Constitutive and β-Naphthoflavone-induced Expression of the Human γ-Glutamylcysteine Synthetase Heavy Subunit Gene Is Regulated by a Distal Antioxidant Response Element/TRE Sequence*

(Received for publication, December 2, 1996)

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Glutathione (GSH) is an abundant cellular non-protein sulphydryl that functions as an important protectant against reactive oxygen species and electrophiles, is involved in the detoxification of xenobiotics, and contributes to the maintenance of cellular redox balance. The rate-limiting enzyme in the de novo synthesis of glutathione is γ-glutamylcysteine synthetase (GCS), a heterodimer consisting of heavy and light subunits expressing catalytic and regulatory functions, respectively. Exposure of HepG2 cells to β-naphthoflavone (β-NF) resulted in a time- and dose-dependent increase in the steady-state mRNA levels for both subunits. In order to identify sequences mediating the constitutive and induced expression of the heavy subunit gene, a series of deletion mutants created from the 5′-flanking region (~3802 to +465) were cloned into a luciferase reporter vector (pGL3-Basic) and transfected into HepG2 cells. Constitutive expression was maximally directed by sequences between ~202 and +22 as well as by elements between ~3802 to ~2752. The former sequence contains a consensus TATA box. Increased luciferase expression following exposure to 10 μM β-NF was only detected in cells transfected with a reporter vector containing the full-length ~3802+465 fragment. Hence, elements directing constitutive and induced expression of the GCS heavy subunit are present in the distal portion of the 5′-flanking region, between positions ~3802 and ~2752. Sequence analysis revealed the presence of several putative consensus response elements in this region, including two potential antioxidant response elements (ARE3 and ARE4), separated by 34 base pairs. When cloned into the thymidine kinase-luciferase vector, pTK-luciferase, and transfected into HepG2 cells, both ARE3 and ARE4 increased basal luciferase expression approximately 20-fold. When cloned in tandem in their native arrangement the increase in luciferase activity was in excess of 100-fold, suggesting a strong interaction between the two sequences. Luciferase expression was elevated in β-NF-treated cells transfected with the ARE4-tk-luciferase vector and all DNA fragments containing ARE4. In contrast, ARE3 did not directly increased luciferase expression in response to β-NF nor did it significantly modify the magnitude of induction directed by ARE4. The influence of the ARE4 oligonucleotide on constitutive and induced expression was eliminated by introduction of a single base mutation, converting the core ARE sequence in ARE4 from 5′-GGTACCTCGG-3′ to 5′-GGGACTCAGG-3′. When introduced into the full-length ~3802+465 segment, the same single base mutation also eliminated both functions. Collectively the data indicate that the constitutive and β-NF-induced expression of the human GCS heavy subunit gene is mediated by a distal ARE sequence containing an embedded tetradecanoylphorbol-13-acetate-responsive element.

Glutathione (L-γ-glutamyl-L-cysteinyl-glycine, GSH),1 a non-protein sulphydryl compound present in millimolar concentrations in virtually all cells, serves a myriad of cellular functions and plays a prominent role as an intracellular protectant (1, 2). GSH is an effective oxygen radical scavenger and serves as a critical co-factor in peroxide detoxification via a reaction catalyzed by glutathione peroxidase. Furthermore, conjugation with GSH is an integral step in the detoxification and elimination of diverse classes of toxic chemical compounds. The formation of hydrophilic glutathionyl conjugates is catalyzed by glutathione S-transferases, a family of isozymes that mediate the conjugation reaction in a substrate-dependent fashion (3). Long the object of interest from a toxicology perspective, the protective properties of GSH have assumed even further significance since GSH not only plays a critical role in protection of normal cells, but it has recently been implicated in protection of neoplastic cells from a number of chemotherapeutic agents that exert their cytotoxic effects via generation of reactive oxygen species or production of electrophilic intermediates (4, 5). The augmentation of GSH and GSH-related detoxification systems has also engendered considerable interest as a possible approach for the chemoprevention of cancer. Many chemical chemopreventive agents have been shown to exert an effect on GSH homeostasis or on other elements of GSH detoxification pathways (6–8).

