An Electrophile Responsive Element (EpRE) Regulates 
β-Naphthoflavone Induction of the Human γ-Glutamylcysteine 
Synthetase Regulatory Subunit Gene

CONSTITUTIVE EXPRESSION IS MEDIATED BY AN ADJACENT AP-1 SITE*

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Helen R. Moinova and R. Timothy Mulcahy‡

From the Department of Human Oncology, University of Wisconsin Medical School, Madison, Wisconsin 53792

Exposure of HepG2 cells to β-naphthoflavone (β-NF) results in time- and dose-dependent increase in the steady-state mRNA levels for both the catalytic (GCSh) and regulatory (GCSr) subunits of γ-glutamylcysteine synthetase (GCS) which catalyzes the rate-limiting step in the de novo synthesis of the cellular antioxidant glutathione (GSH) (Mulcahy, R. T., Wartman, M. A., Bailey, H. B., and Gipp, J. J. (1997) J. Biol. Chem. 272, 7445–7454). Cloning and sequencing of the GCS promoter region is reported. Regulatory sequences mediating basal and β-NF induced expression of the GCS gene were identified using a series of promoter/reporter fusion genes transfected into HepG2 cells. Sequences directing basal and β-NF induced expression were localized between nucleotides –344 and –242 (numbered relative to the translation start site).

Mutational analyses indicate that basal expression of the GCS gene is directed by a consensus AP-1-binding site located 33 base pairs upstream of a consensus electrophile responsive element (EpRE) sequence; both cis-elements are capable of supporting β-NF inducibility. Elimination of the inducible response requires simultaneous mutation of both sequences, however, in the presence of an intact EpRE the upstream AP-1 site is irrelevant to induction. Regulation of expression of both human GCS subunit genes in response to β-NF is therefore mediated by cis-elements satisfying the consensus core EpRE motif.

Glutathione (L-γ-glutamyl-cysteinyl-glycine; GSH) is the prominent cellular non-protein thiol, typically present in millimolar concentrations in most cell types (1, 2). GSH is a predominant cellular antioxidant and as such serves critical functions in the maintenance of cellular redox balance, provides protection against reactive oxygen species, and is involved in the detoxification of xenobiotics either through direct conjugation with reactive species or through enzymatic reactions catalyzed by glutathione S-transferases (3). GSH is frequently elevated in normal cells upon exposure to xenobiotics and has been implicated in tumor cell resistance to alkylating agents, Pt compounds, and anthracyclines (4, 5). Recent evidence (6–13) suggests that increase in steady-state GSH levels in stressed cells is related to increased activity of γ-glutamylcysteine synthetase (GCS), which catalyzes the rate-limiting step in the de novo synthesis of GSH from its constituent amino acids (14).

In vivo the functional GCS holoenzyme exists as a heterodimer consisting of catalytic (heavy, M₀ = 73,000) and regulatory (light, M₀ = 27,700) subunits which can be dissociated under nondenaturing conditions (15). Studies by Meister (14) have demonstrated that all the catalytic activity and the site of GSH feedback inhibition reside with the heavy subunit. However, the kinetic properties of the heavy subunit under physiological conditions are greatly influenced by association with the light subunit, presumably mediated by a redox-sensitive disulfide bond between the two subunits (16). The influence of the regulatory subunit on the kinetic properties of the catalytic subunit is so profound that Huang et al. (16) hypothesized that the monomeric catalytic subunit would be nonfunctional at the substrate (glutamate) and inhibitor (GSH) concentrations typically existing in cells. Our laboratory has cloned the cDNAs for the human liver GCS (17) and GCSr (18) subunits and recently reported the cloning and sequencing of the GCSr gene (19). We now report cloning and sequencing of the promoter and 5’-flanking sequence of the GCS light subunit gene.

Steady-state levels of mRNA corresponding to the heavy and the light subunits of GCS have been reported to be elevated after exposure of cells to various xenobiotics such as β-NF (19), methyl mercury (20), tert-butyl hydroquinone (11), and butylated hydroxyanisole (13). Since several of these treatments also induce the expression of key Phase II detoxifying enzymes (21), we hypothesized that the transcriptional up-regulation of the GCS subunit genes and genes of the Phase II battery may be mediated by common regulatory elements. Regulation of several Phase II enzymes in response to a wide variety of inducing agents, including several of those which induce GCS expression, is mediated, at least in part, by the presence of electrophile responsive elements (EpRE) within the 5’-flanking region of the gene (21–28). Our laboratory has recently demonstrated that basal and β-NF-inducible expression of the GCSr gene is mediated by a consensus EpRE sequence located

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‡ To whom all correspondence should be addressed: University of Wisconsin-Madison, Dept. of Human Oncology, 600 Highland Ave., Madison, WI 53792. Tel.: 608-263-3695; Fax: 608-263-9947; E-mail: mulcahy@mail.bascom.wisc.edu.

