Overlapping antioxidant response element and PMA response element sequences mediate basal and β -naphthoflavone-induced expression of the human γ -glutamylcysteine synthetase catalytic subunit gene

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γ -Glutamylcysteine synthetase (GCS), the rate-limiting enzyme in the *de novo* synthesis of GSH, is a heterodimer, consisting of a catalytic (GCS_h) and a regulatory subunit (GCS_r). We previously demonstrated that the constitutive and β -naphthoflavone (β -NF)-induced expression of the GCS_h gene is mediated by a distal antioxidant response element (ARE), ARE4, located 3.1 kb upstream of the transcriptional start site [Mulcahy, Wartman, Bailey and Gipp (1997) *J. Biol. Chem.* **272**, 7445–7454]. ARE4 consists of a consensus ARE sequence (5'-GTGACTCAGCG-3') containing an embedded PMA-responsive element (TRE, underlined). The relative significance of the two overlapping response elements to constitutive and β -NF-induced expression of the GCS_h gene was determined by mutational analyses. The internal activator protein-1 (AP-1)-binding sequence mediated constitutive expression of promoter/reporter transgenes, but was not required for β -NF responsiveness. In gel-shift experiments,

the TRE was necessary for binding of proteins from nuclear extracts prepared from untreated HepG2 cells. In contrast, induction by β -NF was dependent on an intact ARE sequence, particularly the terminal GC box of ARE4. The GC box of ARE4 was shown to be essential for both basal and β -NF-induced expression of reporter constructs. This element also influenced binding of nuclear proteins to ARE4, specifically in extracts isolated from β -NF-treated HepG2 cells. Because previous studies indicated that ARE4 may co-operate with a separate putative ARE, the role of the neighbouring sequence (ARE3), located 34 bases downstream of ARE4, was also evaluated. Mutation of this element within a GCS_h promoter/reporter did not modify the basal or β -NF-induced expression of the transgene, demonstrating that ARE3 does not influence the constitutive or β -NF-induced expression of the GCS_h gene.

INTRODUCTION

The enzyme γ -glutamylcysteine synthetase (GCS) catalyses the rate-limiting step in the *de novo* synthesis of glutathione (GSH). GCS is a heterodimer, composed of a catalytic (73 kDa) and a regulatory (28 kDa) subunit, referred to as the heavy (GCS_h) and light (GCS_r) subunits respectively. We have been interested in identifying *cis*-acting elements that mediate the regulation of these two genes for basal expression and in response to a wide variety of agents, including those which generate a pro-oxidant state in the cell, either by producing reactive oxygen species or by affecting the level of GSH in the cell.

Increases in the expression of the gene encoding the catalytic subunit of GCS (GCS_h) have been reported in response to many agents, often associated with increases in GCS enzyme activity and GSH. Specifically, increased GCS_h steady-state RNA levels have been observed following exposure to compounds including dimethoxy-1,4-naphthoquinone (DMNQ) [1,2], menadione [2,3], t-butylhydroquinone [4], dietary 2,3-t-butyl-4-hydroxyanisole (BHA) [5], methyl mercury hydroxide [6,7], diethylmaleate [8], and β -naphthoflavone (β -NF) [9]. In some cases, evidence of increased transcription of the GCS_h subunit gene has been demonstrated [1,4,8]. Many of these agents have been shown to induce genes encoding Phase II enzymes via antioxidant

response elements (AREs), supporting a hypothesis that an ARE is operative in the regulation of the GCS_h gene.

We recently demonstrated that an antioxidant response element, ARE4, was necessary for constitutive and β -NF-induced expression of the GCS_h gene [9]. Located approximately 3.1 kb upstream of the transcriptional start site, this element also contains an embedded PMA-responsive element (TRE) within the consensus ARE sequence (5'-GTGACTCAGCG-3'). Site-directed mutagenesis of a single base (5'-GTGACTCAGCG-3' to 5'-GGGACTCAGCG-3'), previously shown to be critical for ARE function [10], abolished both the constitutive and β -NF-induced expression of a reporter gene driven by the 3.8 kb ARE4-containing fragment of the GCS_h 5'-regulatory region. However, the T-to-G mutation introduced into ARE4 to create the mutant sequence, ARE4m1, ablated not only the ARE consensus, but also disrupted the internal TRE sequence, complicating the assignment of relative functional significance to the two *cis*-elements.

On the basis of the observation that AREs contain activator protein-1 (AP-1) or AP-1-like binding sites, it has been proposed that AP-1 binding proteins recognize and bind ARE sequences. The human NAD(P)H:quinone oxidoreductase 1 (NQO1) ARE sequence, like the GCS_h ARE4, includes a consensus TRE site [11]. In electrophoretic-mobility super-shift experiments, nucleo-

Abbreviations used: AP-1, activator protein-1; ARE, antioxidant response element; NF, naphthoflavone; bZIP, basic leucine zipper; GCS, γ -glutamylcysteine synthetase; GCS_h, catalytic (heavy) subunit of GCS; GCS_r, regulatory (light) subunit of GCS; GST, glutathione S-transferase; NQO1, NAD(P)H:quinone oxidoreductase 1; *tk-luc*, thymidine kinase-luciferase; T-MARE, TRE-type Maf recognition element; TRE, PMA-responsive element

