GLUTATHIONE AND GLUTATHIONE S-TRANSFERASES IN CHLORAMBUCIL RESISTANT MOUSE CELL LINES

BY

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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For my Wife, Aiping, with Love,

Whose sustaining support and buoyant Energy

Made it Possible for Me to Complete My Ph.D. Training, and

For my Daughter, Jane, with Love,

Who gave me much of the Inspiration with her Songs and Music

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ABSTRACT

Chlorambucil (CLB) is a cancer chemotherapeutical agent and is widely used in the treatment of chronic lymphocytic leukemia (CLL), Hodgkin's disease, ovarian carcinoma, breast cancer and other lymphomas. Because of acquired drug resistance which frequently limits its clinical application, studies searching for the mechanisms of CLB resistance have been carried out for the past 20 years. Investigations of this study and others strongly suggest that inactivation of CLB via glutathione (GSH) and glutathione S-transferase (GST) detoxication system is one of the important mechanisms in CLB resistance.

In the first part of my study, we observed significant increases in intracellular GSH content, GST enzyme activity and steady-state levels of GST alpha-class mRNA in the CLB-resistant N50-4 cells, compared with the wild-type NIH-3T3 cells. We then carried out experiments to test the roles of GSH and GST in CLB resistance using a number of GSH- and GST-blocking agents. We showed that the resistance of N50-4 cells to CLB was partially reversed by administration of either GSH depleting agent or GST inhibitor, and was almost completely abolished by combination pretreatment of the GSH depleting agent and the GST inhibitor. The findings support the hypothesis that both GSH and GST play major roles in CLB resistance in N50-4 cells.

To further investigate the increase in steady-state levels of the GST alpha-class mRNA in the CLB-resistant cells, we examined the regulation of the GST alpha-class gene expression. Amplification of the GST Ya and Yc (alpha-class) genes was demonstrated in one of the CLB-resistant cell lines, HUCLB, by In-gel hybridization and Southern blot analysis. Increased transcriptional activity of both GST Ya and Yc genes was shown in both CLB-resistant cell lines by nuclear "run-on" assay. Increased GST Ya mRNA stability was also shown in both CLB-resistant cell lines by mRNA decay assay. We also observed that the wild-type cells transcribed trace amount of GST Ya_1 mRNA, but not GST Ya_2 mRNA. However, the CLB-resistant cells transcribed significant amount of GST Ya_1 and GST Ya_2 mRNAs. Amplification of GST Ya_2 gene and increased steady-state level of GST Ya_2 mRNA were found in the CLB-resistant HUCLB cell line, compared to the CLB-resistant N50-4 cell line. The findings that the transcription of the GST Ya_1 and Ya_2 gene in CLB-sensitive and -resistant cells is differentially regulated may suggest different roles of GST Ya isozymes in CLB resistance. We further demonstrated that the 3' untranslated region (UTR) of the GST Ya_1 gene destabilized the mRNA when linked to the CAT (chloramphenicol acetyl transferase) reportor gene, compared to the 3' UTR of the GST Ya_2 gene and to the CAT gene without the 3' UTR sequence. The results indicate a functional role of the GST Ya isozymes in terms of affecting the mRNA stability in CLB resistance. Our studies suggest that regulation of the GST Ya gene in CLB-sensitive and -resistant cells is a

complex process, and that multiple factors are responsible for the increases in steasy-state levels of the GST Ya mRNA in the CLB-resistant cell lines.

HISTORICAL REVIEW

INTRODUCTION

There has been a rapid evolution in understanding the multistage process of cancer (Weinberg, 1989; Baker et al., 1990; Levine et al., 1991), unfortunately, developing clinical strategies to exploit these findings have not followed as quickly (Antman, 1991; Perez et al., 1991). In addition, both natural and acquired resistance to cancer chemotherapeutic agents have also limited the effectiveness of treatment. Patients treated with these agents have approximately a 50% response rate, but within 12 to 24 months the majority of these patients relapse (DeVita, 1983; Frei, 1985; Louie et al., 1986). Understanding the mechanisms for drug resistance may provide us with new therapeutic options.

1. GENERAL MECHANISMS OF DRUG RESISTANCE.

The process by which cells acquire drug resistance is very complex. For example, cells selected for resistance against a single cytotoxic drug may acquire cross resistance to a range of structurally unrelated drugs (Van der Bliek and Borst, 1989), or may simultaneously induce a number of mechanisms of drug resistance (Moscow et al., 1989). Evidence from a variety of sources implicates a genetic basis for most drug-resistant phenotypes. These mechanisms of drug resistance may involve genetic changes, such as mutations, deletions, gene amplifications, chromosomal

rearrangements and translocations (Piller, 1989). Such genetic change may result in alteration of the coding protein, and may subsequently affect cellular function of the protein. Some of the well known examples are:

1) Decreased drug accumulation. The P-glycoprotein (PGP) is a membrane protein that can selectively extrude a wide range of substances, including anticancer drugs, and is a key protein responsible for multidrug resistance (Bradley et al., 1989). PGP exists in normal tissues, usually in tissues that have an excretory function: kidney, liver, spleen, colon, jejunum, rectum and esophagus. Increased gene expression and enhanced PGP exporter function were shown to contribute to the increased drug excretion, which renders the cells resistance to a group of structurally unrelated drugs, e.g., vinca alkaloids, anthracyclins, podophyllotoxins and actinomycin D (Alexander et al., 1989). Amplification of the PGP gene (Scotto et al., 1986), increased transcription of the PGP mRNA (Hsu et al., 1989), increased PGP mRNA stability (Marino et al., 1990), altered protein properties by phosphorylation (Chambers et al., 1990; Staats et al., 1990), and mutations within the gene (Choi et al., 1988; Safa et al., 1990) were found in the drug resistant cells.

2) Increased drug inactivation. Glutathione (GSH) and glutathione S-transferases (GSTs) represent a major part of the detoxication system in the body (Ketterer et al., 1988). GST

catalyzes GSH conjugation to chemically reactive agents, a process that can inactivate or neutralize the toxicity of the chemicals. Increases in GSH content or GST enzyme activity will increase the ability of the cells to inactivate the drugs, which is the major mechanism of the drug resistance (Waxman, 1990). There are three major classes of GSTs in the cytoplasm of the cells: Alpha (α), Mu (μ) and Pi (π). They have been implicated in resistance to doxorubucin, melphalan, chlorambucil (CLB), cisplatinum and other alkylating agents (Waxman, 1990; Schisselbauer et al., 1990; Nakagawa et al., 1990).

3) Altered target protein function. Topoisomerase II (topo II), the eukaryotic homologue of bacterial DNA gyrase, plays an important role in DNA replication, chromosome scaffold formation, chromosomal segregation, and possibly recombination and gene transcription (Wang, 1985). Drugs that inhibit topoisomerase function or that bind directly to topoisomerase exert their cytotoxic effects by interfering with topoisomerase function. Formation of the drug-topoisomerase II complex prevents chromosome segregation, inhibits DNA synthesis and induces topoisomerase II associated DNA strand breaks (Liu, 1989; Davies et al., 1988). These drugs include intercalating agents such as acridines (mAMSA), anthracylines (doxorubicin, daunomycin), anthracene-diones (mitoxantrone) and ellipticines, and non-intercalating agents such as teniposide (VP26) and etoposide (VP16). Mechanisms of the drug resistance include generation of abnormally functioning

topoisomerases by mutations (Huff et al., 1990; Fernandes et al., 1990; Pommier et al., 1976), methylation (Tan et al., 1989), and switch of topo II (α) to a novel form of topo II (β) (Drake et al., 1989; Chung et al., 1989; Woessner et al., 1990).

4) Increased repair of DNA damage. It is well established that cisplatin binds to DNA and these cisplatin-DNA adducts contribute to cellular toxicity (Ducore et al., 1982; Sherman and Lippard, 1987; Eastman, 1991). A protein that recognizes cisplatin-GG or cisplatin-GA adducts has been identified recently. The protein, termed damage recognition protein, recognizes the cisplatin-DNA adducts, which is a critical step for DNA repair (Donahue et al., 1990; Fujiwara et al., 1990). Enzymes responsible for repairing cisplatin-DNA damages include the DNA polymerases α and β, dihydrofolate reductase, dTMP synthase and topoisomerases (Lai et al., 1988; Scanlon et al., 1989). Evidence suggested that expression of the genes and proteins responsible for repairing cisplatin-DNA adducts was induced or increased in cisplatin resistant cells, which plays an important part for the cisplatin resistance (Eastman and Schulte, 1988; Scanlon et al., 1990, 1991).

5) Enhanced protein function. It has been known that N-phosphonacetyl-L-aspartate (PALA) blocks proliferation of mammalian cells by inhibiting the aspartate transcarbamylase activity, and blocking de novo pyrimidine nucleotide biosynthesis (Kempe et al.,

1979). CAD is a trifunctional protein carrying carbamyl-P synthetase, aspartate transcarbamylase, and dihydro-orotase, the first three enzymes of UMP biosynthesis (Padgett et al., 1979). Amplification of the CAD gene, which leads to an increase in the target gene expression and to an enhancement of the target protein function, has been shown to be an important mechanism for the PALA resistance (Stark et al., 1989; Wahl et al., 1979; Smith et al., 1990).

2. CHLORAMBUCIL.

Chlorambucil is a bifunctional alkylating agent and an aromatic derivative of nitrogen mustard. Chlorambucil has been used for the treatment of many neoplastic diseases, such as chronic lymphocytic leukemia (CLL), ovarian carcinoma, Hodgkin's disease and non-Hodgkin's lymphoma (Mandelli et al., 1991; Price et al., 1991; Rankin et al., 1992). Like most of the chemotherapeutic agents, its clinical application is limited by acquired drug resistance. Understanding the mechanisms of CLB resistance and finding effective ways to overcome the resistance have become an important aspect of medical research.

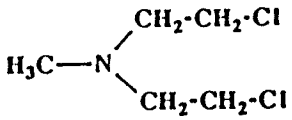
2.1 Chlorambucil and Alkylating agents

Alkylating agents were the first compounds to be used in

cancer chemotherapy, dating from the introduction of nitrogen mustard in 1946 (Gilman and Philips, 1946), and are still important chemotherapeutic agents in cancer treatment today. Nitrogen mustard (N,N-bis(2-chloroethyl)-methylamine, HN2) is the simpliest form of the clinically used mustard compounds. Chlorambucil (4-[bis(2-chloroethyl)amino] phenyl butyric acid, Leukeran), synthesized by Everett et al. (1953), is an aniline mustard derivative with a butyric acid side-chain (Figure 1).

Alkylating agents are compounds containing an alkyl group with an electron-deficienct (electrophilic) centre, which will react with an electron-rich (nucleophilic) cellular sites, such as nucleic acids and proteins. The reaction results in formation of a covalent bond between the alkylating chemical and the cellular component (alkylation). Amongst the consequences of such alkylations are cytotoxicity, mutagenicity, teratogenicity and carcinogenicity (Palmer et al., 1984; Russell et al., 1989; Bank, 1992).

Alkylation of nitrogen mustard with nucleic acids occurs extensively at the N7 positions of guanine residues (Brookes and Lawley, 1961). The N7-alkyl adducts are unstable and decompose by both chemical and enzymic reactions, leaving apurinic sites. Such apurinic sites may promote mis-pairing or DNA strand breakage (Rutman et al., 1969). An alkylation of DNA by two chloroethyl groups of nitrogen mustard may result in a cross-link between two sites in DNA or between DNA and protein. Cross-linking of DNA by alkylation causes a much greater threat to cell survival than



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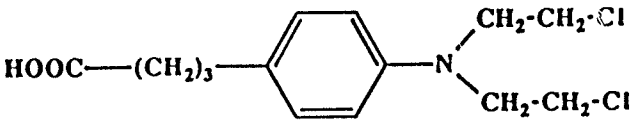
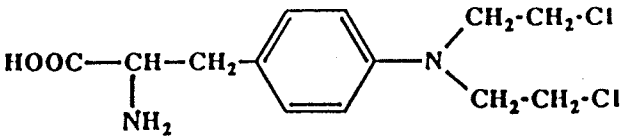


Chlorambucil



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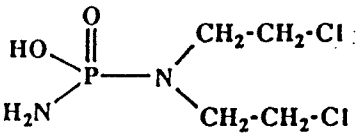

Phosphoramide Mustard

Figure 1. Nitrogen mustard and its derivatives.

monofunctional alkylations (Kohn, 1979).

2.2 Clinic applications of Chlorambucil

CLB is frequently administered orally in human and the drug is rapidly absorbed from the gastro-intestinal tract (Farmer, 1987). CLB is taken up into cells by passive diffusion (Begleiter and Goldenberg, 1983). Metabolism involves hydrolysis of CLB in aqueous solution to yield bis(2-hydroxyethyl) analogue (Ehrsson et al., 1980; Owen and Stewart, 1979) and β-oxidation of the butyric acid side-chain of CLB to 2-(4-N-[2-chlorothyl]aminophenyl) acetic acid (McLean et al., 1980).

Because of its cytotoxicity, CLB has become established as a drug of major importance in the treatment of cancers, such as chronic lymphocytic leukaemia (Knospe et al., 1974; Mandelli et al., 1991), lymphoma (Price et al., 1991; Mayou et al., 1991), ovarian carcinoma (Rankin et al., 1992) and Hodgkin's disease (McElwain et al., 1977). CLB has also been used in the treatment of a variety of non-cancer diseases, such as Behcet's disease (O'Duffy et al., 1984), nephrotic syndrome (Grupe, 1973; Elzouki and Jaiswal, 1990), chronic progressive multiple sclerosis (Chiappelli et al., 1991), and rheumatoid arthritis (Thorpe et al., 1976).

Because of its cytotoxicity, CLB administration frequently causes serious side-effects. Some of the side-effects observed in the clinic include nausea, vomiting, pulmonary fibrosis (Cole et

al., 1978), spermatogenesis (Richter et al., 1970), jaundice (Robert et al., 1968), bleeding (Goodrick et al., 1992) and immune-allergic reaction (Zervas et al., 1992).

There is considerable evidence from animals that CLB is carcinogenic. Skin application to mice resulted in 58% (11/19) development of papillomas (Salaman and Roe, 1956). Intraperitoneal administration increased the incidence of lung tumors, lymphosarcomas and ovarian tumors (Shimkin et al., 1966; Weisburger et al., 1975). In rats, an increase in the incidence of lymphomas was seen in males but not in females following i.p. administration of CLB. Although there is not sufficient evidence to classify CLB as a human carcinogen, individual case reports have been published associating the development of leukaemia or other tumors in patients receiving CLB (Catovsky and Galton, 1971; Reimer et al., 1977). The occurrence of secondary tumors following anti-cancer drug treatment has been reviewed by Schmahl et al., 1982, with the conclusion that more than 50% of these tumors were acute leukemia.

2.3 Mechanisms of Chlorambucil cytotoxicity

The mechanism of CLB cytotoxicity is not clear at the present. It is generally believed that covalent binding of CLB to DNA and forming cross-links by CLB-DNA adducts are the major causes of the pharmacological effect (Bank, 1992).

Inter-strand DNA crosslinking. CLB can bind to two

complementary strands of DNA (crosslinking), which correlated very well with its cytotoxicity (Sunters et al., 1992; Kohn et al., 1966). The inter-strand DNA crosslinking would affect DNA replication and gene transcription, especially in the rapidly dividing cells. (Kohn, 1983). The major site of crosslinking in DNA is shown to be between two guanines at a position of N-7, which have the highest electronegativity (Kohn et al., 1987; Mattes et al., 1986).

Intra-strand DNA crosslinking. CLB has also been shown by the Maxam-Gilbert DNA sequencing technique, to induce thermolabile, alkaline-stabilized intra-strand DNA adducts at the N-3 rather than N-7 position of adenine (Wang et al., 1991). CLB induced adenine lesions appear to terminate transcription by their ability to form unique adducts at neighboring guanines in the DNA (Pieper et al., 1989; Pieper and Erickson, 1990). The exact nature of the transcription terminating lesion induced by CLB is not completely understood. CLB induced intra-strand adenine adducts were also shown to cause A:T to T:A transversions (Wang et al., 1991). A correlation between CLB-adenine adduct sites and mutation sites was observed (Wang et al., 1991).

Deletion and base-pair substitution mutations. CLB was found to induce high yields of mouse germ-line mutations that appear to be deletions or other structural changes. The deletion frequency caused by CLB was higher than that caused by x-ray or any chemical

investigated to date (Russell et al., 1989). CLB was also shown to cause DNA base pair transversion after formation of intra-strand adducts (Wang et al., 1991).

Induction of apoptosis. Apoptosis, or programmed cell death, is an important mechanism of maintenance of homeostasis in the body under physiological conditions. Compared to necrotic death, apoptosis is a relatively slower process, and is characterized by nuclear chromatin condensation and DNA fragmentation (Compton, 1992). It has been shown that programmed cell death can be induced in cultured human tumor cells by chlorambucil (Lennon et al., 1991; Hickman, 1992). Apoptosis can also be induced by a variety of anticancer drugs with diverse chemical structure and different mechanism of action, including melphalan, cis-platinum, BCNU, vinca alkaloids, methotrexate and 5-fluorouracil (Hickman, 1992; Sen and D'Incalci, 1992). The fact that apoptosis can be induced by a variety of antineoplastic drugs suggests that this biological process can be triggered by multiple mechanisms, or, less probably, by a common mechanism which is activated by all these drugs (Wyllie, 1992; Lennon et al., 1991).

Inhibition of DNA synthesis. Ribonucleotide reductase is a rate-limiting enzyme in DNA synthesis. It catalyzes the conversion of ribonucleotides to their corresponding deoxyribonucleotides, the precursors of DNA synthesis (McClarty et al., 1988; Wright, et al., 1990). Ribonucleotide reductase consists of two subunits, R1 and

R2, both of which are required for the enzyme activity (Thelander and Berg, 1986). CLB has been shown to inhibit partially purified mouse ribonucleotide reductase activity, both R1 and R2 subunits. The result indicates that chlorambucil can inhibit DNA synthesis and cell proliferation by inhibitiing the ribonuleotide reductase activity (Hurta and Wright, 1992).

2.4 Mechanisms of Chlorambucil resistance

Although several mechanisms of CLB resistance have been proposed, the importance of each is still under intensive investigation. 1) It has been shown that there is no difference in drug transportation across the cell membrane, either import or export, between CLB-sensitive and -resistant CLL cells (Bank et al., 1989; Silber et al., 1989); 2) Harrap and Hill (1970) found that the hydrolysis of the mustard group of CLB was faster in CLB-resistant Yoshida sarcoma cells. In contrast, Bank et al. (1989) showed no correlation between cellular metabolism of CLB and sensitivity to CLB in CLL cells; 3) Reduced levels of DNA crosslinks have been found in CLB-resistant CHO (Robson et al., 1987; Jiang et al., 1989), Yoshida sarcoma (Harrap and Gascoigne, 1976) and CLL cells (Panasci et al., 1988). The reduction of DNA crosslinking appeared to be due to an increase in the rate of removal of interstrand DNA crosslinks in CLB-resistant Yoshida sarcoma (Harrap and Gascoigne, 1976) and CLL cells (Panasci et al., 1988). In contrast, no difference in DNA repair was detected

between CLB-sensitive and -resistant CHO cells (Robson et al., 1987; Jiang et al., 1989); 4) A correlation was shown between intracellular GSH levels, GST activity and CLB resistance (Robson et al., 1986; Tew et al., 1988; Hall et al., 1989), and an inverse correlation was observed between GSH content, GST activity, and CLB-induced DNA crosslinks in cultured CHO cells (Robson et al., 1987) and human leukemic (CLL) cells (Johnston et al., 1990), which indicated the involvement of GSH and GST in CLB resistance (Black et al., 1990).

3. GLUTATHIONE AND GLUTATHIONE S-TRANSFERASES.

3.1 Glutathione

GSH, a tripeptide composed of L-glutamyl-L-cysteinylglycine (Figure 2), is the most abundant low-molecular-mass thiol compound found in cells (Arrick and Nathan, 1984). Under physiological conditions, more than 98% of intracellular glutathione is reduced by glutathione reductase. GSH plays an important role in a variety of cellular functions, including detoxication of exogenous and endogenous compounds such as reactive electrophiles and toxic oxygen metabolites. GSH also plays a role in metabolism of some biological substances such as leukotriene and prostaglandin (Ketterer et al., 1983; Coles and Ketterer, 1990). Recent studies suggest that GSH may be involved in regulation of proliferation of normal human T lymphocytes (Suthanthiran et al., 1990). The

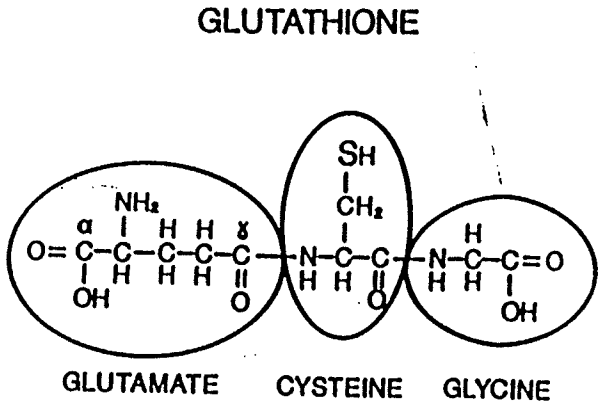

Figure 2. The structure of tripeptide glutathione (Taken from DeLeve and Kaplowitz, 1990).

involvement of GSH in drug resistance has been well documented (Ketterer, 1988). The mechanisms of GSH in drug resistance include GSH-drug conjugation, DNA repair and detoxification of reactive oxygen (Ozols et al., 1990; Lai et al., 1989; Kramer et al., 1988).

3.2 Glutathione S-transferases

GSTs (E.C.2.5.1.18) are widely distributed in cells of animals, plants and bacteria (Arca et al., 1988). Multiple forms of GSTs have been discovered in virtually every organism (Mannervik, 1985). Mammalian cells contain both soluble (cytosolic) and membrane-bound isozymes of GST. Three structurally and immunologically distinct forms of cytosolic GSTs have been identified and are named Alpha (α), Mu (μ) and Pi (π) classes. The cytosolic GST proteins exist either in homo- or hetero-dimers, and only subunits within the same class can form heterodimers (Mannervik, 1985). Membrane-bound microsomal GST shows no obvious homology with any of the cytosolic enzymes and appears to form trimers composed of identical subunits (Morgenstern et al., 1982).

GSTs catalyze conjugation to an extraordinarily diverse group of electrophilic, hydrophobic compounds with glutathione (Mannervik, 1985). It is an important part of intracellular detoxication of endogenous and exogenous substances, including mutagens, carcinogens, and other noxious chemical substances. GSTs also act as intracellular binding proteins for a variety of organic

compounds such as bilirubin and steroids, and may serve as intracellular storage sites or transport depots for hydrophobic compounds, including both noxious xenobiotics and endogenous metabolites (Litwack et al., 1971; Wolkoff et al., 1979). Another effect of GST-catalyzed reactions appears to be the inactivation of drugs used in cancer chemotherapy. Studies suggest that GSH and GSTs are involved in resistance to a wide range of anti-neoplastic chemicals (Batist et al., 1986; Dulik et al., 1986).

3.3 Nomenclature of Glutathione S-transferases

The nomenclature of GST enzymes has been problematic from initial observations as to their ability to catalyze the reaction between GSH and a number of eletrophiles. An early attempt to classify different forms of GSTs was made by Boyland and Chasseaud (1969). They introduced the terms aryltransferase, epoxide transferase, alkyltransferase, aralkyltransferase, and alkenetransferase for the GSTs based on the specificities of the GSTs towards their electrophilic substrates. However, separation and extensive purification of several forms of the enzyme demonstrated conclusively that the GST enzymes displayed overlapping substrate specificities and that their activities were not limited to a single functional group of the substrate, which suggested that identifying the GSTs by the substrate they metabolized was imprecise (Pabst et al., 1973).

Jakoby and his coworkers (1978) named the hepatic rat GST

isozymes as AA, A, B, C, D and E, based on their reverse order of elution from a carboxymethyl-cellulose ion-exchange matrix column. This system of nomenclature also proved inadequate, as rat liver soon was shown to contain many more forms than originally found by Jakoby et al..

Bass et al. (1977) attempted to identify the different isozymes based on their subunit structure, and they named the different subunits Ya, Yb and Yc, with the latter being the largest based on SDS-PAGE (SDS-polyacrylamide gel electrophoresis). However, it was soon observed that more than one Ya, Yb and Yc existed in the cells. Mannervik et al. (1985) suggested that the isozymes in the rat be identified by subunit structure but that each unique subunit receive a number (1, 2, 3, etc.). By 1986, eight subunits had been identified in the rat which formed 14 different GST isozymes (Hayes and Mantle, 1986).

It was observed that some forms of GST appeared to be more closely related than other forms. The isozymes formed by the Ya and Yc subunits have similar substrate specificities and are able to form hybrids with each other. GST formed by Yb_1 and Yb_2 subunits differ from the Ya/Yc isozymes in their substrate specificities. The GST Yb_1/Yb_2 subunits do not form hybrids with the Ya/Yc subunits (Boyer et al., 1983). In addition, antibodies formed against rat Ya/Yc isozymes cross-react with human and mouse GST Ya/Yc isozymes, but not with the GST Yb_1/Yb_2 isozymes (Mannervik et al., 1985). Based on these functional and structural relationships, a new nomenclature has been suggested which separates the mammalian

cytosolic GSTs into three classes of enzymes, Alpha, Mu and Pi (Mannervik et al., 1985). The Alpha class isozymes tend to have alkaline (>8.0) isoelectric points, the Mu class isozymes have near neutral pI values, and the Pi class isozymes have acidic (<7.0) values of pI (Mannervik, 1985). However, these are not absolute, i.e., some isozymes within a class may have an unusual pI value. The nomenclature and characteristics of the rat GSTs are summarized in Table 1.

A uniform system of nomenclature for other species does not exist. In mouse, the nomenclature system applied to the rat has been gradually adopted, as more GST genes have been cloned using rat GST DNA probes or antibodies. The use of numbers for the various subunits may not necessarily be related to the subunits between the two species. In man, a different nomenclature system has been used. Human GST isozymes are formed by combinations of at least four different subunits (Singh et al., 1985).

