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Transcriptional Up-regulation of γ-Glutamylcysteine Synthetase Gene Expression in Melphalan-Resistant Human Prostate Carcinoma Cells

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SUMMARY

Tumor cell resistance to many chemotherapeutic agents, including alkylating agents, cisplatin, and doxorubicin, is frequently associated with increased intracellular levels of the nonprotein sulfhydryl glutathione (GSH). Recent evidence has demonstrated that increased GSH levels can be accompanied by an increase in the activity of γ-glutamylcysteine synthetase (GCS), which catalyzes the rate-limiting step in *de novo* synthesis of GSH, and by an increase in the steady state level of mRNA for the catalytic subunit of GCS. Using melphalan-resistant DU 145/M4.5 human prostate carcinoma cells, which express elevated GSH levels, GCS enzyme activity, and GCS mRNA levels, we sought to determine the mechanism(s) responsible for the increased GCS mRNA expression. As determined by Northern analyses and RNase protection assays, the steady state level of GCS message in the resistant cells was increased 10–20-fold, in comparison with the drug-sensitive parent DU 145 cells. No significant difference in gene copy number or evidence of rearrangement was detected in the resistant cell line by Southern analyses. The GCS-specific mRNA isolated from the resistant cells was less stable than that isolated from the drug-sensitive cells (half-lives of 6 hr and 9 hr, respectively), indicating that this difference does not contribute to the increased steady state levels in the resistant cells. Nuclear run-on experiments revealed that the GCS transcription rate in the DU 145/M4.5 cells was increased approximately 12-fold, in comparison with that detected in the DU 145 cells. This difference in transcription rate was comparable in magnitude to the difference in steady state mRNA levels detectable in the two cell populations. Similar correlations between steady state GCS mRNA levels and transcription rates were also observed in other DU 145 lines expressing intermediate degrees of resistance to melphalan and correspondingly intermediate GCS mRNA elevations. These data suggest that GCS expression is transcriptionally regulated in these melphalan-resistant tumor cells.

One of the most common biochemical changes detected in tumor cells resistant to the cytotoxic effects of alkylating or platinating agents or anthracyclines, such as doxorubicin, is a significant increase in the intracellular concentration of the tripeptide GSH (1, 2). This abundant sulfhydryl has been implicated as an important component of the drug-resistant phenotype by virtue of its prevalence, its nucleophilic properties, its ability to protect against radical damage, and its ability to form conjugates with alkylating species either directly or via enyzmatic reactions catalyzed by various classes of glutathione *S*-transferases, which are themselves frequently elevated in resistant cells (3). A prominent role for GSH elevations in the expression of the drug-resistant phenotype is also suggested by studies that have demonstrated that reduction of GSH concentrations can sensitize resistant cells *in vivo* as well as *in vitro* (4–6). Despite a rather long history documenting elevations of GSH in drug-resistant cells, until recently mechanisms responsible for the GSH increases were not well characterized, particularly at the molecular level.

GSH is synthesized from its constituent amino acids in two sequential, ATP-dependent, enzymatic reactions, catalyzed by GCS and GSH synthetase (7). GCS is the rate-limiting step in *de novo* GSH synthesis and is the point of GSH feedback inhibition. The functional GCS holoenzyme consists of regulatory (light, M_r 27,700) and catalytic (heavy, M_r 73,000) subunits (8, 9). We cloned the cDNA for the human liver GCS catalytic subunit (10) and recently reported that elevated levels of GSH were accompanied by an increase in GCS activity in a series of melphalan-resistant prostate carcinoma cell lines (11), as well as in melphalan-resistant myeloma cells (12). These changes in enzyme activity were associated with a corresponding increase in the steady state levels of mRNA specific for the heavy catalytic subunit of GCS. A similar increase in GCS heavy subunit mRNA has been observed in a panel of cisplatin-resistant human ovarian cell lines (13), as well as in a chlor-

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ABBREVIATIONS: GSH, glutathione; GCS, γ-glutamylcysteine synthetase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; bp, base pair(s).

