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Posttranscriptional Regulation of *MRP/GS-X* Pump and γ-Glutamylcysteine Synthetase Expression by 1-(4-Amino-2-methyl-5-pyrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea and by Cycloheximide in Human Glioma Cells

Akira Gomi,*,† Toshio Masuzawa,† Toshihisa Ishikawa,‡ and M. Tien Kuo*,[1]

**Department of Molecular Pathology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Box 89, Houston, Texas 77030; †Department of Surgical Neurology, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi-machi, Kawachi-gun, Tochigi 329-04, Japan; and ‡Department of Medicinal Biology, Central Research, Pfizer Inc., 5-2 Taketoyo, Aichi 470-23, Japan*

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Treatment of human glioma A172 cells with 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethy-3-nitrosourea (ACNU) for 2 to 4 hr resulted in a 2- to 3-fold increase in steady-state levels of multidrug resistance-associated protein (*MRP*) and γ-glutamylcysteine synthetase (*γ-GCS*) mRNA. Nuclear run-on assays revealed a less than 0.5-fold increase in transcription rates of these genes under the same treatment conditions, suggesting that posttranscriptional regulation plays an important role for the increased mRNA levels. In the absence of ACNU, rates of *MRP* and *γ-GCS* mRNA degradation were similar in A172 cells as determined by incubating cells with the RNase inhibitor, Actinomycin D. ACNU treatments resulted in increased *MRP* mRNA stability. Induction of *MRP* and *γ-GCS* mRNA by ACNU apparently did not require *de novo* protein synthesis as determined by the use of protein synthesis inhibitor cycloheximide (CHX). However, CHX alone could induce accumulation of *γ-GCS* mRNA, also by posttranscriptional mechanism. Taken together, these results demonstrate that (i) posttranscriptional regulation is primarily involved in the induction of *MRP* and *γ-GCS* expression by ACNU and CHX in human glioma cells; and (ii) despite the fact that these two genes have been reported to be frequently co-expressed, their responses to the treatments of RNA and protein synthesis inhibitors are not the same. © 1997 Academic Press

Expression of multidrug resistance-associated protein (*MRP*) is elevated in many multidrug-resistant cell variants (1, 2). Moreover ectopic expression of *MRP* by transfecting expression vectors containing *MRP* cDNA confers resistance in otherwise drug-sensitive cells to many antitumor agents, including doxorubicin, vincristine, taxol, and etoposide as well as heavy metals, e. g., arsenite, antimonials and cadmium (3, 4). MRP contains multiple transmembrane domains and two intracellularly localized ATP-binding cassettes. It is generally believed that MRP functions as an efflux pump from which intracellular cytotoxic compounds are eliminated. However, the mechanisms by which MRP-mediated drug elimination are largely unknown.

Recent transport studies using isolated membrane vesicles have demonstrated that endogenous metabolites, especially those containing glutathione (GSH) moieties, e.g. $_{LTC4}$ (5-11), are good substrates for MRP-mediated transport, suggesting that intracellular GSH levels may influence overall *MRP*-mediated transport. While intracellular glutathione homeostasis is regulated by multiple enzyme systems, the first step of *de novo* GSH biosynthesis, catalyzed by γ-glutamylcysteine synthetase (γ-GCS), is the rate-limiting step (12). We have recently reported that the expression patterns of *MRP* and *γ-GCS* mRNA encoding the heavy subunit (hereafter referred to as γ-GCS) are coordinated in many drug-resistant variants (13, 14). Ciaccio et al. also reported a coordinate increase of *MRP* and *γ-GCS* mRNA in ethacrynic acid-resistant HT29 human colon cancer cells (15). Strikingly, frequent coordinate expression of *MRP* and *γ-GCS* was also observed in human colorectal cancers (16). More recently, we have demonstrated that 1-(4-amino-2-methyl-5-pyrimidinyl)-methyl-3-(2-chloroethyl)-3-nitrosourea (ACNU), a nitrosourea-based antitumor alkylating agent, can tran-

[1] To whom correspondence should be addressed. Fax: 713-794-4672. E-mail: t-Kuo@path.mdacc.tmc.edu.

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siently co-induce the expression of *MRP* and *γ-GCS* in human glioma cells (17).

