Regulation of γ-Glutamylcysteine Synthetase Subunit Gene Expression by the Transcription Factor Nrf2*

(Received for publication, June 29, 1999, and in revised form, August 21, 1999)

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Exposure of HepG2 cells to β-naphthoflavone (β-NF) or pyrrolidine dithiocarbamate (PDTC) resulted in the up-regulation of the γ-glutamylcysteine synthetase catalytic (GCS₇₃) and regulatory (GCS₃₁) subunit genes. Increased expression was associated with an increase in the binding of Nrf2 to electrophile response elements (EpRE) in the promoters of these genes. Nrf2 overexpression increased the activity of GCS₇₃ and GCS₃₁ promoters/reporter transgenes. Overexpression of an MafK dominant negative mutant decreased Nrf2 binding to GCS EpRE sequences, inhibited the inducible expression of GCS₇₃ and GCS₃₁ promoters/reporter transgenes, and reduced endogenous GCS gene induction. β-NF and PDTC exposure also increased steady-state levels of MaG mRNA. In addition to Nrf2, small Maf and JunD proteins were detected in GCS₇₃EpRE-protein complexes and, to a lesser extent, in GCS₃₁EpRE-protein complexes. The Nrf2-associated expression of GCS promoter/reporter transgenes was inhibited by overexpression of MaG. Inhibition of protein synthesis by cycloheximide partially decreased inducibility by PDTC or β-NF and resulted in significant increases in GCS mRNA at late time points, when GCS mRNA levels are normally declining. We hypothesize that, in response to β-NF and PDTC, the GCS subunit genes are transcriptionally up-regulated by Nrf2-basic leucine zipper complexes, containing either JunD or small Maf protein, depending on the particular GCS EpRE target sequence and the inducer. Following maximal induction, down-regulation of the two genes is mediated via a protein synthesis-dependent mechanism.

The heterodimeric enzyme γ-glutamylcysteine synthetase (GCS) catalyzes the rate-limiting reaction in glutathione (γ-glutamylcysteinylglycine; GSH) biosynthesis (1). The steady-state messenger RNA levels corresponding to the GCS catalytic and regulatory subunit genes are increased following exposure of cells to a wide variety of xenobiotics, including β-naphthoflavone (β-NF) (2), menadione (3), butylated hydroxyanisole (4), methyl mercury hydroxide (5), tert-butylhydroquinone (6), and pyrrolidine dithiocarbamate (PDTC) (7). Many of these agents are also potent inducers of phase II enzymes and have been reported to initiate a protective response, designated the "electrophile counterattack" (8), characterized by both the increase in intracellular glutathione and the up-regulation of detoxification genes via antioxidant response element (ARE)/electrophile response elements (EpRE) located in the promoter regions of the respective genes. The increase in intracellular GSH levels and the increased expression of the GCS genes following exposure to phase II enzyme inducers implicates GCS subunit gene induction as an important event in mobilizing the xenobiotic stress response. Consequently, we have been investigating the regulation of the genes encoding the catalytic (or heavy, 73 kDa) and regulatory (or light, 31 kDa) subunits of GCS.

We previously demonstrated that the basal and β-NF-induced expression of the gene encoding the human GCS catalytic subunit (GCS₇₃) is mediated via an electrophile response element, designated EpRE4, located approximately 3.1 kb upstream of the transcriptional start site (2). Expression of the GCS regulatory subunit gene (GCS₃₁) was likewise shown to be under the control of an EpRE sequence, located 0.3 kb upstream of the translational start site (9). In contrast to the GCS₇₃ gene, in which β-NF induction is mediated solely by EpRE4, induction of the GCS₃₁ gene in response to β-NF is also influenced by a second cis-element, an AP-1 binding sequence 32 base pairs upstream of the EpRE.

Originally identified in the promoter of the rat NAD(P)H:quinone oxidoreductase (NQO1) gene, the ARE was shown to mediate gene induction by phenolic antioxidants and was named accordingly (10). Talalay and colleagues (11, 12) later demonstrated that many of the inducers capable of mediating gene induction via ARE sequences were potent electrophiles or could be metabolized to electrophilic intermediates. They further established a strong correlation between electrophilicity and potency as inducing agents and hence preferred to designate elements satisfying the ARE consensus sequence as EpRE (13), as previously suggested by Friling and colleagues (14). Since these original reports, EpREs have been identified and characterized in the promoter regions of a number of genes, including human and rat NQO1 (15, 16), rat and murine glutathione S-transferase Ya (10, 14), murine heme oxygenase-1 (13), as well as the genes encoding the catalytic and regulatory subunits of human GCS (2, 9).