3.4 Glutathione and Glutathione S-transferase in Chlorambucil resistance

Some recent studies strongly suggest the involvement of the GSH and GST in CLB resistance. Evidence supports for this involvement include amplification of the GST α-class gene in CLB-resistant CHO cells (Lewis et al., 1988), overexpression of mRNA and protein of the α-class GST gene in CLB-resistant CHO cells (Hall et al.,1989; Robson et al., 1987), and incresed levels of

Table 1.Nomenclature for cytosolic rat glutathione S-transferases

Class[a]	Isozyme[b]	Subunit[c]	Other Names[d]	Other Names[e]	pI value
Alpha	1-1	Ya Ya	Ligandin	L_2	10.0
Alpha	1-2	Ya Yc	B	BL	9.9
Alpha	2-2	Yc Yc	AA	B_2	9.8
Mu	3-3	Yb_1 Yb_1	A	A_2	8.9
Mu	3-4	Yb_1 Yb_2	C	AC	8.0
Mu	3-6	Yb_1 Yn	P		7.4
Mu	4-4	Yb_2 Yb_2	D	C_2	6.9
Mu	4-6	Yb_2 Yn	S		6.1
Mu	6-6	Yn Yn	M_T		
Pi	7-7	Yp Yp (Yf Yf)	P	209	7.0
Alpha	8-8	Yk Yk	K		6.0

[a]: Mannervik et al., 1985.

[b]: Jakoby et al., 1984.

[c]: Bass et al., 1977; Hayes and Mantle, 1986.

[d]: Jakoby, 1978.

[e]: Mannervik and Jensson, 1982.

cellular GSH content, GST gene expression, and GST enzyme activity in CLB-resistant Walker tumor (Tew et al., 1988; Wang and Tew, 1985), CHO (Robson et al., 1986; Hall et al., 1989), and CLL cells (Schisselbauer et al., 1990). More direct evidence comes from enzyme inhibition studies and gene transfer experiments. Robson et al. (1986) demonstrated a significant enhancement of CLB cytotoxicity in CHO cells by pretreating the cells with the GSH synthesis blocking agent, L-buthionine-SR-sulfoximine (BSO). Tew et al. (1988) observed an increase in CLB sensitivity in rat and human tumor cell lines by pretreating the cells with the GST inhibitor ethacrynic acid. Hall et al. (1989) showed a partial reversal of CLB resistance in CHO cells with preincubation of the cells with the GST inhibitor indomethacin. Black et al. (1990) showed that transfection of alpha-class GST gene induced 3-16-fold resistance to CLB in yeast cells.

However, there were some studies, which did not support the notion that GSH and GST played an important role in CLB resistance. Puchalski et al. (1990) showed induction of 1.3-fold resistance to CLB in monkey kidney cells after GST-alpha gene transfection, which did not correlate well with the expression of the GST-alpha gene in the transfectants. Leyland-Jones et al. (1991) failed to induce CLB resistance in GST-alpha gene transfected human breast cancer cells. Johnston et al. (1990) did not find a significant increase in intracellular GSH content and GST enzyme activity in CLB-resistant CLL cells. These studies indicate that more than one mechanism may be involved in CLB resistance in vitro and in vivo.

3.5 Regulations of Glutathione S-transferase Ya gene expression

GST Ya is a subunit of alpha-class GST isozymes. GST Ya gene spans about 11 kilobases and contains seven exons (Figure 3). At least six GST Ya genes in rat (Hayes et al., 1991; Pearson et al., 1988), two GST Ya genes in mouse (Daniel et al., 1987; Pearson et al., 1988) and four GST alpha-class genes in human cells (Ahmad et al., 1993) have been identified. The mouse GST Ya_1 gene, identified by Daniel et al. (1987), encodes an mRNA of 841 nucleotides and a polypeptide of 223 amino acid. The GST Ya_2 gene, identified by Pearson et al. (1988), encodes an mRNA of 838 nucleotides and a polypeptide of 222 amino acid.

Cellular levels of cytosolic GSTs in rodents are known to be readily increased in response to a wide range of exogenous compounds, such as polycyclic aromatic hydrocarbons, Aflatoxin B_1 and aromatic amines (Benson et al., 1989; Primiano et al., 1992). Also included are the antineoplastic alkylating agents, such as CLB, melphalan and nitrosourea. Studies on regulation of the GST subunit gene expressions have been carried out in the past twenty years and are still not understood. For most of the cases studied, expression of GST Ya gene is regulated at the transcriptional level (Ding and Pickett, 1985; Rushmore and Pickett, 1990). Induction of one of rat GST Ya genes involves the interaction of xenobiotic compounds with a cytosolic receptor protein, Ah or dioxin receptor, which can subsequently bind to specific xenobiotic-regulatory elements in the 5' flanking region of the Ya gene (Rushmore and

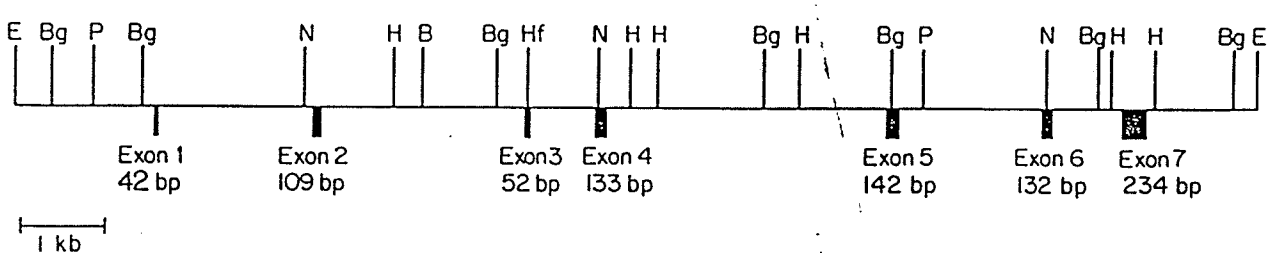

Figure 3. Structure of the mouse GST Ya gene. Exons are denoted by black boxes and introns by the lines between the boxes. The restriction enzyme sites are indicated and abbreviated as follows: B, Bam HI; Bg, Bgl II; E, Eco RI; H, Hind III; Hf, Hinf I; N, Nco I; P, Pst I. All Eco RI, Bam HI and Hind III sites of the gene are indicated. Other restriction enzymes may have more sites than presented (Taken from Daniel et al., 1987)

Pickett, 1990). Another distinct regulatory element in the 5' flanking region of the rat GST Ya gene is also needed for maximum basal level expression (Telakowski-Hopkins et al., 1988; Rushmore et al., 1990). Paulson et al. (1990) demonstrated that the sequences from -980 to -650 of the GST Ya gene were necessary and sufficient for cell-specific and inducible expression. Within this region, four nuclear protein-binding sites were identified. One site was bound by a protein induced by 3-methylcholanthrene, whereas the two other sites were bound by proteins similar to or identical to the constitutive hepatocyte nuclear factors HNF1 and HNF4, and the fourth site was bound by a non-liver-specific nuclear protein (Paulson et al., 1990). Similar findings were also shown in mouse cells. At least two cis-regulatory elements were identified in the 5'-flanking region of the mouse GST Ya gene. One is located within the sequence up to -200 bp, and is responsible for the basal level of gene expression. Another is located within the region from -200 bp to -1.6 kbp, and is responsible for the inducible expression by aromatic conpounds such as β-naphthoflavone and 3-methylcholanthrene (Friling et al., 1990; Daniel et al., 1989).

Post-transcriptional regulation of GST Ya has also been reported. Taniguchi and Pyerin (1989) demonstrated that GST Alpha class isozymes, GST 1-1, 1-2 and 2-2, are good substrates of the Ca^{++}-phospholipid-dependent protein kinase (protein kinase C) in vitro, and can be phosphorylated by the protein kinase C purified from rabbit brain. Pyerin et al. (1987) also showed

phosphorylation of the GST 1-2 and 4-4 isozymes by the protein kinase C purified from chick oviduct. Phosphorylation of the GST Alpha class isozyme showed decreased affinity for bilirubin, which indicates a functional significance for this post-translational modification (Taniguchi and Pyerin, 1989).

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THE ROLE OF GLUTATHIONE AND GLUTATHIONE S-TRANSFERASE IN CHLORAMBUCIL RESISTANCE

FOREWORD

The following chapter is the full-paper format of a manuscript published in the Molecular Pharmacology: Wen Z. Yang, Asher Begleiter, James B. Johnston, Lyonel G. Israels, and Michael R. A. Mowat. 1992. Role of glutathione and glutathione S-transferase in chlorambucil resistance. Mol. Pharmacol. **41**:625-630.

Resistance to chlorambucil (CLB) is a significant problem in the treatment of neoplastic diseases such as chronic lymphocytic leukemia (Gale and Foon, 1985). A better understanding of the mechanisms leading to CLB resistance could indicate ways to more effective treatment. The mechanisms of resistance to CLB have been extensively investigated in tissue culture. Harrap and Hill have shown that hydrolysis of the mustard group of the CLB was increased in CLB-resistant Yoshida sarcoma cells (Harrap and Hill, 1970). Reduced levels of CLB-DNA cross-linking were found in CLB-resistant Chinese hamster ovary (CHO), Yoshida sarcoma and CLL cells (Robson et al., 1987; Jiang et al., 1989, Harrap and Gascoigne, 1976, Panasci et al., 1988). The reduction of CLB-DNA cross-linking may be due to enhanced DNA repair (Harrap and Gascoigne, 1976). Recent studies suggest that the involvement of glutathione (GSH) and glutathione S-transferases (GSTs) may be an important mechanism of CLB resistance. Resistance to CLB has been associated with increased intracellular levels of sulfhydryl containing compound GSH (Johnston et al., 1990; Begleiter et al., 1991; Robson et al., 1987). Increased levels of GST enzyme activity and GST gene

expression have been also shown in CLB-resistant Walker tumor, CHO and CLL cells (Tew et al.; Robson et al., 1986, 1987; Hall et al., 1989; Schisselbauer et al., 1990). GSTs represent a family of multifunctional cellular proteins composed of dimers with a molecular mass of 45-50 kDa (Mannervik et al., 1985). These enzymes catalyze conjugation reactions between GSH and a variety of organic molecules (Mannervik et al., 1985; Schecter et al., 1992). Recently studies have shown that GSTs, mainly alpha class isozymes, catalyze the conjugation of GSH with CLB leading to the inactivation of the CLB (Dulik et al., 1990; Schecter et al., 1992). In our study, we also found that GSH and GST play important roles in CLB resistance.

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THE ROLE OF GLUTATHIONE AND GLUTATHIONE S-TRANSFERASE IN CHLORAMBUCIL RESISTANCE*

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Running Title: GSH and GST in Chlorambucil Resistance

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ABSTRACT

A chlorambucil (CLB)-resistant cell line, N50-4, was developed from the established mouse fibroblast cell line NIH-3T3, by multi-step drug selection. The mutant cells exhibited > 10-fold resistance to CLB. Alterations in glutathione (GSH) and glutathione S-transferase (GST) were found in CLB-resistant variants. A 7-10-fold increase in cellular GSH content and a 3-fold increase in GST activity were detected in N50-4 cells, compared with parental cells, as determined by enzymatic assays. An increase in steady-state levels of the GST-α isozyme mRNA was found in CLB-resistant cells, as analyzed by Northern blotting. No GST gene amplification or rearrangement was shown by Southern blot analysis. To test the role of GSH and GST in CLB resistence, a number of GSH- and GST- blocking agents were used. The CLB toxicity was significantly enhanced in N50-4 cells by administration of either the GSH-depleting agent buthionine sulfoximine (BSO) or the GST inhibitors ethacrynic acid or indomethacin. The resistance to CLB cytotoxicity in N50-4 cells, however, was still higher than that of parental cells. The resistance of N50-4 cells to CLB was almost completely abolished by combination pretreatment yielding both GSH depletion and GST inhibition. The results indicate that both increased cellular GSH content and increased GST activity play major roles in CLB resistance in N50-4 mutant cells.

The abbreviations used are: CLB, chlorambucil; GSH, glutathione; GST, glutathione S-transferase; BSO, L-buthionine-S,R-sulfoximine; CDNB, 1-chloro-2,4-dinitrochlorobenzene.

INTRODUCTION

CLB is a bifunctional alkylating agent that has been widely used for the treatment of neoplastic diseases such as chronic lymphocytic leukemia (CLL), Hodgkin's disease, ovarian carcinoma, and non-Hodgkin's lymphomas (McElwain et al., 1977; Knospe et al., 1974; Gale and Foon, 1985). The clinical application of CLB is, however, limited by acquired drug resistance (Wheeler, 1963; Harrap et al., 1980). Mechanisms of CLB cytotoxicity have been extensively investigated for the past twenty years but remain unclear. CLB is taken up into cells by passive diffusion (Begleiter and Goldenberg, 1983; Harrap and Hill, 1970). The major pathways of CLB metabolism have been found to involve hydrolysis of the mustard group (Bank et al., 1989; Hill and Harrap, 1972). Chlorambucil can bind to DNA and induce DNA cross-links (Bank et al., 1989; Silber et al., 1989), a process that may prevent DNA replication and cause cell death (Farmer, 1987; Foon et al., 1990).

Several mechanisms of CLB resistance have been proposed, but the importance of each is not fully defined at this time. No difference in drug uptake was observed between CLB-sensitive and -resistant CLL cells (Bank et al., 1989; Silber et al., 1989). Harrap and Hill (1970) found that the hydrolysis of the mustard group of CLB was faster in CLB-resistant Yoshida sarcoma cells. In contrast Bank et al. (1989) showed no correlation between cellular metabolism of CLB and sensitivity to CLB in CLL cells. Reduced

levels of DNA cross-links have been found in CLB-resistant Chinese hamster ovary (CHO) (Robson et al., 1987; Jiang et al., 1989), Yoshida sarcoma (Harrap and Gascoigne, 1976), and CLL cells (Panasci et al., 1988). The reduction of DNA crosslinking appeared to be due to an increase in the rate of removal of interstrand DNA cross-links in CLB-resistant Yoshida sarcome (Harrap and Gascoigne, 1976) and CLL cells (Panasci et al., 1988). In contrast, no difference in DNA repair was detected between CLB-sensitive and -resistant CHO cells (Robson et al., 1987; Jiang et al., 1989). However, an inverse correlation was observed between GSH content, GST activity, and CLB-induced DNA cross-links in cultured CHO cells (Robson et al., 1987) and CLL cells (Johnston et al., 1990).

GSH is an intracellular cysteine-containing tripeptide and is present at high concentrations in most mammalian cells (Arrick and Nathan, 1984). It has been suggested that GSH plays a critical role in cellular defense against a variety of injurious agents, including antineoplastic alkylating agents such as nitrogen mustard (Evans et al., 1987) and its derivatives Melphalan (Suzukake et al., 1983) and chlorambucil (Robson et al., 1986). GSTs (EC 2.5.1.18) are a family of isozymes that catalyze the conjugation of the electrophilic compounds with GSH. Three structurally distinct gene families of cytosolic GSTs have been identified in rat, as well as in mouse and human, and have been termed α (basic), μ (neutral), and π (acidic) (Mannervik et al., 1985).

The involvement of GSH and GST in CLB resistance was suggested by the observations that the gene encoding GST-α isozyme was

amplified in CLB-resistant CHO cells (Lewis et al., 1988). Messenger RNA and protein of α-subclass GST in the CLB-resistant cells were also shown to be over-expressed (Robson, et al., 1987; Hall et al., 1989). Increased levels of cellular GSH content, GST gene expression, and GST enzyme activity were also detected in CLB-resistant Walker tumor (Wang and Tew, 1985; Tew et al.,1988), CHO (Robson et al., 1986; Robson et al., 1987; Hall et al., 1989), and CLL cells (Schisselbauer et al., 1990). More direct evidence supporting the role of GSH and GST in CLB resistance came from enzyme inhibition studies and gene transfer experiments. Robson et al. (1986) demonstrated a significant enhancement of CLB cytotoxicity in CHO cells by pretreatment of the cells with the GSH synthesis inhibitor BSO. Tew et al. (1988) observed an increase in CLB sensitivity in rat and human tumor cell lines after pretreatment of the cells with the GST inhibitor ethacrynic acid, and Hall et al. (1989) showed a partial reversal of CLB resistance in CHO cells by preincubation of the cells with the GST inhibitor indomethacin. Black et al. (1990) showed that transfection of α-class GST recombinants induced 3-16-fold resistance to CLB in yeast cells. However, Puchalski et al. (1990) showed induction of 1.3-fold resistance to CLB in monkey kidney cells after GST-α gene transfection, which did not correlate well with the expression of the GST-α gene in the transfectants, and Leyland-jones et al. (1991) failed to induce CLB resistance in GST-α gene-transfected human breast cancer cells. These studies indicate that a number of mechanisms may contribute to CLB resistance in vitro and in vivo

and that the relative contributions of these mechanisms may vary considerably.

In the present study, we have established a CLB-resistant cell line from NIH-3T3 mouse fibroblast cells. This CLB-resistant cell line is characterized by increased intracellular GSH content and elevated GST enzyme activity. We have investigated the relative roles that GSH and GST play in this CLB-resistant cell line, by altering the intracellular GSH pool and GST activity using known GSH- and GST-inhibitory agents. These studies indicate that both increased GSH content and GST activity are major factors involved in CLB resistance in this cell line.

MATERIAL AND METHODS

CELLS AND CELL CULTURE

CLB-resistant cell lines were developed from NIH-3T3 mouse fibroblast cell line by stepwise drug selection. NIH-3T3 cells were exposed to increasing concentrations of CLB, from 30 μM to 100 μM, over 6 months. The degree of CLB resistance was monitored every 2 months by clonogenic assay. The CLB-resistant cell line N50-4 was cloned by glass ring cloning technique (Freshney, 1983). All cells were grown as monolayers in α-minimal essential medium (α-MEM), supplemented with 10% (v/v) fetal bovine serum and antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml), at 37°C in a humidified atmosphere containing 5% CO_2.

CELL SURVIVAL AND CLB CYTOTOXICITY

Cells (ranging from 500 to 5,000) were plated on 100-mm^2 Petri dishes and incubated for 4 hrs at 37°C, to allow cell adhesion. For cell survival measurements, CLB was dissolved in acidified ethanol, added to the cell culture medium for 3 hr, and then replaced with fresh drug-free medium. For BSO-mediated GSH depletion experiment, cells were pretreated with BSO for 18 hr and then treated with CLB for 3 hr. For GST inhibitor experiments, cells were preincubated with ethacrynic acid for 10 min, or with indomethacin for 1 hr, followed by treatment with CLB for 3 hr. After 1-week incubation, medium was aspirated and surviving cells

were visualized by methylene blue staining. Colonies of 50 cells or more were scored. Results are expressed as percentage of survival of CLB-treated cells, compared with CLB-untreated cells. In drug combination studies involving BSO, ethacrynic acid, or indomethacin with CLB, relative survival was determined by the percentage of survival of CLB-treated cells, compared with cells treated with these agents without CLB.

GSH MEASUREMENT

Total cellular GSH content was measured by using the enzyme-recycling assay (Griffith, 1980). Cells were lysed by sonication, proteins were precipitated by 4% 5-sulfosalicyclic acid, GSH content in the supernatant was measured in the presence of 0.5 unit/ml glutathione reductase at 25°C. Protein content was determined by the modified Lowry method (Bradford, 1976). GSH is expressed as nanomoles per milligram of protein or nanomoles per 10^6 cells. For the BSO-mediated GSH depletion assay, cells were treated with BSO for 18 hr and were harvested by rubber policeman for GSH analysis.

GST MEASUMENT

GST activity was measured by the method of Habig et al. (Habig et al., 1974). Cells were harvested, sonicated, and centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was assayed for GST activity by using 1 mM CDNB as the electrophilic substrate. GST activity is expressed as nanomoles of GSH-CDNB conjugates formed

per minute per milligram of protein or nanomoles per minute per 10^6 cells.

NORTHERN AND SOUTHERN BLOT ANALYSIS

Cytoplasmic RNA was isolated by the method of Gough (Gough, 1988). RNA samples were electrophoresed in 1.0% agarose containing 2.2 M formaldehyde and were transferred to nylon membrane in 20x SSPE (1x SSPE is 3 M NaCl, 0.2 M NaH_2PO_4 and 20 mM EDTA) (Maniatis et al., 1982).

Genomic DNA was extracted from the cells by the method of Maniatis et al. (1982) and was digested to completion with the appropriate restriction endonucleases at 37°C. After electrophoresis in 1.0% agarose, the gel was treated with 0.25 M of HCl, rinsed with water, and the DNA samples were blotted to nylon membrane in 0.4 M NaOH and 0.6 M NaCl (Reed and Mann, 1985).

The membranes of RNA or DNA blots were rinsed with 2x SSPE, air dried, and fixed by baking at 80°C for 2 hr. Prehybridization and hybridization were carried out in 50% of formamide, 4x SSPE, 5x Denhardt's solution (50x Denhardt's solution is 5 g Ficoll, 5 g polyvinyl-pyrrolidone, 5 g bovine serum albumin per 500 ml H_2O) (Maniatis et al., 1982), 1% SDS, at 42°C. cDNA probes for GST Ya (pGTB 38) and GST Yb (pGTA/C48) were obtained from Dr. C. B. Pickett (Pickett, 1987), and GST Yp (pTSS1-2) was obtained from Dr. W. D. Henner (Wang et al., 1989). cDNA fragments, namely, a 521-bp Pst1 fragment from pGTB 38, a 845-bp Pst1 fragment from pGTA/C48, and a 334-bp EcoR1/Sau3a fragment from pTSS1-2, were labeled with

[α-^{32}P] dCTP to a specific activity of 2 x 10^8 cpm/μg DNA, by the random primer method (Maniatis et al., 1982). After hybridization, the filters were washed twice at room temperature in 2x SSPE, 0.1% SDS, for 0.5 hr and twice at 65°C in 0.1x SSPE, 0.1% SDS, for 0.5 hr. Filters were then exposed to Kodak XAR-5 film at -70°C, with intensifying screens.

STATISTICAL ANALYSIS

The changes in CLB cytotoxicity in cells either with or without pretreatment were compared by analysis of co-variance. Values at $p < 0.05$ were deemed statistically significant.

RESULTS

In order to study the mechanisms of CLB resistance, a CLB resistant cell line, N50-4, was selected from NIH 3T3 mouse cells, as described in "Materials and Methods". The CLB concentrations required to kill 90% of the cells were 13 μM for NIH-3T3 cells and 140 μM for N50-4 cells; thus the resistance of N50-4 cells to CLB was about 10-fold, compared with the parental cells (Table 1; Fig. 1). The resistant phenotype of N50-4 cells was very stable and has been maintained in continuous culture in the absence of CLB for more than 12 months. A 7-10-fold increase in intracellular GSH content and a 3-fold increase in GST activity were detected in the CLB-resistant cells, by enzymatic assays (Table 1). The growth rates of the two cell lines were similar, as determined by cell-doubling time (Table 1). No difference in cell volume was detected between N50-4 and parental NIH-3T3 cells, as measured by a Coulter counter (Coulter JT2; Coulter Electronics of Canada, Ltd).

To understand the mechanism for the increase in GST activity, we investigated the expression of the GST genes. by Northern blot analysis. A steady state accumulation of GST α-class mRNA was found in CLB-resistant N50-4 cells, and theGST α-class mRNA was not readly detected in NIH-3T3 cells (Fig. 2A). No significant differences were observed in mRNA level of GST μ- and π-class genes between the two cell lines. We further screened the genomic DNA of these cell lines by Southern blot analysis after Pst1 restriction

endonuclease digestion, using cloned cDNA fragments of GST Ya (α), Yb (μ), and Yp (π) gene as probes. No evidence of amplification or rearrangement of the GST genes was found in CLB-resistant cells (Fig. 2B). Similar results were obtained by digestion of the genomic DNA with other restriction enzymes, such as HindIII and EcoR1 (data not shown).

To test the relative contribution of GSH and GST to CLB resistance, we altered the intracellular GSH content or GST activity, using known GSH or GST enzyme inhibitors (Griffith and Meister, 1979; Ahokas et al., 1985; Ploemen et al., 1990; Wu and Mathews, 1984; Nicholls and Ahokas, 1984). BSO, a potent GSH synthesis inhibitor, was used to deplete GSH from the cells. With BSO preincubation for 18 hr, a dramatic decrease in intracellular GSH content was detected in both CLB-sensitive and -resistant cells (Fig. 3). By treating the CLB-resistant N50-4 cells with 50 μM BSO, which resulted in a 72% reduction in intracellular GSH content and a 18.8% cytotoxicity, the sensitivity to CLB was significantly increased (Fig. 4). However, the resistance of the N50-4 cells to CLB with BSO pretreatment was still greater than that of wild-type cells. This difference in CLB sensitivity was not due to higher intracellular GSH content in CLB-resistant variants, because similar results were obtained with pretreatment with a higher doses of BSO (100 μM).

The inhibition of GST activity by ethacrynic acid and indomethacin was demonstrated using cell lysates from N50-4 cells (data not shown). Pretreatment of CLB-resistant cells with 50 μM

ethacrynic acid and 100 μM indomethacin produced 5.3% and 6.3% cytotoxicity, respectively, and these concentrations were used as pretreatment doses. The CLB cytotoxicity was significantly increased in N50-4 cells with either ethacrynic acid or indomethacin pretreatment (Fig. 5). However, under these conditions, N50-4 cells were still more resistant to CLB than the parental cells (Fig. 5).

These results suggested that both increased intracellular GSH content and GST activity may play important roles in CLB resistance. We further tested this hypothesis by a combination pretreatment of the CLB-resistant cells with both BSO and GST inhibitor, ethacrynic acid or indomethacin. Pretreatment of the N50-4 cells with BSO plus ethacrynic acid or BSO plus indomethacin produced 26% and 28% cytotoxicity, respectively. The resistance to CLB in N50-4 cells was almost fully reversed to the phenotype of the parental cells by combination treatment with the GSH-depleting agent BSO and the GST inhibitors ethacrynic acid or indomethacin (Fig. 6).

DISCUSSION

In the present study, we have observed a significant increase in intracellular GSH content and GST activity in CLB-resistant N50-4 cells, compared with the parental NIH-3T3 cells. Also, we have assessed the role of GSH and GST in CLB resistance by using known GSH- and GST-inhibitory agents to reduce the intracellular GSH content or to inhibit the GST activity in the CLB-resistant cell line.

Pretreatment with BSO to deplete intracellular GSH resulted in a significant enhancement of CLB cytotoxicity in CLB-resistant cells. The resistance of the N50-4 cells to CLB, however, was still much higher than that of wild-type cells. Treatment of N50-4 cells with a higher concentration of BSO (100 μM) did not abolish the CLB resistance, indicating that the intracellular GSH content is not the only factor responsible for the CLB resistance.

The role of GST in CLB resistance was studied using the GST inhibitors ethacrynic acid and indomethacin. Pretreatment with ethacrynic acid or indomethacin increased the sensitivity to CLB in N50-4 cells, but these cells were still significantly more resistant to CLB than were the wild-type cells. Pretreatment of the CLB-resistant cell line with both a GSH-depleting agent (BSO) and a GST inhibitor (ethacrynic acid or indomethacin) resulted in the N50-4 cells being almost as sensitive to CLB as the parental cells, suggesting that both GSH and GST play important roles in CLB

resistance in this cell line.