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protein complexes binding the human NQO1 ARE sequence have been shown to include AP-1 family members JunD, c-Fos [12] and JunB [13]. The transcription factor Nrf1 was also included in complexes binding to this sequence [14]. The ARE (or electrophile response element) in the mouse glutathione S-transferase (GST) Ya subunit gene contains two AP-1-like elements, and is reported to be regulated by AP-1 family members. Overexpression of c-Jun and c-Fos resulted in the *trans*-activation of a reporter gene driven by the mouse GST Ya electrophile response element [15]. In contrast, both the rat NQO1 gene and rat GST Ya subunit gene AREs, which lack internal consensus TRE sequences, do not appear to present high-affinity binding sites for AP-1 family members [16–18]. c-Jun, c-Fos, JunB and JunD translated *in vitro* were unable to bind the rat NQO1 ARE in gel-shift studies [17,18]. More recently, the involvement of other basic leucine zipper (bZIP) proteins, including members of the small Maf family [19,20] and Nrf1 and Nrf2 [14,20], were implicated in binding ARE sequences from rat and human promoters. Owing to the differences observed for the potential involvement of AP-1 family members in the regulation of ARE-containing genes, we were interested in further evaluating the functional significance of the internal AP-1-binding site within ARE4. Because the original ARE4m1 mutation did not discriminate between the importance of the overlapping ARE and TRE motifs within ARE4, a new series of mutations differentially affecting the ARE and TRE binding sites were engineered and applied to resolve this question.

We have also previously noted that ARE4 is located approx. 34 base pairs upstream of another potential ARE (ARE3) having the sequence 5'-GTGACTGCG-3' [9]. Although the ARE3 sequence does not function as an independent ARE when assayed in an enhancer construct, it significantly enhanced basal expression when cloned in tandem with ARE4. In addition to delineating the requirements for the ARE and TRE sequences in ARE4, this study was also designed to characterize the potential interaction of ARE3 and ARE4 in the regulation of constitutive and β -NF-induced expression of the GCS_h gene.

MATERIALS AND METHODS

Recombinant plasmids

Recombinant expression vectors were created by cloning restriction fragments isolated from the 5'-flanking sequence of the GCS_h subunit gene into pGL3Basic (Promega) for the determination of promoter activity, or by introducing synthetic oligonucleotides into pT81 (A.T.C.C. 37584) for determination of enhancer activity. As described previously [9], a 4.2 kb genomic DNA fragment was isolated from the 5'-flanking region of GCS_h by *HindIII*/*NcoI* restriction digestion, and cloned into the *HindIII*/*NcoI* sites of pGL3Basic, creating the recombinant plasmid -3802/GCS_h5'-*luc*. This plasmid was further digested with *SacI*/*HindIII*, and re-ligated to create -2754/GCS_h5'-*luc*. The promoter/reporter transgene containing the ARE4m1 mutation (-3802ARE4m1/GCS_h5'-*luc*) was created by site-directed mutagenesis [9]. Promoter constructs containing GCS_h ARE mutations (ARE4m2, ARE4m3 and ARE3m) were also prepared by site-directed mutagenesis of the wild-type -3802/GCS_h5'-*luc* plasmid, using the MORPH site-specific plasmid DNA mutagenesis kit (5' → 3'). Because we were concerned that a consensus TRE located near the ampicillin-resistance gene in the pGL3Basic vector might potentially influence expression of the luciferase gene, the pGL3Basic vector was mutated with a single-base substitution to eliminate the AP-1-binding site (K.-S. Chen and M.-N. Gould, personal communication), and was then referred to as pGL4Basic. The ARE4 and ARE3 mutant sequences were

re-cloned into pGL4Basic by inserting the 1093 bp *SacI* fragment, containing the ARE3 and ARE4 sequences, into a pGL4Basic construct containing 4.2 kb of GCS_h 5'-sequence, digested with *SacI*. Because expression of the pGL3Basic and pGL4Basic luciferase constructs containing individual GCS_h regulatory sequences was not significantly different (results not shown), we pooled the results obtained with the two plasmids (see Figure 5).

Enhancer reporter transgenes, including those containing ARE4, ARE4m1, a consensus TRE (Promega) and an ARE from the NQO1 gene, were originally constructed by cloning specific oligonucleotides corresponding to those sequences into pT81. The sequence used for the human NQO1 ARE was 5'-CAGTCACAGTGACTCAGCAGAATCTG-3'. To prepare the oligonucleotides, complementary oligonucleotides were synthesized, annealed, phosphorylated and then cloned into pT81. Construction of the enhancer reporters containing ARE4m2 and ARE4m3 was carried out with the MORPH site-specific plasmid DNA mutagenesis kit, using the wild-type ARE4-thymidine kinase-luciferase (*tk-luc*) as a template. Sequencing reactions were carried out to confirm the sequence of all mutants.

Cell culture and transfection

HepG2 cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% (v/v) fetal bovine serum and 50 μ g/ml gentamicin (complete medium). Cells were transfected with recombinant plasmids using a standard calcium phosphate-glycerol shock procedure. HepG2 cells were plated at 7.5×10^5 cells/35 mm dish on day 0. On day 1, medium was replaced with fresh complete medium. After 2 to 4 h, cells were transfected by the addition of appropriate DNA expression vectors. To correct for transfection efficiency, the reporter plasmid pCMV β [21], 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. At 4 h after addition of DNA, cells were shocked with media containing 10% (v/v) glycerol for 3 min at room temperature, re-nourished 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 DMSO (0.1% v/v) or 10 μ M β -NF (Sigma) dissolved one-thousandfold in DMSO. Cells were harvested 16 h later, and prepared for determination of luciferase and β -galactosidase activity and protein concentration. For cell harvesting, transfectants were washed twice with PBS (Mg²⁺- and Ca²⁺-free), and incubated at room temperature for 15 min in 250 μ l 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

β -Galactosidase activity was quantified as described by Rosenthal [22]. Briefly, this assay monitors cleavage of *o*-nitrophenyl- β -D-galactopyranoside and yields β -galactosidase units as ($A_{420} \times 380$)/ t , where t is time in min at 37 °C, and 380 is a conversion factor to convert absorbance to μ mol of *o*-nitrophenyl- β -D-galactopyranoside.