The present investigation was initiated to elucidate mechanisms of *MRP* and *γ-GCS* induction by ACNU. We report here that posttranscriptional control is primarily responsible for the induction. Furthermore, we demonstrate that *γ-GCS* mRNA levels could be induced in A172 cells treated with cycloheximide (CHX) and posttranscriptional control is also involved in the induction. Thus, our present study demonstrates the importance of posttranscriptional regulation for the expression of *MRP* and *γ-GCS* mRNA in cells exposed to antitumor nitrosourea and CHX.

MATERIALS AND METHODS

Cell culture. The human glioblastoma cell line A172 was obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). The cells were maintained in DMEM supplemented with 10% fetal calf serum at 37 °C in a 5% CO2 atmosphere.

ACNU, actinomycin D, and cycloheximide treatments. A172 cells at exponential growth conditions were treated with 10 μM ACNU (Sankyo Co., Japan) together with either 0.05 μg/ml actinomycin D (Act D, Sigma, St. Louis, MO) or with 50 μg/ml CHX (Sigma, St. Louis, MO) at different time intervals. The ACNU concentration used was that would kill 50% of cells in a 3-d exposure (IC_{50}) (17). Likewise, the concentrations of Act D and CHX used were predetermined so that about 90% of overall RNA and protein syntheses, respectively, were inhibited in 3-d treatments, as determined by [^{3}H]uridine and [^{3}H]leucine incorporations into 5% trichloroacetic acid-insoluble fractions in cell labeling experiments.

RNA isolation, RNase protection assay, and nuclear run-on assay. Total RNA was extracted using the acid guanidium thiocyanate-phenol-chloroform method. The procedure for RNase protection assay has been described previously (13, 16). The reliability of this assay was established previously by comparing the results with those obtained by northern blot hybridization (13). Nuclear run-on assays followed the procedure described previously (18), except that full-length *MRP* cDNA (4), *γ-GCS* heavy subunit cDNA (19), and actin cDNA were used for hybridization. Densitometric analyses of the autoradiographs were after the procedures described previously (16).

RESULTS

Transient induction of MRP and γ-GCS by ACNU in A172 cells. The steady-state levels of *MRP* and *γ-GCS* mRNA in A172 cells treated with 10 μM ACNU for different time intervals were measured by the RNase protection assay. Consistent with our previous finding (17), about 2- to 3-fold increases in steady-state levels of *MRP* and *γ-GCS* mRNA were seen in the first 2 to 4 hr of treatment. Levels of these mRNA were reduced thereafter (Fig. 1A), probably due to the short half-life (about 5 hr) of ACNU. These results demonstrated that *MRP* and *γ-GCS* mRNA were transiently induced by ACNU treatment. Furthermore, the rise and fall of *MRP* and *γ-GCS* mRNA levels were coordinated.

Nuclear run-on assay demonstrated posttranscriptional regulation of MRP and γ-GCS expression by ACNU. The elevated steady-state levels of *MRP* and *γ-GCS* mRNA could be due to transcriptional and/or posttranscriptional controls. To differentiate these possibilities, nuclear run-on assays were performed to measure the relative rates of *MRP* and *γ-GCS* tran-