These findings support the previous observations that CLB can react with GSH both nonenzymatically and enzymatically. Dulik et al. (1990) reported that CLB can conjugate with GSH nonenzymatically but at levels 2-5-fold lower than microsomal GST-mediated conjugation. Ciaccio et al. (1990) demonstrated a significant increase in CLB conjugation with GSH by a GST-mediated process, compared with spontaneous CLB-GSH conjugation. Our results showing residual resistance after GST activity was decreased with ethacrynic acid or indomethacin may be due to nonenzymatic conjugation of CLB with the elevated GSH levels found in the resistant cells.

Observations from Northern blot analysis showed that the mRNA level of the GST-α gene in the CLB-resistant cells was greatly increased, which would explain the increased intracellular GST activity. No significant differences in mRNA of GST π- and μ-class genes were found in the two cell lines, which is consistent with the findings in CHO cells (Lewis et al., 1988; Hall et al., 1989). Our results support the findings that the GST-α isozyme is more efficient in conjugating CLB than are other GST isozymes (Ciaccio et al., 1990). Robson et al. (1987) showed that expression of GST α- and π-class proteins was significantly increased in CLB-resistant CHO cells. Schisselbauer et al. (1990) found a 2-fold increase in GST activity in CLB-resistant CLL cells, compared with nonresistant CLL cells and cells from normal individuals, but no obvious correlation between different isozymes of GST expressed and

degree of CLB resistance in CLL cells. Ciaccio et al. (1990) showed that GST-α was more efficient than GST-π and -μ isozymes purified from mouse liver cytosol for GSH conjugation with CLB. Black et al. (1990) demonstrated that expression of both GST-α and -π in yeast cells could confer resistance to CLB. These observations suggest that GST-α, as well as π and μ isozymes, may play a role in CLB resistance. In the present study, we found that GST α-class gene expression was predominantly increased in CLB-resistant cells, as is found in other CLB-resistant cell lines established in vitro (Robson et al., 1987; Lewis et al., 1988). These results indicate that GST-α protein may be most important for CLB resistance in vivo.

By using genomic DNA analysis, we found no evidence of GST gene amplification or rearrangement in the CLB-resistant N50-4 cells. In other studies, Robson et al. (1986) were also unable to detect GST gene amplification in the CLB-resistant CHO cells but found an abnormal karyotype with a modal chromosome number of 29 in the resistant cells, compared with 22 for the parental cells, which appears to have resulted largely from chromosome rearrangements. Lewis et al. (1988), however, found that the genes coding for GST α-class proteins were amplified 4-8-fold in CLB-resistant CHO cells by Southern blot analysis. Our study indicates that mechanisms other than gene amplification can produce an increase in GST mRNA level. Experiments are now underway to determine whether the increased GST-α gene expression is occurring at the transcriptional or post-transcriptional level of control.

In summary, this study gives strong evidence that both increased intracellular GSH content and GST activity, mainly α-class isozyme, are major mechanisms of resistance to CLB in the N50-4 mouse fibroblast cell line. In addition, because these two mechnisms appear to be responsible for most of the resistance to CLB in these cells, the N50-4 cell line may serve as a valuable model for studies investigating methods for reversing alkylating agent resistance.

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Table 1. Comparison of CLB sensitivity, GSH content, and GST activity in NIH-3T3 and N50-4 cell lines

Values are mean ± standard error of the number of determinations in parentheses.

	NIH-3T3	N50-4
Doubling time (hr)	17.5 ± 0.1 (4)	17.4 ± 0.4 (4)
chlorambucil LD_{90} (μM)[a]	13	140
Fold resistance (at LD_{90})		10.8
GSH content (nmol/mg of protein)	6.1 ± 1.1 (5)	57.9 ± 5.3 (6)
GSH content (nmol/10^6 cells)	1.9 ± 0.3 (5)	14.7 ± 1.7 (6)
GST activity (nmol/min.mg of protein)	29.8 ± 1.3 (4)	108.6 ± 2.1 (4)
GST activity (nmol/min.10^6 cells)	10.2 ± 1.1 (4)	36.7 ± 4.0 (4)

[a] Concentrations of CLB that kills 90% of the cells.

Figure 1: Dose-response curves of NIH-3T3 and N50-4 cells to CLB. NIH 3T3 (•) or N50-4 (▲) cells were exposed for 3 hr to various concentrations of CLB. Cell survival was determined as described in "Materials and Methods". Points, mean of four determinations; bars, standard error (SE). On occasion the confidence intervals were too small to be shown.

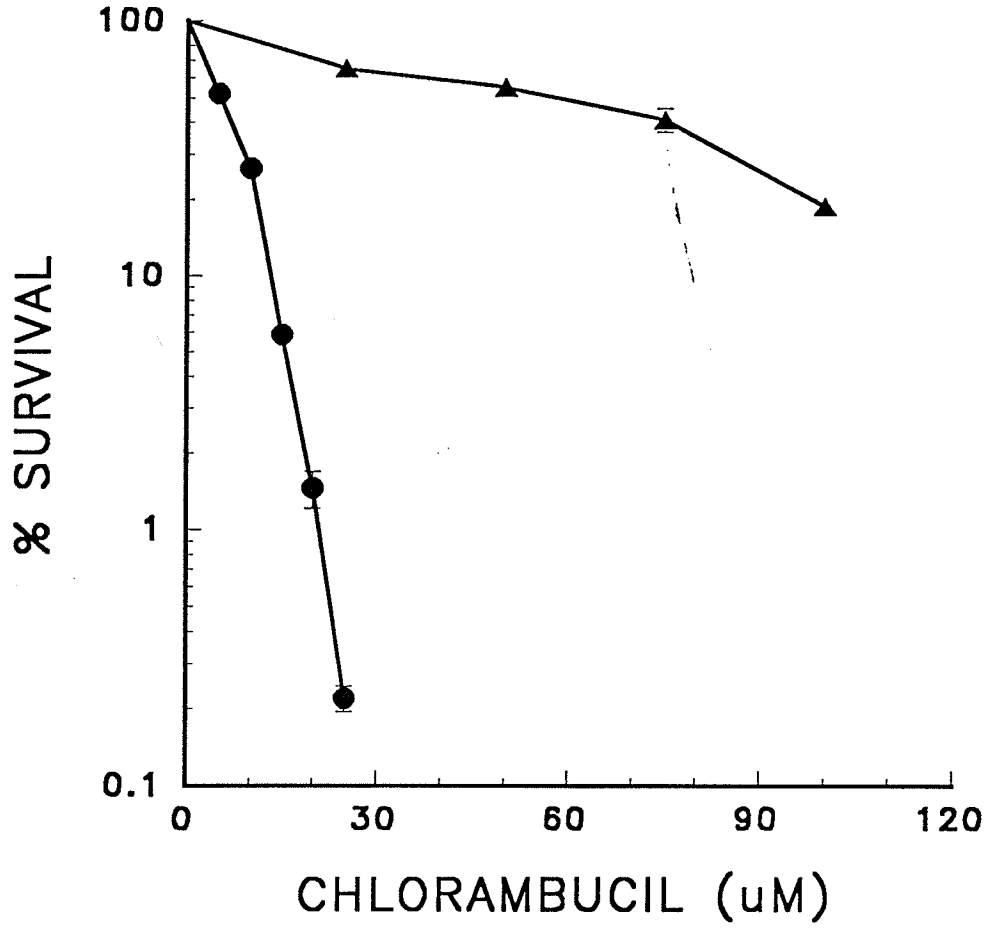
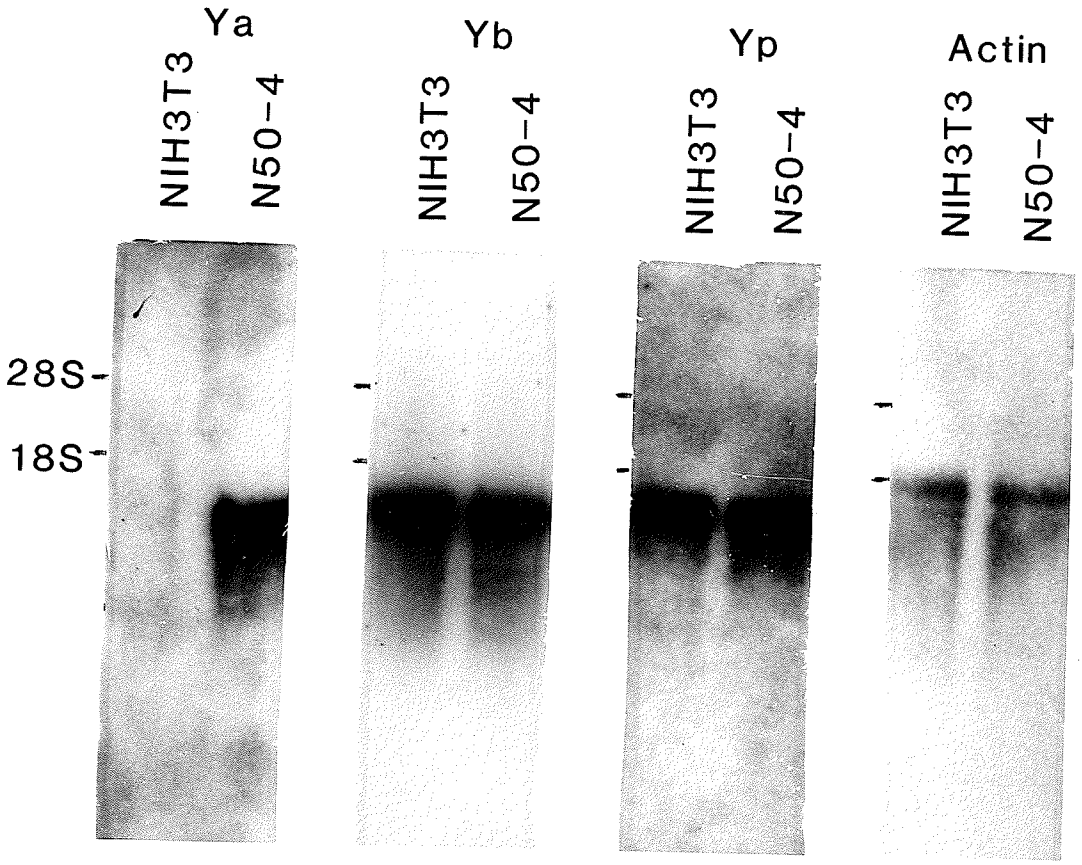

100
10
1
0.1
% SURVIVAL
0
30
60
90
120
CHLORAMBUCIL (uM)

Figure 2: Northern and Southern blot analysis of CLB-sensitive and -resistant cell lines. A, Cytoplasmic RNA (20 μg) from NIH-3T3 and N50-4 cell lines was separated in agarose gels containing formaldehyde and was blotted onto nylon filters. The filters were probed with radioactively labeled cDNA to the Ya (α), Yb (μ), and Yp (π) GST genes and the β-actin gene. B, Genomic DNA (20 μg) was digested with the restriction enzyme Pst1, separated in 1.0% agarose gels, and blotted. The filters were hybridized to the GST cDNA fragment probes as described in "Materials and Methods".

A


Ya
Yb
Yp
Actin
NIH3T3
N50-4
NIH3T3
N50-4
NIH3T3
N50-4
NIH3T3
N50-4
28S
18S

B

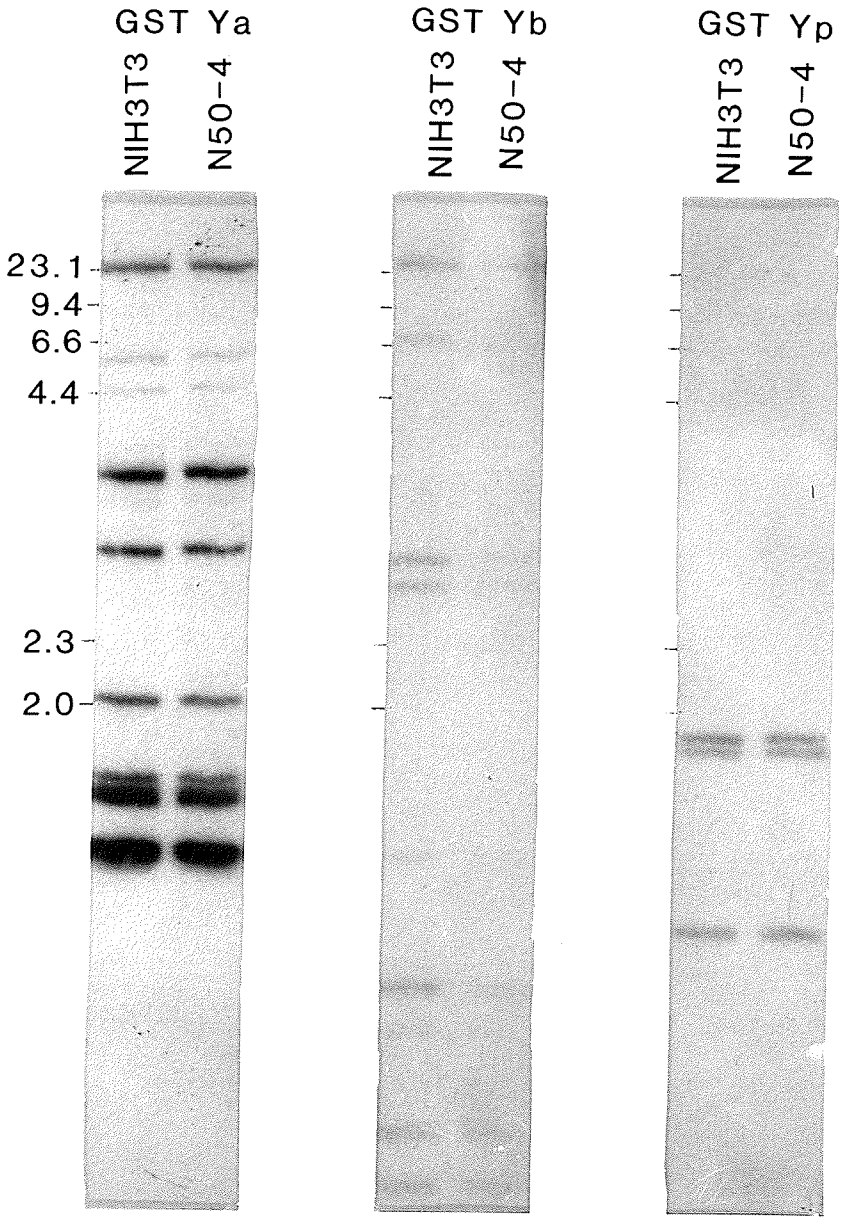

GST Ya
NIH3T3
N50-4
GST Yb
NIH3T3
N50-4
GST Yp
NIH3T3
N50-4
23.1
9.4
6.6
4.4
2.3
2.0

Figure 3: Effect of BSO on intracellular GSH content. Cells growing in monolayers were treated with BSO for 18 hr. The cells were then harvested and GSH content was measured by the enzyme-recycling assay. •, NIH 3T3 cells; ▲, N50-4 cells. Points, mean of four determinations; bars, standard error. On occasion the confidence intervals were too small to be shown.

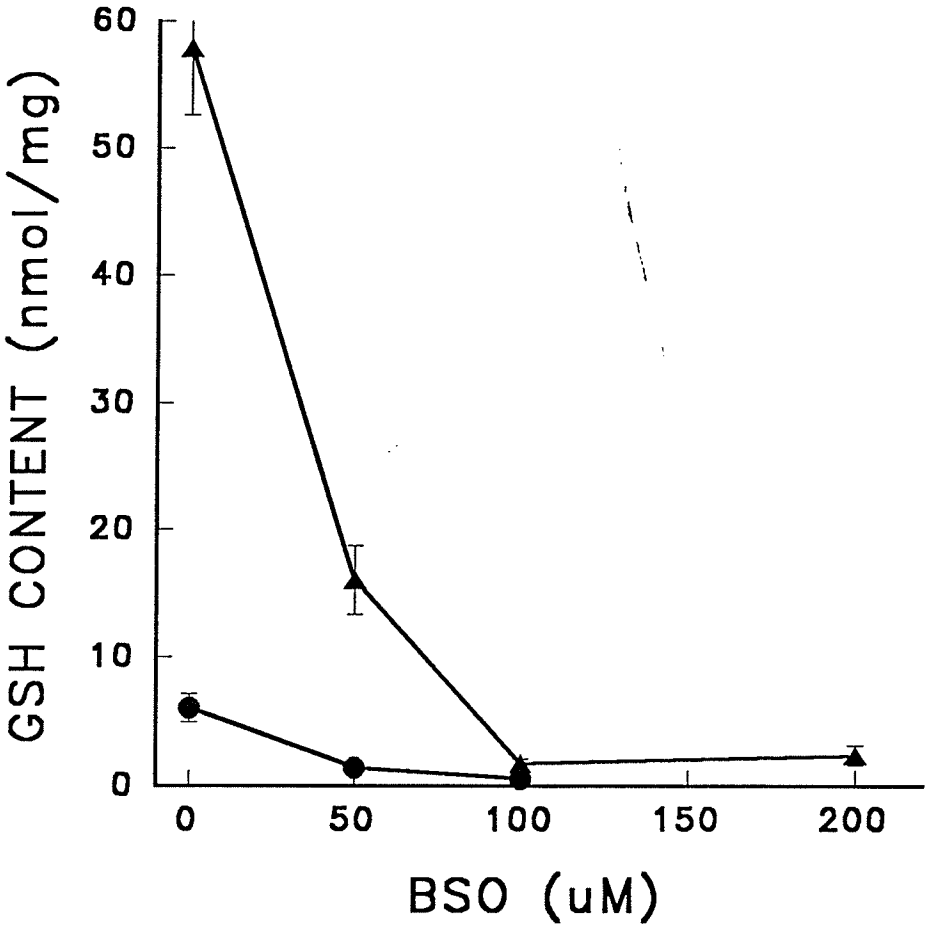

GSH CONTENT (nmol/mg)
60
50
40
30
20
10
0
0
50
100
150
200
BSO (uM)

Figure 4: Effect of BSO-mediated GSH depletion on CLB cytotoxicity. N50-4 cells were pretreated with BSO (50 μM) for 18 hr and then exposed to CLB for 3 hr. Cell survival with BSO and CLB treatment is expressed as a percent of the number of cells surviving in the presence of GSO (50 μM) alone. NIH-3T3 and N50-4 cells were exposed to CLB for 3 hr as non-BSO-treated controls. Points, mean of four determinations; bars, standard error. On occasion, the confidence intervals were too small to be shown.

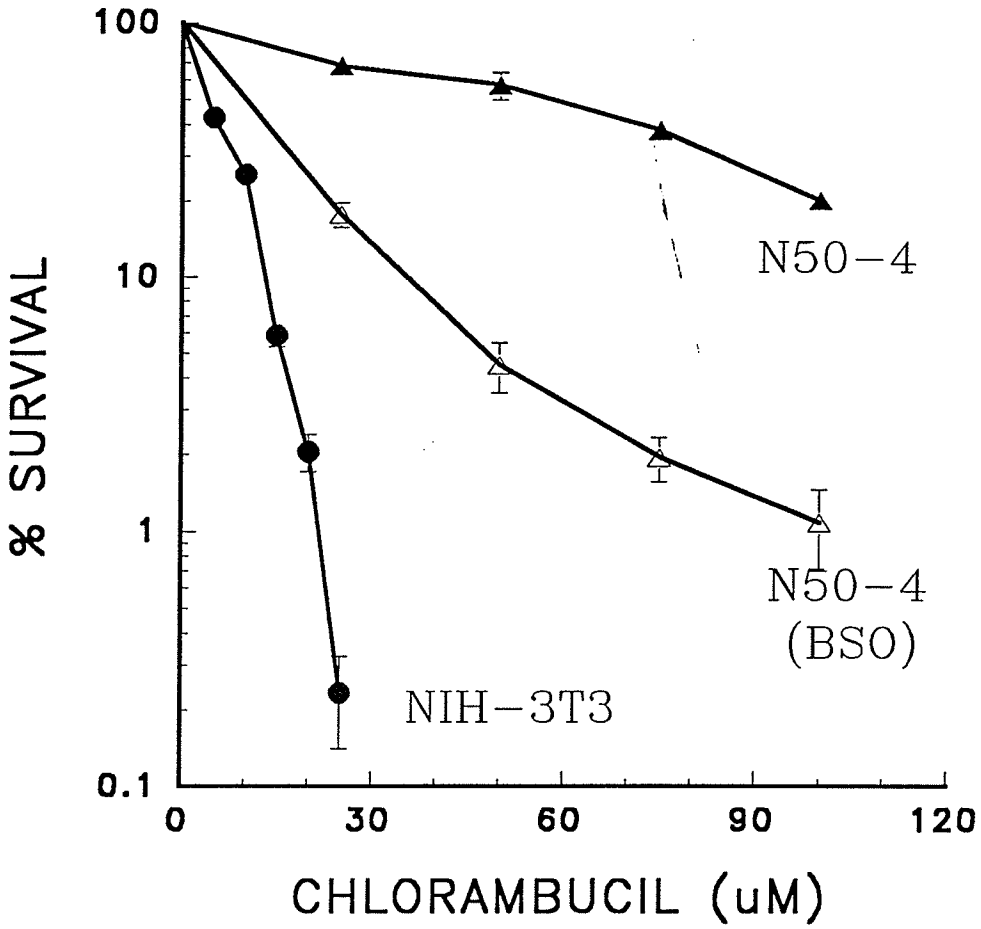
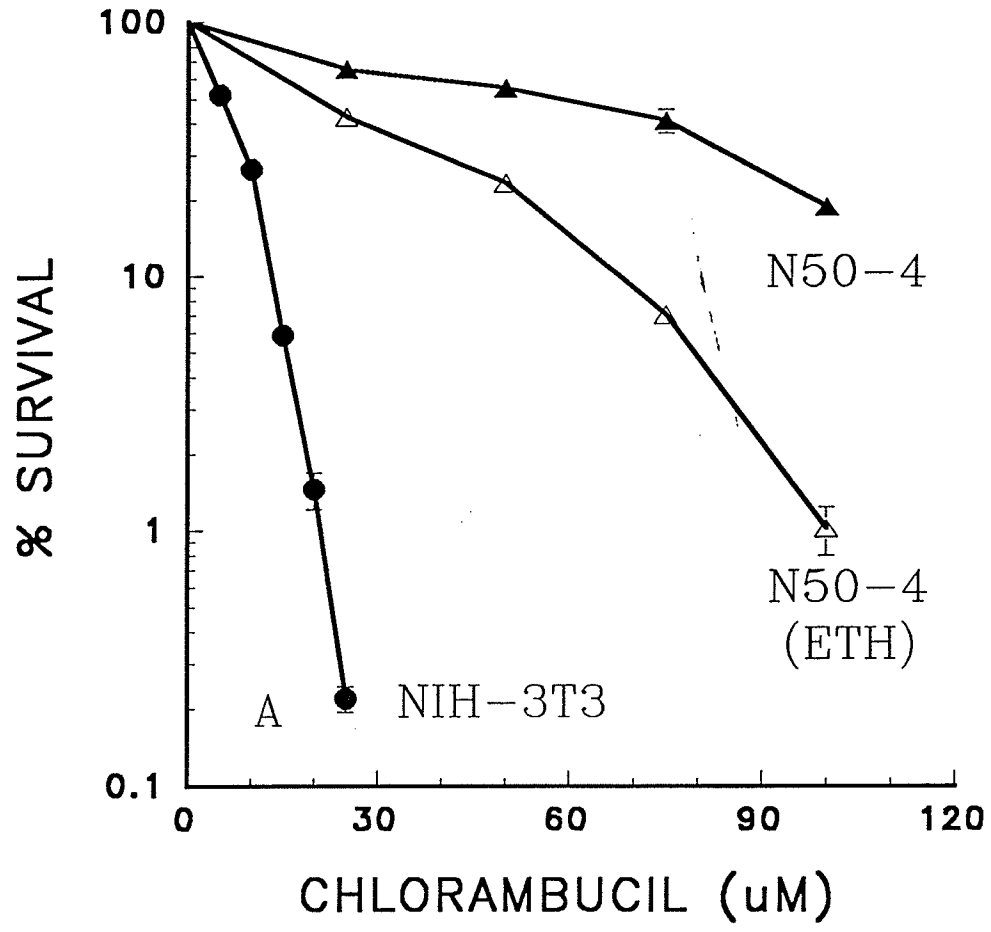

100
10
1
0.1
% SURVIVAL
0
30
60
90
120
CHLORAMBUCIL (uM)
N50-4
N50-4
(BSO)
NIH-3T3

Figure 5: Effect of ethacrynic acid (A) or indomethacin (B) on CLB cytotoxicity. N50-4 cells were pretreated with the GST inhibitors ethacrynic acid (50 μM), for 10 min, or indomethacin (100 μM), for 1 hr, and were then exposed to CLB for an additional 3 hr. Percentage of cell survival with both a GST inhibitor and CLB is based on the survival of the cells treated with GST inhibitor alone. NIH-3T3 and N50-4 cells were exposed to CLB for 3 hr without GST inhibitor, as untreated controls. Points, mean of four determinations; bars, standard error. On occasion, confidence intervals were too small to be shown.


