Free Rad. Res., Vol. 32, pp. 281–301
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Invited Review

Regulation of γ-Glutamylcysteine Synthetase Subunit Gene Expression: Insights into Transcriptional Control of Antioxidant Defenses

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Accepted by Prof. V. Darley-Usmar

(Received 30 July 1999; In revised form 20 August 1999)

γ-Glutamylcysteine synthetase (GCS; also referred to as glutamate-cysteine ligase, GLCL) catalyzes the rate-limiting reaction in glutathione (GSH) biosynthesis. The GCS holoenzyme is composed of a catalytic and regulatory subunit, each encoded by a unique gene. In addition to some conditions which specifically up-regulate the catalytic subunit gene, expression of both genes is increased in response to many Phase II enzyme inducers including oxidants, heavy metals, phenolic antioxidants and GSH-conjugating agents. Electrophile Response Elements (EpREs), located in 5′-flanking sequences of both the GCS_h and GCS_l subunit genes, are hypothesized to at least partially mediate gene induction following xenobiotic exposure. Recent experiments indicate that the bZip transcription factor Nrf2 participates in EpRE-mediated GCS subunit gene activation in combination with other bZip proteins. An AP-1-like binding sequence and an NF-κB site have also been implicated in regulation of the catalytic subunit gene following exposure to certain pro-oxidants. Potential signaling mechanisms mediating GCS gene induction by the diverse families of Phase II enzyme inducers include thiol modification of critical regulatory sensor protein(s) and the generation of the reactive oxygen species. This review summarizes recent progress in defining the molecular mechanisms operative in transcriptional control of the genes encoding the two GCS subunits, identifying areas of agreement and controversy. The mechanisms involved in GCS regulation might also be relevant to the transcriptional control of other components of the antioxidant defense battery.

Keywords: Glutathione, oxidative stress, gene regulation, glutamate-cysteine ligase

Abbreviations: AP-1, activator protein-1; bZip, basic leucine zipper; β-NF, β-naphthoflavone; DEM, diethyl maleate; EpRE, Electrophile Response Element; GCS, γ-glutamylcysteine synthetase; GCS_h, GCS heavy subunit; GCS_l, GCS light subunit; GSH, glutathione; H_2O_2, hydrogen peroxide; MRE, metal response element; MTF-1, metal-responsive transcription factor; NQO1, NAD(P)H quinone oxidoreductase; PEITC, phenethyl isothiocyanate; PDTC, pyrrolidine dithiocarbamate; O_2^-, superoxide anion; tBHQ, *tert*-butylhydroquinone; T-MARE, TRE-type Maf Recognition Element; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; TRE, TPA responsive element; YRE, yAP-1 responsive element

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INTRODUCTION

Glutathione (GSH) is the most abundant cellular non-protein thiol, being present in millimolar concentrations in most prokaryotic and virtually all eukaryotic cells. GSH is an important cellular antioxidant and as such serves critical functions in the maintenance of cellular redox balance, provides protection against reactive oxygen and nitrogen species and is involved in the detoxication of electrophiles either through direct reactions with reactive intermediates or via enzymatic conjugation reactions catalyzed by glutathione S-transferases.[1] Cellular levels of GSH are influenced by multiple factors, including the activities of enzymes in the γ-glutamyl cycle, the availability of the precursor amino acid cysteine and by the rate of consumption or efflux of GSH. Another primary determinant of intracellular GSH levels is the rate of *de novo* synthesis. Glutathione is synthesized from its three substituent amino acids by two sequential ATP-dependent reactions, catalyzed by GCS and GSH synthetase, respectively:

$$\text{L-glutamate} + \text{L-cysteine} + \text{ATP} \Rightarrow \text{L-}\gamma\text{-glutamyl-L-cysteine} + \text{ADP} + P_i \quad (1)$$

$$\text{L-}\gamma\text{-glutamyl-L-cysteine} + \text{glycine} + \text{ATP} \Rightarrow \text{glutathione} + \text{ADP} + P_i \quad (2)$$

GCS catalyzes the rate-limiting step in GSH synthesis and is also the site of feedback inhibition by GSH. In response to conditions generating oxidative or electrophilic stress, the intracellular content of GSH is often significantly increased. In many cases where it has been examined, the increase in GSH has been attributed to an increase in the activity of GCS. Consequently, elucidating the regulation of the GCS holoenzyme is critical for understanding the dynamics of GSH homeostasis.

γ-Glutamylcysteine Synthetase: Catalytic and Regulatory Subunits

The mammalian GCS holoenzyme is a heterodimer which can be dissociated under non-denaturing conditions into light (GCS_l) and heavy (GCS_h) subunits of 31,000 and 73,000 Da, respectively.[2] Catalytic activity and GSH feedback-inhibition are properties of the heavy subunit, but the kinetic properties of the heavy subunit can be significantly influenced by association with the light, or regulatory, subunit.[3] Specifically, the K_m for glutamate is 1.4 mM versus 18.2 mM for the rat GCS holoenzyme and GCS catalytic subunit, respectively, while the corresponding K_i for glutathione for the two catalytic configurations is 8.2 or 1.8 mM. In the original studies with rat GCS, the kinetic consequences of association with the light subunit were so profound that Huang *et al.* hypothesized that the rat catalytic subunit would be non-functional at typical intracellular concentrations of glutamate (~1–3 mM) and GSH (~1–10 mM)[4] if not associated with the regulatory subunit. An analysis of GCS enzyme activity in mammalian cells transfected with the cDNA corresponding to the human GCS heavy subunit alone or in combination with the human regulatory cDNA at various molar ratios[5] also suggested a functional benefit of expression of the light subunit. This conclusion is also supported in principle by kinetic analyses of recombinant human GCS proteins,[6,7] although minor differences in the kinetic parameters for the human holoenzyme and monomeric catalytic subunit were reported. While the magnitude of the modulatory effect of the light subunit on the catalytic properties of the heavy subunit apparently varies depending on the species or model system examined, to date all studies confirm that the availability of the light subunit clearly enhances the catalytic efficiency of the heavy subunit and reduces its sensitivity to feedback inhibition of GSH. Consequently, evaluation of GSH homeostasis under steady-state conditions or following perturbations requires assessment of the relative contribution of each subunit to the composite response and an examination of expression of the two respective subunit genes. This review will summarize recent experimental findings pertaining to GCS subunit gene

transcription and will critically evaluate molecular mechanisms involved following pro-oxidant challenge. Excellent reviews of other aspects of GSH homeostasis have recently been published and readers interested in a comprehensive overview of GSH metabolism are encouraged to consider these additional sources.[7–10]

