INCREASED EXPRESSION OF CYTOSOLIC GLUTATHIONE S-TRANSFERASES IN DRUG-RESISTANT L5178Y MURINE LYMPHOBLASTS

BY

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INCREASED EXPRESSION OF CYTOSOLIC GLUTATHIONE S-TRANSFERASES IN DRUG-RESISTANT L5178Y MURINE LYMPHOBLASTS

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ABSTRACT

The expression of cytosolic classes of glutathione S-transferases has been examined in L5178Y murine lymphoblasts either sensitive or resistant towards the model antitumor agents hydrolyzed benzoquinone mustard (HBM) or aniline mustard (AM). HBM is a quinone-containing compound with no alkylating activity, while AM is an aromatic alkylating agent. HBM-resistant cell lines L5178Y/HBM2, L5178Y/HBM10, and L5178Y/HBMR are 2.5-, 6-, and 2.9-fold less sensitive towards HBM, and demonstrate 3-, 11-, and 9-fold elevated GST activity, respectively, than the sensitive L5178Y parental cell line. The AM-resistant cell line, L5178Y/AM, is 10-fold less sensitive towards the cytotoxic activity of AM and shows a 3.6-fold elevated level of GST activity, relative to L5178Y.

Using class specific antibody probes, Western blot analysis showed elevated expression of class Alpha, class Mu, and class Pi cytosolic GSTs in both HBM- and AM-resistant cell lines, relative to expression in the sensitive L5178Y cell line. Using class specific cDNA probes, Northern blot analysis demonstrated steady-state levels of GST mRNAs that parallel closely, the protein levels of the corresponding cytosolic classes of GSTs in

these cell lines. Southern blot analysis revealed no evidence for either amplification or rearrangement of GST genes in either HBM- or AM-resistant cell lines.

In HBM-resistant cell lines the expression of class Alpha and class Mu GSTs correlated with level of resistance. However, expression of the class Pi GST, which correlates more closely with GST activity, questions the relative importance of this GST class in resistance towards HBM. Unlike L5178Y/AM, HBM-resistant cell lines are sensitive to the aromatic alkylator AM. In L5178Y/AM the class Mu, class Pi, and class Alpha GSTs showed relative levels of expression that were similar, reduced, and greater than 3-fold, respectively, the expression observed in the HBM-resistant cell line, L5178Y/HBM10. This difference in the expression profile of cytosolic GSTs between HBM- and AM-resistant cell lines emphasizes how the structural character of inducers, and hence the nature of their inductive electrophilic signal, may influence the pattern of GST induction and perhaps resistance, in cell lines resistant to different cytotoxic agents.

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LIST OF ABBREVIATIONS

Ah	aryl hydrocarbon
AHH	aryl hydrocarbon hydroxylase
AM	aniline mustard
AP-1	activator protein 1
ARE	antioxidant responsive element
BaP	benzo(a)pyrene
BaPDE	benzo(a)pyrene-(anti)-7,8-dihydrodiol-9,10 epoxide
BCIP	5-bromo-4-chloro-3-indoyl phosphate
BCNU	N,N′-bis-(2-chloroethyl)-N-nitrosourea
BDM	benzoquinone dimustard
BHA	butylated hydroxyanisole
BM	benzoquinone mustard
bp	base pair
BSA	bovine serum albumin
BSO	buthionine sulfoximine
CAT	chloramphenicol acetyltransferase
CDNB	1-chloro-2,4-dinitrobenzene
DEPC	diethyl pyrocarbonate
EpRE	electrophile responsive element
ER	estrogen receptor
g	gram
GSH	glutathione (reduced)

GSH-Px	selenium-dependent glutathione peroxidase
GST	glutathione S-transferase
GST-Px	selenium-independent glutathione peroxidase
HBM	hydrolyzed benzoquinone mustard
HHM	hydrolyzed hydroquinone mustard
L	liter
LB	luria-bertani
M	molarity
mA	milliampere
mL	milliliter
mm	millimeter
mM	millimolar
NBT	nitro blue tetrazolium
NF	naphthoflavone
nmol	nanomole
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PMA	phorbol 12-myristate 13-acetate
RE	restriction enzyme
RFLP	restriction fragment length polymorphism
SDS	sodium dodecyl sulphate
SE	standard error
SS	salt-saturated
SSPE	NaCl, NaH_2PO_4, disodium ethylenediaminetetra-

	acetate
TBE	tris base, boric acid, disodium ethylene-diaminetetraacetate
t-BHQ	*tert*-butylhydroquinone
TBS	tris buffered saline
TE	tris base, disodium ethylenediaminetetra-acetate
TPA	phorbol 12-O-tetradecanoate 13-acetate
µg	microgram
µL	microliter
µm	micrometer
µM	micromolar
V	volt
v/v	volume/volume
w/v	weight/volume
XRE	xenobiotic responsive element

1. INTRODUCTION

1.0 Mammalian cytosolic glutathione S-transferases: nomenclature, structure, function, and distribution

1.0.0 Nomenclature

The systematic naming of the multiple isozymes of glutathone S-transferases (GSTs) is an area of confusion. This has resulted from the introduction and use of a number of different systems of nomenclature by various authors and the lack of concensus on adoption of a single system.

Early attempts at classifying GSTs were based on substrate specificity and resulted in a system of nomenclature based on the type of substrate that was conjugated to GSH: aryltransferase, epoxidetransferase, alkyltransferase, aralklytransferase, and alkene-transferase (1). However, this system was abandoned when it became clear that GSTs exhibited broad and therefore overlaping substrate specificites.

On the basis of their reverse order of elution from a carboxymethylcellulose exchange resin, Jacoby and co-workers used letter designations (AA, A, B, C, D, E) to identify the six distinct isozymes that had been purified

from rat liver (2,3,4).

One of the most useful systems was initially proposed by Mannervik and Jensson (5) who, upon recognition that cytosolic GSTs are dimeric enzymes composed of subunits with distinct substrate specificities, suggested that the multiple forms of GST should be named on the basis of their subunit composition. This system was subsequently adopted for the naming of the multiple forms of glutathione S-transferases in the rat (6). Distinct subunits were to be identified by an Arabic numeral, with new subunits numbered in order as they are identified and characterized. On this basis, the naming of distinct GST isozymes would reflect their subunit composition. Therefore, transferase A, which is composed of two identical subunits, is now known as glutathione S-transferase 3-3 and transferase C, composed of two different subunits (subunits 3 and 4), is now named glutathione S-transferase 3-4. This system of nomenclature has also been adopted for the multiple forms of GST in the mouse. However, in mice the numerical designation is preceeded by a letter that designates the particular strain from which the isozyme was isolated (7). Thus glutathione S-transferases C 1-1, D 1-1, and N 1-1 represent the same isozyme, but were isolated from three different strains of mice.

Another system that is commonly used in naming

cytosolic GSTs from the rat and mouse is based on the relative mobilities of different subunits in an SDS-polyacrylamide gel (8). This system was originally applied to the three separable isozymes from rat liver which were named Ya, Yb, and Yc. On the basis of immunological, catalytic, and other properties, isozymes distinct from the original three classes have been given new letters (Yk, Yn, Yp, etc.), while isozymes which are distinct but closely related to the original classes are identified by adding a numerical subscript (Yc_1, Yc_2, etc.). Difficulties exist since the application of this system has not been uniform in the rat and mouse species. For example, two closely related subunits in the mouse have been named Ya_1 and Ya_3. Although the mouse Ya_1 subunit is closely related to the rat Ya subunit, the mouse Ya_3 subunit is more closely related to the rat Yc rather than the rat Ya subunit. Therefore direct comparisons between species in the literature must be approached with caution. A further problem with this approach is that the relative mobilities of some subunits (rat Ya, Yk, and mouse Ya_1) are dependent upon the degree of cross-linking in the polyacrylamide gel (9,10).

GSTs in the mouse have also been named using other systems which, although not in general use, require familiarity since they are often refered to in the literature. Benson *et al.* (11) named isozymes purified

from rat liver on the basis of their isoelectric points (GT8.7, GT8.8a, GT8.8b, GT9.0, GT9.3, GT10.3, and GT10.6), while Warholm *et al.* (7) named isozymes purified from rat liver in order of their elution from an affinity column (MI, MII, and MIII). Isozymes purified from rat liver by Lee *et al.* (12) were named according to their increasing isoelectric points (F1, F2, F3). The relationships of these isozymes with those named according to the nomenclatures of Jakoby *et al.* (6) and Bass *et al.* (8) are outlined in Table 1.

GSTs in man have been classified according to their isoelectric points using Greek letters. Originally, five basic isozymes were identified and named in order of increasing isoelectric point: α, β, γ, δ, and ε (13). Near-neutral and acidic isozymes have been designated as μ (14) and π (15), respectively. Both the μ and π isozymes are distinct from one another and from the basic isozymes, but the basic isozymes have virtually identical immunologic, catalytic, and physiochemical properties and were thought to represent charge isomers (13). Stockman *et al.* (16,17) have subsequently shown that the basic isozymes, GST(α-γ) (B_2B_2), GSTδ (B_1B_2), and GSTε (B_1B_1) result from the dimeric combination of two subunits termed B_1 and B_2. Human GSTs have also been named by numbering them according to their gene loci (18,19). Using this approach the μ, α-ε, and π isozymes, were named GST1,

GST2, and GST3, respectively. Isozymes B_1B_1 and B_2B_2 have been named GST2-type1 and GST2-type2, respectively, given that the subunits are now believed to be the products of two distinct genes (20) encoded at the GST2-1 (B_1) and GST2-2 (B_2) loci (18,21). An allelic variant of the near-neutral isozyme (μ) exists and has been designated ψ. These allelic variants also have been termed GST Mu1a-1a (μ) and GST Mu1b-1b (ψ) (22). In addition, GST4, GST5, and GST6, believed to be products of additional gene loci, have been recently identified (23,24,25). GST1, GST4, GST5, and GST 6 belong to a family of cytosolic isozymes that have been designated as class Mu GSTs.

In addition to the systems of nomenclature for individual isozymes of glutathione S-transferase, studies suggest that isozymes from both man and mouse can be grouped into three distinct classes based on their immunologic, enzymatic, and physiochemical characteristics (7,26,27,28). A comprehensive review of substrate specificities, sensitivities to inhibitors, immunologic reactivities, and available primary protein sequences led Mannervik *et al*. (29) to propose a division of the cytosolic GSTs of the rat, mouse, and man into three distinct species-independent classes. Borrowing from the nomenclature of the human isozymes, the three classes of the species-independent system of classification were designated class Alpha, class Mu, and class Pi. Although

members of a given class represent separate and distinct isozyme species, they share similar enzymatic, immunologic, and structural characteristics, but differ significantly from isozymes belonging to other classes. A fourth class of cytosolic GST has been recently identified and has been designated class Theta (30,31). Finally, microsomal GST (32,33,34,35) appears to have characteristics that are significantly different from the four cytosolic classes of GST that have been identified and may therefore represent a fifth class of GST (29,31).

Table 1 summarizes the nomenclature and class divisions of the cytosolic GSTs in the rat, mouse, and man.

1.0.1 Structure-Function

The glutathione S-transferases (EC 2.5.1.18) comprise a family of multi-functional isozymes that catalyze the conjugation of the tripeptide glutathione (L-γ-glutamyl-L-cysteinyl-L-glycine) with a wide variety of electrophilic endogenous and xenobiotic compounds. Conjugation involves nucleophilic attack by the sulfur atom of glutathione (GSH) at an electrophilic site of the substrate. Numerous reviews on glutathione S-transferases and the multitude of electrophiles capable of being conjugated to glutathione have been published (36-43). The cytosolic GSTs are

Table 1. Nomenclature and class divisions of glutathione S-transferases in the rat, mouse, and man.

Species	CLASS			
	Alpha	Mu	Pi	Theta
	Subunit	Subunit	Subunit	Subunit
	1a (Ya$_1$) 1b (Ya$_2$) 2 (Yc) 8 (Yk) 10 (Yl,Yc$_2$)	3 (Yb$_1$) 4 (Yb$_2$) 6 (Yn$_1$,Yb$_3$) 9 (Yn$_2$) 11 (Yo)	7 (Yf)	5 12 13
	Isozyme	Isozyme	Isozyme	Isozyme
Rat[a,b]	1-1 (YaYa) (L$_2$) (Ligandin)	3-3 (Yb$_1$Yb$_1$) (A$_2$) (A)	7-7 (YpYp) (YfYf) (P)	5-5 12-12
	1-2 (YaYc) (BL) (B)	3-4 (Yb$_1$Yb$_2$) (AC) (C)		13-13
	2-2 (YcYc) (B$_2$) (AA)	4-4 (Yb$_2$Yb$_2$) (C$_2$) (D)		
	8-8 (YkYk) (K)	6-6 (Yn$_1$Yn$_1$) (Yb$_3$Yb$_3$)		
	10-10 (YlYl) (Yc$_3$Yc$_3$)	9-9 (Yn$_2$Yn$_2$)		
		11-11 (YoYo)		

[a] Data compiled from the following references: 30, 31, 105, 108, 126, and 203.

[b] Data in parenthesis represents alternative nomenclature for a given GST subunit or isozyme.

[c] Data compiled from the following references: 7, 10, 11, 57, 58, and 120.

[d] Sub., subunit.

Table 1 continued.

Species	CLASS						
	Alpha		Mu		Pi		Theta
	Sub.[d]	Isozyme	Sub.	Isozyme	Sub.	Isozyme	----
Mouse[b,c]	Ya1	Ya1Ya1	Yb1	Yb1Yb1 (GT8.7)	Yf	YfYf (GT9.0)	
	Ya2			(GT8.8a)		(F1,F2)	
	Ya3	Ya3Ya3 (MI) (GT10.6)		(GT8.8b) (MIII) (F3)		(MII)	NI[e]
			Yb2				
		GT10.3	Yb4	GT9.3			
			Yb5				

Species	Alpha		Mu	Pi	Theta
		Isozyme	Isozyme	Isozyme	Isozyme
Man[b,f]	αβγ	(B2B2) (GST2-type2)	μ(GST Mu1a-1a)	π(GST3)	θ(T1-1*)
			ψ(GST Mu1b-1b)		?(T2-2*)
	δ	(B1B2)	GST4		
	ε	(B1B1) (GST2-type1)	GST5		
			GST6		

[e]NI; not identified. Although not yet formally identified and characterized, mouse (and hamster) liver does contain a GST fraction that does not bind to GSH-agarose and which shows substantial reactivity towards 1,2-epoxy-3(p-nitrophenyl)propane. These characteristics are consistent with class Theta GST (30).

[f]Data compiled from the following references: 16-25, 31, and 131.

dimeric enzymes and subunits that belong to the same class can associate to form both homo- and heterodimeric species. Heterodimers composed of subunits belonging to different classes are not known. The kinetic and catalytic aspects of GSTs have been summarized in reviews by Mannervik (38) and Mannervik and Danielson (40). Each subunit of a dimeric cytosolic GST contains a single active site. The active sites are kinetically independent from each other and are comprised of separate binding sites for GSH (G-site) and the electrophilic substrate (H-site). The H-site is hydrophobic, which is consistent with the overall hydrophobicity of elecrtophilic substrates in general. Catalysis by GSTs is thought to involve: a) a base assisted deprotonation of the thiol group of the cysteine residue of GSH, which increases the nucleophilicity of the attacking sulfur atom and b) activiation of the electrophilic site of the substrate, possibly by a protonation reaction (40). Many of the electrophilic substrates of GSTs are α,β-unsaturated carbonyl compounds and protonation of the carbonyl oxygen would enhance the electrophilicity of the β-carbon. While nucleophilic attack by GSH was once thought to occur only at an electrophilic carbon, Jakoby and Habig established that oxygen, nitrogen, and sulfur could serve as alternative electrophilic sites in compounds such as hydroperoxides, nitrate esters, thiocyantates, and

disulfides (44).

The primary structures for many of the GST subunits in the rat (45-53), mouse (54-59), and man (25,60-64) have been determined. Where complete amino acid sequences are known, many have been deduced from the nucleotide sequence of the corresponding cDNA. However, standard protein sequencing techniques were used to elucidate the primary structures of rat microsomal (65), rat Yb_2 (48), and mouse Ya_1, Ya_3, Yb_2, and Yb_5 (57,58) subunits. Details regarding amino acids of critical importance to the binding and catalytic activity of the acitive site remain unclear, but along with the available primary structures, the recent determination of the three-dimensional structures of pig class Pi (66) and rat class Mu (67) GSTs are beginning to shed light on this issue. The binding of GSH to the G-site is thought to involve ionic interactions with the charged amino and carboxyl groups of glycine and glutamic acid, respectively. Using site-directed mutagenesis, Stenberg *et al*. (68) replaced arginine residues (known from primary sequence data to be conserved in all cytosolic classes of GST) with alanine at postitions 13, 20, and 69 in human GST α and found a significant loss in catalytic activity. Kinetic data indicated that the enzyme affinity for GSH was significantly reduced in the mutants, suggesting the importance of these amino acids in the binding interaction with GSH. The three-dimensional structure of a pig GST-π:

glutathione-sulfonate crystal, as reported by Reinemer *et al.* (66), implicated residues Arg13, Gln64, and Asp96 as important for binding glutathione, whereas the close proximity of the hydroxyl group of Tyr7 to the sulfhydryl group of GSH implicated a role for this residue in catalysis. Site-directed mutagenesis studies of similiar residues (Arg13, Gln64, Asp98, and Tyr7) in a human GST-π (69,70) and of Tyr6 in rat GST 3-3 (67), support both these predictions. The study by Liu *et al.* (67) indicated that Tyr6 appears to play an important role in catalysis by lowering the pKa of the sulfhydryl group of GSH and by stabilizing the thiolate anion after deprotonation.

Booth *et al.* (71) were among the earliest to demonstrate the enzyme catalyzed conjugation of electrophiles with glutathione. Later, Boyland and Chasseaud suggested that glutathione and glutathione S-transferases function to protect cellular constituents from the toxicity of reactive electrophiles (1,72). Due to their electrophilic nature many endogenous metabolites and xenobiotics (including carcinogens) can react with critical intracellular targets such as DNA, RNA, or protein, causing cellular toxicity or perhaps carcinogenesis. Intracellular glutathione acts to protect such intracellular targets from reactive electrophilic compounds by acting as an alternative nucleophilic site. Conjugation to glutathione results in the stabilization

and hence reduced cytotoxicity (or carcinogenicity) of toxic electrophiles and renders these compounds more hydrophilic and therefore more easily excretable from the cell. In the liver, glutathione conjugates may be directly excreted into the bile or they may be metabolized to mercapturic acids, classical excretion products of xenobiotics, which are then excreted and eliminated from the body in the bile or urine (37,38). Alternatively, cysteine conjugates resulting from the enzymatic cleavage of glycine and glutamic acid from the original glutathione conjugate may be converted into mercaptans, and after glucuronosylation or methlyation, the resulting thioglucuronide or methylthio derivates are then excreted from the cell (38). These pathways are summarized in Figure 1.

In addition to their role in the detoxication of xenobiotics and endogenous metabolites, GSTs are also intracellular binding proteins. Litwak *et al.* (73) were the first to recognize that in liver, the cytosolic binding proteins known as Y-protein (which binds bilirubin), corticosteroid 1 binding protein, and azo dye carcinogen binding protein, were one and the same. This protein was subsequently renamed ligandin, a name which reflects the wide range of compounds that bind to it, including bilirubin, certain steroids, heme and its

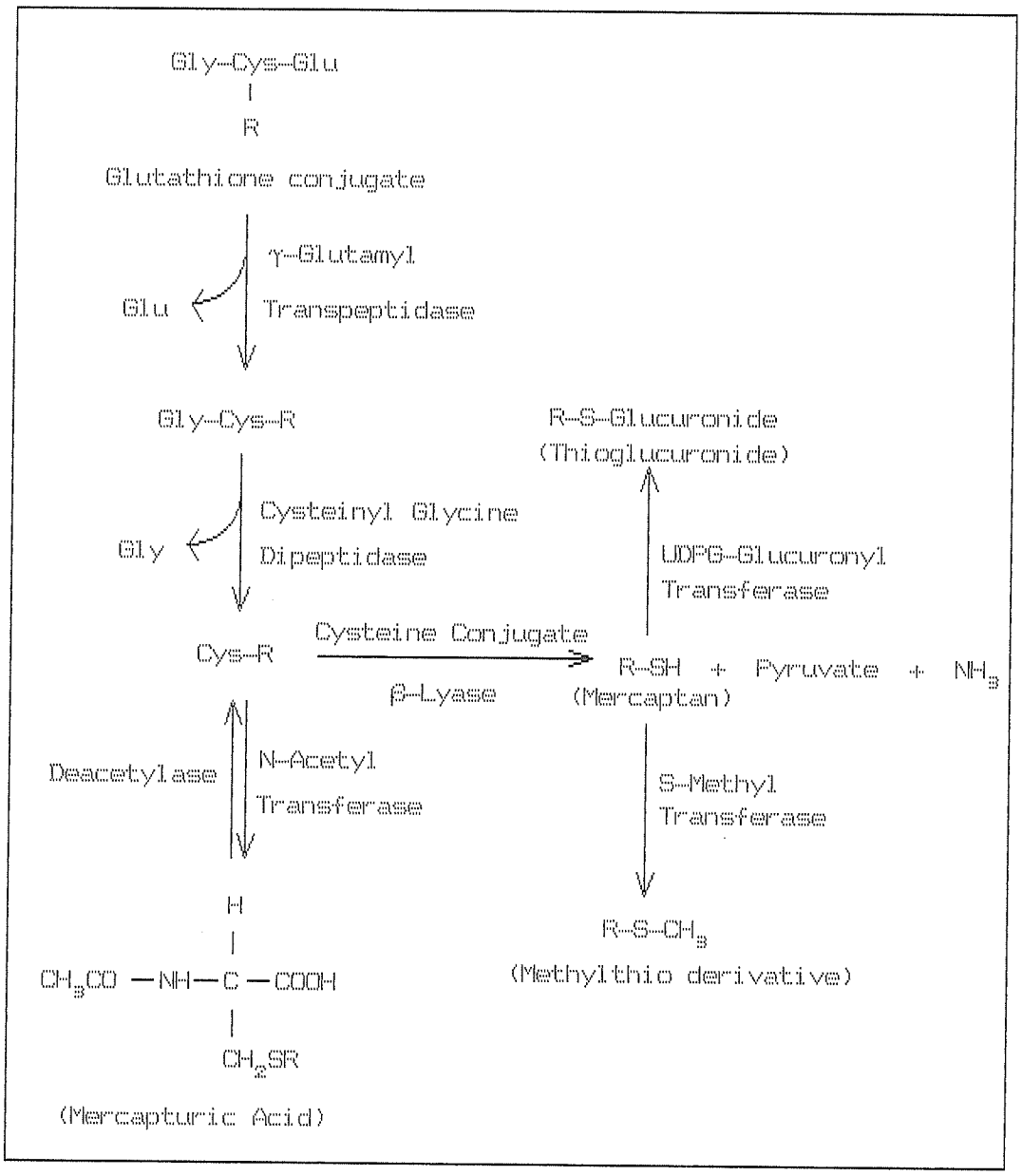

Figure 1. Pathways involved in the conversion of glutathione conjugates to soluble excretion products. The formation of mercapturic acids, thioglucuronides, and methylthio derivatives (From Pickett and Lu (126)).

derivatives, lipophilic antibiotics, and numerous others (74-77). The link between ligandin and the ability of GSTs to serve as intracellular binding proteins came when Habig *et al.* (78) showed that ligandin was identical to rat liver glutathione S-transferase B. Subsequently, a similar binding capacity for GSTs other than transferase B was demonstrated by Ketley *et al.* (79). GSTs are thought to play an important role in hepatocytes through their ability to bind and solubilize bilirubin, much as does albumin in plasma. In addition, through their capacity as binding proteins, GSTs are thought to serve as intracellular storage sites and may therefore influence the net uptake and efflux of compounds from the cell (74).

Left unchecked, cellular lipid peroxidation will lead to increased leakiness and decreased fluidity of membranes, damage to membrane proteins, and eventual loss of membrane integrity. Fatty acid hydroperoxides are natural products of the peroxidation process and can further stimulate the chain propagation steps of lipid peroxidation. Both selenium-dependent (GSH-Px) and selenium-independent glutathione peroxidases (GST-Px) exist in the cytosol and mitochondria of cells and GST-Px activity is known to be associated with cytosolic glutathione S-transferases. Together, these enzymes detoxify H_2O_2 (which also stimulates lipid peroxidation in cells) and organic hydroperoxides by reducing them to more

stable alcohols (ie. ROOH to ROH and H_2O, where R = H in the case of H_2O_2; for a review of free radicals and lipid peroxidation, see ref. 80). Although both organic hydroperoxides and H_2O_2 are substrates of GSH-Px, H_2O_2 is not a substrate of GST-Px. Tan *et al.* (81) have demonstrated the inhibition of microsomal lipid peroxidation by class Alpha GST and both linoleate and arachidonate hydroperoxides are known substrates for GSTs (82-84). Thus GSTs may be of importance in the inhibition of lipid peroxidation by detoxication of fatty acid hydroperoxides. In addition, aldehydes such as malonaldehyde and hydroxyalkeneals can react with amino and sulfhydryl groups of proteins, causing enzyme inhibition, and through cross-linking, the aggregation of membrane proteins with subsequent loss of function. These and many other low molecular weight products from the breakdown of fatty acid hydroperoxides are known to inhibit protein systhesis and to interfere with cell growth (80). Some like cholesterol α-oxide (81) and the very toxic 4-hydroxyalkeneals (85) are known substrates of GSTs. Therefore, GSTs may also be important in the detoxication of at least some of the breakdown products of fatty acid hydroperoxides and overall, may play an important role in protecting cells from the effects of oxidative stress.

Finally, the discovery that both leukotrienes and prostaglandins can serve as substrates for GSTs has

created interest in the role of GSTs in the synthesis of these physiologically significant compounds (38,86-90).

1.0.2 Species and tissue specific distribution of cytosolic GSTs

Although extensively studied in the rat, mouse, and man, GSTs have also been investigated in the hamster, guinea pig, cow, monkey, sheep, trout, shark, little skate, grass hub, American cockroach, earthworm, pea seedlings, and corn (38) and also in adult and embryonic toad (91,92), squid (93,94), chicken (38,95,96) and insects (38,97). GSTs therefore appear to be widely distributed in plants and animals, and have also been identified in bacteria (98-102) and yeast (103).

The distribution of GSTs in the tissues of the rat, mouse, and man have received significant attention. Given their role in detoxication, the tissue specific distribution of GSTs is of interest, particularily in tissues such as the gastrointestinal tract, kidneys, liver, and lungs, which represent portals of entry or exit for foreign compounds and their metabolites. Although overlapping substrate specificities exist, individual isozymes vary in their relative activities towards different substrates, particularily for isozymes belonging to different GST classes. Therefore, the isozyme content

of a tissue may be a determinant of its relative sensitivity and/or capacity for detoxication of potentially toxic agents.

Early studies concentrated on the measurement of GST activities in different tissues and species using a variety of substrates (37). With improved methods of purification and an increasing availability of class specific antibody and cDNA probes, the tissue specific expression of distinct isozymes could be examined in greater detail.

The tissue specific distribution of GSTs has been most extensively studied in the rat, mouse, and man. Tissues examined include brain (b,c,d,e,i,k), gastro-intestinal tract (b,e,h,l), kidney (b,e,h,j,k), liver (a,b,c,e,f,g,h,j,k), lung (b,e,h,j,k), and spleen (b,e,j) in the rat (38[b],42[e],49[d],104[j],105[k]), mouse (10[a],55[e],57[f], 58[g],59[h]), and human (38[b],25[i],106[l]), the adrenal gland (b,c), erythrocyte (b,c), heart (b,c,j,k), and testis (b,j,k) in the rat (38[b],42[e],104[j],105[k]) and human (38[b]), the epididymis (b,j), olfactory epithilium (m) and biliary epithilial cells (n) in the rat (38[b],104[j],105[k],107[m],108[n]), and lens (b), ovary (b), pancreas (c), placenta (b), platelet (c), prostate (q), skeletal muscle (o), skin (p), and thyroid (c) in humans (38[b],42[e],109[e], 110[p],111[q]).

Given its importance in the detoxication of foreign compounds, liver tissue has been the subject of a vast

number of studies. Rat liver has been shown to contain significant amounts of subunits 1(Ya), 2(Yc), 3(Yb$_1$), and 4(Yb$_2$) and minor quantities of subunits 6(Yb$_3$), 8(Yk), and 10(Yl)(105,112). Subunit 7(Yp) is generally absent or found only in trace quantities in adult rat liver, but is expressed significantly during hepatocarcinogenesis (82, 113,114). Subunits designated Ya$_1$, Ya$_2$, Ya$_3$, Yb$_1$, Yb$_2$, Yb$_3$, and Yf have been demonstrated in mouse liver (57,58) and in human liver, basic (α-ϵ) and near-neutral (μ and ψ) isozymes are present (13,14,22). Similar to the rat Yp subunit, the human anionic (π) isozyme (both belong to class Pi) is either absent or expressed at a low level in the liver (115).

The examination of liver and extrahepatic tissues has demonstrated a number of important conclusions. First, it is apparent that the isozyme content can vary significantly from tissue to tissue. For example, the class Pi GST in the rat (Yp) and human (π), which are absent or found only at trace levels in liver, are expressed at significant levels in most extrahepatic tissues that have been examined (38,50,105). In the rat, subunit 1 is generally absent in lung, heart, testis, and brain (38,105), whereas subunits 3 and 6 are absent or present only in trace amounts in kidney (38,105). Although rat testis is rich in subunits 6 and 9, in rat liver, subunit 9 is absent and subunit 6 is present in only minor

amounts (42,105). In humans, skeletal muscle has been shown to contain only class Pi and class Mu GSTs (109) whereas the class Mu isozyme, GST5, has been identified only in human lung and brain (23,117). Many other examples of the variability in the isozyme content of tissue exist. One of the more interesting findings from studies of human tissues is that approximately 50% of the human population expresses the null phenotype at the GST1 locus and therefore expresses neither μ or ψ (class Mu) GSTs (18,19, 116,117). It is interesting, given the high specificity of these isozymes towards mutagenic epoxides (14,26), that smokers who lack these enzymes appear to have a higher susceptibility towards the development of lung cancer (118,119).

