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Evidence for Altered Regulation of γ-Glutamylcysteine Synthetase Gene Expression among Cisplatin-sensitive and Cisplatin-resistant Human Ovarian Cancer Cell Lines[1]

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ABSTRACT

We have shown previously that tumor cell resistance to cisplatin is associated with elevated intracellular levels of glutathione, which is accomplished at least in part by increased expression of the heavy subunit of γ-glutamylcysteine synthetase (γ-GCS). To investigate the mechanism by which γ-GCS expression is elevated, we have examined four related human ovarian cancer cell lines with increasing cisplatin resistance. Relative amounts of steady-state γ-GCS mRNA in CP70, C30, and C200 were 4.8, 6.0, and 10.6, respectively, compared to the parental A2780 cell line, and a proportional increase in the transcriptional rate but not RNA stability was demonstrated. In contrast, no increase in mRNA for the γ-GCS light subunit was found. To determine the mechanism of up-regulation of this mRNA, we have cloned the promoter of the gene that encodes the heavy subunit of γ-GCS. This region contains AP-1, NF-κB, XRE, AP-2, EpRE, CAAT, and TATA box elements upstream of the transcription initiation site and two MREs between this site and the start codon for the protein. Using gel mobility shift assays, we have found nuclear extract binding activity to the AP-1 response element to be closely associated with the level of γ-*GCS* gene expression. A supershift assay showed that the AP-1 DNA-binding complexes are predominantly formed by dimers of JUN family members. Consistent with this finding, the expression of c-*JUN* was found to be elevated in the resistant cells. In contrast to AP-1 binding, AP-2 and NF-κB binding were inversely related to resistance. Furthermore, we have examined a partial revertant of the C200-resistant cells, which shows lower glutathione levels, and found decreased γ-GCS expression associated with decreased AP-1 binding activity.

INTRODUCTION

It is generally considered that GSH,[3] as the most prevalent cellular nonprotein thiol, is critical to homeostasis by nature of its ability to assist in maintaining a reducing environment within the cell and its ability to inactivate many xenobiotics (1, 2). As such, it is not surprising that up-regulation of GSH is often associated with resistance of cancer cells to many chemotherapeutic agents including cisplatin and classical alkylating agents (3–7). There are many data to indicate that GSH contributes to resistance. The conjugation of drugs to GSH may inactive them (8, 9) and in some cases facilitate their excretion from cells (9, 10). GSH may also contribute to resistance indirectly by acting as a cofactor for DNA repair enzymes (11) and maintenance of DNA damage recognition proteins in a reduced/active state (12). It is of interest that induction/selection of cancer cells for alkylating agent and cisplatin resistance results in the constitutive up-regulation of GSH. This is in contrast to the increasingly well characterized induced response to environmental stress, which involves the transient increased expression of a large number of gene products including GSH (13–16).

The significance of GSH to homeostasis, along with its role in the major clinical problem of anticancer drug resistance, creates an important rationale for investigation of the mechanism of up-regulation of this abundant tripeptide. The synthesis of GSH is accomplished by the sequential actions of γ-GCS and GSH synthetase with γ-glutamyltranspeptidase at least in part responsible for maintenance of precursor pools (1, 2, 17). In normal cellular systems, the rate-limiting enzyme in GSH synthesis is γ-GCS, which is composed of a light and heavy subunit (18). It is believed that the heavy subunit contains the catalytic activity of the enzyme, whereas the light subunit has been suggested to serve a regulatory function (18). In ovarian cancer cell lines selected *in vitro* for low to high levels of cisplatin resistance (~10–1000-fold), we have shown that GSH increases linearly with resistance culminating in a near 50-fold increase in steady-state cellular GSH in the most resistant cells (5, 19). These data in part support the causal role in alkylating agent and cisplatin resistance generally attributed to GSH. As a step toward gaining an understanding of the mechanism of this up-regulation, we have measured steady-state mRNA levels for the enzymes involved in GSH synthesis. We observed greater than 10-fold increases in γ-glutamyltranspeptidase and the heavy subunit of γ-GCS transcript levels (5). Here, we describe the mechanism of up-regulation of the heavy subunit of γ-GCS including the cloning and evaluation of the 5′-regulatory sequences upstream of the first exon of the gene.

MATERIALS AND METHODS

Materials. Cisplatin was obtained from Bristol-Myers Squibb (Syracuse, NY). Chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. Cell culture reagents were obtained from GIBCO (Grand Island, NY).

Cell Lines and Culture. To initiate CP70, C30, and C200, the A2780 ovarian cancer cell line derived from an untreated patient was exposed to cisplatin using protocols of varying intensity. CP70 was selected by intermittent exposure to incrementally increasing amounts of drug (8, 20, and 70 μM), culminating in exposure to 70 μM cisplatin on 3 separate occasions for 3 days. C30 and C200 were derived from CP70 by near continuous exposure to drug, culminating with 30 μM or 200 μM cisplatin for >4 years. Efforts to develop revertants of C200 were initiated in January 1991 by removal of the selection pressure. The partial revertant cells utilized here, PREV, were maintained in cisplatin-free medium for 3.5 years, whereas the control for PREV referred to as CPREV were C200 cells subjected to cryopreservation in January 1991 at the time PREV was initiated.

Cell lines were maintained at 37°C in a humidified incubator containing 5% CO_2 in air. The culture medium, RPMI 1640, was supplemented with 10% (v/v) FCS, 100 μg/ml streptomycin, 100 units/ml penicillin, 0.3 mg/ml glutamine, and 0.3 unit/ml insulin (porcine). Unless otherwise stated, cells were utilized 40–48 h after subculture.

Cytotoxicity. Cisplatin cytotoxicity was determined using the micro tetrazolium assay as we have described previously (20). The reported IC_{50}s are the result of triplicate determinations on at least two separate occasions.

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[3] The abbreviations used are: GSH, glutathione; γ-GCS, γ-glutamylcysteine synthetase; kbp, kilobase pair; PMSF, phenylmethylsulfonyl fluoride; EMSA, electrophoretic mobility shift assay.

Gene Expression Analysis. The total RNA was isolated from cells based on the one-step guanidinium method described by Chomczynski and Sacchi (21), and Northern blots prepared and analyzed as we have described previously (5). The probes used were as follows: (*a*) a 3.7-kbp insert of a human γ-GCS cDNA probe containing the full-length cDNA for the coding region of γ-GCS heavy subunit gene (5) and (*b*) a 503-bp PCR product generated from a C30 human cDNA library based on the cDNA sequence for the rat γ-*GCS* light subunit gene (Genbank/EMBL Data Bank No. L22191). Additionally, we utilized: (*a*) a 1.8-kbp *Bam*HI-*Eco*RI insert of a human c-*JUN* probe containing a 1.0-kbp coding sequence and 0.8 kbp of 3′-untranslated sequence (22); (*b*) a 1.0-kbp *Xho*1 fragment of a human c-*FOS* cDNA (23); and (*c*) a 2.0-kbp *Pst*1 cDNA fragment of chicken β-actin (20). Autoradiography was carried out at −70°C for 3–4 days. The blot was subsequently stripped and reprobed. The intensity of β-actin labeling was used in normalizing values to provide a measure of the relative expression of the mRNA of interest.