Exposure of cells to a number of xenobiotic agents has been demonstrated to result in an increase in the total intracellular GSH content. In several cases (9–16) where it has been examined, the increase in GSH has been attributed to an

*This work supported by National Institutes of Health Grants RO1-CA57549 (to R. T. M.) and RO1-Ca01749 (to H. H. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1The abbreviations used are: GSH, glutathione; ARE, antioxidant response element; β-NF, β-naphthoflavone; EpRE, electrophile response element; GCS, γ-glutamylcysteine synthetase; GCS, heavy subunit of GCS; GCS, regulatory subunit of GCS; MeSO, dimethyl sulfoxide; TRE, 12-O-tetradecanoylphorbol-13-acetate-responsive element.
increase in the activity of γ-glutamylcysteine synthetase (GCS, EC 6.3.2.2), the rate-limiting enzyme in its de novo synthesis (17). The GCS holoenzyme is a heterodimer that can be dissociated under non-denaturing conditions into light (GCS_l) and heavy (GCS_h) subunits of 28,000 Da and 73,000 Da, respectively (18). Catalytic activity resides entirely with the heavy subunit, but it has recently been demonstrated that the kinetic properties of the heavy subunit can be profoundly influenced by association with the light, or regulatory, subunit and the redox state of a single disulfide linkage between the two subunits (19, 20). The heavy and light subunits are encoded for by two distinct genes located on chromosomes 1 and 6, respectively (21–23). The cDNAs for each subunit of human GCS have been cloned and sequenced (24, 25), as has the 5′-flanking sequence of the heavy subunit gene (26). Northern analyses reveal that xenobiotic-induced GCS enzyme activity is frequently accompanied by an increase in the steady-state levels of GCS_h-specific mRNA transcripts (10, 12–14, 16). Similar analyses for the light subunit genes have only recently been completed. Exposure to the antioxidant butylated hydroxyanisole (16), cDNA has only recently been completed. Exposure to the antioxidant butylated hydroxyanisole (16), tert-butyl hydroquinone (14), and methyl mercury (10) results in transcriptional up-regulation of the heavy subunit gene, suggesting that GCS_h gene expression in response to these toxic insults is regulated via as yet unidentified specific cis- and trans-acting factors.

Since several treatments that induce expression of key phase II detoxifying enzymes also result in elevated GCS activity as well as increased intracellular GSH levels, we hypothesized that the heavy subunit gene is regulated by as yet unidentified specific cis- and trans-acting factors. Repression of ARE4 (tissue-specific ARE) of the human hepatic GCS gene by the antioxidant catalase (35) suggests that other ARE sequences (5) might also participate in transcriptional regulation of these genes. AREs have been demonstrated to be present in the promoter region of the human GCS gene and in the 5′-flanking region of the gene (27–29). Recent evidence suggests that other cis-acting elements might also participate in concert with the ARE in transcriptional regulation of these genes (30). We identified numerous potential response elements and/or enhancer sequences, including a putative ARE, in the 5′-flanking sequence of the human GCS_h gene (26). However, the involvement of any specific elements in constitutive or induced expression of the GCS_h gene has not been established by functional analyses.

In the present study, we utilized a deletion mutagenesis analysis strategy to identify those sequences that modulate constitutive expression of the human GCS_h gene and to begin to decipher mechanisms involved in the induced expression of the gene. To this end, HepG2 cells transfected with a series of deletion mutant/reporter fusion genes were exposed to a flavone, an inducing agent which activates phase II gene expression in response to these toxic insults and then cloned into pT85. Oligonucleotides were dissolved in 1x Sequenase reaction buffer and annealed by heating to 90–100°C, cooled slowly to 35°C, extracted once with phenol/chloroform/isoamyl alcohol and once with chloroform/isoamyl alcohol, ethanol-precipitated, and finally resuspended in water and ligated into pT85. 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 1 x 10^6 cells/5-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 concentrations of plasmid DNA were used to compensate for variations in plasmid size. To correct for transfection efficiency, 1.25 μg of the reporter plasmid pCMVβ (33) containing the luc2 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 shocked with media containing 10% glycerol for 3 min at room temperature and then maintained at 37°C for an additional 24 h. At the conclusion of this incubation period, the medium was once again replaced with fresh complete medium containing MeSO (0.1%) or 10 μM β-NF (Sigma) dissolved at 1000 x in MeSO. Sixteen hours later cells were harvested and assayed for determination of luciferase, β-galactosidase activity, and protein content. For cell harvest, transfecants were washed twice with phosphate-buffered saline (Mg^2+ - and Ca^2+ -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 top speed in a microcentrifuge for 2 min at 4°C. The resultant supernatants were transferred to Eppendorf tubes and stored on ice pending assay.