§ The abbreviations used are: GSH, glutathione; AhRE, aromatic hydrocarbon responsive element; AP-1, activation protein-1; β-NF, β-naphthoflavone; EpRE, electrophile responsive element; GCS, γ-glutamylcysteine synthetase; GCSr, GCS heavy subunit; GCSr, GCS light subunit; hp, base pair(s); kb, kilobase pair(s); NQO1, human NADPH quinone oxidoreductase.

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* In this report we use the designation EpRE to indicate elements matching the consensus sequence 5’-A/G(A/T)GACNNNGCA-3’. These elements have also been referred to as antioxidant responsive elements (AREs).
in the distal portion of the promoter of the GCS heavy subunit gene (19). We have also identified numerous potential regulatory elements, including a putative EpRE in the 5′-flanking sequence of the GCS gene, suggesting the possibility that the expression of the regulatory subunit gene may similarly be mediated by one or more EpRE or EpRE-like sequences. We have also demonstrated that exposure of HepG2 cells to β-NF, a planar aromatic compound capable of inducing gene expression via EpREs, results in increased expression of the GCS gene, as well as the GCSβ subunit gene (19). Although this observation is consistent with the hypothesis, the involvement of any specific cis-acting elements in constitutive and/or induced expression of the GCS gene has not been established.

Therefore, in this study we utilized a deletion mutagenesis strategy to identify the regulatory elements required for β-NF inducible expression of the GCS gene. Using a series of progressive promoter deletion/reporter transgenes transfected into HepG2 cells, we have been able to discern that the EpRE sequence identified in the GCS promoter is required for maximal induction in response to β-NF and that a neighboring AP-1 site located 33 bp upstream of the EpRE mediates constitutive expression of the gene and is also capable of directing increased expression following β-NF exposure should the core EpRE sequence be mutated.

EXPERIMENTAL PROCEDURES

GCS, Genomic DNA and Sequencing—Two clones containing genomic sequence corresponding to the light subunit of human GCS and its 5′-flanking region were isolated from a human foreskin fibroblast P1 library by Genome Systems, Inc. (St. Louis, MO) as described previously (29), utilizing polymerase chain reaction primers corresponding to specific sequences present in GCS cDNA (18). A PstI fragment containing 63 nucleotides of GCS, coding sequence and approximately 6-kb of 5′-flanking sequence was subcloned into pSKII-Bluescript (CLONTECH) and subsequently sequenced. Sequencing was performed by the dideoxynucleotide sequencing method using Sequenase (U. S. Biochemical Corp.) and synthetic oligonucleotide (20-mers) primers corresponding to internal sequences. The nucleotide sequence was verified by multiple bidirectional sequencing reactions.

Recombinant Plasmids—Expression vector constructs were created by cloning restriction fragments isolated from the 5′-flanking sequence of the GCS gene into pGL3-Basic vector (Promega) for determination of transcriptional orientation of the promoter activity or by introducing GCS, DNA fragments or synthetic oligonucleotides into pTS1 (ATCC 37584) for determination of enhancer activity. A 6.0-kb genomic DNA fragment was isolated from 5′-flanking region of GCS by XhoI restriction digestion and cloned into the XhoI site of pGL3-Basic creating the recombinant plasmid 6000/ GCS5′-luc. This construct was subjected to digestion with additional restriction enzymes to generate a series of deletion mutants as detailed in Fig. 2. A series of promoter/reporter transgenes containing mutations in regions of interest within the GCS promoter (Fig. 4A) were prepared by site-directed mutagenesis. The sequence of the final constructs were verified by diodeoxyxucleotide sequencing.

The sequence of the pGL3-Basic vector includes a consensus AP-1 site. In order to rule out any confounding influences of this potential enhancer element several of the deletion mutants (−1927, −1633, −712, −344, and −208) were cloned into a pGL3-Basic backbone in which the AP-1 site had been deleted. No significant differences were detected, therefore results with the two reporter vectors were combined.

