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Frequent Coexpression of MRP/GS-X Pump and γ-Glutamylcysteine Synthetase mRNA in Drug-Resistant Cells, Untreated Tumor Cells, and Normal Mouse Tissues

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ABSTRACT. Expression of the multidrug-resistance protein gene *MRP*, which confers non-P-glycoprotein-mediated multidrug resistance, has been found in many drug-resistant variants and tumor samples. Recent studies have demonstrated that MRP functions as an ATP-dependent transporter functionally related to the previously described glutathione-conjugate (GS-X) pump. We have shown recently that the MRP and γ-glutamylcysteine synthetase (γ-GCS) heavy subunit mRNA levels are coordinately overexpressed in cisplatin (CP)-resistant human leukemia cells (Ishikawa *et al., J Biol Chem* **271:** 14981–14988, 1996) and frequently co-elevated in human colorectal tumors (Kuo *et al., Cancer Res* **56:** 3642–3644, 1996). In the present study, we showed the coexpression patterns of thirteen additional human drug-resistant cell lines representing different tumor cell origins selected with different agents, except for one doxorubicin-selected line which demonstrated minor elevation in MRP mRNA with no detectable increase in γ-GCS mRNA, suggesting that the increase of MRP mRNA preceded the increase in γ-GCS mRNA. Furthermore, in seventeen randomly selected untreated tumor cell lines, the overall correlation coefficient between MRP and γ-GCS mRNA levels was 0.861. In normal mice, the correlation coefficient of mrp and γ-gcs mRNA was 0.662 in fourteen tissues (kidney and liver were not included) analyzed. Kidney and liver expressed low levels of mrp relative to γ-gcs; however, these two tissues expressed high levels of a functionally related mrp homologue, mrp2 (cMoat or cMrp), which may have compensated for the underexpressed mrp in maintaining the total GS-X pump activities. Altogether, these results demonstrated the frequent coexpression of these two genes in various cell settings. BIOCHEM PHARMACOL 55;5:605–615, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. multidrug-resistance protein; γ-glutamylcysteine synthetase; antitumor drugs; chemotherapy; gene regulation

Development of drug resistance in tumor cells is the major obstacle to effective tumor cell killing by chemotherapy. Accumulating evidence indicates that increased drug efflux capacity and consequent reduction in intracellular drug accumulation can be an important mechanism for the development of simultaneous resistance to many different drugs. Recent studies have revealed that the *MRP*¶ encoding a multidrug-resistance protein [1, 2], in addition to the previously described *MDR* that encodes the multidrug-resistance transporter P-glycoprotein [for review, see Ref. 3], are associated with this type of drug resistance. Like P-glycoprotein, MRP contains ATP-binding cassettes and belongs to the ABC transporter superfamily. While the exact mechanisms of MRP-mediated drug resistance remain to be determined, many recent studies strongly suggest that MRP may transport substrates containing glutathione, glucuronate, and sulfate moieties [4–8]. These findings are consistent with the idea that MRP is functionally related to the previously described GS-X pump [9].

The demonstration that MRP functions as an ATP-dependent transporter for glutathionated substrates supports an important scenario in which GSH metabolism may play an important role in overall MRP-mediated drug

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¶ *Abbreviations:* ACNU, 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)3-nitrosourea; BSO, buthionine sulfoximine; CP, cisplatin; cMoat, canalicular multispecific organic anion transporter; cMrp, canalicular multidrug-resistance protein; DMEM, Dulbecco's modified Eagle's medium; Dox, doxorubicin; γ-GCS, γ-glutamylcysteine synthetase; γ-*gcs*, murine γ-glutamylcysteine synthetase gene; GS-X pump, ATP-dependent glutathione S-conjugate efflux pump; HyR, hydroxyrubicin; LTC_4, leukotriene C_4; *MRP*, multidrug-resistance protein gene; *mrp*, murine multidrug-resistance protein gene; nt, nucleotide; RT–PCR, reverse transcription–polymerase chain reaction; and SCLC, small cell lung cancer.

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resistance. It has been reported previously that depletion of intracellular GSH by BSO results in a complete reversal of drug resistance in *MRP*-transfected lung carcinoma cells [10]. Furthermore, ATP-dependent transport of some anti-tumor drugs in membrane vesicles prepared from MRP-overproducing cells can be enhanced by the presence of GSH [7]. We have demonstrated increases of intracellular GSH levels in the MRP mRNA-overexpressing human CP-resistant HL-60 leukemia cell line HL-60/R-CP [11]. While intracellular GSH homeostasis is regulated by multiple factors, the first step in *de novo* GSH biosynthesis, catalyzed by γ-GCS, is the rate-limiting step [12]. γ-GCS consists of a catalytic heavy subunit and a regulatory light subunit [12]. We have reported recently that steady-state levels of MRP and γ-GCS mRNA encoding the heavy subunit (hereafter referred to as γ-GCS unless otherwise indicated) are elevated in the human CP-resistant cell line, HL-60/R-CP, in comparison with those in the parental HL-60 line [13]. We have also observed frequent coordinated overexpression of these genes in human colorectal cancers [14]. Finally, Ciaccio *et al.* [15] also reported a coordinate increase of MRP and γ-GCS mRNA in the ethacrynic acid-resistant HT29 human colon cancer cell line.

In light of these results, the present study was undertaken to investigate whether such coordinate expression also occurs in other independently established drug-resistant variants, in untreated human tumor cell lines, and in normal tissues. We demonstrate here that MRP and γ-GCS are coexpressed frequently in these various cell settings.