TRANSCRIPTIONAL CONTROL OF GSH SYNTHESIS

While it has been reported that GCS enzyme activity can be modulated positively or negatively by post-transcriptional mechanisms, including phosphorylation,[11,12] oxidation[13] or S-nitrosation,[7] the increased GCS activity documented in a majority of cases involves a transcriptional component. The mouse heavy and light GCS subunits are encoded by two distinct genes located on chromosomes 9D-E and 3HI-3, whereas the human genes are mapped to chromosomes 6p12 and 1p21, respectively.[14–17] The human cDNAs[18,19] and genes for both subunits have been cloned and sequenced and potential *cis*-regulatory sequences of the 5′-flanking regions of both subunit genes cataloged.[20–24] As might be expected by their ubiquitous expression, the 5′-flanking regions of the catalytic and regulatory genes share characteristics common to many typical "housekeeping gene" promoters. These features include the presence of putative CpG islands, multiple transcription initiation sites and numerous Sp-1 binding sites. Both genes contain Electrophile Response Elements (EpREs), multiple AP-1 or AP-1-like binding sites and metal response elements (MRE). A consensus NF-κB site is also present in the GCS_h subunit gene promoter but is lacking in the GCS_l subunit gene. The relevance of these and other putative regulatory sequences to transcriptional control of GCS gene expression is currently the subject of intense investigation. Progress in the field over the past several years will be summarized in the remainder of this review.

The Regulation of GSH1, the *Saccharomyces Cerevisiae* GCS Homolog

The functional GCS homolog in the budding yeast *S. cerevisiae* is a monomeric protein of ~78.2 kDa, encoded by the *GSH1* gene. Regulation of *GSH1* expression has been shown to involve a yAP-1 responsive element (YRE) having a sequence identical to the mammalian AP-1 motif.[25] The yeast yAP-1 protein, a homolog of mammalian AP-1,[26,27] binds the YRE to direct gene expression. yAP-1 has been shown to play a prominent role in directing the expression of multiple target genes as part of an adaptive response to oxidative stress, such as that induced by exposure to pro-oxidants including H_2O_2, diamide, diethyl maleate and cadmium.[28–30] Constitutive expression of *GSH1* is reduced in strains deleted for yAP-1 and inducible expression of the gene in response to H_2O_2 is also lost.[31] The yAP-1-dependent activation of *GSH1* in response to oxidative stress is believed to result from increased accumulation of the yAP-1 protein in the nucleus.[32] Nuclear accumulation of the transcription factor is hypothesized to be a consequence of modification of the cysteine rich domain in the carboxy terminus of the protein. This oxidative alteration of the protein decreases its recognition by Crm1, a nuclear export protein which constitutively transports yAP-1 from the nucleus.[33–35] Hence, as a consequence of oxidant-induced modifications, nuclear yAP-1 levels increase, and *GSH1* is up-regulated. Although the H_2O_2 data provide irrefutable evidence of a central role for yAP-1 factors in modulating the induction of *GSH1* in response to this particular pro-oxidant, it should be noted that the yAP-1 deficient strain noted above still supports *GSH1* inducibility in response to menadione,[31] a prototypic superoxide anion generator. Clearly then, yAP-1-independent mechanisms can also affect yeast *GSH1* expression in response to some types of oxidant challenges. Elucidation of the molecular characteristics of these alternative regulatory

pathways remains an important experimental objective.

Regulation of Mammalian GCS: Constitutive Expression

GCS enzyme activity is frequently difficult to measure in many tissues and in cells in culture. Nevertheless, mRNA transcripts corresponding to the two GCS subunits can be detected in most cell types. Transcript levels can be low abundance in some cell types, requiring isolation of mRNA for visualization by Northern blotting or use of more sensitive RNA detection methods like Ribonuclease Protection assays. cDNA probes corresponding to the rat GCS_h sequence hybridize to a single 4.1 kb transcript, while GCS_l specific probes recognize two distinct transcripts ~1.8 and 5.2 kb in length.[36] The corresponding mRNA transcripts for human GCS_h are ~3.2 and 4.1 kb in length, while the GCS_l transcripts are estimated to be 1.4 and 4.1 kb.[19] The difference in transcript size in the case of human GCS_h reflects differences in the length of the 3′-untranslated region of the gene (E.L. Dahl and R.T. Mulcahy, unpublished observation).

Steady-state mRNA levels corresponding to the human GCS_h and GCS_l subunit genes vary in different tissues and in relationship to each other in any individual tissue.[19] Consequently, it is likely that constitutive and inducible expression of the two GCS subunit genes involve tissue-specific mechanisms, perhaps dependent on distinct sets of tissue-specific transcription factors. Characterization of tissue-dependent control mechanisms of GCS subunit gene expression is expected to receive increasing attention from investigators interested in the relationship between GSH homeostasis and tissue-specific pathologies. Similar motivation is sparking investigations into the developmental regulation of GCS subunit gene expression as well.

As is true for *S. cerevisiae*, the prevailing evidence suggests that AP-1 family members play a functional role in the regulation of basal expression of the mammalian GCS subunit genes. For example, total intracellular GSH levels and the constitutive expression of the GCS catalytic subunit gene were decreased in SV40 immortalized fibroblasts derived from c-Jun null mice when compared to fibroblasts originating from c-$Jun^{+/+}$ mice, implicating Jun family members in the regulation of basal expression of the murine GCS_h subunit gene.[37] The involvement of Jun family members in human GCS_h and GCS_l constitutive expression is supported by studies which examined reporter gene expression in HepG2 cells transfected with promoter/reporter transgenes corresponding to wild-type or mutant human GCS promoter sequences. Selective mutation of an AP-1 binding site (−3144 to −3138) embedded within an EpRE located in the GCS_h promoter (see Figure 1) significantly decreased the basal activity

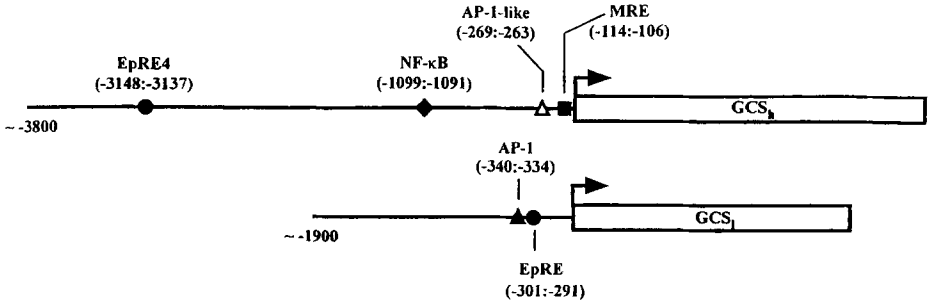

FIGURE 1 Schematic representations of potential regulatory sequences in the human GCS_h and GCS_l genes.

of the GCS_h reporter transgene.[38] The 5′-regulatory region of the GCS_l gene also contains an EpRE sequence (−301 to −291) downstream from a consensus AP-1 motif (−340 to −334). Mutation of the AP-1 sequence decreased the basal activity of the GCS_l promoter transgene.[24,39] Furthermore, co-expression of either GCS_h or GCS_l reporter transgenes with AFos, an AP-1 dominant negative mutant,[40] which significantly inhibits AP-1 activity, reduced the basal expression of the human GCS_h and GCS_l reporter transgenes by 33% and 58%, respectively,[41] providing compelling evidence that AP-1 family members exert an effect on constitutive expression of the two subunit genes.