EpREs confer high basal expression and mediate xenobiotic inducibility of genes possessing the sequence within their promoters (17). The EpRE core sequence (5'-G(C/T)G/A/C/ (G)NNGC(A/G)-3') may contain an embedded AP-1-binding site and is often flanked by AP-1 or AP-1-like sequences. It has been noted that the primary sequence of the EpRE is similar to that of other response elements, including the TRE-type Maf

* This work was supported in part by National Institutes of Health Grant CA57549 and NIEHS Grant ES09749. 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.

‡ Supported by National Institutes of Health Grant 5T32-CA09471.

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The abbreviations used are: GCS, γ-glutamylcysteine synthetase; PAI-1, activator protein-1; ARE, antioxidant response element; βZip, basic leucine zipper; β-NF, β-naphthoflavone; Me₃SO, dimethyl sulfoxide; EpRE, electrophile response element; GCS₃₁, GCS heavy subunit; GCS₇₃, GCS light subunit; MafK, Maf dominant negative; NQO1, NAD(P)H quinone oxidoreductase; PDTC, pyrrolidine dithiocarbamate; T-MARE, TRE-type Maf recognition element; TPA, 12-O-tetradecanoylphorbol-13-acetate; TRE, TPA responsive element; kb, kilobase pairs; CMV, cytomegalovirus.
recognition elements (T-MARE) and the NF-E2-binding sites, suggesting that transcription factors recognizing these sequences may bind to and regulate expression through EpRE sequences (18, 19). The sequences are so comparable that it has been suggested that EpRE sequences may, in fact, constitute a subset of the T-MARE regulatory sequences (9, 20).

The interpretation of studies designed to identify those trans-acting factors capable of specifically recognizing the EpRE regulatory sequences to affect gene expression has been complex. Several experimental approaches to address this question have been utilized, although most investigators have relied on electromobility supershift assays and the evaluation of EpRE-directed reporter gene expression following manipulation of transcription factor expression. These studies have implicated AP-1 family members JunB, JunD, c-Fos, Fra1, Fra2, and NF-E2 family members NrF1 and NrF2 as regulators of genes containing EpRE promoter sequences (21–28). A number of investigators have also identified novel EpRE-binding proteins by direct purification, describing a 74.3-kDa protein (YABP) recognizing the rat GST Ya subunit gene EpRE (29, 30), a 160-kDa protein complex binding the human NQO1 EpRE isolated from Hela nuclear extracts (31), an 80-kDa protein from murine smooth muscle cells recognizing an EpRE in the c-Ha-ros gene promoter (32), and a unique 160-kDa protein, identified as ARE-binding protein 1 (33).

As mentioned above, the similarity of the EpRE sequence and that of the T-MARE has led to the proposal that small Maf and its heterodimerization partners, such as NrF1 and NrF2, can mediate expression through EpRE sequences. NrF1 and NrF2 belong to the Cap “n” Collar subfamily of bZip transcription factors (34, 35). As is the case for p45 NF-E2, the founding member of this family, neither NrF1 nor NrF2 form homodimers that effectively bind DNA (27, 36, 37). Rather, NF-E2 family members, including NrF1 and NrF2, heterodimerize with bZip factors, including the small Maf proteins (27, 36–40). Small Maf proteins compose a family of transcription factors identified by their homology to the avian transforming retroviral oncogene, v-maf (musculo-aponeurotic fibrosarcoma) (36, 41). The small Maf proteins, including MafK, MafG, and MafF, are distinct from the large Maf family members in that they lack amino-terminal transactivation domains. The small Maf proteins can form maf:maf homo- and heterodimers to negatively regulate transcription (20, 36, 38, 40, 42). When heterodimerized with bZip factors containing transactivation domains, such as Fos, Bach1, Bach2, NF-E2 p45, NrF1, and NrF2, the small Maf factors facilitate DNA binding to T-MARE sequences and result in transcriptional inhibition (20, 36–40, 42–44). In support of the hypothesis that small Maf/NrF heterodimers bind to and direct expression through phase II enzyme EpRE sequences, Itoh and colleagues (27) demonstrated that the induction of phase II enzymes by the phenolic antioxidant butylated hydroxyanisole was decreased in the liver and intestine of nrf2-/-null mice.