MATERIALS AND METHODS

Cell and Tissue Sources

Thirteen human drug-resistant cell lines and seventeen randomly selected human tumor cell lines were used in this study. Among the thirteen drug-resistant cell lines (see Table 1), three (SR2, SR2A, and SR3A) were established from human small cell lung carcinoma cell lines (SCLC) and ten (HyR0.5-1 to -10) from the human cervical carcinoma cell line HeLa. SR2 is a CP-resistant SCLC, whereas SR2A and SR3A are Dox-resistant derivatives of SR2 cells, selected using stepwise increasing concentrations of Dox in the absence of CP. Establishment of the HyR0.5 series using HyR as a selecting agent was described previously [16]. HyR is an analog of Dox in which the C3′-amino group of the sugar moiety is replaced by a hydroxy group, while the original configuration of the drug is retained. Briefly, HeLa cells were treated with 0.5 μg/mL of HyR, after which the surviving colonies were isolated and expanded as independent cell lines. SCLC and their drug-resistant variants were cultured in RPMI-1640 medium, whereas HeLa, the HyR0.5 series, and all randomly selected tumor cell lines were cultured in DMEM. Both types of media were supplemented with 10% fetal bovine serum containing appropriate concentrations of selecting drugs, if applied.

Various tissues were resected from female C57/B6 mice, except testes and prostates, which were resected from male Balb/C mice. All tissues were processed immediately for RNA extraction.

RNA isolation and RNase Protection Assay

The procedures for the preparation of total RNA and for the measurement of MRP and γ-GCS mRNA levels by the RNase protection assay using ^{32}P-labeled antisense RNA probes for MRP and γ-GCS were described previously [13]. For the γ-GCS light-chain probe, a 165-nt fragment of γ-GCS light-chain cDNA spanning from the translation start site [17] was synthesized by RT–PCR using forward primer 5′-GAGGATCCTCATGATTGTATCCAA-3′ and reverse primer 5′-GTTGAATTCTACAATGAA CAG-3′. The PCR products were cloned into a pSPT18 vector. The resultant plasmid was linearized with *Xba*I, and the probe was synthesized using T7 RNA polymerase. For mouse *γ-gcs* probe, a 990-nt fragment of cDNA was synthesized using forward primer 5′-GGAGGAG GAGGGGGCGG-3′ and reverse primer 5′-TCT TCAGGGGCTCCAGTCC-3′. (These primer sequences were selected because they are conserved in the human [18] and rat [19] homologues.) The PCR product was subcloned into a pSPT18 vector and sequenced. The plasmid was linearized by digestion of the internal *Pst*I site, and the probe was synthesized with T7 polymerase to generate a 183-nt fragment in the RNase protection assay. To prepare the mouse *mrp* and *mrp2* antisense probes, forward primer 5′-GCTGGGAAATCATCCCTCAC-3′ and reverse primer 5′-GGATCCTGTGGAATGATG-3′, derived from the two conserved regions in Sequence I and Sequence II of rat mrp and mrp2 cDNA, respectively [20], were used. The resultant PCR products were cloned similarly into pSPT18, and several clones were sequenced. One clone containing 74 nt with a sequence identical to that reported for the mrp cDNA sequence [21] and the other a sharing striking similarity to sequences of rat mrp2 [20] and generating 70 nt of a protection fragment by the subsequent RNase protection assay were used for assaying murine mrp and mrp2 mRNA levels, respectively.

For the RNase protection assay, 20 μg RNA was hybridized with either the *MRP* or *γ-GCS* probe alone or together, and 1 μg RNA was hybridized with an 18S rRNA probe (2×10^5 cpm each). After annealing, the reaction mixtures were combined and digested with RNase A and T1, followed by phenol-chloroform extraction. The protection products were ethanol precipitated and analyzed by PAGE. We found that the primary factors contributing to experimental variations in the RNase protection assay were those in many subsequent steps after probe annealing. The use of the 18S probe in the combined reaction mixtures described here served as a reference for monitoring such

TABLE 1. Drug-resistant cell lines used in this study

Cell line	Origin	Selecting agent (fold resistance)	Fold expression*	
			MRP mRNA	γ-GCS mRNA
HL-60	Leukemic		1	1
HL-60/R-CP†	Leukemic	Cisplatin (10×)	5	5
SCLC	SCLC		1	1
SR2	SCLC	Cisplatin (35×)	0.1	0.2
SR2A	SCLC	Doxorubicin (60×)‡	2	1.5
SR3A	SCLC	Doxorubicin (120×)‡	3	1.5
HeLa	Cervical carcinoma		Undetectable	
HyR0.5-1 through	Cervical carcinoma	Hydroxyrubicin (5×)	Undetectable	
HyR0.5-9	Cervical carcinoma	Hydroxyrubicin (5×)	Undetectable	
HyR0.5-10	Cervical carcinoma	Hydroxyrubicin (5×)	High	High

* Data were collected from at least two independently prepared RNA samples.
† Data were published previously (see Ref. 13).
‡ Fold increase in reference to SR2 cells.

variations. All the RNase protection assays were repeated at least twice.

Preparation of Antipeptide Antibody and Western Blot

A peptide containing C-terminal 23 amino acid residues of MRP with single letter sequence GAPSDLLQQRGLFYS MAKDAGLV was synthesized on a Vega Coupler 250 C synthesizer using *tert*-butoxycarbonyl chemistry. Synthesized peptides were deprotected and cleaved from the supporting resin with hydrogen fluoride in the presence of 5% anisole, solubilized in 26% glacial acetic acid, and lyophilized. Peptides were characterized by amino acid analysis and high performance liquid chromatography. Peptides were reconstituted in PBS at 1 mg/mL, mixed with an equal volume of keyhole limpet hemocyanin (1 mg/mL in PBS), and an aliquot of 2.5% glutaraldehyde was added to a final concentration of 0.04%.

Polyclonal antipeptide antibody was produced in Balb/C mice by injecting the coupled peptide mixed with RIBI adjuvant. Animals were boosted every 2 weeks for 6 weeks. One week later, sera were withdrawn from the animals, and the titer was determined by ELISA against synthetic peptide and/or Western blot against membrane vesicles prepared from a high MRP-producing cell line (SR3A) versus a low MRP-producing cell line (SR2).