To initiate the luciferase assay, cell lysate (1–5 μ l) was added to reaction buffer [14 mM MgCl₂/14 mM glycylglycine/0.1 mg/ml BSA/18 mg/ml ATP (pH 7.8)], vortex-mixed, and placed in an Analytical Luminescence Laboratory luminometer (400 μ l total

vol.). Following injection of 100 μ l of luciferin (4 mg/ml in 10 mM Na_2CO_3 , 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 concentration of the lysate, yielding a final value of (relative light units/ β -galactosidase)/ μ g of protein.

Protein concentration was determined by the Bradford method [23], using BSA as a standard.

Electrophoretic mobility-shift analysis

Complementary oligonucleotides containing the ARE and TRE sequences were annealed as described by Tilburn et al. [24] with an initial incubation at 95 °C for 5 min, and were purified by non-denaturing PAGE. The section of the gel containing the double-stranded oligonucleotide was excised, and DNA was recovered using Spin-X (Costar) filter units. DNA concentration was estimated by A_{260} . Purified double-stranded oligonucleotides were ^{32}P -end-labelled with T4 polynucleotide kinase, followed by the removal of non-incorporated label with Bio-Spin 6 (Bio-Rad) column chromatography.

Nuclear extracts were prepared from HepG2 cells after 16 h treatment with 0.1 % DMSO, 25 μM β -NF or 60 ng/ml PMA, as described by Dignam et al. [25]. Nuclear extract (10 μ g) was incubated for 10 min at room temperature in a buffer containing 4 % (v/v) glycerol, 1 mM MgCl_2 , 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris/HCl, pH 7.5, and 75 $\mu\text{g}/\text{ml}$ poly(dI-dC), with or without competitor oligonucleotide at 100 $\times$, 50 $\times$ or 20 $\times$ molar concentrations. Following the initial incubation, 50 000–100 000 c.p.m. of ^{32}P -labelled oligonucleotide was added, and incubation continued for an additional 20 min at room temperature. Loading buffer was added, and the reaction products were analysed on a 4 % non-denaturing polyacrylamide gel (80:1 acrylamide:bisacrylamide). Shifted bands were revealed with a Phosphorimager, following overnight exposure.

Northern-blot analysis

Total cellular RNA was isolated using TRI Reagent (Molecular Research Center, Inc.), according to the manufacturer's procedure. RNA was isolated after a 16 h treatment of HepG2 cells with 0.1 % (v/v) DMSO, 25 μM β -NF or 60 mg/ml PMA, both dissolved one-thousandfold in DMSO. mRNA was isolated with the Poly(AT)tract mRNA Isolation System (Promega). For Northern-blot analysis, 0.8 μg of mRNA was size-fractionated in formaldehyde-agarose gels, transferred to a charged nylon membrane (Hybond N+, Amersham Corp.), and hybridized with random-prime-labelled probes specific both for the AP-1 family members and for the GCS_h gene transcript. The GCS_h subunit gene probe is the 800 bp *Pst*I fragment from the GCS_h cDNA sequence. The complete cDNAs for *c-fos*, *fosB*, *fra1*, *fra2*, *c-jun* and *junD*, and a 1.6 kb fragment from the *junB* cDNA, were labelled for probes. The *fos* family member cDNAs were a generous gift from Dr. Inder M. Verma (The Salk Institute). The *jun* family cDNAs were obtained from A.T.C.C. A radiolabelled probe specific for 36B4 [26] was used to normalize for RNA loading.

Statistical analysis

Results are presented as the means $\pm$ S.E.M. Statistical comparisons were performed by paired Student's *t* tests. A value of $P < 0.05$ was considered statistically significant.

RESULTS

GCS_h ARE4 acts as an enhancer

The sequences of the GCS_h mutants used in these studies are shown in Figure 1. $\text{GCS}_h/\text{ARE4m1}$, the mutant employed in our previous studies [9], contains a mutation simultaneously disrupting the ARE and TRE sequences within ARE4. The $\text{GCS}_h/\text{ARE4m2}$ mutant discriminates between the two sequences, conserving the ARE consensus while mutating the AP-1-binding site. A similar mutation was described by Prestera et al. [27] in mutation of embedded TRE sites within two AREs in the haem oxygenase-1 promoter. $\text{GCS}_h/\text{ARE4m3}$ is a sequence that contains a mutation of the GC box of ARE4, yet preserves the internal PMA-responsive element. The GC box of an ARE (GCA or GCG) has been reported to be necessary for the ARE-inducible response [10,28,29].