While assessments of the involvement of AP-1 family members in GCS gene expression have predominated, data implicating other transcription factors is growing. For example, it has been noted that expression of the catalytic subunit gene is decreased in mouse embryos null for MTF-1,[42] the metal-responsive transcriptional activator. It is postulated that a pair of MRE sequences located in the GCS_h promoter (at −114 and +296[42]) function to direct basal expression of the gene. Interestingly, expression of the light subunit gene was equivalent in wild-type and MTF-1 null mice. Presumably, MTF-1 is not involved in the control of the murine light subunit gene basal expression. There is also evidence that AP-1-independent regulatory mechanisms are operative in the basal expression of the human GCS_l gene. Sequences responsible for approximately 50% of the constitutive expression of the human GCS_l reporter transgene are localized to a fragment in the GCS_l promoter sequence (−712 to −344), upstream of the EpRE and AP-1 sites. This fragment contains multiple Sp-1 and AP-2 sites,[24] but transactivating potential is distributed throughout the entire fragment and not assignable to individual elements. Further studies are required to identify the total complement of specific GCS_l *cis*-elements required for full basal expression of the gene and to understand the potential cooperativity of these elements in mediating expression.

Regulation of Mammalian GCS: Induced Expression

Increased expression of the mammalian GCS subunit genes has been observed following exposure to a wide variety of agents and experimental conditions as summarized in Tables I–III.[22,23,41–111] Casual inspection of the diverse array of the prooxidant chemical agents (in Table I) capable of up-regulating GCS gene transcription provides few obvious clues about potential mechanisms of activation. However, upon closer inspection, many of the agents can be recognized as inducers of Phase II detoxicating enzymes. Despite their inherently distinct chemical structures, Phase II gene inducers are all capable of reacting with sulfhydryl groups via oxidation reactions or electrophilic attack.[112,113] The majority of these inducers are themselves electrophilic or are metabolized to electrophiles, and for these agents, inducing potency correlates well with electrophilicity.[114,115]

Other GCS inducing agents capable of producing oxidative or nitrosative stress, including ionizing radiation,[67–71] TNFα,[85,87,89] prostaglandin A2[81] and nitric oxide,[77,78] are not classical Phase II enzyme inducers. However, it is possible that some of these inducers may still mediate gene expression via mechanisms similar to those of classic Phase II inducers. For example, Ohno and Hirata hypothesize that an electrophilic α,β-unsaturated ketone moiety in prostaglandin A2, capable of reacting with cellular sulfhydryls,[81,116] is responsible for its effect on GCS gene induction.

cis-Elements Mediating Induction of the Catalytic Subunit Gene

EpRE

Inducible expression of many detoxicating enzymes, including glutathione S-transferase Ya and NAD(P)H quinone-oxidoreductase, in response to the Phase II inducing agents has been shown to be mediated by EpREs (also referred

TABLE I Effect of pro-oxidants on GCS mRNA expression

	Subunit		Reference(s)
	GCS_h	GCS_l	
Adriamycin	+*	NR	[43]
1-(4-Amino-2-methyl-5-pyrimidinyl)-methyl-3-(2-chloroethyl)-3-nitrosourea	+	NR	[44,45]
Apocynin	+	NR	[46]
β-Naphthoflavone (β-NF)	+	+	[22]
Buthionine sulfoximine (BSO)	+	+	[47–51]
Butylated hydroxyanisole (BHA)	+	+	[52–54]
Butylated hydroxytoluene (BHT)	+	NR	[55]
Cigarette smoke condensate	+	NR	[56]
Ciprofibrate	−	NR	[57]
Copper chloride	+	NR	[58]
Diethyl maleate (DEM)	+	+	[50,52,54,59]
2,3-Dimethoxy-1,4-naphthoquinone (DMNQ)	+	+	[51,60,61]
Diquat	−	NR	[57]
Ethacrynic acid	+	NR	[62]
Ethoxyquin	+	NR	[53]
Hydrogen peroxide (H_2O_2)	+	+	[63–65]
6-Hydroxydopamine	+	NR	[65]
4-Hydroxy-2-nonenal	+	+	[66]
Iodoacetamide	+	NR	[58]
Ionizing radiation (0.05–30 Gy)	+	NR	[67–71]
Menadione	+	+	[54,60,63]
Methyl mercury hydroxide	+	NR	[72–76]
Nitric oxide (direct exposure and IL-1-induced)	+	+	[77,78]
Oltipraz	+	NR	[79]
Oxidized low density lipoprotein	+	NR	[80]
Phorone	+	NR	[52]
Prostaglandin A2	+	NR	[81]
Pyrrolidine dithiocarbamate (PDTC)	+	+	[82]
Sodium arsenite	+	NR	[58]
tert-Butyl hydroquinone (tBHQ)	+	+	[23,50,54,83,84]
Zinc chloride	+	NR	[58]

*+ = increase, − = decrease, 0 = no effect, NR = not reported.

TABLE II Effect of cytokines and hormones on GCS mRNA expression

	Subunit		Reference(s)
	GCS_h	GCS_l	
Dexamethasone	−*	NR	[85]
Erythropoietin	+	NR	[58]
Hydrocortisone	+	0	[50,86]
Insulin	+	0	[50,86]
Interleukin-1β (IL-1β)	+	NR	[87]
Transforming growth factor β1 (TGF-β_1)	−	NR	[88]
Tumor necrosis factor-α (TNFα)	+	NR	[85,87,89]

*+ = increase, − = decrease, 0 = no effect, NR = not reported.

to as Antioxidant Response Elements) localized within the 5′-flanking regions of the corresponding genes.[117–119] Recognition of the high coincidence of induction of the GCS genes with those encoding the Phase II enzyme battery prompted the hypothesis that the transcriptional up-regulation of the GCS subunit genes were likewise mediated by EpREs or their functional equivalents.[52,53,61,120] This possibility was strengthened when cloning and sequencing of the human genes culminated in the identification of EpRE sequences in the 5′-flanking regions of both GCS_h and GCS_l genes.[20–24]

TABLE III Effect of miscellaneous treatments on GCS mRNA expression

	Subunit		Reference(s)
	GCS_h	GCS_l	
L-Azetidine-2-carboxylic acid	+*	NR	[58]
Chronic ethanol and high-fat diet	+	NR	[90]
Cisplatin	+	NR	[91]
Cycloheximide	+	NR	[45]
↑ Days in culture	+	NR	[92]
Heat shock	+	NR	[58]
High glucose	−	NR	[87]
Hypoxia	+	NR	[93]
Lipopolysaccharide (LPS)	−	NR	[94]
Low density plating	+	0	[50,86]
Methionine or protein-supplemented diet	−	NR	[95]
Okadaic acid	+	NR	[37]
Partial hepatectomy	+	NR	[96]
Conditions associated with stable expression			
BSO-resistant cell lines	+, −	NR	[97–99]
Copper-deficient rats	+	NR	[100]
Drug-resistant cell lines	+	+	[21,101–110]
Eisai hyperbilirubinemic rats	+	NR	[111]
MTF-1 null mice	+	0	[42]

*+ = increase, − = decrease, 0 = no effect, NR = not reported.