Other Procedures

Procedures for Southern blot, preparation of plasma membrane vesicles, and measurement of the transport activity of [^{3}H]LTC$_4$ have been described previously [13]. Densitometric scans of autoradiographs and statistical analyses of the digitized image using the Statistica program (Statsoft) have been described elsewhere [14].

RESULTS

Expression of MRP and γ-GCS in Human Drug-Resistant Cell Lines

Using Northern blot hybridization and RNase protection assays, we demonstrated previously that HL-60/R-CP cells exhibited an approximately 5-fold increase in the steady-state levels of MRP and γ-GCS heavy-chain mRNA as compared with those in the drug-sensitive HL-60 line [13]. To investigate whether *MRP* and γ-*GCS* heavy-chain genes are also coordinately expressed in other drug-resistant cell lines, we first analyzed the levels of MRP and γ-GCS mRNA in SCLC cells and their drug-resistant variants. In comparison with SCLC cells, SR2 cells exhibited a 35-fold increase of resistance to CP, but no cross-resistance to Dox. SR2A and SR3A showed 60- and 120-fold higher resistance, respectively, to Dox in reference to SR2, but only marginal resistance (1.5-fold) to CP (Table 1). Figure 1 shows that in SR2 cells, levels of MRP and γ-GCS mRNA were about 10- and 5-fold lower, respectively, than those in the drug-sensitive SCLC line. In SR2A and SR3A cells, however, the levels of MRP mRNA were approximately 20- and 30-fold higher, respectively, than those in the SR2 line (2- and 3-fold higher than those in SCLC cells). Western blot analyses using anti-MRP peptide antibody revealed that levels of MRP in the membrane fractions prepared from these cell lines generally paralleled the levels of MRP mRNA (Fig. 2). The levels of γ-GCS mRNA in SR2A cells were about 7.5-fold higher than those in SR2 cells (or an approximately 1.5-fold increase in reference to those in SCLC cells), but there was no further increase in SR3A cells. Our failure to see a further increase of γ-GCS mRNA in SR3A cells may have been due to the low increment of MRP mRNA in comparison with that in SR2A cells. Nevertheless, these results show frequent coordinate expression of MRP and γ-GCS mRNA in this panel of cell lines.

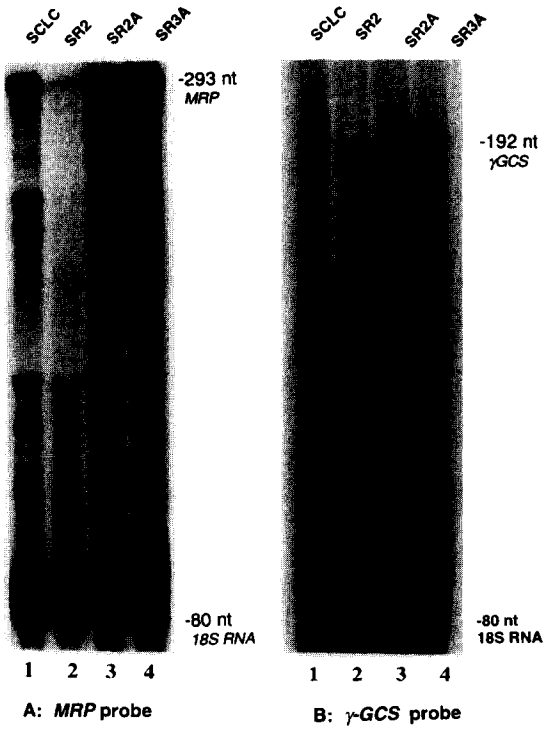

FIG. 1. RNase protection assay of MRP (A) and γ-GCS (B) mRNA in a small cell lung cancer line (SCLC) and its resistant variants (SR2, SR2A, and SR3A). Twenty micrograms of total RNA was used in the RNase protection assay using MRP and γ-GCS heavy-chain probes. 18S rRNA probe was used as a reference. Note that MRP and γ-GCS mRNA levels were both lower in SR2 cells but higher in SR2A and SR3A than in SCLC cells.

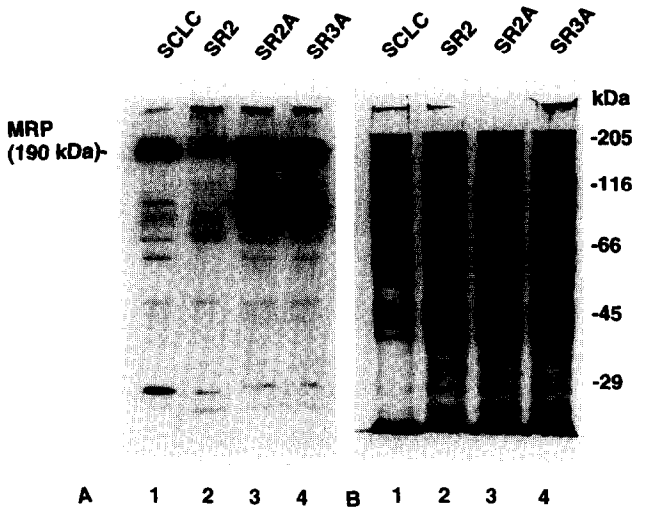

FIG. 2. Western blot analyses of MRP in SCLC, SR2, SR2A, and SR3A cell lines. (A) For each lane, 50 μg of membrane vesicles was separated by SDS–PAGE and probed with an anti-MRP peptide antibody. The signals were developed by an ECL detection kit (Amersham Life Science) according to the vendor's specifications. (B) Duplicated gel was stained with Coomassie blue to view the protein loading.

To investigate whether the variations in MRP and γ-GCS mRNA levels in SCLC, SR2, and SR3A cells were due to alterations in gene copy number, we carried out a Southern blot analysis. DNA from these cell lines was digested with *Eco*RI restriction endonuclease and Southern hybridized with ^{32}P-labeled MRP and γ-GCS cDNA probes [13]. Our analysis revealed that the copy numbers of both genes in these three cell lines were not significantly different (data not shown), indicating that the changes in mRNA abundance in the drug-resistant variants were not due to either DNA amplification or under-replication in these cells.