Biochemical Assays—Total (oxidized and reduced) glutathione content was determined using the technique described by Tietze (34) as modified by Bump et al. (35).

GCS activity was determined as described previously using a modification (36) of a high performance liquid chromatography technique described by Nardi (37). The rate of γ-glutamylcysteine formation was quantitated by comparison to γ-glutamylcysteine standards and normalized on the basis of protein concentration. β-Galactosidase activity was quantitated as described by Rosenthal (38). Briefly, this assay monitors cleavage of o-nitrophenyl-β-D-galactopyranoside and yields β-galactosidase units as (A_420 - 380)/t where t = time in min at 37°C and 380 is a conversion factor to convert absorbance to μmol of o-nitrophenyl-β-D-galactopyranoside.

To initiate the luciferase assay, cell lysate (1–5 μl) was added to reaction buffer (14 mM MgCl₂, 14 mM glycylglycine, 0.1 mg/ml bovine serum albumin, 18 mg/ml ATP, pH 7.5), 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 assayed for transfection efficiency on the basis of β-galactosidase activity and protein content of the lysate yielding a final value of (relative light units/β-galactosidase) μg protein.

Protein content was determined by the Bradford method (39) using bovine serum albumin as a standard.

Northern Analysis and RNase Protection—Total cellular RNA was isolated using TRI Reagent (Molecular Research Center, Inc.) according
**GCS Heavy Subunit Expression Involves a Distal ARE/TRE**

**Fig. 1.** β-NF induces GCS activity and elevates GSH. HepG2 cells were incubated in complete medium for 24 h. At the conclusion of this incubation period, the medium was replaced with fresh complete medium or with medium containing 10 μM β-NF, 25 μM β-NF, or 0.1% Me2SO. At various intervals up to 24 h, cells were harvested for measurement of GCS activity (A) or total GSH levels (B). Results are mean and standard errors of three or more determinations.

**RESULTS**

**Induction of GCS Activity by Exposure to β-NF**—To examine regulation of GCS, induction, we first elected to investigate the effect of exposure of HepG2 hepatocarcinoma cells to β-NF, a potent inducer of several phase II enzymes and an agent that exerts its effects, at least in part, through activation of AREs in the case of 25 μM β-NF exposure. The results illustrated in Fig. 1A indicate that exposure to 10, 25, and 40 μM β-NF resulted in progressive increases in GCS activity, culminating in a moderate, although significant (p < 0.05), increase in enzyme activity by 24 h after addition of β-NF to the culture medium (Fig. 1A). Induction of GCS expression occurred sooner and was greater following exposure to 25 μM β-NF.

These changes in GCS expression were accompanied by a progressive increase in intracellular GSH levels at each β-NF dose over the 24-h observation period (Fig. 1B). By 24 h GSH levels had increased significantly, reaching in excess of 2- and 4-fold over controls for 10 and 25 μM exposures, respectively. In the case of 25 μM exposure, a significant increase was evident as early as 2 h after addition of β-NF.

**Increase in Steady-state mRNA Levels for Heavy and Light Subunits**—Exposure of HepG2 cells to increasing concentrations of β-NF also resulted in a dose-dependent increase in steady-state mRNA levels for both subunits of the GCS holoenzyme (Fig. 2, A and B). In all cases, the two heavy subunit transcripts increased proportionally (Fig. 2A). Changes in the relative abundance of the transcripts for the light and heavy subunits as a function of time after addition of β-NF or Me2SO was quantitated by a ribonuclease protection assay engineered to permit quantitation of both transcripts simultaneously. A representative radiograph is shown in Fig. 2C. Quantitation by nuclease protection assay was particularly important in the case of the light subunit transcripts that are typically present at low abundance. Quantitative and temporal changes in transcript abundance in β-NF-treated cells are illustrated in Fig. 2, D and E. These data confirm the dose-dependent increase in mRNA levels observed by Northern analysis. The β-NF-induced increase in steady-state mRNA levels for the GCS heavy and GCS, subunits displayed similar kinetic patterns, reaching a maximum at approximately 12 h after initiation of exposure. Interestingly, the increase in GCS transcripts levels was consistently higher than that observed in the case of the GCS heavy mRNA.