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ambucil-resistant HT-29 human colon carcinoma line.[1] Although light subunit mRNA levels were not examined in previous studies, we have also recently detected increased levels of mRNA species for the GCS light subunit in both the DU 145 prostate carcinoma series and chlorambucil-resistant HT-29 cells.[2]

These data suggest that increased expression of the genes coding for both of the subunits of the GCS holoenzyme may be involved in the increase in GCS activity and the corresponding increase in GSH levels observed in these models of resistance. However, the mechanisms responsible for the increased mRNA steady state levels have not been determined. In the current study we used the melphalan-resistant DU 145/M4.5 prostate carcinoma line to examine possible mechanisms responsible for the increase in steady state GCS heavy subunit mRNA levels. The DU 145/M4.5 line was the most resistant line derived from wild-type DU 145 cells by growth in the presence of progressively increasing concentrations of melphalan over a 2-year period (14). And, although resistance to melphalan in these cells was undoubtedly multifactorial, the cells expressed increased GSH levels, elevated GCS activity, and a 10–20-fold increase in GCS mRNA (11). Therefore, in an initial attempt to define the molecular events associated with drug-induced GSH elevations, we compared gene copy number, message stability, and rate of transcription in drug-resistant cells with those measured in the drug-sensitive DU 145 parent line. The results indicate that the increase in mRNA expression is the result of an increased rate of heavy subunit gene transcription.

Experimental Procedures

Cell culture. The parent and drug-resistant DU 145 human prostate carcinoma cells were maintained as detailed previously (14). Briefly, the cells were maintained in exponential growth in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum and gentamycin. Cultures were maintained at 37° in a humidified atmosphere of 95% air/5% CO_2. Cells were passaged weekly, and medium was changed at 3–4-day intervals. Twenty-four hours after passage, melphalan was added to the culture medium to final concentrations of 10^{-6}, 10^{-5}, and 5×10^{-5} M for the DU 145/M6, DU 145/M5, and DU 145/M4.5 drug-resistant lines, respectively. An additional cell line, designated DU 145/$M5_{cst}$, was derived from DU 145/M5 cells by growth in culture without drug selection pressure for a period of >2 years.

DNA analysis. Genomic DNA was isolated from DU 145 cells using the Qiagen genomic DNA kit (Qiagen, Chatsworth, CA). For Southern analysis, genomic DNA was incubated with restriction enzymes, electrophoresed on 1.2% agarose gels, and transferred overnight to GeneScreen-Plus membranes (DuPont, Boston, MA). After prehybridization under standard conditions (15), the membranes were hybridized at 42° overnight with a ^{32}P-labeled 762-bp probe isolated from the GCS cDNA after digestion with *Pst*I (10). The probe was labeled using the Prime-a-Gene random primer technique (Promega, Madison, WI). The membranes were washed twice in 2× SSC at room temperature for 15 min each, twice in 2× SSC with 1% SDS at 65° for 30 min each, and twice in 0.1× SSC at room temperature for 15 min each. Bands were visualized using a PhosphorImager, and relative intensities were calculated using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). To correct for possible differences in the amount of DNA loaded for the sensitive and resistant cells, the membranes were stripped in boiling distilled water and hybridized, as described above, to a β-actin-specific probe labeled with ^{32}P, as described above. Comparisons of relative GCS signal intensities were made after normalization to the β-actin signal.

RNA analysis. Total RNA was isolated from cultured cells using TRI-Reagent (Molecular Research Center, Inc., Cincinnati, OH), according to the manufacturer's protocol. Levels of GCS mRNA were quantitated by RNase protection using the RPA II protection assay (Ambion, Austin, TX), essentially as described by the manufacturer. A ^{32}P-labeled 301-bp RNA probe was generated by T7 polymerase transcription of a 764-bp *Pst*I GCS cDNA fragment cloned into the pALTER-1 vector (Promega) and linearized with *Nco*I. When incubated with RNA isolated from the DU 145 cells, this probe produced a 253-bp protected fragment corresponding to positions +1375 to +1628 of the GCS heavy subunit cDNA (10). Human GAPDH mRNA levels were quantitated using the pTRI-GAPDH-human antisense control template linearized with *Hind*III (Ambion). After hybridization of the probes with cellular RNA and digestion with RNase A and T1, the protected fragments were separated on 5% acrylamide/8 M urea gels. The bands were visualized and intensities were quantitated using a PhosphorImager and ImageQuant software.