For short DNA fragments of interest complementary synthetic oligonucleotides of the proper sequence were annealed, phosphorylated, and cloned into the pTS1 vector. Oligonucleotides dissolved in 1 × Sequenase reaction buffer were annealed by heating to 95 °C, cooled slowly to 35 °C, and desalted by using Bio-Spin chromatography columns (BioRad). These double-stranded fragments were then phosphorylated using T4 polynucleotide kinase, extracted once with phenol/chloroform/isoamyl alcohol and once with chloroform/isoamyl alcohol, ethanol precipitated, and redissolved in water for ligation into the pTS1 vector.

Cell Culture and Transfection—HepG2 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 50 μg/ml gentamicin (complete medium). Cells were transfected with recombinant plasmids using a standard calcium phosphate-glycerol shock procedure. HepG2 cells were plated at 4 × 10⁵/35-mm dish on day 0. On day 1, medium was replaced with fresh complete medium. Two to four hours later the cells were transfected by the addition of appropriate DNA expression vectors. Equimolar amounts of plasmid DNA were used to compensate for variations in plasmid size. To correct for transfection efficiency, 1.5 μg of the reporter plasmid pCMVβ (30) containing the lacZ gene encoding β-galactosidase under the control of the human cytomegalovirus immediate-early promoter/enhancer was co-transfected with each recombinant plasmid. Four hours after addition of DNA, cells were washed by the addition of 63% glycerol for 3 min at room temperature and then maintained at 37 °C for an additional 24 h in complete medium. At the conclusion of this incubation period, the medium was again replaced with the complete medium containing Me2SO (0.1%) or 10 μM β-NF (Sigma) dissolved at 1000 × in Me2SO. Sixteen hours later cells were harvested and prepared for determination of luciferase, β-galactosidase activity, and protein content. For cell harvest, plates were washed twice with phosphate-buffered saline (Mg²⁺- and Ca²⁺-free) and incubated at room temperature for 15 min in 250 μl of reporter lysis buffer (Promega). Cells were then scraped from the plates and the resulting lysates spun at the top speed in a microcentrifuge for 2 min at 4 °C. The resulting supernatants were transferred to Eppendorf tubes and stored on ice pending assay.

Luciferase Assays—β-galactosidase activity was quantified as described by Rosenthal (31). Briefly, this assay monitors cleavage of o-nitrophenyl-β-D-galactopyranoside and yields β-galactosidase units as (OD₄₂₀ × 380)/t, where t = time in minutes at 37 °C and 380 converts OD₄₂₀ to micromoles of o-nitrophenyl-β-D-galactopyranoside.

To assay for luciferase activity, 5 μl of cell lysate was added to the reaction buffer (14 mM MgCl₂, 14 mM glycyglycine, 0.1 mg/ml bovine serum albumin, 18 mg/ml ATP, pH 7.8), vortexed, and placed in an Analytical Luminescence Laboratory luminometer (400 μl total volume). Following injection of 100 μl of luciferin (4 mg/ml in 10 mM Na₂CO₃, pH 6.0) luminescence was recorded as relative light units. Luciferase activity was normalized for transfection efficiency on the basis of β-galactosidase activity and protein content of the lysate yielding a final value of (relative luciferase units/β-galactosidase units/μg of protein). Protein content was determined using the Bradford method (32) with bovine serum albumin as a standard.

Statistics—Differences between experimental groups were compared by analysis of variance using Fisher’s protected least difference significance.

RESULTS

Sequence Analysis—Two genomic clones containing the full-length human GCS gene were obtained by polymerase chain reaction screening of a human foreskin fibroblast P1 genomic library (Genesystem, Inc.) using a pair of polymerase chain reaction primers spanning a 300-bp region of the 3′-end of the GCS cDNA. A 6-kb PstI fragment from one clone was identified by in-gel hybridization using three oligonucleotide probes complimentary to sequences in the 5′-region of the GCS cDNA, isolated from agarose gels, and subcloned into pSKII-Bluescript. Sequence analysis revealed that this fragment contained 316 bp corresponding to the 5′-end of the GCS cDNA and −5.7-kb of 5′-flanking sequence. 3.3-kb of the 3′-half of the fragment was sequenced (Fig. 1). The 5′-flanking region of the GCS light subunit gene shares several characteristics typical of many “housekeeping” genes, including a large proportion of GC residues and several putative Sp-1 binding sites. Multiple consensus AP-1, as well as several AP-1-like, sites were also identified. No nuclear factor-κB-binding sites and no core sequence corresponding to the AhRE were identified. Sequence analysis did, however, identify a putative EpRE motif at −290 to −301. The 5′-flanking sequence of the GCS light subunit gene also contains a consensus metal responsive element, which directs induction of some genes, such as metallothionein-1, in response to exposure to heavy metals and H₂O₂. Since the gene utilizes multiple start sites which vary among cell lines examined and which are not associated with consensus TATA sequences, we have adopted a numbering convention which assigns the number +1 to the first base of the translation start codon.