The $\text{GCS}_h/\text{ARE4}$ mutants described in Figure 1 were each cloned into a thymidine kinase-luciferase reporter vector, pT81. The resultant ARE4-*tk-luc* constructs were transfected into HepG2 hepatocarcinoma cells, and reporter activity was measured in cell extracts. The normalized luciferase activity of each construct after treatment of HepG2 cells for 16 h with vehicle alone (DMSO) or with 10 μM β -NF is shown in Figure 2. NQO1hARE-*tk-luc*, containing a well-characterized ARE isolated from the human NQO1 gene [11], was used as a positive control. As expected, this construct resulted in an increase in basal luciferase expression of approximately 20-fold in comparison with pT81 alone, and in a further increase of threefold in response to β -NF exposure for 16 h. $\text{GCS}_h\text{ARE4-}tk-luc$ likewise resulted in a 30-fold increase in basal activity, and a further twofold increase in activity in β -NF-treated cells.

The activities of the pT81 enhancer constructs containing the mutant ARE4 sequences are also plotted in Figure 2. By comparison with the wild-type $\text{GCS}_h\text{ARE4-}tk-luc$ vector, the ARE4m1 mutant resulted in a significant loss of basal expression, and was not inducible by β -NF, as reported previously [9]. The reporter construct containing the mutation specific for the AP-1-binding site (ARE4m2) increased constitutive activity approximately fivefold over pT81 alone, but was significantly less effective than the wild-type sequence in this regard. β -NF responsiveness was likewise significantly reduced as a result of mutating the AP-

Figure 1 Sequence of the wild-type and mutant response elements

The wild-type ARE4 sequence corresponds to the functional ARE located in the distal region of the 5'-flanking sequence of the human GCS catalytic subunit gene [9]. Core ARE motif and internal TRE sequences are indicated by the bracketed nucleotides. Nucleotides included in the GC box are underlined. The sequences of the mutants (arrow heads; lower-case) used in these studies are also shown, as is the sequence of an oligonucleotide containing a consensus TRE sequence.

Figure 2 Enhancer activity of oligonucleotides containing the wild-type and mutant ARE/TRE sequences

The indicated enhancer vectors were co-transfected into HepG2 cells along with the plasmid pCMV β . Luciferase activity was measured after a 16 h incubation with 10 μ M β -NF (open bars) or without 10 μ M β -NF (filled bars). The luciferase activities of each enhancer construct, corrected for β -galactosidase activity and protein, were normalized to the activity of the pT81 vector alone. The values represent the means $\pm$ S.E.M of 3 determinations.

1-binding site. The ARE4m3 mutant, containing an intact AP-1 site but a mutated ARE GC box, was slightly less effective at directing basal expression of the reporter gene than was the wild-type ARE ($P = 0.1$), increasing luciferase expression approximately 20-fold relative to the pT81 alone. Mutation of the ARE GC box, however, completely eliminated β -NF inducibility.

These enhancer studies suggest that both ARE- and TRE-associated sequences are important for full constitutive and β -NF-induced expression of the GCS_n subunit gene. Mutation of either the core ARE or TRE sequence within ARE4 leads to partial reduction of enhancer activity. In contrast, mutation of both elements, as in the ARE4m1 mutation, results in virtually complete elimination of both constitutive and inducible expression.

Binding of the ARE4 mutant sequences by components of HepG2 nuclear extract

The ability of HepG2 nuclear proteins to recognize and bind the wild-type and mutant ARE4 sequences was examined by electrophoretic mobility-shift assays. Figure 3 reveals the presence of DNA binding of proteins in nuclei from HepG2 cells incubated with labelled ARE/TRE probes. Three distinct bands (a, b, and c) were detected when a probe containing an AP-1-binding site was incubated with nuclear extracts from control, β -NF- and PMA-treated cells (lanes 1–3). The fast-migrating complex (band a) represented non-specific binding to the probes, deduced after a thorough examination of gel-shift competition experiments (J. Gipp and R. Mulcahy, unpublished work). Increased binding activity in extracts prepared from β -NF- and PMA-treated cells was evident by increased radioactivity associated with the slower-migrating band (band c). Similarly, incubation of the labelled wild-type GCS_n ARE4 probe (lane 4) or the human NQO1 hARE

Figure 3 Electromobility gel-shift assays of ARE/TRE probes with nuclear extracts from HepG2 cells

Nuclear extracts were prepared from HepG2 cells exposed to DMSO (C, control), 25 μ M β -NF (β) or 60 ng/ml PMA (T) for 16 h. Synthetic oligonucleotides containing the ARE and TRE sequences described in Figure 1 were annealed, end-labelled and then incubated with nuclear extract preparations, followed by analysis by gel electrophoresis. Three major complexes were observed, labelled a, b and c.

probe (lane 16) resulted in detection of three bands when the radiolabelled probes were incubated with nuclear extracts from untreated HepG2 cells. Treatment with either β -NF (lane 5 or 17) or PMA (lane 6 or 18) resulted in an increase in band c intensity. These changes were completely eliminated when the ARE4m1 probe was utilized (lanes 7–9). In nuclear extracts from untreated cells, only two bands (a and b) were detected, and no changes relative to the control were observed following exposure to either inducing agent.

When nuclear extracts from untreated cells were reacted with the ARE4m2 probe, containing a consensus ARE but mutated internal AP-1-binding site, little evidence of binding corresponding to band c was detected (lane 10). Similarly, extracts isolated from PMA-treated cells did not result in a substantial band c complex when incubated with the ARE4m2 probe (lane 12). However, incubation of the probe with extracts from β -NF-treated cells (lane 11) resulted in increased intensity of the slow migrating complex (band c), suggesting that mutation of the embedded AP-1 site did not abrogate binding of nuclear proteins from cells exposed to β -NF. When extracts prepared from untreated cells were incubated with the ARE4m3 probe, lacking the GC box, the pattern of binding was similar to that detected using the wild-type ARE4 probe (lanes 4 and 13). Increased binding of nuclear proteins from extracts of PMA-treated cells resulting in the band c complex was evident using the ARE4m3 probe. However, less activity was associated with the band c complex following incubation with nuclear extracts from β -NF-treated HepG2 cells (lane 14). The lack of increased binding to the ARE4m3 sequence in extracts from β -NF-treated cells suggests that the GC box nucleotides may either influence or stabilize binding, particularly inducible binding, but clearly are not fully responsible for binding to the ARE4 sequence.