Four putative sequences matching the EpRE consensus sequence were described in the promoter of the human GCS_h gene. Three of these (EpRE1, 2 and 3) were determined to be non-functional by promoter analysis studies.[22,38] However, the most distal EpRE sequence (EpRE4), located ~3.1 kb upstream of the transcription start site (−3148 to −3137), was identified as a key GCS_h regulatory sequence. An EpRE4 point mutation introduced into a promoter/reporter transgene containing 3.8 kb of the GCS_h 5′-flanking sequence disrupted both basal and β-naphthoflavone (β-NF)-induced expression of the reporter, solidifying EpRE4's role in the constitutive and β-NF inducible expression of the gene.

β-NF, a bifunctional Phase II inducer, was selected for study in early investigations of the GCS promoters because it was originally used in identifying the first EpRE sequence[117] and served as a prototypic inducer of EpREs. However, despite compelling evidence that β-NF-responsiveness of GCS_h is mediated by EpRE4 (Figure 2A), several other Phase II enzyme inducers, including *tert*-butylhydroquinone (tBHQ), menadione, hydrogen peroxide (H_2O_2), phenethyl isothiocyanate (PEITC), diethyl maleate (DEM) and pyrrolidine dithiocarbamate (PDTC), all failed to induce a meaningful increase (~1.3–1.7-fold) in reporter activity in HepG2 cells transfected with the "full-length" 3.8 kb GCS_h promoter/luciferase transgene.[54] In contrast, endogenous GCS_h transcript levels increased dramatically following exposure to these same agents. For example, 100 μM PDTC increased GCS_h steady-state mRNA levels ~8-fold (Figure 2B), yet resulted in an inconsequential increase (1.3-fold) of reporter gene expression.[41,54,82] The failure to direct expression of reporter transgenes harboring up to 5.5 kb of the GCS_h promoter (J.J. Gipp, A.C. Wild and R.T. Mulcahy, unpublished observation) leads us to conclude that one or more regulatory elements essential for mediating response to these agents resides outside of this expanse of the GCS_h 5′-flanking sequence.

AP-1-Like Sequence

Besides EpRE4, two additional sequences within the GCS_h 5′-flanking region have been identified as potential GCS_h regulatory elements (Figure 1). A proximal AP-1-like sequence (5′-TGATTCA-3′) located at −269:−263 has been implicated in up-regulation of the human GCS_h gene in response to a number of agents including cigarette smoke condensate,[56] menadione,[63,121] H_2O_2,[63,121] TNFα,[85,89] oxidized low density lipoprotein[80] and ionizing radiation.[69] However, arrival at consensus regarding the regulatory significance of this particular AP-1 site is hampered by marginal inductions (some of questionable biological relevance) in some cases, as well as by inconsistencies among groups in others. While reasonably robust induction (~2–3-fold) of GCS_h