**Regulation of Constitutive Expression**—We recently reported (26) the cloning and partial sequence (−1460: +547) of a genomic clone, designated P1-GCSh, containing approximately 4.7 kb of 5′-flanking sequence and 0.5 kb of exon I of the human GCS catalytic subunit gene. A 4.2-kb fragment from this clone, spanning the sequence −3802 to +465, was cloned into the pGL3-luciferase reporter vector to create the GCSh luciferase fusion gene. −3802 GCS5′-luc (Fig. 3). A series of deletion mutants was then generated from this parent chimeric gene and used to identify functional response elements directing constitutive and β-NF-induced expression of the GCS gene. The various reporter constructs shown in Fig. 3 were transfected into HepG2 cells and luciferase expression examined 48 h later. Transfection of −3802/GCSh5′-luc increased the luciferase expression approximately 35-fold relative to the expression detected in cells transfected with pGL3-luciferase alone. Progressive deletions from −3802 to −510 resulted in significant reductions in luciferase expression when compared with that produced by −3802/GCSh5′-luc. However, the differences in luciferase expression among these three intermediate constructs were not statistically significant (p > 0.05) from each other. Transfection of −202/GCSh5′-luc resulted in significantly increased luciferase activity, attaining levels comparable with those observed following transfection with the full-length fusion gene. Deletion of sequences between −202 and +22 resulted in a significant reduction in luciferase expression, whereas further deletion to +358 completely eliminated promoter activity. Collectively these results suggest that the fragment between −202 and +22 contains a positive regulatory sequence(s) capable of directing constitutive expression of the gene in HepG2 cells; negative regulatory sequences exist between −202 and −814, and one or more positive regulators exists between −2752 and −3802. Further analyses revealed that the enhanced activity associated with inclusion of sequences from −202 to +22 corresponded to the presence of the TATA sequence previously identified in this region of the promoter (data not shown).

**Regulation of β-NF-induced GCS Heavy Subunit Expression**—To determine whether the increase in steady-state mRNA levels observed following β-NF exposure was the result of increased transcription and to identify response elements that might mediate β-NF-induced GCS heavy gene expression, HepG2 cells were transiently transfected with the GCS heavy promoter/reporter constructs and then exposed to 10 μM β-NF for 16 h. As shown in Fig. 3, β-NF failed to increase luciferase expression in cells transfected with any of the recombinant reporter genes other than the full-length −3802/GCSh5′-luc. The magnitude of induction was approximately 2-3-fold, comparable with the magnitude of increase in endogenous GCS heavy mRNA expression following similar β-NF exposure. Hence, the response element(s)
mediating GCS\textsubscript{h} gene induction in response to \(\beta\)-NF is present between \(-2752\) and \(-3802\).

**Potential Response Elements Present in the Distal 5' Flanking Region of the GCS\textsubscript{h} Gene**—Transfection experiments therefore suggest that the DNA sequence from \(-2275\) to \(-3802\) contains elements that influence both constitutive and \(\beta\)-NF-induced expression of GCS\textsubscript{h}. This region is beyond the 5' distal end of the sequence we had previously reported, and therefore we extended our original sequencing to include an additional 2342 bp of this 5' distal fragment in order to identify potential enhancer sequences. The complete sequence of \(-3491\) to \(-2438\) is shown in Fig. 4. In addition to the potential regulatory sequences identified previously, the distal genomic fragment includes a consensus NF-kB binding site, a consensus AP-1 site, Sp-1 sites, and three additional potential AREs.

In order to approximate the position of elements or enhancers that contribute to the constitutive and induced expression of the heavy subunit gene, a series of enhancer/reporter constructs were developed by cloning various restriction fragments or synthetic oligomers (Fig. 5) into the reporter vector, pTS1, in which luciferase expression is under the control of the herpes simplex thymidine kinase (tk) minimal promoter. Fig. 6A illustrates the structure of the individual enhancer vectors and their effect on constitutive luciferase expression. The fragment \(-3491\) to \(-2438\), representing the majority of the distal region of GCS\textsubscript{h}5' responsible for increased constitutive expression, increased luciferase expression 5-fold relative to that observed in pTS1 transfected cells. When this 1-kb fragment was subdivided by SacI digestion, the fragment corresponding to the distal 0.7 kb (\(-3491\) to \(-2275\)) maintained enhanced luciferase expression, whereas the proximal 0.3-kb fragment (\(-2275\) to \(-2438\)) failed to do so.