Stability of GCS mRNA. Logarithmically growing DU 145 and DU 145/M4.5 cells were treated with actinomycin D (10 μg/ml) to inhibit RNA synthesis. Total RNA was then isolated from cells harvested at various times after addition of actinomycin D and levels of GCS mRNA were quantitated by RNase protection assay as described above. Message half-life was calculated by linear regression of the fraction of GCS-specific transcripts present as a function of time, after normalization to GAPDH expression to control for potential loading differences. The slopes of the resulting regression lines were compared by analyses of covariance (16).

Nuclear run-on assays. Relative GCS transcription rates for the drug-sensitive and -resistant DU 145 cells were determined essentially as described by Celano *et al.* (17, 18). Nuclei were isolated according to the method of Ausubel *et al.* (15) and were stored at −70° in glycerol storage buffer (50 mM Tris·HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA) before assay. Nuclei ($0.5–1.0 \times 10^7$) were resuspended in 200 μl of reaction buffer consisting of 35% glycerol, 10 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, 80 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 4 mM ATP, 4 mM GTP, 4 mM CTP, and 200 μCi of [α-^{32}P]UTP (3000 Ci/nmol) and were incubated at 26° for 10 min. Nuclei were then digested with 10 μl of RNase-free DNase I and 10 μl of $CaCl_2$ (20 mM) at 26° for 5 min, followed by the addition of 2 μl of proteinase K (10 mg/ml), 25 μl of 10× SET (5% SDS, 50 mM EDTA, 100 mM Tris·HCl, pH 7.4), and 5 μl of yeast tRNA (10 mg/ml) and incubation at 37° for 30 min. After digestion, the RNA was isolated by the addition of 550 μl of solution G (4 M guanidinium thiocyanate, 25 mM sodium citrate, (pH 7.0), 0.5% sarcosyl, 0.1 M 2-mercaptoethanol), 90 μl of 2.0 M sodium acetate, (pH 4.0), 900 μl of water-saturated phenol, and 180 μl of chloroform/isoamyl alcohol (19:1). This mixture was incubated on ice for 15 min and then centrifuged at 12,000 × *g* at 4°. The supernatant was combined with an equal volume of isopropanol and incubated overnight at −70°. After centrifugation, pellets were solubilized in 300 μl of solution G and reprecipitated with 300 μl of isopropanol. Pellets were washed in 70% ethanol and dissolved in 10 mM Tris·HCl (pH 7.2), 1 mM EDTA, 0.1% SDS.

Twenty-five micrograms of the plasmids pSK-Bluescript (Promega), pSK-GCS50, and pβ-actin were vacuum-blotted onto nitrocellulose membranes using a BRL Hybri-Slot manifold, according to the manufacturer's protocol. The plasmid pSK-GCS50 contains the full length cDNA for human liver GCS (10) cloned into the *Eco*RI/*Xho*I sites of pSK-Bluescript. Squares of nitrocellulose membrane containing each of the three DNA samples and a slot containing 25 μg of rRNA were prehybridized in scintillation vials overnight at 65°, in 1.0 ml of prehybridization buffer consisting of 10 mM TES, 0.2% SDS, 10 mM EDTA, 0.6 M NaCl, and 250 μg of yeast tRNA in 1× Denhardt's solution. rRNA was slotted to help minimize nonspecific binding. After addition of equal counts of labeled RNA from the transcription assays,

[1] R. T. Mulcahy and H. H. Bailey, unpublished observations.

[2] R. T. Mulcahy, Over-expression of mRNA for light and heavy subunits of γ-Glutamylcysteine synthetase in drug-resistant human tumor cell lines, manuscript in preparation.

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the hybridization mixture was brought to a final volume of 2.0 ml by the addition of TES. The membranes were hybridized for 48 hr at 65°, washed twice for 1 hr at 65° in 2× SSC and once for 30 min at 37° in 2× SSC, and air-dried. The slots were visualized and intensities were quantitated using a PhosphorImager and ImageQuant software.