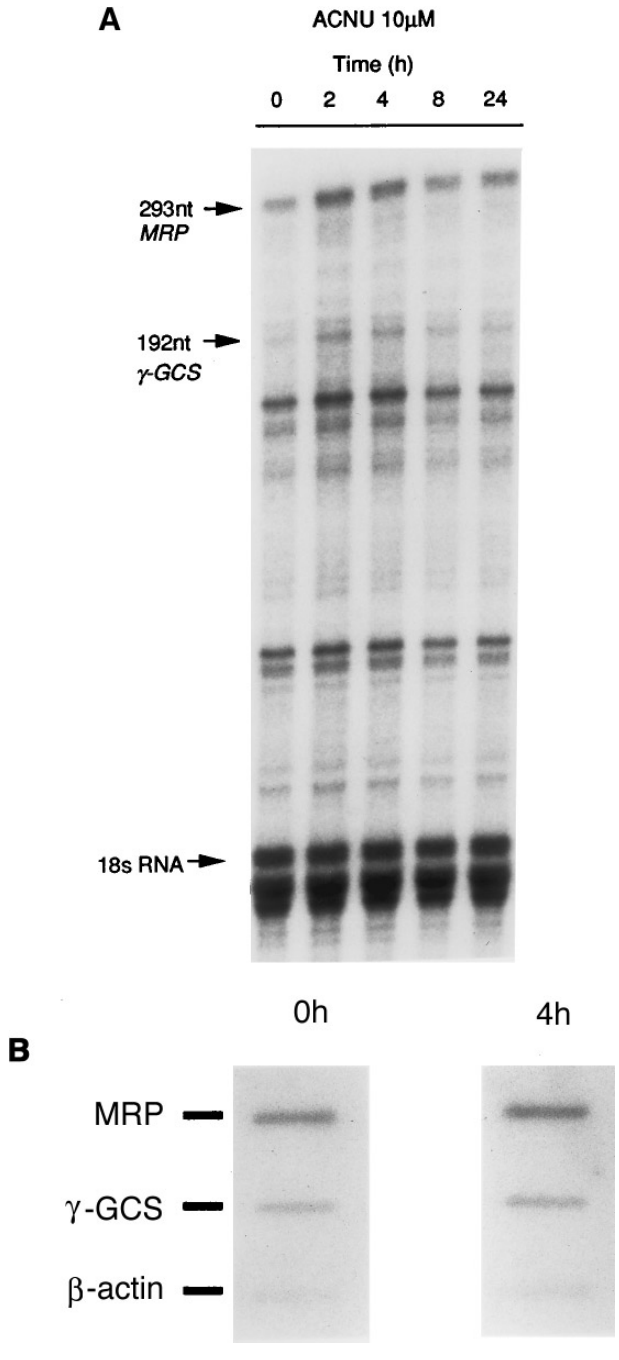

FIG. 1. (A) Time-course induction of *MRP* and *γ-GCS* mRNA by ACNU. The steady-state levels of *MRP* and *γ-GCS* mRNA in A172 cells treated with 10 μM of ACNU were determined by RNase protection assay. *MRP* and *γ-GCS* probes give 293 nt and192 nt protected fragments, respectively. 18S rRNA probe was used as reference to monitor sample variation. (B) Nuclear run-on assays of transcription rates of *MRP* and *γ-GCS* in ACNU-treated (10 μM, for 4 hr) and in untreated cells.

scription in A172 cells treated with ACNU for 4 hr and in untreated controls. ^{32}P-labeled RNA synthesized in the isolated nuclei of these cells were hybridized to *MRP*, *γ-GCS*, and actin cDNA immobilized onto a nitrocellulose filter. The signals for *MRP* and *γ-GCS* were compared using the actin signal as an internal reference. Although slight increases in the hybridization signals were detected in the treated sample, the increase was no more than on-half fold at best (Fig. 1B) (three experiments). These results suggest that posttranscriptional regulation was primarily responsible for the elevated levels of *MRP* and *γ-GCS* mRNA in the ACNU-treated cells.

Alteration of Intrinstrinc MRP and γ-GCS mRNA stabilities by ACNU treatment. Since alteration of mRNA stability may be an important factor contributing to the posttranscriptional accumulation of *MRP* and *γ-GCS* mRNA in the ACNU-treated cells, the stability of *MRP* and *γ-GCS* mRNA in ACNU-treated cells was compared with that in the untreated control using the RNA synthesis inhibitor Act D. In the absence of ACNU, the rates of *MRP* and *γ-GCS* mRNA degradation were similar (Fig. 2, right) with the half lives for both mRNA ($t_{1/2}$) of about 4 hr, as judged by the coordinate disappearance of the corresponding signals in the RNase protection assay. Addition of ACNU to the Act D-treated cells reduced the rates of *MRP* mRNA degradation ($t_{1/2} \cong 8$ hr). Furthermore, no increase of *MRP* mRNA was seen in the 2 hr and 4 hr-treated samples, whereas increased *γ-GCS* mRNA levels was seen in the 2 hr-treated sample. These results suggest that induction of *MRP* mRNA accumulation by ACNU may require RNA synthesis. These results also suggest that the effects of Act D on the induction of *MRP* and *γ-GCS* expression are different, despite the fact that ACNU can transiently induce both mRNA levels.