In the experiments presented here, we demonstrate that the transcription factor NrF2 is a positive regulator of EpRE-dependent GCS subunit gene expression in cells exposed to β-NF and PDTC. Our findings suggest that JunD and small Maf proteins may mediate GCS gene inducibility under certain conditions, following heterodimerization with NrF2. Consistent with this hypothesis, we report that the expression of MafG is increased in response to β-NF and PDTC, at time points preceding GCS subunit gene induction. We provide evidence that the small Maf proteins may also negatively regulate GCS gene expression; the opposing positive and negative effects of the small Maf proteins on GCS gene expression may reflect changes in the dynamic equilibrium between the formation of small Maf/bZip hetero- and Maf/Maf homodimers, respectively. Furthermore, we find that protein synthesis is required for full inducibility of the GCS subunit genes by β-NF or PDTC and may be necessary for the subsequent down-regulation of GCS subunit gene expression observed following gene induction by these agents.

**EXPERIMENTAL PROCEDURES**

**Recombinant Plasmids**—The recombinant expression vectors—3802/ GCSb, 5'-lac and 1927/GCS, 5'-lac were constructed by cloning the 4.2-kb HindIII/NcoI and the 1.9-kb NcoI genomic restriction fragments flanking the 5'-flanking sequences of the GCSb and GCSl genes, respectively, into pGL3-Basic (Promega Corp., Madison, WI) (2, 9). The pGL3-Basic vector used in these studies was mutared by a single base substitution to eliminate the AP-1-binding site located near the ampicillin resistance gene (45). pCI-NrF2neo was constructed by isolating the 2.3-kb EcoRI fragment containing the human NrF2 cDNA by partial digestion of pCRII (obtained from Dr. Etsuro Ito, Hiroaki University) and ligating the fragment into pCIneo (Promega Corp.). A 1.6-kb EcoRI fragment of the human MafG cDNA was excised from the plasmid pMT2 containing the MafG cDNA (obtained from Dr. Nancy Andrews, Harvard University) and was cloned into pCIneo to generate pCI-MafGneo. The pCMV-JunD expression vector was created by blunt-end ligating the 1.7-kb BamHI-NrF1 fragment containing the c-DNA (ATCC, Rockville, MD) into the vector pCMVβ (CLONTECH Laboratories, Inc., Palo Alto, CA), following NrF1 excision of the β-galactosidase cDNA insert. The MafK dominant negative mutant (MafK DN) was generated by mutation of conserved bases in the MafK DNA binding domain by Kotok and Orkin (46). For the current studies, the dominant negative mutant cDNA was excised with NcoI from the plasmid neo-plasmid in which it was provided (from Dr. Stuart Orkin, Harvard University) and recloned into the pcDNA3 expression vector (Invitrogen, Carlsbad, CA). pcDNA3 expression vectors containing the dominant negative mutant cDNA in the reverse and forward orientations were isolated and were used in transient transfection studies. The expression vector, pRc-CMV-FAFs, containing the AP-1 dominant negative mutant, AFos, was designed by Olive et al. (47) by elimination of the DNA binding domain of c-Fos and the addition of an amphotropic acid extension to the leucine zipper region following by cloning of the resultant mutant sequence into pBR-CMV (Invitrogen). We monitored the function of the transiently expressed AFos mutant by measuring the pTS1–4XAP-1 reporter constitutive activity, and its expression in response to 50 ng/ml 12-O-tetradecanoylphorbol 13-acetate (TPA) for 16 h. The pTS1–4XAP-1 plasmid was generated by annealing complementary synthetic oligonucleotides containing the sequence 5′-GGAGAGTGAATCCGAACCGTTACTAAAGG-3′ (AP-1-binding site, underlined) and ligating the double-stranded product into the pTS1 vector (ATCC) digested with SmaI, as described previously (2). The resulting construct contains four tandem AP-1-binding sites cloned upstream of the tk promoter, driving luciferase expression. Plasmids used for stable transfection experiments include the pHUD0–3-hyg plasmid, created by ligation of a 1.8-kb Mael fragment, from the vector pRS3 (Stratagene, La Jolla, CA), containing the hygromycin resistance gene, into the HindIII site of the pHUD0–3 (48). The pHUD0–3/MafK DN plasmid was generated by cloning the 0.5-kb EcoRI fragment containing the MafK DN mutant cDNA from pcDNA3/MafK DN into the EcoRI site of pHUD0–3.