Gel-shift competition assays (Figure 4) were carried out to define further the nucleotide requirement for protein binding to GCS_n ARE4. The wild-type ARE4-labelled probe was used in these experiments. Attempts to compete away the binding to the ARE4 probe were made by incubating nuclear extracts with the

Figure 4 Electromobility shift competition assays

Electromobility-shift competition assays were carried out using the mutant ARE4 sequences and the TRE probe as competitors for binding of HepG2 nuclear extract components to the labelled wild-type ARE4 probe. Unlabelled competitor probes were incubated with HepG2 nuclear extracts prepared from β -NF (β)- or PMA (T)-treated cells at 100-fold, 50-fold and 20-fold molar excess of the labelled ARE4 probe for 10 min. This was followed by incubation with the labelled wild-type ARE4 probe and gel electrophoresis.

Figure 5 Promoter activity of $-3802/GCS_5'$ -luc transgenes containing wild-type or mutant ARE3 or ARE4 sequences

Site-directed mutagenesis of the pGL4Basic luciferase reporter containing the 3.8 kb *HindIII/NcoI* fragment of the *GCS_h* gene 5'-flanking region was performed to create reporter transgenes containing the mutations described in Figure 1. In addition to the ARE4 mutations, mutation of ARE3, a consensus ARE sequence located 34 bp downstream of ARE4, was carried out (5'-GTGACTGCGG-3' to 5'-GGGACTGCGG-3', where the bold T is mutated to the bold G), singularly and in combination with the ARE4 mutants. The large open boxes represent ARE3 and ARE4. The smaller rectangle contained in ARE4 corresponds to the internal TRE. The filled boxes correspond to mutations of the indicated response elements. Transient co-transfection of HepG2 cells with each reporter transgene and the pCMV β plasmid expressing β -galactosidase was performed in triplicate, followed by a 16 h exposure to 0.1% DMSO or 10 μ M β -NF. The resulting luciferase activity was corrected for β -galactosidase activity and protein concentration, and was plotted after normalization for vector activity alone. Values are presented as the means $\pm$ S.E.M. for 3–7 determinations. Abbreviation: Me₂SO, DMSO.

radiolabelled ARE4 probe in the presence of 100-fold, 50-fold and 20-fold molar excess of cold competitor probes. Lanes 1 and 2 in Figure 4 show binding to ARE4 in response to 25 μ M β -NF (β) or 60 ng/ml PMA (T) for 16 h. Incubation with unlabelled wild-type ARE4 efficiently competes for binding of the nuclear HepG2 proteins to labelled probe, even when present at only 20-fold excess (lanes 3–8). In contrast, mutation of both the ARE and TRE (ARE4m1) resulted in a probe which was unable to compete with labelled ARE4 for binding, even at 100-fold excess (lanes 9–14). The TRE-containing probe competed well for binding to the wild-type ARE4 (lanes 15–20). However, competition by the TRE sequence was not as effective as the wild-type ARE4 sequence. Although the TRE contains an identical internal AP-1-binding site, it also includes different flanking nucleotides, which may affect the binding of nuclear proteins. The TRE probe was also a less effective competitor in extracts

from β -NF-treated cells than in those from PMA-treated cells, suggesting the presence of binding protein(s) recognizing the ARE4 sequence that are distinct from those which bind the TRE sequence after PMA exposure.

The ARE4m2 sequence was not as effective as the wild-type ARE4 as a competitor of ARE4 binding (lanes 21–26). This sequence, containing the disrupted AP-1-binding site, did exhibit some competition, but only if added at 100-fold excess, again indicating that nucleotides within the AP-1-binding site are required for protein binding to ARE4. The ARE4m2 probe also appeared to be a better competitor in extracts from β -NF-treated cells compared with its competition in extracts from PMA-treated cells. The unlabelled ARE4m3 probe was also less effective than the wild-type ARE4 in terms of competition, particularly in extracts from β -NF-treated cells (lanes 27–32). In summary, the ARE and TRE mutants were not as effective as the wild-type

Figure 6 Effect of β -NF and PMA on the steady-state mRNA levels of AP-1 family members and the GCS_n transcript

mRNA was isolated from HepG2 cells exposed to 0.1% (v/v) DMSO (C, control), 25 μ M β -NF (β) or 60 ng/ml PMA (T) for 0, 1, 3, 6, 12 and 18 h, and analysed by Northern-blot analysis. Random prime-labelled probes containing cDNA sequences for *c-jun*, *junB*, *junD*, *c-fos*, *fosB*, *fra1*, *fra2* and GCS_n were hybridized to unique membranes following transfer from formaldehyde-agarose gels. Subsequently, the membranes were hybridized to a probe corresponding to the loading control, 36B4. The 36B4-labelled blots are placed directly below the corresponding membrane labelled for an AP-1 family member. The *c-fos* and *fosB* transcripts were not detected at these conditions, and their blots are not shown.