100
10
1
0.1
% SURVIVAL
0
30
60
90
120
CHLORAMBUCIL (uM)
N50-4
N50-4
(ETH)
A
NIH-3T3

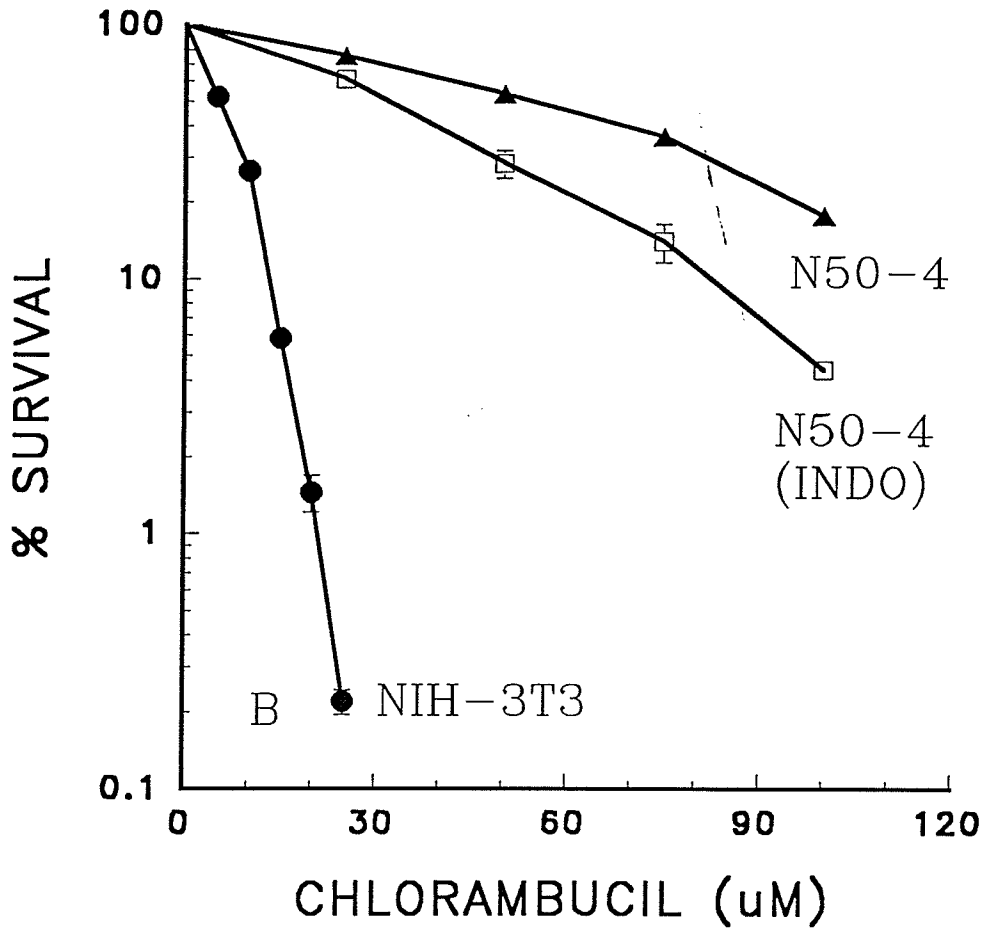
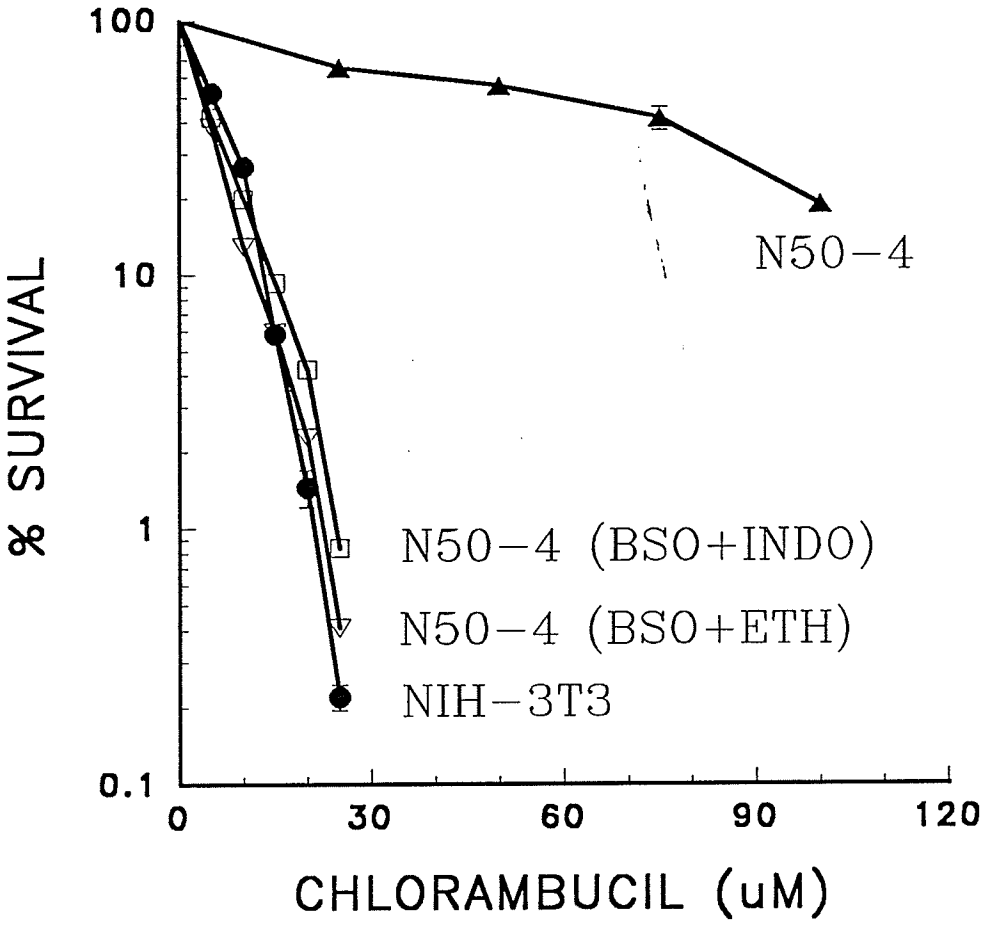

100
10
1
0.1
% SURVIVAL
0
30
60
90
120
CHLORAMBUCIL (uM)
N50-4
N50-4
(INDO)
B
NIH-3T3

Figure 6: Effect of GSH depletion and GST inhibition on CLB resistance. N50-4 cells were pretreated with BSO (50 μM) for 18 hrs, followed by pretreatment of ethacrynic acid (50 μM) for 10 min or indomethacin (100 μM) for 1 hr; cells were then exposed to CLB for 3 h. The percentage of cell survival with the combination treatment is based on the number of surviving cells pretreated with BSO and GST inhibitor alone. NIH-3T3 and N50-4 cells were exposed only to CLB, without any pretreatment, served as control. Points, mean of four determinations; bars, standard error. On occasion, the confidence intervals were too small to be shown.


100
10
1
0.1
% SURVIVAL
0
30
60
90
120
CHLORAMBUCIL (uM)
N50-4
N50-4 (BSO+INDO)
N50-4 (BSO+ETH)
NIH-3T3

REGULATION OF GLUTATHIONE S-TRANSFERASE ALPHA-CLASS GENE EXPRESSION IN CHLORAMBUCIL RESISTANT CELL LINES

FOREWORD

The following manuscript is currently being prepared for submission to J. Biol. Chem.: Wen Z. Yang, Asher Begleiter, James B. Johnston, Lyonel G. Israels, and Michael R. A. Mowat. 1994. Transcriptional and post-transcriptional regulation of glutathione S-transferase α-class gene expression in chlorambucil resistant cell lines.

We have shown increased levels of intracellular glutathione (GSH) content and glutathione S-transferases (GSTs) in chlorambucil (CLB) resistant N50-4 cells, compared with the wild-type NIH-3T3 cells. We have also demonstrated that both GSH and GSTs play important roles in CLB resistance using GSH synthesis inhibitor, l-buthionine-S,R-sulfoximine (BSO) and GST enzyme inhibitors, ethacrynic acid and indomethacin (Yang et al., 1992).

GSTs represent a functionally related group of isozymes that catalyze the nucleophilic attack of the sulfur atom of GSH on electrophilic centers in a wide variety of organic molecules (Schecter et al., 1992). The GST isozymes are subdivided into four cytosolic forms, alpha (α), mu (μ), pi (π) and theta (θ),) and a microsomal form, each with differing structural and functional characteristics (Mannervik et al., 1985; Mannervik and Danielson, 1988; Ogura et al., 1991). The cytosolic GST isozymes are usually homo- or heterodimers with subunits encoded by the same class of the gene family: Ya and Yc of alpha class; Yb_1 and Yb_2 of mu class and Yp or Yf of pi class. Microsomal GST exists in trimeric form (Rahilly et al., 1991).

A large number of effective inhibitors for the GSTs have been reported in the recent twenty years. Because of the overlapping

substrate specificities, discrimination between multiple forms of the GST isozymes and distinction between homo- and heterodimers of the GSTs are difficult (Mannervik and Danielson, 1988). Our previous study can, therefore, only suggest the importance of the GST in CLB resistance, but can not distinguish which GST isozyme is more critical for the CLB resistance. However, our finding that a significant increase in expression of the GST alpha class genes in the CLB-resistant N50-4 cell line (Yang et al., 1992), as well as the similar findings of others that amplification and overexpression of the GST alpha class gene in CLB resistant cells may suggest the important role of the GST alpha class gene in CLB resistance (Hall et al., 1989; Robson et al., 1986, 1987; Tew et al., 1988). To further study the significance of the GST alpha class isozyme in CLB resistance, we conducted experiments investigating the regulation of the GST alpha class gene expression in CLB-sensitive and -resistant cell lines.

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TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL REGULATION OF GLUTATHIONE S-TRANSFERASE α-CLASS GENE EXPRESSION IN CHLORAMBUCIL RESISTANT CELL LINES

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Running Title: Regulation of GST Ya gene expression

Key Words: Glutathione S-Transferase, amplification, transcription rate, mRNA stability.

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ABSTRACT

We used two chlorambucil resistant cell lines, N50-4 and HUCLB, derived from NIH-3T3 cells in this study. Increased steady-state levels of glutathione S-transferase (GST, EC 2.5.1.18) α-class (Ya and Yc) mRNAs, GST enzyme activity and glutathione content were found in both N50-4 and HUCLB cell lines, compared with wild-type NIH-3T3 cells. Studies were carried out to examine the regulation of the GST α-class gene expression in these cell lines. Two closely related GST Ya mRNAs have been identified in these mouse cell lines. One is the GST Ya_1 gene described by Daniel et al. (DNA **6**:317-324, 1987), and the other is the GST Ya_2 gene as described by Pearson et al. (J. Biol. Chem. **263**:13324-13332, 1988). Specific amplification of the GST α-class genes both Ya_2 and Yc was shown for the HUCLB cells but not the N50-4 cell line. Nuclear "run-on" transcription assays showed an increase in transcription of the GST Ya genes for both chlorambucil-resistant cell lines, whereas the transcription of the GST Ya gene was not detected in the NIH-3T3 cells by this assay. Using the sensitive reverse transcriptase-polymerase chain reaction and ligase chain reaction techniques, only transcription of the GST Ya_1 gene was observed in NIH-3T3 cells. GST Ya mRNAs half-lifes were greatly increased in both chlorambucil resistant cell lines. The effect of the GST Ya 3' sequences on mRNA stability was analyzed using the chloramphenicol acetyl transferase (CAT) reporter gene. Cell lines containing the CAT gene plus GST Ya_1 3' sequences showed a significantly shorter mRNA half-life, compared to the GST Ya_2 3'

sequences, or without 3' sequences. These findings show that the GST Ya_1 3' sequences contain a mRNA destabilizing element(s). Also the mRNA half life for both GST Ya-CAT hybrid genes was greater in the N50-4 cell line compared to NIH-3T3 cells, suggesting possible alteration of trans acting factors involved in mRNA turnover in the resistant cells. Our results indicate that gene copy number and both transcriptional and post-transcriptional regulation of the GST Ya genes are important mechanisms for the increased expression of the GST Ya genes in these CLB-resistant cell lines.

INTRODUCTION

The emergence of antineoplastic drug resistance frequently limits the effectiveness of chemotherapy and thus is a major obstacle to cancer treatment. Extensive investigations of experimental and clinical drug resistance have revealed several mechanisms that render cells refractory to cytotoxic agents. Elevated expression of glutathione S-transferases (GSTs) that catalyze the conjugation of glutathione (GSH) to electrophilic compounds, is an important mechanism in acquired drug resistance, especially resistance to alkylating agents (Robson et al., 1987; Kramer et al., 1987; Suzukake et al., 1983; Adams et al., 1985; Evans et al., 1987; Hamilton et al., 1989). Increased levels of GST and GSH have also been associated with multi-drug resistance (Batist et al., 1986; Dusre et al., 1989; Kramer et al., 1988).

GST isozymes are widely distributed in bacteria, plants and animals (Arca et al., 1988; Mannervik, 1985). Mammalian cells contain both soluble (cytosolic) and membrane-bound isozymes. At least four structurally and immunologically distinct gene families of GSTs (α, μ , π and θ), have been identified (Mannervik et al., 1985, Ogura et al., 1991). Among the GSTs, increased levels of the GST α-class mRNA and protein have been reported to play a role in chlorambucil (CLB) resistance (Yang et al., 1992; Hall et al., 1989; Lewis et al., 1988; Schisselbauer et al., 1990; Wang and Tew, 1985). It has also been reported that increased levels of GST α-class isozymes are associated with resistance to other

antineoplastic agents, such as melphalan (Bolton et al., 1991) and Adriamycin (Schisselbauer et al., 1989).

Studies on regulation of the GST α-class gene expression, mainly Ya, have been carried out in the rat (Ding and Pickett, 1985; Paulson et al., 1990; Pickett, 1987; Tee et al., 1992; Telakowski-Hopkins et al., 1988) and in the mouse liver cells (Daniel et al., 1989). Most of these studies were focused on regulation of gene transcription. There has been no direct study of GST α-class mRNA stability, as the low abundance of the GST α-class mRNA in cells makes the detection of mRNA decay extremely difficult (Vickers et al., 1989; Vandenberghe et al., 1991).

In a previous study, we showed increased levels of the GST α-class mRNA in a CLB resistant cell line, N50-4, without amplification of the GST genes (Yang et al., 1992). To understand the mechanisms responsible for the increased level of the GST α-class mRNA, we undertook experiments to examine the transcriptional and post-transcriptional regulation of the GST α-class gene expression in sensitive and resistant cell lines. We observed amplification of the GST Ya_2 and Yc α-class genes in the CLB-resistant HUCLB cell line, which is a more resistant varient derived from the N50-4 cell line. In the resisitant cell lines an increase in transcription of the GST Ya and Yc genes and an increase in GST Ya mRNA stability were seen compared to CLB-sensitive cells. This increased GST mRNA stability is partially due to increased expression of the GST Ya_2 gene in the resistant cells with its intrinsically stable mRNA. Also, the N50-4

-reisistant cell line shows an increased stability of mRNAs compared to the parental NIH-3T3 cell independent of the mRNA examined. Our results indicate that the regulation of the GST α-class gene is a complex process, and that both transcriptional and post-transcriptional control of gene expression are involved in the steady-state accumulations of the GST α-class mRNA in CLB-resistant cells.

MATERIALS AND METHODS

Cell lines. A CLB-resistant cell line, N50-4, was developed from the NIH-3T3 mouse fibroblast cell line by stepwise drug selection (Yang et al., 1992). The HUCLB cell line was derived from the N50-4 cell line, using the method of Johnston et al. (1986) to induce DNA amplification. The N50-4 cells were arrested at S-phase with 2.0 mM of hydroxyurea for 18 hr and the cells were cultured in fresh medium containing 200 μM of CLB for two weeks. A resistant cell line was cloned and named HUCLB.

CLB cytotoxicity assays. CLB cytotoxicity was measured by clonogenic assay as previously described (Yang et al., 1992).

GSH and GST measurement. Cellular GSH content was assayed by the method of Griffith (1980), and the GST enzyme activity was measured by the method of Habig et al. (1974).

Northern and Southern blot analyses. Cytoplasmic RNA was isolated by the method of Gough (1988). RNA samples were electrophoresed in 1.0% agarose containing 2.2 M formaldehyde and were transferred to nylon membrane in 20x SSPE (1x SSPE is 3 M NaCl, 0.2 M NaH_2PO_4, 20 mM EDTA) (Sambrook et al., 1989).

Genomic DNA was extracted from the cells by the method of Sambrook et al. (1989) and was digested to completion with the appropriate restriction endonucleases. After electrophoresis in 1.0% agarose, the gel was treated with 0.25 M HCl and rinsed with water, and the DNA samples were blotted to nylon membranes in 0.4 M NaOH, 0.6 M NaCl (Reed and Mann, 1985).

Prehybridization and hybridization were carried out as

described previously (Yang et al. 1992). cDNA probes for GST Ya (pGTB38), GST Yc (pGTB42) and GST Yb (pGTA/C48) were kindly provided by Dr.C.B.Pickett (1987), and GST Yp (pTSS1-2) was obtained from Dr. W.D.Henner (Wang et al., 1989). The GST cDNA fragments, namely, a 521-bp Pst1 fragment from pGTB38 (Ya), a 572-bp Pst1 fragment from pGTB42 (Yc), an 845-bp Pst1 fragment from pGTA/C48 (Yb), and a 334-bp EcoR1/Sau3a fragment from pTSS1-2 (Yp), were labelled with [α-^{32}P] dCTP to a specific activity of 2x 10^8 cpm/μg DNA, by the random primer method (Feinberg and Vogelstein, 1983). The filters were washed twice for 30 min each in 2x SSPE and 0.1% SDS at room temperature, then twice for 30 min each in 0.1x SSPE and 0.1% SDS at 65°C. The filters were exposed to Kodak XAR-5 film at -70°C, with intensifying screens. For loading controls, the glutathione peroxidase probe was a 700-bp EcoR1 fragment from plasmid pGSHPX, kindly provided by Dr. P.R. Harrison (Chambers et al., 1986).

For Northern blot analysis using oligonucleotide probes, GST Ya_1 and Ya_2 specific oligonucleotides (see Table 1) were 5' end-labelled with [γ-^{32}P] ATP by T4 polynucleotide kinase. Hybridizations were carried out at 37°C for 18 hr as previously described (Yang et al., 1992). GST Ya_1 and Ya_2 positive control RNAs were in vitro transcribed from pCR-TA plasmids (Invitrogen, San Diego, CA, USA) containing GST Ya_1 or Ya_2 cDNA. The in vitro transcription reaction was carried out by T7 RNA polymerase using Sp6/T7 Transcription Kit (Boehringer, Mannheim, Montreal, Canada). Filters were washed at room temperature in 2 x SSPE, 0.1% SDS twice for 10 min each and at 55°C twice in 0.1 x SSPE, 0.1% SDS for 10

min each.

In vitro transcription assays. Transcription assays were done by modification of the method of Greenberg and Ziff (1984). In brief, logarithmically growing cells (1x 10^7) were harvested and lysed in 200 μl of NP40 lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP40), and nuclei were then precipitated. The nuclei were suspended in 100 μl storage buffer (50 mM Tris-HCl pH 8.3, 40% (v/v) glycerol, 5 mM $MgCl_2$ and 0.1 mM EDTA), mixed with 100 μl reaction buffer (10 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$ and 300 mM KCl), 0.5 mM each of ATP, GTP, UTP and 100 μCi of [α-^{32}P] CTP (760 Ci $mmol^{-1}$), and incubated at 30°C for 30 min. The nuclei were lysed in 250 μl of lysis buffer (7 M urea and 1% SDS), and 50 μl of Na acetate (pH 4.0) and 10 μl of tRNA (10 mg/ml) were added. The [α-^{32}P] labelled RNA was purified by two cycles of phenol/chloroform extraction and ethanol precipitation.

For preparation of the slot blot filters, plasmid DNA (5 μg each) was linearized by restriction enzyme digestion and the DNA was heated at 100°C for 5 min, quickly cooled on ice and spotted onto Nylon membrane in 10 x SSPE. The GST Ya gene was obtained by PCR (polymerase chain reaction) amplification of genomic DNA. Oligonucleotide primers (Table 1) containing restriction sites were synthesized on an Applied Biosystem DNA synthesizer. A 2-kb DNA genomic fragment of the GST Ya gene from exon 1 to exon 2 was amplified with Perkin-Elmer/Cetus thermal cycler for 35 cycles and each cycle consisted of denaturing for 1 min at 94°C, annealing for 45 sec at 65°C and primer extension for 2 min at 72°C. PCR was carried out at a final concentration of 1x PCR buffer (20 mM

Tris.HCl pH8.3, 50 mM KCl, 2.5 mM $MgCl_2$), 0.2 mM dNTPs, 1 μM each of 5' and 3' primers, 0.5 μg of genomic DNA as template and 2.5 unit of Taq polymerase (Perkin-Elmer/Cetus, Norwalk, CT. USA) in a total volume of 100 μl. To increase the reaction specificity, the hot-start technique using a wax bead to separate the template DNA and the Taq polymerase from the rest of the reaction reagents before the PCR reaction was started. The amplified DNA fragment was treated with proteinase K, followed by phenol chloroform extraction and ethanol precipitation and was digested with HindIII and BamH1 restriction enzymes to create ligation sites. The DNA fragment was then directionally cloned into plus (+) or minus (-) forms of pBSK bluescript plasmids (Stratagene, La Jolla, CA. USA). Double-stranded (ds) DNA was converted to single-stranded (ss) DNA by M13 phage infection. Equivalent amounts (2-5x 10^6 c.p.m.) of [α-^{32}P] CTP labelled nascent RNA probe were applied to the filters. Hybridizations were performed as described previously. An additional final wash was carried out in 0.3 M NaCl containing 10 μg/ml RNase A at 37°C for 5 min. The radioactive signal was visualized by autoradiography and assessed by optical densitometry. The signal was converted to relative transcription rate by subtracting the background signal (plasmid or phage alone) and correcting for the percentage activity of GAPDH or β-actin signal for the respective filters.

Measurement of mRNA half-life. The transcription inhibitors, either actinomycin D (5 μg/ml) or α-amanitin (10 μg/ml), was added to the culture medium, and cytoplasmic RNA was extracted from the cells at various times (Dani et al., 1984). RNA samples for N50-4

and HUCLB cells were analyzed by the Northern blot technique as previously described. The membrane was re-hybridized with [α-^{32}P] dCTP labelled β-actin or GAPDH cDNA for loading calibration. mRNAs were quantitated by densitometry of the autoradiographs.

For measuring the mRNA half-life in NIH-3T3 cells, which contain few GST Ya transcripts, a quantitative reverse transcription-polymerase chain reaction (RT-PCR) was used to amplify the target mRNA (Wang, Doyle and Mork, 1989). Cytoplasmic RNA, isolated from the NIH-3T3 and N50-4 cell lines at various times after actinomycin D treatment, was reverse transcribed to cDNA in a 50-μl reaction mixture containing 5 μg RNA, 5 μl DMSO, 10 units of RNasin (Promega Biotec, Madison, WI. USA), 2 μM each of dNTPs, 1x reaction buffer (50 mM Tris-HCl, pH 8.15, 6 mM $MgCl_2$, 40 mM KCl and 1 mM DTT), 20 nM 3' primers for GST Ya or β-actin (as an internal standard), and 10 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD. USA) at 42°C for 1 hr (Frohman, 1990). PCR amplification was carried out for 20 cycles as described previously using pairs of GST Ya primers also β-actin primers (Table 1). Twenty μls of each PCR reaction were analysed by Southern blot using the rat GST Ya 521-bp Pst1 cDNA fragment from pGTB38 plasmid and β-actin cDNA as probes.

Cloning and sequencing of GST Ya 3' region. The mouse GST Ya fragments, containing the 3' end of the gene and untranslated region (UTR), were PCR amplified from either mRNA using the (T) primer (Belyovsky et al., 1989) and GST Ya primers (Table 1) or genomic DNA. The PCR products were cloned into pCRII vector using

the TA cloning system (Invitrogen, San Diego, CA.). The cDNA and genomic DNA clones were sequenced by the dideoxy chain termination method using Sequenase 2.0 kit (United States Biochemical, Cleveland, OH. USA). DNA sequences were searched using the BLAST programme at the National Center for Biotechnology Information (Benson et al., 1993).

Ligase chain reaction (LCR) assay. Four primers that perfectly match the GST Ya_2 nucleotide sequence (Table 1) were 5' end-labelled with [γ-^{32}P] ATP by T4 polynucleotide kinase. The unincorporated [^{32}P] ATP was removed by chromatography with Sephadex G-25. The LCR assay was carried out in a 10-μl reaction mixture containing 1x reaction buffer (20 mM Tris-HCl, pH 7.6, 25 mM potassium acetate, 10 mM magnesium acetate, 0.6 mM NAD, 0.1% Triton X-100 and 10 mM dithiothreitol), ^{32}P-labelled primers, DNA and 15 units of thermostable DNA ligase (Epicentre Technologies, Madison, WI. USA), and overlaid with a drop of mineral oil. A total of 50 cycles was performed. Each cycle consisted of denaturing for 1 min at 94°C, annealing and ligation for 4 min at 60°C (Kalin et al., 1992; Barany, 1991). The ligated oligo nucleotides were separated from the unligated nucleotides in 8% acrylamide gel containing 7 M urea in 1x TBE buffer (0.1 M Tris borate, pH 8.9 and 1 mM EDTA) for 1 hr at 60-W constant power. The radioactive signal was visualized by autoradiography.

In-gel hybridization. To detect specific amplification of GST Ya genes, in-gel hybridizations using gene specific oligo nucleotides were performed as described (Wallace and Miyada, 1987). Briefly, genomic DNA was extracted from the cells and was digested

to completion with the appropriate restriction endonucleases. After electrophoresis in 1.0% agarose, the DNA samples were denatured by soaking the gel in 0.5 M of NaOH and 1.5 M of NaCl for 30 min at room temperature with gentle shaking. The gel was neutralized in 0.5 M of Tris-HCl (pH 8.0) and 1.5 M of NaCl for 30 min at 4°C for 30 min. The gel was then dried at 60°C under vacuum for 2 hr. The dried gel was hybridized in 5x SSPE, 0.1% SDS, 10 μg/ml denatured salmon sperm DNA and 2x 10^6 cpm/ml of [γ-^{32}P] ATP labelled GST Ya_1 or Ya_2 specific oligo nucleotides (Table 1) at 60°C for 18 hr. The gel was washed with 6x SSPE on ice twice for 10 min each, and then with 3 M tetramethylammonium chloride, 2 mM EDTA and 50 mM Tris-HCl (pH 8.0) twice at room temperature for 2 hr each (Diella and Woo, 1987). The gel was exposed to Kodak XAR-5 film at -70°C, with intensifying screens.

mRNA stability analysis of the GST Ya_1 and GST Ya_2 3' sequences.

The KpnI-XbaI fragments from the pCRII plasmids containing the GST Ya_1 or GST Ya_2 cDNAs were ligated to the 3' end of the chloramphenicol acetyl transferase (CAT) gene in the pCAT-3E plasmid (Chen et al., 1993). These fragments span from the last third of exon 6 including exon 7 and all of the 3' UTR (276 bp) and include about 50 bp of the poly A tail and 108 bp from the plasmid multi-cloning site. The pCAT-3E plasmid containing the CAT gene alone was used as a control named $pCAT_0$ for this study. The recombinant plasmids were transferred along with the hygromycin resistance plasmid pY3 (Blochlinger and Diggelman, 1984) into the CLB-sensitive NIH-3T3 cells and the CLB-resistant N50-4 cells by

the calcium phosphate technique (Chen and Okayama, 1987). Hygromycin resistant clones containing the CAT plasmids were identified by the CAT assay (Sambrook et al., 1989). The stabilities of the CAT RNAs containing either Ya_1 or Ya_2 GST 3' end regions or no insert were analyzed by RNA decay assays after actinomycin D treatment as described previously.