Effects of CHX on the induction of MRP and γ-GCS by ACNU. To investigate whether protein synthesis is required for posttranscriptional regulation of *MRP* and *γ-GCS* expression by ACNU, we measured *MRP* and *γ-GCS* mRNA levels in ACNU-treated cells in the presence of protein synthesis inhibitor CHX. As a control, levels of *MRP* and *γ-GCS* mRNA were also measured in cells treated with CHX alone (Fig. 3B). In contrast to that shown by the use of Act. D. (Fig. 2), induction of *MRP* and *γ-GCS* mRNA by ACNU could still be seen in the presence of CHX (Fig. 3A). These results indicate that the induction did not require *de novo* protein synthesis. The time-course dependence of rise and fall of *MRP* mRNA levels was also similar regardless whether CHX was added to the ACNU-treated samples (comparing between Figs. 3A and 1A). In contrast, levels of γ-GCS mRNA continued to increase throughout the entire 36 hr CHX treatments (Fig. 3A). This increase was due to the presence of CHX, since CHX alone could produce the same effect (Fig. 3B). These results demonstrate that

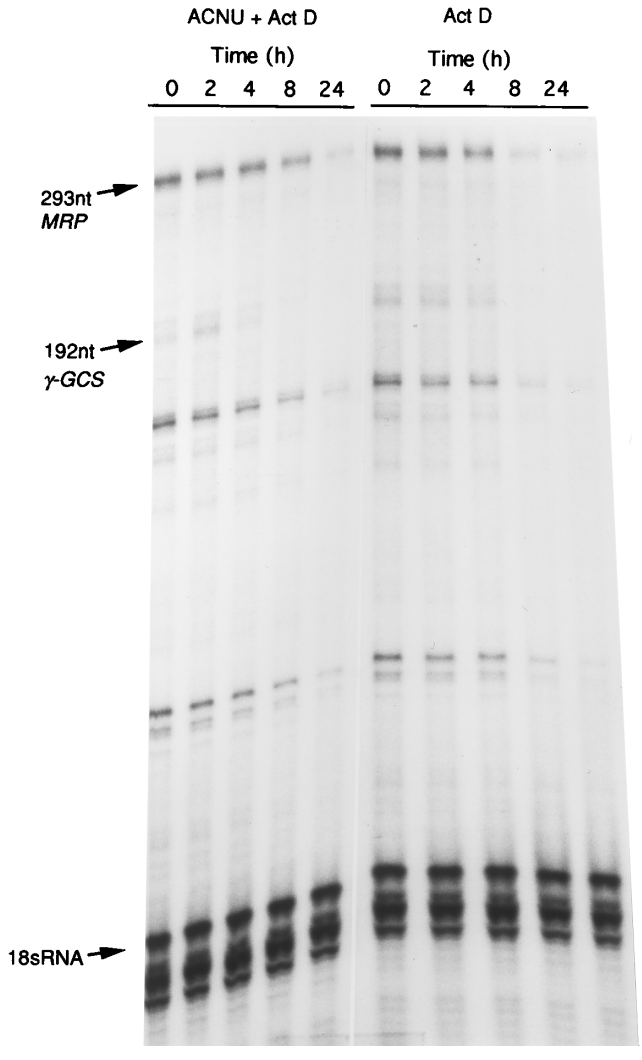

FIG. 2. Time-dependent changes in *MRP* and *γ-GCS* mRNA in A172 cells treated with ACNU together with Act D (left) or Act D alone (right). mRNA levels were measured by RNase protection assay as described in Fig. 1.

CHX has different effects on the expression of *MRP* and *γ-GCS* in A172 cells.