γ-GCS mRNA Stability. Cells were treated with actinomycin D (10 μg/ml, Sigma) to inhibit RNA synthesis. Total RNA prepared from cells harvested at various times after treatment was separated on a 1% denaturing agarose gel and transferred to a nylon membrane as described previously. Blots were probed for γ-GCS and β-actin and quantitated by densitometric scanning. The half-life of γ-GCS mRNA was calculated by linear regression of the fractions of γ-GCS-specific transcripts present as a function of time, after normalization to β-actin expression. The slopes of the resulting regression lines were compared by analyses of covariance.

Nuclear Run-on Assay. A simplification of the nuclear run-on assay described by Greenberg (24) was used. Nuclei (10^8/reaction) in a total volume of 200 μl of transcription buffer containing 4 mM ATP, GTP, and CTP and 200 μCi of [α-^{32}P]UTP (3000 Ci/nmol; Amersham, Arlington Height, IL) were incubated at 26°C for 10 min, followed by the addition of 10 μl $CaCl_2$ (20 mM) and digestion by addition of 10 μl of RNase-free DNase 1 (10 mg/ml) at 26°C for 5 min. Samples were then heated with 2 μl of proteinase K (10 mg/ml), 15 μl of 10× 5% SDS-50 mM EDTA-10 mM Tris-HCl (pH 7.4), and 5 μl of yeast tRNA (10 mg/ml) at 37°C for 30 min. nRNA was isolated by the addition of 550 μl of 4 M guanidinium isothiocyanate-25 mM sodium citrate (pH 7.0)-0.5% sarkosyl-0.1 M 2-mercaptoethanol-9.0 μl of 2 M sodium acetate (pH 4.0) containing 0.1 mM aurintricarboxylic acid (pH 4.0), followed by extraction with 900 μl of aurintricarboxylic acid-buffered-phenol (pH 4.0), and followed by extraction with 180 μl of chloroform:isoamyl alcohol (24:1). The mixture was incubated on ice for 15 min and centrifuged for 15 min at 12,000 × *g* at 4°C, and the aqueous layer was collected and precipitated by an addition of an equal volume of isopropanol. The RNA was dissolved in 300 μl of guanidinium isothiocyanate solution and reprecipitated with 300 μl of isopropanol. After the sample was centrifuged, the pellet was washed by 70% ethanol and dissolved in TE buffer [10 mM Tris-HCl (pH 7.2)-1 mM EDTA-0.1% SDS].

Quantitation of the γ-GCS transcriptional rate was measured by the slot blot method. The DNA probes for γ-GCS and β-actin used for these studies were purified as described above. The probes were denatured by boiling and blotted (2 μg/blot) onto a prewet (6× SSC) nylon membrane. After application of the DNA, the membrane was washed again with 5.0 ml of 6× SSC and dried at room temperature. The membrane was baked at 80°C for 1 h in a vacuum oven. The α-^{32}P-labeled nRNA described above was added to 3 ml of hybridization buffer and hybridized to the membrane for 24 h at 42°C. The membranes were washed in 2× SSC-1% SDS at 65°C for 1 h, and in 0.1× SSC-0.1% SDS at room temperature for 1 h. Autoradiography was performed at −70°C. Quantitation was achieved by densitometric scanning, and data were normalized based on the signal for β-actin.

Cloning of the Human γ-GCS Promoter Region. To clone the 5′-regulatory portion of the gene, which encodes the heavy subunit of γ-GCS, we screened a human fetal brain genomic library (Stratagene) with a probe generated from the 5′-untranslated region of our cDNA clone for the heavy subunit of γ-GCS. This screening yielded a genomic clone bearing a 9.5-kbp insert. Partial sequencing revealed this fragment contained the first exon and first intron of the heavy subunit gene, along with nearly 2 kbp of sequence upstream of the transcription start site (see Fig. 1*A*).

Nuclear Extract Preparation. The nuclear extracts were prepared by the procedure of Dignam *et al.* (25). Briefly, the pelleted cells were resuspended in 1.5 volumes of lysis buffer [25 mM HEPES (pH 7.5)-70 mM KCl-1.5 mM $MgCl_2$-0.5 mM sodium orthovanadate-0.4 mM NaF-0.5 mM PMSF-1.0 mM DTT]. The mixture was incubated on ice for 20 min and then extracted by adding of 1.6 volumes of extraction buffer [25 mM HEPES (pH 7.5)-0.5 mM EDTA-20% glycerol-1.66 M KCl-0.4 mM NaF-0.4 mM sodium orthovanadate-0.1 mM PMSF-1.0 mM DTT] with constant shaking at 4°C for 4–5 h. Samples were centrifuged at 55,000 × *g* for 1 h at 4°C, and the supernatant was dialyzed at 4°C for 4–6 h in 20 mM HEPES (pH 7.5)-50 mM KCl-0.1 mM EDTA-10% glycerol-0.4 mM NaF-0.4 mM sodium orthovanadate-0.1 mM PMSF-1.0 mM DTT. Samples were stored at −80°C. Protein content was determined by the Bradford assay (Bio-Rad, Richmond, VA).

Oligonucleotide Labeling. The oligonucleotide sequences used in the following electrophoretic mobility shift assay were synthesized based on sequence analysis of the 5′ region of the γ-GCS heavy subunit gene fragment cloned as described above. Among the regions with high homology for known transcription factor-binding elements, AP-1- and NF-κB-like sites were identified along with a classical AP-2 site. Thus, we prepared oligonucleotides of 29, 24, and 32 bases (Oligonucleotide Synthesis Facility, Fox Chase Cancer Center), which encompassed the respective sites (Fig. 1). The oligonucleotides were purified and annealed by standard procedures (26). The double-stranded oligonucleotides were labeled with [α-^{32}P]ATP by phosphorylation with bacteriophage T_4 polynucleotide kinase and then precipitated with ethanol to remove the bulk of the unincorporated radioactivity. Oligonucleotides that contained the accepted consensus sequence for AP-1, AP-2, and NF-κB were obtained from Santa Cruz Biotechnology, Inc. and used in competition studies described below.

EMSA. The nuclear extracts were analyzed for transcription factor binding activity by gel mobility shift assays. The binding reaction mixture contained 10 μg of nuclear extract and 1.8 μg of poly(dI-dC) in a 30 μl final volume of binding buffer [20 mM HEPES (pH 7.5)-40 mM KCl-1.0 mM $MgCl_2$-0.1 mM EGTA-0.5 mM DTT]. Each reaction mixture contained 10,000 cpm of an individual double-stranded oligonucleotide transcription factor binding element. In cases where competition was investigated, the reaction also contained the appropriate competitor at 100-fold excess.