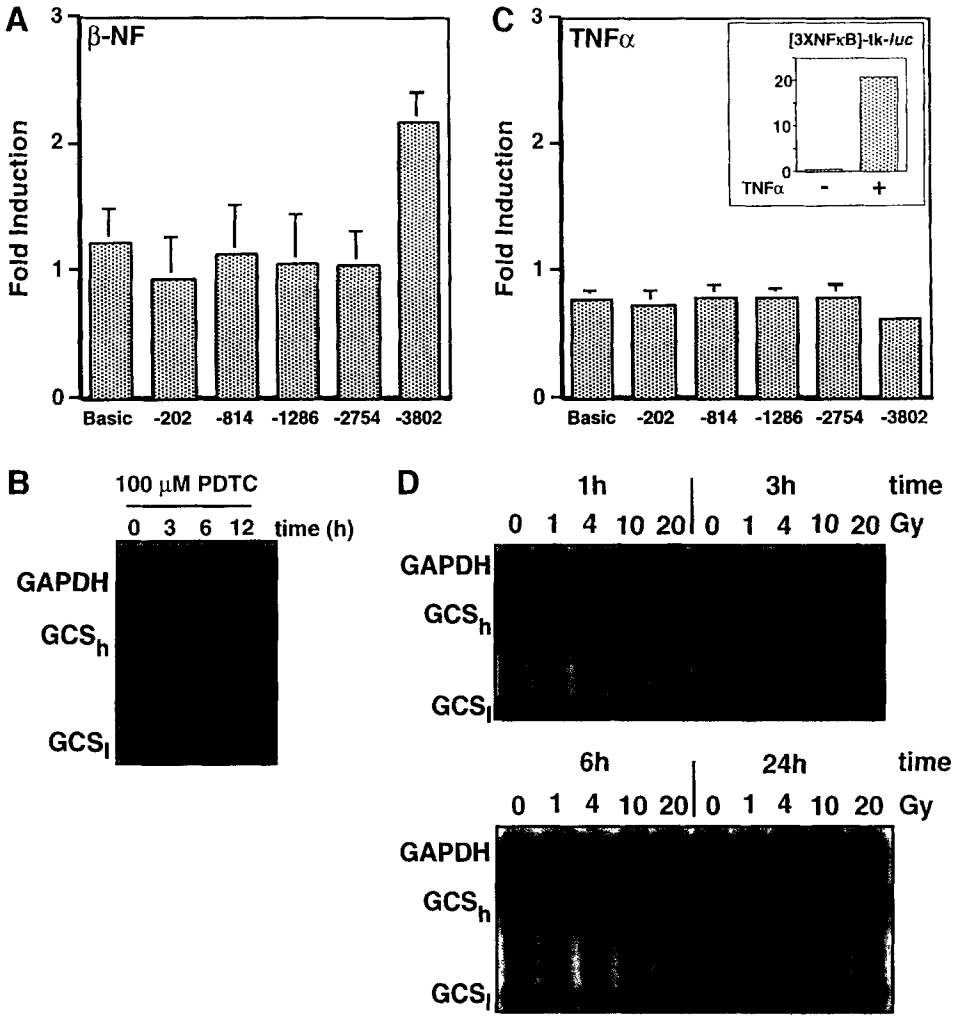

FIGURE 2 The effect of β-NF, TNFα, ionizing radiation and pyrrolidine dithiocarbamate on GCS_h gene expression. (A) and (C) HepG2 cells were co-transfected with GCS_h promoter/reporter transgenes containing progressively longer fragments of GCS_h 5′-flanking sequences and the plasmid pCMVβ, expressing β-galactosidase. The numbers on the *x*-axis refer to nucleotide sequences from the GCS_h 5′-flanking region contained in pGL3-Basic reporter vectors. Sixteen hours after the addition of medium containing 10 μM β-NF (A) or 20 h following treatment with 500 units/ml TNFα (Promega Corp., Madison, WI) (B), cells were harvested, and supernatants prepared for luciferase, β-galactosidase and protein determination. Fold induction was calculated as the ratio of luciferase expression in treated cells to luciferase expression in untreated cells after correction for β-galactosidase activity and protein content. The results are mean ± standard deviation of three determinations. Inset in (C): HepG2 cells were transfected with a recombinant plasmid containing three consensus NF-κB sequences upstream of *tk*-luciferase in the pT81 (ATCC) reporter vector. (B) and (D) Steady-state message levels of GCS_h and GCS_l subunits were evaluated following exposure of HepG2 cells to 100 μM pyrrolidine dithiocarbamate for 0–12 hours (B) or to ionizing radiation (D). HepG2 cells were treated with 1–20 Gy ionizing radiation using a J.L. Shepherd Cesium 137 Irradiator, at a dose rate of 5.6 Gy/min. Total RNA was harvested at 1, 3, 6 and 24 h following irradiation. Twenty micrograms of total RNA per sample was analyzed by Ribonuclease Protection Assay, as previously described.[22]

promoter/reporter transgenes was reported following H_2O_2 and oxidized low density lipoprotein treatment, only marginal inductions were demonstrated for ionizing radiation and cigarette smoke condensate (~1.4–1.8-fold). Although each group of investigators cited above interpret their data as providing evidence that the AP-1-like binding site at −269:−263 mediates up-regulation

of GCS_h in response to their respective agents, this hypothesis was tested by site-directed mutagenesis of the AP-1-like sequence only in experiments evaluating oxidized low density lipoprotein inducibility.[80] Furthermore, even though the same agents were examined, some investigators failed to detect the involvement of this AP-1-like binding site in GCS_h induction at all, attributing up-regulation in response to ionizing radiation[71] or TNFα,[87] for example, to activation of NF-κB. Similarly, our own studies using HepG2 cells transfected with GCS_h promoter/reporter transgenes failed to confirm a functional role for the AP-1-like sequence in response to menadione, H_2O_2 or TNFα (Figure 2C). Furthermore, we have been unable to confirm significant induction of endogenous GCS_h message levels following treatment of HepG2 cells (Figure 2D) or A549 cells with TNFα or ionizing radiation. Unfortunately, these disparate findings undermine confidence in the assignment of a key role for this specific AP-1-like site in GCS_h regulation at this time.

NF-κB Sequence

As already mentioned above, some reports implicate NF-κB as mediating GCS_h inducibility. Cai *et al.* demonstrated that treatment with the protease inhibitors TPCK and TLCK, which have been shown to inhibit NF-κB activation, reduced NF-κB binding to its cognate recognition sequence while also decreasing GCS_h mRNA induction following treatment with tBHQ or buthionine sulfoximine.[50] In similar experiments, Urata *et al.* found that the TNFα-dependent increases in GCS_h message levels were likewise reduced by concurrent treatment with either of these two protease inhibitors.[87] While providing indirect evidence to support a role of NF-κB in GCS gene regulation, neither of these studies examined specific *cis*-elements present in the GCS_h promoter that might be involved. On the other hand, Iwanaga *et al.* did identify an NF-κB binding sequence (5′-GGAAATCCC-3′, −1099 to −1091) in the GCS_h 5′-flanking sequence that they report is involved in mediating GCS_h induction by ionizing radiation.[71] This conclusion, however, is contradictory to that reported by Morales *et al.* who concluded that the radiation response was mediated by the proximal AP-1-like sequence[69] and to our own studies which failed to detect a radiation-induced increase in endogenous GCS_h expression.

In summary, multiple *cis*-elements, including an EpRE, NF-κB and AP-1-like binding site, are hypothesized to mediate up-regulation of human GCS_h subunit gene expression in response to various inducing agents. However, in all but a few cases, the current data are too inconsistent to allow definite assignment of relevance to any specific element. Furthermore, although large fragments of the GCS_h promoter (up to 5.5 kb) have been evaluated, a strong possibility exists that an additional regulatory element(s) contributing to activation of the GCS_h subunit gene in response to several Phase II inducing agents has yet to be identified.

cis-Elements Mediating Induction of the Regulatory Subunit Gene

Although far fewer studies investigating the role of specific *cis*-elements in the promoter of the GCS_l gene have been published, they are not without their differences. In contrast to the situation with the GCS_h subunit, all elements required for full induction of the GCS_l subunit gene in HepG2 cells by PDTC, menadione, PEITC, tBHQ, H_2O_2 and DEM are contained within the proximal 712 bp of the promoter. Inclusion of up to 6 kb of additional 5′-flanking sequence does not alter basal or inducible transgene expression.[24,39,54]

β-NF-induced expression of a 1.9 kb GCS_l promoter/reporter transgene in HepG2 cells was disrupted only by simultaneous mutation of the GCS_l EpRE sequence (−301 to −291) and the upstream AP-1 binding site (−340 to −334).[24] The mutation of these sequences individually did not eliminate inducibility, illustrating that either element could direct GCS_l expression.

Similarly, PDTC- and tBHQ-induced expression of the GCS_l reporter transgene was also eliminated only by mutation of both the EpRE and upstream AP-1 binding sequences (Mulcahy *et al.*, unpublished observation).

Similar mutational analyses of the GCS_l 5′-regulatory region were performed in HepG2 cells by Galloway and McLellan.[39] These investigators localized sequences important for inducible expression in response to tBHQ and β-NF to the same general region as reported by Moinova and Mulcahy (−411 to −256),[24] but disagree with the conclusion that the EpRE or upstream AP-1 sequences support gene induction. When a 156 bp fragment (between −411 and −256) containing the AP-1, EpRE and intervening sequences was deleted from GCS_l promoter/reporter transgenes, induction was completely abolished. In contrast to the previous report,[24] however, site-directed mutagenesis of both the EpRE and upstream AP-1 binding sites did not abolish induction by either tBHQ or β-NF. Furthermore, removal of a 42 bp fragment between −344 and −303, which eliminated the AP-1 binding site and the intervening bases between the AP-1 and EpRE, severely reduced inducibility. Since this mutant reporter retained an intact EpRE core sequence (−301 to −291), Galloway and McLellan argued that the EpRE sequence was unable to support induction, as previously demonstrated. Based on these observations, these investigators hypothesize that tBHQ and β-NF inducible expression is not mediated by either the AP-1 or EpRE sequence, but rather by an unidentified element resident in the 42 bp region between −344 and −303.