RESULTS

Amplification of GST α-class genes in the CLB-resistant HUCLB cell line. Our previous study showed no evidence of GST gene amplification in the CLB-resistant N50-4 cell line (Yang et al., 1992). Since amplification of the GST Ya gene was shown in other CLB-resistant cells (Lewis et al., 1988), a cell line was established to examine the effect of gene amplification on CLB resistance. According to the study of Johnston et al., transient inhibition of DNA synthesis can selectively induce amplification of a functional gene (Johnston et al., 1986). The N50-4 cells were treated with hydroxyurea, followed by CLB selection, and the HUCLB cell line was then cloned. The HUCLB cell line showed 13.3-fold reistance to CLB with an LD_{90} of 170 μM and the N50-4 cell line showed 10.8 fold reisitance with an LD_{90} of 140 μM, compared to parental NIH-3T3 cells with an LD_{90} of 13 μM (Figure 1, Table 2). The CLB-resistant cells contained elevated levels of intracellular GSH content and GST enzyme activity. The GSH content in the HUCLB cell line was not increased significantly compared to the N50-4 cell line, being 59.9 ±7.8 and 57.9 ±5.3 nmol/mg of protein respectively, which are 10 fold greater than NIH-3T3 cells (Yang et al., 1992). The GST enzyme activity in the HUCLB cells was 162.3 ±5.4 nmol/min·mg of protein versus 108.6 ±2.1 nmol/min·mg in the N50-4 cells and was increased by 5-fold compared to the NIH-3T3 cell line (Table 2).

We next determined if the increase in GST activity in the HUCLB line was due to amplification of any members of the GST gene

family by Southern blotting annalysis. Amplification of the GST Ya α-class genes (Figure 2 A), amplification and possible rearrangement and mutation of the GST Yc α-class gene (Figure 2 B) were shown in the CLB-resistant HUCLB cell line, with approximately a 6- and a 3-fold increases in copy number for the GST Ya and Yc genes, as estimated by densitometry. Hybridization with the GSH peroxidase cDNA to the same blot was used as a loading control (Figure 2 C). No evidence of GST μ- and π-class gene amplification was found (data not shown).

In the mouse two closely related GST Ya genes have been cloned which show 93% nucleic acid sequence homology (Daniel et al., 1987, Pearson et al., 1988). We have named the GST gene cloned by Daniel et al., 1987, Ya_1 (Genbank ascension # M19251-6, M17336) and the gene cloned by Pearson et al., 1988, Ya_2 (acension # J03958). To distinguish between these two genes we designed GST Ya_1 and Ya_2 gene specific oligonucleotide probes (Table 1) for in gel analysis of genomic DNA. Hybridization to DNA in dried agarose gels was chosen since oligonucleotide probes produce a stronger signal in gels compared to hybridization on membranes (Wallace and Miyada, 1987). Plasmid DNAs containing GST Ya_1 and GST Ya_2 genomic sequences were included as positive controls. These results show a specific 2-fold amplification of the GST Ya_2 gene, but not the GST Ya_1 gene, in the HUCLB cell line (Figure 3).

Transcriptional activity of GST α-class genes in CLB-resistant cell lines.

Northern blot analysis showed increased steady-state levels of the GST Ya and Yc α-class mRNAs for both N50-4 and HUCLB cell lines

(Figure 4). No significant difference was detected in the mRNA levels of GST μ-(Yb) and π-(Yp) class genes between the CLB-sensitive and -resistant cell lines (Figure 4). Northern blot analysis was also carried out using GST Ya_1 and Ya_2 specific oligonucleotides probes to quantitate the steady-state levels of the GST Ya mRNAs in the CLB-resistant cell lines. In vitro transcribed RNAs from plasmids containing the GST Ya_1 and Ya_2 fragments were included to show the specificity of the oligonucleotide probes. GST Ya_1 or Ya_2 mRNAs were not detected in NIH-3T3 cells whereas equal amounts of the GST Ya_1 mRNA were detected in both CLB-resistant cell lines (Figure 5, upper panel). A 3-fold increase in the steady-state level of GST Ya_2 mRNA was observed in the HUCLB cells, compared with the N50-4 cells, consistant with amplification of this gene in the HUCLB cells (Figure 5, lower panel).

The increase in steady-state levels of the GST α-class mRNAs in both CLB-resistant cell lines compared to NIH-3T3 cells suggested altered regulation of the GST α-class gene expression. Since transcriptional activation is a very important mechanism for regulating mRNA levels (Greenberg and Ziff, 1984; Vickers et al., 1989), we examined the transcriptional activity of the GST Ya genes in CLB-sensitive and -resistant cells by the nuclear "run-on" assay (Greenberg and Ziff, 1984). When [α-^{32}P] CTP labelled nascent RNAs isolated from the three cell lines were hybridized to nylon membranes containing cDNA of the GST genes, a one-fold increase in transcriptional activity of the GST Ya and Yc α-class genes was detected for both CLB-resistant cell lines, compared with the

parental NIH-3T3 cells. No significant difference in transcription of the GST μ-class (Yb) and π-class (Yp) genes was found between the CLB-sensitive and -resistant cell lines (Figure 6).

Recent studies indicated that transcription of anti-sense mRNA can be detected in a number of cells (Armstrong and Krystal, 1992). To examine the possibility that anti-sense GST Ya mRNA is transcribed, we first obstained the sense and anti-sense genomic DNA probes for the nuclear "run-on" assay. The GST Ya genomic DNA fragment was amplified by PCR and was directionally cloned into either pBSK$^+$ or pBSK$^-$ plasmids which contains an F1 origin of replication to obtain opposite orientation. Single-stranded DNA fragment was obtained by M13 virus infection, as described in Methods and Materials. The orientation of the cloned DNA fragments was confirmed by using upper- or lower-strand specific primers to direct the second-strand DNA synthesis in the presence of [α-^{32}P] dCTP (Data not shown). Nuclear "run-on" assays using single-stranded DNA showed that the anti-sense GST Ya RNA was transcribed in all three cell lines. In contrast, the sense-strand GST Ya RNA was detected only in the CLB-resistant N50-4 and HUCLB cell lines (Figure 7). This assay does not discriminate between transcription of GST Ya_1 and Ya_2 genes but both genes are transcribed in the resistant cells (Figure 7). Thus, the CLB resistant cell lines showed increased transcription of the sense GST Ya mRNAs compared with the wild-type NIH-3T3 cell lines.

mRNA stability of GST Ya genes in CLB-resistant cell lines.

Since mRNA stability also plays an important role in regulating gene expression (Carter and Malter, 1991), we examined

mRNA stability of the GST Ya mRNAs in the CLB-resistant cells. We measured the half-life of the GST Ya mRNAs in these cell lines, by blocking mRNA synthesis with actinomycin D (5 μg/ml) and following the decay of the mRNA by Northern blot analysis. Graphs of densitometer tracings of Northern blots, summarizing three separate experiments, are shown in Figure 8. The half-life of the GST Ya mRNA was about 5 hr for the N50-4 cell line and 10 hr for the HUCLB cell line. The biphasic nature of the decay curves at early time points is probably due to reciprocity failure of the X-ray film. Because of the low level of the GST Ya mRNA in the NIH-3T3 cells, it was not possible to measure the GST Ya mRNA half-life by Northern analysis. A quantitative RT-PCR was performed to amplify the gene transcripts after actinomycin D treatment and then subjecting the products to hybridization analysis. The half-life of the GST Ya mRNA was estimated to be approximately 50 min for the NIH-3T3 cells, and approximately 4 hr for the N50-4 cells (Figure 9), These results are comparable but not identical to the values obtained by Nothern analysis in Figure 8. Thus, the half-life of the GST Ya mRNA was greatly increased in the CLB-resistant N50-4 and HUCLB cells, compared to the CLB-sensitive NIH-3T3 cells.

Sequencing of four independent RT-PCR products from the previous experiments, we detected only GST Ya_1 mRNA in NIH-3T3 cells (data not shown). We wanted to find out whether the GST Ya_2 gene was also transcribed in NIH-3T3 cells. To detect the GST Ya_2 mRNA in NIH-3T3 cells a ligase chain reaction (LCR) assay was used (Kalin et al., 1992; Barany, 1991). Two pairs of oligonucleotide primers (Table 1) that perfectly pair with the GST Ya_2 nucleotide

sequence were chosen. These oligos will be ligated to each other at their junction by the thermostable DNA ligase only when hybridized to the GST Ya_2 sequence. However, the mismatch of the GST Ya_1 sequence at the junction of the oligos will prevent their ligation by DNA ligase (Kalin et al., 1992; Barany, 1991). The LCR assay showed positive bands of ligated oligos using cDNAs as templates from the two CLB-resistant cell lines, but not from the CLB-sensitive NIH-3T3 cells (Figure 10). This shows that the GST Ya_2 mRNA was not present or below the level of detection for this assay in the CLB-sensitive NIH-3T3 cells.

Differential effect of the GST Ya_1 and Ya_2 3' sequences on mRNA stability.

Since only the GST Ya_1 gene was expressed in NIH-3T3 cells and the message stability was short compared to the reisistant cell lines, this difference in message stability may be due to the expression of the GST Ya_2 gene in the resistant cells. Previous studies have suggested that sequences from the 3' untranslated region (UTR) of a gene are important for conferring mRNA stability (You et al.,1992; Bernstein et al.,1989; Swartwout et al., 1987; Jones and Cole, 1987, Atwater et al., 1990). More recent data has suggested that sequence elements thoughout the message may also influence stability (for review see Sachs, 1993). Therefore, the sequence differences between the GST Ya_1 and Ya_2 genes may be responsible for the dissimilarity in GST Ya message stability between the cell lines. To test this hypothesis, we chose the CAT (chloramphenicol acetyl transferase) as a reporter gene for this analysis and cloned the 3' ends of the GST Ya_1 and Ya_2 cDNAs

including the 3' UTR sequences into the pCAT-3E plasmid as described in the materials and methods. The 3' GST Ya_1 and Ya_2 fragments are 97% identical at the nucleotide level with most differences being in the 3' UTR (Figure 11). The pCAT-3E plasmid based on the pECE vector (Ellis et al., 1986) contains the SV40 early promoter, the CAT gene and removal of the SV40 poly A site, named $pCAT_0$ for this study, was used as a control. Plasmids were transferred into the NIH-3T3 or the N50-4 cell lines and two clones expressing each CAT gene plasmid were selected for analysis. After actinomycin D treatment, the decay of the CAT mRNA in the transformed cell lines was determined by Northern blot analysis and quantitated by densitomtry. The CAT mRNA had a half-life longer than 6 hr in both NIH-3T3 and N50-4 cells when expressed from the $pCAT_0$ plasmid (Figures 12 and 13). A slight decrease in CAT mRNA stability was seen in cell lines transformed with the $pCAT_2$ plasmid compared to the $pCAT_0$ plasmid (Figures 12 and 13). A significant decrease in CAT mRNA stability was observed in both NIH-3T3 and N50-4 cells when transformed with the $pCAT_1$ plasmid DNA, compared to the cell lines transformed with either $pCAT_2$ or $pCAT_0$ plasmid. (Figures 12 and 13). However, when the CAT mRNA stability in NIH-3T3 cells was compared to N50-4 cells, the mRNA half-life of both $pCAT_1$ and $pCAT_2$ genes was longer in the N50-4 cells. The CAT mRNA half-life of $pCAT_0$ gene was the same when transformed into either NIH-3T3 cells or N50-4 cells (Figures 12 and 13).

DISCUSSION

Since GST was first discovered in 1961 (Booth et al., 1961; Coombs and Stakelum, 1961), multiple forms of GSTs in various organs and different species have been identified and characterized (Mannervik, 1985; VanderJagt et al., 1985; Boyer, 1989). The number of genes encoding for GST isozymes is still growing (Boyer, 1989; Ahmad et al., 1993). The emphasis of current research is to figure out the relationship between structure and function of the different GST isozymes. Also, to understand the regulation of gene expression for each GST subunit in terms of function in different tissues (Batist et al., 1991; Telakowski-Hopkins et al., 1988; Pickett, 1989).

To date three alpha-class GST subunit genes have been identified in the mouse, Ya_1 (Daniel et al., 1987), Ya_2 (Pearson et al., 1988) and Yc or Ya_3 (Hayes et al., 1992; Buetler and Eaton, 1992). The Ya_1 and Ya_2 subunits are very similar with only nine amino acids being different. Also, the Ya_1 subunit is one amino acid longer than the Ya_2 subunit. In mouse liver, three forms of alpha-class GSTs are expressed (McLellan et al., 1991). One form is a homoduplex of Ya_1 subunits, whereas the second is a possible heteroduplex of Ya_1 and Ya_2 subunits and the third is a homoduplex of Yc subunits (McLellan et al., 1991). The Ya subunits are expressed at a low constitutive level in mouse liver (Pearson et al., 1988; Mclellan et al., 1991; Buetler and Eaton, 1992) but, at higher levels in kidney and intestine (Pearson et al., 1988; Buetler and Eaton, 1992). The Yc subunit is expressed at a high

constitutive level in liver cells (Mclellan et al., 1991; Buetler and Eaton, 1992).

Many factors control or regulate transcription of the GST alpha-class genes (Daniel et al., 1989; Friling et al., 1990; Rushmore et al., 1990). Induction by xenobiotics or changes in development can alter expression of the GST genes (Tee et al., 1992; Pickett et al., 1984; Ding and Pickett, 1985). Recent studies have identified the promoter regions and transcription factors involved in basal control and xenobiotic induction of the GST Ya gene (Daniel et al., 1989; Manoharan et al., 1987; Rushmore et al., 1990). We found altered expression of all three alpha-class GST genes in the N50-4 cells. One possible explanation is that a common transcription factor may be activated for all three genes in this cell line. A similar activation of transcription without amplification has also been seen for the mdr3 multidrug resistance gene in a resistant lymphoid cell line (Lepage et al., 1993). We also found constitutive transcription of the anti-sense strand of the GST Ya genes in all three cell lines in nuclear "run on" assays. This may be due to the presence of a cyrptic promoter on the antisense strand, possibly within the intron, or non specific hybridization to other RNAs when using this fragment as a hybridization target. These results emphasize the importance of using single stranded probes when doing these assays.

In the CLB resistant cell lines, a significant increase in GST Ya mRNA stability was observed. Our data suggests that this is partially due to the expression of the GST Ya_2 gene with its inherently stable mRNA compared to the Ya_1 gene. This is supported

by the findings of specific amplification of the Ya_2 subunit gene and increased steady-state levels of the GST Ya_2 mRNA in the HUCLB cells, compared to the N50-4 cells. Our results also show that mRNA destabilizing elements exist at the 3' end of the Ya_1 mRNA. We also observed that the CAT-Ya_1 hybrid mRNA half life was greater than the endogenous GST Ya_1 gene in NIH-3T3 cells (~2 hrs vs. ~1 hr). This suggests that other sequences besides the 3' end of the GST Ya_1 mRNA are also important for message stability. Future work will concentrate on more precisely defining these destabilizing sequences.

It was found that the mRNA half-life of the hybrid CAT-3' Ya genes was greater in the resistant N50-4 cells compared to the NIH-3T3 cells. We also found that we could not amplify GST Ya_1 mRNA from NIH-3T3 cells the using the oligo dT primer but, only with internal GST primers, in contrast to mRNA from the N50-4 cell line. These results suggest that the GST Ya_1 poly A tail is much shorter in NIH-3T3 cells. An initial rate limiting step in mRNA degradation is removal of the poly A tail (Sachs 1993, for review). These results may be due to an alteration in expression of proteins involved in mRNA turnover in the resistant cells. Another possibility is that the increase in stable GST Ya_2 mRNA in the resistant cells may sequester RNA binding proteins involved in mRNA turnover that then prevents other mRNAs being degraded.

It is not known if the various alpha-class GSTs have different affinities toward CLB. Our finding of specific amplification of the Ya_2 and Yc GST genes in the resistant HUCLB cell line would support such a proposal. Alternatively the increased half-life of

the GST Ya_2 mRNA compared to Ya_1 may provide a selective advantage to a cell by amplifying a GST gene with stable mRNA that in turn results in increased GST activity. Studies in the rat looking at induction of GST mRNA by phenobarbital have shown an increase in GST mRNA stability with longer term exposure to phenobarbital (Vandenberghe et al., 1991). This may be due to increased transcription of a GST gene with a stable mRNA. There are at least six GST Ya genes identified in the rat (Hayes et al., 1991; Pearson et al., 1988), and four GST Ya genes have been identified in humans (Ahmad et al., 1993). It is not know at this time which of the rat or human GST genes are equivalent to the mouse GST Ya_2 gene. The rat GST Ya 3'UTRs that have been sequenced more closely resemble the mouse GST Ya_1 3'UTR than the Ya_2.

In summary, increases in intracellular GSH content, GST enzyme activity and steady-state levels of the GST alpha-class mRNAs were shown in both CLB-resistant cell lines, N50-4 and HUCLB. The present study suggests that many factors are involved in the de-regulation of GST alpha-class gene expression in the CLB-resistant cells. These include gene amplification, increases in gene transcriptional activity and mRNA stability including both cis and possibly trans acting factors. Combined, these changes appear responsible for the increased steady-state accumulations of the GST alpha-class mRNA in the CLB-resistant cell lines.

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Figure 1: Relative colony-forming ability of NIH-3T3, N50-4 and HUCLB cells in the presence of increasing concentrations of CLB. Bars indicate standard errors from four independent determinations. On occasion, the confidence intervals were too small to be shown.

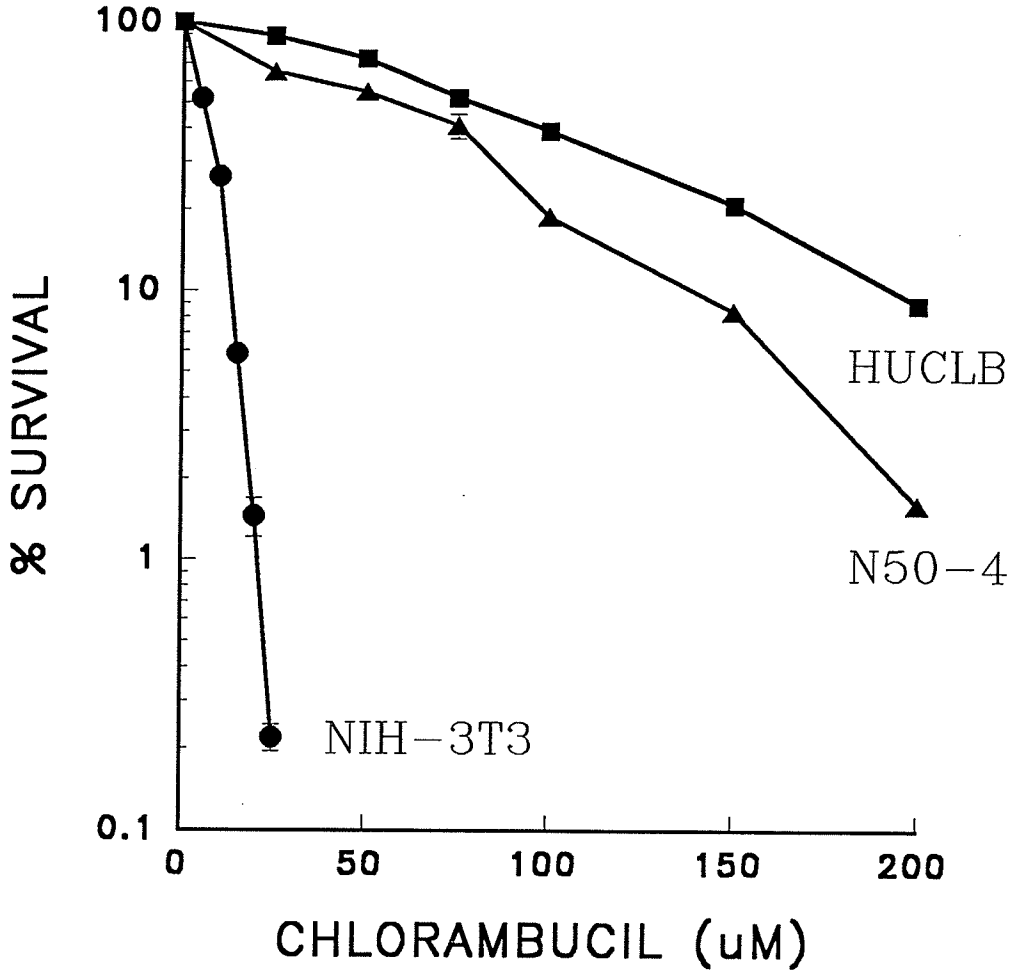
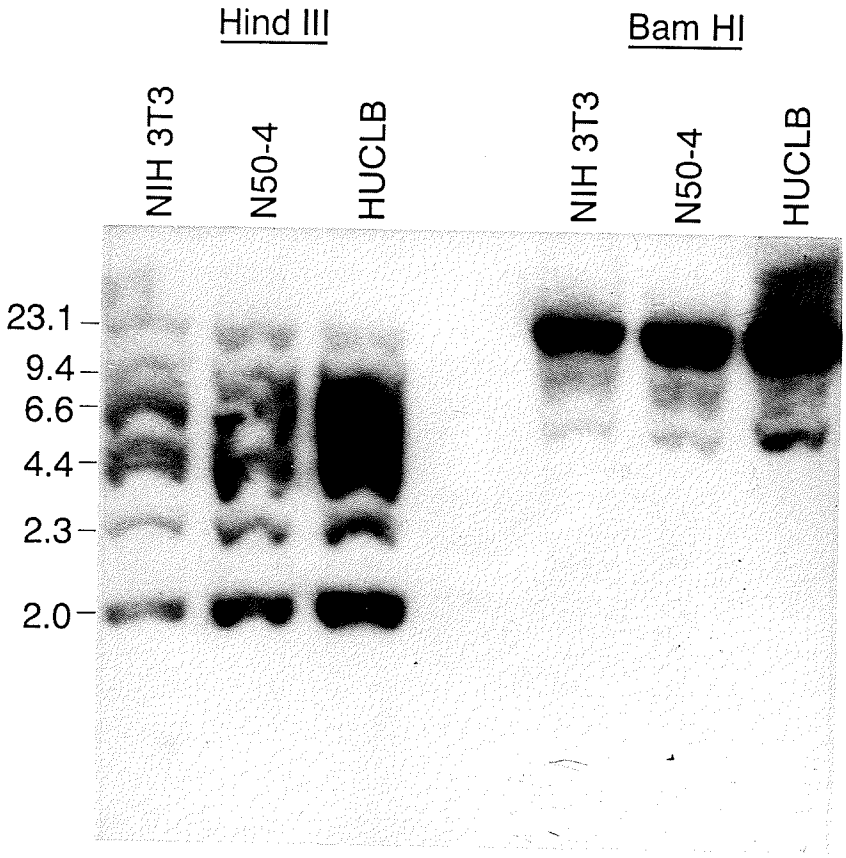
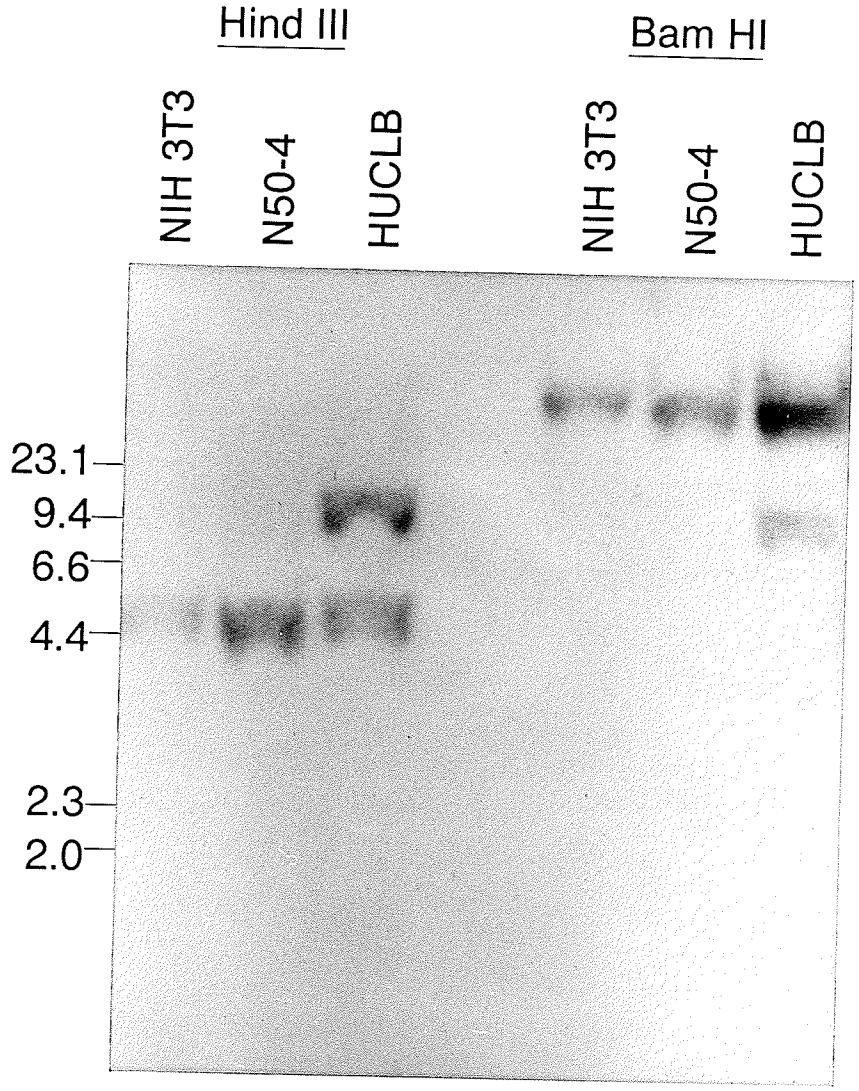

100
10
1
0.1
% SURVIVAL
0
50
100
150
200
CHLORAMBUCIL (uM)
HUCLB
N50-4
NIH-3T3

Figure 2: Southern blot analysis of the CLB-sensitive and -resistant cell lines. Genomic DNA (20 μg) was digested with the restriction enzymes HindIII or BamHI and separated in 1.0% agarose gels and blotted onto nylon filters. The filters were hybridized to the rat GST Ya (A), the rat GST Yc cDNA (B) and the mouse glutathione peroxidase (GSH Px) cDNA (C) probes as described in Materials and Method.