The reaction mixture was allowed to stand for 25 min at room temperature. After the addition of 5 μl of loading buffer (20% Ficoll-0.25% bromophenol blue), samples were loaded onto a 6% polyacrylamide gel and electrophoresed at room temperature for 3 h at 150 V. The gel was dried under vacuum and exposed to X-ray film overnight at −70°C to visualize the results.

Supershift Assay. The nuclear extracts were preincubated with antiserum at room temperature for 20 min before analysis by EMSA as described above. The human anti-c-Jun, anti-c-Fos, anti-AP-2, anti-NF-κB, anti-p50, and anti-p65 sera were obtained from Santa Cruz Biotechnology, Inc. These sera specifically detect the presence of the corresponding transcription factor and do not interfere with nuclear factor binding (Santa Cruz Biotechnology, Inc.).

RESULTS

In this study, we have used a related series of ovarian cancer cell lines selected for low to high levels of cisplatin resistance and also resistant cells (CPREV) allowed to partially revert to a cisplatin-sensitive phenotype (PREV). These cells have increases in GSH that correlate well with their degree of resistance (Table 1). Hence, we have used them to examine the mechanism of the increased synthesis of this tripeptide. As shown in Table 1, the cells vary in their IC_{50}s for cisplatin from 0.2 μM for A2780 to 147 μM for C200, yielding a maximum level of resistance of 735-fold. It is noteworthy that the C200 cells that were subjected to continued cisplatin selection after January 1991, when CPREV was cryopreserved, continued to increase in resistance and that PREV over the ~3.5 years without selection pressure lost ~¾ of its resistance based on comparison of its IC_{50} (24 μM) to that of CPREV (IC_{50} = 98 μM). As we have reported previously, increases in cellular GSH parallel cisplatin resistance (Table 1). Furthermore, there is a substantial decrease in GSH (~3-fold) when resistant cells (CPREV) partially revert to a more cisplatin-sensitive phenotype (PREV; Table 1).

Consistent with our previous reports (5) and those of others (27, 28), we show that increases in GSH are accompanied by increases in steady-state mRNA levels for the heavy subunit of the γ-GCS enzyme (Table 1). Here, we demonstrate that reversion to a more cisplatin-

Fig. 1. *A*, nucleotide sequence of the human γ-GCS heavy subunit gene 5′-flanking region. A total of 1442 bp of the 5′-flanking region and the region of the start site of transcription designated as +1 to the first amino acid codon are shown. The position and sequences of TATA and CCAAT boxes, metal response element (*MRE*), AP-1, electrophile response element (*EpRE*), AP-2, xenobiotic response element (*XRE*) and NF-κB like binding sites are denoted in bold letters. The oligonucleotides used in gel mobility shift assays were designed based on the underlined sequences. *B*, outline of the position of transcription factor binding sites in the γ-GCS heavy subunit gene 5′-flanking region.

sensitive phenotype in PREV is related to a decrease in cellular GSH, accompanied by a decrease in the steady mRNA level for the γ-GCS heavy subunit (Table 1). It is apparent from Table 1 and Figs. 2 and 3 that the increase in steady-state mRNA levels for the γ-GCS heavy subunit may be in large part attributed to a >10-fold increase in transcription rate. The failure to detect any major difference in mRNA half-life (range, 7.5–9 h) for the gene, as shown in Fig. 3, additionally supports this view. These data allowed us to determine the correlation between the degree of resistance and rate of γ-GCS heavy subunit transcription ($r = 0.93$), degree of resistance and steady-state mRNA levels for the γ-GCS heavy subunit, ($r = 0.93$), GSH levels and rate of γ-GCS heavy subunit transcription ($r = 0.94$), and GSH levels and steady-state mRNA amounts for the γ-GCS heavy subunit ($r = 0.94$). As shown in Fig. 4, there was no relationship between steady-state mRNA levels for the light subunit of γ-GCS and GSH levels or resistance to cisplatin.

As a step toward determining how the γ-GCS heavy subunit gene is regulated, we cloned and sequenced a portion of the 5′ region of the gene. The 9.5-kbp genomic clone we isolated was determined to encompass the transcription start site by sequencing, RNase protection, and primer extension methods. Hence, this clone also contained ~2 kbp of upstream nucleotides with a possible regulatory role. The nucleotide sequence of this region is shown in Fig. 1*A*. Analysis of these data including sequence between the transcription start site and

Table 1 *Summary of GSH and γ-GCS characteristics of cisplatin-sensitive and cisplatin-resistant ovarian cancer cell lines*

Cell line	Cisplatin IC_{50} (μM)[a]	Fold increase	GSH level[b]	Fold increase	Relative γ-GCS transcription rate[c]	Relative γ-GCS mRNA level[d]	mRNA $t_{1/2}$[e]
A2780	0.2	1	9.2	1	1	1	8.5 ± 0.6
CP70	5.5	28	22.3	2.4	4.8	3.8	7.5 ± 0.7
C30	54	270	121.5	13.2	6.0	4.7	8.0 ± 0.4
C200	147	735	252.8	27.4	10.6	14.1	9.0 ± 1.0
CPREV	98	490	63.6	6.9	ND[f]	6.2	ND
PREV	24	120	21.6	2.3	ND[f]	3.5	ND

[a] Cytotoxicity was determined by MTT assay. The IC_{50} is the concentration that inhibited growth by 50%.
[b] Total GSH (GSH + 1/2 GSSG) is shown as nmol/mg protein.
[c] The relative transcriptional rate was determined by nuclear run-on assay.
[d] The relative mRNA level was determined by Northern blot.
[e] The mRNA half-life $t_{1/2}$ h was determined by actinomycin D-Northern blot assay.
[f] Not determined.

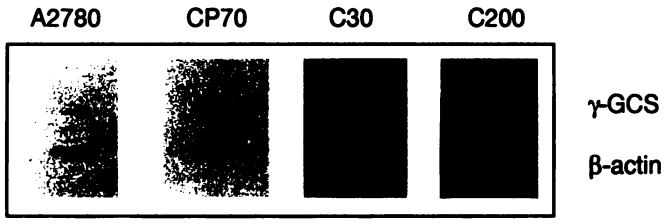

Fig. 2. Nuclear run-on assay of mRNA transcripts of γ-GCS genes in cisplatin-sensitive and cisplatin-resistant human ovarian cancer cell lines. RNA transcripts from the nuclei of four cell lines hybridizing to γ-GCS, and β-actin cDNAs are shown.