Previous studies have demonstrated that membrane vesicles prepared from MRP-overexpressing cells exhibited increased ATP-dependent transport activity of the glutathione conjugate LTC_4 [4–8]. Thus, it would be of importance to investigate whether the transport activities of LTC_4 would correlate with the levels of MRP in these cells. Figure 3 shows that ATP-dependent LTC_4 transport activities were reduced in SR2 cells but increased in SR2A and SR3A cells in comparison with those in SCLC cells. Moreover, the transport activities in these cell lines paralleled the steady-state levels of MRP, consistent with the idea that levels of MRP expression correlate with the LTC_4 transport activities in drug-resistant cell lines.

Analyses of MRP and γ-GCS mRNA levels were extended to another panel of drug-resistant cell lines. We previously established a series of ten HyR-resistant HeLa cell lines, designated HyR0.5-1 through -10 [16]. RNase protection assay demonstrated that among the ten HyR0.5 cell lines, only one (HyR0.5-10) showed detectable MRP mRNA. Concurrently, only this cell line displayed elevated levels of γ-GCS mRNA. A representative RNase protection assay is shown in Fig. 4. The exact fold increase could not be accurately estimated due to the very low level of MRP and γ-GCS expression in the drug-sensitive HeLa cells. Moreover, why only HyR0.5-10 overexpressed MRP and γ-GCS mRNA and why HyR0.5-10 still displayed the same levels (5x) of resistance as other lines are not known. These cell lines also expressed various amounts of MDR1 [18]. Thus, the drug-resistance mechanisms among these cell lines appear to be complex. Nevertheless, the results presented here further substantiate the notion that MRP and γ-GCS mRNA levels are frequently coexpressed in the drug-resistant variants.

Expression of MRP and γ-GCS in Human Tumor Cell Lines

After establishing the frequent coordinated expression of MRP and γ-GCS mRNA in the drug-resistant human tumor cell lines, we wished to investigate whether a similar phenomenon would also occur in the drug-sensitive tumor cell lines. We randomly chose seventeen cell lines: three breast cancer (MDA-MB-468, MDA-MB-453, and MDA-MB-361), three glioma (U251, D54, and T98), three SCLC (H-226 and H-460, SCLC), two pancreas cancer (PANC3

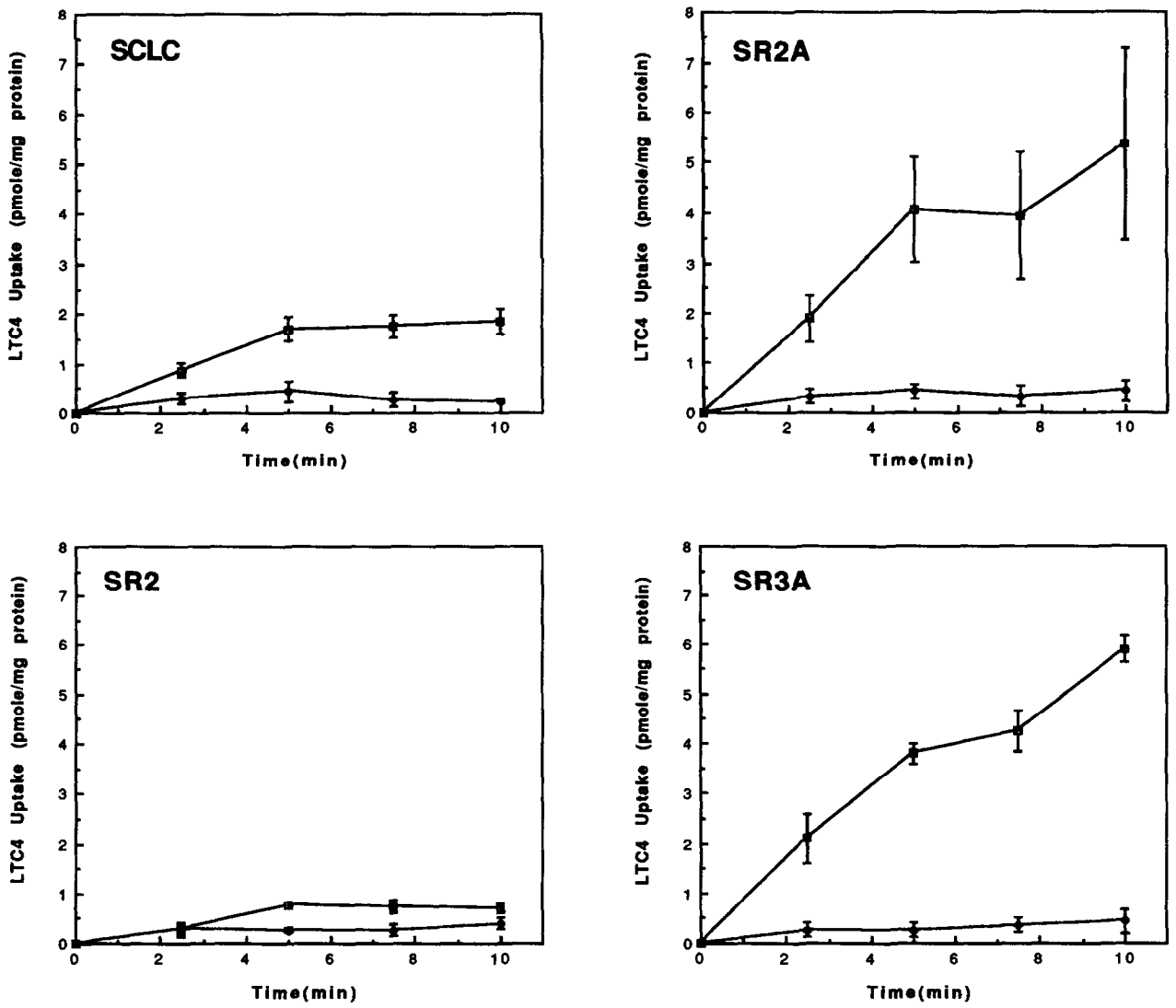

FIG. 3. GS-X pump activities in plasma membrane preparations from SCLC, SR2, SR2A, and SR3A cells. Uptake of LTC_4 by plasma membrane vesicles in the presence of ATP (open squares) or in the absence of ATP (closed diamonds) was determined. Bars represent standard deviations in three independent experiments.