It should be noted, however, that the −344:−303 deletion mutation used by Galloway and McLellan to assess the GCS_l EpRE function did not preserve bases flanking the 3′-end of the EpRE core, nucleotides which have been shown to influence maximal EpRE function.[122–124] Resolution of these differences and confirmation of the specific nucleotide sequence ultimately responsible for induction of the GCS_l gene by these agents requires further analysis.

Technical Considerations

Perhaps multiplicity of *cis*-elements is to be expected considering the varied types of inducing agents capable of up-regulating GCS gene expression. However, as already described, there are differences of opinion, and in some cases even controversies, regarding specific *cis*-acting elements responsible for transactivation of the respective GCS genes and the data cited to support their involvement. Some of these discrepancies may reflect differences in cell types utilized, as commonly suggested, but other factors, some technical in nature, might also contribute to observed differences. One major technical factor that requires careful analysis in this regard is the specific plasmid vectors used in the various promoter studies. Many of the studies reported to date used early versions of the pCAT- and pGL-series of reporter vectors offered by Promega.[39,56,63,85,121] The manufacturer has since modified the original vectors in an effort to, among other things, "eliminate consensus sequences recognized by genetic regulatory binding proteins" present in the original vector backbone.[125,126] Specifically, AP-1 sequences have been eliminated from each vector, reducing the number present in pCAT-Basic and in pGL2-Basic from three to one in the newer reporter vectors. In our experience, significant differences in expression were detected using pGL2- and pGL3-based luciferase reporters containing the same GCS 5′-sequences.

Another issue to consider in making comparisons among the various studies is the array of functional elements included in the reporter construct itself. For example, the presence or absence of different enhancers or minimal promoters could influence outcome in unpredictable ways. Such effects may not invalidate group comparisons in any given study, but may confound comparisons across studies.

Finally, it is important to recognize that many of the studies examining the GCS_h 5′-regulatory region only include the first ~2 kb upstream of

the transcriptional start site, so the potential role of the distal elements such as EpRE4 are not assessed. It would seem prudent to include as much of the 5′-flanking sequence as possible in assessment of *cis*-regulatory elements, especially when the sequence and evidence of functional distal elements are available.

trans-FACTORS INVOLVED IN GCS GENE INDUCTION

Once specific *cis*-regulatory sequences have been identified, sequence specific probes can be utilized to identify transcription factors binding these sequences in response to experimental manipulations. Identification of *trans*-factors can in turn yield information about signaling pathways involved in transduction from drug exposure to response. Perhaps the best characterized regulatory sequences in the GCS genes in terms of transcription factor analysis are the EpRE sequences in the promoters of the two subunit genes. Progress in this area has been accelerated by complimentary analyses of EpREs in various other Phase II gene promoters.

Because EpRE sequences often contain embedded AP-1 binding sites or are flanked by these sequences,[127] it is not surprising that investigators have hypothesized a prominent role for the redox sensitive AP-1 family members in gene induction via EpRE sequences.[128–134] More recently, it was recognized that EpREs bear a remarkable sequence similarity to NF-E2 binding sites and TRE-type Maf Recognition Elements (T-MAREs). In fact, it is suggested that EpRE sequences may actually represent a subset of the more extended responsive element, T-MARE.[24,135] Consequently, EpRE sequences may be recognized by bZip transcription factors which commonly bind T-MARE sequences, including members of the AP-1, NF-E2 (Nrf1 and Nrf2) and small Maf families. In support of this hypothesis, Itoh *et al.* presented data from Nrf2 knock-out mice, demonstrating that Nrf2 heterodimers were responsible for the EpRE-mediated induction of Phase II enzymes by agents generating electrophilic or oxidative stress.[136] Their data further suggested that small Maf proteins represented the major Nrf2 heterodimerization partner involved in this response. Furthermore, Nrf1, Nrf2 and certain AP-1 family members have been shown to bind EpRE sequences to influence EpRE-driven reporter expression.[133,134]

In light of these experiments, the EpRE transactivating potential of NF-E2 family members, including Nrf1 and Nrf2, and the small Maf proteins have been examined in greater detail. Depending on the specific bZip dimer composition, the resulting complexes differ with respect to EpRE sequence specificity, binding affinity and transactivating potential. For example, homo- or heterodimers composed of small Maf proteins, which lack transactivation domains, transcriptionally repress gene expression via EpRE/T-MARE sequences, whereas heterodimerization of Nrf2 and small Maf proteins results in positive transactivation of gene expression.[137–140] The relatively large number of possible bZip homo- and heterodimer combinations contributes to a remarkably flexible response system accounting for what Kerppola and Curran[141] refer to as a "combinatorial determination of target gene specificity." Even in the case of a single gene, these combinations of factors could account for activation by a broad range of inducing agents through common *cis*-elements.

The roles of the bZip factors Nrf2, small Maf proteins and JunD in GCS subunit gene regulation have recently been evaluated.[41,142] In these experiments, increased GCS gene expression in response to β-NF or PDTC treatment was associated with an increase in the binding of Nrf2 to EpREs in the promoters of both GCS genes. Evidence of small Maf and JunD proteins in complexes binding the GCS_h EpRE sequences was also detected; evidence for Maf was more ambiguous in the case of the light subunit EpRE. Nrf2 overexpression increased the activity of GCS_h

and GCS_l promoter/reporter transgenes, and enhanced GCS_l reporter transgene expression when co-transfected with either MafG or JunD. Transfection of MafG alone, however, decreased GCS_h and GCS_l promoter/reporter activity. The most convincing data supporting the conclusion that Nrf2 is a positive regulator of GCS gene transcription was provided by experiments which utilized a MafK dominant negative mutant. This mutant, which lacks a DNA binding domain, was previously shown to sequester Nrf2 protein, inhibiting its ability to form heterodimers capable of binding DNA.[143] Expression of the MafK dominant negative mutant in HepG2 cells decreased Nrf2 binding to GCS EpRE sequences, inhibited the inducible expression of GCS_h and GCS_l promoter/reporter transgenes and reduced endogenous GCS gene induction by β-NF and PDTC. Collectively, these data strongly suggest that Nrf2 participates in mediating GCS_h and GCS_l gene induction by these Phase II gene inducers. A role for small Maf proteins and JunD, both potential Nrf2 heterodimerization partners, is also suggested, but evidence in support of their involvement is far more tenuous than that for Nrf2.