A


Hind III
Bam HI
NIH 3T3
N50-4
HUCLB
NIH 3T3
N50-4
HUCLB
23.1
9.4
6.6
4.4
2.3
2.0

B


Hind III
Bam HI
NIH 3T3
N50-4
HUCLB
NIH 3T3
N50-4
HUCLB
23.1
9.4
6.6
4.4
2.3
2.0

c

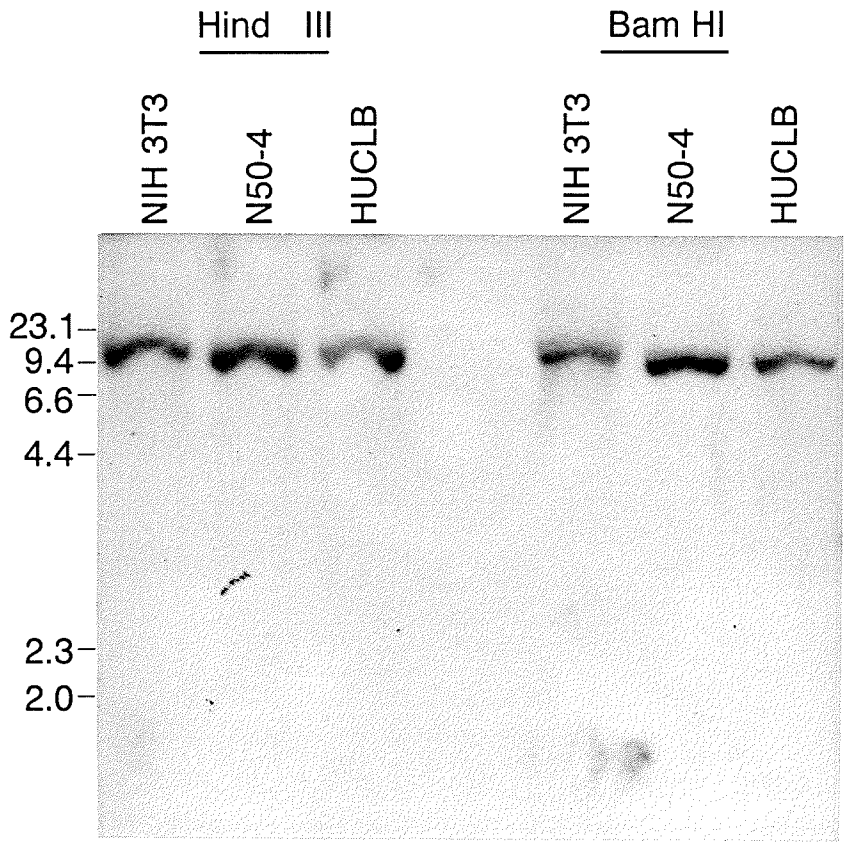

Hind III
Bam HI
NIH 3T3
N50-4
HUCLB
NIH 3T3
N50-4
HUCLB
23.1
9.4
6.6
4.4
2.3
2.0

Figure 3: In gel hybridization of the GST Ya_1 and Ya_2 genomic DNAs in NIH-3T3, N50-4 and HUCLB cells. Genomic DNA (20 μg) was digested with BamH1, separated in 1.0% agarose gel. The gel was dried and hybridized to [γ-^{32}P] ATP labelled GST Ya_1 and Ya_2 specific oligonucleotides as described in the Materials and Methods. The GST Ya_1 and Ya_2 cDNAs were also included as positive controls.

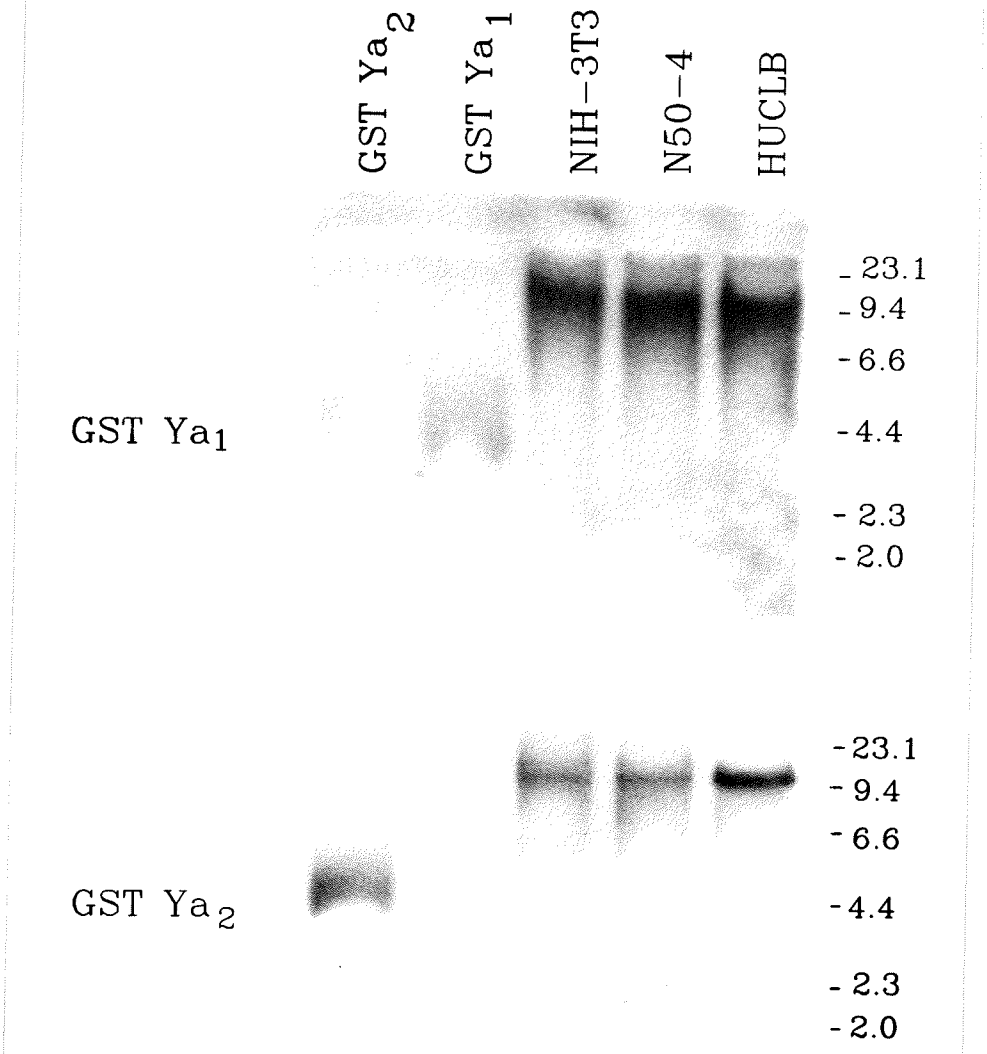

GST Ya2
GST Ya1
NIH-3T3
N50-4
HUCLB
GST Ya1
- 23.1
- 9.4
- 6.6
- 4.4
- 2.3
- 2.0
GST Ya2
- 23.1
- 9.4
- 6.6
- 4.4
- 2.3
- 2.0

Figure 4: Northern blot analysis of the CLB-sensitive and -resistant cell lines. Cytoplasmic RNA (20 μg) was separated in 1.0% agarose gels containing formaldehyde and was blotted onto nylon filters. The filters were hybridized to the GST Ya and Yc (α class), Yb (μ class) and Yp (π class) cDNA probes as described in Materials and Methods.

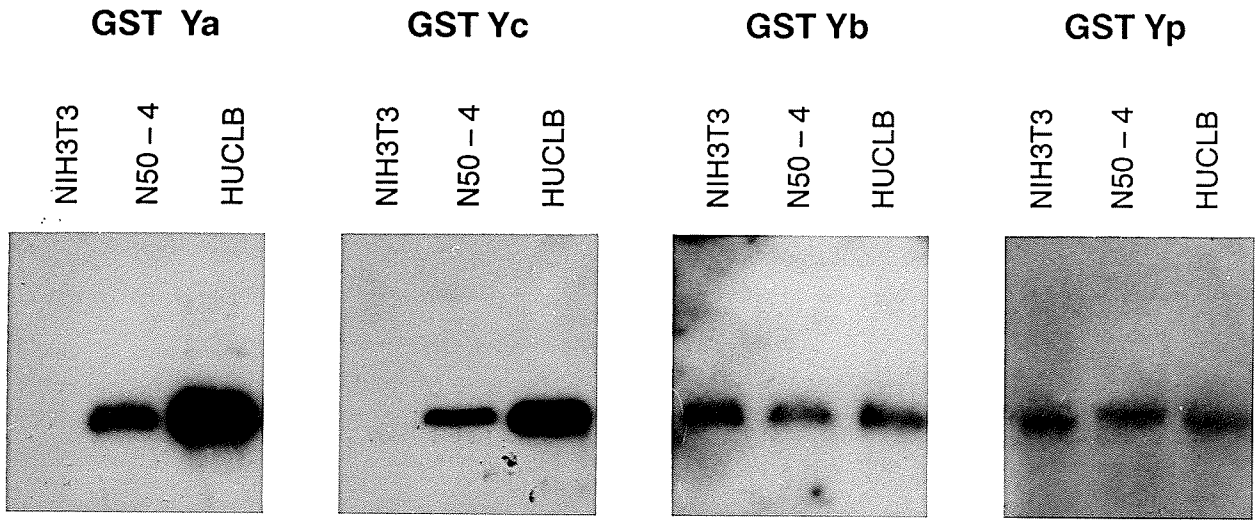

GST Ya
NIH3T3
N50 – 4
HUCLB
GST Yc
NIH3T3
N50 – 4
HUCLB
GST Yb
NIH3T3
N50 – 4
HUCLB
GST Yp
NIH3T3
N50 – 4
HUCLB

Figure 5: Northern blot analysis of the GST Ya_1 and Ya_2 mRNAs in NIH-3T3, N50-4 and HUCLB cells. The GST Ya_1 and Ya_2 specific oligo nucleotides were 5' end-labelled with [γ-^{32}P] ATP by T4 polynucleotide kinase and hybridized to nylon filters containing cytoplasmic RNAs isolated from the three cell lines. The membranes also contained GST Ya_1 and Ya_2 mRNAs, which were <u>in vitro</u> transcribed from the plasmids containing the GST Ya_1 and Ya_2 cDNAs using the T7 RNA polymerase as described in the Materials and Methods.

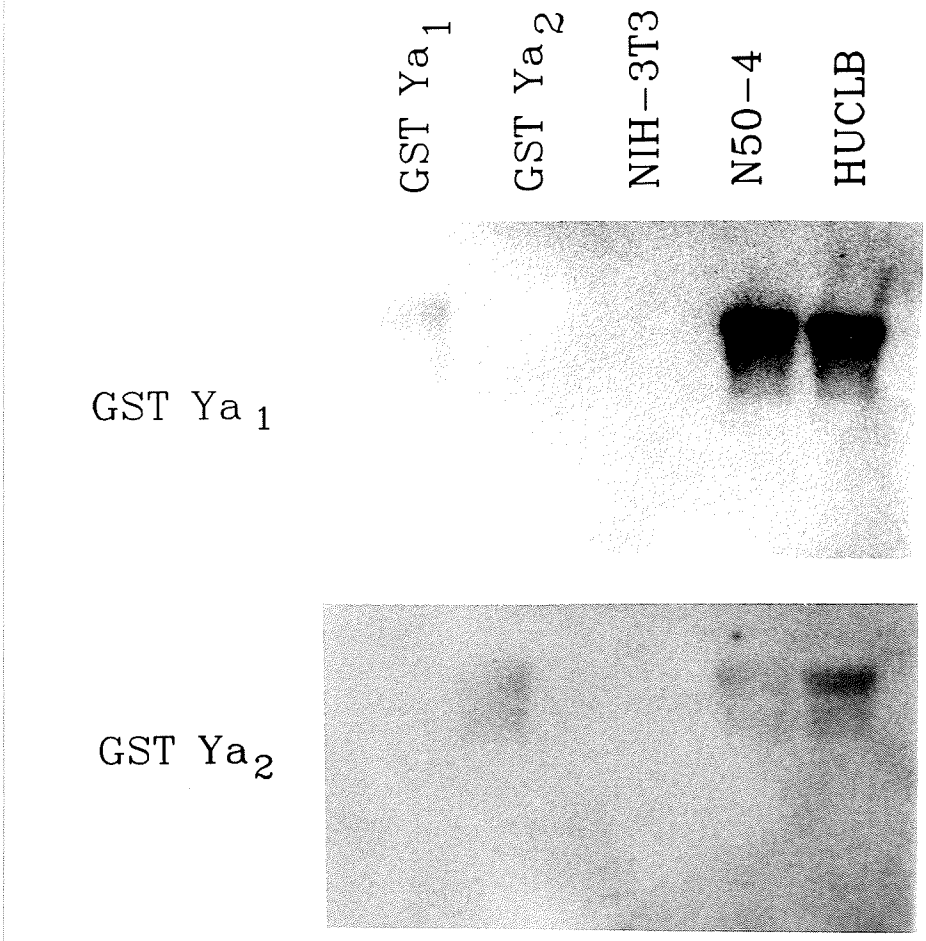
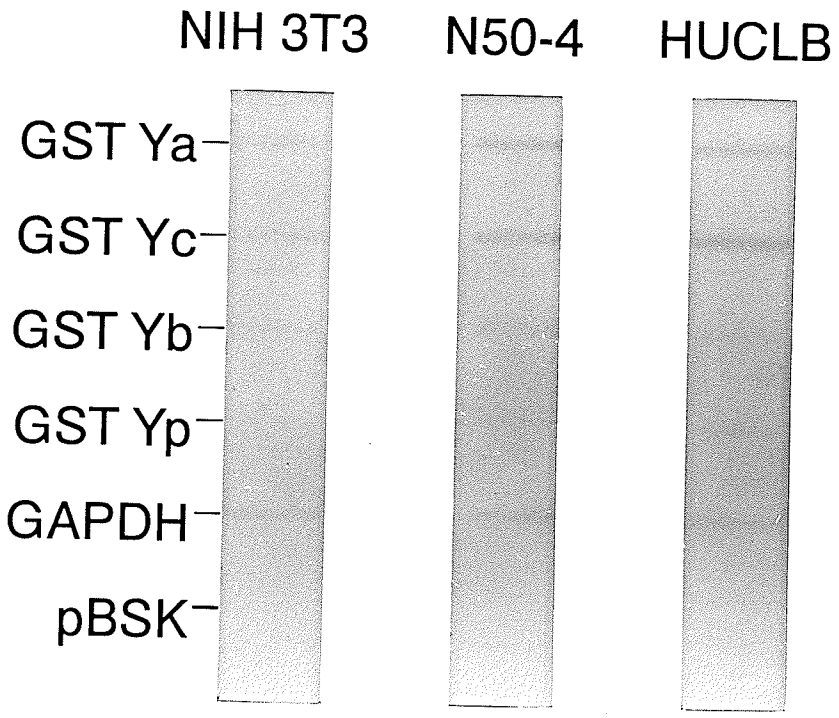

GST Ya 1
GST Ya 2
NIH-3T3
N50-4
HUCLB
GST Ya 1
GST Ya 2

Figure 6: Transcriptional activity of GST genes in CLB-sensitive and -resistant cells. [α-^{32}P] CTP labelled RNA was hybridized to nylon filters containing double-stranded GSTs Ya, Yc, Yb and Yp cDNAs as described in Materials and Methods (A). GAPDH cDNA and pBSK plasmid DNA were included as positive and negative controls (A). A histogram summarizing three separate experiments of the nuclear "run-on" assays (B). Results were expressed as percentage activities of the average value of the positive control, GADPH. *, significantly different from the values for the NIH-3T3 cells with $p < 0.001$. Bars indicate standard error (B).

A



B.

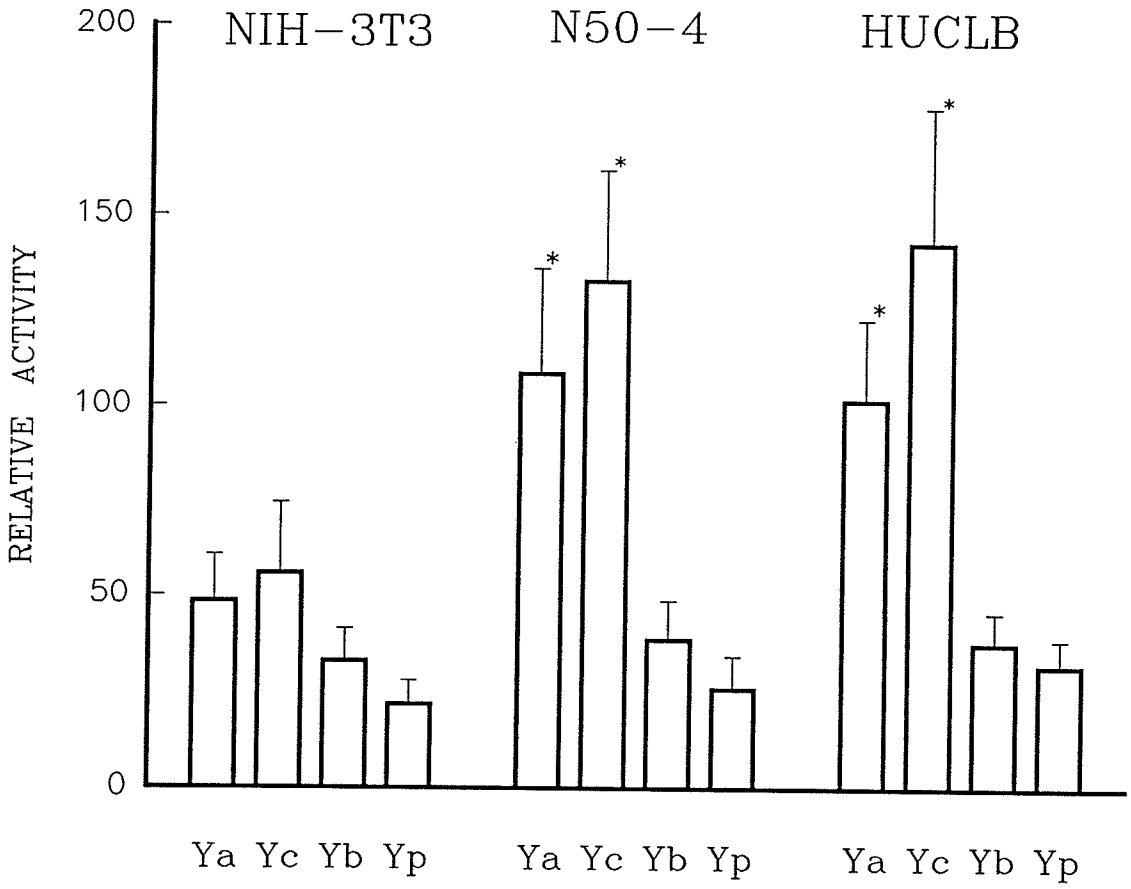
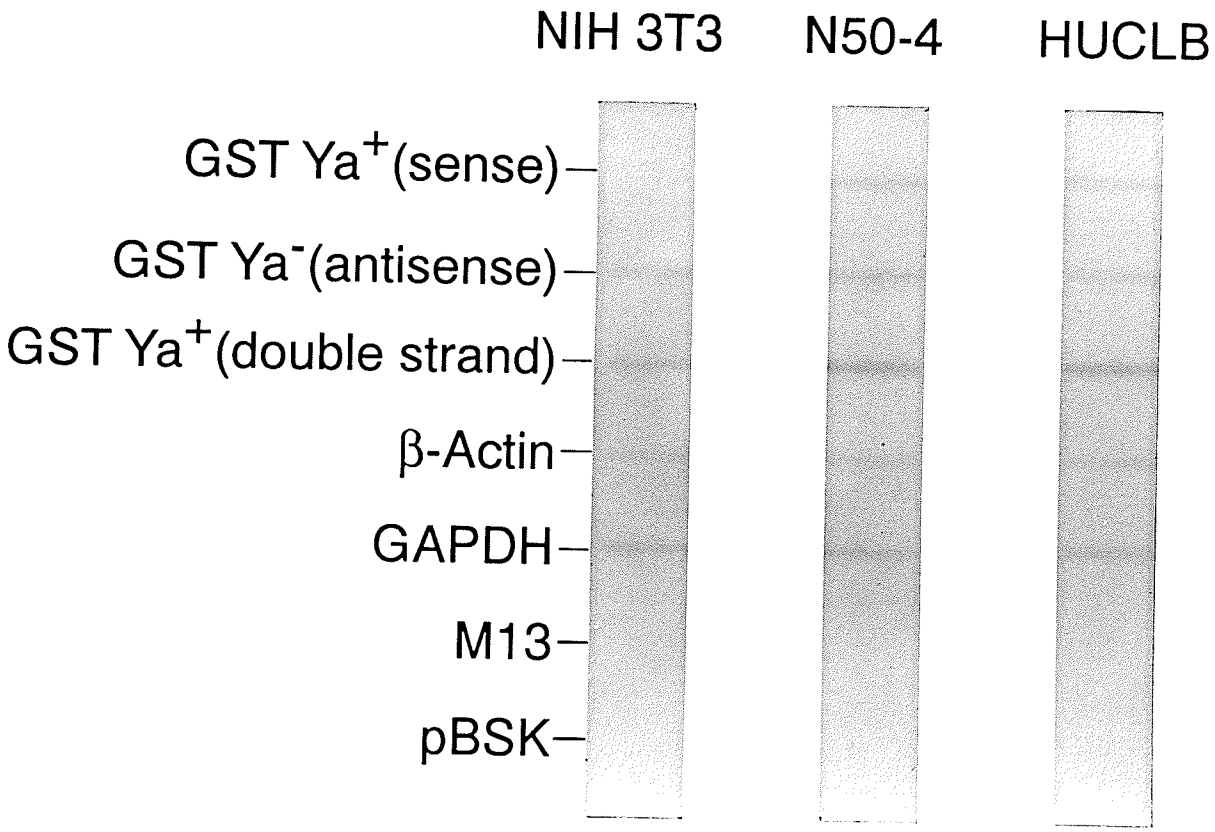

NIH−3T3
N50−4
HUCLB
RELATIVE ACTIVITY
200
150
100
50
0
*
Ya Yc Yb Yp
Ya Yc Yb Yp
Ya Yc Yb Yp

Figure 7: Transcriptional activity of GST Ya sense and antisense strands in CLB-sensitive and -resistant cells. [α-^{32}P] CTP labelled RNA was hybridized to nylon filters containing sense and antisense single-stranded, and double-stranded GST Ya genomic DNAs as described in Materials and Methods (A). β-actin (single-stranded) and GAPDH (double-stranded) cDNAs were included as positive controls. M13 (single-stranded) and pBSK (double-stranded) DNAs were included as negative controls. Sense: GST Ya DNA cloned into pBSK$^+$ vector detects sense-strand GST Ya RNA; antisense: GST Ya DNA cloned into pBSK$^-$ vector detects antisense-strand GST Ya RNA; both: GST Ya double-stranded DNA cloned into pBSK$^+$ vector detects both sense and antisense GST Ya RNAs (A). A histogram summarizing three separate experiments of the nuclear "run-on" assays (B). Results were expressed as percentage activities of the average value of the two positive controls, β-actin and GADPH. *, significantly different from the values for the NIH-3T3 cells with $p < 0.001$. Bars indicate standard error (B).

A


NIH 3T3
N50-4
HUCLB
GST Ya+(sense)
GST Ya-(antisense)
GST Ya+(double strand)
β-Actin
GAPDH
M13
pBSK

B.