and BXPC3), two leukemia (pre-B and T-ALL), and one each prostate cancer (PC3), ovarian cancer (SK-OV-3), hepatoma (HepG2), and colon cancer (HT-29). As shown in Fig. 5, substantial variations in MRP and γ-GCS mRNA levels were seen in these tumor cell lines. Cell lines expressing low levels of MRP and γ-GCS mRNA included PC3, H-226, T-ALL, and MDA-MB-468; high expressers were PANC3, HT-29, BXPC3, T98, MDA-MB-361, and pre-B cells. The signals on the autoradiographs were densitometrically scanned and converted into digitized values. The levels of MRP mRNA were then plotted against those of γ-GCS mRNA, and a correlation coefficient of 0.861 was obtained (Fig. 6). These results suggest that, even in drug-sensitive tumor cells, there was a good correlation between the levels of MRP and γ-GCS mRNA expression.

Expression of mrp and γ-gcs in Normal Mouse Tissues

The observed frequent coexpression of MRP and γ-GCS in drug-resistant cell lines and in drug-sensitive cancer cell lines prompted us to investigate the expression patterns of these two genes in normal tissues. Total RNA was extracted from various tissues of normal mice and subjected to RNase protection assays using murine *mrp* and *γ-gcs* probes. Figure 7A shows tissue-variable expression of mrp and γ-gcs mRNA in sixteen tissues investigated. The expression patterns of mrp generally agreed with those published by Stride *et al.* [21] using Northern hybridization. Moreover, examination of the expression patterns revealed a coordinate expression of *mrp* and *γ-gcs* in many tissues except for kidney and liver. For example, elevated mrp and γ-gcs mRNA levels were seen in appendix, ovary, prostate,

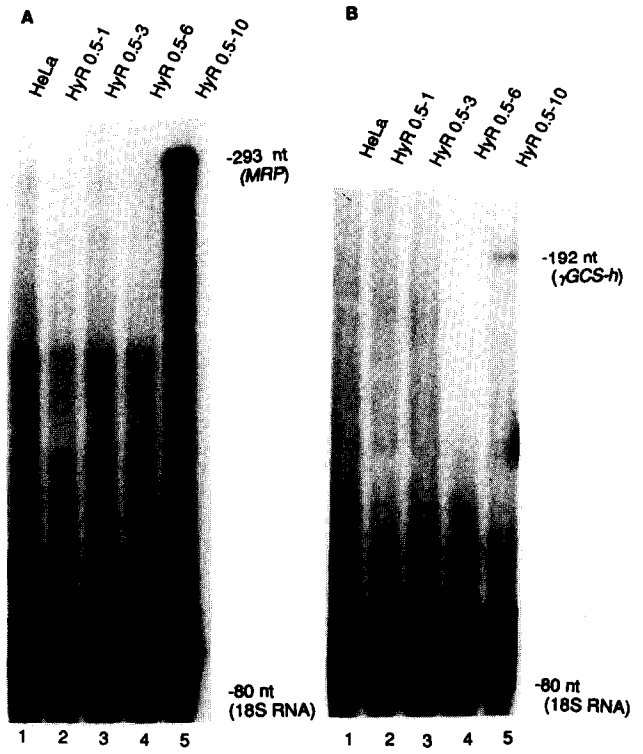

FIG. 4. RNase protection analyses of MRP (A) and γ-GCS (B) transcripts in HeLa and its hydroxyrubicin-resistant cell lines (HyR0.5-1, -3, -6, and -10). Note that only the cell line that overexpressed MRP (A) also overexpressed γ-GCS (B).

stomach, and testis, whereas low levels of expression were seen in colon, heart, ileum, muscle, spleen, and thymus. Densitometric analyses revealed a coexpression coefficient (r) of 0.662 for all the tissues excluding kidney and liver (samples 6 and 7 in Fig. 7A) (Fig. 8). When the values for kidney and liver were included, the overall r value was 0.278, owing to the high levels of γ-gcs mRNA but low values of mrp mRNA in these two tissues. Liver and kidney, but not the other tissues, expressed high levels of mrp2 (or cMrp or cMoat) mRNA (Fig. 7B). Because mrp2 encodes a liver canalicular multi-specific organic anion transporter efflux protein that shares sequence similarities and substrate specificities with those of mrp [22–24], it is tempting to hypothesize that the mrp2 may have been functionally compensated for by the underexpressed mrp for the overall GS-X pump activities. Thus, if the expression of *mrp2* were included, the overall correlation coefficient for all the tissues investigated would be improved substantially. Thus, we conclude that even in normal tissues (except liver and kidney) the expression pattern between mrp and γ-gcs mRNA is still coordinated frequently.