INITIATING SIGNAL FOR GCS INDUCTION

Two alternatives, generation of reactive oxygen species and modification of protein thiols, have been proposed as the biochemical signals initiating the sequence of events culminating in transcriptional up-regulation of EpRE-containing genes, including those encoding the two GCS subunits.[112,113,122] Many of the agents shown to up-regulate the two GCS genes are capable of generating reactive oxygen intermediates either directly or as a result of redox cycling, fueling speculation that gene induction is in response to increases in specific reactive oxygen intermediates. However, in characterizing the mechanisms of action of the diverse chemical compounds comprising the Phase II enzyme inducers, Talalay and colleagues noted that the sole universal property of these agents was their ability to react with sulfhydryl groups. They therefore speculated that thiol modification of a key regulatory protein represented the ultimate signaling mechanism for EpRE-mediated Phase II enzyme induction. While indirect evidence and speculation abound, few experiments designed to discriminate between these and other possible signaling mechanisms in the regulation of the GCS subunit genes have been reported.

A number of investigators have proposed possible regulatory signals required for GCS gene induction, based on the chemical properties of the various inducing agents.[51,52,60,61,66,82,83,144] In perhaps the most direct approach to this question, Shi and colleagues present data supporting the involvement of reactive oxygen intermediates in GCS gene inducibility.[60,61] These investigators compared the ability of the two related quinones, menadione and 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), to up-regulate, GCS gene expression in rat lung epithelial L2 cells or bovine pulmonary artery endothelial cells. DMNQ is a non-conjugating quinone, and its toxicity is thought to result from generation of $O_2^{\bullet}$ and H_2O_2. In contrast, menadione is capable of generating reactive oxygen species via redox cycling and of thiol conjugation. Since both quinones were equally effective at inducing GCS subunit gene expression, it was concluded that formation of an electrophile–glutathione conjugate was not required for gene induction. Although the mechanisms of DMNQ and menadione induction are still not fully characterized, these data provide compelling evidence that the generation of reactive oxygen species is sufficient in some cases to initiate GCS gene induction by the quinones DMNQ, menadione and perhaps, tBHQ.[51]

Studies examining GCS induction following exposure to the dithiocarbamate PDTC, on the other hand, suggest a signaling mechanism involving the activation of a critical regulatory protein(s) by thiol modification, as opposed to the

primary involvement of reactive oxygen species as ultimate effectors.[82] Such a model is consistent with Talalay's proposed mechanism of gene induction by EpRE activators. The formation of either PDTC thiuram disulfides or copper–PDTC complexes, both of which are capable of oxidizing GSH or modifying protein sulfhydryl groups, is hypothesized to occur following exposure of HepG2 cells to PDTC. Significant increases in oxidized glutathione (GSSG) were observed in PDTC-treated cells, but there was no evidence of the generation of reactive oxygen species nor evidence suggesting that reactive oxygen species were required for induction.

Although the DMNQ and PDTC data appear to support different conclusions regarding the identity of the proximate GCS gene inducing signal, it should be noted that these two hypothesized activation schemes are not necessarily mutually exclusive. While it is certainly possible that distinct, agent-specific regulatory signals are capable of inducing the GCS genes, it is conceivable that these signals converge at a common downstream effector, for example, a regulatory sensor protein which is thereby activated as a result of oxidation or thiol modification, as proposed for other EpRE inducers by Talalay.

Interestingly, activation of the Nrf2 transcription factor may represent such a thiol-modified regulatory protein. It has been demonstrated that Nrf2 activation does not involve increases in the total cellular levels of the protein, but rather increases in nuclear localization.[145] It is hypothesized that Nrf2 is sequestered in the cytosol by its interaction with the cytoskeletal-binding protein, Keap1. Itoh *et al.* postulated that the pro-oxidant-induced alteration of the Nrf2–Keap1 interaction resulting in release and activation of Nrf2 is due to either electron transfer reactions generated within the repressor Keap1 protein or oxidation of key cysteine residues in either Nrf2, Keap1 or both proteins. Activation of other transcription factors, such as the proposed Nrf2 heterodimerization partners, small Maf proteins[41,146] and JunD, may also fit this paradigm. Identification and characterization of the specific effectors sensing and transducing the pro-oxidant regulatory signal that initiates GCS gene induction represents one of the most important areas of investigation in the field of stress-induced transcriptional regulation.

A WORKING MODEL FOR EpRE-MEDIATED INDUCTION OF THE GCS SUBUNIT GENES

Based on evidence from the literature and from recent studies completed by our laboratory,[41,142] we have developed a working model for GCS subunit gene expression in response to β-NF, PDTC and perhaps other inducers which function via the EpRE sequences (Figure 3). Following β-NF or PDTC exposure of HepG2 cells, Nrf2 is released from Keap1 and the expression of MafG (and possibly other small Maf proteins) is induced. The formation and binding of specific Nrf2–bZip heterodimers to GCS EpRE sequences results in increased GCS gene transcription. Currently, we hypothesize that Nrf2 heterodimerizes with one or more of the small Maf proteins or JunD. However, different bZip factors are likely to be up-regulated by different inducing agents, hence the specific composition of the Nrf2/bZip heterodimer is likely to be agent-specific. Furthermore, since different Nrf2/bZip heterodimers can recognize subtle differences in EpREs and flanking nucleotides and bind with differing affinities, the transactivating potential of particular Nrf2/bZip dimers might be expected to vary.

The existence of regulatory mechanisms to down-regulate EpRE-mediated gene expression have also been proposed.[133,147] We hypothesize that the GCS gene down-regulation[41] involves the formation of homo- or heterodimers composed of bZip factors lacking transactivation domains, such as small Maf proteins and Fra1. Small Maf factors and Fra1 are up-regulated by β-NF and PDTC exposure,[38,41,148] and as these factors accumulate and dimerize, they compete

