrophilic metabolites derived from carcinogens. The glutathione transferases (GSH transferases, GSTs; EC 2.5.1.18) are therefore important as a family of multifunctional proteins that act as enzymes and also as binding proteins in various detoxication processes (see reviews in References 3 and 4). Recent studies have, however, also shown that some glutathione and cysteine conjugates can be of toxicological concern.[5,6] Since GSH transferases catalyze the reaction of nucleophilic reduced glutathione (GSH) (glutathione conjugation reaction) with electrophilic compounds biotransformed from xenobiotics, including carcinogens, and also from endogenous substances,[7,8] some of them can act to prevent initiation of the carcinogenic process by inactivating or detoxifying electrophilic proximate or ultimate carcinogens.[9,10] During the promotion stage, preneoplastic cells are known to occur in all organs studied, and some of them show resistance to cytotoxic agents, including carcinogens. In these preneoplastic cells as well as neoplastic cells developing from some preneoplastic cells during the progression stage, specific molecular forms (isoenzymes) of GSH transferase are known to be expressed that have been known to participate in the mechanisms of their resistance to drugs.[11,12] Thus, GSH transferases have been investigated for elucidation of the mechanisms of (multi)drug resistance.[13,14] Furthermore, some of the forms such as rat GST 7-7 (P) and human GST-π have attracted attention as reliable preneoplastic or

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neoplastic marker enzymes, the detection of which facilitates analysis of carcinogenic processes and provides the basis for new methods of screening for carcinogens and carcinogenic modifiers. Moreover, their mechanisms of expression are current topics in relation to oncogene activation. Recent advances in the multiplicity of GSH transferases and their cDNA sequencing have been reviewed by other investigators.[5,15]

In this article, following a brief review of the molecular forms of GSH transferase, including newly described ones, with summaries of primary and gene structures and of enzymatic properties and functions, we review recent findings of GSH transferases acting for detoxication or activation (or stimulation) of individual chemical carcinogens and anticancer drugs; GSH transferases as markers for (pre)neoplastic tissues and their gene expression mechanisms, enzymatic properties, and possible functions in (pre)neoplastic tissues; role(s) of GSH transferases in the mechanisms of (multi)drug resistance; and, finally, GSH transferases as markers for susceptibility to cancer.

II. MOLECULAR FORMS OF GLUTATHIONE TRANSFERASE

A. Classification of GSH Transferases

Many molecular forms of GSH transferase have been identified from various organs in a variety of species (see reviews in References 3, 6, 12, and 15). In particular, rat, human, and mouse GSH transferases have been investigated extensively. Although particulate-bound GSH transferases are known, most of the purified forms are localized in the cytosol as homodimeric or heterodimeric proteins. The majority of known cytosolic forms can be purified by *S*-hexylglutathione-[16] or glutathione-bound Sepharose affinity chromatography,[17] followed by chromatofocusing, which allows separation of individual forms.[18] Other methods have also been utilized for purification (see reviews in References 12 and 19). Except for a few forms, most GSH transferases exhibit an activity toward 1-chloro-2,4-dinitrobenzene (CDNB). Each form can be defined by an isoelectric point, subunit molecular weight on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunological properties using the immunoblot method or the double immunodiffusion test, although final identification relies on amino acid sequencing.

Mannervik et al. demonstrated that the major isoenzymes of cytosolic GSH transferases from the rat, mouse, and man share common structural and catalytic properties, on the basis of which they proposed a species-independent classification of GSH transferases.[20] Isoenzymes from these species were grouped with respect to N-terminal amino acid sequences, substrate specificities, sensitivities to inhibitors, and immunological cross-reactivities into three (Alpha, Mu, and Pi) classes. A new class, Theta, which includes rat GST 5-5, 12-12, and possibly YrsYrs and also human GST-θ, has been added recently.[21,22] This classification is useful for comparative identification of new forms. As already described, cytosolic forms are homo- or heterodimers, which can be formed between the subunits belonging to the same class. Whole amino acid sequences of GSH transferase subunits obtained from base sequences of the cDNAs are supplying more precise information on homology or diversity among the subunits in the same and different species. Recent discoveries of several new forms in man and the mouse, and studies on their properties, have gradually disclosed more correspondence of respective forms among these three species, including the rat. Table 1 summarizes the possible correspondence of man and mouse GSH transferase forms to the respective rat forms, estimated from similarities in nucleotide or amino acid sequences and, in some forms for which nucleotide sequences are not available, from similarities in enzymatic properties, or from immunological cross-reactivity. Corresponding forms often, but not always, exhibit similar catalytic properties and distribution in particular organs of these species.

So far, four cDNAs encoding the rat subunit 3 (Yb_1) have been cloned from liver,[23,24] prostate,[25] and testis;[26] replacement in a few bases was observed between the coding region nucleotide sequences, although with conservation of amino acid sequences. The 5′-noncoding sequence of subunit 3 from the liver or testis demonstrates partial differences. Similar replace-

TABLE 1
Correspondence of Human and Mouse Glutathione Transferase Subunits to Rat Subunits

Class		Rat	Human	Mouse
Alpha	Subunit	1 Ya_1 (pGTR261)[42]/Ya_2[57] (pGTB38)[41]	B_1 (pGTH1,[30] pGST2-3[31])/B_2[144] (pGTH2)[427]	MI (genomic DNA)[101]
		2 (pGTB42)[43]	2[204]	
		8		
		10[64]		
Mu		3 (pGTA/C44,[23] pGTR200,[24] pGTR201[26])		MIII (pGT875,[33] pmGT10)[34]
		4 (pGTR187,[44] pGTA/C48)[45]	μ (pGTH4),[156] Ψ (pGTH411)[157]	7.1 (pmGT2)[34]
		6[39]	4[165]	
		9[37]		
		11[64]	5.2 (HTGT-6)[166]	
		Yb_4[50]		9.3 (pGT55)[33]
Pi		7 (pGP5)[46]	π (pGPi2)[27]	MII (pGM211)[219]
Theta		5		
		12[21]	θ[21]	
		Yrs[22]		

ments in a few bases have also been reported for human GST-π cDNAs cloned from the placenta,[27] a breast cancer cell line,[28] and lung,[29] for GST-α (subunit B_1) cDNAs from the liver[30,31] and kidney,[32] and for mouse MIII cDNAs from the liver[33] and a fibroblastic cell line.[34] It is not yet clear whether a few replacements in the nucleotide sequences of particular GSH transferase forms in different organs are due to alteration in sequences during the process of developmental organ formation from germ cells, or to interindividual differences. Available results, however, indicate that individual GSH transferase forms expressed in different organs possess identical primary structures.

B. GSH Transferases in the Rat

1. Primary Structure and Gene Structure

a. Molecular Forms

Rat GSH transferases have been investigated most extensively. Most of the rat forms are localized in the cytosol of various tissues, with the exception of one form purified from rat liver microsomal fractions.[35] At least 19 molecular forms of cytosolic GSH transferase and 14 different subunits have been identified from rat tissues, and new forms continue to be reported. These forms and their subunits are grouped in four classes: Alpha, Mu, Pi, and Theta (Table 1; see also our previous reviews in References 12 and 19). In the nomenclature proposed by Jakoby et al.,[36] rat GSH transferase subunits have been assigned a number that refers to the order in which they were isolated and characterized. One form (GST 6-6, a major form in the testis) named in this manner, however, was corrected to GST 6-9 after the finding that it is, in fact, a heterodimer, Yn_1Yn_2.[37] A homodimer, GST-Yn_1Yn_1 (6-6), with high leukotriene C_4 synthase activity was found in brain cytosol[38] and seems to be identical with Yb_3Yb_3, as deduced from cDNA analysis.[39,40] cDNAs of rat GSH transferase subunits 1 (Ya),[41,42] 2(Yc),[43] 3 (Yb_1),[23,24] 4 (Yb_2),[44,45] and 7 (Yp),[46,47] and genomic DNAs of subunits 1,[48] 3,[49] 4,[50] Yb_4,[50] and 7[51,52] have been cloned (Table 1; see also reviews in References 5, 12, and 15). Some cDNAs encoding subunits 1, 2, and 3 have been expressed in *Escherichia coli*, the respective recombinant forms exhibiting properties very similar to those of forms purified from the liver.[53–55]

The cDNA of the rat microsomal GSH transferase has also been cloned.[56] Within a class, protein-coding regions are highly homologous (70 to 80%), while the 5′- and 3′-untranslated regions are very divergent. Deduced amino acid sequences of cytosolic forms exhibit about 30% homology between subunits belonging to different classes. The amino acid sequence of microsomal GSH transferase is not homologous to those of any of the cytosolic forms so far reported. Subunits 1 and 8 are each separable into two distinct polypeptides by high-performance liquid chromatography.[6,57] Two Ya cDNAs had been isolated, pGTB38[41] and pGTR261.[42] Recently, Hayes et al. have isolated two Ya-type subunits, Ya_1 and Ya_2, and suggested that these subunits are encoded by pGTR261 and pGTB38, respectively.[57]

Pickett and colleagues have investigated the gene structure of subunit 1 (7 exons and 6 introns)[48] and the mechanisms of regulation of its expression.[58,59] Rushmore et al.[60] have identified three regulatory regions in the 5′-flanking sequence of the gene. The first region, located from −867 to −857, contributes to the maximum basal level of the gene; and the second, localized from −908 to −899, is the xenobiotic-response element (XRE); and the third, localized from −722 to −682 (antioxidant-responsive element), is important for antioxidant- or β-naphthoflavone-inducible expression.[61] The second element contains the XRE core sequence (5′-T-GCGTG-3′) found in the 5′-flanking region of the cytochrome P-450 IA1 gene. The nucleotide sequence of the XRE of the subunit 1 gene exhibits the imperfect palindrome structure illustrated below.

5′-CAGGCATGTTGCGTG-3′
GTCCGTACAACGCAC

Such a palindrome structure has been reported in the enhancer of the GST-P gene,[62] as will be described later. Paulson et al. have reported four nuclear protein factors possibly *trans*acting these elements: two factors similar to the constitutive hepatocyte nuclear factors, HNF1 and HNF4, *trans*act enhancer(s) involved in the basal and liver-specific expression of the gene. Other *trans*-acting factors for XRE and ARE are suggested to be involved in the inducible expression of subunit 1.[63]

In the Mu class, five subunits (3, 4, 6, 9, and 11[64]) have been isolated, and one subunit, Yb_4, was predicted from a genomic DNA sequence (Table 1).[50] In the Pi class, only subunit 7 has been reported. The mechanisms of regulation of GST-P (7-7) gene expression will be described later. The fourth class, Theta, recently introduced by Meyer et al.,[21] includes subunits 5, 12, and possibly the Yrs described by Hiratsuka et al.[22] In addition to these forms, an as yet unidentified form involved in the synthesis of leukotriene C_4, which catalyzes the glutathione conjugation of leukotriene A_4, has been reported to be present in membrane fractions of leukemia cells.[65] Prostaglandin-H to -D isomerase purified from the cytosol of rat spleen also exhibits glutathione transferase activity toward CDNB, but it differs immunologically from other known cytosolic GSH transferases.[66]

b. Posttranslational Modification

Many molecular forms of GSH transferase have been identified from various organs in the rat, and these are products of different genes. On the other hand, posttranslational modifications have been suggested to occur in several forms. *In vitro* experiments revealed that rat GSTs 1-1, 1-2, and 2-2 could be phosphorylated by protein kinase C[67] and that 3-3, 3-4, and 4-4 could be methylated, the reaction being stimulated with calmodulin.[68] The phosphorylated 1-1 exhibited a decreased affinity for bilirubin.[67] It is not clear, however, whether these modifications occur *in vivo*, and the biological significance of modifications remains to be clarified. Recent studies also suggest that rat GST 7-7 and human GST-π are glycosylated, and that glycosylation may be involved partly in the microheterogeneity of these subunits observed on isoelectric focusing.[69]

2. Gene Expression

a. Tissue Distribution

Each organ possesses a unique profile of GSH

transferase forms; the liver has the highest activity and number of molecular forms. Other organs share some, but not all, forms expressed in the liver and also specifically express particular forms. These include GST-Yn_1Yn_2 and 11-11 in the testis,[37,64] and 6-6(Yn_1Yn_1) in the brain.[38] Thus, the expression of GSH transferase subunits is, to a certain extent, tissue specific[70,71] (see reviews in References 4, 5, and 12). In the kidney, the Alpha class (subunits 1, 2, and 8) is abundantly expressed, while the Mu class (subunits 3 and 4) occurs at a low level. In the lung, subunits 2, 8, 3, and 4 are expressed, but subunit 1 is undetectable. In some tissues, one subunit predominates, e.g., subunit 7 in the small intestine or subunit 2 in the lactating mammary gland, adrenal gland, and spleen. Immunohistochemically, although subunits 1, 2, 3, and 4 are detectable in all hepatocytes in normal liver, they are more abundant around the central vein than in the periportal region.[72] Induction of GSH transferase isoenzymes by drugs is described in Section III.A.