As shown in Figs. 3 and 4, \(-3491\) to \(-2755\)/GCS\textsubscript{h}5' contains two putative AREs (3 and 4). A third (ARE2) is present in the \(-2754\) to \(-2438\)/GCS\textsubscript{h}5' fragment. Individually, ARE3 and ARE4 each significantly enhance pTS1 luciferase expression, attaining levels of expression (20-fold) comparable with that produced by the ARE-positive control vector, NQO1ARE\(4k\)-luc (32), containing the ARE from the human NQO1 gene (Fig. 6A). When cloned into pTS1 in their normal tandem arrangement (GCS\_ARE3,4), ARE3 and ARE4 increase luciferase expression in excess of 100-fold. In contrast, ARE1, the putative ARE identified previously on the basis of sequence similarity, did not function as an enhancer in this system. Although an oligomer corresponding to ARE2 was not cloned into pTS1, the 0.3-kb \(-2754\) to \(-2438\)/GCS\textsubscript{h}5' fragment, containing ARE2, did...
not increase luciferase activity, suggesting that ARE2 was likewise not functional as an enhancer of constitutive expression. The consensus NF-κB sequence also failed to influence luciferase expression.

When a similar series of experiments was conducted with transfected HepG2 cells exposed to 10 μM β-NF for 16 h, GCSh ARE4, GCShARE3,4 (containing the tandem combination of ARE3 and ARE4), and those larger fragments containing them directed increased luciferase expression in treated cells (Fig. 6B). Luciferase expression in cells transfected with ARE4-tk-luc. However, in the presence of ARE3, which did not itself respond to β-NF induction, the β-NF inducibility of ARE4 was diminished slightly. Nevertheless, the induction of luciferase expression in β-NF-treated cells transfected with any vector containing the combination of ARE3 and ARE4 was similar in magnitude to 1) the increase in endogenous GCSh transcripts in HepG2 cells exposed to the same dose of β-NF, and 2) to the β-NF-induced increase in luciferase expression in cells transfected with the full-length −3802/GCS₅'-luc. Other potential elements, including NF-κB, ARE1, and ARE2, failed to direct a β-NF-induced increase in luciferase expression.

Of the four putative ARE sequences identified in the 5'-flanking sequence of the GCSh gene, only ARE4 influenced both constitutive and induced gene expression in a fashion typical of other functional AREs. In contrast, ARE3 enhanced constitutive expression only, although it did modulate both the constitutive and inducible properties of ARE4. In order to confirm that ARE4 functions as a true ARE, a synthetic oligonucleotide containing a single base mutation in the consensus core ARE sequence of ARE4 (converting the sequence from 5'-GGTGCAGCAC-3' to 5'-GGGACTCGAC-3'; Fig. 5) was synthesized and cloned into pT81. The T in this position has been shown to be a required element for the maintenance of both the constitutive and the inducible properties of AREs. When this vector (GCShARE4m) was transfected into HepG2 cells, constitutive expression was significantly reduced (Fig. 6B) in cells transfected with the mutated ARE4 fusion gene.

To determine whether ARE4 functions in the same capacity when present at its distal location in the genomic sequence of the GCSh gene, 3.1 kb upstream of the transcription start site, the same single-base mutation was introduced into the full-length fusion reporter gene −3802/GCS₅'-luc by site-directed mutagenesis. The mutation of this single base within this 3.8-kb GCSh fragment likewise reduced constitutive expression and eliminated induction in response to β-NF (−3802mAA4/GCS₅'-luc in Fig. 3).