DISCUSSION

In the present study, we found that many MRP-overexpressing cell lines also overexpressed γ-GCS mRNA

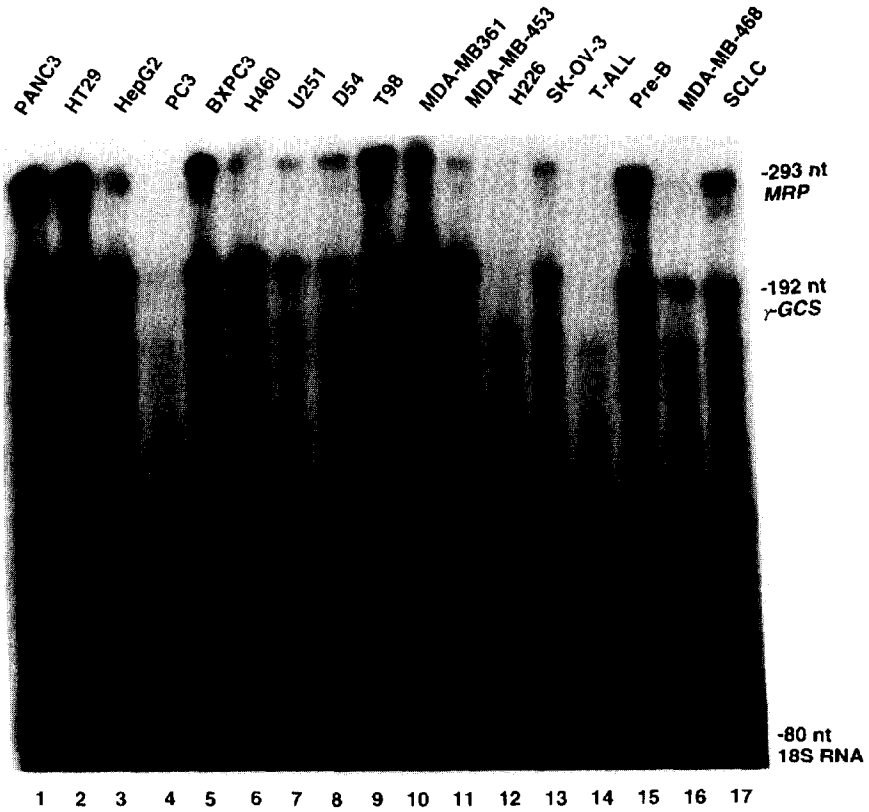

FIG. 5. RNase protection analyses of MRP and γ-GCS transcripts in various tumor cell lines. See "Materials and Methods" for derivations of these cell lines. This autoradiograph was intentionally overexposed in order to see signals in some lanes where signals were weak.

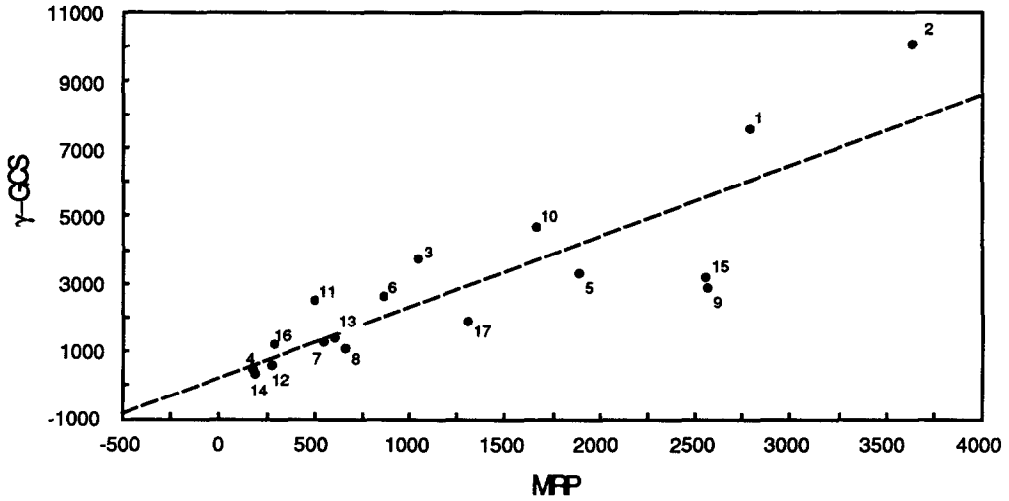

FIG. 6. Coordinate expression of MRP and γ-GCS heavy chain in various tumor cell lines. Relative levels of MRP and γ-GCS mRNA (in arbitrary units) derived from densitometric analyses of autoradiographs shown in Fig. 5 were plotted as shown. Numbers 1 through 17 correspond to the cell lines shown in lanes 1 through 17. The correlation coefficient was 0.861.

(SR2A and HyR0.5-10). Likewise, we found that cell lines in which MRP expression was down-regulated also displayed reduced γ-GCS mRNA levels (SR2 cells). Frequent coexpression of MRP and γ-GCS was also observed in glioma cells lines treated with ACNU, a nitrosourea-based antitumor agent.* These results, together with those shown previously [13, 15], demonstrate the frequent coexpression of MRP and γ-GCS in drug-resistant cell variants. In some cell lines, e.g. SR3A, that display minor increases of MRP mRNA levels, no detectable increase of γ-GCS mRNA levels was observed. Similar results were found in some ACNU-treated glioma cells (unpublished data). These results suggest that increases in MRP mRNA levels may precede the expression of γ-GCS mRNA. Using the RNase protection assay, we found no similar correlation between MRP2 and γ-GCS mRNA in these cell lines (data not shown). Nor did we see a similar correlation between levels of MRP mRNA and γ-GCS mRNA encoding the light-chain subunit (unpublished data). These results suggest that the expression of MRP, MRP2, and γ-GCS light-chain subunit is controlled independently in these drug-resistant variants. Although overexpression of MRP or γ-GCS mRNA in drug-resistant cell lines has been described by others, coexpression of these genes was not described in these studies.