**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 μg/ml gentamicin. Cells were transiently transfected with recombinant plasmids using a standard calcium phosphate-glycerol shock procedure, as described previously (2). When varying concentrations of the transcription factor expression vectors were used within an experiment, as indicated in the figure legends, total DNA concentration was corrected by addition of the pCIneo vector (Promega Corp.). To normalize for transfection efficiency, either pCMVβ (49) or pRSβ-galactosidase, each containing the lacZ gene encoding β-galactosidase, was co-transfected with each recombinant plasmid group. β-NF (Sigma) was dissolved in Me2SO at a concentration of 10 mM and diluted 1000-fold (final Me2SO concentration = 0.1%) by addition to the plating medium. PDTC (Sigma) was dissolved in water and diluted 1000-fold in medium to a final concentration of 10 μM. To initiate treatment HepG2 cells with either agent, culture medium was replaced with fresh complete medium containing Me2SO (0.1% v/v), 10 μM β-NF, or 10 μM PDTC. Cells were harvested at various times after addition of PDTC or β-NF and prepared for determination of luciferase and β-galactosidase activity and protein concentration, as described previously (2).
To establish a HepG2 cell line conditionally expressing the MafK DN mutant, we used the tetracycline-regulated expression system (Life Technologies, Inc.). HepG2 cells were initially co-transfected with the tetracycline-responsive transactivator plasmid pTet.tTAk and with pCMV-neo (GLONTECH Laboratories, Inc.), conferring resistance to gen- (Life Technologies, Inc.). Following G418 selection, pTet.tTAk-positive clones were selected by screening for transactivation of the transiently expressed pTet.tTAk-responsive pUHC13-3 luciferase reporter in the absence of doxycycline treatment. The resulting HepG2 cell was then stably co-transfected with pUHD10–3-byg, conferring resistance to hygromycin B (Calbiochem) and either the empty pUHD10–3 vector or the pUHD10–3/MafK DN construct in a 1:10 molar ratio, respectively. Transfectants were exposed to 600 μg/ml hygromycin B followed by clonal selection.

**Electrophoretic Mobility Supershift Analysis**—Complementary oligonucleotides containing GCS EpRE sequences were prepared as follows, as specified previously (45). Sequences of the probes were as follows: GCSh EpRE4, 5′-CTCCCCGACTCAAGCGCCTTTG-3′; GCSh EpRE4, 5′-GGAGAGCAATTGACTAAAGGAAATCTGAGCCG-3′; and GCSh EpRE4 m1, 5′-CTCCC GGAGACTACGCGGTTTG-3′ (base substitution underlined). Nuclear extracts were prepared, as described by Dignam et al. (50), from HepG2 cells treated with 0.1% MeSO or 25 μM β-NF for 6 h or with 100 μM PDTC for 1–3 h. For supershift analyses, the 32P-labeled oligonucleotide probe and 10 μg of nuclear extract were incubated for 20 min prior to the addition of antibody which was followed by a 20-min incubation at room temperature (45). The Nrf2 and JunD antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and were used according to the recommended procedure. We obtained antiserum to MafG protein from two sources, from Dr. Nancy Andrews and Dr. Volker Blank (51) and from Dr. Kazuhiro Igarashi.

**RNA Isolation and Analysis**—Total cellular RNA was isolated from HepG2 cells using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the recommended procedure. RNA quantita- tion was accomplished using the RPAII system (Ambion Inc., Austin, TX), as described (2). For Northern blot analyses, 2 μg of mRNA (iso- lated with the Poly(AT) Tract mRNA Isolation System (Promega Corp.)) or 10 μg of total RNA was size-fractionated in agarose formaldehyde gels, transferred to a charged nylon membrane (Hybond N−, Amersham Pharmacia Biotech), and hybridized with either random primer labeled probe or with an in vitro transcribed RNA probe. The DNA templates prepared for random primer labeling (Amersham Pharmacia Biotech, Rediprime II kit) included the 1.1-kb Smal/EcoRI fragment from pCI-MafGneo encompassing the MafG 3′-untranslated region, the 1.3-kb Nrf2 EcoRI fragment isolated from pCI-Nrf2neo, the 0.5-kb Nrf2 PCR fragment containing the MafK DN mutant cDNA from pcDNA3/MafK DN, and the 0.7-kb PstI 36B4 fragment. To generate run-off, transcripts corresponding to the 0.4-kb Smal fragment from the coding region of the MaGe cdna, in vitro transcription reactions were performed using T7 RNA polymerase and [32P]UTP.