An examination of mouse liver has demonstrated that isozyme expression in certain tissues can be sex-specific. Similar to the YpYp and π isozymes in rat and human liver, respectively, the mouse YfYf isozyme is found only in trace amounts in female liver, but is the major isozyme present in male liver (120). A recent study has also demonstrated the differential expression of GSTs in foetal versus adult rat liver (121). In contrast to adult liver, class Pi GST and mRNA are expressed significantly in foetal liver, but decline during development, becoming virtually absent in adult liver. Concentrations of isozymes and mRNA corresponding to classes Alpha and Mu

increase during foetal development, decline at birth, and then increase to levels normally found in adult liver. In addition, a significant switch in the predominance of class Alpha subunits was observed, with subunit 2 predominating in foetal liver and subunit 1 in the adult. Finally, GST isozymes show not only tissue specific patterns of distribution but also specific patterns of distribution within cell types that comprise a given tissue. For example, in rat brain, GSTs have been found only in astroglial and ependymal cells but not in neuronal stroma (122).

In examining the expression of GST mRNAs, specific cDNA probes are capable of distinguishing between members of different classes. However, due to their high sequence homology, cross-hybridization often makes it difficult to adequately discriminate between individual members of the same class. A recent approach to this problem has been the development and use of gene-specific oligonucleotide probes, which offers promise as a more efficient method for distinguishing between closely related mRNAs (123).

1.1 Glutathione S-transferases: a multigene family of isozymes

The GSTs represent a supergene family of isozymes.

Each cytosolic class of GST (Alpha, Mu, Pi, and Theta) represents a distinct gene family. Many cDNA and genomic DNA clones have been isolated and characterized, particularily in the rat. Early studies of the rat genome by Southern blot analysis suggested the presence of at least seven class Alpha genes and multiple class Mu genes (124,125). By Southern blot analysis, Okuda *et al.* (51) also demonstated the presence of multiple class Pi genes in the rat genome. Sequence analysis of five non-identical genomic clones suggested the presence of only one active gene. The others were shown to be processed-type pseudo-genes. This is consistent with the present state of knowledge which recognizes only a single class Pi GST (7-7) in the rat. GST genes in the rat have been reviewed by Pickett (50) and Pickett and Lu (126).

In comparison to the rat, GST genes have not been as well characterized in the mouse. Southern blot analysis of the mouse genome by Czosnek *et al.* (127) also indicated the presence of multiple (4-5) class Alpha genes. To date, a single GST-Ya gene (λmYa1) has been isolated (54,128) and is believed to represent the mouse Ya_1 subunit (57). A class Alpha cDNA clone (pGT41) has also been isolated (55). Its amino acid sequence is distinct from that predicted by λmYa1 and is thought to represent the mouse Ya_2 subunit (57). Three distinct class Mu cDNAs, pGT55, pGT875, and pmGT2, corresponding to subunits Yb_4, Yb_1, and

Yb$_2$, respectively, have been isolated and indicate that the mouse class Mu GSTs are encoded by a minimum of three genes (55,56). The isolation of cDNA or genomic clones has not been reported for the class Pi GST, but similar to the rat, Southern blot analysis suggests the presence of multiple genes in the mouse genome (51). Since only a single class Pi isozyme (YfYf) has been identified in mouse tissue, the presence of multiple class Pi genes in the mouse genome might reflect the situation found in the rat, ie. the existence of a single functional gene and numerous non-functional pseudogenes.

In characterizing the GSTs of human liver, Board (18) originally identified three distinct gene loci which were designated GST1, GST2, and GST3. Isozymes encoded by GST1, GST2, and GST3 have been shown to correspond to class Mu (μ,ψ), class Alpha (α,β,γ,δ,ϵ), and class Pi (π) GSTs, respectively (23,61,129,130). The patterns of GST activity after starch gel electrophoresis of tissue extracts (zymogram analysis) led Board to the conclusion that the GST1 and GST2 loci were polymorphic, with isozyme patterns obtained from liver samples of different individuals accounted for by the presence of three alleles at the GST1 locus (GST1-type1, GST1-type2, and GST1-type0 (the null allele)) and two alleles at the GST2 locus (GST2-type1 and GST2-type2) (18). However, a subsequent study by Strange *et al.* (19) was in conflict with the conclusion of genetic

polymorphism at the GST2 locus since zymogram analyses of different tissues from the same individual showed a pattern of GST activity that was not constant from tissue to tissue (expected for an allelic relationship), but rather, tissue specific. This lead to the conclusion that the multiple isozymes encoded by the GST2 locus were more likely a result of post-translational modifications of the gene product of this locus, a conclusion supported by the work of Laisney *et al.* (117). Stockman *et al.* (16,17) have now shown that the multiple basic human isozymes (α, β, γ, δ, and ε) identified by Kamisaka *et al.* (13) result from homo- and heterdimeric combinations of two distinct subunits termed B_1 and B_2, with isozymes B_1B_1 and B_1B_2 corresponding to GSTε and GSTδ, respectively. Although not completely clear, it was also suggested that isozymes GSTα, GSTβ, and GSTγ might represent autoxidation products of the B_2B_2 dimer generated during the purification process. Contrary to the conclusions of Strange *et al.* (19) and Lainsey *et al.* (117), Stockman *et al.* (16) also favoured the existence of allelic variants of the GST2 locus and suggested that subunits B_1 and B_2 represented the respective gene products of these two alleles. Southern blot analysis and *in-situ* hybridizations to human chromosomes have suggested the presence of multiple class Alpha genes in the human genome (61) and consistent with this conclusion it has now been shown that subunits B_1 and

B□ are not the products of allelic variants (GST2-type1 and GST2-type2) of the GST2 locus, but are themselves the products of distinct genes (20,60,129,131).

In addition to GST1, GST2, and GST3, a number of distinct extrahepatic isozymes have been identified. Laisney *et al* (117) first described the existence of a GST abundant in skeletal muscle (GST4) and a second isozyme (GST5) found in brain and lung tissue. Zymogram studies indicated that these gene loci were distinct from GST1, GST2, and GST3, and were not polymorphic. A third isozyme, GST6, has been described by Suzuki *et al*. (24). On the basis of immuological and N-terminal amino acid sequence data, GST4, GST5, and GST6 appear related to GST1 and therefore represent additional human class Mu GSTs (24,25,132). The existence of multiple class Mu GSTs in man is consistent with Southern blot data of genomic DNA which also indicate the presence of at least three class Mu genes in the human genome (132).

At present, the situation for human class Pi GST genes appears to be similar to that seen in the rat and mouse. The anionic class Pi GST that is expressed in a majority of human tissues appears to be the product of a singe gene encoded at the GST3 locus. The human GST-π gene is very similar to rat GST-P (63,64). Both contain 7 exons separated by 6 introns, there is a high degree of sequence homology among the exons, and the sizes of exons 2-7 are

precisely preserved between the two species. In addition to a functional class Pi gene, like the rat and mouse, class Pi pseudogenes are also apparent in the human genome (51,63,64).

1.2 Induction and regulatory control of glutathione S-transferases

Many carcinogens and other xenobiotics are metabolized through at two step process which involves Phase I and Phase II drug detoxication enzymes (133,134). Phase I enzymes, which include the cytochromes P-450, convert compounds to reactive electrophiles through the introduction of functional groups by oxidations, reductions, or hydrolysis. This process is responsible for the conversion of many carcinogens to what is their active carcinogenic form. Phase II enzymes, which include conjugating enzymes such as glutathione S-transferases, detoxify reactive electrophiles to more stable, less reactive forms, by conjugation to endogenous ligands such as glutathione, glucuronic acid, or sulfate. Quinone reductase is also classified as a Phase II enzyme and detoxifys quinones by reducing them to the more stable hydroquinone form.

In response to an appropriate inductive signal the

activities and corresponding isozyme and mRNA levels of glutathione S-transferases will significantly increase in tissues (11,55,135-141). Inducers of Phase II enzymes have been classified by Wattenberg as either type A or type B (134), or alternatively the terms monofunctional (type A) or bifunctional (type B) have been used (133). Bifunctional inducers are large planar aromatic compounds such as polycyclic aromatic hydrocarbons, flavinoids, azo dyes, and TCDD, which induce both Phase I and Phase II enzymes. In contrast, monofunctional inducers, which include a wide range of structurally diverse compounds (142), induce Phase II enzymes only.

There is a great deal of interest in compounds (chemoprotectors) which possess the ability to confer protection against chemical carcinogenesis (134,143). Many of these compounds are efficient inducers of Phase II enzymes and the over-expression of these enzymes in tissues after the administration of chemoprotectors is believed to be an important mechanism through which such compounds offer protection against chemical carcinogens (134,144). In fact, studies have demonstrated a correlation between the induction of GSTs and protection against chemical carcinogenesis (145,146). In contrast to the chemoprotective effect of elevated Phase II enzyme activities in normal tissue, elevation of these enzymes in tumor tissue may contribute to resistance towards chemo-

theraputic agents. Indeed, there is mounting evidence for an involvement of Phase II enzymes such as GSTs in the development of drug resistance in tumor cells.

Given the potential importance of Phase II enzymes in the areas of chemoprotection and drug resistance, a significant amount of research has focused on understanding: a) the cellular mechanisms involved in the induction of Phase II enzymes by mono- and bifunctional inducers, b) the underlying structural feature(s) that unite a wide range of compounds through their ability to induce Phase II enzymes, and c) the regulatory elements controlling the activation of genes that encode Phase II enzymes such as GSTs.

Monofunctional inducers derive from a wide range of structurally diverse compounds and include: a) hydroquinones, catechols, and their derivatives, b) acrylate, crotonate, and cinnamate analogues, c) methyl and ethyl esters of fumarate, maleate, and itaconate, and d) sulfur compounds such as isothiocyanates, 1,2-Dithiol-3-thiones, allyl sulfides, and thiocarbamates (133,142). Studies by Talalay and co-workers have identified a common structural feature of monofunctional inducers that unites them in their capacity to induce Phase II enzymes (139,142,147). These compounds contain or aquire through subsequent metabolism, an electrophilic center. Many, including quinones, catechols, and their derivatives are recognized

as Michael reaction acceptors and contain an olefinic bond rendered electrophilic through conjugation with an electron withdrawing substituent. This structural feature was shown to be a required feature of compounds capable of inducing the activity of the Phase II enzyme, quinone reductase, in Hepa 1c1c7 murine hepatoma cells (142).

Based on studies with Hepa 1c1c7 cells and mutants possessing either a defective Ah receptor or a defective AHH gene transcript, Prochaska and Talalay (133) proposed a model for the induction of Phase II enzymes by mono- and bifunctional inducers (Figure 2). In this model, bifunctional inducers are thought to induce Phase II enzymes through two alternative pathways. In the Ah receptor dependent pathway the bifunctional inducer binds to the Ah receptor and the receptor-ligand complex then binds to specific regulatory region(s) of the gene, causing transcriptional activation and increased expression of Phase II enzymes. In the Ah receptor independent pathway, Ah receptor mediated induction of AHH activity by bifunctional inducers leads to the eventual metabolism of these inducers. Similar to monofunctional inducers, metabolites resulting from the metabolism of bifunctional inducers contain an electrophilic center which acts as a signal, through an as yet incompletely understood mechanism, to cause the transcriptional activiation of Phase II enzymes.

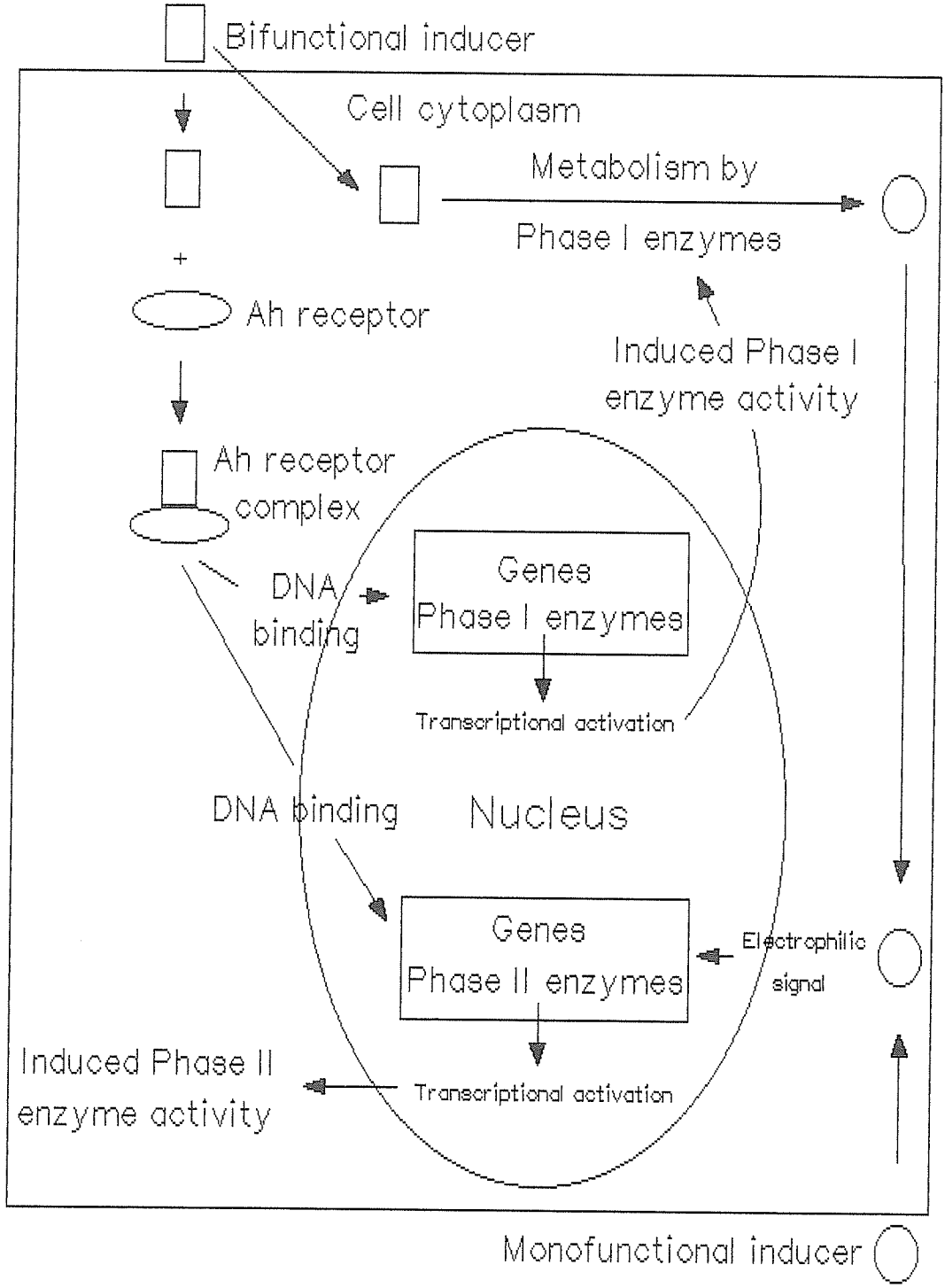

Figure 2. Mechanisms of induction of Phase I and Phase II detoxication enzymes by bifunctional and monofunctional inducers (Prochaska and Talalay (133)).

Strong support for the basic tenants of this model has come from studies involving CAT assays of HepG2 and variant mouse cell lines that were transfected with constructs prepared by fusing the 5′-flanking region (or deletional mutants thereof) of the GST-Ya gene with the pSVoCAT structural gene. These studies demonstrated the presence of regulatory elements in the 5′-flanking region of the rat GST-Ya gene that have been termed the XRE and ARE (148-150). GST-CAT constructs which contained both the XRE and ARE were responsive to both mono- and bifunctional inducers. A number of deletional mutants of the 5′-flanking region of the GST-Ya gene, lacking either the ARE (XRE-CAT) or XRE (ARE-CAT) regulatory elements, were also prepared. The XRE-CAT constructs were found to be Ah receptor dependent, contain the core XRE sequence found in multiple copies of the P-450 IA1 gene, and were responsive only to bifunctional inducers. In contrast, ARE-CAT constructs were shown to be responsive to both mono- and bifunctional inducers, but in an Ah receptor independent manner. The ARE-CAT constructs were responsive to mono-functional inducers in both normal cells and mutants which lack functional Ah receptors or which possess a non-functional P-450 IA1 gene product. The ARE-CAT constructs were also responsive to bifunctional inducers but only in normal cells with funtional Ah receptors and P-450 IA1 protein. Being Ah receptor independent, the responsiveness

of ARE-CAT constructs to bifunctional inducers in normal but not in mutant cells was consistent with the model proposed by Prochaska and Talalay, in which bifunctional inducers acting via the ARE must first be metabolized to electrophilic compounds resembling monofunctional inducers. Similar studies of the 5′-flanking region of a mouse GST-Ya gene have been carried out by Friling *et al.* (151). Results demonstrated the presence of a regulatory element termed the EpRE, which was similar to the ARE in its ability to cause increased activity of GST-CAT constructs in response to monofunctional and metabolizable bifunctional inducers. However, the 5′-flanking region of the mouse GST-Ya gene did not appear to contain an XRE like that found in the rat GST-Ya gene. Further characterizations of both the ARE and EpRE have shown that these regulatory elements contain a core sequence, different from that of the XRE, but similar to the consensus sequence of the AP-1 binding site found in both PMA- and TPA-responsive elements of phorbol ester inducible genes (152,153). A similar AP-1 like sequence has also been found in the GPEI enhancer element of the rat GST-P gene (154,155).

Talalay *et al.* (142) have previously shown the ability of compounds with wide structural diversity, but united through their electrophilic character, to induce Phase II enzymes. Prestera *et al.* (156) have recently

demonstrated the capacity of a 41 bp enhancer element, which contains the core Ap-1-like sequence from the EpRE of the mouse GST-Ya gene, to respond to just such a wide variety of compounds. This 41 bp enhancer element was fused to a growth hormone reporter gene and the construct (p41-284GH) transfected into HepG2 cells. Growth hormone production was induced in the transfected cell line after exposure to 28 out of 34 compounds. It is therefore clear that GST genes contain regulatory elements that are responsive to an appropriate chemical (inductive) signal, causing transcriptional activation of the gene. Whether such regulatory elements also exist in class Mu GST genes remains to be determined, but the identification of both the XRE and ARE in the 5′-flanking region of both the rat and human quinone reductase genes (157,158) lends further support for the importance of these regulatory elements in the transcriptional activation of Phase II enzymes by mono- and bifunctional inducers.

The mechanism by which the electrophilic character of monofunctional inducers mediates the transcriptional activation of GST and quinone reductase genes through regulatory elements such as the ARE, EpRE, or GPEI, is not clearly understood at this time. It is possible that the inducers could interact directly with these regulatory elements or alternatively, they might modulate the abundance or binding activity of specific trans acting

factor(s) which then bind to these regulatory elements to cause transcriptional activation of the gene. Studies have indicated the existence of nuclear factors capable of binding to these regulatory elements, therefore favoring the latter possibility. DNase I footprinting experiments have shown protection by nuclear protein(s) over the regions of DNA containing the EpRE and ARE sequences of mouse and rat GST genes, respectively (151,159). Gel shift assays also demonstrate the ability of the ARE and EpRE sequences of GST-Ya genes and the GPEI sequence of the rat GST-P gene, to bind protein(s) from nuclear extracts (151,155,159,160). The transcription factors c-Jun and c-Fos form homo- and heterodimeric species which are known to bind to AP-1 sites (161). Given that the ARE, EpRE, and GPEI enhancer elements have been shown to contain a core sequence that is similar to the AP-1 consensus sequence, the potential involvement of c-Jun and c-Fos as binding factors that interact with these regulatory sites has been investigated. Using gel shift assays, Friling *et al.* (153) demonstrated that c-Jun and c-Fos together, but not separately, were able to bind to synthetic oligo-nucleotides corresponding to either a consensus AP-1 sequence or a 41-bp EpRE sequence containing the AP-1-like binding site. Using the ARE sequence from the rat GST-Ya gene, similar studies by Nguyen and Pickett (159) demonstrated the binding of nuclear factors to the ARE,

but did not favor the involvement of Jun or Fos in the binding interaction with the ARE. Using nuclear extracts of Hepa-1 cells and anti-c-Jun, -c-Fos, and -Jun-D antisera in gel supershift assays, Li and Jaiswal (160) demonstrated the interaction of c-Fos and Jun-D, but not c-Jun, with the ARE sequence from the human quinone reductase gene. However, not all the nuclear protein in control bands (ARE + Hepa-1 nuclear extract only) could be supershifted, suggesting that unrelated proteins or other members of the Fos and Jun families might be involved in the binding interaction with the ARE. At this point the exact nature of the nuclear protein(s) involved in the binding interaction with the ARE and EpRE remain unresolved.

The relationship between mono- and bifunctional inducers and the XRE and ARE regulatory elements of GST and quinone reductase genes is summarized in Figure 3.

1.3 Glutathione S-transferases and drug resistance in tumor cells

1.3.0 The correlation between GSTs and drug resistance

The over-expression of Phase II enzymes such as GSTs

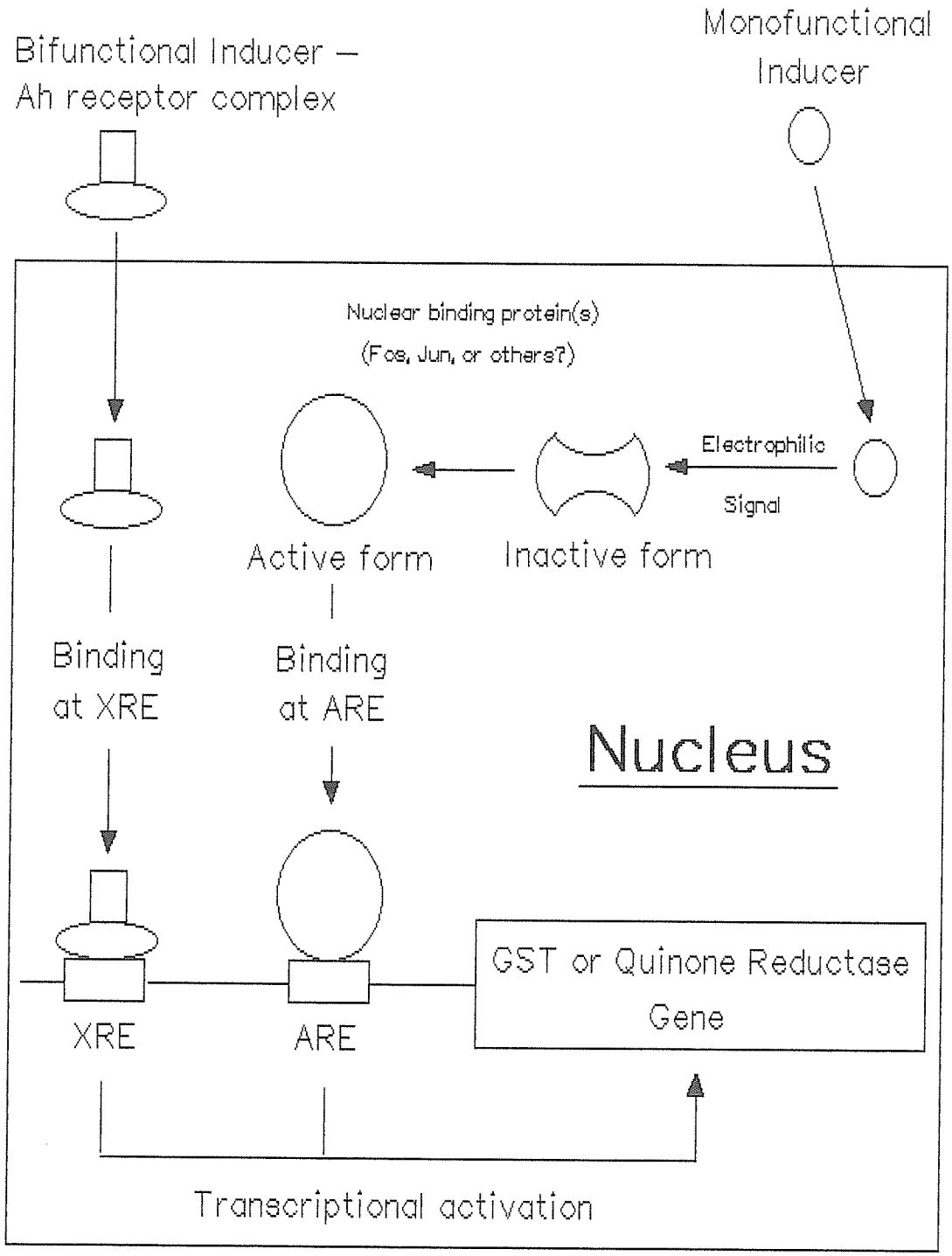

Figure 3. Transcriptional regulation of GST and quinone reductase genes via the xenobiotic (XRE) and antioxidant (ARE) responsive elements.

can represent a double edged sword. In normal cells, an increased expression of drug detoxication enzymes has been shown to protect against the cytotoxic effects of many xenobiotics, including chemical carcinogens, and has caused a significant interest in the field of chemoprotection (134). However, in tumor cells, this same process may contribute to the development of drug resistance. In the treatment of malignant disease, this phenomenon, whether intrinsic or acquired, may represent a significant barrier to a successful outcome of chemotheraputic treatment regimens. Understanding the cellular mechanisms contributing to the development of drug resistance may therefore aid in the design of more effective therapies to circumvent such resistance. In this regard GSTs are of interest since numerous studies have shown that activities and expression levels of these isozymes are often increased in drug resistant cell lines.

Elevated expression levels of GSTs have often been correlated with resistance towards alkylating agents. A previous study of a Yoshida cell line resistant to cyclophosphamide demonstrated a 6-fold elevated GST activity relative to the sensitive cell line (162). The use of chlorambucil is standard in the treatment of chronic lymphocytic leukemia (CLL). After *in-vitro* exposure to chlorambucil, Johnston *et al*. (163) found that GSH levels and GST activity correlated inversely with

levels of DNA cross-linking in CLL cells obtained from patient blood samples. This suggested a role for GSTs in the protection of CLL cells from chlorambucil cytotoxicity. A correlation between chlorambucil resistance and GSTs has also been demonstrated in drug resistant cell lines. Robson *et al.* (164-166) have shown increased GST activity and an increased expression of class Alpha GSTs in a chinese hamster ovary cell line (CHO-Chl^{r}) that was 20-fold resistant towards the cytotoxic activity of chlorambucil. An Alpha class subunit with a similar electrophoretic mobility to the rat Yc (class Alpha) subunit was estimated to be 40-fold elevated in the CHO-Chl^{r} cell line. Despite a similar rate of intracellular drug acculmulation and apporxiately equivalent levels of DNA cross-linking in isolated nuclei, CHO-Chl^{r} cells demonstrated a significantly lower level of DNA cross-linking relative to the sensitive CHO-K1 cell line, after exposure to chlorambucil. Given similar rates of drug accumulation and DNA repair, a reduced level of chlorambucil induced DNA damage in CHO-Chl^{r} cells was attributed to an increased detoxication of chlorambucil, possibly resulting from the increased expression and activity of class Alpha GST. Walker 256 rat mammary carcinoma cells resistant to chlorambucil (WR) have also been shown to have elevated GST activity (167). Walker sensitive (WS) and resistant (WR) cells demonstrated

equivalent levels of expression of class Mu and class Pi GST subunits, but a specific Yc (class Alpha) subunit was over-expressed in the resistant (WR) cell line (168). Both GST activity and the over-expressed Yc subunit were reduced to levels comparable to the sensitive cell line in WR cells that had lost the resistant phenotype. The cross-resistance of CHO-Chl^{r} cells to melphalan and mechlor-ethamine (164) and the correlation between chlorambucil resistance and increased expression of class Alpha GST in CHO-Chl^{r} and WR cells suggests that class Alpha GSTs may be of significance in conferring resistance towards bifunctional nitrogen mustards. This notion is reinforced by a number of studies that have demonstrated the GST catalyzed conjugation of nitrogen mustards to GSH. Dulik *et al.* (169) have demonstrated the formation of both mono- and diglutathionyl adducts resulting from the GST catalyzed conjugation of melphalan with glutathione. A subsequent study demonstrated that the conjugation of melphalan to glutathione was catalyzed by class Alpha but not by class Mu or class Pi GSTs (170). Class Alpha GST from human liver was also shown to catalyze the conjugation of chlorambucil to glutathione more efficiently than an ovarian class Pi GST and formation of the glutathione conjugate was inhibited by the GST inhibitor ethacrynic acid (171). The role of GSTs in resistance towards alkylating agents has been reviewed by

Waxman (172).

Although class Alpha GSTs may play a role in resistance towards nitrogen mustards, their ability to confer resistance towards other alkylating agents may be of lesser significance. For example, although cross-resistant towards the bifunctional nitrogen mustards melphalan and mechlorethamine, CHO-Chl^{r} cells remain sensitive to other alkylators such as cisplatin, mytomycin C, and BCNU (164). Nitrosoureas such as BCNU are important agents in the treatment of human brain tumors but intrinsic and aquired resistance to this drug frequently hampers its effectiveness as an antitumor agent (173). Studies with rat gliosarcoma cell lines sensitive (9L) and resistant (9L-2) towards BCNU have suggested a role for class Mu GSTs in resistance against this agent (174,175). Characterization of the isozyme profile in 9L-2 cells indicated levels of class Alpha and class Pi GSTs that were similar and reduced, respectively, relative to the sensitive 9L cell line. In contrast, 9L-2 cells contained a greater quantity of class Mu GST relative to 9L cells. The inactivation of BCNU is known to occur through a GST mediated denitrosation reaction (172,176,177) and rat class Mu GSTs (4-4 and 3-4) have been shown to catalyze this reaction far more efficiently than class Alpha (1-1 and 1-2) or class Pi (7-7) GSTs (175). These results suggest that increased expression of class Mu GSTs may

offer protection against the cytotoxic effects of BCNU and perhaps other nitrosoureas.