In the present study, we report that the CP-resistant SCLC cell line SR2 displayed reduced levels of MRP and γ-GCS mRNA in comparison with those in the parental cell line (SCLC). We previously reported that MRP and γ-GCS mRNA levels were increased coordinately in HL-60/R-CP cells, a CP-resistant human leukemia cell lines [13]. These combined results suggest that MRP/γ-GCS mRNA levels can be either up-regulated or down-regulated in different CP-resistant variants. While levels of MRP and γ-GCS mRNA were increased in HL-60/R-CP cells, it remains to be seen whether the overexpressed mRNA is responsible for the observed CP resistance. Many MRP-overproducing cell lines, established either by selection against Dox toxicity or by transfection with MRP cDNA in expression vectors, failed to display resistance to CP [25–27]. The two MRP-overexpressing Dox-resistant cell lines (SR2A and SR3A) that were selected in the absence of CP from CP-resistant SR2 cells also exhibited diminished cross-resistance to CP (data not shown). MRP-overexpression is often associated with Dox resistance; however, the mechanisms underlying how MRP mediates Dox resistance in these cell lines remain to be investigated. For instance, Dox is a very poor substrate in membrane vesicle transport using vesicles prepared from MRP-overexpressing variants [Refs. 6, 7, and 10, and our unpublished data, but also see Ref. 28], suggesting that other cellular factors may also contribute to the overall MRP-mediated drug resistance. In addition, it is possible that multiple factors may be necessary for conferring the entire spectrum of MRP-related drug resistance in mammalian cells and that such factors may be cell type specific. Alternatively, specific factors may be co-induced in various drug selection schemes to satisfy MRP-mediated resistance to specific drugs. This may explain why some MRP-overexpressing cells fail to exhibit cross-resistance to certain drugs such as CP, while others do. Thus, it is critically important to evaluate the roles of MRP/γ-GCS in the ACNU- as well as HyR-resistant cell lines described here.

Frequent coordinate expression of MRP and γ-GCS mRNA was also seen in a number of tumor cell lines without previous exposure to antitumor agents (Figs. 5 and 6) and in colorectal cancer biopsy specimens obtained from patients who had not been treated with chemotherapy [14].

*Gomi A, Shinoda S, Masuzawa T, Ishikawa T, and Kuo MT, Transient Induction of the MRP/GS-X pump and γ-Glutamylcysteine Synthetase by 1-(4-Amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea in Human Glioma Cells. *Cancer Res*, In press.

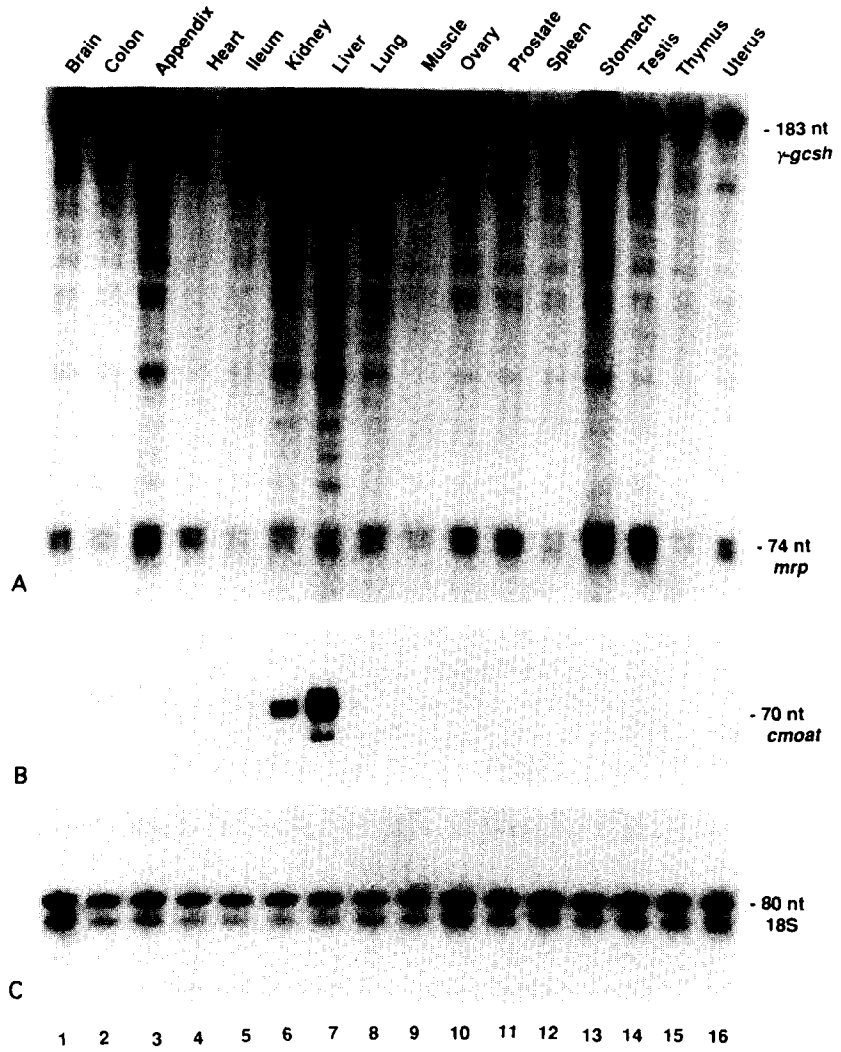

FIG. 7. RNase protection assays of RNA levels in various mouse tissues as indicated. Determinations of mrp (74 nt signal), and γ-gcsh (γ-gcs heavy subunit, 183 nt) mRNA are shown in panel A, cMoat mRNA (70 nt) in panel B, and 18S rRNA (80 nt) in panel C.

Strikingly, frequent coexpression patterns of γ-gcs and mrp were also observed in many normal mouse tissues, though not in kidney and liver, which express functionally overlapping mrp2. Concerted regulation of detoxifying genes by xenobiotics has been well-documented [for reviews, see Ref. 29], and perhaps the best examples are the gene battery encoding the phase I (e.g. *CYP1A1* and *CYP1A2*) and phase II (*Nmo1*, *Aldh-1*, *Ugt-1* and *Gt-1*) enzymes, and those encoding oxidative stress-inducible proteins (e.g. DT-diaphorase [30], glutathione transferase Ya [31], and heme oxygenase 1 [32]). Our present results add important information to the steadily accumulating evidence that various genes involved in the same detoxification pathways can be regulated in concert. These observations also suggest that the underlying concerted expression mechanism(s) for *MRP* and γ-GCS may have coevolved to accommodate the functional need.