Developmental changes in isoenzyme composition in the liver have been noted.[6,73] In late fetal liver, subunit 10 (Yfetus) together with subunit 2 is representative of the Alpha class having high Se-independent glutathione peroxidase activity.[64,74,75] Subunit 7 is not expressed in adult liver except in the bile ducts, but is expressed in fetal liver.[73]

b. Hormonal Regulation of GSH Transferase Expression

Sex differences in subunit composition in the liver have been described.[76,77] In males, subunits 3 and 4 in the Mu class are more abundant than in females. Many reports have revealed that various hormonal treatments effect an alteration in the expression of GSH transferase forms in rat organs. Ovariectomy followed by diethylstilbestrol administration resulted in a decrease of the Mu class subunit mRNAs, including those for subunit 6 and for subunits 2 and 7 in rat pituitary gland.[78] Androgens repressed the level of subunit 3 mRNA in rat prostate.[25] Hypophysectomized rats exhibited large increases in the level of subunit 4 in the adrenal gland, and lack of adrenocorticotropic hormone (ACTH) was apparently related to this increase.[79] Furthermore, subunit 1 mRNA was greatly reduced in the liver, but not in the kidney, of adrenalectomized rats.[80] Thus, effects of hormonal treatments on the expression of GSH transferases are organ specific and isoenzyme specific, suggesting that organ-specific expression of these forms may be regulated by the levels of hormones as well as other factors.

The profile of GSH transferase isoenzymes in cultured hepatocytes differs from that of adult liver. Subunit 1 is decreased and subunits 3 and 7 are increased in cultured hepatocytes, and these changes are also reflected in expression of the mRNAs.[81–84] Higher amounts of subunit 7 are induced by the addition of fetal calf serum to culture media. Expression of subunit 7 is also enhanced by insulin and/or epidermal growth factor,[85] but depressed by dexamethasone.[86]

Cultured rat brain C6 glioma cells showed increased GSH transferase activity in the G_2 and G_1 phases, and the GSH transferase inhibitors, ethacrynic acid and caffeic acid, inhibited growth of the cell line.[87] The growth of K562 cells was also inhibited by ethacrynic acid or bromosulfophthaleine.[88] Although depletion of glutathione content is known to correlate with growth inhibition, glutathione levels are increased in K562 cells treated with these inhibitors. These reports suggested an involvement of GSH transferase in cell growth. However, GSH transferase inhibitors per se did not show significant effects on the growth of many other cell lines.[89–92]

3. Enzymatic Properties and Functions

a. Functions

Glutathione transferases basically catalyze the glutathione conjugation reaction of electrophilic compounds, which are primarily produced from many exogenous xenobiotics by biotransformation,[6,8] but also arise from endogenous substances.[4,15,93] The glutathione conjugation reaction is the first step of the mercapturic acid pathway,[5,7,8,94] which is one of the most important detoxication processes. The second step of

this pathway is catalyzed by γ-glutamyltransferase, which has also been used as one of the markers for putative preneoplastic hepatic foci in the rat. Molecular forms involved in the metabolism of carcinogens are in Section III.A.

In addition to this conjugation reaction, some forms such as GSTs 1-2, 2-2, 5-5, and 7-7 are known to possess selenium-independent glutathione peroxidase activity toward lipid peroxides[95,96] and, in the case of GST 5-5, also toward DNA hydroperoxides[97] (see reviews in References 98 and 99). GST 8-8 has a high conjugation activity toward 4-hydroxynonenal, which is one of the most potent aldehyde products of lipid peroxidation.[100,101] Some cytosolic GSH transferases are also known to be involved in leukotriene (e.g., GST 6-6),[38] prostaglandin (GST 1-1),[102,103] and hepoxilin metabolism (4-4).[104] Ligandin (GSTs 1-1 and 1-2), and especially GST 1-1, has Δ^5-3-ketosteroid isomerase activity toward Δ^5-androstene-3,17-dione.[105]

Furthermore, ligandin and other forms, including GSTs 3-3 and 4-4, act as binding or carrier proteins for several dyes (bilirubin, bromosulfophthalein, and indocyanine green), cholic acid, steroid and thyroid hormones,[106-108] and hematin (heme).[109] Ligandin also binds carcinogens (azodyes and 3-methylcholanthrene) and leukotriene C_4[110,111] (see reviews in References 9, 112, and 113).

b. Structure of Active Site

With respect to catalytic sites, the GSH transferase enzyme possesses a binding site for GSH (G-site) and a hydrophobic binding site for electrophilic substrates (H-site) (see reviews in References 4 and 15). GSH transferase forms show significant activities toward a wide range of electrophiles with different chemical structures, but a high specificity toward glutathione. The H-site is estimated to be specific for each subunit, whereas the G-site may be common to all GSH transferase subunits. However, the *Km* values of the homodimers for GSH are different, and forms in the Mu class show significant activity toward glutathione derivatives, while the Alpha class forms do not,[114,115] suggesting that the glutathione structure requirement for expression of activity may differ between respective forms.

Many substances, including bilirubin, heme, bile acid, and steroid hormone, are not substrates of GSH transferases, but bind to the enzymes and thereby inhibit their activity uncompetitively or competitively[116-119] (see reviews in References 15 and 113). The binding of these substances results in conformational changes that seem to occur at locations other than the G- or H-sites of the enzymes.[120] Although the forms in the Pi class also bind dyes or bile acids, the associated inhibition of their activity is less than for other forms in other classes.[121]

Each subunit of all GSH transferase forms possesses a G-site and an H-site, and exhibits catalytic activity independent of the other subunit. A heterodimeric form shows intermediate activities toward many substrates between those of the respective homodimers. The sensitivities of heterodimers toward many inhibitors are also intermediate.[4,15] Amino acid modification studies have suggested that arginine, histidine, cysteine, and tryptophan residues are involved in the expression of activity.[122-125] However, the localization of active sites in the primary structure remains to be clarified.

After binding to the G-site of GSH transferase, GSH is converted to a thiolate anion (GS^-) by interaction with basic amino acid residues such as arginine or histidine in the vicinity of the G-site.[126,127] Recent studies using site-directed mutagenesis has revealed that the replacement of arginine residues of human GST-α with other amino acids results in significant alterations in *Km* values for GSH,[128] and replacement of the histidine residue by other amino acids at position 159 of the rat GST 1-1 also results in decreased activity. However, replacement of the histidine residue with asparagine does not change the activity, suggesting that histidine residues in 1-1 are not absolutely essential for catalytic activity.[129]

Photoaffinity labeling experiments utilizing *S*-(*p*-azidophenacyl)glutathione revealed that C-terminal sequences of rat subunits 1 and 2 incorporated the ligand, suggesting that C-terminal regions form a portion of the active site, although those regions do not possess arginine residues.[130] Furthermore, a mutant of the human Alpha class

form (B_1B_1), with 12 residues deleted from its C terminus, exhibits diminished specific activity, suggesting that the C-terminal segment may form a component of the H-site rather than of the G-site.[131] In the rat Mu-class forms, chimeric enzymes have been developed by substitution of small pieces (nine or eight amino acid residues) of N- or C-terminal domains from subunit 4 into the corresponding regions of subunit 3.[132] The kinetic properties of the chimeric enzymes are significantly different from those of the native enzyme, suggesting that the active site of the enzyme responds to modifications in both the N- and C-terminal domains of the polypeptide.

GSH transferase forms in the Pi class have in common a sensitivity to SH modifiers[124,133–137] and active oxygen metabolites.[125,134,138] SH modification of the 47th cysteine residue of rat GST-P with *N*-ethylmaleimide results in inactivation, and such a modified enzyme cannot bind to *S*-hexylglutathione-Sepharose,[133] suggesting that the vicinity of the 47th cysteine residue may constitute the G-site of the Pi-class forms. However, the residue itself is not essential for activity, since a mutant enzyme replaced with alanine still retains enzyme activity.[139] Since the involvement of GSH transferases in drug resistance has been indicated, information on the active sites of these enzymes will provide the basis for the development of specific inhibitors. This area is of obvious importance from the viewpoint of overcoming drug resistance. Several inhibitors have been shown to be promising for this purpose, as is described later. A comprehensive list of inhibitors is available in the review by Mannervik and Danielson.[15] The glutathione conjugate of tetrachloro-1,4-benzoquinone[122,123] and a γ-glutamyl-modified analogue of glutathione[140] have been reported to be specific inhibitors for the Mu-class GSH transferases.

C. GSH Transferases in Man

1. Primary Structure and Gene Structure

Human GSH transferases were divided into basic, neutral, and acidic groups. However, the recent discovery of several new forms makes this classification inadequate, and a new unifying nomenclature has been proposed.[141] In this nomenclature, human GSH transferases are divided into the Alpha, Mu, Pi, Theta, and Microsomal classes, and the subunits are numbered within the class using Arabic numerals. Thus, molecular forms are named by capital letter to indicate the class, followed by a combination of numerals to indicate the subunits. Human GSH transferase forms so far reported are summarized in Table 2 (see review in Reference 142). In the alpha class, five forms with pI values of 7.5 to 9.0 (α-ε) were resolved from human liver by Kamisaka et al.,[143] but the subunit structures were not clarified. Stockman et al. identified two basic homodimers and one heterodimer consisting of two subunits, B_1 and B_2, with the same molecular weights but different pIs.[144] These two subunits were also reported by Soma et al.[145] A basic form with a pI of 9.9 detected in the skin[146] and GST-ω with a pI of 5.0 are members of this class.[147]

Two GSH transferase forms have been expressed in *E. coli* from two cDNA clones, λGTH1 and λGTH2, encoding the Alpha class forms.[148] One form from λGTH2 shows catalytic activities similar to those of GST-B_2B_2,[149] suggesting that λGTH2 encodes the B_2 subunit. The deduced amino acid sequence from λGTH1 is identical to that from pGST 2-3,[31] which encodes the B_1 subunit.[150] These two subunits are different in 11 out of 221 amino acid residues and exhibit different activity toward various substrates, as described below. The subunit B_1 gene has been mapped at chromosome 6p12,[31] but the gene structures of these subunits remain to be studied.

Eleven molecular forms have been identified in the Mu class. GST-μ (pI 6.6), a homodimer with a subunit molecular weight of 27,000, which has been reported as a neutral form by Warholm et al.,[151,152] is present in about 60% of adult livers. GST-ψ, which is closely related to GST-μ in catalytic properties but more acidic (pi 5.5), has also been reported by Awasthi et al.[153,154] On the other hand, Board separated three GST bands by starch gel electrophoresis: GST 1-type 2, -type 2-1, and -type 1.[155] GST-μ and -ψ seem to correspond to GST 1-type 2 and -type 1, respectively, GST 1-type 2-1 being a heterodimer of GST-μ and -ψ. Two cDNAs coding the Mu class

TABLE 2
Molecular Forms of Human Glutathione Transferase

Class	Form	Subunit M_r	New name	Other name				
				Jakoby	Board	Sato	Hayes	Awasthi
Alpha	B_1B_1[144]	26,000	A1-1	ε[143]	2-type 1[155]	I[145]		
	B_1B_2		A1-2	δ		II		
	B_2B_2	26,000	A2-2	α-γ	2-type 2	IV		
	9.9[146]	28,500	A3-3					
	ω[147]	26,000						ω[147]
Mu	μ[151]	27,000	Ma1-1a		1-type 2	III, M_3M_3[163]	N_1N_1[164]	
	1-type 2-1[155]		M1a-1b					
	Ψ[153]	27,000	M1b-1b		1-type 1			
	4[159]	26,500	M2-2		4[159]	N_2N_2	N_2N_2	ζ[161]
	5[167]		M3-3		5[167]			
	M_1M_2[163]	27,000				M_1M_2		
	M_2N_1	27,000/26,500				M_2N_1		
	M_3N_2	27,000/26,500	M1a-2			M_3N_2	N_1N_2	
	5.2[166]							
	6[168]				6[168]			
	N_2N_3[164]						N_2N_3	
Pi	π[173]	24,500	P1-1	ρ[172]	3	V	λ	
Theta	θ[21]		T1-1					
Microsomal	Microsomal[180]	17,300	MIC					

forms have been cloned,[156,157] and their deduced amino acid sequences are different in only 1 of 217 residues, at position 172. Recombinant enzymes expressed in *E. coli* from two cDNA clones, pGTH4 and pGTH411, showed the same properties as GST-μ and -ψ, respectively,[158] indicating that one clone, pGTH411, encodes GST-ψ. The GST-4 noted in human muscle[159] is a homodimer with a subunit molecular weight of 26,500, slightly smaller than that of GST-μ.[160] This form was also noted as GST-ζ[161,162] and GST-N_2N_2.[163,164] The cDNA encoding GST-4 has been cloned recently.[165] A heterodimer consisting of GST-μ and -4 subunits has been reported.[163,164] GST-5.2 has been isolated from the testis and brain, and its cDNA clone was also reported by Campbell et al.[166] Its subunit molecular weight is larger than that of GST-μ. In the new nomenclature, this form is identified with GST-5 detected in the brain by electrophoresis.[159,167] However, there is no convincing evidence to support identity.[166] GST-5 reported by Laisney et al.[159] is rather similar to the heterodimer consisting of GST-μ and -4 subunits. Other forms belonging to this class are GST-M_1M_2 and -M_2N_1 isolated from the aorta,[163] GST-N_2N_3 from the skeletal muscle,[164] and GST-6 from the brain.[168] N-terminal amino acid sequences of these subunits have been reported, but information on their entire primary structures is not available yet. Thus, at least ten, apparently independent, subunits have been reported in this class. In addition, two genomic DNA clones have been reported[169] that do not seem to correspond to any of the subunits reported so far. Chromosome mapping revealed the GST-μ gene to be located at 1p31[156] and possibly other Mu-class form(s) at chromosome 3.[170] As described above, GST-μ is not expressed in about 40% of individuals, and hereditary differences in the expression of this form are due to deletion of the gene.[157,171]