Malignant melanoma is a tumor characterized by a high degree of intrinsic drug resistance. Investigations of melanoma biopsy material (AH) and melanoma cell lines (RPMI 8322, A375, TD, and SM) indicated low or undetectable levels of class Alpha and class Mu GSTs, but a significantly increased level of class Pi GST relative to normal liver, fibroblasts, and naevi (178). The over-expression of class Pi GST may therefore be a contributing factor in the intrinsic drug resistance characteristic of malignant melanoma.

Studies also suggest an involvement of GSTs, most notably the class Pi isozyme, in resistance towards adriamycin. In an adriamycin resistant murine mammary adenocarcinoma cell line (16C/ADR^{R}), a lack of detectable P-glycoprotein expression and sensitivity towards vinca alkaloids argued against a typical multidrug resistant phenotype and lack of cross-resistance towards VP-16 suggested that altered topoisomerase II activity was not an important mechansim of resistance in this cell line (179). In contrast, both GSH-Px and GST activities were significantly increased and may therefore contribute to the detoxification of adriamycin in the 16C/ADR^{R} cell line. Increased GST activity and the over-expression of a class Pi GST were sited, along with other mechanisms, as

potential contributing factors in the resistance of P388 leukemia and ES-2R ovarian carcinoma cell lines towards adriamycin and cyanomorpholino doxorubicin (a structural analogue), respectively (180,181). GST activity in an adriamycin sensitive MCF-7 human breast cancer cell line results largely from the expression of a single cationic (class Alpha) GST. However, in an adriamycin resistant subline, ADR^{R}MCF7, the major GST species present is an anionic class Pi isozyme. ADR^{R}MCF7 cells are characterized by a 40-fold increase in GST activity relative to MCF-7, greater than 90% of which is attributed to the over-expression of this anionic class Pi GST (182,183). Moscow *et al.* (184) have also demonstrated an inverse correlation between the expression of class Pi GST and estrogen receptor (ER) content in ADR^{R}MCF7 cells as well as in other breast cancer cell lines and primary breast cancer tumors. The higher expression level of class Pi GST in ER negative tumors, perhaps confering greater protection against cytotoxic antitumor agents, was suggested to be a potential factor in the poorer prognostic outcome generally seen in the treatment of ER negative versus ER positive tumors. Independant confirmation of these results were provided in a subsequent report by Howie *et al.* (185), who also showed a similar relationship for class Alpha, but not class Mu GSTs. Peters and Roelofs have recently investigated mechanisms of resistance in human

colon adenocarcinoma cell lines (186). Levels of GSH and the detoxication enzymes GSH-Px and UDP-glucuronyl-transferase were similar in both Sw1117 and Caco-2 cell lines sensitive and resistant, respectively, towards adriamycin. Caco-2 cells were also resistant towards a structurally similar antitumor agent mitoxantrone. While P-glycoprotein levels were only slightly increased (1.6-fold) in the Caco-2 cell line, GST activity was highly (13-fold) elevated and was due to a significantly increased expression of both class Pi (6.3-fold) and class Alpha GSTs. Recently, class Pi GSTs have also been shown to correlate with resistance towards: a) the alkylator hepsulfam, in a number of human breast cancer cell lines (187), b) ethacrynic acid in HT29 human colon carcinoma cells (188), and c) sodium arsenate in a chinese hamster ovary cell line, SA7 (189).

In contrast to the aformentioned studies, the expression level of class Pi GST correlates poorly with resistance towards other alkylators and to the antimitotic agent colchicine. Human melanoma cell lines selected for resistance against the alkylators cisplatin (G3361/CP), 4-hydroxyperoxycycolophosphamide (G3361/HC), BCNU (G3361/BCNU), or melphalan (G3361/PAM) were all found to over-express class Pi GST at levels significantly higher than in the sensitive G3361 cell line (191). However, cross-resistance towards these antitumor agents was

relatively poor among the cell lines although they each showed similar elevations in class Pi GST (3.4- to 4.5-fold relative to G3361). These results suggested that class Pi GST was not the predominant mediator of resistance towards these alkylators. Other than G3361/PAM and G3361/BCNU, which were directly selected for resistance against these agents, G3361 resistant cell lines showed poor cross-resistance towards melphalan and an increased sensitivity towards BCNU. This is consistent with the idea that class Alpha and class Mu GSTs play a greater role in resistance towards nitrogen mustards and nitrosoureas, respectively, than does class Pi GST. Unfortunately, neither class Alpha or class Mu GSTs were investigated and compared with expression levels of the class Pi GST in these resistant melanoma cell lines. In the MCF7/CL10 cell line, a colchicine resistant subline of MCF-7 breast cancer cells, Yusa *et al.* (190) have described a 70-fold increase in GST activity that was attributed to an increased expression of a class Pi GST. MCF7/CL10 cells also exhibit a multidrug resistant phenotype, showing cross-resistance towards both adriamycin and vinblastine. Examination of GST activity in a revertant (MCF7/CL10R) of the MCF7/CL10 cell line suggested that the class Pi GST, despite its over-expression in MCF7/CL10 cells, does not appear to influence resistance towards colchicine, adriamycin, or vinblastine. Although the

revertant cell line had lost the multidrug resistant phenotype and resistance against colchicine, GST activity remained elevated to the same extent (70-fold) as observed in MCF7/CL10 cells.

1.3.1 Isozyme inhibition and transfection studies

In many if not most cell lines, the multifactorial nature of drug resistance makes it difficult to accurately evaluate the relative contribution of GSTs to this process on the basis of correlative evidence alone. Two approaches have been used in an attempt to obtain more direct evidence for an involvement of GSTs in the drug resistance phenomenon. The effect of GST inhibitors on the sensitivity of drug resistant cell lines has been examined. Alternatively, other studies have investigated the ability of GSTs to confer drug resistance in sensitive cell lines that have been transfected with a specific GST expression vector.

GST inhibitors such as ethacrynic acid, piripost, and indomethacin have been shown to potentiate the cytotoxic effects of chlorambucil and other bifunctional nitrogen mustards (192-196). Treatment of the chlorambucil resistant (WR) Walker 256 rat breast carcinoma cell line with chlorambucil in combination with non-toxic doses of the GST inhibitors ethacrynic acid or piripost, reduced

cell survival to a level similar to that seen for the sensitive (WS) cell line, exposed to chlorambucil alone (197). Resistance in WR cells has previously been correlated with the over-expression of a specific (Yc) class Alpha GST subunit (171). Wang *et al* (198) have described a chlorambucil resistant cell line (N50-4) derived from mouse NIH 3T3 fibroblasts. Resistance in N50-4 cells was associated with increased GSH content (7- to 10-fold), increased GST activity (3-fold), and a significant elevation in the steady-state level of class Alpha, but not class Mu or class Pi GST mRNAs. Pre-incubation with either ethacrynic acid or indomethacin significantly increased the sensitivity of N50-4 cells towards chlorambucil, as did the depletion of GSH levels by pre-incubation with the GSH synthesis inhibitor buthionine sulfoximine (BSO). Although pre-treatment with either BSO or GST inhibitors alone could not completely reverse the resistance of N50-4 cells, a combination of these treatments almost completely restored the chlorambucil sensitivity of N50-4 cells to that of the parental NIH 3T3 cell line. These results suggested that elevated GSH content and class Alpha GST expression were the primary mediators of chlorambucil resistance in the N50-4 cell line. Also consistent with previous reports (164-166) that correlated chlorambucil resistance with a 40-fold increased expression of class Alpha GST, a pre-

incubation of CHO-Chl^{r} cells with indomethacin potentiated the cytoxicity of chlorambucil 5.5-fold in this resistant cell line (199). Hansson *et al.* (200) have also shown a 2-fold potentiation of melphalan toxicity in RPMI 8322 cells by pre-incubation with a non-toxic dose of ethacrynic acid. Elevated GST activity in this melphalan resistant human melanoma cell line (201,202) was previously shown to be attributable to the predominant expression of a class Pi GST (178). Although more frequently assiciated with the expression of class Alpha GST, the results of this study suggest that in melanoma cells the class Pi GST may influence sensitivity towards nitrogen mustards.

Although transfection experiments offer a direct method for accessing the relationship between GST expression and drug resistance, the ability of GST expression vectors to bestow resistance to antitumor agents in drug sensitive transfected cell lines has yielded conflicting results.

Giaccia *et al.* (203) have described the restoration of bleomycin resistance in BL-10 cells, a hypersensitive mutant of the wild-type CHO cell line. Used in the treatment of testicular carcinomas and squamous cell carcinomas of the head and neck (204), bleomycin is 25- to 30-fold more toxic to BL-10 cells relative to the wild type CHO cell line. BL-10 cells possess an inactive class

Alpha gene product responsible for the 36% loss in total GST activity seen in this cell line. In BL-10 revertants, increased GST activity correlated with increased bleomycin resistance and transfection of BL-10 cells with a class Alpha GST expression vector significantly increased the resistance of BL-10 cells towards this agent. Class Alpha GST may therefore be of significance in cellular resistance towards bleomycin.

As previously discussed (section 1.0.1), the inherent GST-Px activity of GSTs may play an important role in the protection of cells from oxidative stress, particularly lipid peroxidation. Lavoie *et al.* have stably transfected a GST-Yc expression vector into human breast T47D cells which express a negligible level of class Alpha GST (205). The rat YcYc isozyme is one of the most catalytically active GSTs towards organic and fatty acid hydroperoxides (206) and T47D transfectants expressed both significantly enhanced levels of Yc protein (4- to 19-fold) and GST-Px activity (2.1- to 9.9-fold) relative to T47D cells and GST-Yc-negative transfectants. Consistent with a role for GST-Px activity in the protection of cells from oxidative stress, the T47D transfectants were shown to be significantly less sensitive to the cytotoxic effects of the free radical generator cumene hydroperoxide and singlet oxygen, a classical inducer of lipid peroxidation. However, over-expression of the class Alpha Yc subunit was

unable to confer resistance against the anthracycline, daunomycin, since T47D transfectants were equally as sensitive as T47D cells towards the cytotoxic activity of this antitumor agent.

Moscow *et al.* (207) have transfected a GST-Pi expression vector into the drug sensitive human breast cancer cell line, MCF-7, which does not normally express anionic class Pi GST (182). The over-expression of class Pi GST in transfected cell lines conferred resistance towards ethacrynic acid and carcinogens such as benzo-(a)pyrene (BaP) and benzo(a)pyrene-(anti)-7,8-dihydro-diol-9,10 epoxide (BaPDE), but not towards the antitumor agents adriamycin, melphalan, or cisplatin. In contrast, transfection of the MCF-7 cell line with an mdr1 expression vector conferred a multidrug resistant phenotype in which the transfected clones were resistant towards adriamycin and cross-resistant against actinomycin-D and vinblastine (208). Co-transfection of the MCF-7 cell line with both mdr1 and GST-Pi expression vectors failed to alter either the level or pattern of resistance from than seen in mdr1 transfectants alone. The resistance of MCF-7 GST-Pi transfectants towards BaP and BaPDE is consistent with the specificity of class Pi GST towards these diol epoxides (209). However, unlike the ADR^{R}MCF-7 cell line, an enhanced expression of class Pi GST in MCF-7 transfectants did not correlate with

resistance towards adriamycin. This suggests that over-expression of class Pi GST is not sufficient in itself to confer resistance towards this agent, at least in the MCF-7 cell line.

In contrast to transfection studies using the MCF-7 cell line, Nakagawa *et al.* (210) have demonstrated a 3-fold increase in resistance towards adriamycin in a clone (RGN2) selected from GST-Pi transfected NIH 3T3 cells. A negligible expression of the mdr1 gene product in NIH 3T3 cells meant that P-glycoprotein expression was not a factor in the resistance towards adriamycin exhibited by the transfected clone. Similarily, resistance towards adriamycin was enhanced 2.3- to 10-fold and 3- to 16-fold, respectively, in GST-Pi and GST-Alpha transfectants of the yeast *Saccharomyces cerevisiae* (211). Wild-type yeast cells had no detectable levels of expression of either class Alpha or class Pi GST prior to transfection. Although transfection studies in NIH 3T3 and yeast cells are consistent with the ability of GST-Pi to confer resistance against anthracyclines such as adriamycin, these studies are in conflict with the negative results obtained with the MCF-7 cell line and leave this issue somewhat unresolved. A point of note however is the fact that GST activity in the MCF-7 transfectants was 3- to 10-fold lower than that observed in ADR^{R}MCF7 cells (182), where the expression level of class Pi GST was found to

correlated with resistance towards adriamycin. Perhaps a higher expression level of class Pi GST than was obtained in the MCF-7 transfectants is required to confer observable resistance towards adriamycin in the MCF-7 cell line.

GST-Pi transfectants of MCF-7 or NIH 3T3 cells also failed to exhibit resistance towards agents such as melphalan, chlorambucil, or cisplatin (207,210). Although the sensitivity of GST-Pi transfectants towards melphalan and chlorambucil is consistent with the view that class Alpha GSTs play a greater role in resistance against nitrogen mustards, GST-Pi transfectants of the yeast *Saccharomyces cerevisiae* (211) were shown to be substantially resistant (2- to 5.2-fold) towards chlorambucil and Miyazaki *et al.* (210) have demonstrated a 3-fold increase in resistance towards cisplatin in a GST-Pi transfectant (β2-5) of the CHO cell line. These results suggest that class Pi GST may also have a role to play in resistance towards alkylating agents in some cellular systems.

The association between class Alpha GST expression and resistance towards nitrogen mustards is also supported by transfection studies in yeast. A 2- to 8-fold resistance towards chlorambucil was reported in GST-B1 (class Alpha) transfectants of *Saccharomyces cerevisiae*, which also demonstrated a 3- to 16-fold resistance towards

adriamycin (211). Transfection studies in the yeast system therefore support a role for both class Pi and class Alpha GST in resistance towards anthracyclines and nitrogen mustards. In a study by Manoharan *et al.* (213), COS monkey cells transiently transfected with a GST-Ya (class Alpha) expression vector were also shown to be more resistant towards the cytotoxic carcinogen benzo(a)pyrene anti-diol epoxide. However, with markedly elevated levels of class Alpha GST and negligible levels of class Mu and class Pi GST, MCF-7 cells transfected with a class Alpha GST expression vector showed no increase in resistance towards chlorambucil, melphalan, adriamycin, cisplatin, BaP, or BaPDE, relative to the sensitive wild-type MCF-7 cell line (214). Thus, similar to results obtained with class Pi GST transfectants of the MCF-7 cell line, a lack of conferred resistance towards antitumor agents in MCF-7 cells transfected with a class Alpha GST expression vector conflicts with transfection studies carried out on other cell lines.

Puchalski *et al.* (215) have also studied resistance towards alkylating agents and adriamycin in cell lines stably (mouse C3H/10T½) and transiently (COS monkey) transfected with either GST-Ya (class Alpha), GST-Yb1 (class Mu), or GST-π (class Pi) expression vectors. While all transfectants, whether stable or transient, conferred moderate (1.1- to 2.9-fold) but clinically relevant levels

of resistance towards the alkylators examined, GST-Pi transfectants were found to confer the greatest resistance towards BaPDE, whereas GST-Ya and GST-Yb1 transfectants conferred the greatest resistance towards chlorambucil and cisplatin, respectively. In addition, the ability of GST-Ya and GST-π, but not GST-Yb1 transfectants, to confer moderate (1.3-fold) resistance towards adriamycin is consistent with the results seen in yeast (211). Although resistance in these transfected cell lines was only moderate at best, clonal variability in factors other than GST expression were unlikely responsible for the resistance observed in transfected COS cells given that GST$^+$ COS transfectants were sorted from the mixed population using a fluorescence activated cell sorter. That resistance was likely attributable to GST over-expression in transfected cell lines was also supported by the complete loss of chlorambucil resistance in GST-Ya$^+$ COS transfectants that had reverted to a GST-Ya$^-$ pheno-type. This result again supports a role for class Alpha GST in resistance towards nitrogen mustards.

Although transfection studies have strengthened the evidence supporting a causal link between GST expression and drug resistance, further work is required to clarify the inconsistencies in results that have been obtained from such studies. GSTs and drug resistance have been reviewed by Wolf *et al.* (216), Tew and Clapper (217), and

Morrow and Cowan (218).

2. RATIONALE AND OBJECTIVES

A growing body of literature implicates the involvement of glutathione S-transferases in the development of drug resistance in neoplastic cells. The correlation between drug resistance and elevated expression of GSTs, as well as transfection and inhibitor studies which demonstrate the ability to alter drug resistance by modulating the expression and activity of GSTs, lend support to this notion. To investigate potential mechanisms of resistance towards quinone antitumor agents, Begleiter *et al.* (219) have previously isolated and characterized L5178Y lymphoblasts resistant to the model quinone compound, hydrolyzed benzoquinone mustard. The HBM-resistant cell lines L5178Y/HBM2, L5178Y/HBM10, and L5178Y/HBMR, were shown to be 2.5-, 6-, and 2.9-fold resistant, respectively, towards the cytotoxic activity of HBM relative to the sensitive parental cell line, L5178Y. Characterization of these cell lines demonstrated a significant increase in the activity of the cell protective enzyme, catalase, and the Phase II drug metabolizing enzymes NAD(P)H: quinone oxidoreductase (DT-diaphorase) and glutathione S-transferase. Another cell line, L5178Y/AM, is 10-fold less senstive than L5178Y towards the cytotoxic activity of the aromatic alkylator

aniline mustard and was also found to exhibit increased glutathione S-transferase activity (Begleiter, unpublished results). Given their increased activity and potential role as a causal mechanism in the development of cellular drug resistance, a more detailed study of GSTs in both HBM- and AM-resistant cell lines was undertaken.

The purpose of this study was to:

1. Characterize the increased GST activity in HBM- and AM-resistant cell lines with respect to the expression of specific cytosolic classes of GST.

2. Examine the molecular mechanisms responsible for enhanced expression levels (and hence elevated GST activity) of cytosolic GSTs in HBM- and AM-resistant cell lines.

3. Investigate the pattern of expression of class specific GSTs in response to agents which differ in both structure and mechanism of cytotoxicity.

3. METHODS

3.0 Cell lines and cell culture

3.0.0 Antitumor agents

Either hydrolyzed benzoquinone mustard [di(2'-hydroxyethyl)amino-1,4-benzoquinone] or aniline mustard [N,N-di(2'-chloroethyl)aniline] were required components of the growth medium used for the culture of drug resistant cell lines. The chemical structures of these two antitumor agents are shown in Figure 4. Both compounds were synthesized by Dr. Asher Begleiter of the Manitoba Institute of Cell Biology, Winnipeg, Manitoba, as previously described (220).

3.0.1 Cell lines

L5178Y lymphoblasts arose as a spontaneous neoplasm in an aged DBA/2 mouse in 1952 and were later adapted by Fischer for growth in tissue culture (221,222). The drug resistant cell lines utilized in this study were developed from the L5178Y parental drug sensitive cell line and were provided by Dr. Asher Begleiter. The HBM-resistant cell lines L5178Y/HBM2, L5178Y/HBM10, and L5178Y/HBMR are

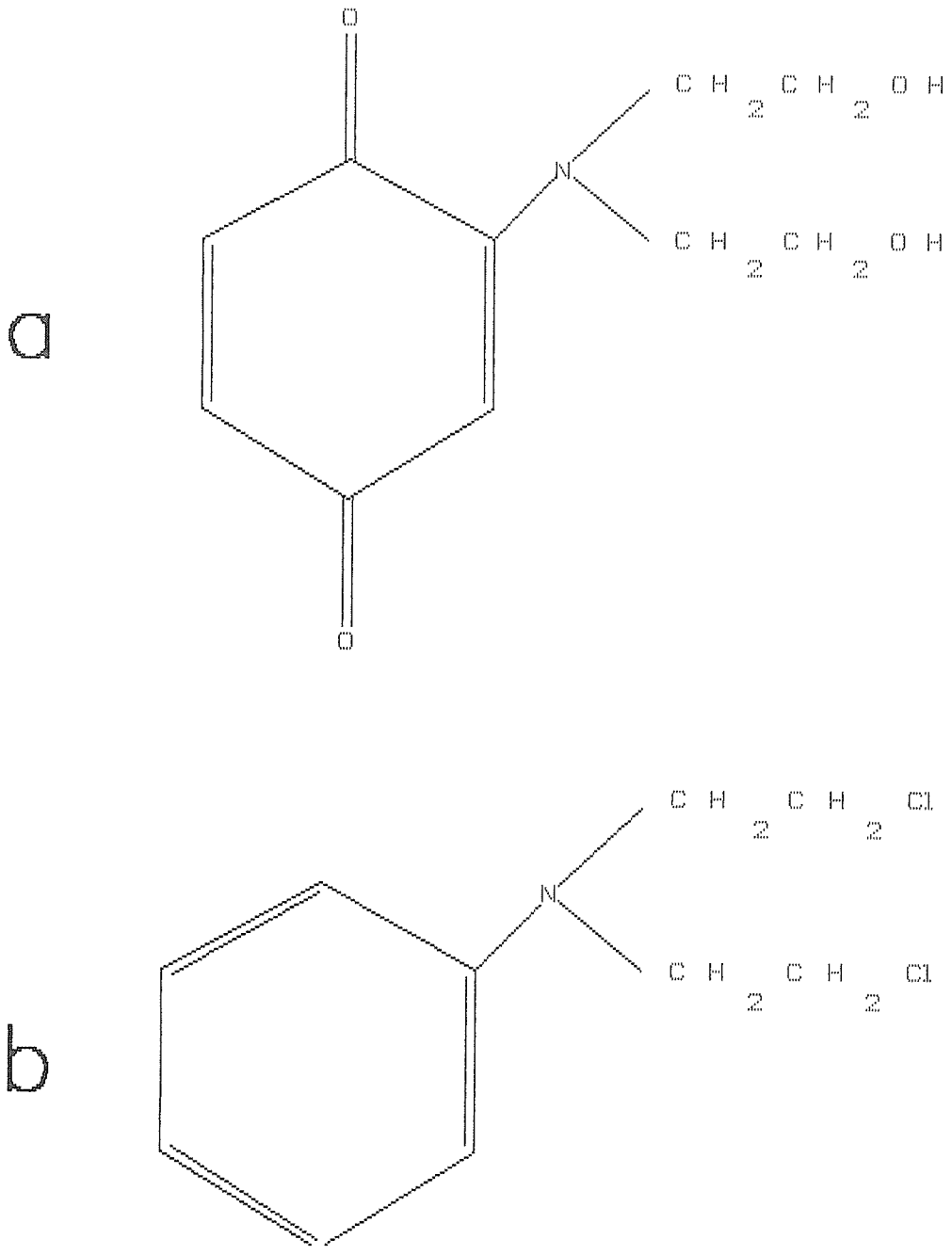

Figure 4. Chemical structures of the antitumor agents: a) hydrolyzed benzoquinone mustard (HBM) and b) aniline mustard (AM).

respectively, 2.5-, 6-, and 2.9-fold less sensitive towards HBM, relative to L5178Y (219). The AM-resistant cell line, L5178Y/AM, is 10-fold less sensitive towards AM than is L5178Y (Begleiter, unpublished results).

3.0.2 Cell culture

Drug-sensitive and -resistant cell lines were grown in suspension culture using Fischer's medium that was supplemented with 12% horse serum (Gibco, Grand Island, N.Y.) and the antibiotics penicillin and streptomycin. Maintenance of the resistant phenotype also requires culture in the presence of drug. Therefore, the medium for L5178Y/HBM2 and L5178Y/HBM10 contained 0.2 and 1.0 mM HBM, respectively, while that for L5178Y/AM contained 1.0 μM AM. L5178Y/HBMR, which is a partial revertant of L5178Y/HBM10, was grown in medium without HBM. Cell cultures were maintained at 37°C and 5% CO_2.

3.1 GST assays and Western blot analysis

3.1.0 Isolation of total cytosolic protein

From each cell line, ~ 1-2 x 10^8 log phase cells were collected by centrifugation at 1000 x g for 5 minutes at

4°C. Pellets were washed twice in cold PBS (pH 7.4) and re-suspended in 3 volumes of cold 1 M potassium phosphate buffer (pH 6.5). Cells were disrupted on ice using a Branson sonifier-cell disruptor with a micro tip fitted to the disruptor horn and the instrument duty cycle and output set at 50% and 6.5 units, respectively. Sonication was carried out using three, five second pulses, with a cooling time of twenty seconds between each pulse. The cell sonicates were then centrifuged at 100,000 x g for 1 hour at 4°C. Samples of the post-microsomal supernatants (cytosolic fractions) were used immediately for GST assays or stored at -70°C until used in Western blot analyses.

3.1.1 Glutathione S-transferase assay

The total protein concentration of cytosolic fractions obtained from each cell line was quantitated by the method of Bradford (223) using the BIO-RAD protein assay kit (BIO-RAD, Richmond, CA) and the glutathione S-transferase activity of these fractions was subsequently assayed by the method of Habig *et al.* (2), using the substrate 1-chloro-2,4-dinitrobenzene (CDNB).

Assays were carried out in quadruplicate, in 1 mL disposable cuvettes containing either 2, 4, 6, or 8 µL (L5178Y and HBM-resistant cell lines) or 4, 8, 10, or 12 µL (L5178Y/AM cell line) samples of the cytosolic fraction

in the assay solution. If too concentrated initially, stock fractions were diluted before addition to the assay solution. Other reagents making up the final assay solution included 500 µL of 0.2 M potassium phosphate buffer (pH 6.5), 50 µL of 20 mM aqueous glutathione, 50 µL of 20 mM CDNB in 95% ethanol, and deionized water to make a total solution volume of 1 mL. The solution was mixed and assayed immediately at 25°C in a DU-8 spectrophotometer (Beckman Instruments Inc.) by monitoring the change in absorbance at 340 nm. Measurements were also carried out on a blank assay solution that contained all reagents less the sample volume of cytosolic protein. With the aid of the Kinetics II software available for use with the DU-8 spectrophotometer, the blank allowed for a correction of the GST activity of cytosolic fractions, to account for the rate of spontaneous conjugation of CDNB to glutathione. The rate of the non-enzymic reaction was then subtracted from that of the enzyme catalyzed reaction to give the corrected GST activity. Specific GST activity is given in units of nmol/min/µg total protein and is reported as the mean ± SE of quadruplicate measurements.

3.1.2 SDS-Polyacrylamide gel electrophoresis

Using the discontinuous buffer system of Laemmli (224), SDS-PAGE was used to separate proteins in the

cytosolic fractions of cell sonicates obtained from the drug sensitive and resistant cell lines. Electrophoresis was carried out using a BIO-RAD Protean II slab cell (BIO-RAD, Richmond, CA).

Polyacrylamide slab gels of 3 mm thickness consisted of a 4% acrylamide stacking gel cast on top of a 12% acrylamide separating gel. The gels were prepared from stock solutions of 30% acrylamide (29% (w/v) acrylamide and 1% (w/v) N,N'-methylenebisacrylamide), 10% SDS, 10% ammonium persulfate, TEMED, 1.5 M Tris (pH 8.8) in the case of separating gels, and 1.0 M Tris (pH 6.8) in the case of stacking gels. The volumes of each stock solution required for the preparation of gels of varying percentage may be found in Sambrook *et al.* (225).

The electrophoresis buffer was 1x aqueous Tris-glycine prepared by dilution of a 5x stock buffer which contained 15g Tris base, 72g glycine, and 5g SDS, per liter of deionized water. Samples were prepared for electrophoresis by dilution with 4 volumes of aqueous SDS reducing buffer (62.5 mM Tris (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, and 1% (w/v) bromophenol blue) and heating the mixture for 4 minutes at 95°C prior to loading onto the gel.

Gels were run under constant current conditions using ~50 mA/gel for the stacking gel and ~70 mA/gel for the separating gel. A 20 μL sample of BIO-RAD low range pre-

stained SDS-PAGE standards was loaded onto the gel and used to monitor the progress of the run. In general, electrophoresis was continued until the hen egg white lysozyme band was near the bottom of the separating gel. BIO-RAD low range SDS-PAGE molecular weight standards were used as molecular weight markers. The standards were prepared by diluting 1 volume of stock solution with 20 volumes of SDS reducing buffer, heating for 5 minutes at 95°C, and loading 10 μL of the mixture onto the gel.

3.1.3 Antibody probes for cytosolic classes of GST

Polyclonal antisera directed against specific cytosolic classes of GST were obtained from various investigators. Polyclonal antiserum against purified rat liver GST, containing antibodies specific for class Alpha (Ya and Yc) and class Mu (Yb) isozymes, were provided by Dr. Kenneth Tew (168). Polyclonal antisera against the purified F3 isozyme (class Mu) from the liver of DBA/2J mice and an anionic isozyme (class Pi) from a human mesothelioma were obtained from Drs. C. -Y. Lee and William D. Henner, respectively (12,226).

3.1.4 Western blotting

Proteins separated by SDS-PAGE were transfered to a

0.2 μm nitrocellulose membrane by semi-dry horizontal electrophoretic transfer using the Multiphor II NovaBlot system (LKB-Produkter AB, Bromma, Sweden). The aqueous transfer buffer was a continuous buffer system composed of 39 mM glycine, 48 mM Tris, 0.0375% (w/v) SDS, and 20% methanol. Electrophoretic transfer was continued for 2 hours using a constant current of 0.8 mA/cm^2 gel. Transfer efficiency was checked by staining the post-transfer gel with Commassie blue dye and by temporarily staining proteins transfered to the membrane with Ponceau S dye (227). Staining with Ponceau S dye also allowed for the visualization of the molecular weight standards such that their positions on the membrane could be marked.