3 K. S. Chen and M. N. Gould, unpublished data.
Functional Analysis of the 5′-Flanking Region of the GCS₁ Gene—In previous experiments, we have demonstrated that the steady-state mRNA levels for both the GCS₁ and GCS₉ genes are increased in HepG2 cells after treatment with as little as 10 µM β-NF (19), reaching peak levels at approximately 12 h (about 3-fold increase compared with the untreated cells) after the addition of β-NF. To localize regions of the GCS₉-flanking region controlling basal and β-NF inducible expression of the GCS₁ gene, a series of deletion mutant/luciferase reporter fusion genes were generated by cloning various length restriction fragments derived from the GCS₁ clone into the luciferase reporter vector, pGL3-Basic (Fig. 2). These constructs were transiently transfected into HepG2 cells and luciferase activity was determined in the presence and absence of 10 µM β-NF.

In terms of basal activity, luciferase expression in cells transfected with the 2,208/GCS₁-luc construct was extremely low; not significantly (p > 0.1) greater than that detected in HepG2 cells transfected with the pGL3-Basic vector (Fig. 2). Cells transfected with the next larger construct, −344/GCS₅₅-luc, expressed a 12-fold higher basal luciferase expression than the 2,208/GCS₁-luc transfectants, suggesting the presence of cis-regulatory element(s) between 2,208 and 2,344 that positively influence constitutive expression of the GCS₁ gene. Basal luciferase expression was maximal in cells transfected with the 2,712/GCS₁-luc vector. Inclusion of an additional 5₉ sequence from 2,712 to 2,600 did not significantly increase constitutive luciferase expression further (data from 2,192 to 2,600 not shown). These data suggest that the major sequences regulating constitutive expression of the GCS₁ gene are localized within the first 712 bp upstream of the translation start codon. They further suggest that there are at least two elements, or a set of elements, influencing basal expression, one contained in the 2,344:2,208 fragment and another in the 2,712:2,344 fragment.

Exposure to β-NF did not result in increased luciferase ex-
pression in HepG2 cells transfected with the −208/GCS,5′-luc fusion gene. However, luciferase expression was induced approximately 3-fold over the basal level when −344/GCS,5′-luc transfectants were incubated with 10 μM β-NF. The magnitude of β-NF induction was not further increased by the inclusion of sequences upstream of the −344 position (Fig. 2, data for fragments > −1927 not shown). Thus, regulatory element(s) controlling β-NF-induced expression are located between nucleotides −208 and −344.

Analysis of Synthetic Oligonucleotides Containing the GCS, EpRE—Analysis of the sequence between −208 and −344 of the GCS, promoter revealed the presence of a consensus (5′-A/GTGACNCAGCA-3′) EpRE core sequence between −290 and −301. Since EpREs have been demonstrated to enhance constitutive and β-NF-induced expression of Phase II enzymes and we previously demonstrated that a distal EpRE was responsible for these activities in the case of the human GCS, subunit gene (19), we considered this element a candidate enhancer of GCS, gene expression. In order to determine whether this potential responsive element contributed to basal and β-NF induced expression of the GCS, gene, an oligonucleotide (GCS,EpRE) spanning nucleotides −308 to −281 of the GCS, promoter was synthesized and subcloned into the SacI site of pT81, a luciferase reporter vector in which luciferase expression is under control of the herpes simplex I thymidine kinase (tk) minimal promoter. A second oligonucleotide containing the functional EpRE sequence (31) from the human NQO1 gene (NQO1hARE) was subcloned into the same vector and used as a positive control. When transfected into HepG2 cells, vectors containing the GCS, EpRE sequence in either orientation directed a significant increase in basal luciferase activity relative to that detected in cells transfected with pT81. The magnitude of the increase was comparable to that observed in HepG2 cells transfected with the NQO1hARE-luc transgene (12–15-fold higher than pT81 alone). β-NF treatment of HepG2 cells transfected with these constructs resulted in a 2.5-fold induction of luciferase activity. The pT81 vector alone did not respond to β-NF treatment.