GST-π, found to be very similar to the acidic GST-ρ isolated from erythrocytes,[172] was purified from the placenta,[173] and is the only form

of the Pi class demonstrated so far. The primary structure of this form has been deduced from cDNA,[27] and protein sequence analysis has confirmed this structure except that one residue at position 104 contains both valine and isoleucine,[174] whereas the deduced sequence identifies only isoleucine. A fatty acid ethyl ester synthetase isolated from myocardium has been shown to be a form of glutathione transferase similar to GST-π in isoelectric point and N-terminal amino acid sequence.[175] GST-π from the placenta, however, does not exhibit this activity,[176] suggesting the presence of another form belonging to this class. GST-π in various human organs has been suggested to exert slight differences in structure.[177] The GST-π gene has been mapped at chromosome 11q13,[178] and a two-allele PstI or EcoRI polymorphism noted.[179]

A novel form termed GST-θ has been isolated from liver cytosol. The form is different in catalytic properties and N-terminal sequence from all the cytosolic GSH transferase forms reported so far, thus constituting a fourth class.[21] From liver microsomes, one form with a subunit molecular weight of 17,300 has also been purified,[180] and its primary structure has been deduced from a cDNA.[56]

2. Gene Expression

a. Tissue Distribution

As described above, several forms of GSH transferase in the liver have been reported by many investigators, although in some cases the results differed considerably regarding expression of the various isoenzymes. Major forms of the liver are GST-B_1B_1, -B_1B_2, and -B_2B_2 as members of the Alpha class, and GST-μ. In addition to GST-μ, interindividual differences have also been noted in the amount of subunit B_2 and, to a lesser extent, in subunit B_1.[181] The distribution of GSH transferases has also been studied in extrahepatic organs, including the kidney,[182,183] lung,[184] brain,[185] skin,[146] intestine,[186] adrenal gland,[187] testis,[188] prostate,[189,190] uterus,[191] heart,[192] blood vessels,[163] and skeletal muscle,[162,164] These studies revealed the expression to be tissue specific.[159] GST-B_1B_1 and -B_2B_2 in Alpha class is expressed in the liver, kidney, and intestine, as is GST-2 in the Alpha class in the skin and uterus, while GST-π is expressed as a major form in many organs other than the adult liver. GST-μ levels are very low in the kidney, lung, and other organs. In considering the expression of GSH transferase forms in cancer tissues, amounts in normal tissues should be taken into account. Localization of GST-π within different organs has been examined by an immunohistochemical technique,[193,194] indicating its presence in luminal epithelia and other tissues.

b. Regulation of Gene Expression

The expression of GSH transferase forms is not only tissue specific, but also regulated by development. For example, GST-π is not expressed in adult liver except in biliary epithelium, but is expressed in fetal liver.[195,196] Developmental change in expression of the Alpha class form(s) as well as GST-π have also been noted in several tissues.[197–199] Clarification of the mechanism(s) of GSH transferase regulation is clearly important to an understanding of altered expression in cancer tissues. As to regulation of GST-π expression, a promoter with a TRE-like sequence as reported for the rat GST-P gene[52] has also been identified in the upstream region of the GST-π gene.[200,201] Unlike the rat GST-P gene, however, this promoter, located from −65 to −58, is not responsive to TPA or to transcriptional activator factors of the *jun* and *fos* family,[202] but is involved in basal expression.[203] Ketterer and colleagues have recently identified a downstream *cis*-acting element located from +8 to +72 that seems to be part of the promoter.[203] Factors *trans*acting on the gene remain to be identified.

3. Enzymatic Properties and Functions

The functions of the various human GSH transferases have not been defined as clearly as those of rat GST forms (see reviews in References 12 and 15). However, human forms are presumed to have activities similar to those of the equivalent rat forms. GST-ε (B_1B_1) possesses

Δ^5-androstene-3,17-dione isomerase[105] and prostaglandin $F_{2\alpha}$ reductase activities.[204] GST-B_2B_2 shows high peroxidase activity toward cumene hydroperoxide[149] as well as prostaglandin D_2 isomerase activity.[204] GSTs-α-ϵ (B_1B_1, B_1B_2, and B_2B_2) possess binding activity to bilirubin.[143,205] GST-μ exhibits high activity toward *trans*-stilbene oxide[206] and prostaglandin E_2 isomerase activity.[207] GST-M_1M_2 and -M_2N_1 purified from the aorta through glutathione-Sepharose have a high activity toward nitroglycerin.[163] Glutathione conjugation of benzo[*a*]pyrene derivatives is catalyzed by GST-μ[151] and GST-π.[208] GST-π is active toward ethacrynic acid and acrolein,[209] which is formed from the anticancer agent cyclophosphamide. GST-θ is a labile form that does not bind to *S*-hexyl-glutathione- or glutathione-Sepharose and has a high activity toward 1,2-epoxy-3-(*p*-nitrophenoxy)propane.[21] The microsomal GSH transferase is active toward 1-chloro-2,4-dinitrobenzene and cumene hydroperoxide, and the activity is enhanced by *N*-ethylmaleimide,[180] as reported earlier for the rat microsomal GSH transferase.[210] The human microsomal enzyme is also active toward a nephrocarcinogen, hexachlorobuta-1,3-diene.[180]

D. GSH Transferases in the Mouse and Other Species

1. Primary Structure and Gene Structure of Mouse GSH Transferases

So far, at least two, one, and four forms of mouse cytosolic GSH transferase have been identified in the Alpha, Pi, and Mu classes, respectively, as summarized in Table 3. The major forms in the liver, GSTs MI, MII, and MIII, are all homodimers with different molecular weights.[211,212] In the Alpha class, three different subunits, Ya_1, Ya_2, and Ya_3, have been isolated recently, a homodimer, Ya_3Ya_3, being a constitutive form in the liver and a heterodimer, Ya_1Ya_2, inducible by *t*-butylated hydroxyanisole (BHA).[213,214] Thus, Ya_3Ya_3 seems to be identical to the GST MI reported earlier. Ya_1Ya_2 may be identical to the form named GST-10.3 by Benson et al.[215] In this class, a cDNA clone (pGT41)[33] and another genomic DNA clone[101,216] have been reported. Although the exact relationship between these clones and the subunits described above has not yet been clarified, partial amino acid sequence analysis suggests that the genomic and cDNA clones may encode the Ya_1 and Ya_2 subunits, respectively.[214] Mouse Ya_1 and Ya_2 subunits have 95% amino acid sequence identity with rat subunit 1, while the Ya_3 subunit is more homologous (86%) to rat subunit 2 than to subunit 1.[214] Daniel and colleagues have studied the gene structure of the Alpha class subunit (Ya, possibly identical with Ya_1[214]) and identified a transcriptional regulation element (electrophile-responsive element), located between −754 and −713 from the start of transcription, which is responsible for induction by both planar aromatic compounds and electrophiles.[217]

In the Mu class, four cDNAs (pGT875, pGT55, pmGT10, and pmGT2) have been cloned.[33,34,218] Nucleotide sequence analysis revealed that pmGT10 and pmGT2 are highly homologous to rat subunit 3 and 4 cDNA, respectively.[34] cDNA pmGT10, isolated from a fibroblastic cell line, is nearly identical to pGT875 encoding the GST MIII in the liver, having only a single silent transition in the coding region and additional differences in the 3′-noncoding region. Homodimers encoded by pmGT10 and pmGT2 have been expressed in *E. coli* and also detected in the fibroblastic cell line. Like rat GST 3-4, a heterodimer of these subunits was also noted, having a pI value of 8.1. A BHA-inducible form, GST-9.3, which is encoded by pGT55, is also a member of this class.[33] The pGT55 shows a marked homology (95%) to the deduced amino acid sequence of the rat genomic Yb_4 gene.[34] The cDNA encoding GST MII belonging to the Pi class also has been cloned,[219] the deduced amino acid sequence sharing 92 and 85% identity with rat GST-P and human GST-π, respectively.

2. Gene Expression

a. Tissue Distribution

Molecular forms expressed in mouse liver differ considerably from those in rat liver. As already described, rat GST-P (7-7) is not expressed in adult liver, but GST MII related to rat

TABLE 3
Mouse Glutathione Transferase Forms

Class	Form	pI	Subunit Mr	cDNA	Origin
Alpha	MI[e 211,212]	9.7	25,500		Liver
	10.3[215]	10.3		pGT41 (25,387)[33]	BHA-administered liver
	Ya_1Ya_2[b 214]				
Mu	MIII[c 211,212]	8.5	27,000	pGT875 (25,825)[33] pmGT10 (25,801)[d 34]	Liver
	9.3[33]	9.3		pGT55 (25,570)[33]	BHA-administered liver
	—	7.1[34]		pmGT2 (25,548)[34]	Kidney, heart, lung
	—	8.1[34]		(heterodimer, pmGT10/pmGT2)[34]	
Pi	MII[e 211,212]	8.7	24,000	pGM211 (23,536)[219]	Liver

[a] Possibly identical to GST 10.6,[215] Ya_3Ya_3.[214]
[b] May be identical to GST 10.3.[215]
[c] Possibly identical to GST 8.7,[218] GST 8.8,[215] or YbYb.[213]
[d] No difference between pGT875[33] and pmGT10[34] in amino acid sequence, but one replacement in nucleotide sequence.
[e] Possibly identical to GST 9.0,[215] YfYf.[224]

GST-P is a major form in adult male mouse liver, and though present at significant levels as a minor form in adult female liver, is further induced by injection of testosterone to females or by administration of various drugs, including BHA.[212] On the other hand, following castration of male mice, the levels are reduced to the lower levels of female mice. Interestingly, GST MII has been reported to possess an isomerase activity toward Δ^5-androstene-3,17-dione, resulting in the formation of Δ^4-androstenedione, an intermediate of testosterone biosynthesis.[211] However, the isomerase activity of mouse GST MII is inhibited by glutathione, while the same reaction in the human GST-α or rat GST 1-1 cases is dependent on glutathione.[15] GST-7.1 encoded by pmGT2, a form equivalent to rat GST 4-4, is not detected in the liver, but is present in the kidney, heart, and lung.[34] Awasthi and colleagues have studied molecular forms in several organs, including the brain, heart, spleen, and muscle, indicating strict tissue-specific expression of the various forms.[220,221] Although activities of three major forms, GSTs MI, MII, and MIII, toward several substrates have been reported (see review in Reference 15), the catalytic properties of other forms remain to be studied.

b. Induction Mechanism

Antioxidants such as BHA and bisethylxanthogen induce mouse liver GSH transferase activity to a much higher extent than in rat liver. The major mouse forms induced by BHA have been identified as GST-9.3 in the Mu class[33] and Ya_1Ya_2 in the Alpha class, the latter being undetectable in normal liver. GST-Ya_3Ya_3, a constitutive form in normal liver, was not significantly induced.[214] However, other studies report that BHA induces GSH transferases (MI and MIII) expressed in normal mouse liver.[222,223] BHA also induces MII in female mouse liver, but not in male liver. β-Naphthoflavone, which is suggested to act through the *Ah* receptor, and phenobarbital are not as strong inducers as BHA, inducing GST-Ya_1Ya_2 and GST MIII to only slight extents.[222,224,225]

The transcriptional regulation element of the mouse Ya gene is reported to be responsible for its induction by *tert*-butylhydroquinone, dimethyl fumarate, or planar aromatic compounds, including β-naphthoflavone, 3-methylcholanthrene, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.[217] β-Naphthoflavone activates the gene through this element, named the electrophile-re-

sponsive element, but the presence of both *Ah* receptors and cytochrome P-450 is required for its transcriptional activation, while *t*-butylhydroquinone activates independently of *Ah* receptors or cytochrome P-450. The nucleotide sequence of this element is highly homologous (95%) to that of the antioxidant-response element of the rat subunit 1 gene.[61] The presence or absence of other regulatory elements analogous to the XRE of rat subunit 1 remains to be clarified in the mouse Ya gene. Lower induction of mouse GSH transferases by 3-methylcholanthrene and other drugs,[222,224,225] in comparison with induction in the rat, suggests that other regulation elements may be different between the two species. The involvement of *Ah* receptor in the induction of GST-Ya_1Ya_2 as well as GST MII by β-naphthoflavone has been supported from *in vivo* experiments utilizing female mice. These forms are not induced in DBA/2 mice that do not possess a functional *Ah* receptor.[214]

3. GSH Transferases in Other Species

Multimolecular forms have also been identified in other species, including monkey, bovine, hamster, guinea pig, dog (see review in Reference 19), and chick livers.[226] Dog liver has three major forms, all of which are immunologically related to rat GST-P (7-7).[227] One form has been purified from *E. coli*,[228] and a GSH transferase from *Tetrahymena thermophilia* has been reported to exist as a monomer.[229]