**Western Blot Analysis**—Total protein was harvested from the HepG2 cells using the single detergent lysis protocol (52). Forty micrograms of total cellular protein was fractionated by SDS-polyacrylamide gel electrophoresis (15% acrylamide), followed by transfer to a nitrocellulose membrane using the Bio-Rad Trans-Blot system (Bio-Rad). Membrane blocking and incubation with the NF-E2 p18 primary antibody (Santa Cruz Biotechnology, Inc.) was performed according to the manufacturer’s protocol. Biotinylated goat anti-rabbit secondary antibody incubation and development was performed with a Western-Star Protein Detection kit (Tropix, Inc., Bedford, MA), according to the manufactur- er’s protocol.

**Pulse Labeling of Cells with L-[35S]Methionine**—To demonstrate inhibition of protein synthesis by cycloheximide treatment, cellular extracts from HepG2 cells labeled with L-[35S]methionine were monitored by trichloroacetic acid precipitation to determine the incorporation of radioactivity into cellular protein (52). Briefly, HepG2 cells grown on 35-mm dishes for 4 days were rinsed with “long term” labeling medium, followed by incubation with the labeling medium, in the absence or presence of 2 μCi/ml cycloheximide (Sigma), to initiate treatment. The long term labeling medium contained 90% Dulbecco’s modified Eagle’s medium lacking methionine (Life Technologies, Inc., Dulbecco’s modi- fied Eagle’s medium catalog number 11965), 10% normal Dulbecco’s modified Eagle’s medium, and 10% fetal bovine serum. After 30 min, long term labeling medium containing 0.04 μCi/ml L-[35S]methion- ine (Amersham Pharmacia Biotech), with or without cycloheximide, was added to cells. Cycloheximide was replenished at 6 and 12 h. Cells were harvested at 3, 6, 12, and 16 h for trichloroacetic acid precipita- tion, as described (52).

**Statistical Analysis**—Results are presented as the means ± S.E. Statistical comparisons were made by paired Student’s t tests. A value of p < 0.05 was considered statistically significant.

**RESULTS**

**Identification of trans-Acting Factors Recognizing GCS EpRE Sequences**—β-NF induction of the GCSδ and GCSγ subunit genes is mediated via EpRE sequences present in the promoter regions of the two genes (2, 4, 45). Consequently, we attempted to identify nuclear proteins isolated from HepG2 cells treated with 25 μM β-NF which were capable of binding oligonucleotides corresponding to the GCSδ, EpRE4 and the GCSγ, EpRE wild-type sequences. Supershift analyses were con- ducted with antisera reactive to the bZip proteins Nrf2, JunD, and the small Maf proteins. As seen in Fig. 1A, with the addition of Nrf2 antisera, a supershift of the EpRE-protein complex was observed (compare lane 1 and 3, Nrf2 antisera), suggesting binding of the bZip transcription factor Nrf2 to the GCSδ EpRE4 probe under constitutive conditions. When nuclear extracts from β-NF-treated cells were incubated with the EpRE4 probe, the intensity of the supershifted EpRE-Nrf2 complex was significantly increased (lane 3 versus 4). When a mutant GCSδ, EpRE4 sequence (m1) that disrupts basal and β-NF inducibil- ity (2, 45) was used as a probe, the binding of HepG2 nuclear protein was diminished (Fig. 1C, lanes 1 and 2 versus 5 and 6). Furthermore, the mobility of the mutant GCSδ, EpRE4-protein complex was not shifted by Nrf2 antibody (compare lanes 3 and 4 with lanes 7 and 8).