ARE4 as competitors for proteins binding to the ARE4 probe, again implicating bases within both the AP-1-binding motif and the ARE GC box in the binding of nuclear proteins to wild-type ARE4. These experiments also suggest that different arrays of binding proteins may exist in the extracts isolated from PMA- and β -NF-treated HepG2 cells.

Mutational analyses of GCS_n ARE4 within the -3802 GCS_n promoter fragment

The ability of a short oligomer containing a potential response element to direct expression of a minimal promoter may differ from the ability of the same sequence to *trans*-activate a gene when cloned into a reporter vector as a large fragment of 5'-flanking sequence. Consequently, we evaluated the functional characteristics of the ARE4 mutants within the -3802 bp fragment of the GCS_n 5'-regulatory region.

HepG2 cells were transfected with luciferase reporter constructs containing the -2754/ GCS_n 5'-fragment and the -3802/ GCS_n 5'-fragment, including the wild-type or mutant ARE4, and treated for 16 h with DMSO alone or 10 μ M β -NF. Figure 5 is a plot of the luciferase activity of each of the constructs after normalizing the activity to the vector alone (pGL4Basic). Consistent with earlier studies, the -2754/ GCS_n 5'-*luc* construct, containing 2.7 kb of the 5'-promoter region of GCS_n and lacking ARE4, displayed low basal, and no inducible, expression. In contrast, the -3802/ GCS_n 5'-*luc* promoter/reporter transgene directed constitutive expression and near-maximal β -NF-induced expression [9].

The mutations of the -3802/ GCS_n 5'-*luc* construct (ARE4m1, ARE4m2 and ARE4m3), disrupting the wild-type ARE4, are identical with the mutations used in the enhancer studies. As previously published [9], the -3802ARE4m1/ GCS_n 5'-*luc* construct reduced basal expression and eliminated inducibility, displaying activity similar to the wild-type -2754/ GCS_n 5'-*luc* construct lacking ARE4 entirely. Disruption of the internal AP-1-binding site in ARE4 (-3802ARE4m2/ GCS_n 5'-*luc*) decreased basal luciferase expression ($P < 0.02$), similar to the ARE4m1-containing promoter construct. This reporter, containing the ARE4m2 mutation, consistently supported β -NF-inducible expression; however, the inducibility was not statistically significant ($P < 0.12$).

-3802ARE4m3/ GCS_n 5'-*luc*, disrupting the ARE4 GC box, also resulted in decreased basal expression ($P < 0.01$), and, as predicted from the enhancer studies, this construct demonstrated a complete loss of β -NF inducibility. The transfection results of the promoter/reporter transgenes in HepG2 cells implicate both the AP-1-binding site and the ARE GC box in the regulation of GCS_n ARE4-mediated basal expression. The internal TRE may influence the β -NF-inducible expression of reporter transgenes; however, the GC box is absolutely required for the response to β -NF.

The role of ARE3 in the constitutive and β -NF-induced expression of GCS_n

AREs are often described as consisting of multiple TRE or TRE-like elements in either a tandem or palindromic configuration [30]. We have noted that the GCS_n ARE4 is 34 bases upstream of a separate consensus ARE sequence, ARE3. Unlike ARE4, the neighbouring ARE3 (5'-GTGACTGCGCG-3') does not contain a consensus TRE. In a previous report, this element directed constitutive expression of the *tk* promoter in pT81, but did not further *trans*-activate the *tk* minimal promoter in response to β -NF. A pT81 enhancer construct containing both ARE3 and ARE4, arranged in their native configuration, greatly augmented constitutive *trans*-activation, but did not lead to increased β -NF-induced expression in comparison with the activity of the enhancer containing ARE4 alone [9]. For these reasons, we examined the potential interaction of ARE3 and ARE4 in directing the expression of the GCS_n gene.

We mutated the ARE3 sequence within the -3802/ GCS_n 5'-*luc* wild-type transgene, creating -3802ARE3m/ GCS_n 5'-*luc*.

This mutation (5'-GTGACTGCGCG-3' to 5'-GGGACTGCGCG-3') is analogous with the initial ARE4m1 mutation, which mutated a nucleotide essential for ARE function. As seen in Figure 5, transfection of the reporter construct -3802ARE3m/GCS_n5'-luc resulted in no change in either basal or β -NF-induced expression, in comparison with -3802/GCS_n5'-luc, containing the wild-type ARE3 sequence. Double-mutant constructs, containing ARE3m and one of ARE4m1, ARE4m2 or ARE4m3, were also created within 3.8 kb GCS_n promoter/reporters. Figure 5 shows that the luciferase activity of the double-mutant constructs was similar to the respective mutant constructs containing only the ARE4 mutations. These data confirm that ARE3 is not required for either basal or β -NF-induced expression of the GCS_n gene.

Steady-state mRNA levels of *jun* and *fos* family members

Analysis of mutations within the AP-1-binding site of GCS_n ARE4 suggested that these bases are required for constitutive expression, and may also influence induction in response to β -NF. Consequently, we examined the steady-state mRNA levels of AP-1 family members in an attempt to correlate their expression with the expression of the GCS_n gene in untreated and β -NF-treated cells. We evaluated the steady-state mRNA levels of AP-1 family members after the treatment of HepG2 cells with 25 μ M β -NF or 60mg/ml PMA. Northern blots shown in Figure 6 illustrate that *c-jun*, *junB*, *junD*, *fra1*, *fra2* and GCS_n RNA steady-state levels were increased early in PMA-treated HepG2 cells, and had begun to return to pretreatment levels within 6 h. In contrast, treatment with β -NF resulted in detectable changes only in the *fra1* and GCS_n steady-state transcripts, and these changes were observed at later time points, 6 h or later after β -NF treatment.