III. GLUTATHIONE TRANSFERASES IN CARCINOGENESIS

A. GSH Transferases for Prevention or Stimulation of Carcinogenesis in the Rat and Mouse

1. Carcinogen Metabolism by GSH Transferases

The covalent binding of electrophiles derived from carcinogens to macromolecules, especially DNA, has been considered as an initial step in chemical carcinogenesis. Such electrophilic compounds were shown to be detoxified by enzymatic or, in some cases, spontaneous conjugation with glutathione (see reviews in References 6 and 8). Recent studies revealed the involvement of specific GSH transferase forms in the conjugation of particular carcinogens. For example, rat GSTs 1-1, 4-4, and 7-7 have conjugation activity toward benzo[*a*]pyrene-7,8-diol-9,10-oxide,[230,231] conjugation by 4-4 and 7-7 being specific for the (+)-enantiomer. While 4-4 is specifically active toward (7R,8S)-(+)9,10-dihydrobenzo[*a*]pyrene-7,8-oxide, the 7-7 is active toward the (7S,8R)-(−)-enantiomer,[232] suggesting that each isoenzyme exhibits different enatio- and regioselectivity. Rat GST 1-1 and mouse Alpha class forms were reported to be active toward aflatoxin B_1-8,9-oxide,[223,233,234] and rat 3-3 and 7-7 have also been shown to possess similar activity.[235] Mouse MII and MIII as well as rat 3-3 and 4-4 have activity toward 4-nitroquinoline 1-oxide.[236,237] The 4-4 also possesses activity toward *trans*-stilbene oxide[157] and denitrosation activity toward 1-methyl-2-nitro-1-nitrosoguanidine,[238] and the rat YrsYrs toward arylmethyl sulfates such as a reactive sulfate ester produced from 7-hydroxymethylbenz[*a*]anthracene by sulfotransferase (Figure 1).[22] *N*-Hydroxy-Trp-P-2, a tryptophan pyrolysis product, is conjugated by cytosolic GSH transferases, but isoenzyme specificity has not been determined.[239]

Some hepatocarcinogens, including *N*-acetyl-2-aminofluorene, *N*-methyl-4-aminoazobenzene, and dimethylnitrosamine, are activated by cytochrome P-450 and then conjugated spontaneously with glutathione.[6]

In addition to these conjugation activities, rat GSTs 1-1 and 1-2 function as binding proteins for azodye carcinogens.[240] Some forms, such as 1-2, 2-2, 5-5, and 7-7, also possess selenium-independent glutathione peroxidase activity toward lipid or DNA hydroperoxides[241,242] (see reviews in References 12, 15, and 243). These functions may play a significant role in the metabolism of carcinogens, but their contribution in specific carcinogen cases largely remains unclear.

2. Induction of GSH Transferases by Drugs and Prevention of Carcinogenesis

Rat GSH transferase subunits are preferen-

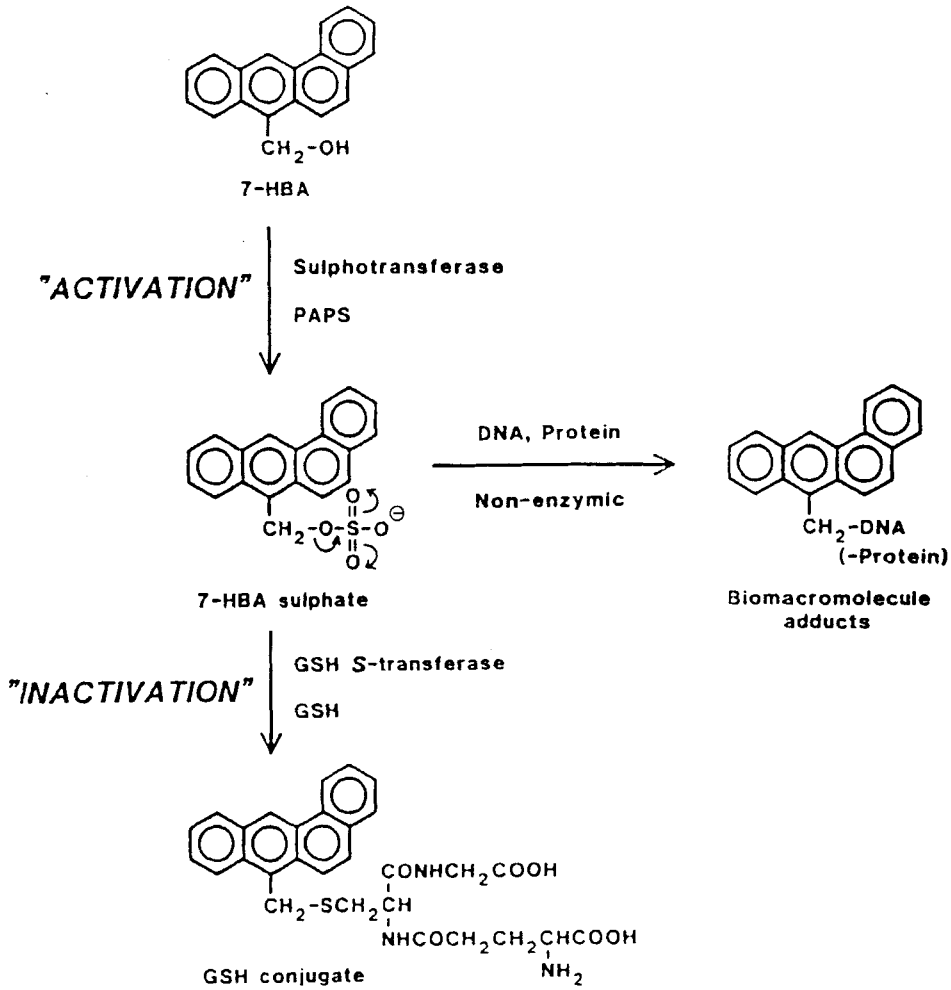

FIGURE 1. Metabolic inactivation of 7-hydroxymethylbenz[*a*]-anthracene (7-HBA) sulfate by GST-YrsYrs. The sulfate ester is produced by a sulfotransferase in the presence of 3′-phosphoadenosine 5′-phosphosulfate (PAPS). (From Watabe, T., Hakamata, Y., Hiratsuka, A., and Ogura, K., *Carcinogenesis (London),* 7, 207, 1986. With permission.)

tially induced by various drugs, including carcinogens (3′-methyldiaminoazobenzene,[244,245] 2-acetylaminofluorene,[244] and *trans*-stilbene oxide[246]) and anticarcinogenic agents such as BHA,[72,244,247] ethoxyquin,[248] and oltipraz.[249] They are also induced by lead nitrate,[11] bacterial protein A,[250] or a selenium-deficient diet[251] (see review in Reference 19). Respective subunits are known to be induced in a different manner by treatment with phenobarbital and 3-methylcholanthrene.[244,247,252] For example, the induction of subunit 3 by 3-methylcholanthrene or β-naphthoflavone is not remarkable,[252] while subunit 1 is inducible by almost all agents examined. As already described, in addition to an enhancer involved in basal expression, two enhancers, XRE and ARE, have been identified in the 5′-flanking sequence of the subunit 1 gene. ARE does not have the XRE core sequence. Rushmore and Pickett have studied the mechanism of induction of subunit 1.[60,61] β-Naphthoflavone, a planar aromatic compound, activates the gene through either XRE or ARE, but the presence of *Ah* receptors and metabolism of β-naphthoflavone by cytochrome P-450 IA1 are required for its transcriptional activation. On the other hand, *t*-butylhydroquinone, a phenolic antioxidant, activates the gene only through ARE, independently of *Ah* receptors or cytochrome P-450 IA1. In the presence of *Ah* receptors, XRE reacts with

2,3,7,8-tetrachlorodibenzo-*p*-dioxin, but ARE does not (Figure 2). These results support the proposal by Talalay et al. that phase II drug-metabolizing enzymes, including GSH transferases, are induced by monofunctional and bifunctional inducers by different mechanisms, and that induction by monofunctional inducers such as *t*-butylhydroquinone is mediated by an electrophilic signal, independently of *Ah* receptors.[253–255]

On the other hand, administration of clofibrate and other peroxisome proliferators to rats results in diminished GSH transferase activity in livers, presumably by reducing the amount of the enzyme.[256] Clofibrate also reduces GST-P levels expressed in rat hyperplastic nodules.[12] Reddy et al. indicated that peroxisome proliferators are hepatocarcinogens,[257] while other investigators have reported that they may act as promoters for liver tumors.[258] Peroxisome proliferator receptors are supposed to mediate the biological effects of these agents,[259] and Issemann and Green have recently cloned the mouse receptor, indicating that it is a putative transcription factor belonging to the steroid hormone receptor superfamily.[260] *Ah* receptors, also members of the same superfamily,[261] are stimulatory for induction of GSH transferases. Thus, expression of GSH transferase forms may be regulated in opposing directions by transcription factors with closely related structures. In this context, it is noteworthy that GSH transferases act as binding proteins for many drugs as well as steroid and thyroid hormones. Although the affinities of GSH transferases for hormones and xenobiotics are less than those of typical receptors, Listowsky et al. raised the possibility that high concentrations of GSH transferases may modulate the binding between hormones and their receptors.[113]

Many studies revealed that chemical carcinogenesis of mouse liver and other organs can be prevented or modulated by antioxidants (see reviews in References 10 and 262). Antioxidants such as BHA and bisethylxanthogen induce mouse liver GSH transferase activity, which has therefore attracted attention with respect to the observed chemopreventive effects. As already described, the electrophile-responsive element of the mouse Ya gene plays an important role in induction by antioxidants or planar aromatic compounds. This element is similar to the rat ARE described above in nucleotide sequence and also in direct activation by electrophilic compounds.

Microsome-mediated aflatoxin B_1 binding to DNA is reduced when rat liver GSH transferases (mainly 1-1 and 3-3) are induced by BHA or phenobarbital.[263–265] Mutagenic response to aflatoxin B_1 is also reduced by BHA treatment.[266] As previously noted, GST 1-1 and 3-3 are active toward aflatoxin B_1-8,9-oxide, produced from aflatoxin B_1 by microsomal cytochrome P-450. A similar reduction in aflatoxin B_1 DNA binding has also been reported in mouse liver treated with BHA, the isoenzyme in the Alpha class being involved in this case.[223,234] In mouse lung, two

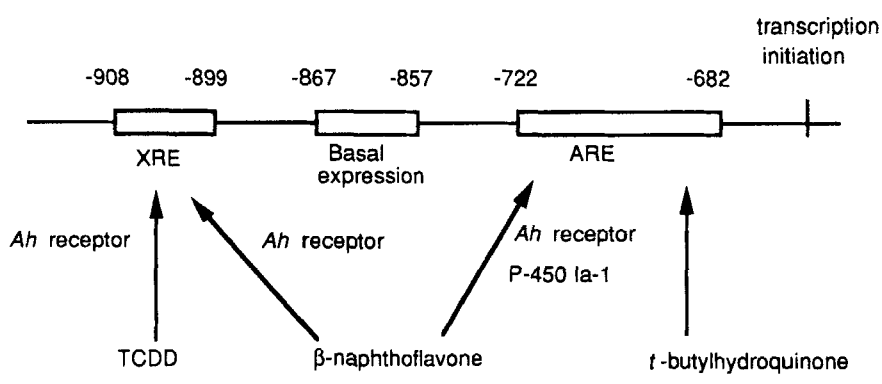

FIGURE 2. *Regulatory elements of the rat subunit 1 gene.*[62] XRE, xenobiotic-response element-like sequence; ARE, antioxidant-responsive element; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

isoenzymes in the Mu class are preferentially induced by BHA, which bound with benzo[*a*]pyrene (BP) metabolites, indicating antineoplastic activity of BHA against BP-induced neoplasia in mouse lung.[267] Thus, induction of GSH transferases by several drugs has been suggested strongly to be directly involved in the prevention or modification of chemical carcinogenesis in rodent livers and other organs. Since antioxidants and other drugs have been noted to inhibit hepatocarcinogenesis induced by many different carcinogens, different isoenzymes are presumably responsible. Only a few isoenzymes, as described above, have been specified in these cases.

3. GSH Transferases for Stimulation of Carcinogenesis

Although, as described above, GSH transferases are generally recognized as detoxifying enzymes, they are also involved in the activation of some carcinogens such as haloalkanes and haloalkenes (see reviews in References 5, 6, 268, and 269). Rat GST 2-2 and 3-3 catalyze the conjugation of ethylene dibromide (1,2-dibromoethane) with glutathione to form 1-bromo-2-*S*-glutathionyl ethane, which reacts with DNA via an episulfonium ion, eventually resulting in the formation of *S*-[2-(N^7-guanyl)ethyl]-glutathione.[270,271] Reaction with DNA is considered to be responsible for carcinogenesis by 1,2-dibromoethane in rats, whereby pretreatment with *t*-butylated hydroxytoluene, an inducer of GSH transferases, markedly increased DNA adduct levels, while depletion of GSH resulted in decreased levels.[272] 1,2-Dichloroethane and 1,2-dibromo-3-chloropropane are also activated by GSH transferase.[273,274] Haloalkenes, including hexachloro-1,3-butadiene and trichloroethene, which induce renal tumors in the rat, are conjugated with glutathione by microsomal GSH transferase.[275] In contrast to haloalkanes, these glutathione conjugates are not mutagenic by themselves, and require further metabolism to unstable thiols via cysteinyl derivatives by the mercapturic acid pathway, followed by the action of the cysteine conjugate β-lyase. These reactive thiols formed by β-lyase-mediated reaction are considered to be involved in the induction of renal tumors.[268]

Many authors have reported increased incidences of hepatomas after administration of various carcinogens, including 2-acetylaminofluorene (AAF) and aflatoxin B_1 to mice suffering from chronic trematode infections such as *Schistosoma japonicum* or *mansoni*, or *Fasciola hepatica* infestations.[276–279] Similar enhancement of liver cancer development is also observed in rats with *Clonorchis sinensis* infection,[280] and in hamsters with *Opisthorchis viverrini* infection.[281] Interestingly, these *Schistosoma* and *Fasciola* have been noted to possess high amounts of GSH transferases.[282–285] Certain mice resistant to infection with *S. japonicum* developed antibodies toward parasitic GSH transferase.[282] Furthermore, immunization of rats and hamsters with GSH transferase of *S. mansoni* resulted in significant protection against infection with the parasite.[283,284,286] Following infection with *S. mansoni*, a decrease in the levels of drug-metabolizing enzymes, including cytochrome P-450, of host livers was reported.[287] In spite of the increased tumorigenicity in schistosome-infected mice, the Ames test demonstrated decreased AAF- and aflatoxin B_1-activating potential,[288] and microsome-mediated binding of AAF to DNA was also decreased in infected mice.[289] However, alteration in host liver GSH transferases, particularly MI with conjugation activity toward aflatoxin B_1, remains to be studied. Since interferon has been reported to change mouse liver GSH transferases,[290] schistosome infection may modulate host liver GSH transferases through production of other mediators, resulting in an alteration in carcinogen metabolism.