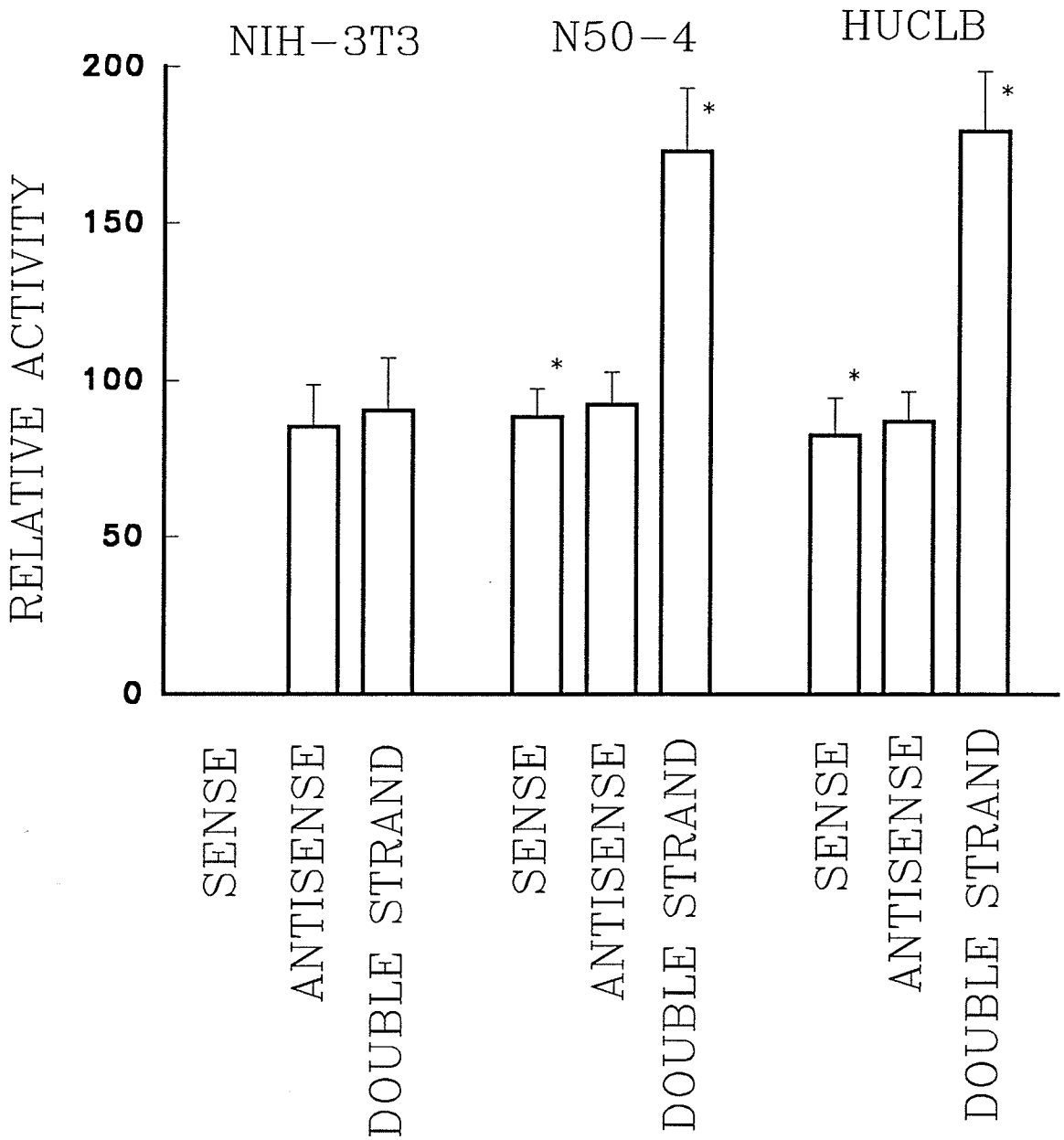
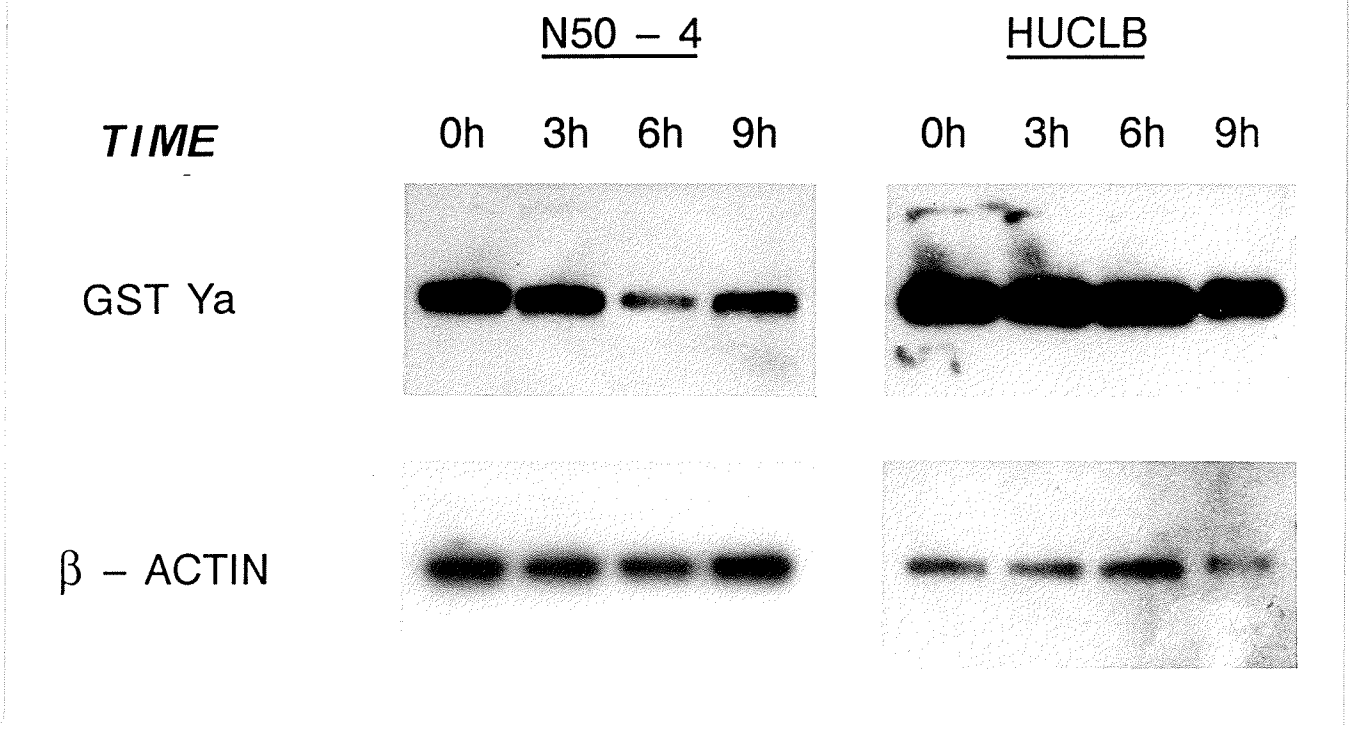

NIH-3T3
N50-4
HUCLB
RELATIVE ACTIVITY
200
150
100
50
0
*
*
*
SENSE
ANTISENSE
DOUBLE STRAND
SENSE
ANTISENSE
DOUBLE STRAND
SENSE
ANTISENSE
DOUBLE STRAND

Figure 8: GST Ya mRNA stability in N50-4 and HUCLB cells. mRNA transcription was blocked with actinomycin D (5 μg/ml) and the GST Ya mRNA turnover was determined by Northern blot analysis as described in Materials and Methods (A). A plot summarizing three separate experiments of the mRNA decay assays; N50-4 (•); HUCLB (▾) cells. Values were standardized with the signals from β-actin hybridization and were expressed as percentage of the untreated sample. Bars indicate standard error (B).

A



B.

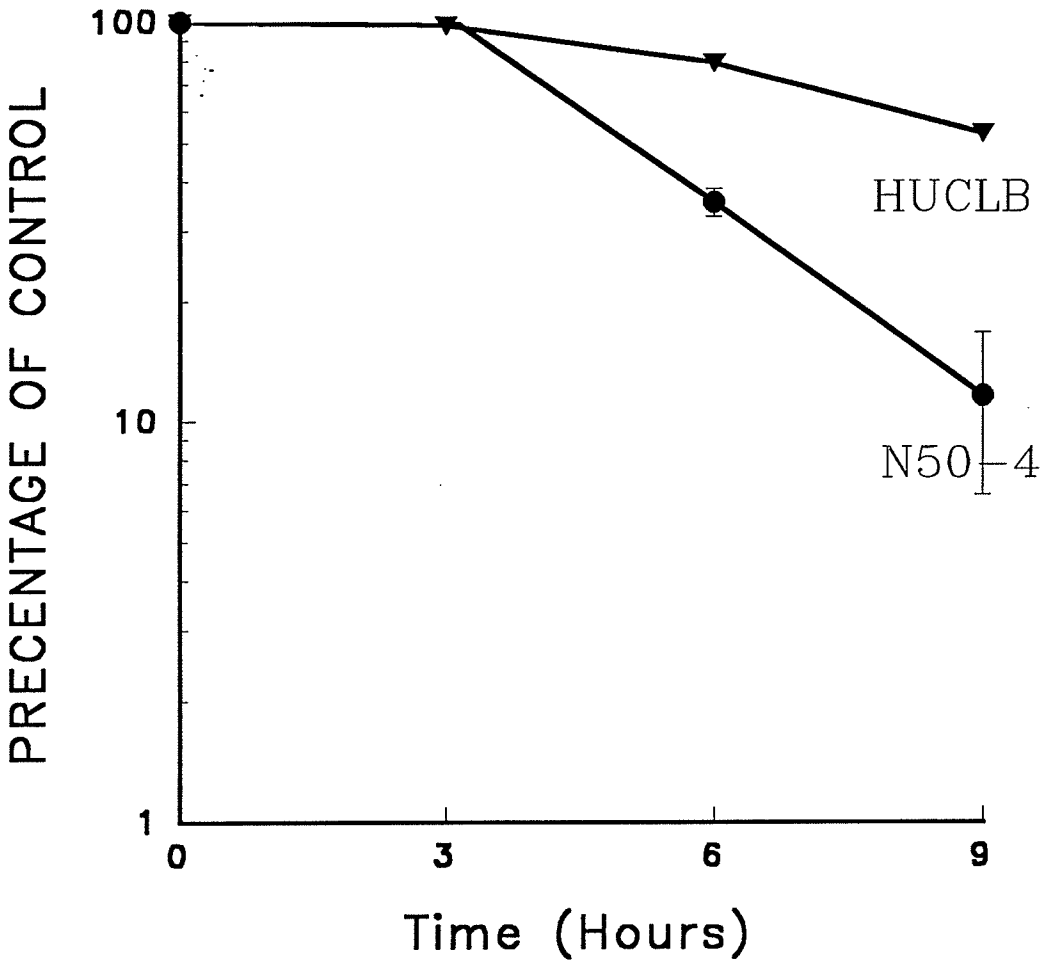
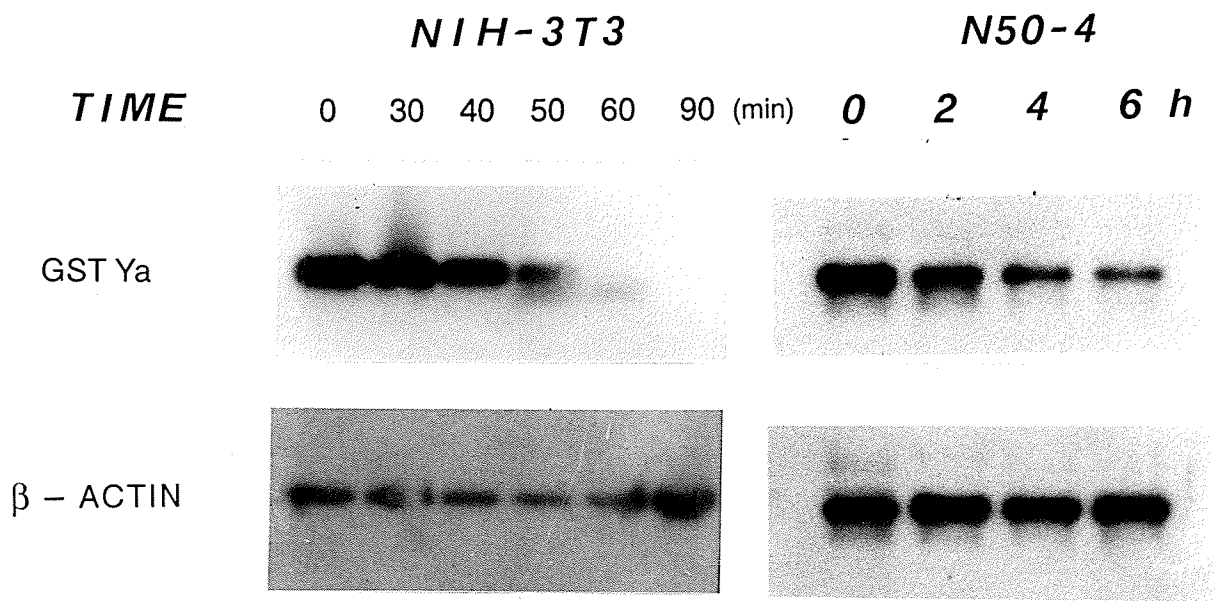


Figure 9: GST Ya mRNA stability in NIH-3T3 and N50-4 cells. mRNA transcription was blocked with actinomycin D (5 μg/ml) and the GST Ya mRNA turnover was determined by RT-PCR analysis as described in Materials and Methods (A). A plot summarizing three separate experiments of the mRNA decay assays; NIH-3T3 (o); N50-4 (•) cells. Values were standardized with the signals from β-actin hybridization and were expressed as percentage of the untreated sample. Bars indicate standard error (B).

A



B.

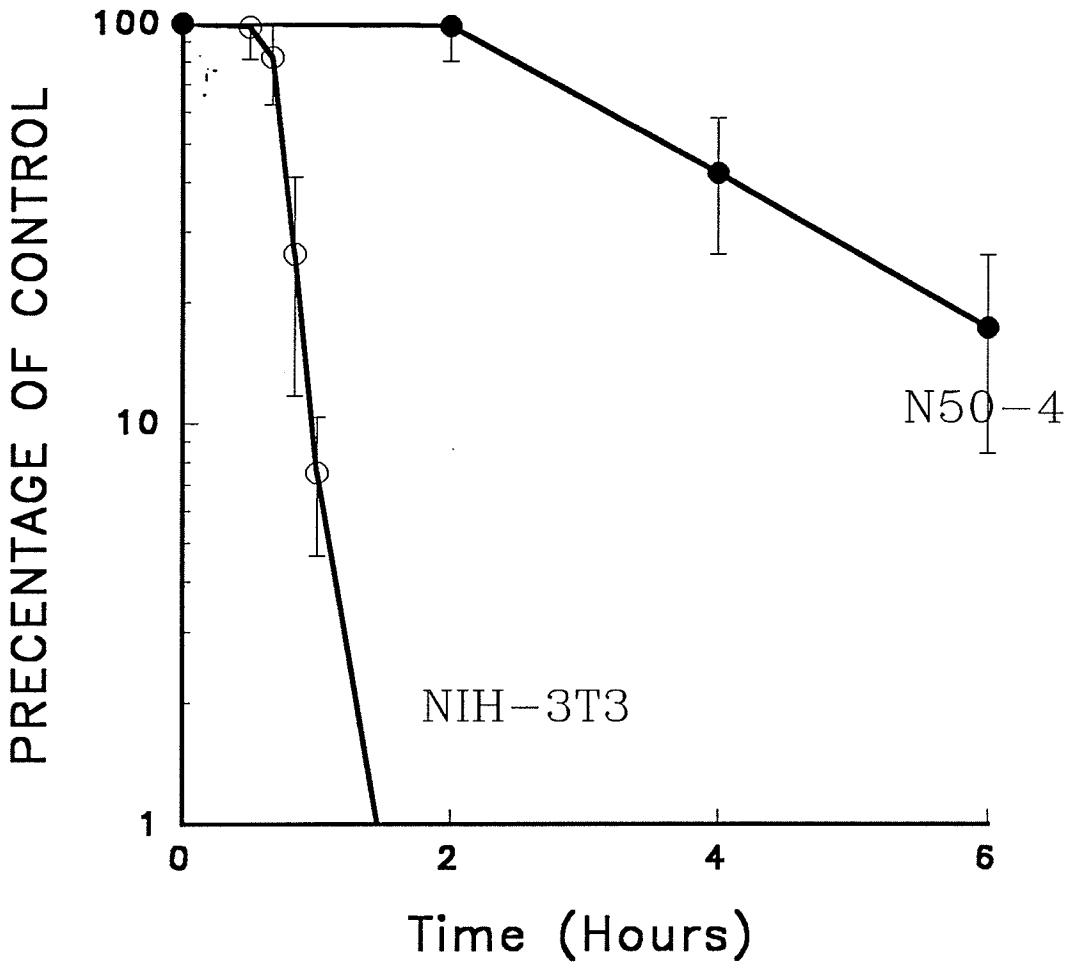


Figure 10: Detection of the GST Ya_2 mRNA by the LCR assay. 50 cycles of LCR were performed using four ^{32}P labeled oligonucleotides that perfectly match the GST Ya_2 sequence as described in the Materials and Methods. The ligated oligonucleotides were separated from the unligated nucleotides in 8% acrylamide gel containing 7 M urea and visualized by autoradiography. Lane 1: LCR with no DNA template; lanes 2 & 3: LCR with cloned GST Ya_1 genomic DNA (lane 2) and cDNA (lane 3) templates as negative controls; lanes 4 & 5: LCR with cloned GST Ya_2 cDNA templates as positive controls; lanes 6, 7 & 8: LCR with templates of RT-PCR amplified cDNAs from NIH-3T3, N50-4 and HUCLB cells, respectively. Arrows show positive bands of GST Ya_2 directed LCR product.

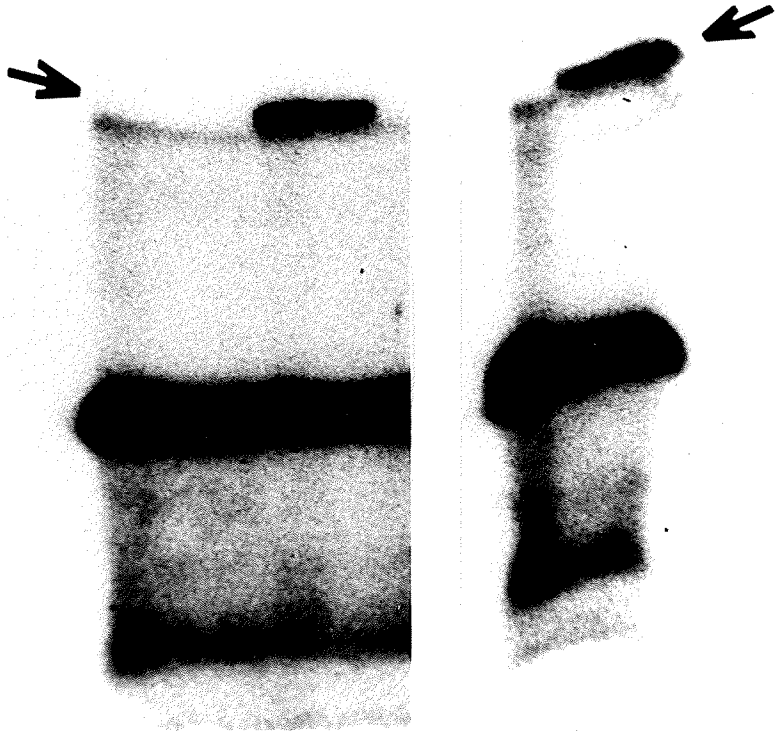
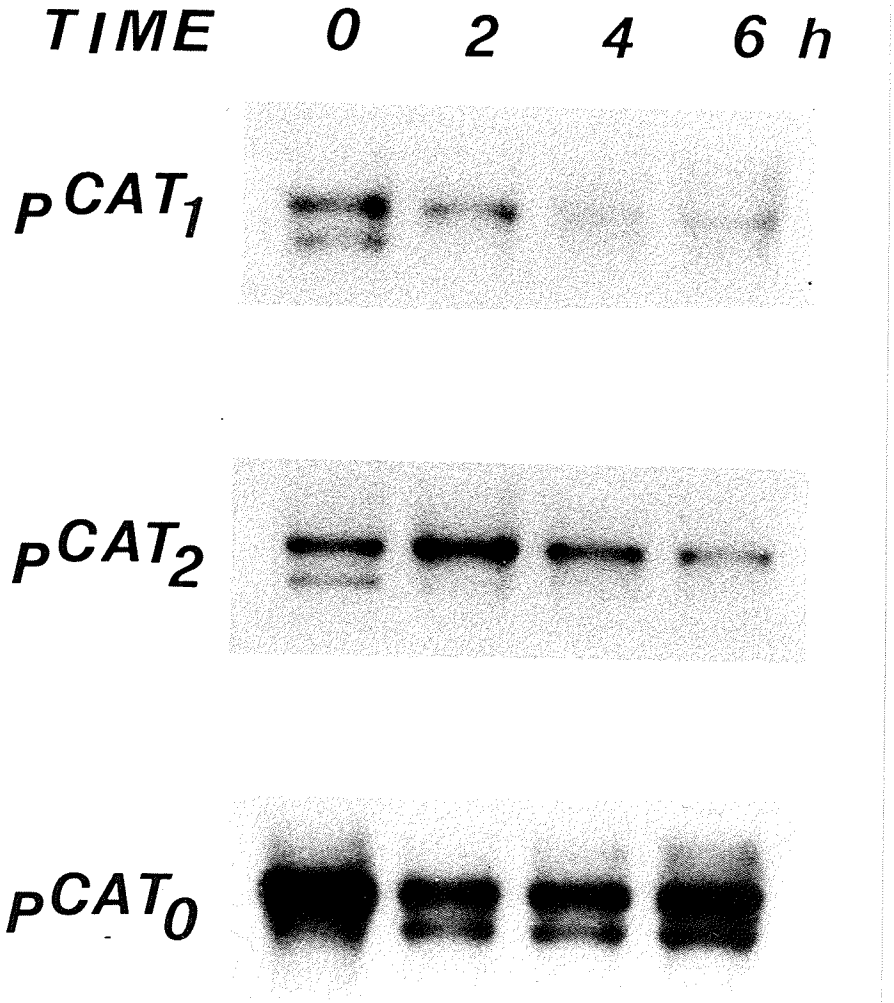

1 2 3 4 5 6 7 8

Figure 11: Comparison of the 3' sequences of the mouse GST Ya_1 and Ya_2 cDNAs. The cDNA fragments were PCR amplified from both CLB-resistant cell lines and were cloned into the pCR TA vector. Top line: GST Ya_1 sequence; bottom line: GST Ya_2 sequence. Primers used for PCR, translation termination sites and poly (A) signal sequence are underlined. *: indicates coding sequence identical to the GST Ya_1 gene, and -: indicates non-coding sequence identical to the GST Ya_1 gene.

```
     540                                                                585
Ya1: TT GAA GAG TTT GAT GCC AGC CTT CTG ACC CCT TTC CCT CTG CTG AAG
Ya2: ** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***

                                                                       636
GCC TTC AAG AGC AGA ATC AGC AGC CTC CCC AAT GTG AAG AAG TTT CTA CAG
*** *** *** *** *** *** *** *** *** *** *** *** *** **C *** ***

                                                                       687
CCT GGC AGC CAG AGA AAG CCT CCC ATG GAT GCA AAA CAA ATT CAA GAA GCA
*** *** *** *** *** *** *** *** T** *** *** *** *** *** G** *** ***

                                                                       738
AGG AAG GCT TTC AAG ATT CAG TGA AGC TGC ATT GAT GGA GCC ACA GAT ACT
*** *** *T* *** *** T** T-- --T G-- --- --- --- --- --- G-- --- ---

                                                                       789
GGC CTC TAA TGG TTT GCA ATT ATA AAA AGC AAT TGT TGA TCC TGG CTA TTT
--- --- --- --- C-- --- --- --- --- TA- --- --- --- -T- --- --- --G

                                    816
TGC AAT AAT AAA AAA TTA ACA ACT GGT. poly (A).
--- --- --- --- --- --- --- --- ---  --------.
```

Figure 12: CAT mRNA stability in stably transformed NIH-3T3 cell lines. $pCAT_1$: pECE plasmid containing the CAT gene and GST Ya_1 3' sequence; $pCAT_2$: pECE plasmid containing the CAT gene and GST Ya_2 3' sequence; $pCAT_0$: pECE plasmid containing the CAT gene without GST Ya 3' sequence. The plasmids were transferred into the NIH-3T3, and two positive transformants for each plasmid were identified by the CAT assay and cloned. Transcription of mRNA was blocked with actinomycin D (5 μg/ml) and the CAT mRNA turnover was analyzed by Northern blotting (A). A plot summarizing three separate experiments of the mRNA decay assays. Plasmids: $pCAT_1$ (•); $pCAT_2$ (▾) and $pCAT_0$ (O). Values were standardized with the signals from β-actin hybridization and were expressed as percentage of actinomycin D untreated sample. Bars indicate standard error (B).

A



B.

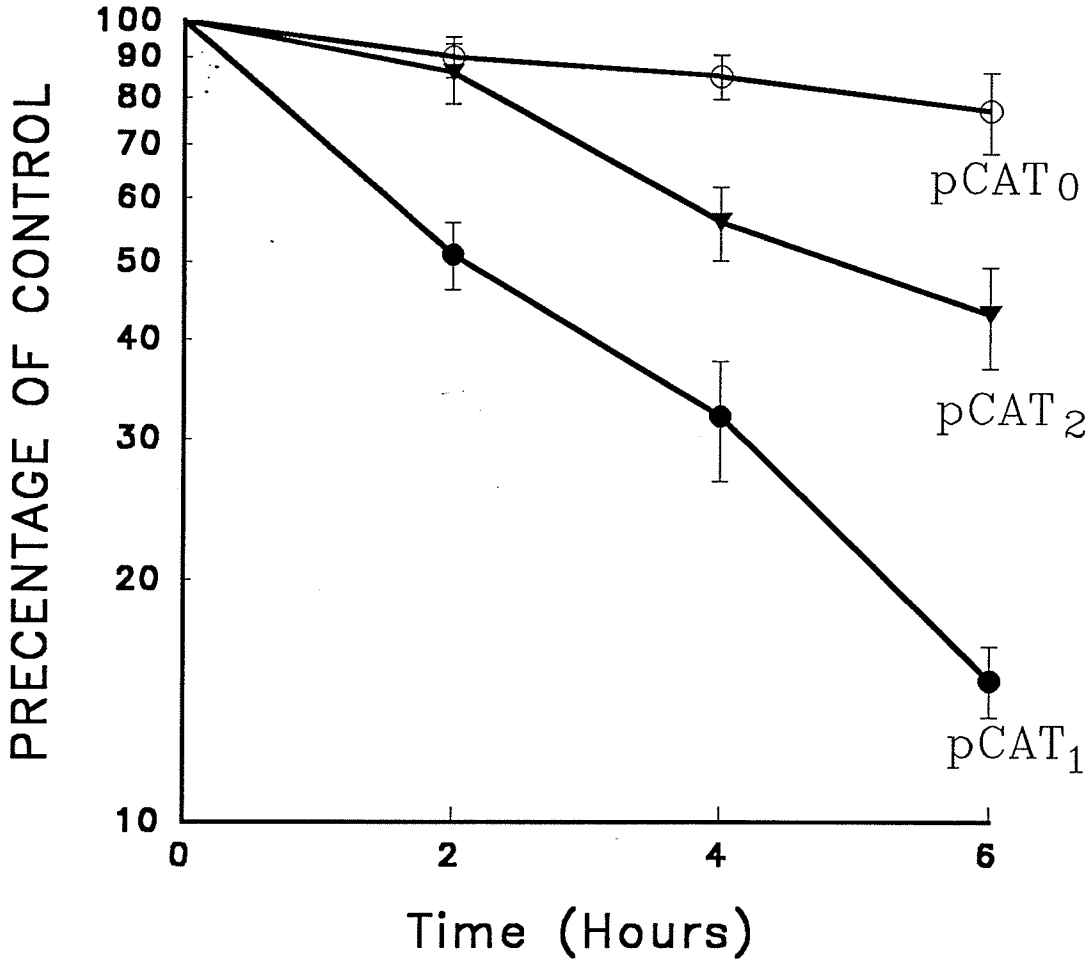
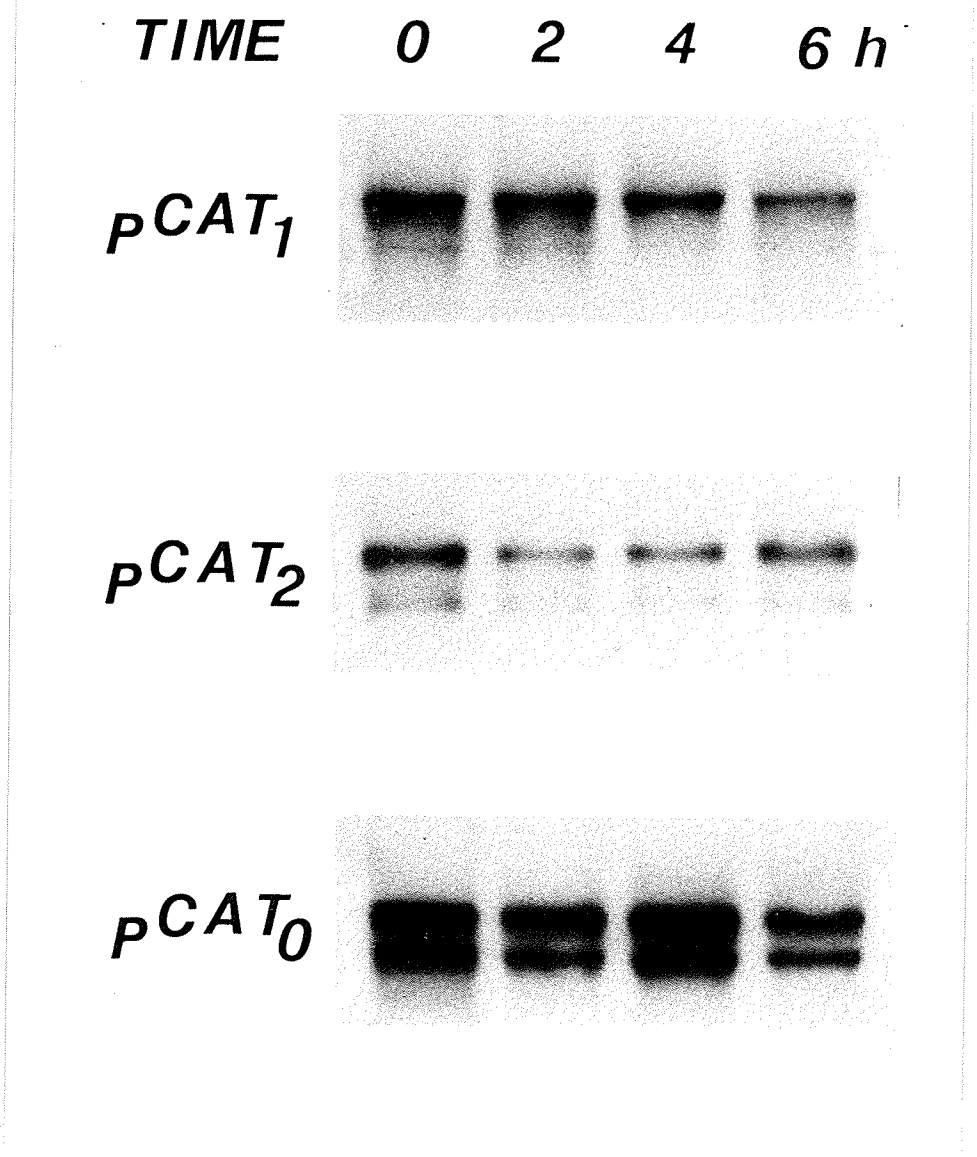

PRECENTAGE OF CONTROL
100
90
80
70
60
50
40
30
20
10
0
2
4
6
Time (Hours)
pCAT0
pCAT2
pCAT1

Figure 13: CAT mRNA stability in stably transformed N50-4 cell lines. $pCAT_1$: pECE plasmid containing the CAT gene and GST Ya_1 3' sequence; $pCAT_2$: pECE plasmid containing the CAT gene and GST Ya_2 3' sequence; $pCAT_0$: pECE plasmid containing the CAT gene without GST Ya 3' sequence. The plasmids were transferred into the N50-4 cells, and two positive transformants for each plasmid were identified by the CAT assay and cloned. Transcription of mRNA was blocked with actinomycin D (5 μg/ml) and the CAT mRNA turnover was analyzed by Northern blotting (A). A plot summarizing three separate experiments of the mRNA decay assays. Plasmids: $pCAT_1$ (•); $pCAT_2$ (▾) and $pCAT_0$ (O). Values were standardized with the signals from β-actin hybridization and were expressed as percentage of actinomycin D untreated sample. Bars indicate standard error (B).