**FIG. 3.** Mutational analysis of GCSh 5'-flanking sequence. A 4.2-kb HindIII fragment (−3802 + 465) from the 5'-flanking region of the GCSh gene was cloned into the HindIII site of the luciferase reporter vector pGL3-Basic to create the recombinant plasmid −3802/GCS₅'-luc. A series of progressively smaller transgenes were created by digesting −3802/GCS₅'-luc with the restriction enzyme indicated on the restriction map at the bottom of the figure. For −3802mA4/GCS₅'-luc, a single base mutation (T → G) was introduced into ARE4 of −3802/GCS₅'-luc (see Fig. 5) by site-directed mutagenesis. These 5'-deletion mutants were co-transfected along with the plasmid pCMV-β into HepG2 cells by calcium phosphate precipitation. Twenty-four hours after transfection the culture medium was replaced with fresh medium containing either Me2SO (control) or 10 μM β-NF. Cells were harvested 16 h later and supernatants prepared for luciferase, β-galactosidase, and protein assays. Values represent the mean ± S.E. luciferase units normalized for β-galactosidase activity and protein content.
Diverse chemical compounds, including Michael reaction acceptors, quinones, diphenols, peroxides, isothiocyanates, mercaptans, heavy metals, arsenicals, and certain planar aromatic and metabolizable polycyclic aromatic hydrocarbons, induce the expression of phase II detoxifying enzymes (8, 30). The induction of several genes in the phase II battery, including the rat (31) and mouse (28) glutathione S-transferase Yα genes, rat (41) and human (42) NAD(P)H quinone oxidoreductase genes, and the rat GST-P gene (43, 44), is mediated through an antioxidant responsive element (or its functional equivalent, an electrophile responsive element, EpRE), in the promoter region of the gene (27). It is hypothesized that these inducing agents share in common the ability to generate reactive oxygen intermediates directly or via redox cycling (28, 31) and result in activation of transcription factors, perhaps through alterations in their redox status. While this mechanism is favored by some, Talalay and colleagues (8) point out that all of these inducers are capable of generating electrophilic intermediates capable of reacting with sulfhydryl groups. They therefore suggest that a better designation for these responsive elements would be EpREs to reflect the nature of the active intermediates.

Interestingly, exposure to several of the agents that induce expression of the ARE-containing phase II genes results in an elevation in intracellular GSH levels as well. These increases are frequently coupled to an increase in the activity of GCS, which led us to hypothesize that the increased GSH levels might result from transcriptional up-regulation of GCS expression mediated by ARE-like elements in the 5'-flanking region of the GCSh gene (26), and three additional potential ARE

FIG. 4. Complete sequence of GCSh. The sequence of the GCSh 5'-flanking region was determined by dideoxynucleotide sequencing using a series of internal primers. The sequence was confirmed by multiple overlapping, bidirectional sequencing reactions. The position of individual sequences referred to in the text are shown. The sequence from -1480 to +546 was published previously (26).

**DISCUSSION**

Diverse chemical compounds, including Michael reaction acceptors, quinones, diphenols, peroxides, isothiocyanates, mercaptans, heavy metals, arsenicals, and certain planar aromatic and metabolizable polyaromatic hydrocarbons, induce the expression of phase II detoxifying enzymes (8, 30). The induction of several genes in the phase II battery, including the rat (31) and mouse (28) glutathione S-transferase Yα genes, rat (41) and human (42) NAD(P)H quinone oxidoreductase genes, and the rat GST-P gene (43, 44), is mediated through an antioxidant responsive element (or its functional equivalent, an electrophile responsive element, EpRE), in the promoter region of the gene (27). It is hypothesized that these inducing agents share in common the ability to generate reactive oxygen intermediates directly or via redox cycling (28, 31) and result in activation of transcription factors, perhaps through alterations in their redox status. While this mechanism is favored by some, Talalay and colleagues (8) point out that all of these inducers are capable of generating electrophilic intermediates capable of reacting with sulfhydryl groups. They therefore suggest that a better designation for these responsive elements would be EpREs to reflect the nature of the active intermediates.