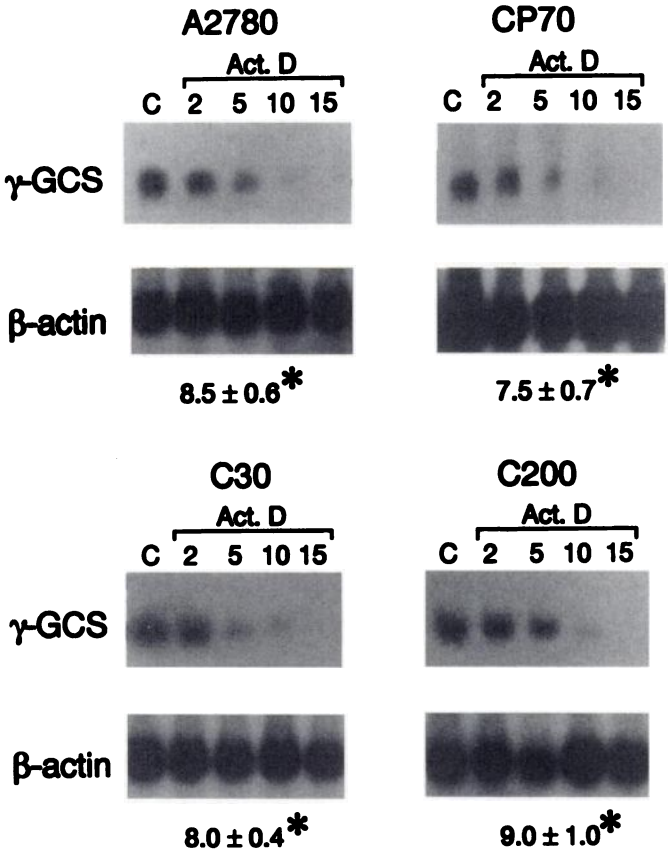

Fig. 3. Northern blot analysis of cisplatin-sensitive and cisplatin-resistant cells to demonstrate mRNA degradation. Total cellular RNA isolated from cells at various times after inhibition of transcription with actinomycin D (*Act. D*; 10 μg/ml). The half-life values are based on the densitometric analysis of autoradiograms. *, $t_{1/2}$ in hours of γ-GCS.

the first ATG visually revealed that it contained several regions with high homology to known transcription factor binding elements. The most studied elements shown in bold type in Fig. 1*A* and schematically in Fig. 1*B* include AP-1, NF-κB, XRE, AP-2, EpRE, two MREs, CAAT, and TATA box elements. We initially evaluated the AP-1, AP-2, and NF-κB elements and their transcription factors. The AP-1-like element (29) located between −291 and −285 bp in the γ-GCS promoter consisted of TGATTCA, and an element, CCCCACGG, located between nucleotides −767 to −760 was consistent with an AP-2 element (30). The sequence GGGGCACATCC located between nucleotides −1067 and −1057 was consistent with an NF-κB element (31).

To examine whether cisplatin-resistant as compared to cisplatin-sensitive cells contained altered amounts of transcription factors capable of binding the AP-1-, AP-2-, and/or the NF-κB-like elements, we constructed oligonucleotides of 29 bp (−278 to −306), 25 bp (−751 to −775), and 32 bp (−1045 to −1077), as shown in Fig. 1*A*, encompassing these respective potential binding regions and performed electrophoretic mobility shift assays and supershift assays. Fig. 5 shows that binding activity for the experimental oligonucleotide incorporating the AP-1-like element of the γ-GCS promoter is increased in the cisplatin-resistant variants and parallels γ-GCS expression. Furthermore, examination of AP-1 binding activity in PREV (partial revertant) revealed a marked decrease (Fig. 6). To ascertain whether the binding observed in resistant cells was consistent with transcription factor binding to a classical AP-1-binding sequence, we used an oligonucleotide containing the accepted consensus sequence for AP-1 as a competitor in the EMSA. This oligonucleotide at 100-fold excess abolished binding to the experimental ^{32}P-labeled AP-1 element (Fig. 7). Using antibodies to JUN and FOS family members in supershift

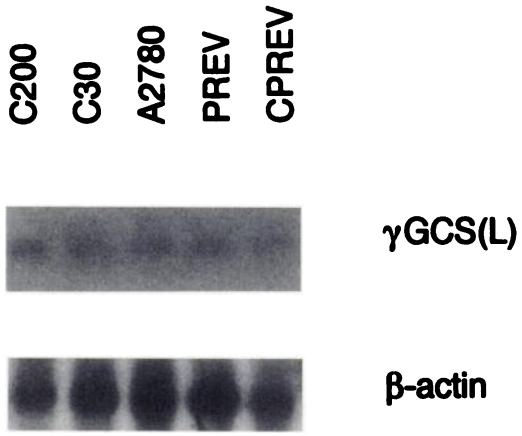

Fig. 4. Expression of γ-GCS gene light subunit mRNA in ovarian cell lines. Total cellular RNA (15 μg) was isolated, separated, blotted, and hybridized to the ^{32}P-labeled γ-GCS light subunit probe. Hybridization to the β-actin probe as an internal control demonstrated equal loading of RNA.

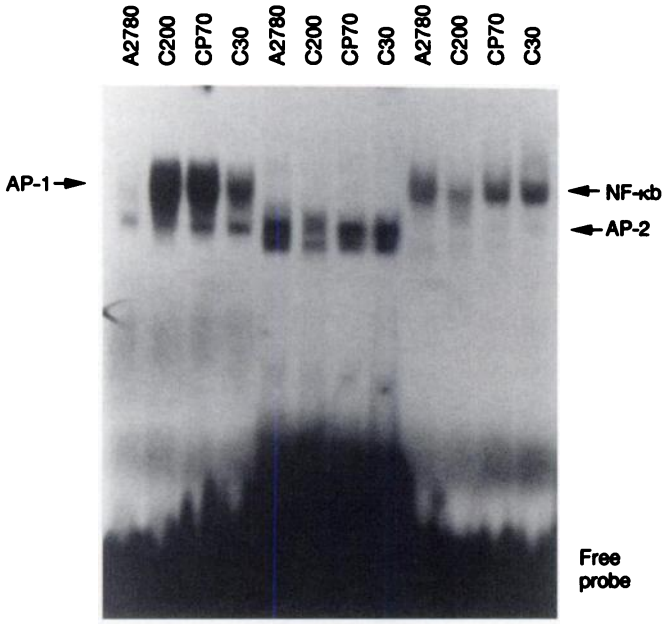

Fig. 5. Gel shift of AP-1-, AP-2-, and NF-κB-binding activity in nuclear extracts from cisplatin-sensitive and cisplatin-resistant cell lines. Gel shifts were performed using a ^{32}P-labeled synthetic double-stranded oligonucleotide containing the γ-GCS promoter sequences of AP-1-, AP-2-, and NF-κB-binding elements as probes. The DNA-protein complexes formed are indicated (*arrow*). The unbound (free) probe in the gel is indicated at the bottom.