The presence of JunD and small Maf factors in GCSδ, EpRE4 DNA-protein complexes was also detected by the appearance of supershifted bands using a JunD antibody (Fig. 1A, lanes 5 and 6) and two different antibodies recognizing the small Maf proteins provided by Drs. N. Andrews and V. Blank (A) (Fig. 1A, lanes 7 and 8) and by Dr. K. Igarashi (C) (Fig. 1A, lanes 9 and 10). The supershift observed with the Igarashi antibody minimally decreases the mobility of the GCSδ, EpRE4-protein complex.

**Binding of nuclear extract components to the GCSδ, EpRE probe** is shown in Fig. 1B. In the case of the GCSδ, EpRE sequence, mutational analyses have provided the identification of a specific DNA-protein complex associated with GCSδ, EpRE function (53). The band corresponding to this complex, designated band b (Fig. 1B, see arrow), is shifted with the Nrf2 antibody. As was true for the GCSδ, EpRE4 Nrf2 supershifts, incubation of the Nrf2 antibody with the GCSδ, EpRE probe and extracts from β-NF-treated cells significantly retarded the mo- bility of this particular complex to the point that the original band b complex was greatly reduced. Inclusion of an antibody reacting against JunD protein resulted in weak supershifts of the GCSδ, EpRE-protein complexes that do not appear to increase in intensity in reactions containing extracts from treated cells (Fig. 1B, lanes 5 versus 6). Small Maf antibodies failed to modify significantly the mobility of the GCSδ, EpRE-protein complexes at this time point (lanes 7–10). Previous electromobility supershift experiments indicated little or no binding of c-Jun, JunB, c-Fos, Fra1, Fra2, Nrf1, or Bach proteins to the GCSδ, EpRE wild-type probes incubated with extracts from cells treated with β-NF for 6, 12, or 16 h (for GCSδ; for GCSγ (53)).

**Pyrrolidine dithiocarbamate (PDTC)** potently increases the steady-state messenger RNA levels corresponding to the GCSδ and GCSγ genes, with kinetics differing from those observed with β-NF induction (7). Thiocarbamates, such as PDTC, have been reported to effect expression of the phase II gene battery

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*Note:* A. C. Wild, H. R. Moïnova, and R. T. Mulcahy, unpublished observations.
and other detoxification genes through EpRE sequences (11). Therefore, we also evaluated binding of nuclear proteins harvested from PDTC-treated HepG2 cells to the GCS EpRE sequences. These electromobility supershift experiments again demonstrated prominent supershifts of GCS EpRE-protein complexes following the addition of Nrf2 antisera (Fig. 1, D and E, lanes 3 and 4), with significantly enhanced binding of Nrf2 following PDTC treatment. In the case of the GCS\textsubscript{h} EpRE4 probe, JunD and the small Maf proteins were again detected in complexes formed from nuclear extracts from untreated and PDTC-treated cells by the formation of supershifted complexes (Fig. 1D, lanes 5–10). DNA-protein complexes containing the GCS\textsubscript{l} EpRE probe did not react noticeably with antibodies to JunD (Fig. 1E, lanes 5 and 6). There is some evidence of a supershifted GCS\textsubscript{l} EpRE-protein complex following incubation with the small Maf antibodies (Fig. 1E, compare lanes 2 and 6 with lanes 8 and 10).

Overexpression of Transcription Factors Potentially Involved in GCS Gene Expression—Since induction of both GCS subunit genes by \(\beta\)-NF and PDTC correlated with increased binding of Nrf2 to the corresponding EpRE sequences, we examined the effect of increased Nrf2 expression on reporter transgenes regulated by the GCS 5'-regulatory regions of the two subunit genes. HepG2 cells were transiently co-transfected with either the GCS\textsubscript{h} or the GCS\textsubscript{l} promoter/reporter transgenes and 150 fmol of pCI-Nrf2neo. As seen in Fig. 2A, overexpression of Nrf2 increased the luciferase activity of the GCS\textsubscript{h} reporter transgene by 2.7-fold relative to the expression of the reporter transgene in cells co-transfected with the empty pCIneo vector (\(p < 0.01\)). When HepG2 cells were co-transfected with 150 fmol of pCI-Nrf2neo and increasing amounts (15–300 fmol) of a second plasmid directing expression of human MafG cDNA (pCI-MafGneo), GCS\textsubscript{h} reporter transgene expression was inhibited in an MafG dose-dependent fashion. Transfection of pCI-MafGneo alone (15 fmol) inhibited GCS\textsubscript{h} transgene expression, decreasing luciferase activity by 30% (\(p = 0.05\)).