B. GSH Transferases as Markers for Preneoplasia and Neoplasia

Changes in GSH transferase isoenzymes during rat chemical hepatocarcinogenesis have been demonstrated. First, rat basic forms in both the Alpha class — such as GSTs 1-1 (YaYa) and 1-2 (YaYc), known as ligandin — and the Mu class — such as GSTs 3-3 (Yb_1Yb_1) and 3-4 (Yb_1Yb_2), earlier named GST A and C, respectively[291] — were noted to be increased (see reviews in References 5, 6, and 11). Since 1984, attention has concentrated on GST-P (7-7), identified as a good

marker for rat hepatic preneoplastic and neoplastic lesions (see review in Reference 12). Human GST-π has also been demonstrated to be increased in preneoplastic and neoplastic lesions in a variety of organs.

1. GSH Transferases in Preneoplastic Foci in Rat Liver

a. Rat GST 7-7 (GST-P)

A new neutral form (pI 7.0), which was first purified from the rat placenta and named the placental form (GST-P) by Sato et al.,[292] was noted to be markedly increased in hyperplastic nodule-bearing livers.[244,245,247] Ketterer and colleagues similarly noted an unidentified form in primary hepatoma induced by *N,N*-dimethyl-4-aminoazobenzene,[293] and later determined this to be GST 7-7.[96] Guthenberg et al. and Robertson et al. demonstrated that their new form (GST 7-7), purified from rat kidney and lung, is immunologically identical to GST-P.[294,295] GST-YfYf purified from the kidney by Hayes was also regarded to be identical with GST 7-7.[296]

GST-P (7-7) has the smallest molecular weight among rat cytosolic forms, and while it is not immunologically cross-reactive with any other forms in the rat, there is considerable cross-reactivity between species, including the rat, mouse, hamster, dog, horse, and man.[11,12,245] GST-P is ubiquitous in normal rat tissues, but in small amounts, even in the placenta. It is especially low in adult rat livers, but significant in fetal liver,[73] though first described as negligible.[12] The kidney, lung, pancreas, small intestine, skin, and brain contain significant amounts.

During rat chemical hepatocarcinogenesis, GST-P (7-7) was found to be markedly increased (30-fold or more, and above 1 mg/g wet weight of the liver) in rat liver bearing hyperplastic nodules induced by several protocols, such as the Solt and Farber model,[297] and in primary and transplantable hepatocarcinomas by several groups.[11,244,247,292,298,299] Eriksson et al. also described a protein (p21) appearing in isolated hyperplastic nodules induced by six different models,[300] including the Solt and Farber model. This polypeptide (later renamed p26) was also identified as the GST-P subunit.[301,302]

The levels of GST-P in primary and transplantable (Morris 5123D) hepatomas induced by different carcinogens were also tenfold higher than in normal liver, but negligible in transplantable Yoshida ascites hepatoma AH 130,[247] indicating that GST-P tends to decrease with dedifferentiation in hepatomas, as is the case for many drug-metabolizing enzymes. However, some strains such as Zajdela ascites hepatoma cells were reported to have significant amounts of only the GST 7-7 form, further inducible by i.p. injection of *trans*-stilbene oxide.[246]

Immunohistochemically, GST-P was demonstrated to be localized in preneoplastic enzyme-altered foci that are also detectable by elevated γ-glutamyltransferase (GGT) activity.[292] The foci are inducible with a large number of different protocols using a variety of carcinogens (diethylnitrosamine, dimethylnitrosamine, 2-acetylaminofluorene, aflatoxin B_1, heterocyclic amines, etc.). Hepatomas induced by these and other genotoxic carcinogens also usually strongly express GST-P.[12] Sex differences are observed in the development of GST-P-positive foci induced by some carcinogens; for example, in the Solt and Farber model, the areas occupied by GST-P-positive lesions are far larger in male rats than in female rats.[12]

GST-P is expressed not only in putative preneoplastic hepatic foci induced by chemicals, but also in spontaneous lesions, for example, in spontaneous "altered cell foci" in the livers of aged Fisher 344 rats,[303] or in LEC rats, without administration of any exogenous carcinogens,[304,305] in both cases particularly in males.

Immunohistochemical staining further revealed that very small GST-P-positive foci or even single cells, appearing 1 or 2 weeks after a single administration of initiator,[11,306] or in one model even within 48 h,[307] are detectable before an increase in GST-P content becomes evident in whole liver preparations. The numbers of these cells increase with increasing doses of initiator (e.g., diethylnitrosamine) and are not induced by promoters of liver carcinogenesis such as phenobarbital.[307] Thus, GST-P-positive single cells are considered to be "initiated cells", indicating

a clonal origin of GST-P-positive foci and hepatomas. GST-P-positive small foci (minifoci) persist for a long time (at least 6 months) after a single injection of diethylnitrosamine, 200 mg/kg[308] or even 10 mg/kg,[309] showing that these lesions are, to a great extent, irreversible and capable of developing into large foci after feeding 2-acetylaminofluorene for 2 or 3 weeks, accompanied by partial hepatectomy (selection pressure).[308]

Unlike the majority of drug-metabolizing enzymes, GST-P is not inducible by administration of a large variety of hepatocarcinogenic promoters or modulators (3-methylcholanthrene, α-hexa-chlorocyclohexane, carbon tetrachloride, cyproterone acetate, phenobarbital, and polychlorinated biphenyls), or even by hepatocarcinogens (diethylnitrosamine, 2-acetylaminofluorene, 3′-methyl-4-dimethylaminoazobenzene, aflatoxin B_1, choline- and methionine-deficient diet, ethionine, and clofibrate), without the appearance of preneoplastic foci and hyperplastic nodules.[247,310] GST-P did, however, prove to be slightly inducible by the antioxidants BHA and butylated hydroxytoluene (BHT),[72,311,312] and by ethoxyquin in periportal areas.[313,314]

Among the other drugs examined, lead nitrate proved to be exceptional in inducing significant levels of GST-P throughout the whole rat liver, with no zonal preference.[315–317] In this case, expression of GST-P is transient and reversible within 2 weeks.[11] Although lead nitrate is a hepatic mitogen, the associated hyperplasia cannot replace compensatory cell proliferation after hepatectomy or carbon tetrachloride poisoning in promoting the development of GST-P-positive foci.[317–319]

In contrast to some other known rat hepatic preneoplastic markers, such as glucose-6-phosphatase, adenosine triphosphatase, GGT, and glucose-6-phosphate dehydrogenase, GST-P appears to be more stable after the withdrawal of carcinogens from the diet.[320] Ease of visualization and this advantage have established GST-P as one of the best markers for detection of early liver lesions, now widely used for analysis of hepatocarcinogenesis[321–325] and in rapid bioassay methods for carcinogens and modifiers of hepatocarcinogenesis (see review in Reference 326).

It has been reported by Reddy and colleagues (see reviews in References 259 and 327) and other groups[328–332] that a new class of carcinogens (nongenotoxic), including peroxisome-proliferating hypolipidemic agents such as clofibrate, nafenopin, ciprofibrate, Wy-14643, tibric acid, and di(2-ethylhexyl)phthalate, can induce GST-P-negative and/or GGT-negative foci and hepatomas. GSH transferase activity is known to be inhibited by clofibrate,[256,333] but this cannot account for the observations, because the mRNA for GST-P is not expressed in foci and hepatomas induced by peroxisome-proliferating agents.[334] Peroxisome proliferators have been considered to be nongenotoxic, but recent studies by Nishimura and colleagues have shown that the adduct 8-hydroxyguanosine is elevated in DNA of the liver and kidney of rats administered these agents, suggesting the involvement of oxidative DNA damage in their hepatocarcinogenesis.[335,336]

The structure of GST-P and the mechanisms underlying regulation of its expression during chemical hepatocarcinogenesis have been investigated by Muramatsu and colleagues.[337] They cloned a rat GST-P cDNA (pGP5) from a cDNA library prepared from poly(A)$^+$ RNA of 2-acetylaminofluorene-induced rat hepatoma and determined the complete amino acid sequence of the rat GST-P subunit (M_r 23,307, excluding methionine at the N terminus).[46] They also showed that GST-P mRNA (about 750 nucleotides) is abundant in hyperplastic nodules, Morris hepatomas, and chemically induced hepatomas, but barely detectable in normal liver, fetal liver, and AH 130 cells. Lower levels of mRNA were detected in lung, testis, kidney, spleen, and placenta, approximately in this order. The GST-P gene is located on rat chromosome 1 at band q43.[338] Okuda et al. have isolated the GST-P gene from a phage library using their GST-P cDNA clone (pGP5); it is about 3000 bp long and consists of seven exons and six introns, with the initiator codon being split between the first and second exons.[51] They also analyzed the *cis*-acting regulatory DNA elements of the rat GST-P gene by the chloramphenicol acetyltransferase (CAT) activity assay method.[52,337] Two enhancing elements (GPEI and GPEII) were located 2500 and 2200 bp upstream from the transcription initiation

site.[52] GPEII contained two simian virus 40 and one polyoma enhancer core-like sequences. A silencing element was found 400 bp upstream from the cap site. 12-*O*-Tetradecanoyl-phorbol-13-acetate (TPA) response element (TRE)-like sequences (TGATTCAG) were present in the GPEI and at position −16.[52,339] Furthermore, they found GPEI to be an imperfect palindrome composed of two TRE-like sequences (Figure 3), each having no activity by itself, but acting synergistically to form a strong enhancer that is active even at the low level of AP-1 (an oncogene *c-jun* product) activity in F9 embryonic stem cells. They also demonstrated that *c-jun* product and a related protein (*c-fos* product) are *trans*-acting on the enhancing elements.[52,62,339,340] It is currently thought that the nuclear oncogene *c-jun* and *c-fos* products form a heterodimer and *trans*act to amplify TRE-containing genes.[341] *c-Jun* and *c-fos* are expressed in livers bearing GST-P-positive foci induced by the Solt-Farber model.[342] Muramatsu and colleagues have also identified a protein *trans*acting on the silencer of the GST-P gene.[62]

GST-P was further demonstrated to be highly expressed, with malignant transformation *in vitro* or primary hepatocytes either by transfection with *ras* oncogenes or by treatment with activated aflatoxin B_1.[343] Transformation of rat liver epithelial cells with *v-H-ras* or *v-raf* also caused expression of GST-P and MDR-1 independent of chemical exposure, resulting in multidrug resistance.[344] Li et al. demonstrated that the expression of a metallothionein-*ras* fusion gene (MT*ras*T24) specifically increases mRNA levels of GGT and GST-P in cultured rat liver epithelial cells, in which these genes have been shown to be expressed together.[345] However, recent studies have suggested that GST-P and GGT are regulated differently.[346,347]

As described above, GST-P possesses conjugation activity toward aflatoxin B_1-8,9-oxide or BP metabolites. However, the actual role(s) and function(s) of GST-P in preneoplastic cells remain to be clarified. It is known that putative preneoplastic foci are resistant to cytotoxic agents, including hepatocarcinogens. Among the basic forms, GSTs 1-1, 1-2, and 2-2 have large capacities to bind bilirubin, heme, and cholic acids, but they markedly lose glutathione transferase activities through binding to these compounds; the activities of GSTs 3-3, 3-4, and 4-4 are less affected.[348] GST-P activity, in contrast, is almost unaffected by binding to such endogenous compounds,[121] suggesting that the increased levels of GST-P in foci might allow replacement of basic forms with regard to GSH transferase activity. GST-P (7-7) also exhibits selenium-independent glutathione peroxidase activity toward lipid hydroperoxides, especially toward arachidonate and linoleate hydroperoxides,[96] and also toward thymine hydroperoxide.[349] Thus, GST-P expression may be related to the prevention of lipid peroxidation, which is considered to play an important role(s) during tumor promotion. Lipid hydroperoxides produced by some promotion regimens can be removed by a series of coupled reactions: selenium-independent glutathione peroxidase activity of GST-P and other forms, followed by reduction of oxidized glutathione by glutathione