Prior to incubation with the primary antibody probe the membrane was blocked with an aqueous solution of 1x TBS (0.02 M Tris and 0.5 M NaCl at pH 7.5) containing 5% (w/v) BSA and 0.02% (v/v) Tween 20. The membrane was left in contact with the blocking solution for 2 hours at room temperature, on a rotating nutator. The membrane was then transfered to fresh blocking solution which contained, in addition, the primary antibody directed against a specific cytosolic class of GST. The primary antibody solutions contained one of the following: a) 1:1000 dilution of antiserum against rat liver (Ya, Yb, and Yc) isozymes, b) 1:300 dilution of antiserum against the mouse liver F3 isozyme, and c) 1:500 dilution of antiserum against the

anionic isozyme from a human mesothelioma. Incubation at 4°C with the primary antibody solution was carried out for 5 hours on a rotating nutator.

After incubation with the primary antibody solution the membrane was washed three times (10 minutes each) with 250 mL of PBS (8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, and 0.24g KH_2PO_4, per liter of deionized water, at pH 7.4). The membrane was then washed for 10 minutes in 200 mL of an aqueous solution of 150 mM NaCl and 50 mM Tris (pH 7.5) and then incubated with the secondary antibody solution.

The secondary antibody was alkaline phosphatase conjugated goat anti-rabbit immunoglobin (Sigma Chemical Co., St. Louis, MO) which was diluted 1:1000 with blocking solution. The membrane was incubated with the secondary antibody solution for 1 hour at room temperature, on a rotating nutator. The membrane was washed three times (10 minutes each) in 200 mL of an aqueous solution of 150 mM NaCl and 50 mM Tris (pH 7.5) and then transfered to a developing solution containing the substrate BCIP/NBT (BRL, Gaithersburg, MD), for visualization of GST protein. The development solution was prepared according to the manufacturer's directions. The volume of blocking, primary antibody, and secondary antibody solutions used was equivalent to 0.3 mL/cm^2 membrane. The general procedure outlined above was based on protocols available from a number of sources (228,229,230).

3.2 Preparation of cDNA probes

3.2.0 Recombinant plasmids

cDNA probes specific for GSTs, quinone reductase, and P-glycoprotein were isolated from plasmids provided by the following investigators. Plasmids pGTB38 and pGTA/C48, containing cDNA complimentary to mRNAs of rat liver Ya (class Alpha) and Yb_2 (class Mu) GSTs, respectively, and plasmid pDTD55, containing the cDNA complimentary to a quinone reductase mRNA, were provided by Dr. Cecil B. Pickett (231,232,233). Plasmid pTSS1-2, containing a cDNA complimentary to the mRNA of an anionic GST (class Pi) obtained from human lung tissue, was received from Dr. William D. Henner (191). The plasmid pCHP1, containing a cDNA complimentary to a mRNA for the plasma membrane protein P-glycoprotein, was a gift from Dr. J. R. Riordan (234).

The bacterial transformants (*E. coli* strain HB101 for pGTB38, pGTA/C48, and pTSS1-2, and *E. coli* strain RR1 for pDTD55) were stored as 20% (v/v) glycerol stock cultures at -20°C.

3.2.1 Amplification of plasmid DNA

E. coli transformants were streaked onto LB agar plates containing 12.5 μg/mL tetracycline (pGTB38 and

pGTA/C48), 10.0 μg/mL tetracycline (pDTD55), or 50 μg/mL ampicillin (pTSS1-2) and incubated at 37°C until colonies were visible. A single colony was inoculated into 100 mL LB medium containing the appropriate antibiotic and incubated overnight at 37°C in a rotary shaker. The culture was then split into two 25 mL volumes and each added to a fresh 1000 mL of the same medium and incubated at 37°C in a rotary shaker until the O.D. of the culture was between 0.6-0.8 absorbance units. At this point chloramphenicol was added to a concentration of 170 μg/mL to allow for amplification of plasmids. The cultures were incubated in a rotary shaker for a further 24 hours at 37°C. LB medium was prepared by dissolving Bacto-tryptone (10 g/L), Bacto-yeast extract (5 g/L), and NaCl (10 g/L) in deionized water and adjusting the pH to 7.5 with NaOH.

3.2.2 Isolation and purification of plasmid DNA

Plasmid DNA was isolated from the *E. coli* transformants by an alkaline lysis procedure as described in Maniatis *et al.* (235). DNA was dissolved in 8 mL of TE buffer (10 mM Tris (pH 7.4) and 0.1 mM EDTA (pH 8.0) in deionized water) and plasmid DNA purified from bacterial DNA by centrifugation to equilibrium in a cesium chloride-ethidium bromide density gradient. The DNA solution was prepared for centrifugation by adding 8 g CsCl/mL DNA

solution and 0.8 g ethidium bromide/10 mL CsCl solution. Centrifugation was carried out at 45,000 rpm for 36 hours at 20°C. After recovery of the lower DNA (plasmid) band, ethidum bromide was removed from the plasmid DNA solution by alternate extractions with n-butanol and water-saturated n-butanol. Subsequently, cesium chloride was removed by dialysing the solution against three changes of 2000 mL TE buffer (pH 8.0), with a 24 hour period of dialysis between each change. After dialysis the plasmid DNA solution was prepared as before and purified a second time by centrifugation to equilibrium in a cesium chloride-ethidium bromide density gradient. After removal of ethidium bromide and cesium chloride, the final purified plasmid preparation was stored in TE buffer at 4°C. The general procedures for CsCl centrifugation and n-butanol extraction are outlined in Maniatis *et al.* (236).

3.2.3 Isolation and purification of cDNA probes

In preparation for recovery and purification, cDNA probes for use in Northern and Southern blot analysis were released from plasmids by digesting 20 µg of plasmid DNA with an appropriate restriction enzyme(s).

For restriction enzyme digestion, plasmid DNA was ethanol precipitated (237) and re-dissolved in 63 µL of TE

buffer (pH 7.4) and 7 µL of an appropriate 10x digestion buffer. Digestion for 2 hours at 37°C was carried out using 3 units of restriction enzyme per µg DNA. Restriction enzymes and digestion (REact™) buffers were from BRL, Gaithersburg, MD. Table 2 lists the restriction enzymes used and the number and size of cDNA fragments released upon RE digestion of specific plasmids. Digested DNA samples were prepared and electrophoresed through a 0.8% agarose slab gel. The preparation of DNA samples, agarose gels, and conditions for electrophoresis, are described in section 3.4.1.

Upon completion of electrophoresis, gel slices containing the cDNA fragments of interest were cut from the gel (Figure 5). The cDNA fragments were recovered from gel slices by electroelution as described in Maniatis *et al.* (238) and were further purified by passage through a minicolumn D (Sigma chemical Co, St. Louis, MO), following the manufacturer's protocol. The purified cDNA probes were ethanol precipitated, washed twice in 80% ethanol, and the air dried pellets taken up in TE buffer (pH 7.4) and stored at -20°C until used. The solutions of purified cDNA probes were checked by electrophoresis of a small sample of these solutions through a 0.8% agarose mini-gel (Figure 6). The purified cDNA probe for P-glycoprotein had been prepared by others and was supplied to me directly.

Table 2. Recombinant plasmids pGTB38, pGTA/C48, pTSS1-2, and pDTD55: cDNA inserts, restriction enzyme digestion, and probe specificity.

Recombinant Plasmid	pGTB38	pGTA/C48	pTSS1-2	pDTD55
Length (bp) of cDNA insert	950	845	334	1900
cDNA Complimentarity	rat Ya mRNA	rat Yb$_2$ mRNA	human π mRNA	QuinRd[c] mRNA
RE used for release of cDNA insert	*Pst*I[a]	*Pst*I	*EcoR*I & *Sal*I	*Pst*I[a]
No. cDNA fragments released	3	1	1	4
Length (bp) of cDNA fragments	521[b],266, 163	845	334	873[b],617 218,192
Probe specificity	class α GST	class μ GST	class π GST	QuinRd[c]
Reference	231	232	191	233

[a]Digestion of the recombinant plasmid with the restriction enzyme (RE) *Pst*I, to release the cDNA insert, will generate multiple cDNA fragments due to the presence of internal *Pst*I sites within the cDNA insert.

[b]Where multiple cDNA fragments were generated after restriction enzyme digestion, only the largest fragment was selected for further purification and use as a cDNA probe in Northern and Southern blot hybridizations.

[c]QuinRd, Quinone Reductase.

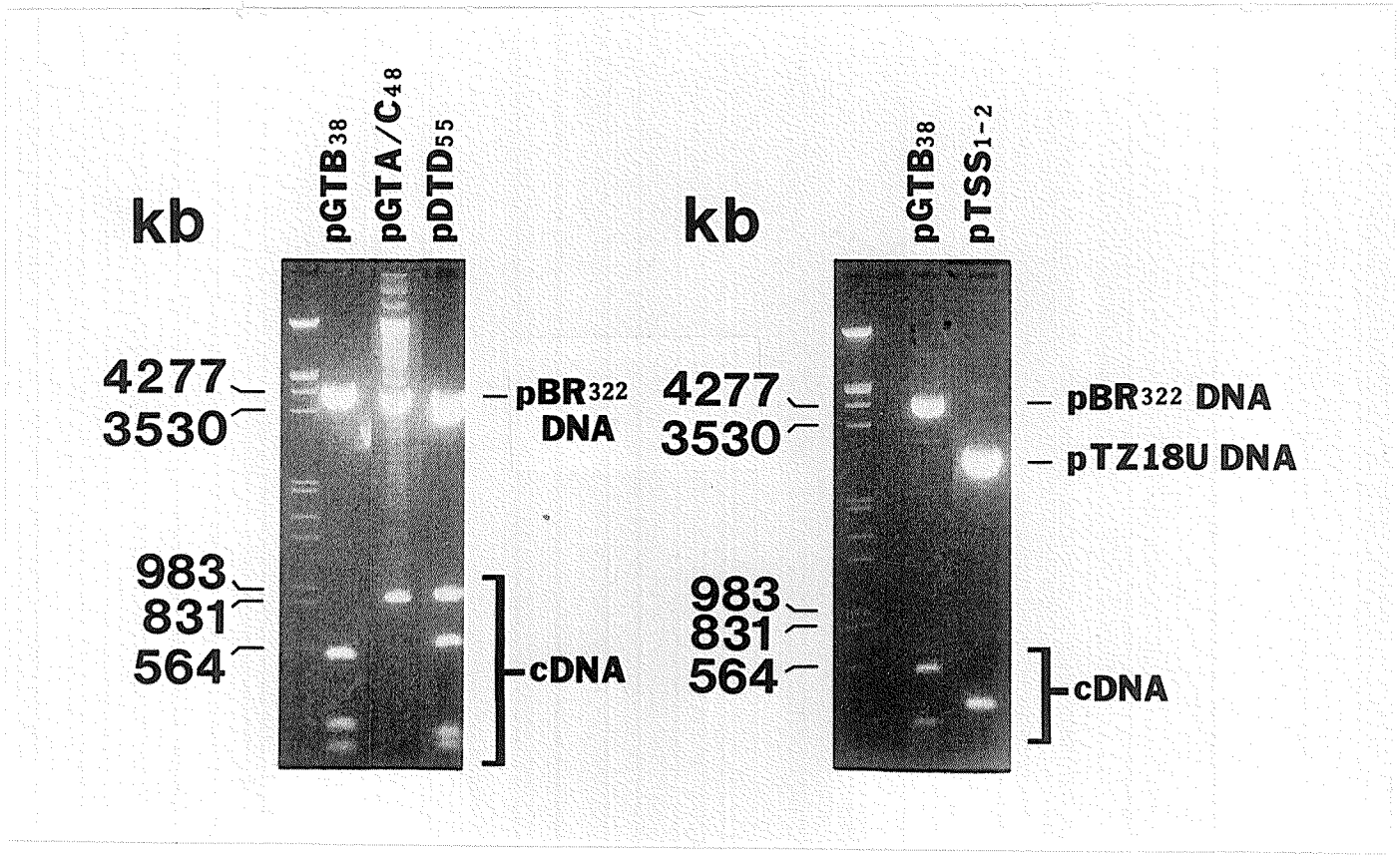

Figure 5. Separated plasmid DNA and cDNA fragments after electrophoresis through 0.8% agarose gels. Recombinant plasmids were digested with the restriction enzyme(s) Pst I (pGTB38, pGTA/C48, and pDTD55) or EcoR I and Sal I (pTSS1–2). DNA size markers (kb) are Hind III digested lambda DNA (Boehringer–Mannheim, FRG).

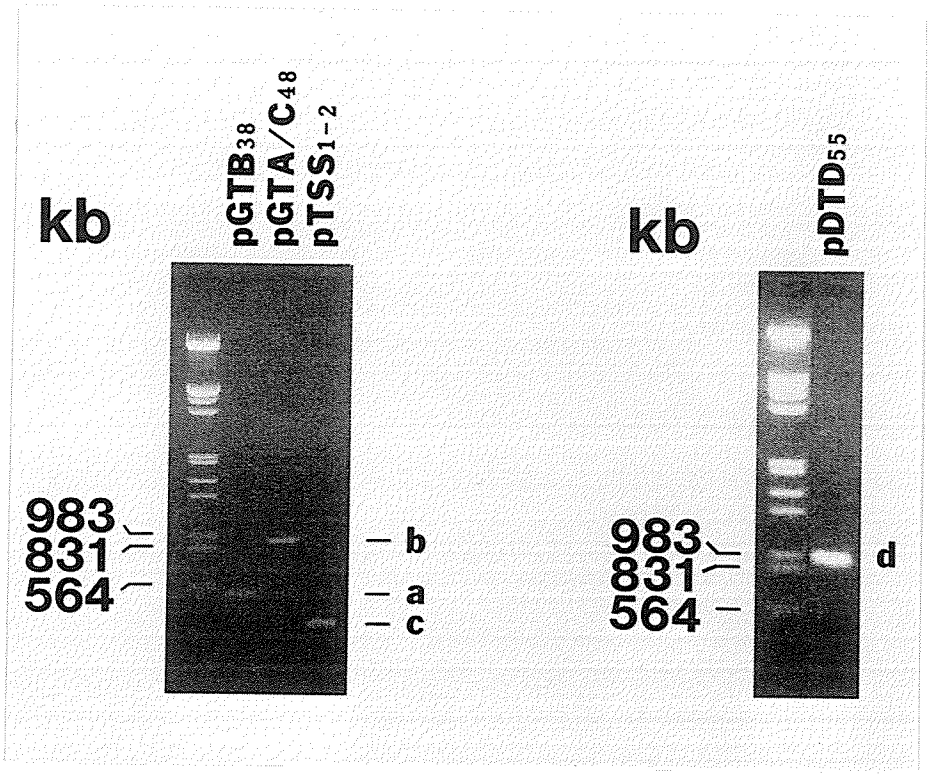

Figure 6. Examination of purified cDNA probes after electroelution from agarose gels and purification using a Minicolumn–D. Purified cDNA probes are shown after electrophoresis through 0.8% agarose mini–gels. Identified cDNA bands are: a) 521 bp fragment of the GST Ya cDNA insert of pGTB38; b) 845 bp GST Yb_2 cDNA insert of pGTA/C48; c) 334 bp GST Pi cDNA insert of pTSS1–2; and d) 873 bp fragment of the quinone reductase cDNA insert of pDTD55. DNA size markers (kb) are Hind III digested lambda DNA (Boehringer–Mannheim), FRG).

3.2.4 Preparation of [α-^{32}P]-dCTP labelled cDNA probes

cDNA probes for use in Northern and Southern blot hybridizations were radioactively labelled with [α-^{32}P]-dCTP using random oligonucleotide primers. [α-^{32}P]-dCTP was obtained from ICN Biochemicals, CA and the labelling kit was from Pharmacia, Uppsala, Sweden. For the oligo-labelling reaction, distilled water was added to 2-10 μL of the stock cDNA probe solution to give a final volume of 29 μL. The solution was boiled for 5 minutes and then quenched on ice for a further 5 minutes before the addition of 10 μL each of reagent mix (from kit) and [α-^{32}P]-dCTP, and finally, 2 μL of klenow fragment (from kit). The reaction was incubated overnight at room temperature and the labelled cDNA probe separated from unincorporated [α-^{32}P]-dCTP by passing the reaction mixture through a Nick-column (Pharmacia, Uppsala, Sweden) as described by the manufacturer.

3.3 Northern blot analysis

3.3.0 Isolation of total cellular RNA

To minimize the chance of RNase contamination, sterile disposable plasticware was used wherever possible.

Glassware and metal spatulas were baked in a drying oven for 4 hours at 250°C and then autoclaved. Centrifuge tubes were autoclaved and deionized water for use in preparation of reagent solutions was treated with 0.1% diethylpyrocarbonate (DEPC) for 12 hours prior to being autoclaved.

From drug-sensitive and -resistant cell lines, 1-2 x 10^8 log phase cells were collected by centrifugation at 1000 x g for 5 minutes and the cell pellets washed twice in a 15 mL volume of cold sterile PBS (pH 7.4). Total RNA was extracted by the single step method of Chomczynski and Sacchi (239). Cells were lysed in 100 µL/10^6 cells, of a denaturing solution (solution D) composed of 4 M guanidinium thiocyanate, 2 mM sodium citrate (pH 7), 0.5% sarcosyl, and 0.1% β-mercaptoethanol. Added sequentially were solutions of 2 M sodium acetate, pH 4 (0.1 mL/mL soln. D), water-saturated phenol (1 mL/mL soln. D), and 49:1 chloroform-isoamyl alcohol (0.2 mL/mL soln. D). After shaking vigorously for 15 seconds the extract solution was placed on ice for 15 minutes and the aqueous and organic phases separated by centrifugation at 12000 x g for 30 minutes. The upper aqueous phase was removed and mixed with 1 volume of isopropanol and RNA precipitated overnight at -20°C. After centrifugation (12000 X g; 10 minutes) the pellet was re-dissolved in 1/3 the original volume of solution D and an equivalent volume of isopropyl alcohol was added to re-precipitate the RNA. After 1 hour

at -20°C the RNA was centrifuged as before, the pellet washed twice with 75% ethanol, air dried, and finally dissolved in a minimum volume of DEPC treated sterile water and stored at -70°C.

3.3.1 Formaldehyde-agarose gel electrophoresis

In preparation for Northern blotting, equivalent amounts of total RNA from each cell line was size fractionated in a 1% agarose-2.2 M formaldehyde slab gel. Preparation of the gel, MOPS electrophoresis buffer, RNA loading buffer, and total RNA samples were as described in Maniatis *et al.* (240). Ethidium bromide (0.8 mg/mL) was also added to the RNA samples prior to heating and loading onto the gel. This has been shown to give a more efficient UV fluorescence, and hence visualization, of formaldehyde denatured nucleic acid (241). Electrophoresis using a BIO-RAD DNA sub-cell (BIO-RAD, Richmond, CA) was carried out at room temperature with 1x MOPS as the running buffer. The gel was run at constant voltage (25V) until the leading dye band had migrated approximately 3/4 the length of the gel.

3.3.2 Northern blotting

Upon completion of electrophoresis the gel was washed

twice for 20 minutes in two changes of 500 mL of 10x SSPE buffer (20x sterile SSPE stock contains 3 M NaCl, 0.2 M $NaH_2PO_4 \cdot H_2O$, 0.02 M EDTA-Na_2, and adjusted to pH 7.4 with NaOH). RNA was Northern blotted to a Zeta-Probe nylon membrane (BIO-RAD, Richmond, CA) using a transfer buffer of 10x SSPE. Prior to blotting, the membrane was wetted by soaking (5 minutes each) in DEPC treated water and then in 10X SSPE transfer buffer. Capillary transfer was continued for 24-36 hours, after which the membrane was rinsed for 5 minutes in 5x SSPE and then oven dried for 1/2 hour at 70°C. To check for completeness of transfer the post-transfer gel was re-stained in a solution of 5 µg/mL ethidium bromide, destained by washing in water, and then examined under UV light.

For Northern blot hybridization the membrane was pre-hybridized at 42°C for 12-24 hours in a solution composed of 50% formamide, 5X SSPE, 5X Denhardt's solution, 2% SDS, and 100 µg/ml denatured salmon sperm DNA. Hybridizations with appropriate cDNA probes were carried out for 24 hours at 42°C in fresh solution containing, in addition, 8% dextran sulfate. cDNA probes added to the hybridization solutions were at a final concentration of apporximately 10^6 cpm/ml. After hybridization the membrane was washed twice for 15 minutes in two changes of a solution of 6X SSPE and 0.5% SDS, at room temperature. Subsequent washes were of increasing

stringency using solutions of 1X and 0.1X SSPE and 0.1-0.5% SDS, at temperatures between 42-65°C. The general procedure outlined above for Northern blot analysis was based on protocols available from a number of sources (229,242).

3.4 Southern blot analysis

3.4.0 Isolation of high molecular weight genomic DNA

From drug-sensitive and -resistant cell lines, 1-2 x 10^8 log phase cells were collected by centrifugation at 1000 x g for 5 minutes and the cell pellets washed in 15 mL of cold PBS (pH 7.4). DNA was extracted using the procedure in Davis *et al.* (243).

Cells were digested for 15 minutes at 65°C in 10 volumes of proteinase K solution (10 mM Tris (pH 7.4), 10mM EDTA-Na_2, 150 mM NaCl, 0.4% SDS, and 1 mg/mL proteinase K) and then incubated in this solution overnight in a shaking water bath at 37°C. The solution was extracted with an equal volume of a 1:1 (v/v) mixture of ss-phenol-chloroform and the aqueous and organic phases separated by centrifugation at 12,000 x g for 10 minutes. The upper aqueous phase was removed and the extractions repeated until white matter at the interface of the two

phases was no longer visible. Phenol was routinely purified by simple distillation prior to use and was stored at -20°C. DNA was precipitated by addition of 1/10 the volume of 3 M sodium acetate (pH 7.4) and 2.5 volumes of absolute ethanol and then pelleted by centrifugation at 12,000 x g for 10 minutes. The pellet was washed twice in 5 mL of 80% ethanol, air dried briefly, and re-dissolved in 4.5 mL of TE buffer over 24-48 hours in a shaking water bath at 37°C. RNA was degraded by incubation of the DNA solution for 1/2 hour at 37°C after the addition of 25 µL of a 10 mg/mL solution of DNase free RNase. 500 µL of 3 M sodium acetate was added and the solution extracted with 5 mL of a 1:1 (v/v) mixture of ss-phenol-chloroform. After centrifugation (12,000 x g, 10 minutes) the upper aqueous phase was removed and extracted with 5 mL of chloroform. A further centrifugation was done and the upper aqueous phase removed and combined with 2.5 volumes of absolute ethanol to precipitate DNA. The DNA was pelleted as before, washed twice with 80% ethanol, partially air dried, and finally re-dissolved in TE buffer (pH 7.4) and stored at 4°C.

3.4.1 Restriction enzyme digestion and agarose gel electrophoresis

Restriction enzyme digestion of genomic DNA was carried out using 3 units of restriction enzyme per µg

DNA. Specific digestion buffers and reaction temperatures were as recommended by the manufacturer for a given restriction enzyme. The digestions were allowed to continue overnight.

DNA was precipitated by cooling at -20°C for several hours after the addition of 1/10 the volume of 3 M sodium acetate (pH 7.4) and 2.5 volumes of absolute ethanol. After centrifugation, DNA pellets were washed twice with 300 µL of 80% ethanol, air dried, and re-dissolved in 30-60 µL of TE buffer (pH 7.4). DNA loading buffer (type II, as described in Maniatis, *et al.* (244), with the exception that the ficoll content was 30%) was added and comprised 20% of the final sample volume. Prior to loading, samples were heated for 2 minutes at 65°C to denature the cohesive ends of the DNA.

DNA fragments were size fractionated in a 0.8% agarose slab gel, prepared as described in Maniatis *et al.* (245). The electrophoresis buffer was 1x TBE (20x stock TBE is 1M Tris base, 1M boric acid, and 20 mM EDTA-Na_2). Both the gel and electrophoresis buffer contained ethidium bromide (0.5 µg/mL) for the visualization on DNA under UV light. Electrophoresis in a BIO-RAD DNA sub-cell was carried out at room temperature and constant voltage (25V) until the leading dye band had migrated approximately 3/4 the length of the gel.

3.4.2 Southern blotting

After electrophoresis, the gel was soaked for 10 minutes in 500 mL of 0.25M HCl to acid depurinate DNA, rinsed for 1 minute in 500 mL of deionized water, and then soaked twice (15 minutes each) in two changes of 500 mL of an alkaline transfer buffer composed of 0.4 M NaOH and 0.6 M NaCl. DNA was Southern blotted to a Zeta-Probe nylon membrane using the alkaline transfer buffer. Prior to blotting, the membrane was wetted by soaking (5 minutes each) in deionized H_2O and then in the alkaline transfer buffer. Capillary transfer was continued for 24-36 hours after which the membrane was rinsed in 5x SSPE for 5 minutes and then dried at 70°C for 1/2 hour. To check the efficiency of transfer, the post- transfer gels were re-stained in a solution of 5 μg/mL ethidium bromide, destained by washing in water, and then examined under UV light.

Hybridizations with appropriate cDNA probes were carried out as described for Northern blotting (section 3.3.2) except that the pre-hybridization solution contained 6X SSPE and 10X Denhardt's solution, while the hybridization solution contained no Denhardt's solution. Membranes were washed as described for Northern blotting.

3.5 Autoradiography

Autoradiograms of hybridized membranes were obtained using Kodak XAR-5 film exposed at -70°C with a Dupont Cronex Lightning-Plus intensifying screen. Film was not pre-flashed prior to exposure to hybridized membranes.

3.6 Densitometry

The photographic record of Western and Northern data presented in the results section depicts clear differences in the relative abundance of protein or mRNA between drug sensitive and resistant cell lines. However, a numerical estimate of the magnitude of these differences was desired. Therefore, Western blots and autoradiograms from hybridized Northern blots were analyzed by densitometry using a BIO-RAD model 620 video densitometer (BIO-RAD, Richmond, CA). Measurements were taken either in reflectance (Western blots) or transmitance (images on autoradiographic film) mode. Densitometric response will likely show an upper limit of linearity for the relationship between densitometric area and sample size. The densitometric area of an autoradiographic image will also vary with exposure time and X-ray film that has not been pre-flashed, to activate the silver halide crystals

in the film emulsion, shows a tendency towards a sigmoidal rather than linear response to increasing amounts of radioactivity (246). To estimate the linear range of densitometric response and to provide a calibration curve for analysis of densitometric data, separate blots were prepared in which individual sample lanes contained sequentially decreasing quantities of either protein or total RNA. Western or autoradiographic bands were measured and the densitometric area (OD·mm) of each band was plotted as a function of the sample quantity (μg) per lane.

For the densitometric analysis of a particular Northern blot, multiple autoradiograms were obtained, each varying in its time of exposure. Only data from film that gave autoradiographic images whose densitometric areas were within the acceptable range of linearity established by the calibration curve, were used. Northern blots were also hybridized with a β-actin cDNA probe. Where membranes contain equivalent quantities of total RNA from drug sensitive and resistant cell lines, densitometric measurement of the β-actin signals allowed for the correction of densitometric data for irregularities in sample loading.

4. RESULTS

4.0 GST assays

4.0.0 GST activity in drug sensitive and resistant L5178Y lymphoblasts

The specific GST activity of the cytosolic fraction of cell sonicates from drug sensitive and resistant L5178Y lymphoblasts was determined with the substrate CDNB. The specific GST activities of the drug sensitive L5178Y, and drug resistant L5178Y/HBM2, L5178Y/HBM10, and L5178Y/AM cell lines were 0.042, 0.108, 0.43, and 0.208 nmol/min/μg cytosolic protein, respectively. Compared to the sensitive L5178Y cell line, the specific GST activity was increased 2.6-, 10.2-, and 5.0-fold in the drug resistant cell lines, L5178Y/HBM2, L5178Y/HBM10, and L5178Y/AM, respectively (Table 3 and Figure 7). Therefore, the relative GST activities between drug sensitive and resistant cell lines used in this study were consistent with previous reports (219; a value of 3.6 nmol/min/μg cytosolic protein was communicated to me by Dr. A. Begleiter).

4.1 Western blot analysis

Table 3. Specific GST activity of the cytosolic fraction of cell sonicates from drug sensitive, HBM-resistant, and AM-resistant L5178Y lymphoblasts.

	Specific GST Activity ($nmol \cdot min^{-1} \cdot \mu g$ cytosolic $protein^{-1}$ ± SE)			
Assay[b]	L5178Y	L5178Y/HBM2	L5178Y/HBM10	L5178Y/AM
1	0.048±0.001[a]	0.107±0.006[a]	0.450±0.009[a]	0.206±0.002[a]
2	0.045±0.002[a]	0.109±0.003[a]	0.418±0.009[a]	0.209±0.004[a]
3	0.032±0.002[a]	----------	----------	----------
Mean[c]	0.042±0.003	0.108±0.007	0.43±0.01	0.208±0.004
Norm	1.0±0.1	2.6±0.2	10.2±0.8	5.0±0.4

[a]Data represent the mean ± SE of four determinations.
[b]For each cell line, cytosolic fractions used for individual assays (1-3) were obtained from replicate cell cultures.
[c]Data is represented graphically in Figure 7.