Interestingly, exposure to several of the agents that induce expression of the ARE-containing phase II genes results in an elevation in intracellular GSH levels as well. These increases are frequently coupled to an increase in the activity of GCS, which led us to hypothesize that the increased GSH levels might result from transcriptional up-regulation of GCS expression mediated by ARE-like elements in the 5'-flanking region of the GCS catalytic subunit and/or light subunit genes. Previous cloning efforts identified an ARE-like sequence in the promoter of the GCSh gene (26), and three additional potential ARE
sequences were found further upstream in the present studies. In addition to sequence data, this hypothesis is supported by several other recent lines of evidence. Based on the demonstration that GCS expression in rat lung epithelial cells is inducible by exposure to the pro-oxidant, 2,3-dimethoxy-1,4-naphthoquinone, Shi et al. (13) suggested the possibility that transcription of the rat GCSh gene is regulated by an ARE-like element. Similarly, Liu et al. (9) predicted the existence of an EpRE in the murine GCS gene. They demonstrated that exposure to the synthetic indolic antioxidant, 5,10-dihydroindeno[1,2-b]indole, increased GCS activity and GSH levels in mouse hepatoma cells. Borroz et al. (16) also postulated the existence of an ARE in the GCSh gene of the rat after demonstrating transcriptional up-regulation of the GCSh subunit in murine liver following dietary treatment with the antioxidant 2(3)-tert-butyl-4-hydroxyanisole.

Functional AREs display two cardinal properties; they enhance basal expression and are inducible by phase II enzyme inducers (27). Using these criteria, the current study provides the first direct evidence that a functional ARE is present in the 5′-flanking region of the human GCSh gene. Despite the fact that four ARE-like sequences were identified on the basis of sequence similarity to the ARE consensus core sequence, only two, ARE3 and ARE4, contributed to basal expression, each augmenting expression of luciferase in the presence of the tk minimal promoter by −20-fold. Interestingly, when cloned into pT81 in tandem in their native configuration, separated only by 34 bp, these two sequences enhance basal expression greater than 100-fold. However, this potent enhancer activity is dampened by the inclusion of additional DNA, such that in the full-length GCSh5′ sequence, presence of the native context ARE3/ARE4 tandem only doubles basal expression (i.e. −3802/GCSh5′-luc versus −2752/GCSh5′-luc).

Both ARE3 and ARE4 match the consensus 11-nucleotide ARE motif described by Pickett et al. (31, 45), yet only the ARE4 oligonucleotide directed enhanced expression following exposure to β-NF. Since these two enhancers only differ by two nucleotides, in the region of the core (NNN) previously demonstrated to be of no consequence in influencing either basal or β-NF induction (31, 45), it is not immediately obvious why ARE3 failed to respond to β-NF induction. Analyses of functional AREs in other genes have revealed that the organization of these enhancers may be more complex than the simple linear arrangement of the “core” nucleotides, often involving proximal nucleotides flanking either side of the core motif itself. Furthermore, functionality of specific AREs may be dependent on the spatial arrangement of these multiple partners. As illustrated in Fig. 7, AREs in the rat GSTP (43) and GST-Ya (31) genes, the murine GST-Ya (28) gene, and rat (41) and human (42) NQO1 genes include an adjacent pair of TRE or TRE-like elements. Favreau and Pickett (45) divided the 31-base pair rat NQO1 gene ARE into three regions as follows: proximal and distal half-sites of a 13-base pair palindromic sequence and a 3′-flanking region containing 4 adenines. Mutations in these various regions influenced basal and induced expression in different ways supporting the conclusion that the two half-sites are not functionally equivalent. While only the proximal half-site was required for binding of nucleoproteins to the ARE, both half-sites contribute to full basal gene expression. However, only three nucleotides in the proximal site were absolutely

**Fig. 5.** Nucleotide sequence of oligonucleotides used to examine enhancer activity. Synthetic oligomers corresponding to the complementary strands for the positive control ARE, NQO1hARE, and the potential response elements in −3802/GCSh5′-luc were annealed, phosphorylated, and ligated into the tk-luciferase enhancer vector, pT81. A single base mutation (lowercase letters) was introduced into the 76-mer GCShARE3,4 creating a SmaI restriction site which was then used to generate ARE3- tk-luc and ARE4- tk-luc from ARE3,4- tk-luc. GCShARE4m was created by introducing a single base change (lowercase) in the corresponding oligomers, converting the consensus ARE sequence 5′-GGTACTCAGC-3′ to 5′-GAGGACTCAGC-3′.
required for maximal basal and induced transcription (45). Considering this complexity, it is possible that selection of the 20 base pairs used to create the ARE3 oligonucleotide might have omitted additional neighboring elements that are required for inducibility in situ. However, no evidence for comparable elements around ARE3 was found. Even though ARE3 doesn’t appear to function as an independent ARE and despite the fact that mutation of ARE4 within the ARE3/ARE4 tandem eliminated basal and β-NF-induced expression by \(23802/GCSh5\), the current data are insufficient to preclude the possibility that an interaction between ARE3 and ARE4 is required for regulation of GCSh gene expression in situ. Further mutational analysis of the ARE3/ARE4 tandem is being conducted to clarify the relationship between these two sequences.