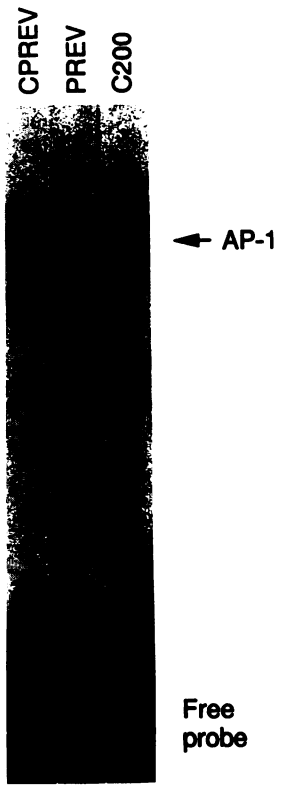

Fig. 6. Gel shift assay to demonstrate AP-1 binding activity in the partial revertant (*PREV*) compared to control cells (*CPREV*). The DNA-AP-1 protein complexes formed are indicated.

members in supershift assays demonstrated that the increased AP-1 binding activity present in resistant cells was predominantly related to binding of JUN (Fig. 8). On the basis of this finding, it was of interest to examine the sensitive and resistant cells for differences in the steady-state mRNA levels for c-*JUN*. As shown in Fig. 9, there is an approximately 10-fold increase in c-*JUN* mRNA in C200 as compared to A2780 cells. Steady-state mRNA levels for c-*FOS* were very low in the various cell lines compared to c-*JUN* (Fig. 9) and showed no variation related to degree of resistance (relative expression compared to A2780: CP70 = 1.02, C30 = 0.93, C200 = 0.086).

In contrast to our finding of increased binding activity for the AP-1 element in cisplatin-resistant cells, binding to AP-2- and NF-κB-like elements decreased (Fig. 5). We confirmed that the pattern observed was consistent with binding to classical AP-2 and NF-κB elements by competition studies with oligonucleotides containing the consensus sequence for the two elements (Fig. 7). Furthermore, we confirmed that these two sequences were AP-2 and NF-κB binding elements by supershift assays using antibodies to the respective transcription factors (Fig. 8 for AP-2 and data not shown for NF-κB).

DISCUSSION

The sulfhydryl moieties of GSH confer nucleophilicity to this abundant tripeptide. As such, GSH reacts with a variety of naturally occurring and synthetic toxic agents to decrease their impact on homeostasis. Such information suggests that GSH could also have the detrimental effect of interacting with chemotherapeutic agents such as cisplatin and classical alkylating agents to subvert their desired cyto-

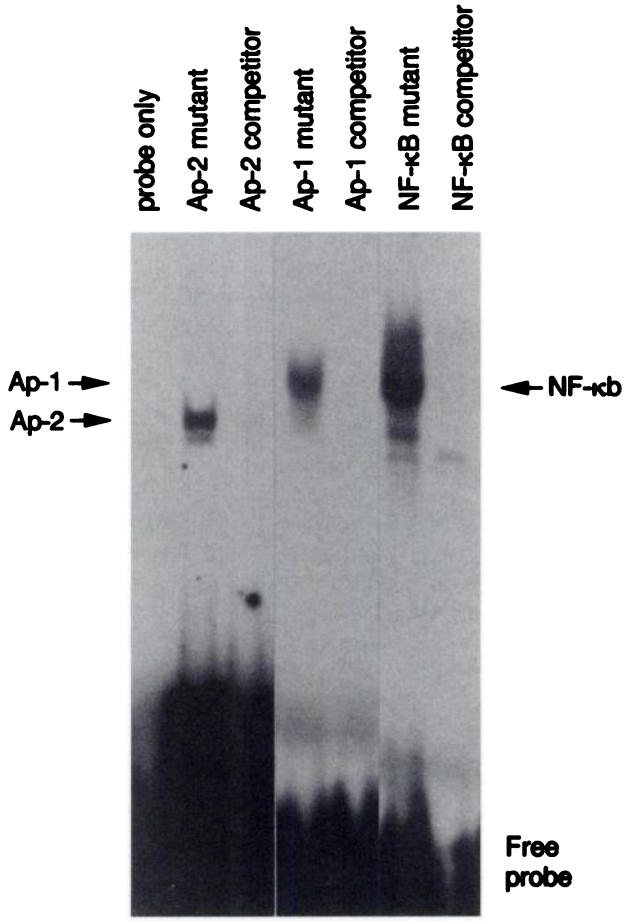

Fig. 7. The binding activities of nuclear extracts for the experimental AP-1, AP-2 and NF-κB elements designed as shown in Fig. 1 are abolished by a 100-fold molar excess of unlabeled consensus AP-1, AP-2, and NF-κB oligonucleotides (*lanes* labeled *competitor*). Additionally, in *lanes* labeled *mutant*, it is seen that mutant AP-1-, AP-2-. and NF-κB-like elements are ineffective competitors for the experimental AP-1, AP-2, and NF-κB elements. In the case of AP-1 analysis, C200 extract was used. In the case of AP-2 and NF-κB analysis, C30 extract was used.

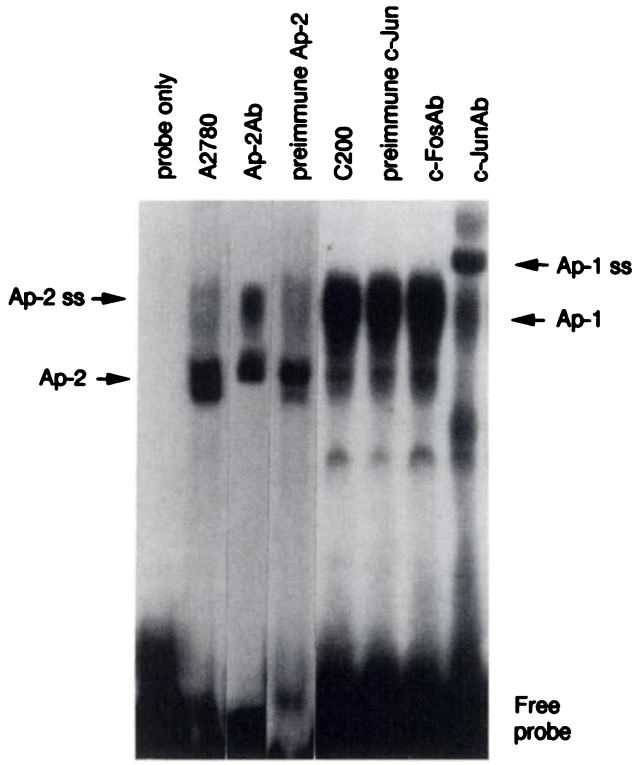

Fig. 8. Supershift assay of binding activity in cisplatin-sensitive (A2780) and resistant (C200) cell nuclear extracts to experimental AP-1- or AP-2-binding elements. Preincubation of 10 μg of nuclear extract from each cell line with preimmune sera is shown as controls. The use of anti-c-FOS, anti-c-JUN, or anti-AP-2 sera before gel shift is seen to result in a super-retardation in band mobility (*arrow* with *ss*).