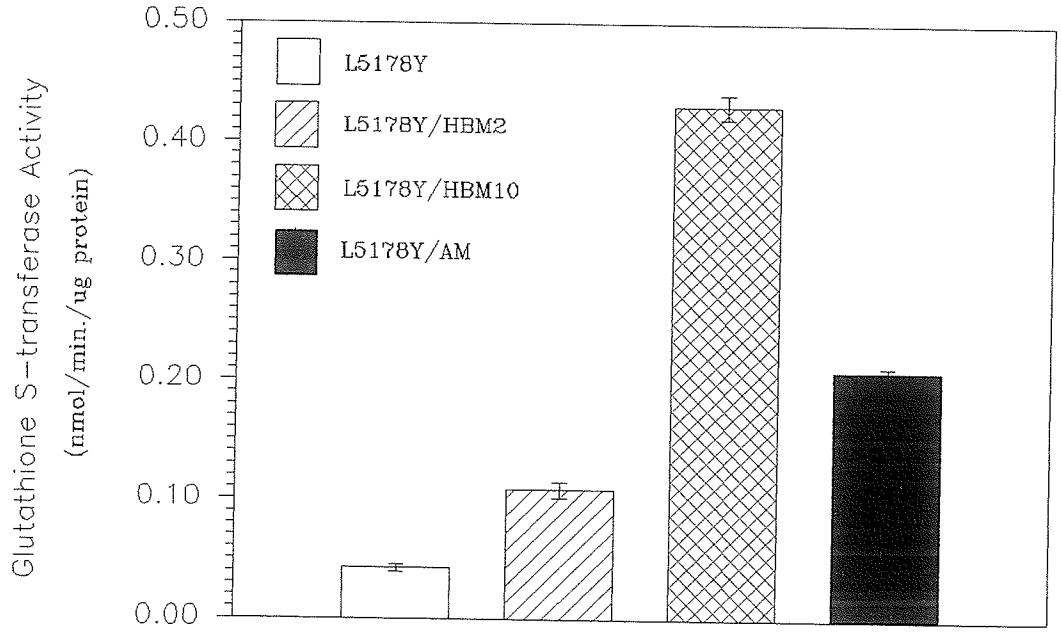

Figure 7. Specific GST activity of the cytosolic fraction of cell sonicates from drug sensitive, HBM-resistant, and AM-resistant L5178Y lymphoblasts.

4.1.0 Densitometry

The Western bands in Figure 8c represent class Pi GST from a series of sequentially decreasing quantities of total cytosolic protein from L5178Y/HBM10 cells. The densitometric area of each band was measured and plotted as a function of the quantity of total cytosolic protein loaded per lane (Figure 8a). The relationship between densitometric area (OD·mm) and sample quantity (μg) approximates linearity over the range 0.0 - 0.9 OD·mm (Figure 8b). The calibration curve shown in Figure 8b was used for the densitometric analysis of Western bands that correspond to cytosolic class GSTs from drug sensitive and resistant L5178Y lymphoblasts.

4.1.1 Identification of GSTs on western blots

Western blots show the presence of other reactive bands in addition to those identified specifically as GSTs. These non-GST bands are a result of two factors. First, SDS-PAGE and Western blotting were carried out using total cytosolic protein from the cytosolic fraction of each cell line. Second, the polyclonal antisera used for Western analysis contain contaminating antibody species that react with the non-GST protein present on these membranes. Unless purified further, antisera raised

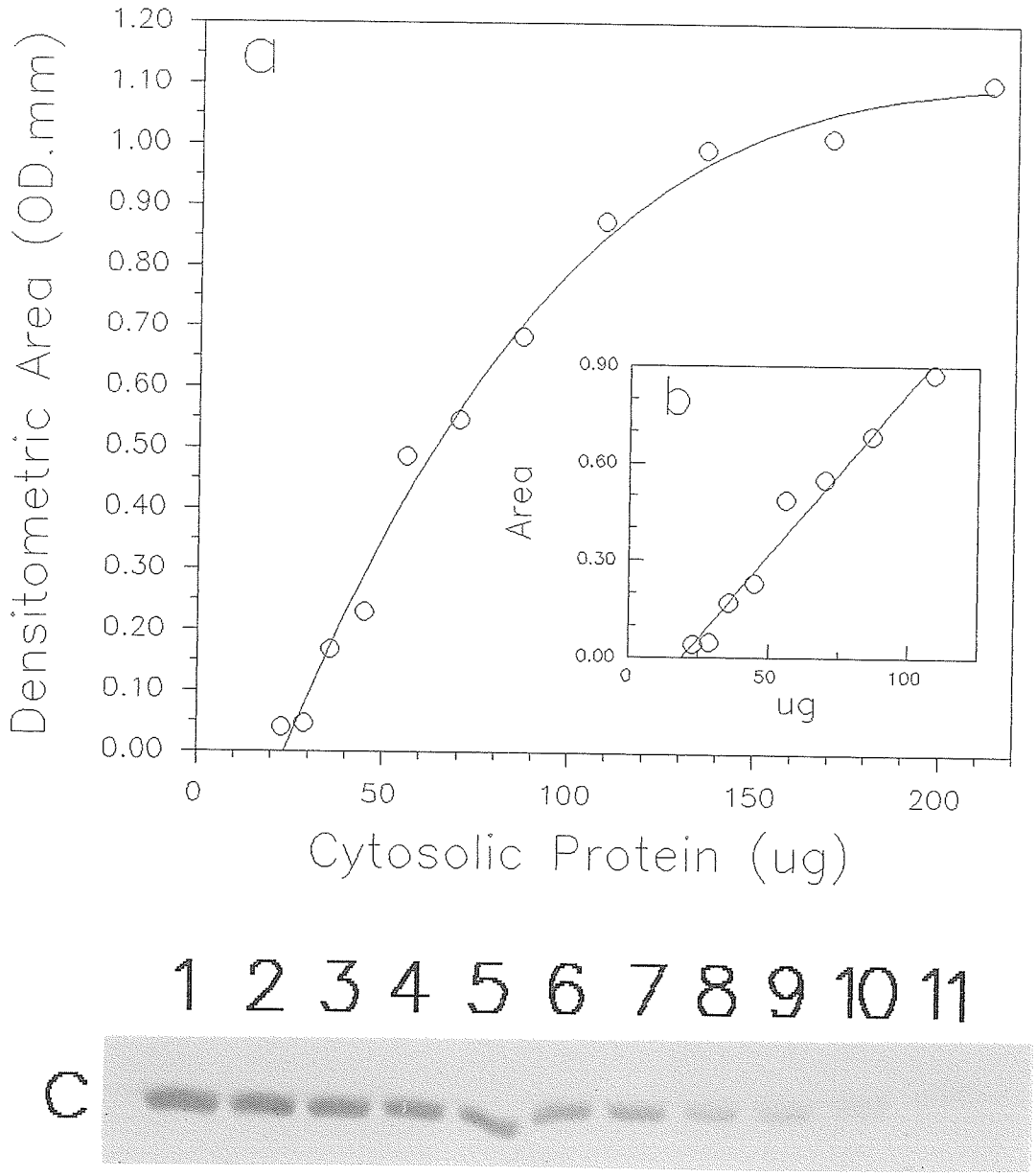

Figure 8. Variation of densitometric area (OD.mm) with quantity of cytosolic protein (μg): a) graph of reflectance data from Western blot in c; b) range over which densitometric area and sample quantity approximate linearity; and c) Western blot of total cytosolic protein from L5178Y/HBM10 probed with class Pi specific anti-GST polyclonal antiserum. Lanes 1-11: 213, 170, 136, 109, 87, 70, 56, 45, 36, 29, and 23 μg protein, respectively.

in animals will normally contain contaminating antibodies that are directed against antigens other than the target antigen. No attempt was made to purify the antisera recieved for use in Western analysis and the extent to which they were purified by the investigators who provided them is unknown. GSTs were identified using a number of criteria. The molecular weights of GST subunits fall in the general range of 21-29 kDal (40), which eliminates bands outside this range. Purified rat liver GSTs were also loaded onto SDS-polyacrylamide gels alongside cytosolic protein samples from drug-sensitive and -resistant cell lines. These purified GSTs were used as standards to confirm the position of GST proteins on Western blots (Figure 14c, lane 5). Finally, as indicated in Table 4, the molecular weights of cytosolic GSTs follow the general order: class Mu > class Alpha > class Pi. This was the order demonstrated by Western bands identified as class Alpha, class Mu, and class Pi GST in L5178Y lymphoblasts.

4.1.2 GST expression in HBM-sensitive and -resistant L5178Y lymphoblasts

Relative to the sensitive L5178Y cell line, the Western blots in Figure 9 demonstrate the increased expression of three cytosolic classes of GST in

Table 4. Subunit molecular weight and specific GST activity towards the substrate CDNB, for class Alpha, class Mu, and class Pi cytosolic GSTs in the mouse.[a]

	Specific GST Activity (μmol/min/mg)				
Isozyme	Class Mu	Class Alpha	Class Pi	Subunit Molecular Weight (kDal)	Ref.
GT-8.7	95			ND[c]	11
GT-8.8a	86			ND	11
GT-8.8b	109			ND	11
GT-9.3	22.2			ND	11
MIII	148			26.5	7
Yb_1Yb_3	74			26.4,26.5	58
Yb_1Yb_1	193			26.4	58
Yb_1Yb_2	81			26.4,26.2	58
GT-10.3		6.0		ND	11
GT-10.6		14.5		ND	11
Ya_3Ya_3		15.1		25.8	10
Ya_1Ya_1		3.14		25.6	10
MI		19		25	7
GT-9.0			69	ND	11
YfYf[b]			75.4	24.8	120
YfYf[b]			85.2	24.8	120
MII			119	23	7

[a]Data compiled from references (Ref.) listed.
[b]The YfYf isozyme was isolated in two separate fractions (PIa and PIb) using hydroxyapatite HPLC.
[c]ND, not determined.

HBM-resistant cell lines. Densitometric analysis indicated, relative to L5178Y, that a class Pi GST was elevated 2.2-, 6.4-, and 5.1-fold, while a class Mu GST was elevated 1.6-, 3.3-, and 1.1-fold in L5178Y/HBM2, L5178Y/HBM10, and L5178Y/HBMR resistant cell lines, respectively (Figure 9a,b). The increased expression of a class Alpha GST is apparent only in the 6-fold resistant L5178Y/HBM10 cell line (Figure 9c, lane 3). A comparison of the expression level of the class Alpha GST between HBM-sensitive and -resistant cell lines could not be obtained by densitometry due to the lack of a detectable reference signal in the sensitive L5178Y cell line (Figure 9c, lane 1). In addition, other bands in close proximity to the identified class Alpha band would likely interfere with densitometric measurements, were they possible. Western analysis was used as an alternative approach to densitometry for the analysis of class Alpha GST expression. A Western blot was prepared which contained equivalent quantities of total cytosolic protein from both L5178Y and L5178Y/HBM10 cell lines, in addition to a series of sequentially decreasing quantities of total cytosolic protein from L5178Y/HBM10. This Western is shown in Figure 10 and demonstrates that an approximate four fold reduction in the initial quantity of cytosolic protein from L5178Y/HBM10 was required to reduce the intensity of the class Alpha isozyme band to background

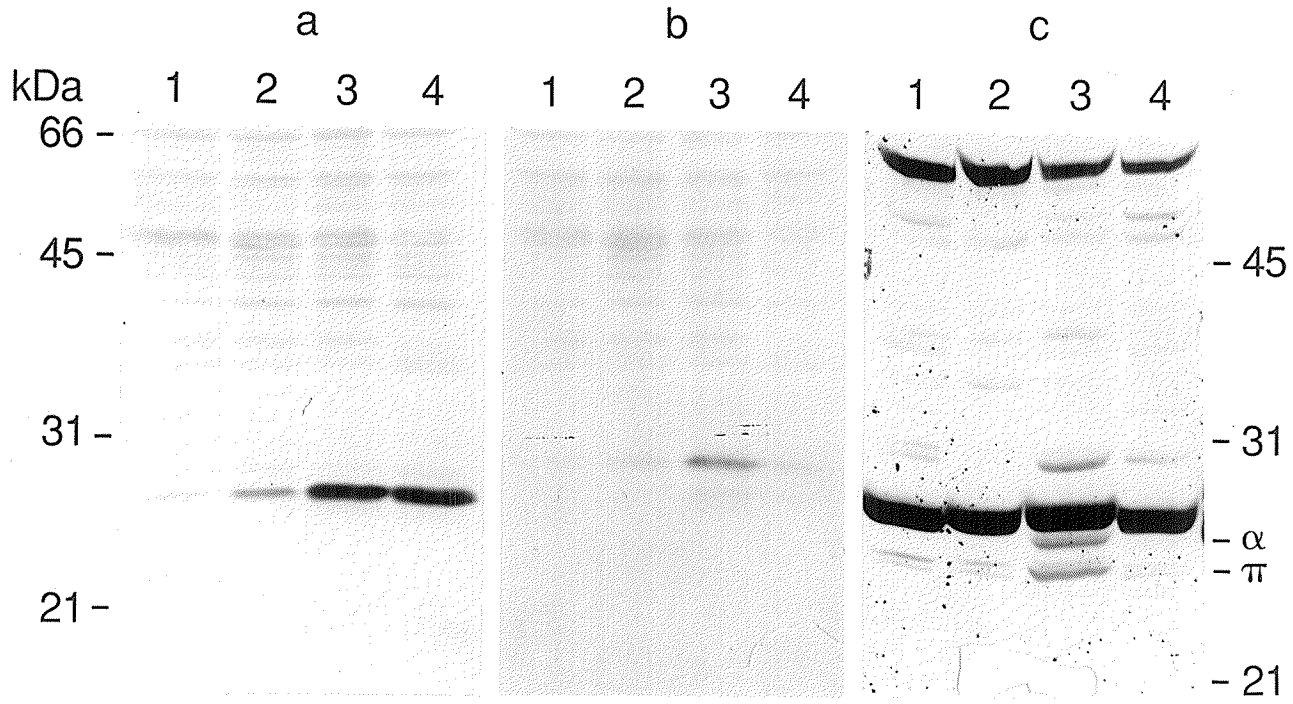

Figure 9. Western blot analysis of GST expression in HBM-sensitive and -resistant L5178Y lymphoblasts. 200 ug of total cytosolic protein from each of the following cell lines was loaded per lane: Lane 1, sensitive L5178Y cells; Lane 2, resistant L5178Y/HBM2 cells; Lane 3, resistant L5178Y/HBM10 cells; and Lane 4, partial revertant L5178Y/HBMR cells. Blots were probed with polyclonal antisera against the following GST antigens: a) human GST Pi (class Pi); b) mouse GST F3 (class Mu); and c) rat liver GST Ya (class Alpha), Yb (class Mu), and Yc (class Alpha). Molecular weight standards were bovine serum albumin (66 kDa), hen egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21 kDa). The gels in panels a and b were run simultaneously and for a shorter time than the gel shown in panel c.

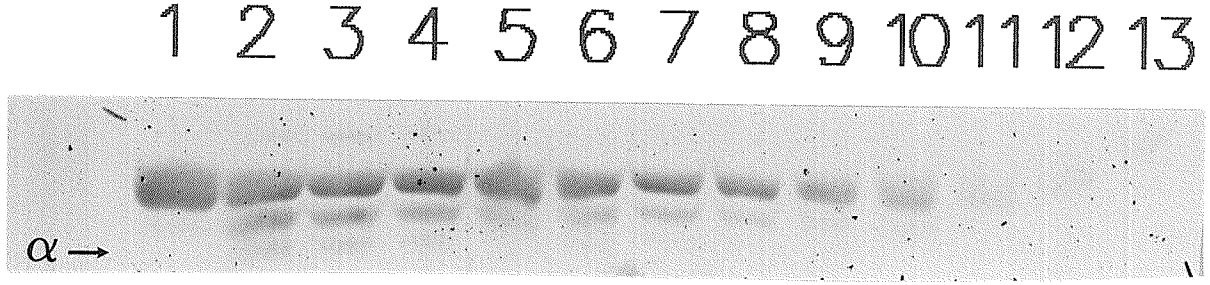

Figure 10. Western blot analysis of the relative expression of class Alpha GST in HBM-sensitive L5178Y and HBM-resistant L5178Y/HBM10 cell lines. The amount of total cytosolic protein loaded per lane was: Lane 1, sensitive L5178Y cells, 250 ug; Lanes 2-13, resistant L5178Y/HBM10 cells, 250, 200, 160, 128, 102, 82, 66, 52, 42, 34, 27, and 21 ug, respectively. The blot was probed with polyclonal antiserum containing antibodies directed against rat liver Ya (class Alpha), Yb (class Mu), and Yc (class Alpha) GST.

Table 5. Relative GST expression between drug sensitive, HBM-resistant, and AM-resistant L5178Y lymphoblasts.[e]

	Glutathione S-transferase		
Cell line	Class Pi	Class Mu	Class Alpha
L5178Y	1	1	NDS[d]
L5178Y/HBM2	2.2±0.1[a]	1.6±0.2[a]	ND[d]
L5178Y/HBM10	6.4±1.0[a]	3.3±0.9[a]	~4[b]
L5178Y/HBMR	5.1±1.3[a]	1.1[a,c]	ND[d]
L5178Y/AM	3.0±0.2[a]	3.4±0.4[a]	>12[b]

[a]Data were obtained by densitometric analysis of Western blots (mean ± S.E. of 2-4 determinations).
[b]Densitometric analysis not possible. Data obtained by Western blot analysis as described in section 4.1.2.
[c]Single determination.
[d]ND, not determined; NDS, no detectable signal.
[e]Data is represented graphically in Figure 11.

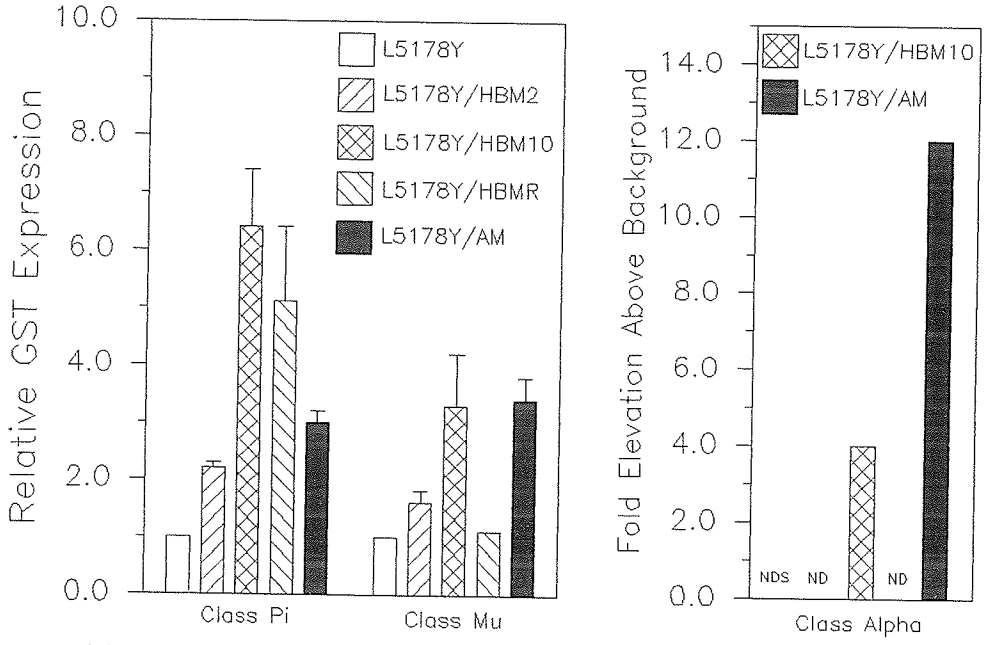

Figure 11. Relative GST expression between drug sensitive, HBM-resistant, and AM-resistant L5178Y lymphoblasts. ND, not determined; NDS, no detectable signal.

level. Thus, relative to L5178Y, there is an approximate 4-fold increase in the expression of the class Alpha GST in L5178Y/HBM10. GST expression in HBM-sensitive and -resistant L5178Y lymphoblasts has been summarized in Table 5 and Figure 11.

4.1.3 GST expression in AM-sensitive and -resistant L5178Y lymphoblasts

Western analysis of GST expression in AM-sensitive and -resistant cell lines also demonstrated, relative to the sensitive L5178Y cell line, that three cytosolic classes of GST have elevated expression levels in the resistant L5178Y/AM cell line (Figure 12). Compared to L5178Y, densitometric analysis indicates that the expression of a class Pi and a class Mu GST are 3.0- and 3.4-fold elevated, respectively, in L5178Y/AM (Figure 12a and 12b). A class Alpha GST also shows a significantly increased level of expression in L5178Y/AM (Figure 12c). Both the lack of a reference signal in L5178Y and the presence of interfering bands precluded a densitometric analysis of the relative expression level of the class Alpha GST between AM-sensitive and -resistant cell lines. Therefore, Western blot analysis as described in section 4.1.2 was employed for this purpose. Figure 13 demonstrates that the class Alpha band remains easily

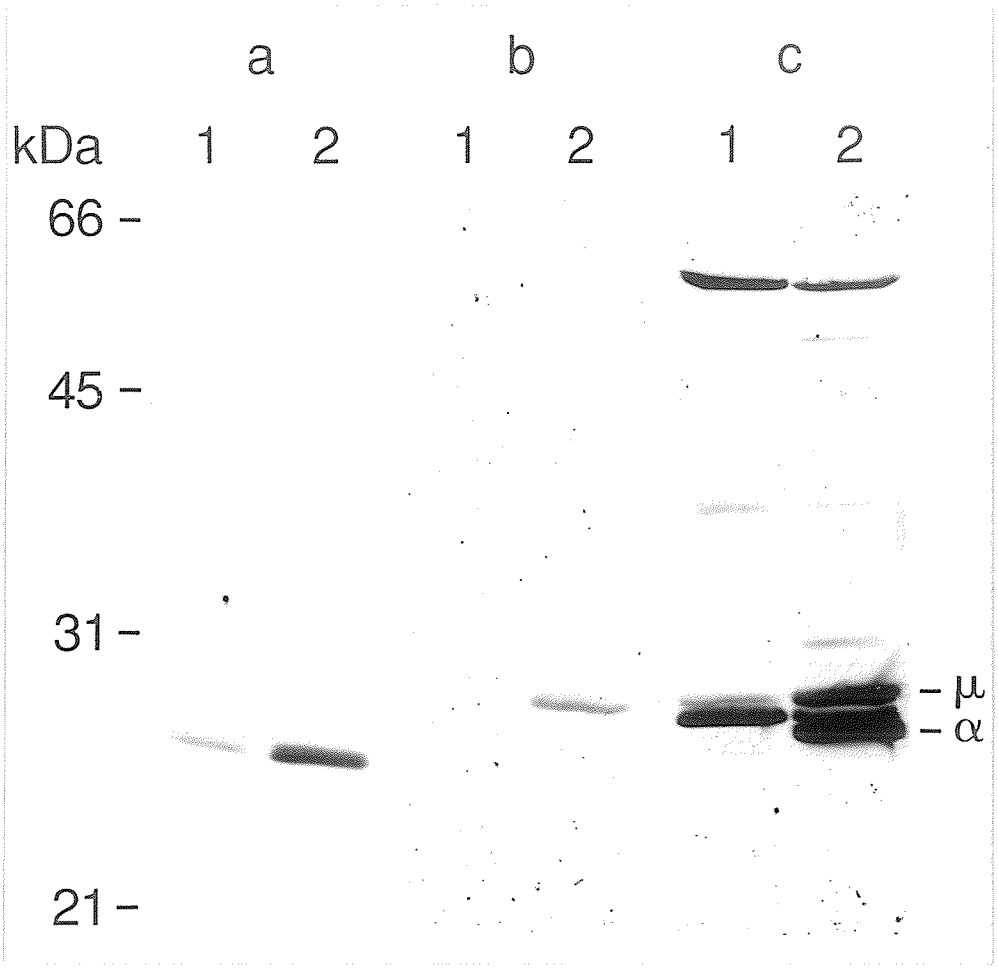

Figure 12. Western blot analysis of GST expression in AM-sensitive and -resistant L5178Y lymphoblasts. 200 ug of total cytosolic protein from each of the following cell lines was loaded per lane: Lane 1, sensitive L5178Y cells; Lane 2, resistant L5178Y/AM cells. Blots were probed with polyclonal antisera against the following GST antigens: a) human Pi (class Pi); b) mouse GST F3 (class Mu); and c) rat liver GST Ya (class Alpha), Yb (class Mu), and Yc (class Alpha). Molecular weight standards were bovine serum albumin (66 kDa), hen egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21 kDa).

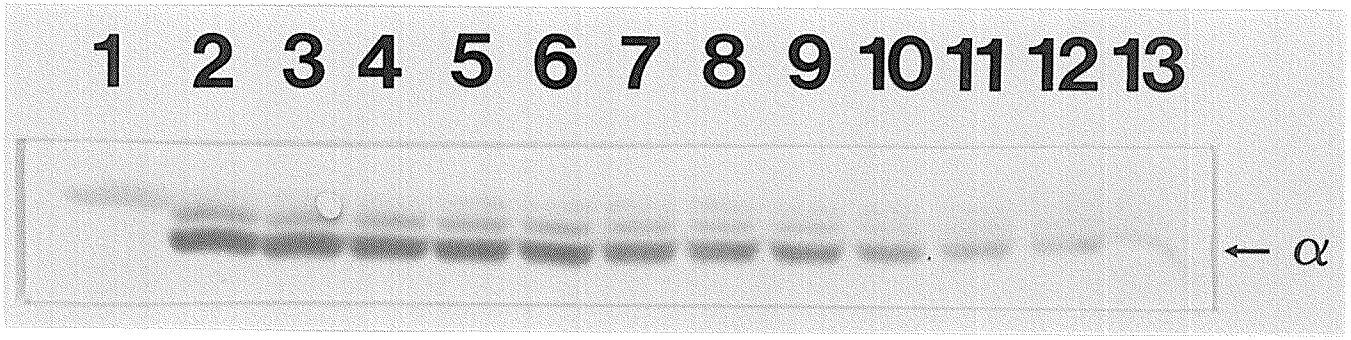

Figure 13. Western blot analysis of the relative expression of class Alpha GST in AM-sensitive L5178Y and AM-resistant L5178Y/AM cell lines. The amount of total cytosolic protein loaded per lane was: Lane 1, sensitive L5178Y cells, 250 ug; Lanes 2-13, resistant L5178Y/AM cells, 250, 200, 160, 128, 102, 82, 66, 52, 42, 34, 27, and 21 ug, respectively. The blot was probed with polyclonal antiserum containing antibodies directed against rat liver Ya (class Alpha), Yb (class Mu), and Yc (class Alpha) GST.

detectable even after a 12-fold reduction in the initial quantity of total cytosolic protein from L5178Y/AM. Therefore, relative to L5178Y, there appears to be a greater than 12-fold increase in the expression of a class Alpha GST in L5178Y/AM. GST expression in AM-sensitive and -resistant L5178Y lymphoblasts has been summarized in Table 5 and Figure 11.

4.1.4 The expression profiles of class specific GSTs in HBM- and AM-resistant L5178Y lymphoblasts

Relative to the sensitive L5178Y cell line, Western blot analysis has shown increased expression levels of class Alpha, class Mu, and class Pi cytosolic GSTs in HBM- and AM-resistant cell lines. However, the data also demonstrate that for GSTs belonging to the same class, their relative expression levels differ in HBM- and AM-resistant cell lines. The expression profiles of class specific GSTs in drug sensitive, HBM-resistant, and AM-resistant L5178Y lymphoblasts are illustrated in Figure 14. In conjunction with Table 5 and Figure 11, the expression levels of class Mu and class Pi GSTs in L5178Y/AM are aproximately similar and one half, respectively, that found in L5178Y/HBM10. In contrast, the expression level of the class Alpha GST in L5178Y/AM is

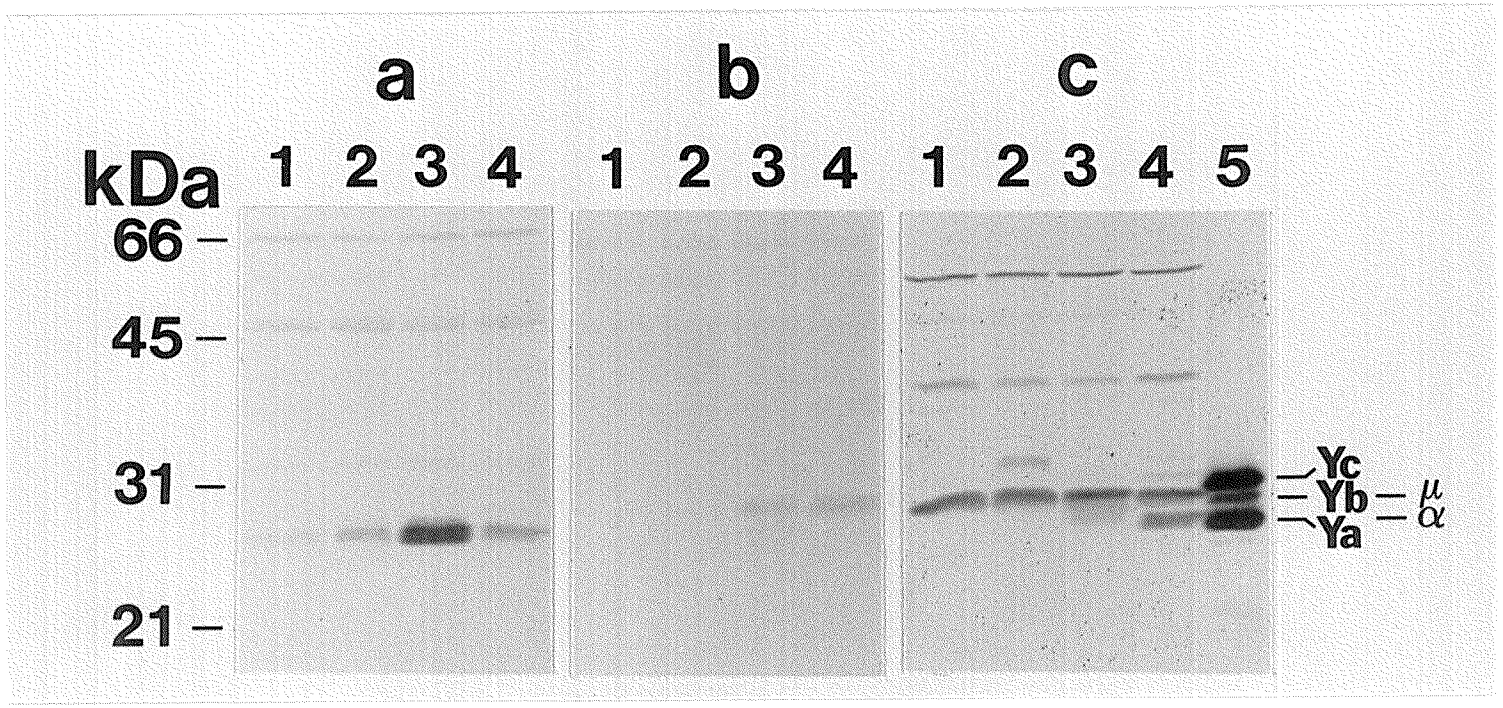

Figure 14. Western blot analysis of GST expression in drug sensitive L5178Y, HBM–resistant, and AM–resistant L5178Y lymphoblasts. 200 ug of total cytosolic protein from each of the following cell lines was loaded per lane: Lane 1, sensitive L5178Y cells; Lane 2, resistant L5178Y/HBM2 cells; Lane 3, resistant L5178Y/HBM10 cells; and Lane 4, resistant L5178Y/AM cells. Lane 5 in panel c contains purified GST corresponding to the Yc, Yb, and Ya subunits from rat liver (Sigma Chem. Co., St. Louis, MO). Blots were probed with polyclonal antisera against the following GST antigens: a) human GST Pi (class Pi); b) mouse F3 (class Mu); and c) rat liver GST Ya (class Alpha), Yb (class Mu), and Yc (class Alpha).

greater than 3-fold that of the L5178Y/HBM10 cell line.