Results

The biochemical properties of the DU 145 prostate carcinoma cells used in these studies are summarized in Table 1. The resistant DU 145/M4.5 cells consistently expressed 10–20-fold higher levels of the message for the GCS heavy subunit, relative to the parent cells. DNA was collected from both the sensitive DU 145 cells and the resistant DU 145/M4.5 cells and was subjected to Southern analyses to compare relative gene copy number and to determine whether gross gene rearrangements existed in the resistant cells. Fig. 1 shows the results of Southern analysis using a 764-bp GCS probe hybridized to genomic DNA cut with each of several restriction enzymes. The same numbers of bands, of comparable sizes, were detected in the DNA isolated from both cell types. After normalization for the amount of DNA loaded in each lane of the gel, as determined by hybridization of the membranes to a β-actin probe, quantitation of the amount of radioactivity hybridized to corresponding bands for each restriction digest revealed no significant differences in GCS-specific DNA sequences. These results suggest that increased steady state mRNA levels in the DU 145/M4.5 cells are not the result of increased gene copy number. The data also suggest that the GCS heavy subunit gene in the resistant cells was not overexpressed as a result of rearrangement or translocation. Such rearrangement would have been expected to result in changes in banding patterns of the Southern blots.

To determine whether the increased levels of GCS mRNA were the result of increased message stability, total RNA was isolated from sensitive and resistant cells at various times after treatment with actinomycin D to inhibit RNA synthesis. Using an RNase protection assay, the amount of GCS-specific message was quantitated as a function of time after actinomycin D addition. The relative amount of GCS mRNA at each time point was quantitated after normalization using GAPDH. No discernible change in the level of GAPDH expression was detected over the time course evaluated in these experiments. The results of three independent determinations are shown in Fig. 2. GCS mRNA levels decreased linearly in both cell types, but the rate of mRNA degradation was greater in the resistant DU 145/M4.5 cells. Analysis of variance revealed that the rates of decay of GCS mRNA in the two cell lines were significantly different ($p < 0.05$). Based on linear regression analysis, half-lifes of 6 and 9 hr were calculated for the resistant and sensitive cells, respectively. The greater instability of the mRNA isolated from the resistant cells indicates that the increased mRNA levels characteristic of the drug-resistant cells are not the result of greater stability.

The GCS probe used in these studies hybridizes with two RNA species, 3.2 and 4.0 kilobases in length (11). Consequently, the RNase protection assay used to measure mRNA stability does not permit discrimination of the stability of the two individual mRNAs but, rather, provides information on the composite stability of both species. We therefore also examined mRNA stability using Northern analysis. These studies revealed that the 3.2- and 4.0-kilobase messages decayed at similar rates in the two cell lines (data not shown).

Relative rates of GCS mRNA transcription were quantitated for each of the five DU 145 cell lines by nuclear run-on analysis (Fig. 3). This analysis revealed that the rate of GCS transcription in the drug-resistant DU 145/M4.5 cells was on average ~12 times greater than that detected in the drug-sensitive parent cells. The magnitude of the difference in transcription rate is comparable to the differences in steady state mRNA levels observed in the two cell types. Similarly, run-on analysis of the other resistant cell lines in the DU 145 series revealed increased transcription rates approximately proportional to the levels of mRNA detected in each line by Northern analysis. The rate of GCS transcription in DU 145/$M5_{out}$ cells, DU 145/M5 cells that have been passaged in the absence of melphalan exposure for several years and that express GCS mRNA levels, GCS enzyme activity, and GSH levels comparable to those of control DU 145 cells (11), had likewise returned to control levels. These data strongly implicate up-regulation of transcription as the basis for the increase in GCS steady state levels detected in this series of melphalan-resistant prostate carcinoma cells.

It should be noted that the rate of GCS transcription in DU 145/M6 cells was approximately 2-fold greater than that detected in the DU 145/M5 cells, despite the fact that steady state mRNA levels historically have been approximately 2-fold greater in the more resistant DU 145/M5 cells (Ref. 11 and Table 1). Recent Northern, RNase protection, and reverse transcription-polymerase chain reaction analyses, however, have indeed revealed that steady state GCS mRNA levels are approximately 2-fold greater in the DU 145/M6 cells than in the DU 145/M5 cells, suggesting that the original DU 145/M5 and DU 145/M6 lines may have been reversed at some time during recent passage.