To confirm that this EpRE motif influences response to β-NF, as well as basal expression of the gene, an oligonucleo-
tide (GCS, mEpRE) containing a single mutation in the EpRE core sequence (5'-AGGACNNNGCA-3' to 5'-AGGACNNNGCA-3') was synthesized and cloned into the pT81 vector. As established by other investigators, preservation of the T in this position is essential for both EpRE-regulated basal and β-NF-induced expression (25, 33). As shown in Fig. 3, this point mutation completely eliminated both the increased basal expression and inducibility by β-NF in cells transfected with vectors harboring the mutated sequence. The results of these enhancer studies were therefore consistent with the hypothesized involvement of the EpRE core motif in the basal and β-NF-induced expression of the GCS, gene.

**Mutation of the Putative EpRE Within the Native Genomic 5'-Flanking Sequence**—In an attempt to confirm that the GCS, EpRE mediates GCS, gene expression in vivo, the same GCS, EpRE point mutation described above was introduced into −1927/GCS5'-luc by site-directed mutagenesis, generating the mutant m1 (Fig. 4A). The transgenes containing the wild-type (wt) and mutated genomic 5'-flanking sequences were then transfected into HepG2 cells and their effects on basal and β-NF-induced expression of the reporter gene were compared. The introduction of the EpRE core-disrupting mutation into the genomic sequence failed to significantly (p < 0.05) alter constitutive expression in comparison to that observed in HepG2 cells transfected with the transgene containing the wild-type sequence (Fig. 4B). More importantly, luciferase expression in cells transfected with the mutant transgene was still significantly (p = 0.04) induced following incubation with 10 μM β-NF, although the level of expression was only ~60% of that detected in β-NF-treated cells transfected with the wild-type −1927/GCS5' sequence. Hence, cis-acting elements within the −344 to −208 region of the gene, other than the core EpRE, apparently contribute to β-NF inducibility.

The GCS, EpRE sequence includes an embedded core AP-1
sequence (Fig. 4A) which was also mutated by the T to G mutation introduced to generate the m1 mutant. A similar EpRE-embedded AP-1 site has been shown to influence basal and β-NF induced expression of the GCS subunit gene. In addition, this region of the GCS promoter also includes a second AP-1 site located 33 bp upstream of the EpRE (Figs. 1 and 4A). To evaluate the possibility that these AP-1 sites contribute to the inducible activity detected in β-NF-treated cells, a series of AP-1/EpRE mutants were prepared (Fig. 4A). Two additional mutations (m2, m3) of the putative EpRE sequence were generated. The m2 mutant disrupts the core EpRE sequence, leaving the embedded AP-1 site intact. The m3 mutant, on the other hand, retains a consensus EpRE motif, but the internal AP-1 site is eliminated. Mutations of the upstream AP-1 site were likewise prepared. Two different mutations (TGAGTAA to gGAGTAA; and TGAGTAA to TGAGTAc) disrupting the upstream AP-1 site at −340:−334 were examined and found to be equivalent in terms of their effect on luciferase expression. For the sake of simplicity, the results obtained with these two mutants were pooled and are collectively referred to as m4. Finally, a series of combined mutants (m1m4, m2m4, and m3m4) were also prepared.

As shown in Fig. 4B, neither mutation of the embedded AP-1 site (m1, m3) significantly altered activity relative to their respective counterpart containing the intact embedded AP-1 sequence (i.e. compare m3 versus wt; m1 versus m2; m3m4 versus m4; m1m4 versus m2m4). These data suggest that maintenance of the internal AP-1 sequence is not required for either basal or induced expression of the gene. However, this was not true of the upstream AP-1-binding site. Basal expression was significantly (p < 0.01) reduced in cells transfected with any of the transgenes containing a mutation of the upstream AP-1 site (m4, m1m4, m2m4, and m3m4), regardless of the status of the core EpRE or embedded AP-1 sequences. Furthermore, there were no significant differences (p > 0.25) in basal expression among these various m4 mutants, suggesting that the upstream AP-1 site is responsible for the increased basal expression previously localized to the −344:−208 region of the promoter.