toxicity to cancer cells, and indeed, there are abundant data that the amount of GSH in a cancer cells relates directly to the amount of drug required to kill the cell (3–7). Furthermore, it is noteworthy that induction of cisplatin or alkylating agent resistance results in constitutive increased expression of GSH (5, 27, 28). Thus, there is strong rationale for investigating the mechanism of regulation of GSH in cancer cells. It should be noted that cisplatin resistance need not solely be mediated by GSH. In the models described here, a portion of the resistance is also accounted for by decreased drug accumulation, altered patterns of DNA platination, enhanced repair of platinum-DNA lesions, and increased tolerance of platinum damage (32, 33). The focus of the present investigation, however, was to use these cells with their high GSH levels as a tool to examine of how large increases in GSH may be accomplished. In this report, we have clearly demonstrated a direct relationship between the steady-state mRNA level for the heavy subunit of γ-GCS, which encodes the rate-limiting enzyme in GSH synthesis, and the relative rate of the transcription of this gene as measured by a nuclear run-on assay in cisplatin-sensitive and cisplatin-resistant cells. These data strongly support transcriptional activation as the major mechanism of up-regulation of the expression of this gene in the resistant cells based on the absence of changes in mRNA stability and are consistent with one previous report (34). As we anticipated, based on the strong relationship between GSH and degree of resistance (5, 19), we found a good correlation based on linear regression analysis between the following parameters: degree of resistance, GSH levels, γ-GCS heavy subunit steady-state mRNA levels, and γ-GCS heavy subunit rate of transcription using all pair-wise combinations for analysis (r = >0.9 in all cases).

It was of special interest that we observed no alteration in the expression of mRNA for the γ-GCS light subunit in cisplatin-resistant as compared to cisplatin-sensitive cells. This finding is consistent with a previous report in which we showed no change in protein amounts between sensitive and resistant cells using Western blot analysis (5). It has been suggested that the light subunit of γ-GCS may serve a regulatory role in GSH synthesis, and one recent report describes a parallel increase in γ-GCS light and heavy subunit mRNA levels in nitrogen mustard-resistant prostate cancer cells as compared to nitrogen mustard-sensitive cells (35). In contrast, such coordinate changes in expression of both subunits do not occur in our model. Hence, it appears that the light subunit of γ-GCS may not necessarily be required to perform a major regulatory role in all cases of increased GSH synthesis.

Knowledge that the increases in γ-GCS heavy subunit mRNA were primarily related to increased rate of transcription in resistant cells provided a strong incentive to investigate the mechanism by which this change was accomplished. Hence, we cloned a portion of the potential 5′ regulatory region of the gene. As described in "Results," sequence analysis revealed many regions with high homology to known transcription factor-binding elements. As noted above, we found the presence of a subgroup of these elements of special interest based on our previous work on detoxication enzyme induction by hypoxia, heat, and alkylating agents (16, 36). This work showed transcriptional induction of mRNAs encoding several enzymes involved in the detoxication of xenobiotics. In this earlier study, we took advantage of the fact that AP-1-, AP-2-, and NF-κB-like elements were known to be present in the promotor of DT diaphorase and showed that exposure of cells to hypoxia resulted in induction of transcription factor binding to these elements (37). Of direct relevance to the present investigation, we also showed increased expression of the γ-GCS heavy subunit as a consequence of hypoxia (16). Hence, when we found that an AP-2 element and AP-1- and NF-κB-like elements were among the potential transcription factor-binding elements present in the 5′-regulatory region of the γ-GCS heavy subunit gene, we focused attention on these sequences. In each case, the sequences we uncovered diverged only slightly or not at all from the respective consensus sequence. As noted above, we identified a sequence, CCCCACGG, identical to a classical AP-2 element (30). The AP-1-like element of the γ-GCS heavy subunit gene, TGA TTCA, contained a T for C substitution (29) and the NF-κB-like element, GGGG CACATCC, contains one substituted base a C for G (31). The

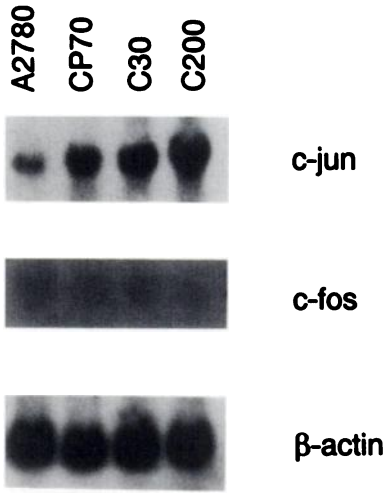

Fig. 9. Expresssion of AP-1 complex genes c-*Jun* and c-*Fos* mRNA in cisplatin-sensitive and cisplatin-resistant cells. Total cellular RNA (15 μg) was separated electrophoretically, and after Northern transfer, the blots were hybridized with ^{32}P-labeled c-JUN or c-FOS cDNAs. Hybridization with a β-actin probe was used to demonstrate equal loading.

T for C substitution in our AP-1 element is considered a minor change in contrast to a terminal CA to TG substitution, and the competitive gel shift results we present support the view that our element is behaving as a classic AP-1 element. Similarly, the substitution in the NF-κB element is considered minor.

Strong evidence favoring up-regulation of transcription factors that bind AP-1 sites and decreased AP-2 and NF-κB activity in cisplatin-resistant cells was provided by gel mobility shift results. To gain an insight into the identity of the factor(s) responsible, we used antibodies directed against potential candidates in an effort to produce supershifts in mobility. Only an antibody that cross-reacts with all JUN family members produced supershifts, supporting the view that the AP-1 gel mobility shift data likely resulted from JUN/JUN homodimer binding to AP-1 sites. Evidence that c-*JUN* was at least one of the *JUN* family members involved was provided by analysis of Northern blots with a cDNA probe with specificity for c-*JUN*. This analysis revealed that steady-state mRNA levels for c-*JUN* were markedly increased in cisplatin-resistant as compared to the relatively cisplatin-sensitive parental cells. Therefore, we suggest that the constitutive up-regulation of c-*JUN* induced by selection for cisplatin resistance may be in large part responsible for the increase in steady-state mRNA for the γ-GCS heavy subunit and in turn the elevation of cellular GSH.