Discussion

Elevations in intracellular levels of the protectant sulfhydryl GSH have long been implicated in resistance to many antican-

TABLE 1

Summary of GSH and GCS characteristics of melphalan-resistant Du 145 human prostate carcinoma cells

Data were compiled from Refs. 11 and 14. Numbers in parentheses refer to fold increases, relative to DU 145 cells. Values are mean ± standard error.

Cell line	IC_{50}	GSH level	GCS activity	Relative GCS mRNA level
	μM	*nmol/mg of protein*	*nmol/min/mg of protein*	
DU 145	3	117.6 ± 12	0.6 ± 0.1	1
DU 145/M6	10 (3.3)	190.7 ± 10[b] (1.6)	1.7 ± 0.3[b] (2.8)	~3
DU 145/M5	20 (6.7)	186.0 ± 8.2[b] (1.6)	1.4 ± 0.2[b] (2.3)	~5
DU 145/M4.5	80 (26.7)	288.0 ± 7.4[b] (2.4)	8.8 ± 1.2[b] (14.7)	~10–20
DU 145/$M5_{out}$	9 (3.0)	120.5 ± 12 (1.0)	0.6 ± 0.1 (1.0)	1

[a] Dose causing 50% inhibition, as determined by tetrazolium dye assay.
[b] Significantly different from DU 145 cells ($p < 0.02$).

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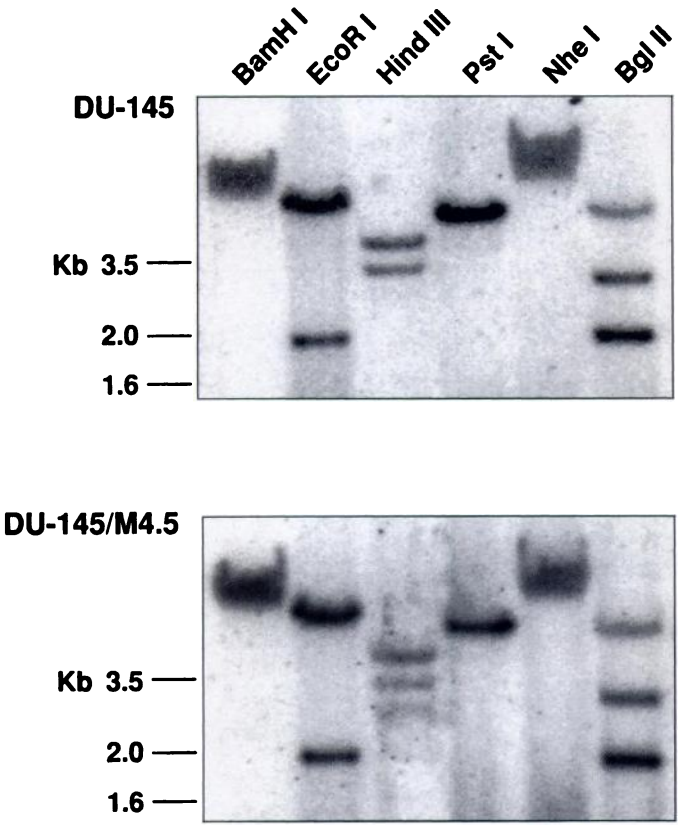

Fig. 1. Southern analysis of genomic DNA isolated from melphalan-sensitive DU 145 prostate carcinoma cells (*top*) and melphalan-resistant DU 145/M4.5 cells (*bottom*). After digestion with the indicated restriction enzymes, 10 μg of DNA were loaded in each well and subjected to electrophoresis through a 1.2% agarose gel. After transfer of the DNA to membranes, the membranes were hybridized with a ^{32}P-labeled GCS-specific probe corresponding to a 764-bp *Pst*I fragment of GCS cDNA. Bands were visualized using a Molecular Dynamics PhosphorImager. *Numbers to the left*, migration of molecular weight markers.