DISCUSSION

Early studies characterizing AREs, also referred to as electrophile response elements, identified common sequences in the rat NQO1 promoter and the rat GST Ya subunit gene, ultimately culminating in the definition of the core sequence for AREs as 5'-RTGACnnnGC-3' [10,28,31]. Subsequently, promoter regions from the mouse GST Ya subunit gene [15] and the human NQO1 gene [11] were also shown to contain ARE consensus sequences. These studies helped to expand the ARE motif beyond the original core sequence, implicating additional flanking sequences as being critical for full expression in the respective genes. Moreover, several of these newly identified AREs contained internal TRE or TRE-like sequences. Consequently, ARE sequences are now generally described as consisting of multiple TRE or TRE-like elements [30].

AREs mediate the induction of Phase II enzyme genes in response to a wide variety of agents. These agents typically mobilize the electrophile counter-attack response [32], characterized by (i) the induction of Phase II enzymes, which detoxify electrophiles by the addition of endogenous ligands (e.g. GSH or glucuronic acid), and (ii) an increase in intracellular GSH. Since this adaptive response involves the up-regulation of GSH synthesis, it is conceivable that GCS, the rate-limiting enzyme in GSH synthesis, is a significant component of the electrophile adaptive response gene battery, although not itself a Phase II enzyme. This possibility has been reinforced by the recent identification of ARE sequences within the promoters of the genes encoding the GCS catalytic and regulatory subunits ([9], H. Moinova and R. T. Mulcahy, unpublished work).

As is typical of several other ARE-containing genes, both

GCS-subunit-gene AREs contain embedded TRE sequences. The current mutagenesis studies were designed to determine the relative significance of the ARE-associated bases and the internal AP-1-binding site in regulation of the gene expression of GCS_n. These experiments indicate that the core consensus TRE sequence is primarily involved in the constitutive expression of the GCS_n subunit gene, and that the ARE-associated sequences, specifically the GC box, are essential for mediating both basal and β -NF-induced expression. We have also addressed the potential for interaction between ARE4 and a neighbouring ARE (ARE3), located 34 bp upstream of ARE4, in the GCS_n promoter. These studies can be summarized as demonstrating that ARE3 is involved in neither the basal nor the β -NF-induced *trans*-activation of the GCS_n gene in HepG2 cells.

As noted above, the internal AP-1 site in ARE4 mediates constitutive expression of GCS_n reporter genes. Basal expression was decreased in cells transfected with either of the promoter constructs containing the AP-1 mutant (-3802ARE4m2/GCS_n5'-luc or -3802ARE3m1:ARE4m2/GCS_n5'-luc). In gel-shift experiments, binding to the ARE4m2 probe was decreased in extracts prepared from untreated HepG2 cells. In contrast, the AP-1 sequence exerts a minimal effect on β -NF inducibility, and does not appear to be required for induction. Both of the GCS_n -3802 promoter constructs containing ARE4m2 consistently mediated increased expression in response to β -NF. In gel-shift studies, extracts from cells treated with β -NF resulted in increased binding to the ARE4m2 oligonucleotide probe, implying that the mutation did not prevent binding of nuclear proteins to the sequence. Similarly, mutation of internal TRE and TRE-like elements of two AREs in the haem oxygenase 1 promoter did not disrupt the inducible expression of relevant promoter reporters [27]. However, conflicting evidence was observed when the ARE4m2 sequence was assayed for enhancer function in the pT81 enhancer vector (Figure 2). In this system, mutation of the internal AP-1 sequence was shown to interfere with β -NF inducibility. The differences observed in β -NF inducibility of ARE4m2 promoter and enhancer reporters may reflect a differential interaction of *trans*-acting factors with the response element alone versus the element within the 'intact' promoter sequence. We have given more credibility to the promoter transgene results, because this model is likely to be a better representation of the native promoter of the GCS_n subunit gene *in situ*. In summary, we conclude that the internal TRE contributes to the basal expression of the GCS_n gene, and does not significantly influence β -NF responsiveness.

The binding of the ARE4 sequences by nuclear proteins from HepG2 cells was consistent with the transfection data, and suggests a correlation between the binding of ARE4 sequences and the ability to direct basal or β -NF-induced *trans*-activation of the reporter gene. Specific binding resulting in the formation of the band c complex was only evident when nuclear extracts prepared from untreated or PMA-treated HepG2 cells were incubated with probes containing intact TRE sites, including ARE4, ARE4m3 and the TRE probe itself, suggesting that the AP-1-binding site is occupied constitutively. Mutation of the AP-1-binding site (ARE4m2) resulted in a loss of binding by proteins in the extracts from untreated cells, again strongly implicating the importance of the TRE in constitutive binding to the ARE/AP-1 response element. Nuclear extracts prepared from β -NF-treated HepG2 cells contained protein(s) recognizing and binding sequences with an intact GC box (i.e. GCS_nARE4, ARE4m2 and human NQO1 hARE). Mutation of the consensus GC box (ARE4m3 probe) resulted in decreased binding of extracts from β -NF-treated cells in comparison with binding to the wild-type ARE4 probe. Collectively, the results of the gel

mobility-shift assays suggest that the internal AP-1 site contributes to constitutive binding of the ARE4 sequence, whereas the GC box is important for binding of proteins in β -NF-treated cells.