The association of transcription factor up-regulation and cisplatin resistance has been reported previously. In those studies, increased expression of c-*FOS* was described (38). The hypothesis was put forward that c-*FOS* up-regulation in turn resulted in the increased expression of several enzymes involved in DNA synthesis and repair and also metallothionein, the most abundant sulfhydryl-rich protein in cells. To examine this concept, studies were performed with an antisense ribozyme directed at c-*FOS* and under control of a dexamethasone-inducible promotor. These experiments showed down-regulation of dTMP synthetase, DNA polymerase β, topoisomerase 1, and metallothionein after induction of expression of the antisense molecule. Hence, it was argued that the promotors of these genes contained c-FOS-binding elements (39, 40).

Our approach was to directly examine the 5′-regulatory sequences of a gene the product of which is important in cisplatin resistance for motifs characteristic of known transcription factor binding elements. In summary, we identified several such elements and by the circumstantial evidence of constitutively increased expression of c-*JUN* and its binding to an AP-1 site in the γ-GCS heavy subunit promotor suggest this change may be pivotal in up-regulation of this important gene. Ongoing functional studies in resistant and parental cells with reporter gene/normal or mutated promotor constructs will support or refute this hypothesis.

It was of interest that c-FOS, which may form heterodimers with c-JUN and bind AP-1 sites, was not constitutively increased in our cisplatin-resistant cells. This is consistent with the failure of anti-FOS antibodies to impact on AP-1 site/nuclear extract mobility (Fig. 8). The apparent specificity for up-regulation of the JUN family of proteins may be peculiar to these cell lines and to our method of selection (see "Materials and Methods"). Under various conditions associated with acute induction of AP-1 binding, the pattern of expression appears to vary with the inducing stimulus. Thus, exposure to hypoxic conditions results predominantly in the formation of JUN-JUN dimers (16), whereas the immediate response to exposure to the DNA-damaging drug, mitomycin C, is the formation of JUN-FOS heterodimers.[4] The basis for these differences presumably lies in the multiple signal transduction pathways through which gene expression can be induced by AP-1.

We have used several strategies to identify potential cisplatin resistance genes and found numerous genes including vimentin, tissue inhibitor of metalloproteinase 1, thymosin β4, *ERCC* 1, and heat shock proteins[5] for which in some cases functional roles in cisplatin resistance are not readily apparent. The discovery that induction of genes that have a clear role in cisplatin resistance may occur by increased expression of transcription factors, which in turn regulate the expression of the relevant gene(s) rather than through direct activation, provides one explanation for the abundant gene expression alterations that often accompany cisplatin resistance. For example, any gene with an AP-1 site in its promotor, the expression of which is primarily up-regulated by binding of JUN/JUN homodimers to the AP-1 site, might be anticipated to be up-regulated in our cisplatin-resistant cells irrespective of whether it contributes functionally to the resistant phenotype.

Note added in proof

During the course of our investigation, an independent report describing the cloning of the potential 5′-regulatory sequences of the γ-GCS heavy subunit gene was published (41). A comparison of our data with those and by use of the computer program TFMAP using the database GenMoreData revealed an additional AP-1 site in our sequence (nucleotides −1361 through −1355; Fig. 1).

REFERENCES

1. Meister, A., and Anderson, M. Glutathione. Ann. Rev. Biochem., *52:* 711–760, 1983.
2. Meister, A. Metabolism and Function of Glutathione. *In:* D. Dolphin, R. Poulson, and O. Avramovic (eds.), Glutathione: Chemical, Biochemical, and Medical Aspects—Part A, pp. 367–474. New York: John Wiley and Sons, 1989.
3. Hamilton, T., Winker, M., Louie, K., Batist, G., Behrens, B., Tsuruo, T., Grotzinger, K., McKoy, W., Young, R., and Ozols, R. Augmentation of Adriamycin, melphalan and cisplatin cytotoxicity in drug-resistant and -sensitive human ovarian cancer cell lines by buthionine mediated glutathione depletion. Biochem. Pharmacol., *34:* 2583–2586, 1985.
4. Mistry, P., Kelland, L., Abel, G., Sidhar, S., and Harrap, K. The relationships between glutathione, glutathione-*S*-transferase and cytotoxicity of platinum drugs and melphalan in eight human ovarian carcinoma cell lines. Br. J. Cancer, *64:* 215–220, 1991.
5. Godwin, A., Meister, A., O'Dwyer, P., Huang, C., Hamilton, T., and Anderson, M. High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. Proc. Natl. Acad. Sci. USA, *89:* 3070–3074, 1992.
6. Hosking, L., Whelan, R., Shellard, S., Bedford, P., and Hill, B. An evaluation of the role of glutathione and its associated enzymes in the expression of differential sensitivities to antitumor agents shown by a range of human tumor cell types. Biochem. Pharmacol., *40:* 1833–1842, 1990.
7. Meijer, C., Mulder, N., Timmer-Bosscha, H., Sluiter, W., Meersma, G., and de Vries, E. Relationship of cellular glutathione to the cytotoxicity and resistance of seven platinum compounds. Cancer Res., *52:* 6885–6889, 1992.
8. Eastman, A. Crosslinking of glutathione to DNA by cancer chemotherapeutic platinum coordination complexes. Chem. Biol. Interact., *61:* 241–248, 1987.
9. Ishikawa, T., and Ali-Osman, F. Glutathione-associated *cis*-diamminedichloroplatinum(II) metabolism and ATP-dependent efflux from leukemia cells: molecular characterization of glutathione-platinum complex and its biological significance. J. Biol. Chem., *268:* 20116–20125, 1993.
10. Müller, M., Meijer, C., Zaman, G., Birst, P., Scheper, R., Mulder, N., de Vries, E., and Jansen, P. Overexpression of the gene encoding the multidrug resistance protein results in increased ATP-dependent glutathione *S*-conjugate transport. Proc. Natl. Acad. Sci. USA, *91:* 13033–13037, 1994.
11. Lai, G-M., Ozols, R., and Hamilton, T. Role of glutathione on DNA repair in cisplatin resistant human ovarian cancer cell lines. J. Natl. Cancer Inst., *81:* 535–539, 1989.
12. Billings, P., Davis, R., Engelsberg, B., Skov, K., and Hughes, E. Characterization of high mobility group protein binding to cisplatin-damaged DNA. Biochem. Biophys. Res. Commun., *188:* 1286–1294, 1992.
13. Devary, Y., Gottlieb, R., Lau, L., and Karin, M. Rapid and preferential activation of the c-*jun* gene during the mammalian UV response. Mol. Cell. Biol., *11:* 2804–2811, 1991.
14. Ausserer, W., Bourrat-Floeck, B., Green, C., Laderoute, K., and Sutherland, R. Regulation of c-*jun* expression during hypoxic and low-glucose stress. Mol. Cell. Biol., *11:* 5032–5042, 1994.
15. Bergelson, S., Pinkus, R., and Daniel, V. Intracellular glutathione levels regulate *fos/jun* induction and activation of glutathione *S*-transferase gene expression. Cancer Res., *54:* 36–40, 1994.