ARE4 is the only element to fulfill the functional requirements of an ARE in terms of both basal and inducible expression. Identification of ARE4 as a functional element was confirmed by mutational analysis in which conversion of the sequence 5'-GTGACTCAGCA-3' to 5'-GGGACTCAGCA-3' in the ARE4 oligonucleotide and in \(-3802/GCSh5'-luc\) effectively eliminated constitutive and β-NF-induced activity in both cases, as demonstrated in the functional characterization of other AREs (31, 45). Abolishment of the basal and induced expression directed by \(-3802/GCSh5'\) by introduction of this mutation provides strong evidence that this sequence is involved in the regulation of expression of the endogenous GCSh subunit gene in situ. Hence, all or part of ARE4 is necessary for basal and β-NF-induced expression of the gene.

Although ARE4 contains a consensus ARE core sequence, this element also includes a canonical TRE motif (5'-TGA(C/G)T(C/A)A-3'), an arrangement also found in AREs from the human NQO1 (42) and murine heme oxygenase (46) genes (Fig. 7). Since the mutation we introduced in ARE4 corrupted both the ARE and TRE sequences, we have not as yet distinguished experimentally which of these responsive elements is involved in the expression of the GCSh gene. Evidence from other ARE analyses favors the involvement of the ARE core, however. In analyzing the elemental requirements of the ARE in the human NQO1 gene, Xie et al. (32) reported that the sequence 5'-GATGAGTCAGCC-3', containing a single TRE motif (in boldface), but not an ARE failed to increase expression in transfected HepG2 cells in the response to β-NF. Similarly,
Preterra et al. (46) demonstrated that maintenance of the ARE consensus was required for the induction of murine HO-1 gene expression in response to a battery of phase II inducers. Mutation of the TRE sequences had no influence on inducibility, provided the ARE core sequence was retained. In a recent characterization of the ARE sequence in the rat NQO1 gene, Favreau and Pickett (46) mutated (underlined) the existing sequence, 5'-GTTGACTTGCA-3', to one containing a TRE motif, 5'-GTTGACTAGCA-3'. When the mutant oligonucleotide was used in electrophoretic band shift assays, unique nuclear proteins recognizing either the ARE or the TRE sequence were detected in HepG2 nuclear extracts. Since this mutated sequence matches the core sequence of ARE4 with the exception of an A to G substitution at the 3' terminal nucleotide, a substitution that does not influence binding or ARE function (45), these data provide evidence that proteins capable of binding the ARE4 sequence are present in HepG2 cells. In preliminary gel shift experiments, binding activity to a probe corresponding to ARE4 is elevated in nuclear extracts from HepG2 cells; however, an increase in TRE-binding activity is not detected. This ARE4 binding activity is effectively competed by unlabeled ARE4 probe but not by a probe containing a consensus TRE sequence, providing further evidence to support the importance of the ARE sequence in mediating basal and β-NF-induced expression of GCSs. Definitive identification requires completion of additional mutational analyses currently in progress.

Finally, the location of a functional ARE in a position 3.1 kb distal to the transcription start site is atypical by comparison to the position of most other AREs, which have been typically identified proximal to the transcription origin in phase II enzymes. However, Preterra et al. (46) recently identified a functional ARE 4.1 kb upstream in the human heme oxygenase-1 gene, capable of regulating constitutive and xenobiotic induced expression of this important stress response gene. Collectively these observations in two different genes indicate that AREs can exert an effect on gene transcription over a long expanse of intervening sequence.

In summary, constitutive and β-NF-induced expression of the GCS heavy subunit gene, at least in part, is mediated by a distal sequence including a consensus ARE motif containing an embedded TRE. Experiments designed to determine specific sequences involved in GCSs expression as well as to identify additional elements that may contribute to the composite regulation of this important gene are in progress.

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