Neither of the EpRE-disrupting mutants with intact upstream AP-1 sites (m1, m2) significantly (p > 0.1) altered basal expression by comparison to the wild-type (wt) promoter. Exposure of these transfectants to β-NF resulted in increased luciferase expression (p < 0.05), although expression was only 60% of that detected in cells transfected with comparable transgenes containing intact EpRE sequences (m2 versus wt; m1 versus m3). If, however, cells were transfected with transgenes containing the m1 or m2 mutations in combination with mutations of the upstream AP-1 site (m1m4, m2m4), β-NF responsiveness was abolished (p > 0.05). When the magnitude of induction is expressed as a ratio of luciferase activity in treated cells to that in untreated cells, the fold-induction produced by the m4 and m3m4 mutants are equivalent to those induced by their upstream AP-1-intact counterparts, wt and m3. These experiments clearly demonstrate that the upstream AP-1 can support increased luciferase expression in response to β-NF treatment in the absence of the EpRE core, accounting for the residual induction observed in the original studies using the m1 mutant. Furthermore, the data suggest that induction of the EpRE sequence is dominant over that of the upstream AP-1 when each is intact, since the magnitude of induction in β-NF-treated cells transfected with mutants having an intact EpRE and mutated upstream AP-1 site (m4, m3m4) was comparable to that detected in cells transfected with EpRE-containing sequences having intact upstream AP-1 sites (wt, m3).

**DISCUSSION**

Constitutive expression of the human GCS, gene is mediated by at least two distinct cis-elements or groups of elements, one within the −344:−242 fragment and a second within the −712:−344 fragment. The former fragment includes a consensus EpRE motif, GCS, EpRE, at −301:−291 and a consensus AP-1 binding motif positioned 32 bp upstream, at position −340:−334. The upstream AP-1 site at −340:−334 was identified as the sole cis-element within the −344:−242 fragment responsible for the basal enhancing properties attributable to this fragment. Additional deletion analyses are required to definitively establish the identity of specific elements responsible for the enhancing properties associated with the −712:−344 fragment. These elements are sufficient to account for the residual basal activity observed in cells transfected with transgenes containing the mutations of the −340:−334 AP-1 site (i.e. all m4 series mutants).

The identification of a functional role for AP-1 in regulation of the constitutive expression of the GCS, subunit gene is consistent with recent evidence from other groups which likewise suggest that AP-1 is an important component in the regulation of GCS gene expression. Transcriptional regulation of the GSH1 gene, the yeast GCS homolog, has been shown to be mediated by yAP-1 (34), the biochemical homolog of mammalian AP-1. In fact, basal and hydrogen peroxide induced expression of the yeast GSH1 gene has been shown to be yAP-1 dependent (35). Sekhar et al. (36) recently suggested a similar AP-1 dependence for expression of the human GCS heavy subunit gene based on the observation that the constitutive expression of the GCS gene and steady-state GSH levels were reduced in fibroblasts derived from c-Jun null mice by comparison to wild-type murine fibroblasts. Yao et al. (37) reported that overexpression of the GCS heavy subunit mRNA in a cisplatinum-resistant ovarian carcinoma cell line was secondary to constitutive overexpression of c-Jun and a concomitant increase in AP-1 binding activity. Similarly, mutational analyses and preliminary gel shift studies performed in our laboratory also suggest a role for AP-1 family members in regulation of basal expression of both GCS genes. It is therefore likely that the basal and induced expression of both GCS subunit genes involves AP-1 transcription factors, acting alone, or more likely, in concert with other trans-acting factors.

Although evaluation of basal regulation was necessary to establish baseline expression levels, the major thrust of the current studies was to define those cis-elements which function to control β-NF induced expression of the GCS subunit gene. By analogy to the GCS, subunit promoter, the EpRE at −301:−291 of the GCS, 5′-flanking sequence was considered a strong candidate for this activity. This possibility was supported by the pT81 enhancer studies which clearly demonstrated that the EpRE sequence could indeed support induction following β-NF exposure. However, mutation of this EpRE sequence in the 1.9-kb 5′-promoter fragment isolated from the GCS, gene resulted in a reduction in β-NF responsiveness, but it did not completely eliminate induction as was true in the case of the EpRE in the heavy subunit promoter (19). The use of individual and tandem mutants unequivocally demonstrated that the residual inducibility characteristic of the GCS, EpRE-disrupting mutants, m1 and m2, was attributable to the presence of the upstream AP-1 site. Addition of a mutation to the upstream AP-1 site to these single mutants (i.e. m1m4, m2m4) abolished the β-NF response. Ablation of the β-NF inducibility therefore required simultaneous mutation of both of these cis-elements. Hence, individually in either the GCS, EpRE or the upstream AP-1 site is sufficient to direct the induced response, and only one of them is absolutely necessary.