4.2 Northern blot analysis

4.2.0 Densitometry

The autoradiographic bands in Figure 15c and 15d represent class Pi GST mRNA from a Northern blot containing a series of sequentially decreasing quantities of total RNA from L5178Y/HBM10 cells. The autoradiograms were obtained by exposure to the same membrane after hybridization and differ only in their time of exposure. The densitometric area (OD·mm) of each band was measured and plotted as a function of the quantity (μg) of total RNA loaded per lane. The plots in Figure 15a and 15b were obtained from the densitometric analysis of the autoradiograms in Figure 15c and 15d, respectively. Figure 15a demonstrates how over exposure of film gives rise to a non-linear response for the relationship between densitometric area and sample quantity. Using an appropriate exposure time, Figure 15b shows that densitometric area and sample quantity bear an approximate linear relationship over the range 0.0 - 15.0 OD·mm. The calibration curve shown in Figure 15b was used for the densitometric analysis of autoradiographic images that

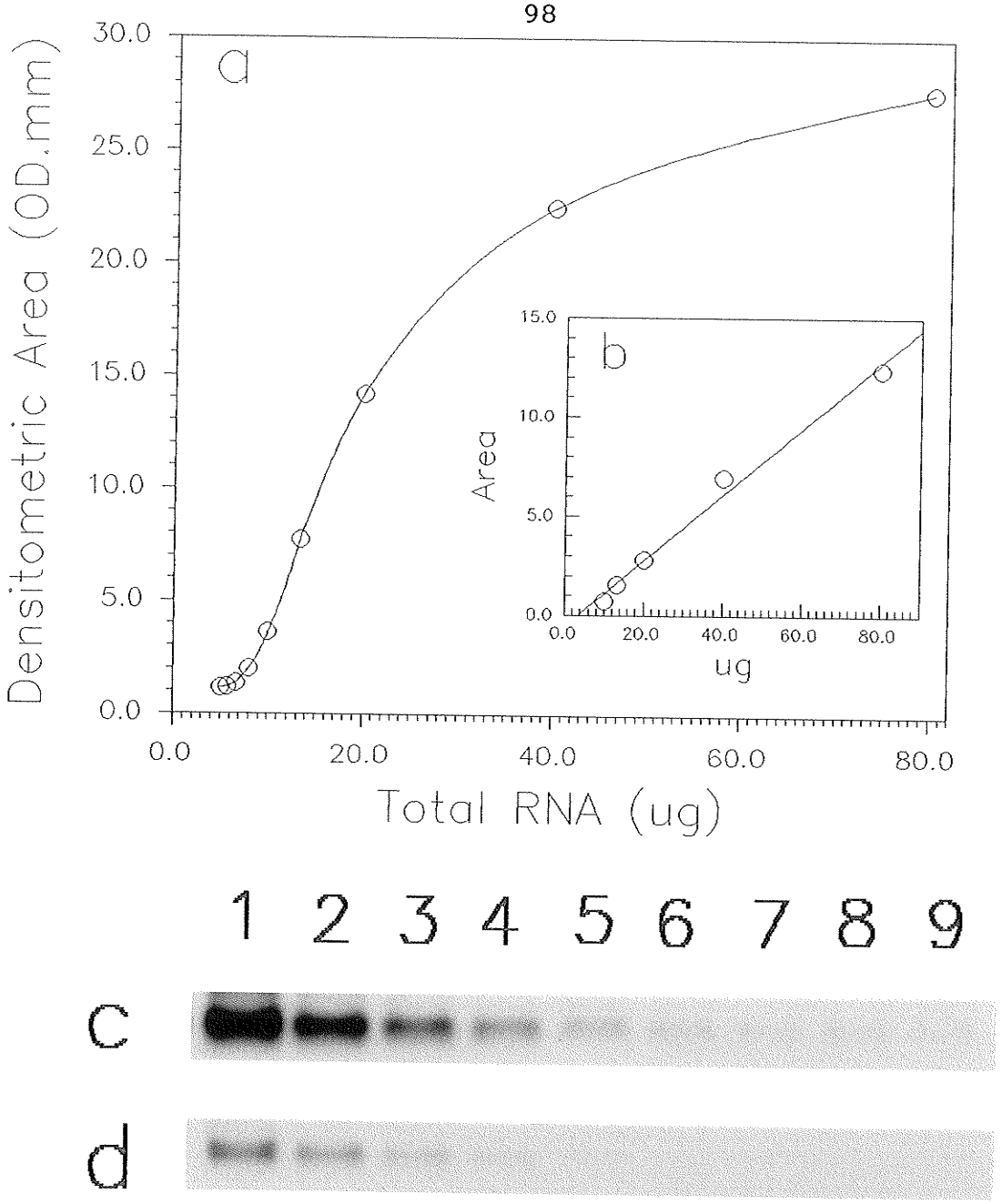

Figure 15. Variation of densitometric area (OD·mm) with quantity of total RNA (µg). Panels a and b are plots of transmittance data from autoradiograms in panels c and d, respectively. The autoradiograms in panels c and d differ only in their exposure time to a Northern blot containing total RNA from L5178Y/HBM10 that was probed with a class Pi cDNA. Lanes 1-9: 80, 40, 20, 13.3, 10, 8, 6.7, 5.7, and 5 µg total RNA, respectively.

represent GST mRNAs in drug-sensitive and -resistant L5178Y lymphoblasts.

4.2.1 GST mRNA expression in HBM-sensitive and -resistant L5178Y lymphoblasts

Northern blot analysis demonstrates the induction of class Alpha, class Mu, and class Pi GST mRNAs in HBM-resistant cell lines (Figure 16). Relative to the sensitive L5178Y cell line, densitometric analysis indicated that a class Pi mRNA was elevated 1.3-, 8.4-, and 5.4-fold, while a class Mu mRNA was elevated 2.5-, 5.7-, and 2.1-fold in the HBM-resistant cell lines L5178Y/HBM2, L5178Y/HBM10, and L5178Y/HBMR, respectively (Fig. 16a and 16b). A class Alpha mRNA, induced in L5178Y/HBM10, was not expressed in the sensitive L5178Y cell line (Figure 16c). Therefore, relative to L5178Y, the expression level of the class Alpha mRNA in L5178Y/HBM10 could not be accessed by densitometry. However, using the technique described in section 4.1.2, Northern blot analysis shows that an approximate 4-fold reduction in the initial quantity of total RNA from L5178Y/HBM10 brought the class Alpha mRNA signal to background level (Figure 17a). This suggests a 4-fold increase in the expression level of the class Alpha mRNA in L5178Y/HBM10, relative to the sensitive L5178Y cell line. GST mRNA expression in

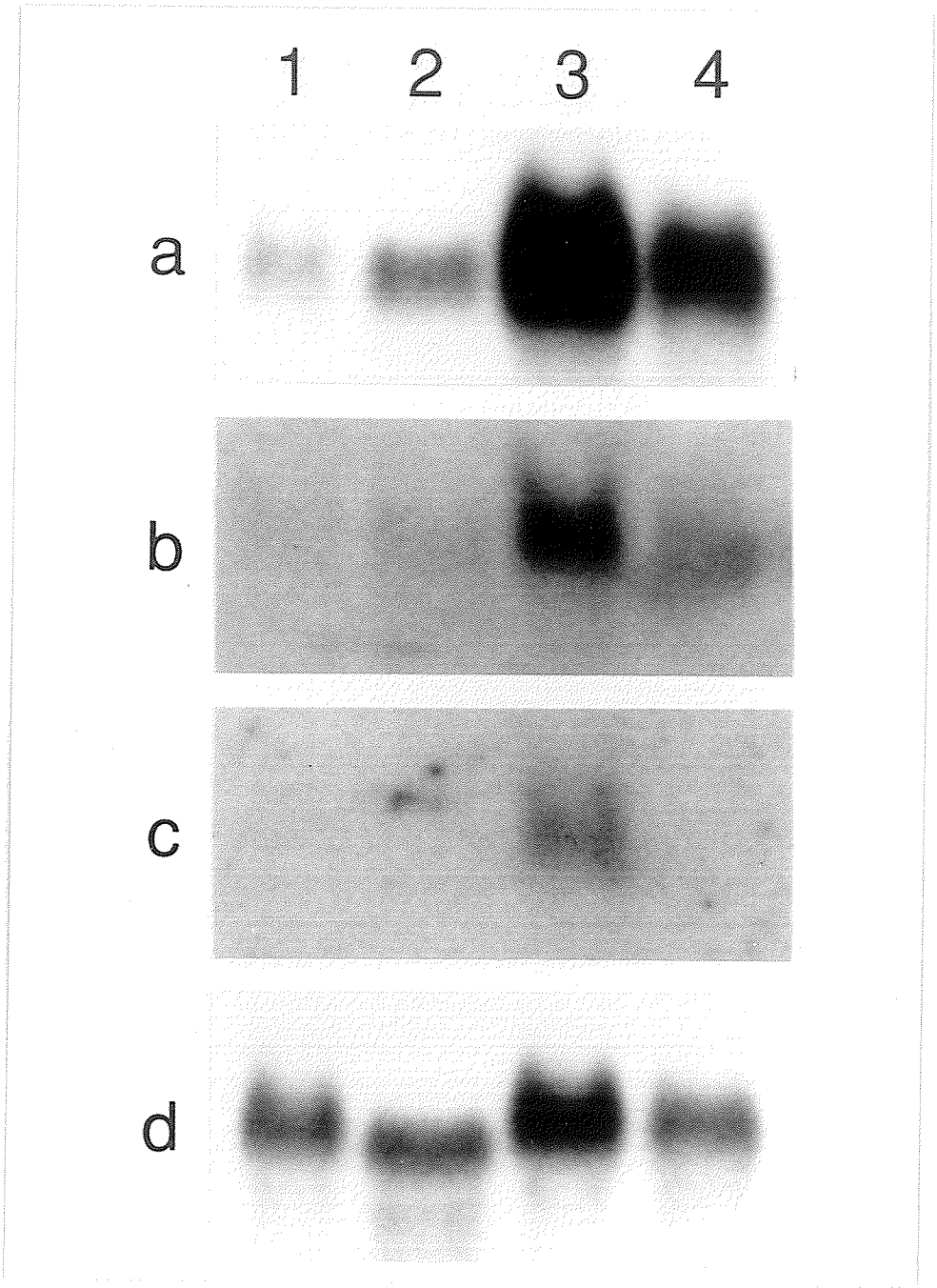

Figure 16. Northern blot analysis of GST mRNA expression in HBM-sensitive and -resistant L5178Y lymphoblasts. 50 ug of total RNA from each of the following cell lines was loaded per lane: Lane 1, sensitive L5178Y cells; Lane 2, resistant L5178Y/HBM2 cells; Lane 3, resistant L5178Y/HBM10 cells; and Lane 4, partial revertant L5178Y/HBMR cells. The blot was probed sequentially with a cDNA complimentary to mRNA from each of the following GST classes: a) human Pi (class Pi); b) rat liver Yb2 (class Mu); c) rat liver Ya (class Alpha); and d) beta-actin. The signal obtained with beta-actin was used to correct for irregularities in RNA loading.

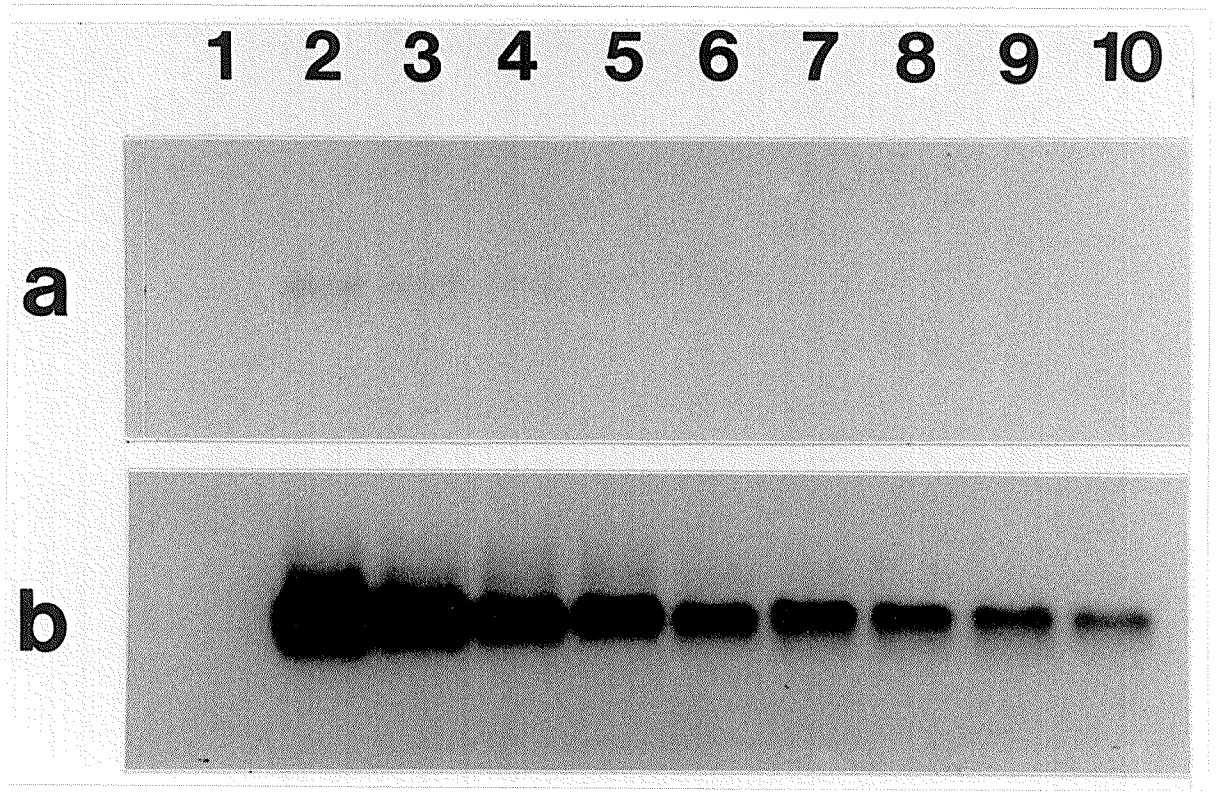

Figure 17. Northern blot analysis of the relative expression of class Alpha GST in drug sensitive L5178Y, HBM-resistant L5178Y/HBM10, and AM-resistant L5178Y/AM cell lines. The lanes contain total RNA from the following cell lines: Panel a) Lane 1, sensitive L5178Y cells; Lanes 2-10, resistant L5178Y/HBM10 cells. Panel b) Lane1, sensitive L5178Y cells; Lanes 2-10, resistant L5178Y/AM cells. The amount of total RNA loaded per lane was: Lane 1, 80 ug; Lanes 2-10, 80, 40, 20, 13.3, 10, 8, 6.7, 5.8, and 5.1 ug, respectively. The blot was hybridized with a 521 bp fragment of the cDNA insert from plasmid pGTB38, which is complimentary to the rat Ya (class Alpha) GST mRNA. After gel electrophoresis, the RNA samples represented in panels a and b were Northern blotted to a single membrane to allow for identical conditions of hybridization. The autoradiograms shown in panels a snd b represent the relevant sections of a single film and therefore conditions for autoradiography were identical.

Table 6. Relative GST mRNA expression between drug sensitive, HBM-resistant, and AM-resistant L5178Y lymphoblasts.[d]

	Glutathione S-transferase mRNA		
Cell line	Class Pi	Class Mu	Class Alpha
L5178Y	1	1	NDS[c]
L5178Y/HBM2	1.3±0.1[a]	2.5±0.2[a]	ND[c]
L5178Y/HBM10	8.4±1.1[a]	5.7±1.3[a]	~4[b]
L5178Y/HBMR	5.4±0.7[a]	2.1±0.5[a]	ND[c]
L5178Y/AM	3.4±1.4[a]	7.9±3.0[a]	>16[b]

[a]Data were obtained by densitometric analysis of autoradiograms from Northern blots (mean ± S.E. of 2-4 determinations).

[b]Densitometric analysis not possible. Data obtained by Northern blot analysis as described in section 4.1.2.

[c]ND, not determined; NDS, no detectable signal.

[d]Data are represented graphically in Figure 18.

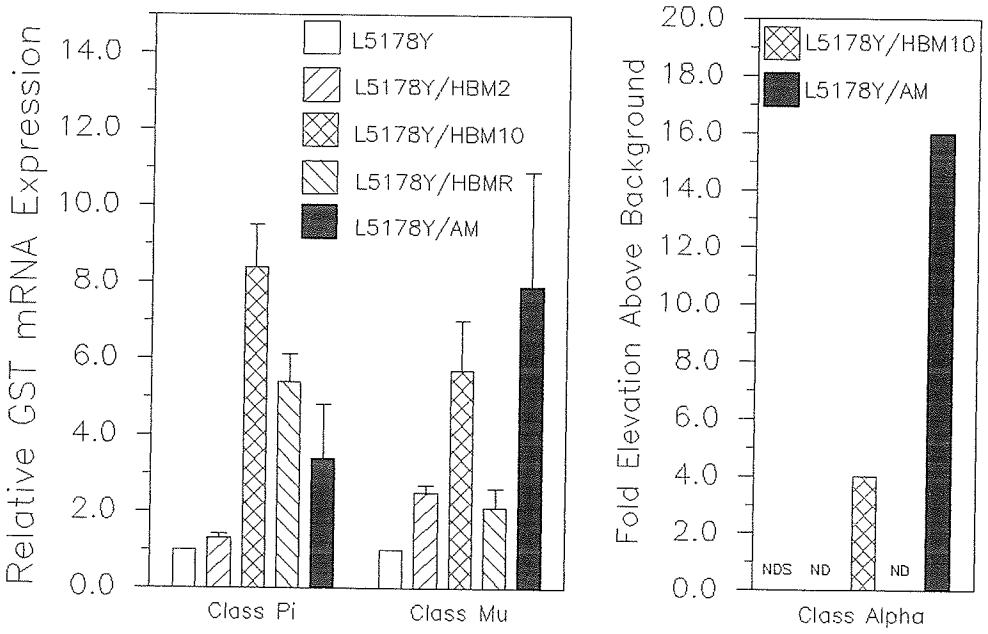

Figure 18. Relative GST mRNA expression between drug sensitive, HBM-resistant, and AM-resistant L5178Y lymphoblasts. ND, not determined; NDS, no detectable signal.

HBM-sensitive and -resistant L5178Y lymphoblasts has been summarized in Table 6 and Figure 18.

4.2.2 GST mRNA expression in AM-sensitive and -resistant L5178Y lymphoblasts

Relative to the sensitive L5178Y cell line, induction of class Alpha, class Mu, and class Pi GST mRNAs was also observed in the AM-resistant cell line, L5178Y/AM (Figure 19). Densitometric analysis of GST mRNA expression in L5178Y/AM indicated that class Pi and class Mu mRNAs were 3.4- and 7.9-fold elevated, respectively, relative to L5178Y (Figure 19a and 19b). Using the technique described in section 4.1.2., the level of induction of the class Alpha mRNA in L5178Y/AM was analyzed by Northern blot analysis (Figure 17b). In L5178Y/AM, the class Alpha band remained easily detectable after a 16-fold reduction in the initial quantity of total RNA from this cell line. Therefore, relative to L5178Y, there appears to be a greater than 16-fold increase in the expression of the class Alpha mRNA in the AM-resistant cell line, L5178Y/AM. GST mRNA expression in AM-sensitive and -resistant L5178Y lymphoblasts has been summarized in Table 6 and Figure 18.

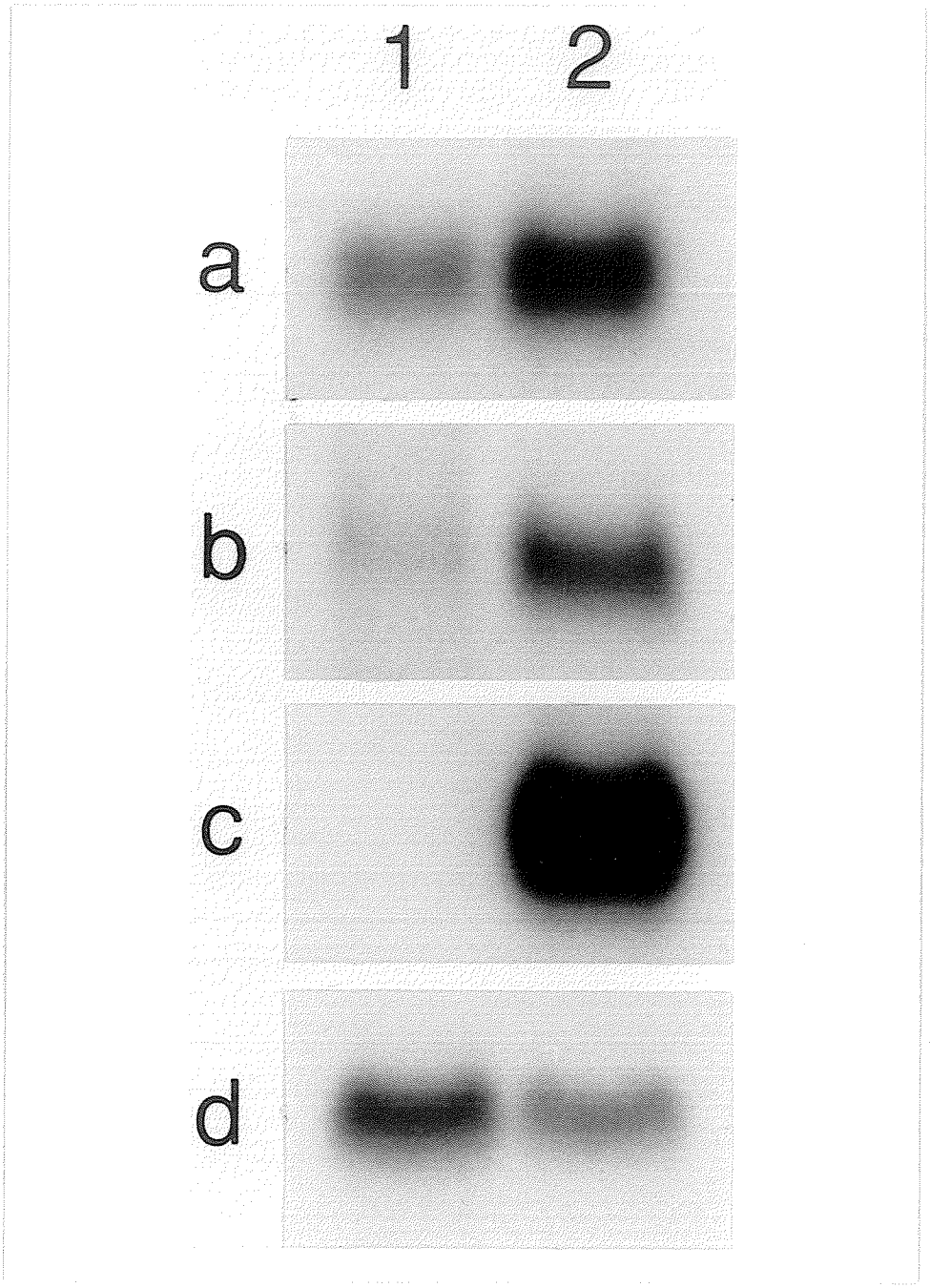

Figure 19. Northern blot analysis of GST mRNA expression in AM-sensitive and -resistant L5178Y lymphoblasts. 100 ug of total RNA from each of the following cell lines was loaded per lane: Lane 1, sensitive L5178Y cells; Lane 2, resistant L5178Y/AM cells. The blot was probed sequentially with a cDNA complimentary to mRNA from each of the following GST classes: a) human Pi (class Pi); b) rat liver Yb2 (class Mu); c) rat liver Ya (class Alpha); and d) beta-actin. The signal obtained with beta-actin was used to correct for irregularities in RNA loading.

4.2.3 The expression profiles of class specific GST mRNAs in HBM- and AM-resistant L5178Y lymphoblasts

Relative to the sensitive L5178Y cell line, Northern blot analysis has shown an increased expression of class Alpha, class Mu, and class Pi GST mRNAs in both HBM- and AM-resistant cell lines. Figures 17 and 18 also demonstrate that mRNAs which belong to the same GST class have expression levels which differ in HBM- and AM-resistant cell lines. A comparison of the expression profiles of class specific GST mRNAs between L5178Y/AM and L5178Y/HBM10 resistant cell lines indicates that class Mu and class Pi mRNAs in L5178Y/AM are expressed at levels roughly equivalent (higher by 1.4-fold) and approximately one half, respectively, that found in L5178Y/HBM10 (Figure 18). In contrast, class Alpha mRNA expression in L5178Y/AM is greater than 4-fold the level of expression in the L5178Y/HBM10 cell line (Figure 18). In fact, a direct comparison of the relative expression of class Alpha mRNA between L5178Y/HBM10 and L5178Y/AM demonstrates that the expression of this mRNA is greater than 16-fold higher in L5178Y/AM (Figure 17a and 17b). Therefore, relative to the sensitive L5178Y cell line, this suggests that the expression level of the class Alpha mRNA is increased 64-fold in the AM-resistant cell line, L5178Y/AM.

4.2.4 Expression of p-glycoprotein and quinone reductase mRNA in drug sensitive, HBM-resistant, and AM-resistant L5178Y lymphoblasts

The expression of P-glycoprotein and quinone reductase mRNA was evaluated by Northern blot analysis of total RNA from drug sensitive L5178Y, HBM-resistant, and AM-resistant cell lines. P-glycoprotein mRNA was not detected in any of the cell lines, drug sensitive or resistant (data not shown). Although not detected in the L5178Y cell line, the induced expression of quinone reductase mRNA in both HBM-resistant (L5178Y/HBM10) and AM-resistant (L5178Y/AM) cell lines is shown in Figure 20.

4.3 Southern blot analysis

4.3.0 Southern blot analysis of GST genes in HBM-sensitive and -resistant L5178Y lymphoblasts

Southern blot analysis of GST genes in HBM-sensitive and -resistant L5178Y lymphoblasts is shown in Figure 21. In comparison to the sensitive L5178Y cell line, class Alpha, class Mu, and class Pi genes in HBM-resistant cell lines showed no evidence of either gene amplification or re-arrangement. A more extensive search for restriction

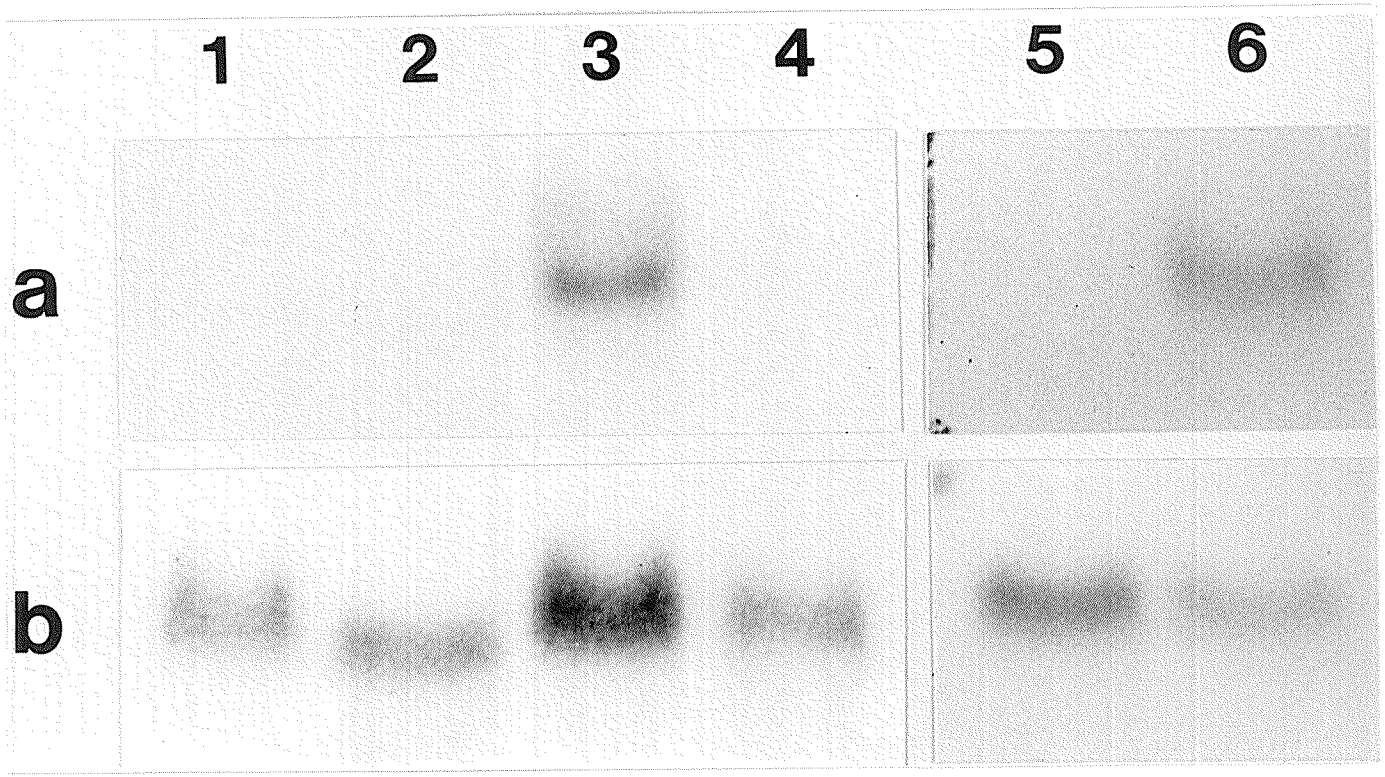

Figure 20. Northern blot analysis of quinone reductase mRNA expression in drug sensitive L5178Y, HBM-resistant, and AM-resistant L5178Y lymphoblasts. 50 ug of total RNA from each of the following drug sensitive and HBM-resistant cell lines was loaded per lane: Lane 1, sensitive L5178Y cells; Lane 2, resistant L5178Y/HBM2 cells; Lane 3, resistant L5178Y/HBM10 cells, and Lane 4, partial revertant L5178Y/HBMR cells. 100 ug of total RNA from each of the following drug sensitive and AM-resistant cell lines was loaded per lane: Lane 5, sensitive L5178Y cells; Lane 6, resistant L5178Y/AM cells. The blots were probed sequentially with: a) an 873 bp fragment of the cDNA insert from plasmid pDTD55, which is complimentary to the quinone reductase mRNA and b) a cDNA for beta-actin. The signal obtained with beta-actin was used to correct for irregularities in RNA loading.

fragment length polymorphism (RFLP) of the class Pi gene in the HBM-resistant cell line, L5178Y/HBM10, was undertaken (Figure 22). Separate digestions with different restriction enzymes were carried out on genomic DNA from L5178Y and L5178Y/HBM10 cells. Again, in comparison with the sensitive L5178Y cell line, Southern blot analysis failed to detect evidence for RFLPs in the class Pi gene of the HBM-resistant L5178Y/HBM10 cell line. The class Mu and class Alpha gene families were not investigated further.