The observed TRE-binding activity evident in nuclear extracts from β -NF treated cells (Figure 3, lane 2) appears to be contrary to the observation that the ARE-related GC box is required for induced binding. However, the TRE probe itself (5'-cgcttg-ATGAGTCAGCCgga-3') resembles a core ARE sequence (upper-case letters) and contains a GC-like sequence (in bold). This partial GC box may allow both recognition and increased binding of ARE-binding protein(s) following β -NF treatment. Subsequent gel-shift studies compared the binding to the TRE sequence used in these studies with that of a second TRE probe lacking the partial GC box element (5'-gggaTGAGTAACGG-ttcaag-3'). Following incubation with nuclear extracts from β -NF-treated cells, increased binding was only evident in the case of the TRE sequence containing the partial GC box (H. Moinova, unpublished work), again underlining the importance of these bases in the recognition of binding proteins induced by β -NF.

Recent evidence suggests that AP-1-related factors might be operative in the regulation of the GCS genes. Regulation of GSH1, the yeast GCS homologue, has been shown to involve the binding of yAP-1, the yeast homologue of mammalian AP-1, to a consensus TRE element in the promoter of the GSH1 gene [33]. Mutation of yAP-1, or changes in yAP-1 gene dosage, resulted in decreased constitutive expression of GSH1 [33,34] and a loss of inducibility following exposure to hydrogen peroxide [34]. A potential role for AP-1 in mammalian GCS expression has been suggested by Sekhar et al. [35], who reported that both the constitutive and okadaic-acid-induced expression of the human GCS_n subunit gene is dependent on Jun transcription factors. We evaluated the steady-state mRNA levels of AP-1 family members to assess potential changes in expression following β -NF and PMA exposure. As expected, many of the family members displayed increased mRNA steady-state levels in response to PMA. In contrast, treatment of HepG2 cells with β -NF resulted in notable increases in only the *fra1* transcript, which displayed induction kinetics that coincided with the GCS_n subunit steady-state mRNA levels after β -NF treatment. It has been previously hypothesized that putative ARE sequences within the *fra1* promoter may be responsible for this type of response [16], providing a reasonable explanation of the similarities in the induction patterns of *fra1* and GCS_n. Although the results from the Northern blot experiments do not clearly identify potential candidates for the regulation of the GCS_n gene, these observations do not eliminate the possibility that AP-1 family members are involved in binding GCS_n ARE4, because post-transcriptional events can influence the binding of these transcription factors to DNA. In support of the hypothesis that AP-1 members may interact with GCS_n ARE4, preliminary supershift assays have implicated JunD, c-Jun, c-Fos and Fra1 in binding to ARE4 (J. J. Gipp and R. T. Mulcahy, unpublished work). The potential involvement of the AP-1 family members in the regulation of the GCS_n gene is currently under further investigation.

Other transcription factors recognizing the general ARE core sequence have also been identified. In addition to certain AP-1 family members, Venugopal and Jaiswal implicated Nrf1 and Nrf2, in regulation of hARE-mediated NQO1 expression [14]. Liu and Pickett isolated a unique nuclear protein from HepG2 cells (YABP) which was shown to specifically bind the rat GST Ya subunit ARE, most likely as a heterodimer [36]. Identification of the two component proteins of YABP has not yet been reported. The most direct characterization of ARE-binding proteins was presented by Itoh et al., who identified a heterodimer consisting

of Nrf2 and a small Maf protein [20], which binds specifically to the ARE sequence in murine GST Ya genes. The study, utilizing *nrf2* knockout mice, demonstrated that the expression of many of the GST family members and the steady-state message level of NQO1 was decreased or lost in null mice. Gel-shift analyses utilizing Nrf2 and MafK bacterial fusion proteins demonstrated binding to the mouse GST Ya subunit gene ARE only when both proteins were present.

This recent observation could have significant implications for defining the specific *trans*-factors involved in GCS_n regulation. The sequence of GCS_n ARE4 overlaps a consensus TRE-type Maf recognition element (T-MARE, 5'-TGCTGACTCAGCA-3'), which supports binding of small Maf proteins [37]. It is also similar to an NF-E2 binding site (5'-TGCTGACTCAC-3'), which binds NF-E2 family members, including Nrf1 and Nrf2 [38,39]. This raises the intriguing possibility that the GCS_n subunit gene may be regulated by Maf-related transcription factors, and perhaps NF-E2 family members. Similar hypotheses have also been proposed for other ARE-containing genes, including the human NQO1 gene [14] and the mouse haem-oxygenase 1 gene [40]. The small Maf proteins have been shown to regulate transcription both negatively and positively, depending on the formation of homodimers or heterodimers with other bZIP transcription factors, such as AP-1 [37,41–43] and NF-E2 family members [44–46].

In summary, the constitutive and β -NF-induced expression of the GCS_n subunit gene is mediated by the distal ARE4 sequence. The ARE4 internal AP-1-binding site is necessary for full constitutive expression. In contrast, the GC box of the ARE4 sequence is required for both basal and β -NF-induced expression of GCS_n. Gel-shift competition studies (Figure 4) suggested the existence of different protein complexes binding to ARE4 in extracts isolated from both β -NF- and PMA-treated cells. This possibility, and the observation that ARE4 may overlap with a T-MARE sequence, suggest that multiple complexes consisting of small Maf factors and other proteins from the bZIP superfamily of transcription factors may bind ARE4 and regulate the expression of GCS_n under varying conditions. Identification of specific GCS_n ARE4 binding factors and elements of the upstream signalling cascade are currently under investigation.

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