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4 A. C. Wild and R. T. Mulcahy, unpublished data.
The magnitude of induction following β-NF exposure in cells transfected with either the m4 or m3m4 transgenes was comparable to that observed in cells transfected with fusion genes containing both intact elements (wt, m3), suggesting that the presence of the EpRE sequence is dominant and able to produce the full induced response regardless of the status of the upstream AP-1 site. It is therefore difficult to ascertain the role that the upstream AP-1 element might play in β-NF responsiveness in the presence of an intact EpRE in situ. However, the apparent regulatory redundancy provided by this arrangement of tandem cis-responsive elements may provide an important safeguard against the potentially catastrophic effects of mutations to the regulatory region of this vitally important gene. Although regulation of their expression does not appear to be entirely analogous, the data for the two GCS subunit genes clearly indicate that the induction of gene expression provoked by β-NF exposure is mediated by similar cis-elements present in the promoter regions of the two subunit genes.

Despite its identity with the core EpRE consensus sequence and its EpRE-like functional properties evident in enhancer studies, it is possible that the GCSβ EpRE, unlike its GCSδ counterpart, does not function as an EpRE in situ. This is suggested by the observation that this element is not involved in the regulation of constitutive expression of the GCSβ gene when cloned into the promoter/reporter vectors as part of a large fragment of genomic DNA. This activity is consistently observed as a functional property of “true” EpREs described in other genes. In this regard, it is interesting to note that the GCSβ EpRE sequence is very similar to the recently defined TRE-type Maf recognition (38, 39) element (T-MARE, 5′-TGCTGACTCAGCA-3′). This motif supports binding of homo- and heterodimers of small Maf proteins. The sequence also resembles an NF-E2-binding site (5′-TGCTGACTCAGCA-3′) which is recognized by the NF-E2 family of transcription factors, including Nrf1 and Nrf2 (40, 41). The small Maf proteins form homodimers which exert negative regulatory influences when bound to their cognate binding sequence (42, 43). On the other hand, small Maf proteins also contribute to positive regulation of gene expression by forming heterodimers with numerous other bZIP transcription factors, including members of the AP-1 superfamily as well as Nrf1 and Nrf2 (38, 39, 42, 43).

It is conceivable, therefore, that regulation of the GCSβ gene might involve binding of Maf and Nrf factors to the sequence we have referred to as the GCSβ EpRE. In recent supershift assays, we have been able to demonstrate the increased binding of Nrf2 to the GCSβ EpRE sequence in response to β-NF treatment, strengthening this supposition. Similar hypotheses have recently been proposed for other genes containing EpRE-related sequences, including the human NQO1 (44) and murine heme-oxygenase 1 genes (45). Itoh et al. (46) recently demonstrated that regulation of several glutathione S-transferase isozymes in the mouse is dependent on the Nrf2 and MaK expression and binding of heterodimers to the GST EpRE sequence, providing the most direct evidence that these factors might contribute to the constitutive and induced expression of genes containing EpRE-related motifs. Perhaps what has been referred to as EpREs are in fact members or subsets of the MARE or NF-E2 family of binding sites.

In summary, basal expression of the GCSβ gene is influenced by a consensus AP-1-binding site located 33 bp upstream of a consensus EpRE sequence in the proximal region of the promoter. The latter element is not required for maximal basal expression. Both of these cis-elements are capable of supporting induction following β-NF exposure, although the EpRE element appears to be the more potent of the two. Elimination of the inducible response requires simultaneous mutation of both sequences. The current studies provide a solid foundation to support current efforts to identify specific trans-acting factors involved in regulation of GCS subunit gene expression and the signaling cascades responsible for their activation. Finally, it is possible that members of the Maf, Nrf, and AP-1 family of proteins are involved in regulation of these important genes.

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