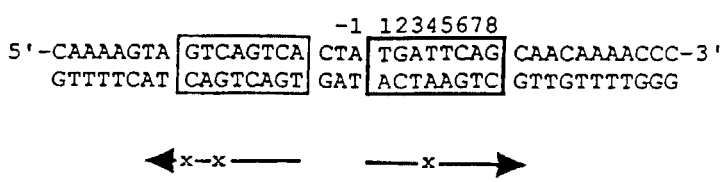

FIGURE 3. TPA-responsive element-like sequences of the enhancer (GPEI) of the rat GST-P gene exhibiting an imperfect palindrome. The upstream and downstream TRE-like sequences are boxed with thin and heavy lines, respectively. X indicates the position of a nucleotide that is different from that of the consensus TRE. (From Okuda, A., Imagawa, M., Sakai, M., and Muramatsu, M., *EMBO J.*, 9, 1131, 1990. With permission.)

reductase [NAD(P)H] using NADPH supplied by the pentose phosphate pathway.[12] The fact that glutathione reductase, NADPH-generating glucose-6-phosphate dehydrogenase, and the total and reduced glutathione levels are all increased in enzyme-altered foci and hyperplastic nodules supports the possibility that the aforementioned reactions might be operating.[12]

On the other hand, it has been noted that GST-P and Pi class forms in other species are irreversibly inactivated by sulfhydryl(SH)-blocking reagents such as *N*-ethylmaleimide and iodoacetamide, as described in Section II.B. The Pi-class forms are also reversibly inactivated by hydrogene peroxide (H_2O_2) or oxidized glutathione (GSSG),[124,350] while other forms in the other classes are hardly affected. Indeed, the microsomal form[351] and Mu class forms[138] are known to be activated by active oxygen species. In the inactivation, intra- or intersubunit disulfide bonds between the 47th cysteine residue and other cysteine residue(s) are involved, GST-P being reactivated by thioltransferase in the presence of GSH.[134] Thus, the thiol redox state, which is influenced by active oxygen metabolites, might regulate GST-P and other Pi class forms, as pointed out for certain key enzymes of other metabolic pathways (see review in Reference 352).

b. Other Forms

During chemical hepatocarcinogenesis, the basic subunits 1/2 (Ya/Yc) and 3/4 (Yb_1/Yb_2), in addition to GST-P (7-7), are increased in foci and nodules, and may perform multiple detoxication functions as enzymes and binding proteins in the resistance of these lesions to cytotoxic agents[353] (see reviews in References 5 and 6). In these foci and nodules, a large range of enzymes are changed, some finding application as marker enzymes (see review in Reference 12). Among the drug-metabolizing enzymes, phase I species are reduced, while phase II forms, including GSH transferases, are increased; such a deviation pattern of drug-metabolizing enzymes is considered to be responsible for the resistance of these lesions to cytotoxic agents. Since Farber and coworkers pointed out that enzyme-altered foci and hyperplastic nodules induced by different carcinogens in rat liver have pleiotropic resistance to structurally unrelated agents, they were termed "resistant foci and nodules".[1,2,354] In aflatoxin B_1-induced hyperplastic nodules, the Ya_2 subunit in the Alpha class was found to be increased and suspected to endow resistance of these cells to this carcinogen.[57,355] It was also noted that subunit Yk (8) with a high activity toward 4-hydroxynonenal, a lipid peroxide metabolite, was also increased in hyperplastic nodules.[355]

2. GSH Transferases as Tumor Markers in Human Tissues

a. Molecular Forms

Since the identification of GST-P as a reliable marker for rat hepatocarcinogenesis,[245,247,292] expression of the human equivalent form, GST-π, has been examined in many cancer tissues. It was soon demonstrated that while human hepatocellular carcinomas do not express GST-π, a significant increase of GST-π levels is evident in metastatic liver tumors originating from the gall bladder, stomach, and colon.[145] Immunohistochemical studies revealed that both colon carcinomas[356] and squamous cell carcinomas of the uterine cervix express GST-π.[357] Other investigators have reported further increased levels of GST-π in several tumors by immunohistochemical, immunoblotting, or Northern blotting methods.

Human primary cancers expressing GST-π, as summarized in Table 4, are histologically classified as adenocarcinomas or squamous cell carcinomas. In the lung tumor case, e.g., GST-π is expressed in both adenocarcinomas and squamous cell carcinomas, but not in small-cell lung cancers.[358–361] Furthermore, it also should be noted that these organs normally express GST-π as a major form, but in lower amounts than in their cancer tissues. In addition to the cancers listed in Table 4, brain (glioma),[362] skin,[363] and Wilms'[364] tumors have been shown immunohistochemically to express GST-π. The properties of GST-π purified from several cancer tissues[365,366] or leukemia cells are not different from those of placental GST-π.[367,368]

TABLE 4
Glutathione Transferase Activity and Molecular Forms in Human Cancer Tissues

Cancer	Activity[a] (n mol/min/mg protein)		Form	Ref.
1. Tumor with increased activity				
Colon	130 ± 30	(30 ± 10)[b]	π	385
	239 ± 76	(174 ± 44)	π	428
	350	(180)	π	376
Stomach	273 ± 24	(200 ± 18)	π	429
	500	(300)	π	376
Urinary bladder	150 ± 130	(29 ± 20)	π	430
Uterine cervix	260 ± 190	(40 ± 10)	π	357
Esophagus	230 ± 160	(40 ± 20)	π	385
Lung	205 ± 131	(112 ± 70)	π	358
	500	(190)	π	376
2. Tumor with decreased activity				
Liver	350	(700)		376
Kidney	327 ± 130	(732 ± 213)	π	378
	600	(350)	π	376
	249 ± 242	(926 ± 390)	π	29
3. Tumor without alteration in activity				
Breast	70 ± 50	(10)	π	431
	67 ± 44	(65 ± 27)	π	381
	121 ± 109	(141 ± 102)	π	382
	200	(150)	π	376
Leukemia (chronic lymphocytic)	51 ± 9	(48 ± 9)	π	367

[a] GSH transferase activity toward 1-chloro-2,4-dinitrobenzene, mean ± SD.
[b] Activity in control normal tissues in parenthesis.

GST-π is not only expressed in cancers, but also in preneoplastic tissues such as colon adenomas,[356] dysplasia of the uterine cervix[357] and esophagus, and cirrhotic liver.[369,370] Although a few reports suggest that GST-π may appear in cervical epithelia under nonneoplastic conditions,[371,372] its expression has been noted in cervical dysplasia accompanying koilocytosis, which suggests infection by human papilloma virus.[357] A strong correlation between infection with cancer-associated human papilloma virus and GST-π expression has been reported.[373]

GST-π expression is more frequent in larger colonic adenomatous polyps than in smaller polyps.[374] This finding is interesting, since genetic alterations, including *ras* gene activation and loss of tumor suppressor genes, are also observed more frequently in larger adenomas.[375] Thus, GST-π

may be a useful marker not only for various cancers, but also for high-risk precancerous lesions.

In view of the striking expression of GST-P in rat hepatocarcinogenesis, the finding that GST-π is hardly expressed in human primary hepatocellular carcinomas[12,376] is surprising. Alterations in GST-μ expression in cancer tissues are also not striking. In fact, it appears that the profiles of GSH transferase forms in cancer tissues are influenced by the respective normal tissue profiles. It should be borne in mind, however, that while many human forms have been isolated from normal tissues, as described in Section II.C, expression of only GST-π, GST-B_1B_1, -B_1B_2, -B_2B_2, and -μ has been studied in cancer tissues, and alterations in other forms remain to be clarified.

b. Tissue Levels

In addition to immunohistochemical and Northern blotting studies, GSH transferase activities have been examined biochemically in many cancers, as summarized in Table 4. Activity toward 1-chloro-2,4-dinitrobenzene is increased two- to sixfold in cancers classified in category 1 of Table 4, compared with the respective control tissue values. Cancer tissues of this group exhibit similar activities with each other, irrespective of origin, and more than 70 to 90% of the activity is due to GST-π. Since smooth muscles also contain GST-π,[191,193] tissue specimens excluding smooth muscles should be used for assaying activity; otherwise, values for noncancerous control tissues might be overestimated. Cancers originating from the liver or kidney (category 2 in Table 4), both of which are organs normally possessing high GSH transferase activity, show less activity than the respective control tissues, mainly due to a decrease of the B_1 and B_2 subunits in the Alpha class,[376–378] GST-π being changed marginally. GST-π becomes the dominant form in renal cancers.[32,378] GSH transferase activities in breast cancers or chronic lymphocytic leukemia cells are not significantly different from those in control tissue or control lymphocytes, respectively. Since comparisons are based on mean values, some breast cancers demonstrate enhanced activity. GST-π and its mRNA levels were reported to be negatively correlated with estrogen receptor content in breast cancer specimens,[178,379,380] but these findings could not be confirmed by other investigators.[381,382] Thus, changes in GSH transferase forms in cancer tissues are not homogeneous. GST-π expression is enhanced in some, but not all, cancers derived from many organs. Schematically, alterations in GSH transferase forms in cancer tissues can be divided into three groups, as shown in Figure 4, where some particular cancers are given as examples.

GSH transferase activity has also been examined in many cancer cell lines (Table 5), the majority established from cancers possessing high GSH transferase activity and also retaining high activity, GST-π being the major form. Expression in various tumor tissues and cell lines indicates that GST-π may be a useful marker for a wide range of cancers. On the other hand, only a limited number of cell lines from breast cancer, small-cell lung cancer, and lymphoma even have low activity, confirming the results obtained in these cancer tissues (Table 4). It should be noted that the MCF-7 cell line used by Batist et al.[383] to examine GST-π expression following acquisition of drug resistance is one of these cell lines with very low activity.

c. Serum Levels

Investigation of GST-π levels in the sera of patients with cancer of the gastrointestinal tract has revealed significant elevation.[384,385] In addition, these high serum values decreased to the normal range after surgical removal of the tumors, suggesting that follow-up of serum GST-π levels may be useful for monitoring such patients during the course of treatment. Since red blood cells contain GST-ρ, a form very similar to GST-π, attention should be paid to avoid hemolysis during measurement.[385] Furthermore, as platelets also contain GST-π,[386] plasma may be more suitable than serum for its assay.[387]

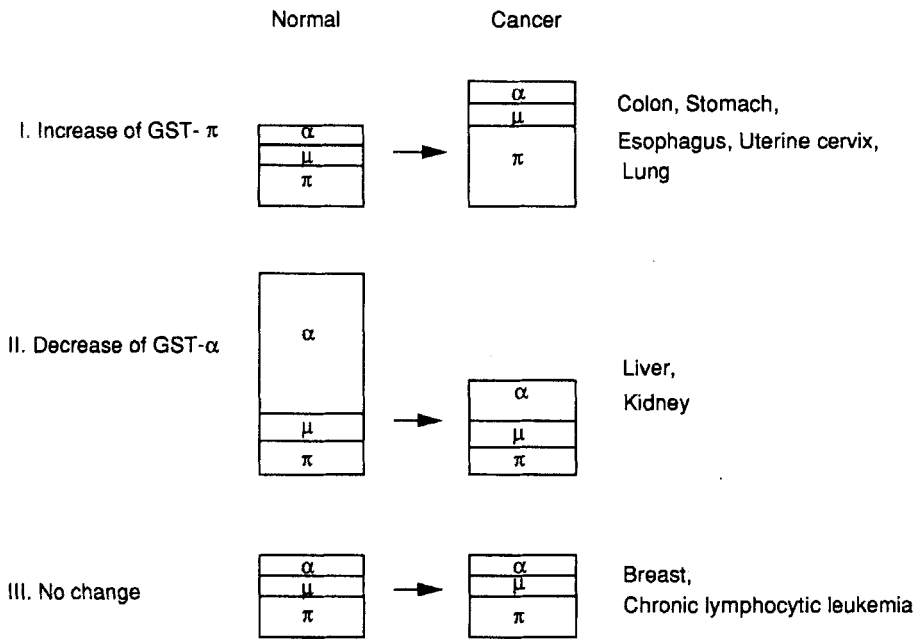

FIGURE 4. Alteration in GSH transferase isoenzymes in human cancers.

C. GSH Transferases for Anticancer Drug Resistance

1. Metabolism of Anticancer Drugs by GSH Transferases

Many anticancer drugs as well as carcinogens have been considered to be detoxified by conjugation with glutathione (see reviews in References 14 and 388 to 391). Thus, several alkylating agents or their metabolites have been shown to be conjugated with glutathione by cytosolic GSH transferases, and some agents also by microsomal GSH transferase.

Three glutathione adducts of melphalan were identified in reactions catalyzed by rabbit or human liver microsomal GSH transferases (Figure 5).[392,393] These adducts were also produced by cytosolic forms, although the isoenzymes involved were not specified. Chlorambucil, a drug with a structure similar to that of melphalan, is also conjugated with glutathione by monkey liver microsomal GSH transferase.[394] A recent study revealed that the mouse MI in cytosol has a higher activity toward melphalan than either MII and MIII,[395] but in the MI-catalyzed reaction, only monoglutathionyl melphalan was identified. The mouse MI also has a higher activity toward chlorambucil than MII or MIII.[396] In this reaction, three glutathione adducts are formed (Figure 6), monochloro-monoglutathionyl chlorambucil being the major product.