Posttranscriptional modulation of γ-GCS mRNA by CHX. The induction of γ-GCS mRNA expression by prolonged CHX treatments prompted us to investigate whether such induction was due to transcriptional or posttranscriptional control. Nuclear run-on analyses were performed to determine rates of transcription of *MRP* and *γ-GCS* in A172 cells treated with CHX for 0, 3, and 24 hr. No significant increase in transcriptional rate of *γ-GCS* was seen in cultured cells treated with CHX for either 3 or 24 hr (Fig. 3C), despite the fact that a several-fold increase in steady-state *γ-GCS* mRNA was detected in the 24 hr-treated cells (Fig. 3B). These results indicate that posttranscriptional regulation was primarily responsible for the increased *γ-GCS*

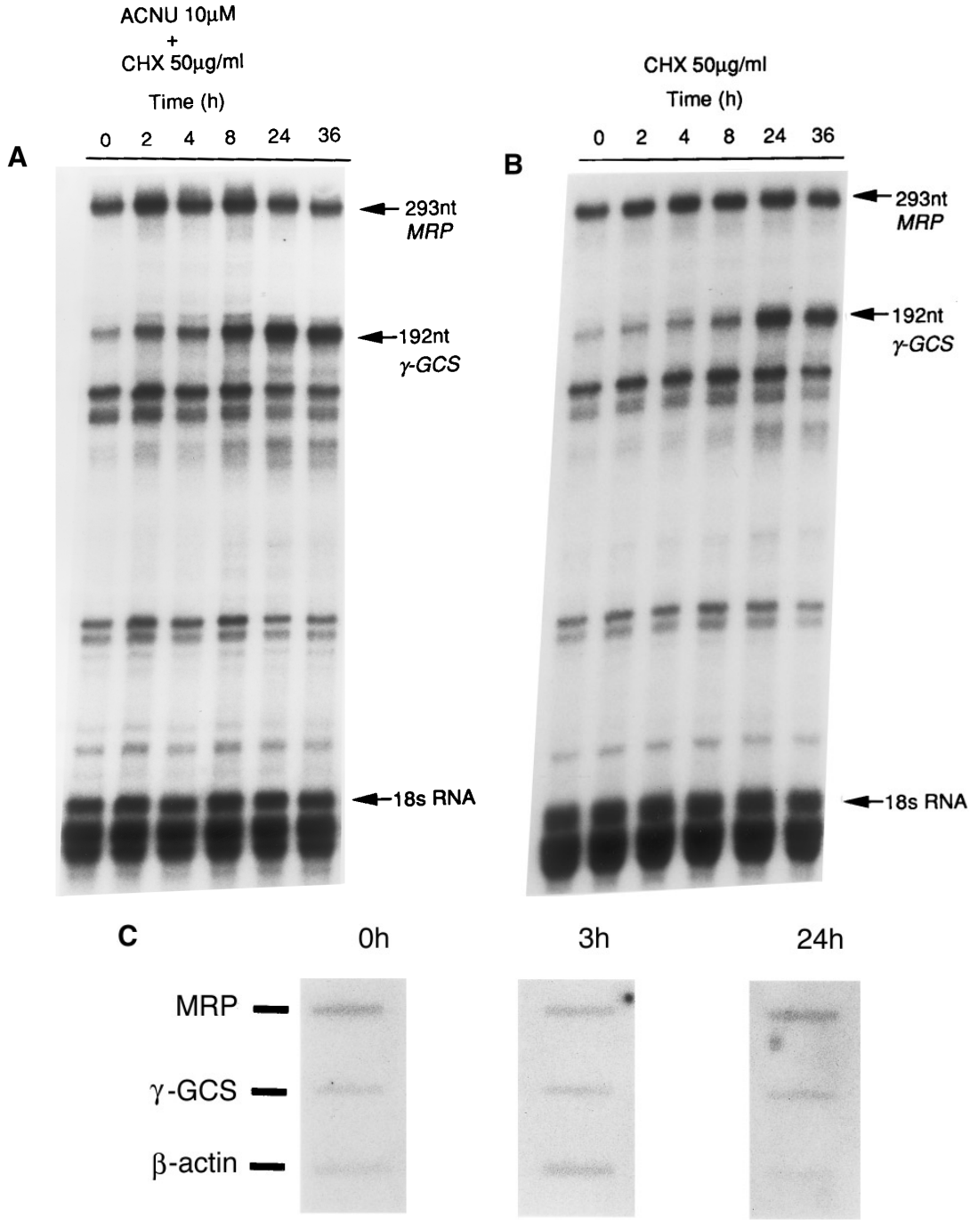

FIG. 3. Determination of steady-state levels of *MRP* and *γ-GCS* mRNA in A172 cells treated with ACNU plus CHX (A) or CHX alone (B). Transcription rates of *MRP* and *γ-GCS* in cells treated with CHX for 0, 3 and 24 hr were determined by nuclear run-on assays (C).

mRNA. Collectively, these results provide important information that posttranscriptional events play an important role for the unregulation of *MRP* and *γ-GCS* mRNA in ACNU- and CHX-treated cells.