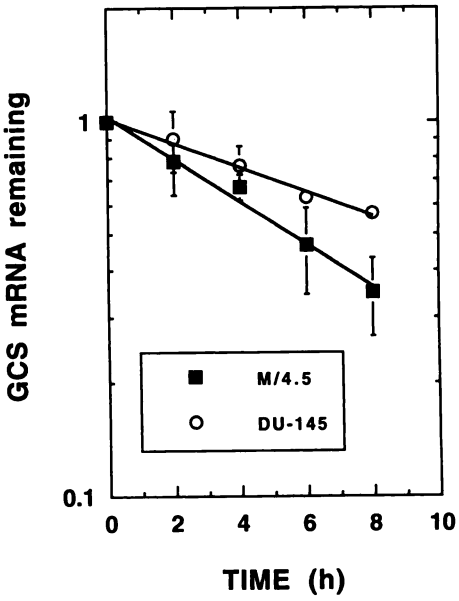

Fig. 2. Stability of GCS-specific mRNA in DU 145 and DU 145/M4.5 cells. DU 145 and DU 145/M4.5 cells were incubated with 10 μg/ml actinomycin D dissolved in culture medium. At the indicated times after actinomycin D addition, total RNA was harvested from the cells. The quantity of GCS-specific mRNA in each sample was determined by RNase protection assay, as described in the text, and plotted as a fraction of the amount present at the time of actinomycin D addition. Each *point* represents the mean of three independent determinations; *error bars*, standard deviations. *Solid lines*, best-fit regression lines determined by linear least-squares analysis.

cer agents, including alkylating agents, cisplatin, and doxorubicin. Until recently, however, the molecular events responsible for GSH elevations typical of many drug-resistant cells have not been characterized. An increase in GCS activity, the rate-limiting step in the *de novo* synthesis of GSH (7), and an increase in steady state mRNA levels for the catalytic subunit of GCS have recently been detected in several cell lines expressing drug-resistant phenotypes characterized by an increase in intracellular GSH levels (11–13). We have also detected a parallel increase in the steady state levels of GCS regulatory subunit mRNA in our drug-resistant models.[3] The evidence suggests that regulation of this key regulatory enzyme in the GSH synthetic pathway might be involved in drug resistance. The current studies are consistent with this hypothesis and strongly implicate up-regulation of transcription of the GCS catalytic subunit gene as an important event in the emergence of a GSH-associated drug-resistant phenotype.

In contrast to the multidrug-resistant phenotype involving overexpression of the *mdr1* gene, selection of melphalan-resistant cell lines by growth in increasing concentrations of drug did not result in amplification of the GCS catalytic subunit gene. Although Southern analyses were restricted to DNA isolated from the parent DU 145 cells and the highly resistant DU 145/M4.5 line, the two extremes of the drug response spectrum, increases in gene copy number or gross rearrangements are not likely to be involved in the increased GCS mRNA expression observed in either the DU 145/M6 or DU 145/M5 line, because these lines represent earlier stages in the selection process culminating in the selection of the DU 145/M4.5 cell line (14).

Although an increase in the stability of the GCS message could result in the observed increase in steady state levels, this is clearly not the case for the DU 145/M4.5 cells. In fact, the GCS message is apparently less stable in these cells than in the wild-type population. Rather, nuclear run-on experiments indicate that the changes in GCS mRNA expression result from an increase in the rate of transcription in the drug-resistant cells. In each of the three resistant cell lines, the transcription rate was increased approximately in proportion to the magnitude of the increase in their respective GCS steady state levels, suggesting that up-regulation of transcription is responsible for increased mRNA expression. This possibility is further supported by the data for the DU 145/M5$_{out}$ revertant line.

The mechanisms responsible for up-regulation of GCS transcription and increased GSH levels in response to chronic drug exposure remain to be identified, but an acceptable working model must accommodate a few observations. First, considering the drug exposure schedule used to maintain the resistant phenotypes (drug exposure once each week), the extremely short half-life of melphalan in culture medium, and the half-life of the GCS message, transcriptional up-regulation must be prolonged in these cells; otherwise, Northern analyses would be expected to reveal cyclic changes in mRNA levels as a function of time between dosing. This has not been the case; mRNA levels are relatively constant for each cell line. This

[3] R. T. Mulcahy and H. H. Bailey, unpublished observations.