Rat GST 4-4 has been shown to detoxify 1,3-*bis*(2-chloroethyl)-1-nitrosourea (BCNU) by a denitrosation reaction.[397] Although the structure of the product in this reaction remains to be clarified, two possible pathways have been proposed in the recent review by Waxman.[391] Acrolein, a genotoxic aldehyde released in the metabolic activation of cyclophosphamide, is conjugated with glutathione by human GST-π (Figure 7),[209] GST-μ and GST-B_1B_1 having a lower activity. Involvement of GSH transferase in the conjugation of 9-deoxy-Δ^9,Δ^{12}(E)-prostaglandin D_2 (Δ^{12}-prostaglandin J_2) has been suggested; this reaction is considered a detoxication process for the compound,[398,399] which exerts a cytotoxic effect on many cancer cell lines.[400,401] Gossypol [1,1′ 6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl-(2,2′-binaphthalene)-8,8′-dicarboxyaldehyde] has been shown to have antitumor properties and to inhibit several enzymes, including GSH transferases. Interestingly, both antitumor properties and the inhibition of GSH transferase activity are stereoselective, the (−)-enantiomer of gossypol being more potent in both cases.[402]

In addition to their conjugation activities, the Se-independent glutathione peroxidase activity of GSH transferase forms may play significant roles in the detoxication of lipid peroxides and DNA

TABLE 5
Activities and Molecular Forms of Glutathione Transferase in Human Cancer Cell Lines

Origin	Cell line	Activity[a]	Cell line	Activity[a]	Cell line	Activity[a]
Colon[366,431,432]	CX-1	100	HT-29	200	LS174T	100 (319)
Breast[366,385,431]	MX-1	91	ZR75-1	10	MCF-7	2
Ovary[431]	PE04	210				
Urinary bladder[385,431,433]	HT1376	170	EJ	90		
	J82	102	SCaBER	459	FCCB-1	91
Lung[360,366,431]	EF484	175	NCIH322	80	NCIH358	180
Small-cell lung cancer	SW2-10S	8	NCIH69	8		
	NOC-361	50	NES	130		
Uterine cervix[385]	CaSki	380				
Esophagus[385]	TE-8	80	TE-9	520		
Head and neck[366]	SCC-25	532				
Liver[431,432]	HepG2	80 (14)	HepA1	120		
Wilms' tumor[385]	G401	180				
Neuroblastoma[385]	IMR32	1,000				
Leukemia[385,432]	K562	210 (298)	HL60	250		
Lymphoma[432]	Raji	1	SKO	198	Jurkat	170
	U937	107				
Melanoma[365]	A375	510	SM	190		

[a] Toward 1-chloro-2,4-dinitrobenzene, n mol/min/mg protein.

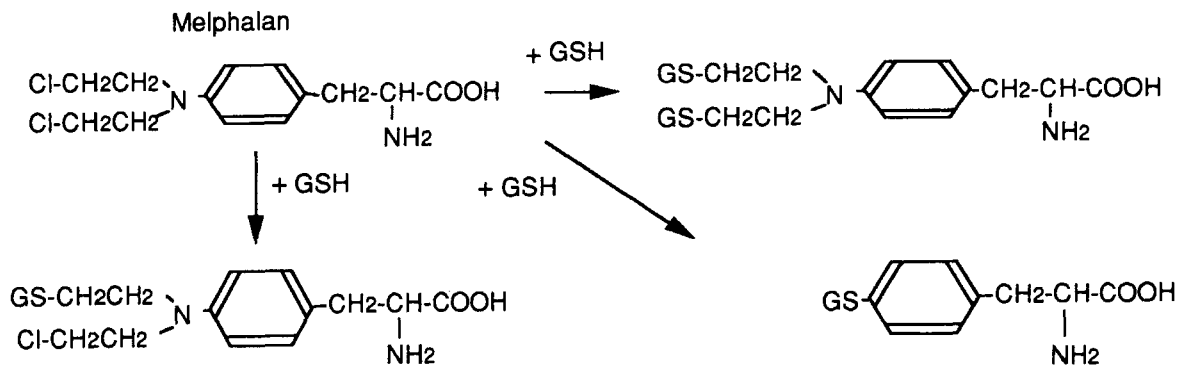

FIGURE 5. Glutathione conjugation of melphalan by GSH transferases.[392,393]

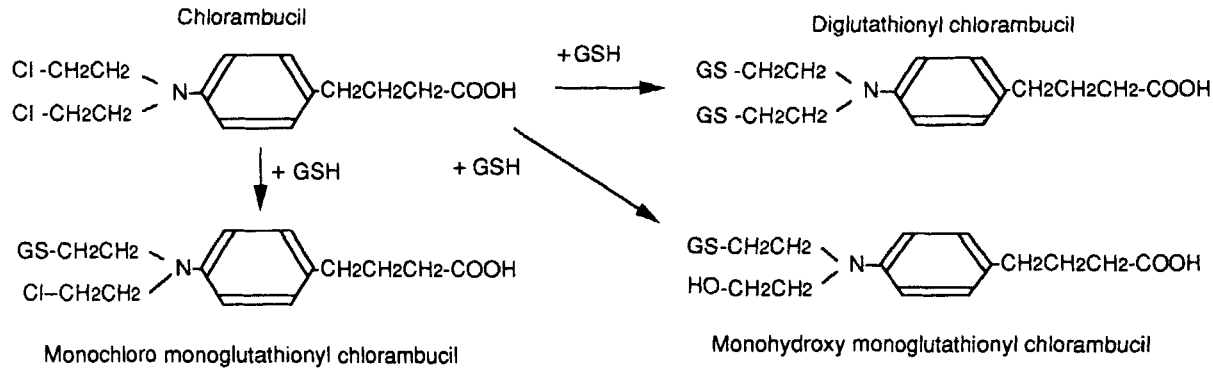

FIGURE 6. Glutathione conjugation of chlorambucil by GSH transferases.[394,396]

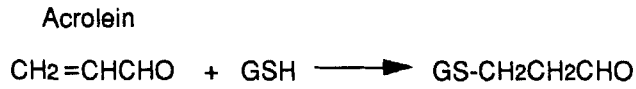

FIGURE 7. Conjugation of acrolein with glutathione catalyzed by GSH transferase.[209]

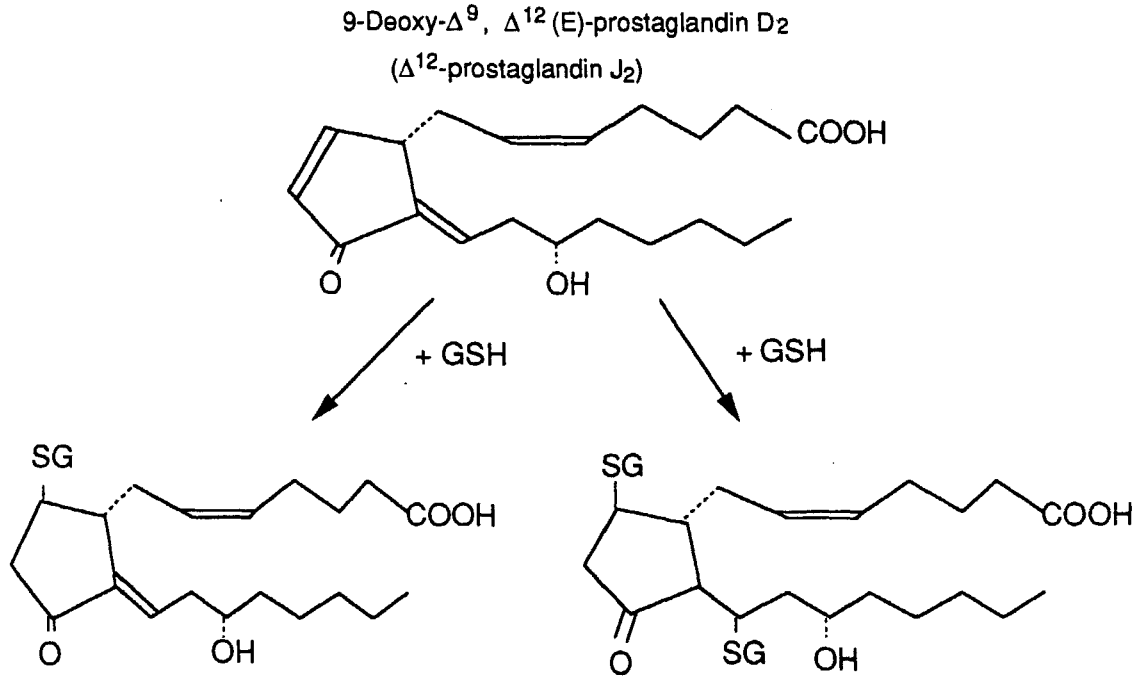

FIGURE 8. Glutathione conjugation of 9-deoxy-Δ^9,Δ^{12}(E)-prostaglandin D_2.[398,399]

hydroperoxides produced by exposure to doxorubicin, mitomycin C, and other drugs. Although several GSH transferase forms share protein-binding properties, it is not evident whether binding to *cis*-platinum or other anticancer drugs occurs.

2. Expression of GSH Transferase Forms in Drug Resistance

An increase in GSH transferase activity was reported in a rat mammary carcinoma cell line resistant to chlorambucil,[403] and GST-π was found

to increase after acquisition of doxorubicin resistance by a human breast cancer cell line, MCF-7.[383] Since then, the relationship between drug resistance and the expression of GSH transferases has been studied extensively in many cancer cell lines. The results are summarized in Table 6, indicating a two- to fivefold increase of activity, — mainly due to the Pi class in cell lines resistant to alkylating agents, doxorubicin, or *cis*-platinum — compared with the respective sensitive cell lines. In some rat cell lines, other forms (e.g., GST 2-2 and 4-4) are increased. On the other hand, some cell lines do not exhibit a significant alteration in GSH transferase activity between resistant and sensitive cells, both possessing very low activities. In cell lines that exhibit increased activities after acquisition of resistance, their activities in sensitive cells (in parentheses, Table 6) are, in general, exceedingly low compared with the values observed in most established cell lines (Table 5) or the values in cancer tissues (Table 4). The activity expressed in most resistant cell lines is comparable to the activities in cancer tissues. Thus, it seems likely that cell lines with low GSH transferase activity show enhanced activity after acquisition of resistance. As illustrated in Table 6, only a few cell lines with high activity exhibit a further increase upon becoming resistant.

Transfection studies with cDNAs encoding GSH transferase forms and studies utilizing inhibitors for GSH transferase have, however, sug-

TABLE 6
Activities and Molecular Forms of Glutathione Transferase in Cancer Cell Lines Resistant to Anti-Cancer Drugs

Cell line (origin)	Resistant drug	Activity[a]		Form	Ref.
8226 (human myeloma)	Doxorubicin	1	(1)[b]	π	434
MCF-7 (human breast cancer)	Doxorubicin	161	(4)	π	383
H69AR (human small-cell lung cancer)	Doxorubicin	202	(20)	π	435
UWR1 (human malignant astrocytoma)	BCNU	89	(33)	π	436
G3361 (human malignant melanoma)	Cisplatin	283	(33)	π	29
	Melphalan	257		π	
	BCNU	178		π	
	4-Hydroperoxy-cyclophosphamide	163		π	
PE04 (human ovarian carcinoma)	Cisplatin Chlorambucil 5-Fluorouracil	215	(74)	π	437
HS-Sultan (human myeloma)	Melphalan	118	(77)	π	438
SCC-25 (human squamous cell carcinoma)	Cisplatin	447	(231)	π	439
KP-N-AYR (human neuroblastoma)	Doxorubicin	564	(312)	π	440
P388 (mouse leukemia)	Doxorubicin	14	(9)	Pi	441
P388	Doxorubicin	97	(54)	Pi	442
16C (mouse mammary carcinoma)	Doxorubicin	48	(10)		443
ARN2 (mouse erythroleukemia)	Doxorubicin	15	(14)	Alpha	444
Walker 256 (rat mammary carcinoma)	Chlorambucil	25	(13)	Alpha	403
Walker 256	Chlorambucil	53	(17)	2-2	411,445
9L (rat gliosarcoma)	Nitrogen mustard	75	(30)	7-7	446
9L-2 (rat gliosarcoma)	BCNU	30	(30)	4-4	397
CHO (Chinese hamster ovary cell)	Chlorambucil	650	(240)	Alpha	150,412

[a] Toward 1-chloro-2,4-dinitrobenzene, n mol/min/mg protein.
[b] Activities in sensitive cell lines in parentheses.

gested the direct involvement of these forms in drug resistance. Several cell lines transfected with cDNAs encoding GST-π, rat subunits 1 or 3 exhibited increased resistance to particular drugs,[404–407] depending on the respective cDNAs transfected. Although the increase in resistance was modest (1.5- to threefold), the extent was nearly proportional to the GSH transferase activity in the transfectants. Since many cell lines possess a high activity, endogenous GSH transferase forms present in parent cells may be involved in the modest increase in these experiments. Treatment with inhibitors of GSH transferases such as ethacrynic acid[89–91] and indomethacin[92] resulted in reduced resistance to alkylating agents in cell lines with high GSH transferase activities. However, transfection of MCF-7 cells with GST-π cDNA did not result in resistance to doxorubicin, irrespective of the expression of amounts of GST-π comparable to those in resistant cells.[408] MCF-7 cells transfected with cDNA encoding the B_1 subunit in the Alpha class, again, did not demonstrate resistance to doxorubicin or chlorambucil,[409] indicating that GST-π expression may not be involved in the resistance, but that other mechanisms, including an increase in P-glycoprotein and glutathione peroxidase, may be responsible in this case.[410]