A



B.

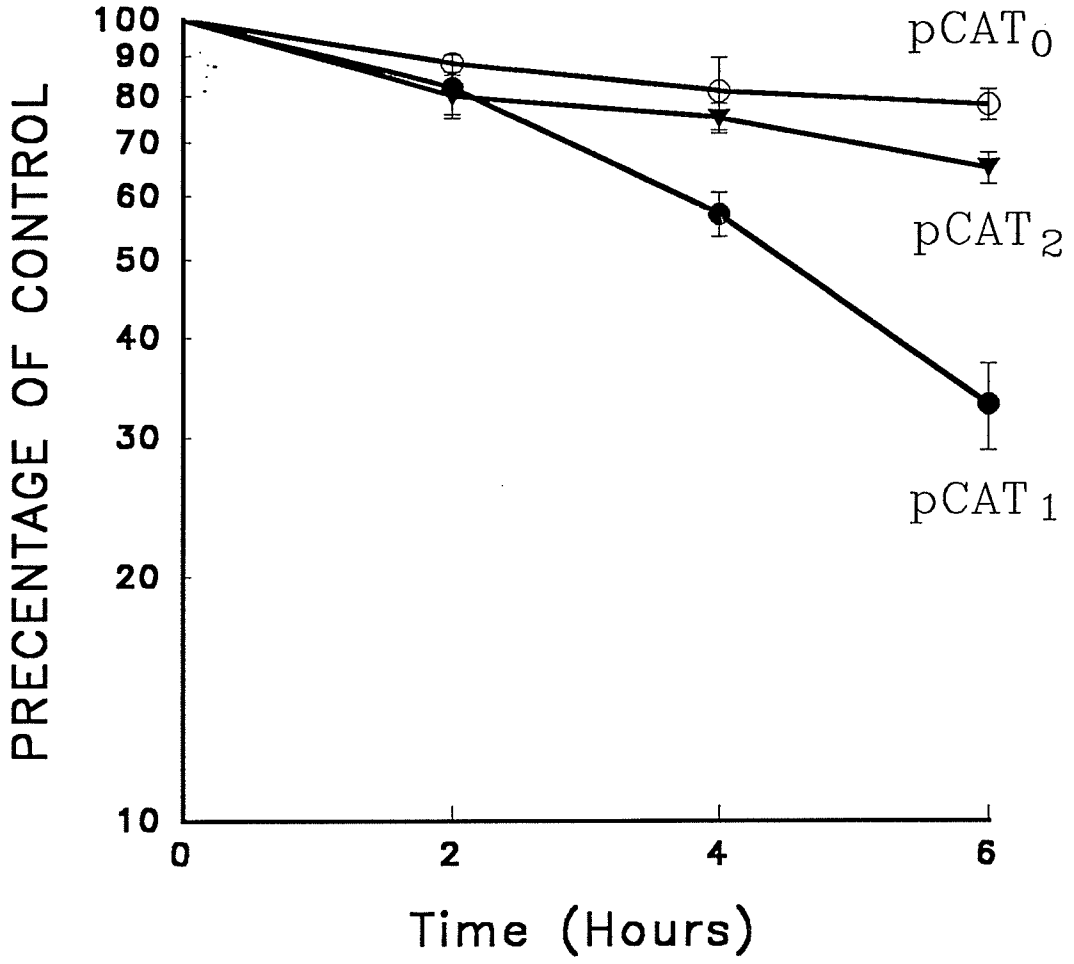

PRECENTAGE OF CONTROL
100
90
80
70
60
50
40
30
20
10
0
2
4
6
Time (Hours)
pCAT0
pCAT2
pCAT1

Table 1 Oligo nucleotide primers used for PCR or LCR.

Primer Sequence (5' to 3')	Usage
Ya.G1: CTCAAGCTTGTGTGTGTCTGTGGGAGGG; Ya.G2: CTCGAATTCGGGCATTGAAGTAGTGAAG.	To amplify a 2-kb genomic DNA of GST Ya from exon 1 to exon 2; Used for nuclear "run-on" transcription assay.
Ya 944: ATGGTGGAGATTGATGGGAT; Ya 2289: ATTGGGGAGGCTGCTGATTC.	To amplify a 410-bp cDNA fragment from mRNA; Used for mRNA decay assay by RT-PCR method.
β-Actin 262: GCCAACCGTGAAAAGATGACC; β-Actin 612: TCCAGGGAGGAAGAGGATGCG.	To amplify a 350-bp cDNA fragment from mRNA; Used for mRNA decay assay by RT-PCR as an internal control.
Ya 1952: TTGAAGAGTTTGATGCCAGCC; (T) primer. $GGGAGGCCC(T)_{18}$	To amplify a 290-bp cDNA fragment from mRNA; Used to clone the GST Ya_1 and Ya_2 3' UTR sequences.
Ya 2385 (GST Ya_2): TTTTCAAGTTTTAGTGTGGCT; Ya 2548: TTGCCTTCCTCTTTGCTATGC.	To amplify a 163-bp fragment from genomic DNA; Used to clone the GST Ya_2 3' UTR sequence.

Primer Sequence (5' to 3')	**Usage**
Ya 1952: TTGAAGAGTTTGATGCCAGCC; Ya 2459: ATAGCCAGAATCAACAATTGT.	To amplify a 260-bp cDNA fragment from mRNA; Used for LCR to identify GST Ya_2 sequence.
Ya_1: GCTTTCAAGATTCAGTGAAGC. Ya_2: GTTTTCAAGTTTTAGTGTGGC.	Used for hybridization of GST Ya_1 and Ya_2 sequences by Northern and Southern blot analyses.
Ya.U1: AATGGCTTGCAATTATAAAAT; Ya.U2: ACAATTGTTGATTCTGGCTAT; Ya.L1: ATAGCCAGAATCAACAATTGT; Ya.L2: ATTTTATAATTGCAAGCCATT.	Used for LCR to identify GST Ya_2 sequence.

Table 2 **Comparison of CLB sensitivity, GSH content and GST activity.**

Values are mean ± standard error of the number of determinations in parentheses.

	NIH-3T3	N50-4	HUCLB
Doubling time (hr)	17.5 ±0.1(4)	17.4 ±0.4(4)	17.8 ±0.3(4)
CLB LD_{90} (μM)[a]	13	140	172
Fold resistance		10.8	13.3
GSH content (nmol/mg of protein)	6.1 ±1.1(5)	57.9 ±5.3(6)[b]	59.2 ±7.8(4)[b]
GSH content (nmol/10^6 cells)	1.9 ±0.3(5)	14.7 ±1.7(6)[b]	15.2 ±1.8(4)[b]
GST activity (nmol/min.mg of protein)	29.8 ±1.3(4)	108.6 ±2.1(4)[b]	162.3±5.4(4)[b,c]
GST activity (nmol/min.10^6 cells)	10.2 ±1.1(4)	36.7 ±4.0(4)[b]	50.4 ±7.6(4)[b,c]

a. Concentrations of CLB that kills 90% of the cells.

b. Significantly different from the values of NIH-3T3 cells ($p < 0.001$).

c. Significantly different from the values of N50-4 cells ($p < 0.01$).

SUMMARY AND PERSPECTIVE

SUMMARY AND PERSPECTIVE

There has been a great progress in cancer chemotherapy since 1946 when nitrogen mustard was first used as a chemotherapeutic agent (Gilman and Philips, 1946). The clinical application of the chemotherapy is having a definite impact on overall cancer survival statistics (Frei, 1982; Jones et al., 1990). While these trends are encouraging, there are still many obstacles in cancer treatment. Acquired drug resistance continues to be a major problem in limiting the effectiveness of cancer chemotherapy. Despite the availability of a wide spectrum of clinically active antineoplastic agents, eventual treatment failure is usually the outcome of chemotherapy in many types of cancer. Even with the most drug-sensitive classes of tumor, a significant proportion of tumor cells may show a poor initial response and ultimately relapse in a drug-resistant state. When patients eventually die of metastatic cancer, their diseases are characterized by an extreme insensitivity to the highest tolerated doses of all of the available anticancer drugs (Goldstein et al., 1992). Evidence from a variety of sources implicates a genetic basis for most drug-resistant phenotypes. Understanding the mechanism of the drug resistance may provide new options for the treatment of cancers (Harris and Hochhauser, 1992).

In the course of my Ph.D. thesis study, I investigated the mechanisms of chlorambucil (CLB) resistance in a mouse fibroblast NIH-3T3 cell line. Previous studies showed that reduced CLB-DNA

cross-linking (Robson et al., 1987; Jiang et al., 1989; Panasci et al., 1988; Harrap and Gascoigne, 1976) and enhanced DNA repair (Harrap and Gascoigne, 1976; Panasci et al., 1988) were partially responsible for CLB resistance in Chinese Hamster Ovary (CHO) cells, Yoshida sarcoma cells and chronic lymphocytic leukemia (CLL) lymphocytes. Investigation from our group and others indicated that glutathione (GSH) and glutathione S-transferase (GST) may play an important role in CLB resistance in CLL lymphocytes (Begleiter et al., 1991; Johnston et al., 1990) and CHO cells (Robson et al., 1986; Lewis et al., 1988). These findings lead us hypothesize that GSH and GST are critical for the CLB resistance. It is possible that GSH-CLB conjugation, partially catalyzed by GST α-class isozyme (Ciaccio et al., 1990), inactivates the CLB, which leads to reduction of CLB-DNA cross-linking. From the finding on mechanisms of cisplatin resistance, it is also possible that GSH could facilitate enzymatic repair of CLB-DNA adducts (Lai et al., 1988, 1989; Ozols, 1992).

Based on these findings and the above hypothesis, I began to investigate the involvement of glutathione (GSH) and glutathione S-transferase (GST) in CLB resistance. I established a CLB resistant cell line, named N50-4, from NIH-3T3 cells by step-wise drug selection. I measured the intracellular GSH content and GST enzyme activity in the wild-type NIH-3T3 and CLB-resistant N50-4 cells by biochemical assays (Griffith, 1980; Habig et al., 1974). Significant increases in GSH content and GST enzyme activity were observed in the N50-4 cells, compared to the NIH-3T3 cells. Pharmacological studies were then carried out to demonstrate the

relative roles of the increased intracellular GSH content and GST activity in CLB resistance. GSH synthesis inhibitor L-buthionine-SR-sulfoximine (BSO), and GST enzyme inhibitors, ethacrynic acid and indomethacin, were used to alter the intracellular GSH pool and GST activity (Robson et al., 1986; Tew et al., 1988; Hall et al., 1989). Pretreatment of the CLB-resistant N50-4 cells with either GSH depleting agent (BSO), or GST inhibitor (ethacrynic acid or indomethacin), significantly enhanced cytotoxicity of the cells to CLB. However, the CLB resistance in the N50-4 cells was still higher than that of the parental NIH-3T3 cells. By a combination pretreatment with both GSH blocking agent (BSO) and GST inhibitor (ethacrynic acid or indomethacin), the resistant phenotype of the N50-4 cells was almost completely reversed. The results suggest that both increased intracellular GSH content and increased GST enzyme activity are important for CLB resistance in the N50-4 cells (Yang et al., 1992).

Recently, there were some reports on clinical trials of GSH synthesis inhibitor BSO or GST enzyme inhibitor ethacrynic acid in combination with alkylating agents to overcome drug resistance in cancer patients. Phase I clinical trials of melphalan (Hantel et al., 1991) or Thiotepa (Schilder et al., 1990) combining with ethacrynic acid have been undertaken to determine optimal schedules and doses. Phase I studies of BSO in combination with alkylating agents are underway to determine the toxicity of optimal doses (Schecter et al., 1991). However, these clinical studies on chemotherapy resistance emphasize on inhibiting either GST enzyme activity or intracellular GSH production. Based on our findings,

it would benefit patients more if a proper agent or agents can be found to inhibit both GSH production and GST enzyme activity.

Analyzing the mRNA levels of the GST subunit genes by Northern blotting, I observed an increase in steady-state accumulation of the GST Alpha-class Ya subunit gene transcripts in the CLB-resistant cells. No significant difference in mRNA level of the GST Mu- and Pi-class genes was found between the CLB-sensitive and -resistant cells. These observations suggest that the Alpha-class GST enzymes may play an important role in CLB resistance. This is consistent with the finding that the GST Alpha-class isozyme is more efficient in catalyzing the conjugation reaction of CLB and GSH than GST-Pi and -Mu isozymes purified from mouse liver cytosol (Ciaccio et al., 1990).

Gene expression is a complex process with each step under regulatory control. Some of the key regulatory controls include transcriptional activation and the mRNA stability. To understand mechanisms for the increased GST Alpha-class gene expression in CLB-resistant cells, I examined transcriptional and post-transcriptional regulation of the GST Alpha-class gene expression in CLB-sensitive and -resistant cell lines.

It has been shown that gene amplification occurs in tumor cells with high frequency (Wright et al.,1990). DNA amplification is also shown to be one of the important mechanisms in development of drug resistance (Rolfe et al., 1988; Stark et al., 1989). In the CLB-resistant N50-4 cell line, we did not detect amplification of the GST genes (Yang et al., 1992). In order to examine the mechanism of gene amplification in CLB resistance, we developed a

CLB-resistant cell line, named HUCLB, from the N50-4 cells under conditions that induce DNA amplification (Johnston et al., 1986). Amplification of the GST Alpha-class Ya and Yc subunit genes was observed in the HUCLB cells by Southern blot analysis. No evidence of GST Mu- and Pi-class gene amplifications was shown (Yang et al., 1993). Northern blot analysis showed a 3-fold increase of GST Ya and 2-fold increase of GST Yc mRNA levels in the HUCLB cells, compared to the N50-4 cells.

Since transcriptional activation is a very important mechanism in regulating the mRNA levels in prokaryotic and eukaryotic cells (Greenberg and Ziff, 1984), I first examined the transcriptional activity of the GST genes in CLB-sensitive and -resistant cells by the nuclear "run-on" assay. A significant increase in transcriptional activity of the GST Alpha-class gene, both Ya and Yc subunits, was detected in the CLB-resistant N50-4 and HUCLB cell lines, compared to the CLB-sensitive NIH-3T3 cells. No difference in transcription of the GST Mu- and Pi-class genes was found between the cell lines. This finding suggests that the increased transcriptional activity of the GST Alpha-class gene is, at least partially, responsible for the increased steady-state accummulations of the mRNA in the CLB resistant cells (Yang et al., 1993).

The stability of mRNA also plays an important role in regulating gene expression. Changes in the turnover rate of a mRNA ensures a proper level of mRNA can be maintained (Carter and Malter, 1991; Brawerman, 1989). To determine whether mRNA stability is also responsible for the accumulations of the GST Ya

mRNA in the CLB-resistant cells, I measured the half-life of the GST Ya mRNA in these cell lines. Because of the low copy number of the GST Ya mRNA in the wild-type NIH-3T3 cells, the quantitative reverse transcription-polymerase chain reaction (RT-PCR) technique was used to amplify the GST Ya mRNA (Wang et al., 1989). By blocking mRNA synthesis using actinomycin D, time-dependent decay of the GST Ya mRNA in the cells was determined. A significant increase in stability of the GST Ya mRNA was observed in both CLB-resistant N50-4 and HUCLB cells, compared to the wild-type NIH-3T3 cells. These results suggested that the mRNA stability is also responsible for the steady-state accumulations of the GST Ya mRNA in the CLB-resistant cells (Yang et al., 1993).

Although some studies have shown that mRNA sequences in the 5' untranslated region (UTR) or the sequences in the coding region can influence mRNA stability, sequences in 3' UTR of the gene affect mRNA stability more frequently (Bouvet and Belasco, 1992; Chen et al., 1992; You et al., 1992). To investigate whether GST Ya 3' UTR sequences would affect the stability of the mRNA, I amplified the GST Ya cDNA containing 3' UTR sequences by RT-PCR. Two GST Ya subunit genes were identified in the CLB-resistant cells, whereas only one GST Ya subunit gene was identified in the CLB-sensitive NIH-3T3 cells. The GST Ya_1 cDNA sequence, which is identical to the mouse GST Ya gene cloned by Daniel et al. (1987), was identified in both CLB-sensitive and -resistant cells. The GST Ya_2 cDNA sequence, which is identical to the GST Ya gene cloned by Pearson et al. (1988), was identified only in the CLB-resistant N50-4 and HUCLB cells. The observation that the GST Ya_2 mRNA was

not present in the NIH-3T3 cells was further confirmed by using the Ligase-chain reaction (LCR) technique with GST Ya_2 specific oligo nucleotides (Kalin, 1992). Northern blot analysis using GST Ya_1 and GST Ya_2 specific oligonucleotides showed a 2-fold increase in the steady-state mRNA levels of the GST Ya_2 gene in the HUCLB cells, compared to the N50-4 cells. No significant difference was detected in the GST Ya_1 mRNA levels between the two cell lines (Yang et al., 1993). This finding indicates that the GST Ya_1 and Ya_2 gene expression was differentially regulated in CLB-sensitive and -resistant cells. These findings raised two important issues:

First, what is the significance of the differential regulation of GST Ya_1 and Ya_2 genes expression in the CLB-sensitive and -resistant cells? In other wards, does the GST Ya_2 gene play a more important role than the GST Ya_1 gene in CLB resistance?

GSTs exist in multiple forms, including Alpha-, Mu- and Pi-class of soluble isoenzymes and membrane bound microsomal isoenzyme, each with different catalytic, physical and chemical properties (Mannervik, 1985; Mannervik et al., 1985; Pickett, 1987). For the Alpha-class GSTs, at least six GST Ya and two Yc subunit genes have been identified from rat cells (Hayes et al., 1991; Pearson et al., 1988), four GST Alpha-class genes have been identified from human cells (Ahmad et al., 1993), and two GST Ya and one GST Yc have been identified from mouse cells (Daniel et al.,1987; Pearson et al.,1988; McLellan et al., 1991; Buetler and Eaton, 1992). These GST Alpha-subclass genes share more than 90% nucleotide and amino acid sequence identity (Pearson et al., 1988; Ahmad et al., 1993). No one has yet investigated whether the

different forms of the GST Alpha-class subunit isozymes have different physical, biochemical or catalytic properties, or what is the exact role for each GST Alpha-class isozymes in the cells. Our findings on amplification and gene rearrangement of the GST Yc gene in CLB-resistant HUCLB cell line and over expression of the GST Yc mRNA in both CLB-resistant cell lines indicate that the Alpha-class GST Yc isozyme also play a part in CLB resistance. These observations open up other future directions for investigation on the regulation of GST Yc gene expression in CLB-resistant cell lines and the relative role of the GST Yc isozyme in CLB resistance.

In the course of my investigation, I tested whether the 3' UTR sequence of the GST Ya_1 or Ya_2 gene has any direct impact on its mRNA stability. I performed gene transfection assays using the CAT reporter gene system. The GST Ya_1 and GST Ya_2 3' UTR cDNA sequences were cloned by RT-PCR. Each of the GST Ya 3' UTR sequences was linked to 3' end of the CAT reporter gene, which was subsequently ligated into the pECE vector. The pECE plasmid containing the CAT gene alone ($pCAT_0$) was also constructed as a reference control. The pECE plasmid contains the SV40 early promoter and SV40 poly A site (Ellis et al., 1986). The recombinant plasmids were transfected into the CLB-sensitive NIH-3T3 cells and the CLB-resistant N50-4 cells, respectively. The stability of the CAT mRNA in the transfected cell lines was determined by mRNA decay assays. After actinomycin D treatment, the half-life of the CAT mRNA is longer than 6 hr in both NIH-3T3 and N50-4 cells when transfected with the plasmid containing the CAT gene without the GST Ya 3' UTR

sequence ($pCAT_0$). Cell lines transfected with the plasmid containing the CAT gene as well as the GST Ya_2 3' UTR sequence ($pCAT_2$) had a CAT mRNA half-life similar to that of the cell lines transfected with the $pCAT_0$ plasmid. A significant decrease in the CAT mRNA stability was observed in both NIH-3T3 and N50-4 cells when transfected with the plasmid containing the CAT gene and the GST Ya_1 3' UTR sequence ($pCAT_1$). These results suggest that the 3' UTR sequence of the GST Ya_1 gene might contain mRNA destabilizing element(s). No known destabilizing sequence, such as AU rich sequence, has been identified in the 3' UTR region of the GST Ya_1 gene (Brewer and Ross, 1989; Adam et al., 1986; DeZazzo et al., 1992; Schek et al., 1992, Wilson and Treisman, 1988). Identifying the mRNA destabilizing sequence at the UTR region of the GST Ya_1 gene would certainly be one of our future studies, which would be expected to contribute to a better understanding of the mechanisms for the mRNA stability of the GST Ya genes (Brawerman, 1987, 1989). Gel mobility shift assay, in vitro mRNA decay assay and UV cross-linking of protein to RNA methods could be carried out using the GST Ya_1 and Ya_2 UTR cDNA constructs to identify protein-binding sequences and proteins that binds to these RNA sequences (Brewer, 1991; Brewer and Ross, 1989; Adam et al., 1986).

Second, how is the expression of the GST Ya_1 and Ya_2 genes differentially regulated in the CLB-sensitive and -resistant cell lines? This would be a future direction of the investigation. To do this experiment, the 5' flanking sequence of the GST Ya_1 or Ya_2 gene could be identified from the mouse genomic DNA library by using GST Ya_1 or Ya_2 specific oligonucleotides as probes. The 5'

flanking sequence of the GST Ya_1 or Ya_2 gene would be linked to the chloramphenicol acetyl transferase (CAT) reporter gene, and then subsequently ligated into an expression vector. The DNA construct could be transfected into the CLB-sensitive or -resistant cell lines, and the CAT enzyme activity could be assayed to monitor the transcriptional activity. CLB responsive elements for transcription regulation would be identified (Manoharan et al., 1987; Telakowski-Hopkins et al., 1988; Rushmore et al., 1990; Paulson et al., 1990). The 5' flanking sequence of the GST Ya_1 gene has been cloned (Daniel et al., 1989; Friling et al., 1990) but the 5' flanking sequence of the GST Ya_2 gene has not yet been cloned.

Summarizing our findings on the regulation of the GST Ya_1 and Ya_2 genes expression in the CLB-sensitive and -resistant cells, we observed a direct correlation of the CLB resistance and the mRNA level of the GST Ya_1, Ya_2 and Yc genes in the cells. In the CLB-sensitive NIH-3T3 cells, transcriptional activity of the GST Ya gene was hardly detected by the nuclear "run-on" assay. Trace amounts of the GST Ya_1 mRNA could be detected only by the RT-PCR technique. No GST Ya_2 mRNA was found in the NIH-3T3 cells. When the cells acquired resistance to chlorambucil by step-wise drug selection, transcription of the GST Ya genes was quite significant, as detected by nuclear "run-on" assay in the CLB-resistant N50-4 cells. Increased steady-state mRNA levels of both GST Ya_1 and Ya_2 genes were readily detected by Northern blot analysis. In another CLB-resistant cell line, HUCLB, specific amplification of the GST Ya_2 and Yc genes was observed. Amplification of the GST Ya_2 and

Yc genes resulted in increased steady-state levels of the GST Ya_2 and Yc mRNAs, as detected by Northern blot analysis. In the CAT gene transfection assays, we found that the GST Ya_2 3' UTR sequence contributed to a longer mRNA half-life of the CAT gene, compared to the GST Ya_1 3' UTR sequence. This finding could explain the longer half-life of the GST Ya mRNA in the HUCLB cells compared with the N50-4 cells, because the HUCLB cells transcribed more GST Ya_2 mRNA. As a result, a 3-fold increase in the steady-state level of the GST Ya mRNA was observed in the HUCLB cells compared to the N50-4 cells, and a higher resistance to CLB was found. These observations suggest that the GST Ya_1, Ya_2 as well as GST Yc subunit isozymes may play important roles in CLB resistance. Further studies should be carried out to elucidate the functional and biological properties of the two GST Ya and GST Yc subunit isozymes in relation to the CLB resistance in mouse cells.

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