DISCUSSION

The role of *MRP* in conferring multidrug resistance has been firmly established. The frequent coexpression of γ-GCS with MRP suggests a positive role of γ-GCS in the overall MRP-mediated drug resistance, although this remains to be critically investigated. At the present, the underlying mechanisms for the co-expression of these two important drug resistance-related genes in various cell settings remain elusive. The demonstration that expression of *MRP* and *γ-GCS* can be transiently induced by antitumor nitrosoureas described

here and in previous account (17) offers a convenient system for studying the regulatory mechanisms involved. The present communication reveals that the upregulation of *MRP* and *γ-GCS* expression by ACNU is primarily controlled at the post- transcriptional levels. A recent report revealed that both transcriptional and posttranscriptional regulations are involved in the induced expression of *γ-GCS* in HepG2 cells treated with radiation sensitizer, diethyl maleate (20). However, the expression of MRP in the diethyl maleate-treated cells was not mentioned in the study. To the best of our knowledge, this is the first report describing posttranscriptional control for the regulation of *MRP* and *γ-GCS* expression by antitumor nitrosourea.

Our present results provide important information to the steadily accumulating evidence that posttranscriptional regulation plays important roles in the modulation of many drug-resistance gene expression in cells treated with various cytotoxic substances and under various physiological conditions. For example, steady-state levels of *MDR* mRNA encoding the multidrug transporter of P-glycoprotein can be transiently induced in cultured cells by many chemotherapeutic agents (21, 22). Although transcriptional regulation has been noted, posttranscriptional regulation is primarily responsible for the observed increases of *MDR* mRNA. As consistent with our finding with γ-GCS reported here (Fig. 3), inhibition of protein synthesis by CHX could also enhance the steady-state level of *MDR* mRNA in cultured cells (23, 24). Although an initial report suggested that the enhancement was due to increased transcriptional rate (23), recent results have demonstrated that posttranscriptional control is primarily responsible (24, 25). Thus, it appears that there are overlapping mechanisms that control *MDR* and *MRP* expression. In experimental animal models, posttranscriptional regulation has also been observed for the upregulation of *mdr* gene expression during partial hepatectomy (21) and in the uterine epithelial cells during pregnancy (18). It would be of importance to determine whether similar mechanism is also involved in the increased *MRP* and *γ-GCS* mRNA levels in colorectal cancers (16).

Although our present results show posttranscriptional involvement in the induced expression of *MRP* and *γ-GCS* by ACNU and CHX, it remains possible that different mechanisms may be used for the regulation of these genes in various cell settings by different cytotoxic agents. It has been demonstrated that transcriptional regulation in primarily involved in the induced expression of *γ-GCS* in cultured cells treated with antioxidant and an antioxidant responsive element has been identified upstream of the *γ-GCS* promoter (26). We found that *MRP*, like *γ-GCS* (25 - 28), can be transiently co-induced in cultured cells by many antioxidants, i.e., butylhydroxyquinone, dimethoxy-1,4,-naphthoquinone, and menadione (our unpublished results). It would be of importance to investigate whether transcriptional and/or posttranscriptional regulation is involved in this regulation. These experiments, as well as those addressing the upregulation of *MRP* and *γ-GCS* in HL-60 cells by cisplatin and heavy metals as reported previously (13), are under way in our laboratories.

Finally, It remains critically important to determine whether *MRP* and *γ-GCS* mRNA levels are transiently elevated during chemotherapy in clinical settings, and if so, whether the induction is controlled by posttranscriptional events. The observations presented in this communication highlight the need to elucidate regulatory mechanisms of drug resistance gene expression. A better understanding of how the *MRP* and *γ-GCS* genes are upregulated under various cytotoxic insults, as well as whether *γ-GCS* actually involves in the *MRP*-mediated drug resistance, might provide valuable insights into circumvent the clinical problem of drug resistance in cancer chemotherapy.

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