[4] A. Hageboutros, P. Ford, K-S. Yao, and P. O'Dwyer. Induction of expression of DT-diaphorase in colon adenocarcinoma cell lines by mitomycin C through the AP-1 and NF-κB elements. Submitted for publication.

[5] A. K. Godwin, S. W. Johnson, and T. C. Hamilton, unpublished observations.

16. Yao, K-S., Xanthoudakis, S., Curran, T., and O'Dwyer, P. Activation of AP-1 and of a nuclear redox factor, Ref-1, in the response of HT29 colon cancer cells to hypoxia. Mol. Cell. Biol., *14:* 5997–6003, 1994.
17. Hanigen, M., Frierson, H., Brown, J., Lovell, M., and Taylor, P. Human ovarian tumors express γ-glutamyl transpeptidase. Cancer Res., *54:* 286–290, 1994.
18. Huang, C-S., Anderson, M., and Meister, A. Amino acid sequence and function of the light subunit of rat kidney γ-glutamylcysteine synthetase. J. Biol. Chem., *268:* 20578–20583, 1993.
19. Hamaguchi, K., Godwin, A., Yakushiji, M., O'Dwyer, P., Ozols, R., and Hamilton, T. Cross resistance to diverse drugs is associated with primary cisplatin resistance in ovarian cancer cell lines. Cancer Res., *53:* 5225–5232, 1993.
20. Cleveland, D., Lopata, M. A., MacDonald, R. J., Rutter, W. J., and Kirschner, M. W. Number and evolutionary conservation of α and β-tubulin and cytoplasmic β- and γ-actin genes using specific cloned cDNA probes. Cell, *20:* 95–105, 1980.
21. Chomczynski, P., and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., *162:* 156–159, 1987.
22. Angel, P., Allegretto, E. A., Okino, S. T., Hattori, K., Boyle, W. J., Hunter, T., and Karin, M. Oncogene *jun* encodes a sequence-specific *trans*-activator similar to AP-1. Nature (Lond.), *332:* 166–171, 1988.
23. Curran, T., McConnell, W. P., Van-Straten, F., and Verma, I. M. Structure of the FBJ murine osteosarcoma virus genome: molecular cloning of its associated helper cells. Mol. Cell. Biol., *3:* 914–921, 1983.
24. Greenberg, M. E. Identification of newly transcribed RNA. *In:* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Seidman, and K. Strahl (eds.), Current Protocols in Molecular Biology, pp. 4.10.1–1.10.8. New York: John Wiley and Sons, 1988.
25. Dignam, J. P., Lebowitz, R. M., and Roeder, R. G. Accurate transcription initiation by RNA polymerase II in a soluble extract from mammalian nuclei. Nucleic Acids Res., *11:* 1475–1489, 1983.
26. Sambrook, J., Fritsch, E. F., and Maniatis, T. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989.
27. Bailey, H., Gipp, J., Ripple, M., Wilding, G., and Mulcahy, R. Increase in γ-glutamylcysteine synthetase activity and steady-state messenger RNA levels in melphalan-resistant DU-145 human prostate carcinoma cells expressing elevated glutathione levels. Cancer Res., *52:* 5115–5118, 1992.
28. Mulcahy, R., Bailey, H., and Gipp, J. Up-regulation of γ-glutamylcysteine synthetase activity in melphalan-resistant human multiple myeloma cells expressing increased glutathione levels. Cancer Chemother. Pharmacol., *34:* 67–71, 1994.
29. Rauscher, F. J., III, Sambucettie, L. C., Curran, T., Dister, R., and Spiegelman, B. M. Common DNA binding site for fos protein complexes and transcription factor AP-1. Cell, *52:* 471–480, 1988.
30. Williams, T., and Tjian, R. Analysis of the DNA-binding and activation properties of the human transcription factor AP-2. Genes & Dev., *5:* 670–682, 1991.
31. Baeuerle, P. A., and Baltimore, D. The physiology of the NF-κB transcription factor. *In:* P. Cohen and J. G. Foulkers (eds.), Molecular Aspects of Cellular Regulation: Hormonal Control Regulation of Gene Transcription, pp. 409–432. New York: Elsevier, 1991.
32. Johnson, S., Perez, R., Godwin, A., Yeung, A., Handel, L., Ozols, R., and Hamilton, T. Role of platinum-DNA adduct formation and removal in cisplatin resistance in human ovarian cancer cell lines. Biochem. Pharmacol., *47:* 689–697, 1994.
33. Johnson, S. W., Swiggard, P. A., Handel, L. M., Brennan, J. M., Godwin, A. K., Ozols, R. F., and Hamilton, T. C. Relationship between platinum-DNA adduct formation, removal, and cytotoxicity in cisplatin-sensitive and -resistant human ovarian cancer cells. Cancer Res., *54:* 5911–5916, 1994.
34. Mulcahy, R., Untawale, S., and Gipp, J. Transcriptional up-regulation of γ-glutamylcysteine synthetase gene expression in melphalan-resistant human prostate carcinoma cells. Mol. Pharmacol., *46:* 909–914, 1994.
35. Untawale, S., Gipp, J., Bailey, H., and Mulcahy, R. Transcriptional up-regulation of the γ-glutamylcysteine synthetase gene in melphalan-resistant prostate carcinoma cells expressing elevated glutathione. Proc. Am. Assoc. Cancer Res., *35:* 377, 1994.
36. Yao, K-S., Clayton, M., and O'Dwyer, P. Interaction of heat and hypoxia in modulating transcription of DT diaphorase in human colon adenocarcinoma cells. Cell Growth & Differ., *5:* 125–131, 1994.
37. O'Dwyer, P., Yao, K-S., Ford, P., Godwin, A., and Clayton, M. Effects of hypoxia upon detoxicating enzyme activity and expression in HT29 colon adenocarcinoma cells. Cancer Res., *54:* 3082–3087, 1994.
38. Scanlon, K., Kashani-Sabet, M., Tone, T., and Funato, T. Cisplatin resistance in human cancer. Pharmacol. Ther., *52:* 385–406, 1991.
39. Scanlon, K., Jiao, L., Funato, T., Wang, W., Tone, T., Rossi, J., and Kashani-Sabet, M. Ribozyme-mediated cleavage of c-*fos* mRNA reduces gene expression of DNA synthesis enzymes and metallothionein. Proc. Natl. Acad. Sci. USA, *88:* 10591–10595, 1991.
40. Funato, T., Yoshida, E., Jiao, L., Tone, T., Kashani-Sabet, M., and Scanlon, K. The utility of an antifos ribozyme in reversing cisplatin resistance in human carcinomas. Advan. Enzyme Regul., *32:* 195–209, 1992.
41. Mulcahy, R. T., and Gipp, J. J. Identification of a putative antioxidant response element in the 5′-flanking region of the human γ-glutamylcysteine synthetase heavy subunit gene. Biochem. Biophys. Res. Commun., *209:* 227–233, 1995.

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