4.3.1 Southern blot analysis of GST genes in AM-sensitive and -resistant L5178Y lymphoblasts

Southern blot analysis of GST genes in AM-sensitive and -resistant L5178Y lymphoblasts is shown in Figures 23 and 24. Comparison with the sensitive L5178Y cell line revealed no evidence for either gene amplification or re-arrangement of class Alpha or class Mu genes in the AM-resistant L5178Y/AM cell line. The class Pi gene was not investigated.

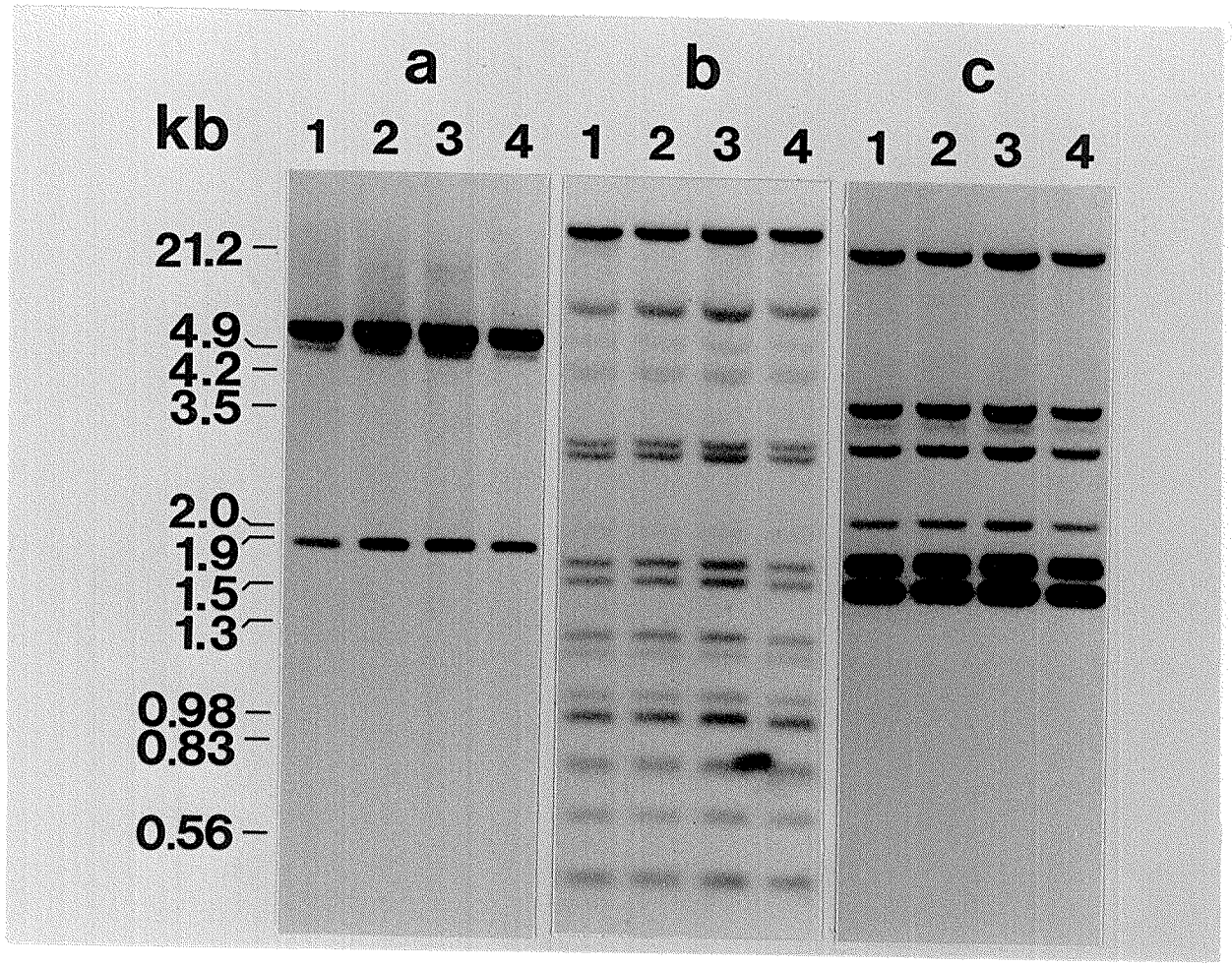

Figure 21. Southern blot analysis of GST genes in HBM-sensitive and -resistant L5178Y lymphoblasts. 20 ug of genomic DNA from each of the following cell lines were loaded per lane: Lane 1, sensitive L5178Y cells; Lane 2, resistant L5178Y/HBM2 cells; Lane 3, resistant L5178Y/HBM10 cells; and Lane 4, resistant L5178Y/HBMR cells. Genomic DNA was digested with the following restriction enzymes: a) Stu I and b-c) Pst I. Blots were probed with a cDNA complimentary to mRNA from each of the following GST classes: a) human Pi (class Pi); b) rat liver Yb_2 (class Mu); and c) rat liver Ya (class Alpha). DNA size markers (kb) are Hind III digested lambda DNA (Boehringer-Mannheim, FRG).

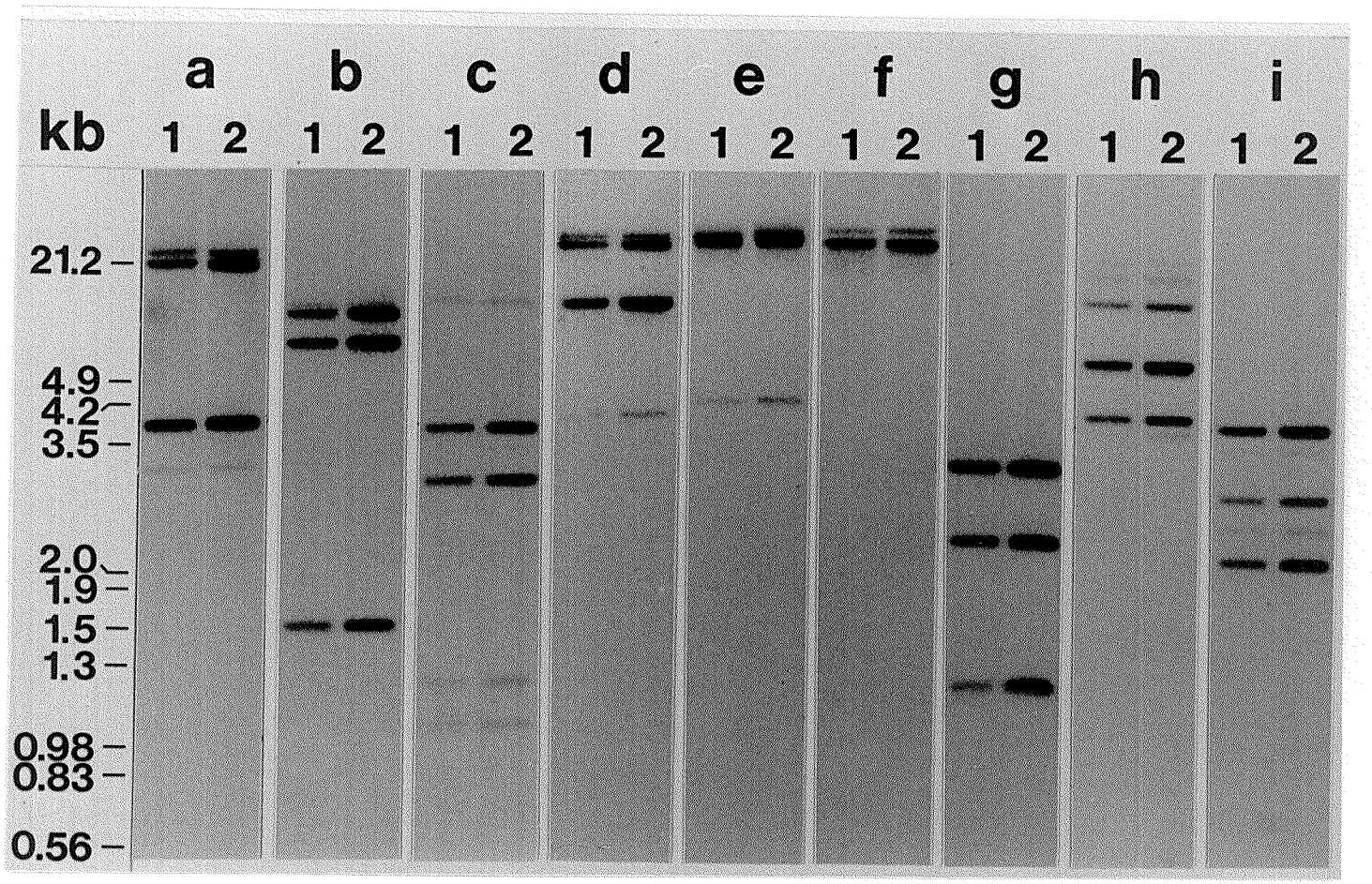

Figure 22: Southern blot analysis of the class Pi GST genes in HBM-sensitive and -resistant L5178Y lymphoblasts. 10 ug of genomic DNA from each of the following cell lines were loaded per lane: Lane 1, sensitive L5178Y cells and Lane 2, resistant L5178Y/HBM10 cells. Genomic DNA was digested with the following restriction enzymes: a) Bcl I, b) Hind III, c) Sst I, d) Xba I, e) Xho I, f) BamH I, g) Bgl II, h) EcoR I, i) Hinc II. Blots were probed with a cDNA complimentary to the human Pi (class Pi) mRNA. DNA size markers (kb) are Hind III digested lambda DNA (Boehringer-Mannheim, FRG).

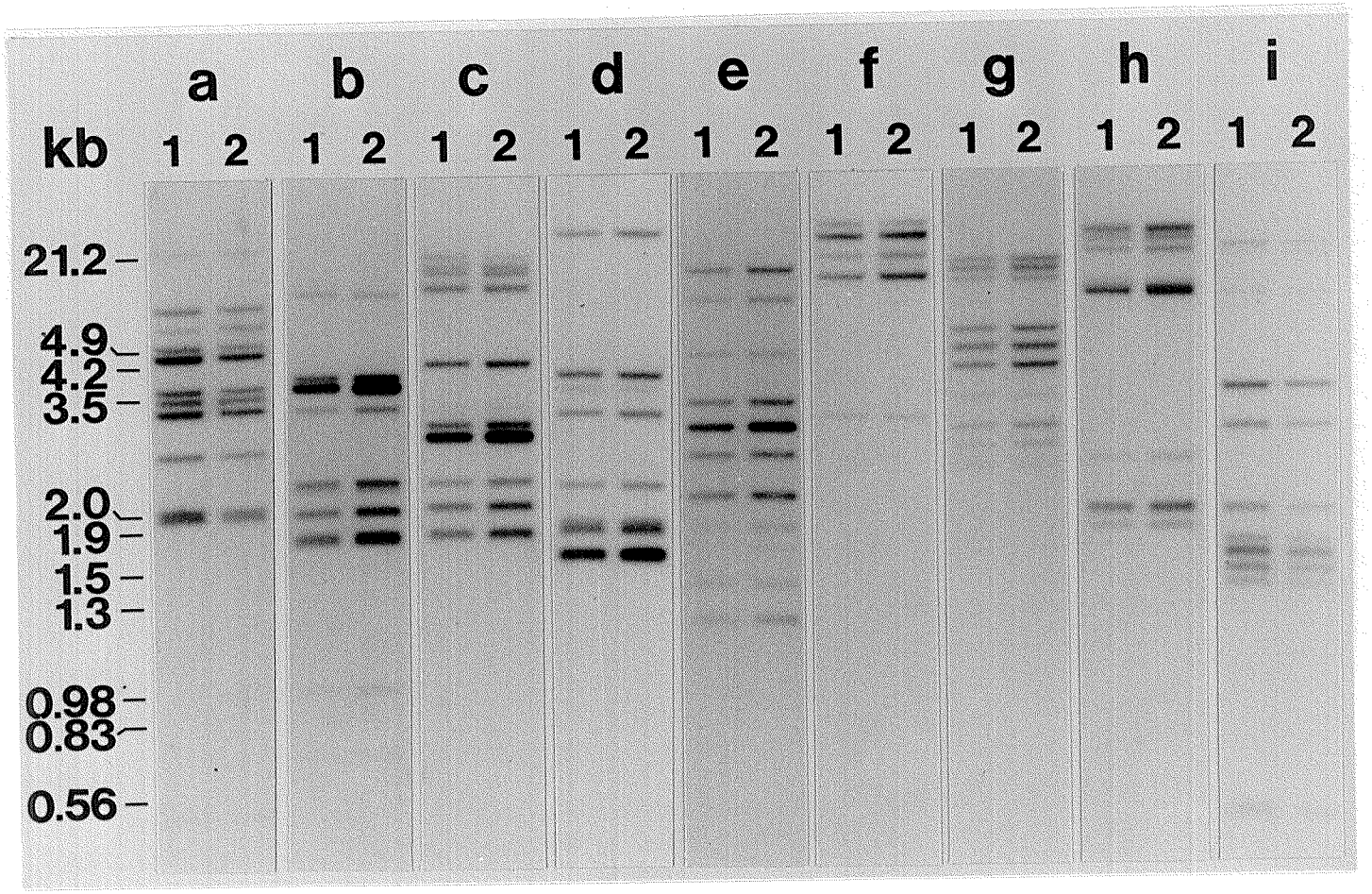

Figure 23. Southern blot analysis of the class Alpha GST genes in AM–sensitive and –resistant L5178Y lymphoblasts. 10 ug of genomic DNA from each of the following cell lines were loaded per lane: Lane 1, sensitive L5178Y cells and Lane 2, resistant L5178Y/AM cells. Genomic DNA was digested with the following restriction enzymes: a) Hind III, b) Stu I, c) Xba I, d) Pst I, e) Bgl II, f) EcoR I, g) Hinc II, h) Bcl I, i) Pvu II. Blots were probed with a cDNA complimentary to the rat liver Ya (class Alpha) mRNA. DNA size markers (kb) are Hind III digested lambda DNA (Boehringer–Mannheim FRG).

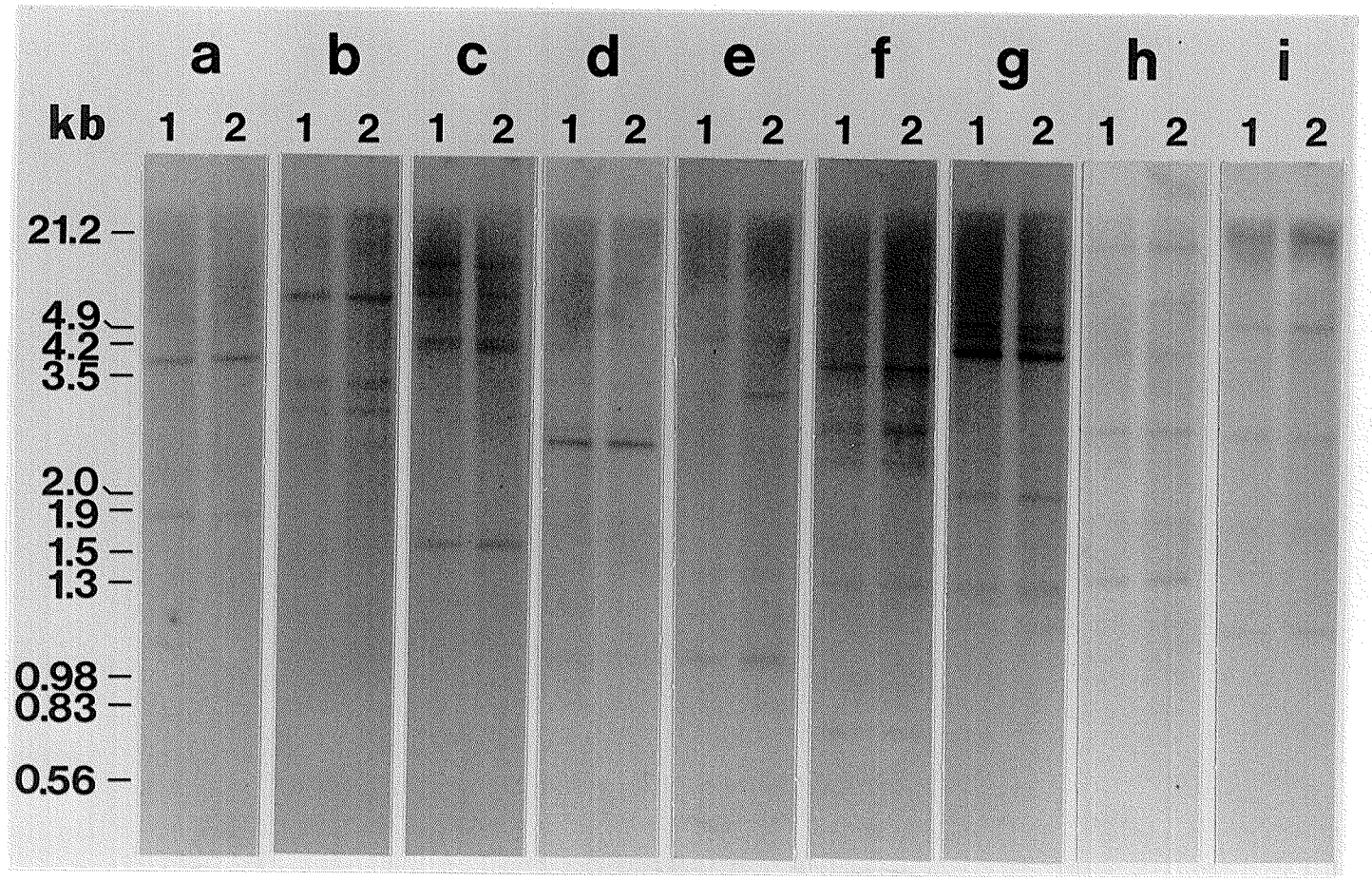

Figure 24. Southern blot analysis of the class Mu GST genes in AM-sensitive and -resistant L5178Y lymphoblasts. 10 ug of genomic DNA from each of the following cell lines were loaded per lane: Lane 1, sensitive L5178Y cells and Lane 2, resistant L5178Y/AM cells. Genomic DNA was digested with the following restriction enzymes: a) Hind III, b) Stu I, c) Xba I, d) Pvu II, e) BamH I, f) Bgl II, g) EcoR I, h) Hinc II, i) Kpn I. Blots were probed with a cDNA complimentary to the rat liver Yb_2 (class Mu) mRNA. DNA size markers (kb) are Hind III digested lambda DNA (Boehringer-Mannheim, FRG).

5. DISCUSSION

The rationale for the present study resulted from earlier characterizations of L5178Y lymphoblasts selected for resistance towards the model antitumor agents hydrolyzed benzoquinone mustard and aniline mustard. HBM- and AM-resistant cell lines demonstrated elevated GST activity. GSTs are a Phase II drug detoxication enzyme and therefore might play a role in the resistance exhibited by these cell lines. Relative to the sensitive L5178Y cell line, GST activity was found to be increased 3-, 11-, and 9-fold, respectively in HBM-resistant L5178Y/HBM2, L5178Y/HBM10, and L5178Y/HBMR cell lines (219), and 3.6-fold in the AM-resistant L5178Y/AM cell line (Begleiter, unpublished data).

To establish whether their relative GST activities were consistent with the activities reported above by Begleiter *et al.*, assays of GST activity were carried out on the cytosolic fractions of drug-sensitive and -resistant cell lines used in the present study. The results indicate that the cell lines used in this study show relative GST activities that are consistent with previous reports (Table 4).

Western blot analysis of GST expression in drug-sensitive and -resistant L5178Y lymphoblasts indicates

that an increased expression of the gene product from three distinct classes of cytosolic GST (class Alpha, class Mu, and Class Pi) contribute to the elevated GST activity observed in HBM- and AM-resistant cell lines (Figures 9 and 12). For the HBM-sensitive and -resistant cell lines the expression of each GST class was also found to correlate with the level of resistance of these cell lines, following the order: L5178Y < L5178Y/HBM2 < L5178Y/HBM10. The expression of the class Alpha and class Mu GSTs also correlated with the loss of resistance seen in the partial revertant, L5178Y/HBMR. However, the class Pi GST remained expressed at a level only slightly reduced from that observed in L5178Y/HBM10 (Figure 9a, lanes 4 and 5).

Although three classes of cytosolic GST are inducible upon exposure to HBM or AM, only the class Pi and class Mu GSTs are expressed constitutively in the sensitive cell line, L5178Y. The class Alpha gene product is inducible but does not appear to be expressed constitutively in L5178Y cells (Figure 9c and 12c). The non-constitutive expression of class Alpha isozymes has also been demonstrated in mouse liver, where three isozymes belonging to the class Alpha GST family are derived from three distinct subunits (10,57,58). Two isozymes, Ya_1Ya_1 and Ya_1Ya_2, are non-constitutively expressed but inducible. The third, a homodimer of the Ya_3 subunit

(Ya_3Ya_3), is constitutively expressed but is not inducible. The Ya_1 and Ya_2 subunits have identical molecular weights and are closely related to the rat Ya subunit, while Ya_3 has a molecular weight greater than Ya_1 and Ya_2 and appears to be more closely related to the rat Yc subunit. The Ya_1 and Ya_2 subunits of mouse liver have also been shown to have electrophoretic mobilities similar to the rat Ya subunit (58), as does the class Alpha subunit identified in HBM- and AM-resistant cell lines (Figure 14c). Its non-consitutive expression in L5178Y, inducibility, and similarity in electrophoretic mobility, suggests that the inducible class Alpha isozyme identified in L5178Y lyphoblasts may be composed of subunits related to the inducible Ya_1 and/or Ya_2 subunits of mouse liver. In mouse tissues that have been examined (primarily liver) there appears to be only a single form of class Pi GST (YfYf), but like class Alpha GSTs, multiple isozymes of class Mu GST exist (Tables 1 and 4). The relatedness between subunits that have already been described in the mouse and those that comprise the cytosolic classes of GST identified in L5178Y lymphoblasts, is not clear at this time. A better understanding on this issue awaits the individual purification and structural, immunological, and catalytic characterization of these classes of cytosolic GST in L5178Y lymphoblasts.

Increased expression of cytosolic GSTs in HBM- and

AM-resistant cell lines could arise from an increase in the steady-state level of mRNA or possibly through altered rates of protein turnover. To investigate whether altered mRNA levels contribute to the induced expression of cytosolic GSTs in HBM- and AM-resistant cell lines, the expression of GST mRNA was studied by Northern blot analysis. Consistent with the expression of their corresponding gene products, elevated levels of gene transcripts for class Alpha, class Mu, and class Pi GST were found in both HBM- and AM-resistant cell lines (Figures 16 and 19). The size of these transcripts followed the order: Mu > Alpha > Pi, which is in agreement with the relative molecular weights of their translation products. In the HBM-sensitive and -resistant cell lines the expression level of mRNA transcripts for each GST class were observed to increase in an incremental manner, following the order: L5178Y < L5178Y/HBM2 < L5178Y/HBM10. This parallels the pattern of expression of the corresponding gene products in these cell lines. In L5178Y/HBMR, the changes in expression of class Alpha, class Mu, and class Pi GSTs were accompanied by similar changes in the expression of the corresponding mRNAs. A comparison of the data in Tables 5 and 6, or Figures 11 and 18, shows that the level of induction of message and gene product are similar for each GST class in the resistant cell lines. These results suggest that the

increased expression of cytosolic classes of GSTs, and hence the elevated GST activity observed in HBM- and AM-resistant cell lines, can be rationalized on the basis of an increased steady-state level of GST mRNAs. Northern analysis (Figures 16 and 19) also demonstrates an immeasurable level of the class Alpha mRNA in L5178Y. This reinforces the notion that the class Alpha gene product is non-constitutively expressed in the sensitive cell line and requires an appropriate inductive signal before detectable levels of expression occur.

The increased steady-state level of GST mRNAs in HBM- and AM-resistant cell lines could arise through a number of mechanisms:

i. Transcriptional activation of the respective gene by an appropriate inductive signal.
ii. An increase in the copy number of the actively transcribed gene (gene amplification).
iii. Altered message stability.

Various studies provide evidence for an involvement of these mechanisms in the regulation of GST expression in cells. In female CD-1 mice, De Long *et al.* (138) and Prochaska *et al.* (139) have demonstrated the ability of the anticarcinogenic antioxidant BHA and various structural analogues, to induce both GST and quinone

reductase activity in the tissues of mice, particularily liver and upper small intestine. Subsequently, Pearson *et al.* (55) described the BHA mediated induction of GST mRNAs in the tissues of CD-1 mice and demonstrated, using nuclear "run on" experiments, that increased rates of gene transcription could account for the observed inductions of class Mu and class Alpha mRNA. Similarily, nuclear "run on" studies also demonstrated that increased transcriptional activity of GST genes accounted for elevated GST Ya, Yc, and Yb mRNA levels in the liver of phenobarbital and 3-methylcolanthrene treated rats and of GST Ya and Yc mRNAs in the liver of selenium deficient rats (140,90). Lewis *et al.* (166) found that the elevated level of class Alpha mRNA in a chlorambucil-resistant CHO cell line (CHO-Chl^{r}) was accompanied by a 4- to 8-fold amplification of DNA corresponding to the class Alpha gene family. In studying regulatory mechanisms of class Pi GST expression in human breast cancer cell lines, Morrow *et al.* (247), using nuclear "run on" and RNase protection experiments, demonstrated that message stability contributed significantly to the high level of the class Pi GST mRNA in estrogen receptor negative cells.

Gene amplification does not appear to be responsible for the elevated message levels of cytosolic GSTs in HBM- or AM-resistant L5178Y lymphoblasts. Southern blot analysis of genomic DNA from HBM-sensitive and -resistant

cell lines demonstrated no significant difference in the intensity of autoradiographic bands corresponding to class Alpha, class Mu, or class Pi genes (Figure 21). A similar situation was found for class Alpha and class Mu genes when genomic DNA from AM-sensitive and -resistant cell lines was examined (Figures 23 and 24). Gene amplification appears to be an infrequent mechanism of altered GST expression. The report by Lewis *et. al* (166), is to date, the only report of GST gene amplification in the literature. The relative contribution of transcriptional activation or altered message stability towards an increased level of GST mRNAs in HBM- and AM-resistant cell lines is unknown at this time. Future work might involve both nuclear "run on" and RNase protection experiments to help clarify this issue.

Studies of GST genes have clearly established the presence of specific regulatory elements (the ARE and EpRE of rat and mouse GST-Ya genes and the GPEI enhancer of the rat GST-P gene) with the capacity to transcriptionally activate those genes under their control, in response to monofunctional inducers of appropriate structural (electrophilic) character (148-155). As previously mentioned, the monofunctional inducer BHA has been shown to induce GST mRNAs in mouse liver via transcriptional activation (55). A major metabolite of this compound is *tert*-butylhydroquinone (147), and its oxidized form, *tert*-

butylquinone, is believed to be the active inducer (142). Figure 25 demonstrates the structural similarily between *tert*-butylquinone and HBM. Thus, despite a lack of direct experimental evidence, it does not seem unreasonable to suggest that HBM may act to transcriptionally activate GST genes through an element such as the EpRE, leading to elevated levels of GST mRNAs in HBM-resistant cell lines. The identification of the ARE (and XRE) in the 5′-flanking region of the rat and human quinone reductase gene is of some interest since it establishes a potential causal link in the coordinate induction of both GSTs and quinone reductase in BHA induced mouse tissue (138,139) and in HBM- and AM- resistant cell lines. Figure 20 demonstrates elevated expression of quinone reductase mRNA in both HBM- and AM-resistant cell lines. Both HBM and AM are electrophilic compounds. The coordinate induction of both GSTs and quinone reductase in HBM- and AM-resistant cell lines is consistent with a process whereby the gradual exposure of sensitive L5178Y lyphoblasts to these monofunctional inducers causes the transcriptional activation and resultant over-expression of Phase II detoxication enzymes.