The mechanisms by which *MRP* and γ-GCS are coordinately expressed in various cell sources are not clear. It is possible that transcriptional and/or posttranscriptional regulation is involved. Likewise, different mechanisms may be utilized for the coexpression of these two genes in drug-resistant variants, in cancer cells, and in normal tissues. We favor the hypothesis that oxidative stress induction mechanisms may play a role in the regulation of these genes, particularly in situations where cytotoxic agents are used, from the following considerations: (i) It has been reported that expression of human γ-GCS can be induced by antioxidants, i.e. 1, 4-naphthoquinone and menadione [33, 34], and we have preliminary evidence that these compounds can induce expression of *MRP* as well (unpublished data). These agents are known to introduce intracellular oxidation-reduction labile conditions by virtue of their capacities to undergo 1- and/or 2-electron valency changes, leading to imbalance of intracellular oxy radicals and generation of oxidative stress to the cells. (ii) In yeast, the *GSH1* gene that encodes γ-GCS [35] and the *YCF1* gene that encodes the human MRP homolog [36, 37] are coordinately regulated by the *yAP-1* gene encoding yeast transcription factor AP-1 [38]. There is considerable evidence that the AP-1 family members are oxidative stress-responsive transcription factors [for reviews see Refs. 39 and 40]. In experiments using mammalian cultured cells, most of the inducers of MRP/γ-GCS expression are known to

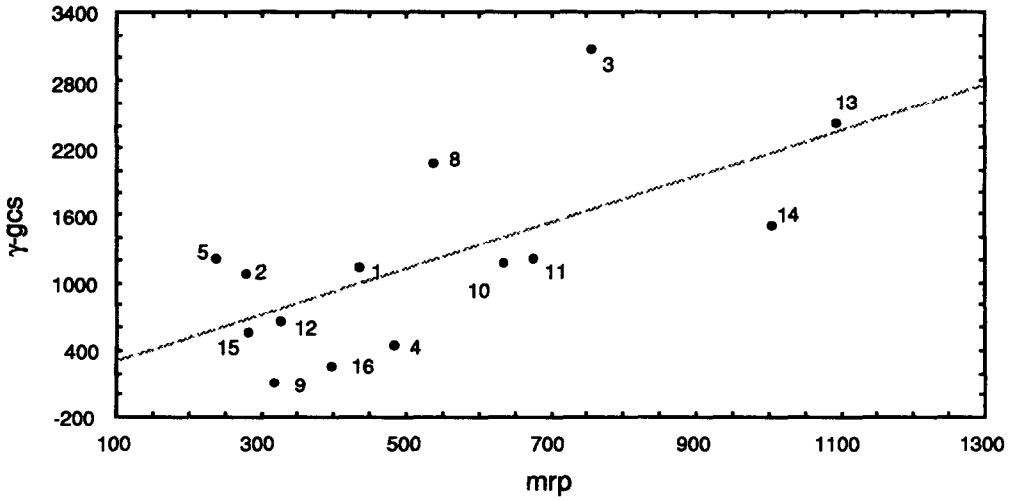

FIG. 8. Coordinated expression of mrp and γ-gcs heavy chain in normal mouse tissues. Signals corresponding to mrp and γ-gcs mRNA (in arbitrary units) derived from densitometric analyses of autoradiographs shown in Fig. 7 are plotted as shown. Numbers 1 through 16 refer to tissues shown in lanes 1 through 16. The correlation coefficient was 0.662. Note that kidney and liver (No. 6 and 7) were not included in the analysis for the reasons indicated in the text.

induce AP-1 and/or NF-κB activities, and we have evidence that in cell lines with high levels of MRP/γ-GCS expression, levels of AP-1 are also increased (in comparisons between HL60/R-CP and HL-60 cells and between SR3A and SR2 cells, unpublished data). (iii) The association of oxy radicals with tumor development, particularly in colorectal carcinogenesis, has long been postulated [for review, see Ref. 41]. (iv) An oxidative stress-responsive element or antioxidant responsive element (ARE), CCGT GACTCAGCGGCGC^{-3148} (underlined nucleotides are functionally important), has been identified at the 5′-flanking sequences of the γ-GCS gene [42]. Strikingly, two similar sequences are located at the 5′-region of the *MRP* gene, i.e. CAGTGACAACGCTTCCT^{-1120} and GTGT GACTCAGCTTTGG^{-454} [43]. Despite these data, further experiments are needed to confirm (or dispute) the involvement of oxidative stress in the coordinate regulation of *MRP* and γ-*GCS*, and whether different mechanisms are involved in the regulation of these genes in different cell sources.

Finally, we would like to stress that, despite the observed frequent co-induction of *MRP* and γ-*GCS* in many drug-resistant and cancer cells and the roles of these genes in the GS-X pump transport pathway, it remains important to evaluate whether γ-GCS up-regulation plays a critical role in MRP-mediated drug resistance. Intracellular GSH is regulated by multiple enzyme systems, including γ-GCS and GSH synthetase for its biosynthesis, γ-glutamyl transpeptidase (γ-GT) for its cleavage into constituent amino acid moieties for GSH salvage, and glutathione-*S*-transferases (GST) in the conjugation reactions for GS-X formation. In a survey of sixty human tumor cell lines, Tew *et al.* [44] recently reported that γ-GCS and GSTP1, by themselves, are important determinants of cellular response to certain antitumor agents. It is conceivable then that interplay among these GSH metabolic enzymes is critical for the maintenance of GSH homeostasis. Thus, the roles of these GSH-associated enzymes as a whole in the overall MRP-mediated drug resistance need to be investigated. These experiments are currently under way in our laboratories.

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