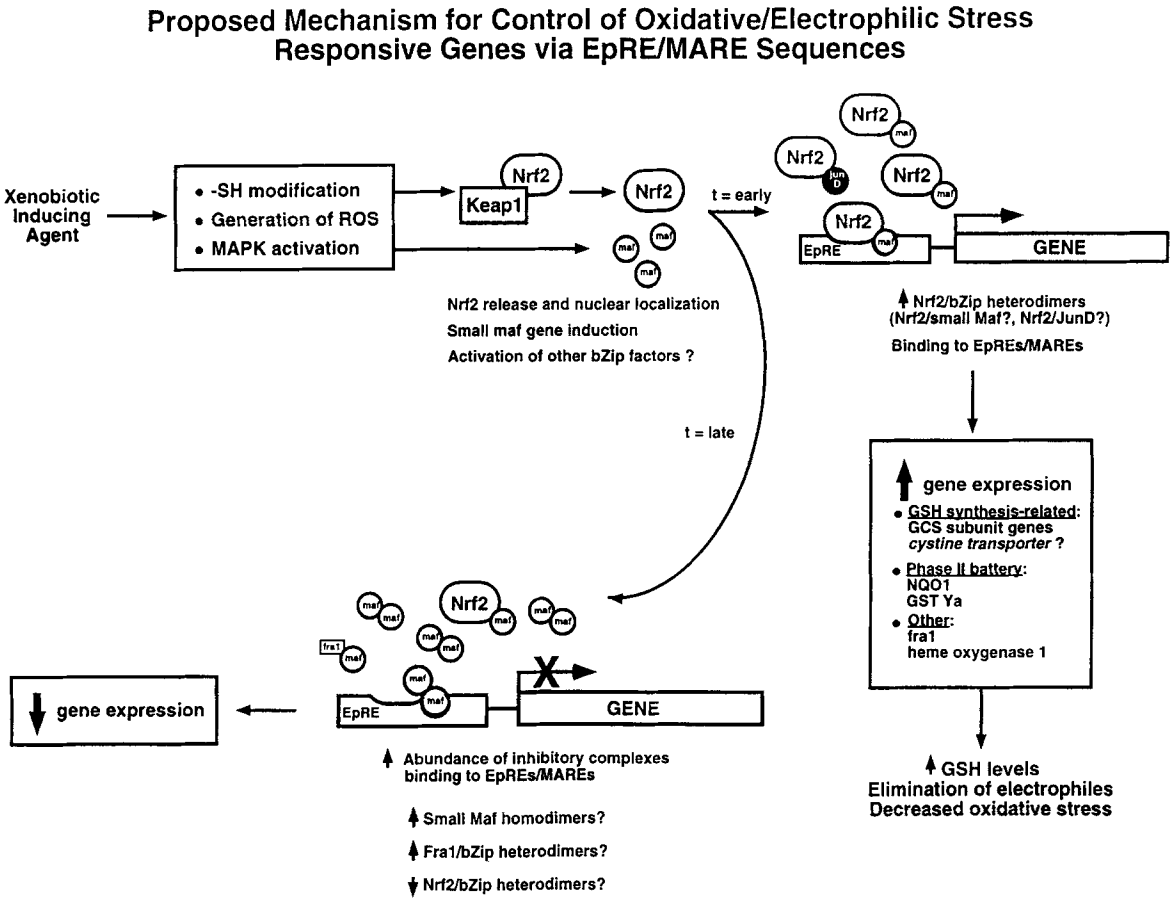

FIGURE 3 Working model for EpRE-dependent GCS gene regulation following xenobiotic exposure.

with positive-regulatory bZip dimers for binding to GCS EpRE sequences, eventually resulting in the inhibition of GCS gene transcriptional activity.

Admittedly, many details need to be filled in, but this working model provides an excellent blueprint for future experiments designed to further define the mechanisms operative in the transcriptional regulation of the GCS genes. Furthermore, it is expected that many of these control elements will participate in the transcriptional regulation of other genes involved in the response to oxidative challenge.

DIFFERENTIAL REGULATION

The model presented above suggests the two subunit genes are coordinately regulated and that transcriptional control of both involves similar elements. However, the GCS subunit genes are differentially responsive to certain stimuli. For example, induction of the catalytic subunit gene alone has been demonstrated in response to hormone exposure,[50] manipulation of cell proliferation[50] and hypoxic conditions (K.S. Gregg, A.C. Wild and R.T. Mulcahy, unpublished observation). And, as mentioned earlier, an MRE directs basal expression of the murine GCS_h gene, but is apparently not involved in GCS_l regulation.[42] Even in some cases where the two genes appear to be co-regulated, subtle differences in gene regulation have been documented. Liu and colleagues recently demonstrated induction of both GCS subunit genes by 4-hydroxy-2-nonenal, but determined that the GCS_l subunit gene was induced to a greater extent than was the GCS_h

gene.[66] Furthermore, they demonstrated that the protein synthesis inhibitor, emitine, only blocked induction of the GCS_l subunit gene, clearly suggesting differences in the transcriptional activation of the two genes. Like Liu *et al.*, we have also observed differences in the magnitude of induction of the two genes in response to some agents, including H_2O_2, PEITC and β-NF.[54] In all cases, the GCS_l gene is induced to a greater extent than the GCS_h subunit gene. We also have noticed differences in GCS gene expression in response to attempts to manipulate the availability of potential regulatory transcription factors. For example, expression of a MafK dominant negative mutant decreased endogenous GCS_h messenger RNA levels following PDTC treatment from 8- to 2-fold, but only reduced GCS_l induction by 50%.[41] Furthermore, although both GCS EpRE sequences are recognized and bound by Nrf2 protein, GCS_h and GCS_l EpRE/protein complexes are differentially recognized by MafG antibodies, suggesting differences in the array of transcription factors bound to the two sequences, even in response to the same inducing agents.[41] These data indicate that understanding the molecular mechanisms responsible for control of GCS expression will in all likelihood require elucidation of subunit-specific regulation.

SUMMARY

The significance of glutathione in the protective response to noxious agents and the up-regulation of the GCS subunit genes following exposure suggests that GCS serves as an important chemoprotective enzyme. A review of the GCS literature suggests that multiple *cis*-elements may be important for mediating GCS gene inducible expression, suggesting the existence of agent-dependent alternative activation pathways. Although there is reason to be cautious in interpretation, the data suggest that the regulation of the catalytic subunit gene can involve a distal EpRE sequence, an MRE binding site, an AP-1-like sequence and an NF-κB binding site. However, despite the fact that $\sim$5.5 kb of the GCS_h promoter has been examined, the inability to induce reporter expression in response to a number of agents capable of inducing endogenous GCS_h expression strongly suggests that other, yet unidentified, sequences function to direct gene expression. Expression of a MafK dominant negative mutant was demonstrated to reduce binding of Nrf2 to EpRE sequences and to inhibit endogenous GCS_h inducibility by both β-NF and PDTC, suggesting that the "missing" GCS_h *cis*-regulatory element(s) is (are) an EpRE or some other member of the extended T-MARE family. Studies evaluating the GCS_l subunit gene promoter have localized key responsive elements to a region of the promoter encompassing an EpRE sequence, an upstream AP-1 binding site and the intervening 32 bp in mediating GCS_l responsiveness. However, the exact sequences within this span of nucleotides which mediate GCS_l inducibility are still the subject of debate. Recent studies provide support for the EpRE-dependent regulation of the GCS subunit genes by the bZip transcription factors Nrf2, small Maf family members and the AP-1 members JunD and Fra1. These data also predict the existence of a negative regulatory mechanism designed to down-regulate GCS gene induction.

Further evaluation of all elements comprising the GCS_h and GCS_l subunit gene regulatory pathways, including the initiating signal, molecular sensors, signal transduction cascades, *trans*-acting factors and GCS-specific *cis*-regulatory sequences, is required before the differential expression of the two genes and the resulting consequences for GCS holoenzyme function are fully understood. The last several years have witnessed key scientific milestones in this quest. The next several years can be anticipated to provide new insight at an even greater pace.

Acknowledgments

This work was supported by NIH grant RO1-CA57549 and NIEHS grant ES09749.

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