The studies presented in this report show that GSTs belonging to the same class have relative levels of expression that differ in HBM- and AM-resistant cell lines. These differences are of interest since they may

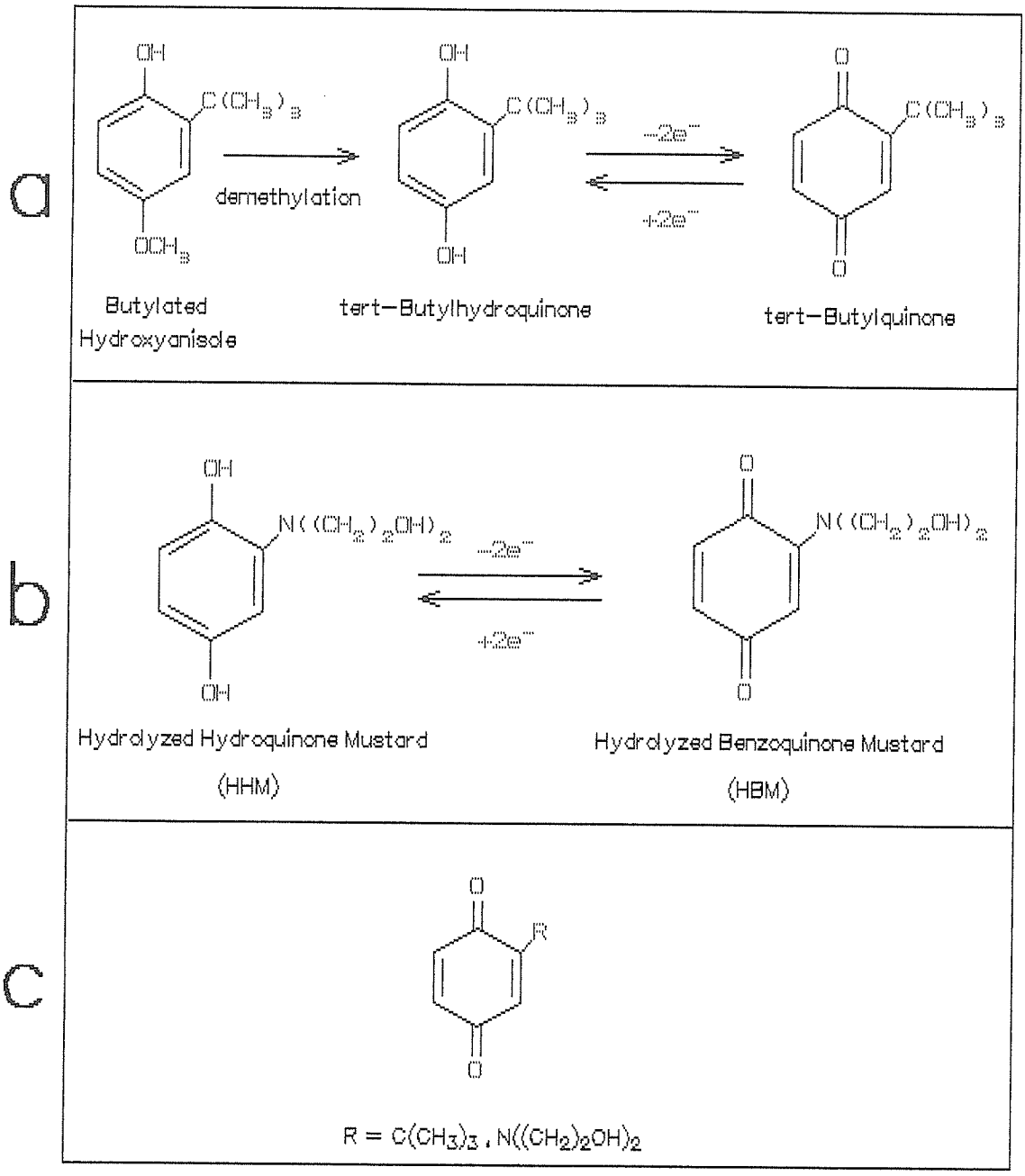

Figure 25. Structural similarity of the Phase II enzyme inducers tert-butylquinone and hydrolyzed benzoquinone mustard. a) Metabolic conversion of butylated hydroxyanisole (BHA) to the ultimate electrophilic inducer, tert-butylquinone (t-BQ); b) Reduced (HHM) and oxidized (HBM) forms of the cytotoxic quinone compound hydrolyzed benzoquinone mustard; c) General formula representing the structurally related compounds t-BQ and HBM.

define the relative importance of specific GST classes in providing protection from the cytotoxic effects of HBM and AM. The HBM-resistant cell line L5178Y/HBM10 and the AM-resistant cell line L5178Y/AM have roughly equivalent expression levels of the class Mu GST, but the class Pi GST in L5178Y/AM is expressed at only half the level observed in L5178Y/HBM10. In contrast, the expression of the class Alpha GST in L5178Y/AM is greater than 3-fold that in L5178Y/HBM10 (Table 5 and Figure 11). Results at the message level were very similar (Table 6 and Figure 18). These findings demonstrate that the regulatory machinery governing the induction of GSTs in HBM- and AM-resistant cell lines responds differently towards two structurally distinct antitumor agents. If derived from unrelated parental cell lines these differences might be attributed to inherent differences in the regulatory machinery characterizing these cells lines (eg. the expression or concentration of specific regulatory factors might differ). However, HBM- and AM-resistant cell lines are derived from the same sensitive parental cell line, i.e. L5178Y.

Both DNase I footprinting and gel shift assays have demonstrated the ability of the ARE and EpRE of the GST-Ya gene of the rat and mouse, respectively, and the GPEI enhancer of the rat GST-P gene, to bind protein factors from nuclear extracts of cells (159,151,155). The

relationship between the modulation of such factors and the expression profiles of class specific GSTs in HBM- and AM-resistant cell lines is of interest. Prestera *et al.* (156) have noted a common property among the varied classes of electrophilic monofunctional inducers that have been shown to transcriptionally activate Phase II enzymes. These structurally varied compounds share a capacity for reaction with sulfhydryl groups, either by alkylation or through redox reactions, suggesting the possibility that the electrophilic properties of monofunctional inducers may activate specific trans acting factors via protein thiol modifications. Favreau and Pickett have also suggested that transcriptional activation of the quinone reductase gene through the ARE might involve the activation of a constitutively expressed DNA binding protein(s) by mechanisms such as alkylation or the altered redox state of such a protein(s) (158). The DNA binding activity of Jun homo- and Fos-Jun heterodimers to AP-1 sites is known to depend on the redox state of a specific cysteine residue (161), while the transcriptional activiation of oxidative stress-inducible genes in *Salmonella typhimurium* and *E. coli* has been shown to depend on the redox state of the *oxy*R protein (248,249). Monofunctional inducers such as p-benzoquinone, catechol, hydroquinone, and 1,2,3-trihydroxybenzene have the ability to undergo redox cycling with the subsequent production of

reactive oxygen species such as superoxide, hydroxyl radical, and hydrogen peroxide. These compounds and the reactive oxygen species they produce have the potential to alter the redox state of proteins. Rushmore *et al.* (152) have demonstrated the ability of such compounds and in fact, hydrogen peroxide, to induce in a dose dependent manner, CAT activity in HepG2 cells transfected with an ARE-CAT construct.

As a result of their modification, perhaps through reaction with electrophilic monofunctional inducers, specific nuclear binding proteins might exhibit an increase in binding activity towards ARE, EpRE, or GPEI regulatory elements, resulting in the transcriptional activation of GST and quinone reductase genes. In support of such a mechanism, Friling *et al.* (151) have demonstrated with DNase I footprinting assays, using the 159 bp (-852 to -693) fragment of the 5′-regulatory region of the GST-Ya gene, that protection over the region of DNA corresponding to the EpRE sequence was substantially increased in nuclear extracts from Hepa 1c1c7 cells pre-treated with t-BHQ, relative to non-treated controls. Also, in a recent study by Pinkus *et al.* (250), HepG2 cells transfected with EpRE Ya-CAT or ARE Ya-CAT constructs showed a significant induction of CAT activity after exposure to phenobarbital. Concurrently, in phenobarbital treated cells, gel shift assays also showed

an increase in the AP-1 binding activity of nuclear extracts with an AP-1 oligonucleotide, relative to non-treated controls. However, in contrast to these results, gel shift assays showed that nuclear extracts from control and β-NF treated HepG2, Hepa-1, or HeLa cells had similar DNA-binding activities with ARE containing oligo-nucleotides, in studies by Rushmore *et al.* (149) and Li and Jaiswal (160). Studies have therefore yielded conflicting evidence that activation of nuclear binding proteins results in an increase in the DNA-binding activity of these factors at the ARE or EpRE regulatory elements of GST and quinone reductase genes.

The reactivity of the two model antitumor agents utilized in this study are distinctly different. HBM is structurally similar to the compounds examined by Rushmore *et al.* (152) and is capable of redox cycling with the production of reactive oxygen species. On the other hand, AM is an alkylating agent. The nature of the interactions between electrophilic monofunctional inducers, nuclear binding proteins, and the ARE, EpRE, or GPEI regulatory elements of GST and quinone reductase genes are complex and as yet, not fully understood. It is intriguing to speculate, upon exposure of the L5178Y parental cell line to either HBM or AM, that the nature of these interactions differs, at least in part due to the dissimilar mechanisms of reactivity (reduction-oxidation versus alkylation) of

these two compounds, and that this might account for the different expression profiles of class specific GSTs in HBM- and AM-resistant cell lines. Additional studies will be required to further elucidate the nature of the signal transduction pathway involved in the transcriptional activation of GST and quinone reductase genes by electrophilic monofunctional inducers.

Elevated quinone reductase activity in HBM-resistant cell lines (219) is accompanied by an induction of the mRNA for this enzyme (Figure 20a). The induction of quinone reductase message in L5178Y/AM has also been demonstrated (Figure 20b) and this cell line would also be expected to show elevated quinone reductase activity. Given the coordinate induction of both GSTs and quinone reductase in HBM- and AM-resistant cell lines, what is the potential relevance of these detoxication enyzmes to the development of resistance towards HBM and AM in these cell lines?

HBM and AM generate their cytotoxic effects through different mechanisms (Figure 26). Although the hydrolyzed mustard group of HBM has no alkylating activity, HBM generates the superoxide radical through redox cycling of the quinone group. Quinone compounds may be reduced to their hydroquinone form through two sequential one electron reductions which may be catalyzed by a number of enzyme systems, including cytochrome P-450 reductase

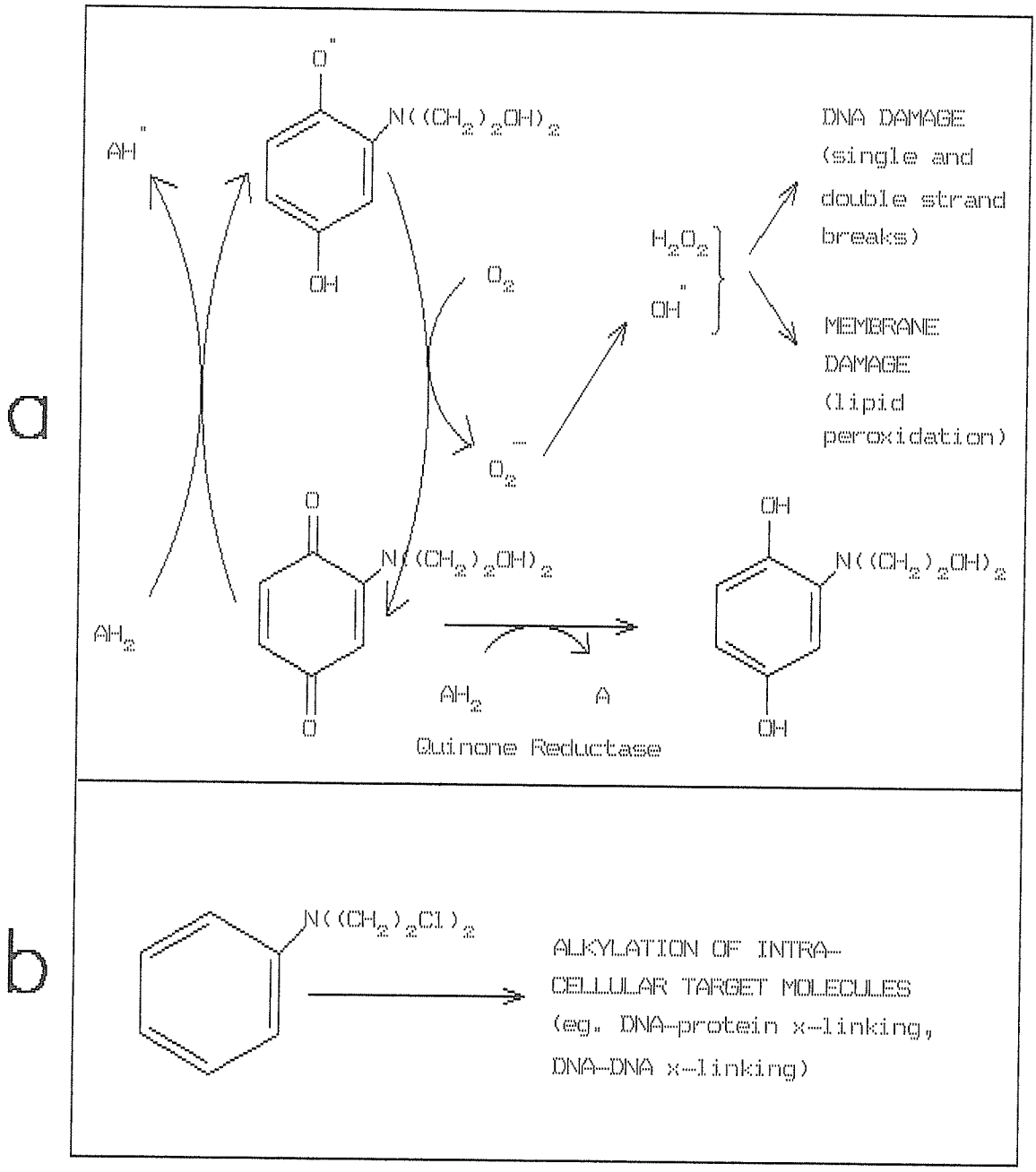

Figure 26. Mechanisms of cytotoxicity of the model antitumor agents: a) hydrolyzed benzoquinone mustard (HBM) and b) aniline mustard (AM). AH_2 signifies a reducing system.

(251). The product of the first reduction is a semiquinone. A reversible redox reaction can occur between the quinone and semiquinone forms (redox cycling), resulting in the generation of the superoxide radical. Superoxide dismutase catalyses the dismutation of this radical to hydrogen peroxide, which in turn, through metal catalyzed Fenton reactions, can react to form the highly reactive and toxic hydroxyl and perhydroxyl radicals (251,252). Hydrogen peroxide and hydroxyl radical are capable of causing the stimulation of lipid peroxidation which can lead to membrane damage in cells (80). These species can also damage DNA through the formation of double and single strand breaks. Aniline mustard has an active mustard group and can alkylate intracellular target molecules including protein and DNA. The formation of DNA single and double strand breaks by HBM and DNA cross-linking by AM have been demonstrated in L5178Y lymphoblasts (220,253).

Quinone reductase catalyzes a single step, two electron reduction of quinone compounds directly to their hydroquinone forms. Quinone reductase therefore has the capacity to inactivate cytotoxic quinone compounds like HBM by reducing them directly to their hydroquinone form. This bypasses the formation of the semiquinone and hence prevents the formation of toxic oxygen species generated through redox cycling. Given that AM is a non-quinone alkylator, the induction of quinone reductase in L5178Y/AM

would not be expected to influence the cytotoxicity of this compound. This suggests that the elevated expression of GSTs in L5178Y/AM may be of greater relevance as a potential mechanism of resistance towards AM. In contrast, the elevated quinone reductase activity in HBM-resistant cell lines would likely play a major role in resistance towards HBM. It has previously been shown that HBM is rapidly converted to the fully reduced hydroquinone form, HHM, in both HBM-sensitive and -resistant cell lines (219). As previously discussed, this may involve two sequential one electron reductions or a direct two electron reduction. In HBM-resistant cell lines where quinone reductase activity is elevated (24-fold in L5178Y/HBM10), the single step, two electron reduction of HBM is likely to be favored and would result in a decreased cytotoxicity of this antitumor agent. Thus in HBM-resistant cell lines both GSTs and quinone reductase are potential factors of importance in the development of resistance towards this agent.

GSTs have the potential to detoxify HBM and AM by catalyzing the conjugation of these drugs to glutathione, with the subsequent excretion of the metabolized conjugate from the cell (Figure 1). Thus elevated expression of GSTs may lead to a reduced intracellular concentration of these drugs in HBM- and AM-resistant cell lines. Although data is not available for L5178Y/AM, previous studies have

shown that the intracellular concentration of total hydrolyzed mustard (HBM and HHM) is lower in HBM-resistant cell lines than in the sensitive L5178Y parental cell line. The expression of P-glycoprotein, an energy-dependent drug efflux pump, may be an alternative mechanism potentially responsible for reduced intracellular concentrations of HBM in HBM-resistant cell lines. This glycoprotein is beleived to play an important role in the phenomenon of multidrug resistance (254). The expression level of P-glycoprotein mRNA was examined in HBM-sensitive and -resistant cell lines by Northern blot analysis. Detectable expression of P-glycoprotein mRNA was not observed in either the sensitive or resistant cell lines (data not shown). Therefore it is doubtful that the reduced intracellular concentration of total hydrolzyed mustard in HBM-resistant cell lines results from increased drug efflux mediated by P-glycoprotein and this favors an involvement of GSTs. Northern analysis of total RNA from L5178Y/AM gave identical results, suggesting that P-glycoprotein does not play a role in the resistance of this cell line towards AM.

The elevated expression of GSTs in HBM-resistant cell lines might offer protection against the damaging effects of lipid peroxidation. Hydrogen peroxide, hydroxyl, and perhydroxyl radicals generated by HBM can damage cellular membrane systems by stimulating lipid peroxidation. The

fatty acid hydroperoxides that result from this process can further stimulate lipid peroxidation (80,255). Detoxication of organic hydroperoxides by reduction to more stable alcohols helps to break the chain propagation stage of lipid peroxidation and is therefore thought to be an important cellular defense mechanism against oxidative membrane damage. Two cytosolic enzymes, a selenium-dependent glutathione peroxidase (GSH-Px) and a selenium-independent glutathione peroxidase (GST-Px), catalyze the reduction of organic peroxides to their corresponding alcohols. Although previous characterizations of L5178Y lymphoblasts demonstrated that GSH-Px activity did not differ significantly between HBM-sensitive and -resistant cell lines (219), GST-Px activity is associated with cytosolic GSTs. Therefore, the increased expression of GSTs in HBM-resistant cell lines could play a role in protection against the membrane damaging effects of HBM by increasing the cellular capacity for detoxication of fatty acid hydroperoxides. Tan *et al.* (256) have demonstrated the inhibition of microsomal lipid peroxidation by class Alpha isozymes (YaYc and YcYc). Lavoie *et al.* (205) have also shown that transfection of human T47D cells with rat GST-Yc expression vectors conferred significant protection against the toxic effects of two well known inducers of lipid peroxidation, cumene hydroperoxide and singlet oxygen. Although the inherent GST-Px activity of GSTs is

most commonly associated with class Alpha GST, on occasion this activity has been demonstrated in the class Pi GST. Meyer *et al.* (82) have demonstrated activity towards linoleate and arachidonate hydroperoxides for a class Pi GST that was isolated from a rat hepatoma. In Adr^{R}MCF7 cells a 45-fold increase in GST activity was associated with a class Pi GST which demonstrated high levels of activity towards the organic hydroperoxide, cumene hydroperoxide (182).

With the exception of the class Pi GST in L5178Y/HBMR, GST expression in drug-sensitive and -resistant cell lines shows good correlation with levels of resistance. However, functional studies have yet to be carried out to more closely establish the relative importance of GST expression to the development of resistance in HBM- and AM-resistant L5178Y lymphoblasts. GST-Px activity has yet to be demonstrated for the cytosolic classes of GST that have been identified in L5178Y lymphoblasts. As well, although the enzyme catalyzed conjugation of GSH with quinones and alkylating agents is known (37,42), direct experimental evidence demonstrating the GST catalyzed conjugation of HBM or AM to glutathione, in L5178Y lymphoblasts, is lacking at this point in time. The separation and purification of the specific classes of GSTs that have been identified in the drug-sensitive and -resistant cell lines investigated in

this study would help to establish both their relative GST-Px activity and relative specificity towards HBM and AM. Future work should also involve direct functional studies. The ability of GSTs to confer resistance towards HBM or AM could be examined by transfection of the sensitive L5178Y cell line with class specific GST expression vectors. Alternatively, treatment of HBM- and AM-resistant cell lines with GST inhibitors such as ethacrynic acid or indomethacin would help to access the relative importance of elevated GST expression as a potential mechanism of resistance in these cell lines.

Previous characterizations of HBM-sensitive and -resistant L5178Y lymphoblasts indicated that resistance towards HBM was unstable. A partial revertant, L5178Y/HBMR, was isolated after culturing L5178Y/HBM10 in the absence of drug for 4 months (219). Resistance in L5178Y/HBMR had decreased to a level similar to the L5178Y/HBM2 cell line. Loss of resistance in L5178Y/HBMR was accompanied by an 8-fold decrease in the activity of quinone reductase, the activity of which was 24-fold elevated, relative to L5178Y, in the L5178Y/HBM10 resistant cell line. In contrast, the loss in GST activity was small. GST activity in L5178Y/HBMR remains 9-fold elevated above the activity in the sensitive L5178Y cell line and represents only a 1.2-fold reduction from the 11-fold increased activity shown by L5178Y/HBM10. This

poor correlation between GST activity and loss of resistance in the revertant, questions the relevance of GSTs as a contributing factor in the resistance of HBM-resistant cell lines. In examining the expression of specific classes of GSTs, the present study has clarified the nature of GST expression in L5178Y/HBMR and shows that it is not GSTs in general, but the class Pi GST in particular, which correlates poorly with resistance towards HBM. A similar finding as regards the class Pi GST and resistance has also been demonstrated in a revertant of the colchicine-resistant MCF7/CL10 cell line (190). This cell line was shown to express a multidrug resistant phenotype in which a 70-fold increase in GST activity was associated with the induction of a class Pi GST. Although a revertant, MCF7/CL10R, no longer expressed the multidrug resistant phenotype and had lost resistance towards colchicine, GST activity remained elevated 70-fold relative to the MCF7 parental cell line.

Figures 9c and 11 indicate a complete loss of expression of the class Alpha GST in L5178Y/HBMR. In addition, Table 4 also indicates that relative to class Mu and class Pi GSTs, CDNB is a poor substrate for class Alpha GST. Therefore, given its poor specificity towards CDNB, the decrease in total GST activity accompanying the loss of expression of the class Alpha GST in L5178Y/HBMR is expected to be small. Expression of the class Mu GST is

also decreased substantially in the revertant (Figure 9b and 11). This suggests that the total GST activity of the revertant is attributable to the expression of the class Pi GST which has a high substrate specificity towards CDNB and which remains significantly elevated in this cell line (Figures 9a and 11).

An only modest reduction of the class Pi GST in L5178Y/HBMR indicates that the induction of this GST is a relatively stable event. The nature of this change is not understood at this time. Evidence for genetic rearrangement of the class Pi gene in HBM-resistant cell lines was not demonstrated by Southern blot analysis (Figures 21 and 22). However, the identification of a silencer element between nucleotides -400 to -140 upstream from the cap site in the rat GST-P gene may be of significance (154). If conserved in the mouse GST-Pi gene, a loss of negative regulatory control via this element might account for the apparently irreversible induction of this GST in HBM-resistant cell lines. Interestingly, the poor correlation between the class Pi GST and resistance in HBM-resistant cell lines may in part be due to an inactivation of this GST by reactive oxygen species generated by HBM. Hydrogen peroxide has been shown to inhibit the activity of class Pi GSTs in both the rat and human (257), while either superoxide or hydroxyl radicals have been implicated in the activation of class Mu GST in

the rat (258). In L5178Y/HBMR, where quinone reductase activity is reduced 8-fold from levels in L5178Y/HBM10, the potential for inhibition of the class Pi GST by reactive oxygen species would be significantly increased. With the reduction in quinone reductase activity, redox cycling and the generation of reactive oxygen species by HBM, including H_2O_2, would be expected to increase. This is in agreement with the increased toxicity of HBM in L5178Y/HBMR as evidenced by the lower level of resistance of this cell line. It is perhaps ironic that an agent (H_2O_2) having the potential to transcriptionally activate GSTs (152) also exhibits the capacity to inhibit their activity.

This study demonstrates that GSTs belonging to the same class show relative levels of expression that differ in HBM- and AM-resistant cell lines. These differences may account for the pattern of cross-resistance towards other antitumor agents that has been observed in HBM-resistant cell lines. Begleiter and Leith have shown that HBM-resistant cell lines are cross-resistant towards the quinone alkylators benzoquinone mustard (BM) and benzo-quinone dimustard (BDM), yet remain sensitive to the non-quinone alkylator, aniline mustard (259). Both BM and BDM were shown to produce significantly lower levels of DNA-DNA crosslinking in HBM-resistant cell lines, whereas the level of crosslinking activity by AM remained

unchanged. The authors proposed that activation of the alkylating activity of BM and BDM requires reduction of these compounds to their semiquinone forms. As previously discussed, elevated quinone reductase activity in HBM-resistant cell lines was believed to cause the preferential reduction of quinones to their hydroquinone forms, causing reduced formation of activated semi-quinones. Therefore, elevated quinone reductase activity in HBM-resistant cell lines would be expected to reduce the alkylating activity of BM and BDM, resulting in the observed cross-resistance towards these agents. Lacking a quinone moiety, the alkylating activity of AM would not be influenced by quinone reductase activity and therefore HBM-resistant cell lines would remain sensitive to this agent. That HBM-resistant cell lines remain sensitive to AM, despite a significant elevation in GST activity, has lead to the suggestion that GSTs do not play a major role in resistance towards AM (259). The sensitivity of HBM-resistant cell lines to AM contrasts with L5178Y/AM, in which the significantly elevated GST activity of this cell line is accompanied by a 10-fold level of resistance towards AM. Therefore, unlike HBM-resistant cell lines, GST activity in L5178Y/AM does correlate with resistance towards AM.

Relative to L5178Y, the present study has demonstrated a greater than 12-fold increase in the

expression of the class Alpha GST in L5178Y/AM, which is more than 3-fold greater than the expression level of this GST in L5178Y/HBM10. In contrast, class Mu and class Pi GSTs in L5178Y/AM are expressed at levels roughly equivalent and one half, respectively, to that seen in L5178Y/HBM10. Therefore, despite elevated GST activity, the lack of resistance towards AM might be a direct consequence of the expression profiles of class specific GSTs in HBM-resistant cell lines. The more greatly enhanced expression of the class Alpha GST in L5178Y/AM may be the important contributing factor in the relative resistance of HBM- and AM-resistant cell lines towards AM. The association between the over-expression of class Alpha GST and alkylator resistance has been demonstrated in numerous studies (section 1.3). Where there is a potential involvement of GSTs in the resistance phenotype, the relative sensitivity of HBM- and AM-resistant cell lines towards AM suggests that resistance against specific antitumor agents might, under certain conditions, be markedly influenced by the expression levels of specific cytosolic classes of GST.

6. CONCLUSIONS

1. The elevated GST activity characteristic of HBM- and AM-resistant cell lines results from the coordinate induction and over-expression of class Alpha, class Mu, and class Pi cytosolic GSTs. Although inducible, the class Alpha GST is non-constitutively expressed in the sensitive L5178Y cell line.

2. In HBM-resistant cell lines the expression levels of class Alpha and class Mu GSTs correlated with resistance, following the order: L5178Y $<$ L5178Y/HBM2 $\cong$ L5178Y/HBMR $<$ L5178Y/HBM10. In contrast to the loss of resistance, the high GST activity of the partial revertant, L5178Y/HBMR, reflects the continued over-expression of the class Pi GST whose induction upon exposure to HBM appears to be largely irreversible. In L5178Y/HBMR, expression of class Alpha and class Mu GSTs are reduced to levels similar to that found in L5178Y. Therefore, expression of the class Pi GST correlates less with resistance and more closely with GST activity, which in HBM-resistant cell lines, follows the order: L5178Y $<$ L5178Y/HBM2 $<$ L5178Y/HBMR $\sim$ L5178Y/HBM10.

3. An increased expression of cytosolic GSTs in HBM- and AM-resistant cell lines was accompanied by the coordinate induction of class Alpha, class Mu, and class Pi GST mRNAs. The level of induction of these mRNAs was similar to that of the corresponding gene products. This suggests that the underlying mechanism responsible for the increased expression of GSTs in HBM- and AM-resistant cell lines is an increase in the steady-state level of mRNA. Southern blot analysis has shown that gene amplification is not responsible for the increased expression of class specific GST mRNAs in HBM- and AM-resisant cell lines, however, the relative importance of transcriptional activation or message stability remains to be determined.

4. The induction of GSTs by HBM and AM is non-specific but class selective. These compounds coordinately induce the expression of class Alpha, class Mu, and class Pi GSTs (non-specific), but the level of induction by these agents differs for each specific GST class (class selectivity). In addition, there is also a difference between the class selectivity exhibited by HBM and that of AM, which is reflected in the dissimilar expression profiles of class specific GSTs between HBM- and AM-resistant cell lines.

5. The sensitivity of HBM-resistant cell lines towards AM may reflect the different expression profiles of class specific GSTs and in particular, a significantly lower level of expression of class Alpha GST, relative to the AM-resistant L5178Y/AM cell line. Relative to L5178Y/HBM10, the AM-resistant cell line L5178Y/AM has roughly equivalent and reduced expression levels, respectively, of class Mu and class Pi GSTs, but a greater than 3-fold higher level of expression of the class Alpha GST. Differences in the relative sensitivity of HBM- and AM-resistant cell lines towards AM suggests that resistance against specific antitumor agents, under certain conditions, might be markedly influenced by the expression levels of specific cytosolic classes of GST.

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