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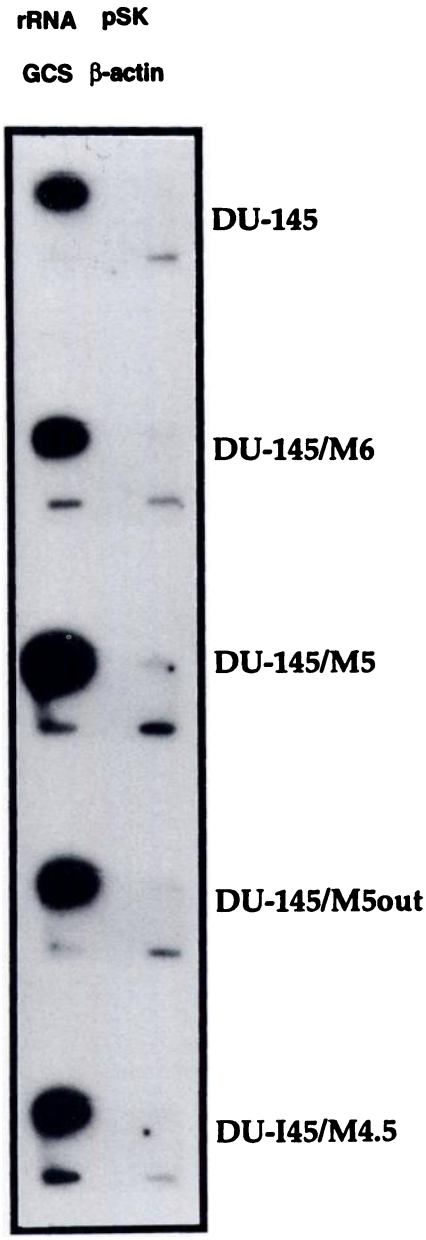

Fig. 3. Increased rate of GCS transcription in melphalan-resistant DU 145 cells, as determined by nuclear run-on analysis. Twenty-five micrograms of the plasmids pSK-Bluescript (*pSK*), pSK-GCS50 (*GCS*), and pβ-actin (*β-actin*) were vacuum-blotted onto nitrocellulose membranes. Squares of nitrocellulose membrane containing each of the three DNA samples and a slot containing 25 μg of rRNA (*rRNA*) were hybridized for 48 hr at 65° with equal counts of ^{32}P-labeled RNA from transcription reactions for each of the cell lines in the DU 145 series. The membranes were washed and air-dried. The slots were visualized and intensities were quantitated using a PhosphorImager and ImageQuant software.

suggests that if *trans*-acting factors are involved in increased GCS gene expression, as we hypothesize they are, then they must persist, be regenerated, or exert long-lasting effects (i.e., changes in methylation patterns) after each drug exposure. This constitutively elevated expression in the absence of continuous exposure to the inducing agent is analogous to the expression of certain phase II enzymes in liver nodules arising during chemically induced hepatocarcinogenesis (19). Second, the molecular changes responsible for increased transcription, at least in the drug-resistant DU 145/M5 cells, are not permanent and can be down-regulated if selection pressure is eliminated. Finally, even though transcription of the GCS catalytic subunit gene and GCS activity are increased, GSH levels do not differ significantly among the resistant lines, indicating that some level of post-transcriptional or post-translational control of GSH levels exists (11, 13).

Based upon the data presented, we hypothesize that specific *cis*- and *trans*-acting factors regulate GCS gene expression in response to chemotherapeutic agents, although their identification will ultimately require cloning and characterization of the GCS catalytic subunit gene and its 5′ flanking sequences. Towards this aim, we recently succeeded in cloning the gene encoding the human GCS catalytic subunit and have identified several putative response elements in the 5′ flanking region of the gene.[3] The functional significance of these putative regulatory sequences in responses to chemotherapeutic drugs is currently being investigated, but it is tempting to speculate that they may be involved in increased GCS expression in alkylator- and cisplatin-resistant cell lines.

Although the current studies clearly do not permit identification of specific mechanisms involved, they do establish that transcription of the gene for the rate-limiting enzyme in GSH synthesis is increased in response to chronic exposure to melphalan. The availability of the 5′ flanking sequences of the GCS catalytic subunit gene should facilitate identification of those *cis*- and *trans*-acting factors that are operative in drug-resistant phenotypes characterized by elevated intracellular GSH levels. Such characterizations are in progress.

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