In drug-resistant cell lines, particular GSH transferase forms are induced that are suitable for detoxification of the respective anticancer drugs; rat GST 2-2 as well as 1-1 are expressed in chlorambucil-resistant cell lines[411,412] and GST 4-4 in a BCNU-resistant cell line.[397] A slight increase in the human GST-μ similar to rat 4-4 has also been reported in (2-chloroethyl)-3-sarcosinamide-1-nitrosourea-resistant glioma cells.[413] The finding that the Pi-class forms are expressed in many cell lines resistant to structurally unrelated drugs is analogous to the expression of rat GST-P in hepatocarcinogenesis induced by many genotoxic carcinogens.[11,12] Although mouse MII belonging to this class and human GST-π have been reported to possess conjugation activities toward chlorambucil and acrolein, respectively, the actual role of the Pi-class forms expressed in drug-resistant cells remains to be clarified. As described in the previous sections, reversible inactivation by active oxygen species, including hydrogen peroxide and superoxide anion,[125,133,134,138] suggests that the Pi-class forms might function as scavengers to remove active oxygen metabolites escaping metabolism by superoxide dismutase, catalase, or selenium-dependent glutathione peroxidase. Since the cytotoxicity of doxorubicin has been suggested to be dependent on the formation of free radicals, this possible scavenger function as well as the glutathione peroxidase activity of Pi-class forms toward lipid hydroperoxides may be important as mechanisms of doxorubicin resistance. Interestingly, an increase in GSH transferase activity has also been reported in some cell lines resistant to hydrogen peroxide.[414]

In resistant cell lines where GSH transferase activities are not increased or detectable, other mechanisms must be involved in the acquisition of multidrug resistance: increased drug output due to changes in transport mechanisms including the 170-kDa P-glycoprotein, decreased activation of prodrugs in phase I, alteration in drug target enzymes, alterations in cellular metabolism and repair mechanisms such as topoisomerases, and increased inactivating enzymes in phase II are all possible candidates (see reviews in References 13, 415, and 416).

The relationship between intracellular glutathione levels and drug resistance has been studied extensively (see reviews in References 388 and 389), glutathione metabolism being suggested as one of the major determinants of therapeutic efficacy. Alterations in enzymes involved in glutathione metabolism in drug-resistant cell lines are summarized in Table 7. Some resistant cell lines with enhanced GSH transferase activities also exhibited increased GGT activities, indicating that the two enzymes are coexpressed in these cells resistant to nitrogen mustards. An increase in glutathione peroxidase activity toward hydrogen peroxide is not obviously related to increased GSH transferase activities in drug-resistant cells. In addition to regulation by the glutathione redox cycle, recent studies revealed that intracellular GSSG and glutathione conjugates of drugs (GS-X) are pumped out by a particular ATPase species whose activity is dependent on GSSG or GS-X and Mg^{2+}. The existence of this ATPase has been demonstrated in human red

ABLE 7
:hanges of GSH-Related Enzyme Activities in Drug-Resistant Cancer Cell Lines

Cell line (origin)	Resistant drug	GSH[a]	GST[b]	GPX[c] CuOOH[f]	GPX[c] H_2O_2	GR[d]	GGT[e]	Ref.
226 (human myeloma)	Doxorubicin	19.2 (11.6)[g,h]	1.2 (1.3)	3.2 (3.7)	2.8 (2.2)	—	—	434
L160 (Human myelogenous leukemia)	Doxorubicin	1.6 (3.1)[h]	114 (117)	—	30 (35)	—	19 (39)	422
69 (human small-cell lung cancer)	Doxorubicin	2.2 (13.8)	202 (20)	6.6 (5.3)	6.0 (6.1)	75 (39)	2.5 (0.5)	435
ICF-7 (human breast cancer)	Doxorubicin	2.3 (9.5)[h]	78 (2)[i]	27 (0.5)[i]	24 (0.4)[i]	—	—	447
LC (mouse erythroleukemia)	Doxorubicin	16.8 (23.8)	14.6 (13.8)	13.6 (9.4)	5.5 (4.1)	43 (31)	—	444
388 (mouse leukemia)	Doxorubicin	—	97 (54)	121 (88)	78 (74)	—	—	442
388	Doxorubicin	4.3 (2.2)[h]	3 (9)[i]	4.0 (1.2)[i]	3.1 (1.0)[i]	—	—	447
uman ovarian adenocarcinoma	Chlorambucil	37 (28)	215 (74)	—	27 (12)	87 (62)	0.4 (0.1)[i]	437
L (rat brain gliosarcoma)	Nitrogen mustard	53 (25)	75 (30)	—	—	12 (9)	14 (5)	446
HO (Chinese hamster ovary cell)	Chlorambucil	33 (18)	638 (239)	83.4 (16.4)	10 (8.9)	130 (97)	2.9 (0.8)[i]	150

Glutathione, n mol/mg protein.
GSH transferase activity, n mol/min/mg protein.
Glutathione peroxidase activity, n mol/min/mg protein.
Glutathione reductase activity, n mol/min/mg protein.
γ-Glutamyltransferase activity, n mol/min/mg protein.
Cumene hydroperoxide.
Values for sensitive cells in parentheses.
n mol/10^6 cells.
n mol/min/10^6 cells.

blood cells,[417] and rat liver [418] and heart,[419] but whether it is present in drug-resistant cells remains to be clarified. This enzyme is similar to P-glycoprotein with respect to the ATP-dependent drug-pumping property. However, glutathione conjugation is not required for P-glycoprotein action, and its subunit molecular weight (about 170,000) is different from that of GSSG-dependent ATPase from red blood cells (85,000 and 65,000).[417,420] It should be noted that a membrane protein with a subunit molecular weight (85,000) similar to that of the GSSG-dependent ATPase has been detected in doxorubicin-resistant human tumor cells.[421] Furthermore, reduction of intracellular glutathione levels has been reported to result in increased retention of daunorubicin.[422] Thus, transport systems for GSSG or GS-X may be active in resistant cells with enhanced GSH transferase activity.

The finding of high amounts of GST-π in most established cell lines (Table 5) and in many cancer tissues (Table 4) as well as in drug-resistant cell lines (Table 6) suggests that GSH transferases may play important roles not only in acquired resistance, but also in natural resistance. Modulation of GSH transferases with inhibitors has shown promise of overcoming this resistance in experimental models,[89–92] although the inhibition does not seem to be sufficiently specific. Thus, more specific inhibitors for GSH transferases, including GST-π, are required. One recent study using a series of glutathione analogues has,

however, identified a selective inhibitor for rat GSTs 4-4 and 3-3 that is potentially suitable for *in vivo* application.[140] In addition to the transfection studies described above, experiments with antisense RNAs may be informative for evaluation of the roles played by GSH transferases in drug resistance, by lowering enzyme expression.

D. GSH Transferases and Susceptibility to Cancer

1. Inverse Relationship between Organ-Specific Distribution of GSH Transferases and Carcinogenesis

GSH transferases possessing conjugation activity toward electrophiles derived from known carcinogens are limited to several forms, such as those composing subunits 1, 3, 5, and 7 in the rat.[230,231,233,235,295] As polyaromatic hydrocarbon epoxides from BP and 3-methylcholanthrene are good substrates for these forms, which are abundant in rat liver, these carcinogens are generally not hepatocarcinogenic. The GST-YrsYrs exhibits activity only toward arylmethyl sulfates, which are known to be carcinogenic in rat skin, but not in the liver, where the isoenzyme is abundant.[22] Similarly, rat isoenzymes in the Mu class catalyze the glutathione-dependent liberation of nitrite from 4-nitroquinoline 1-oxide,[236,237] which is tumorigenic to the lung, esophagus, and other organs where these isoenzymes are not expressed or expressed only marginally. Thus, the organ-specific distribution of isoenzymes involved in detoxication of carcinogens seems to be one of the factors that can suppress carcinogenesis in particular organs.

2. GST-μ as a Marker for Susceptibility to Human Lung Cancer

GSH transferase activity toward *trans*-stilbene oxide was observed less frequently in mononuclear leukocytes from lung cancer patients than from a matched control group of smokers.[423,424] The difference was only modest, but nevertheless statistically significant. The responsible form was identified as GST-μ,[206] its express in mononuclear leukocytes being parallel to that in liver from the same individuals. GST-μ as well as GST-π are known to have a high activity toward epoxides from BP.[151,208] Thus, loss of GST-μ has been suggested as a possible marker for greater susceptibility to lung cancer among smokers, hereditary differences in the expression of this form being due to deletion of the gene.[157] Consonant with this finding, a correlation between GST-μ deficiency and sister chromatid exchange induction by *trans*-stilbene oxide has been demonstrated.[425] However, the frequencies of GST-μ expression in patients with breast or colon cancers are not significantly different from those in normal controls.[381,382,426] On the other hand, a recent report has suggested that individuals lacking GST-μ expression have a greater risk of developing adenocarcinoma of the stomach or colon.[426a]

IV. CONCLUSIONS

GSH transferase isoenzymes that catalyze glutathione conjugation reactions exhibit overlapping activities toward a variety of xenobiotics. However, recent studies have revealed that the respective forms show different activities and different stereoselectivity toward particular chemicals, including carcinogens and anticancer drugs. Although many compounds are known to be detoxified by the enzyme, only limited examples have been clarified in terms of the specific isoenzymes involved. Furthermore, the distribution of these isoenzymes is tissue specific, and the expression of particular isoenzymes in some organs seems to be inversely related to the induction of cancers by carcinogens, as described in Section III.D. For aflatoxin B_1, the amounts of particular isoenzymes (rat GSTs 1-1 or 3-3) present in the liver are not sufficient to detoxify the electrophiles derived from the compounds. However, when sufficient isoenzymes are induced by antioxidants, detoxification is enhanced to prevent hepatocarcinogenesis.

Studies on the rat subunit 1 and mouse Ya subunit genes have revealed the molecular mechanisms of their induction and tissue-specific expression; in both cases, upstream *cis*-acting elements were suggested to be responsible. The

antioxidant-responsive or electrophile-responsive elements are responsible for induction by antioxidants, while the xenobiotic response element is involved in induction by bifunctional inducers such as β-naphthoflavone, their induction being dependent on the presence of *Ah* receptors and P-450 activity. Tissue specificity in the expression of rat subunit 1 is considered to be attained by transcriptional *trans*acting factors for which distribution is tissue specific. However, less homology in the nucleotide sequences of noncoding regions than of coding regions suggests that noncoding regions might also be involved in tissue-specific expression of GSH transferases. While the nucleotide sequences in coding regions of the Pi class forms are highly homologous among the rat, mouse, and man, the structures of *cis*-acting elements are markedly different between the rat GST-P and human GST-π genes, the latter lacking several enhancers observed in the former. These differences may be related to different expression of the Pi class forms in hepatomas between two species.

The possible correspondence of the respective cytosolic forms among the rat, mouse, and man is described in Table 1. Although further studies are required to establish exact correspondences, such a summary may be useful for the preliminary consideration of interrelationships.

GSH transferases act as binding proteins for many drugs as well as steroid and thyroid hormones. These ligands are diverse in their chemical structures, and common properties have not been established. Some drugs that exhibit affinities for GSH transferases also bind to *Ah* receptors that belong, together with steroid hormone receptors and thyroid hormone receptors, to the steroid hormone receptor superfamily. Categorization from this viewpoint might suggest that GSH transferases may also bind retinoic acid and vitamin D_3, whose receptors are also members of the steroid receptor superfamily.

Rat GST-P is strongly expressed in hepatic foci, nodules, and hepatomas induced with a large number of genotoxic carcinogens, and is regarded as one of the most reliable markers for preneoplastic lesions in rat livers. Furthermore, recent studies have revealed that it is also expressed in putative initiated cells. Such cells could not be distinguished at very early stages until single GST-P-positive hepatocytes were demonstrated within a few days after injection of a single dose of carcinogens. The detection of these initiated cells by GST-P should enable the investigation of processes involved in the initiation stage. The protooncogene products *c-JUN* and *c-FOS* have been suggested to bind to TPA-responsive, element-like sequences of the GST-P gene and thus activate its expression. The related human GST-π is also expressed in preneoplastic and neoplastic tissues such as in colon, uterus, and esophagus. In addition, Pi class and other forms are increased in cell lines resistant to anticancer drugs. Although the actual roles of Pi class forms in these two cases remain to be clarified, the individual species are known to possess unique enzymatic properties: broad substrate specificity, glutathione peroxidase activity toward lipid hydroperoxides, low sensitivity to organic anion inhibitors, and high sensitivity to sulfhydryl modifiers and active oxygen species. These properties suggest that they may be related to the prevention of lipid peroxidation or oxidative stress, which are considered to play an important role during tumor promotion or in cytotoxicity due to some anticancer drugs. The finding of high amounts of GST-π in many cancer tissues as well as in drug-resistant cell lines, suggests that elevated GSH transferase expression may be of direct relevance not only to acquired resistance, but also in natural resistance. To clarify the relationship between GSH transferases and drug resistance, and to overcome the resistance related to these isoenzymes, specific inhibitors are needed. Recent progress in the definition of active sites has provided a favorable environment for the development of such inhibitors. Further studies on this enzyme family are clearly needed to obtain a better understanding of cancer prevention and therapy.

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