The luciferase expression in HepG2 cells transfected with the GCS\textsubscript{h} reporter transgene (Fig. 2B) was likewise increased (5.2-fold) by transfection of the pCI-Nrf2neo expression vector (\(p < 0.01\)). However, in contrast to the GCS\textsubscript{h} transgene, co-transfection of 150 fmol of pCI-Nrf2neo and 15 fmol of pCI-MafGneo significantly increased GCS\textsubscript{l} reporter luciferase ac-
Luciferase activity was corrected for decreased with higher levels of MafG expression. Overexpression of MafK and AP-1 

**Fig. 3. Effect of the MafK dominant negative mutant (MafK DN) on the GCS reporter transgene activities.** The GCS (−1927/GCS, 5′-luc) (A and C) or GCS (−3802/GCS, 5′-luc) (B and D) reporter transgenes were co-transfected along with the pCDNA3 expression vector containing MafK DN cloned in the forward (+) or reverse (−) orientation. Following transfection, cells were exposed to 0.1% Me2SO or 10 μg β-NF for 16 h (A and B) or to 100 μg PDTC for 6 h (C and D). Luciferase activity was corrected for β-galactosidase expression and normalized to the reporter expression in untreated cells, not transfected with the dominant negative mutant. Values are presented as the means ± S.E. for 4–6 determinations.

In a similar series of experiments, an AP-1 dominant negative mutant, AFos (47), was employed to evaluate the potential role of AP-1 family members in GCS gene regulation. In control experiments, AFos overexpression decreased the basal expression and eliminated TPA inducibility of a luciferase reporter vector (pT81–4X AP-1) containing four tandem AP-1-binding sites, demonstrating the ability of this dominant negative mutant to inhibit AP-1 activity (Fig. 4A), as reported (47). Following co-transfection of the GCS transgenes along with the AFos expression vector, the basal expression of the GCS and the GCS subunits were significantly decreased (−58% (p < 0.01) and −33% (p < 0.01), respectively) (Fig. 4, B–E). β-NF induction of GCS was partially decreased (3- to 1.7-fold, p < 0.01), following co-transfection of AFos (Fig. 4B). Similarly, induction of the GCS transgene by β-NF dropped from 2- to 1.4-fold in cells transfected with the AFos vector (p < 0.01) (Fig. 4C). For both the GCS and GCS subunit reporter transgenes, PDTC induction was not significantly altered by AFos expression (Fig. 4, D and E). However, since PDTC induction of the GCS subunit promoter/reporter transgene was so slight, the use of this transgene was unrewarding in the case of PDTC inducibility.

Stable Expression of the MafK Dominant Negative Mutant Reduces GCS Subunit Gene Induction In Vivo—In order to determine whether the inhibitory effect of the MafK DN mutant on GCS reporter gene expression would be similarly manifested in endogenous GCS gene expression in intact cells, stable transfectants expressing the MafK DN mutant under the control of a tetracycline-regulated expression system were generated. Northern and Western blot analyses were used to identify stable HepG2 clones expressing the MafK DN transgene. The HepG2 clones designated mdnL, mdnM, and mdnN all express increased levels of MafK DN mRNA which were repressed upon addition of 0.1 μg/ml of the expression inhibiting tetracycline derivative doxycycline (Fig. 5A). The mdnL clone expressed high levels of the MafK protein when incubated in the absence of doxycycline; no expression of the dominant negative protein was detected following the addition of doxycycline (Fig. 5B). No MafDN expression was detected in a cell line established by transfection with empty vector (TetB; Fig. 5B).

Evaluation of messenger RNA isolated from the mdnL clone (Fig. 6B) revealed a decreased responsiveness of endogenous GCS and GCS subunit gene expression following treatment with PDTC (lanes 2 versus 5) or β-NF (lanes 7 versus 9), under conditions in which the MafK DN mutant is expressed. Induction of GCS message levels following PDTC treatment decreased from −8- to −2-fold (p < 0.01) in cells expressing the MafK DN mutant (Fig. 6C), whereas PDTC induction of GCS mRNA was reduced from −8- to −4-fold (p < 0.01) (Fig. 6D). Similarly, induction in response to β-NF was reduced from −6- to −3-fold for transcripts corresponding to the GCS subunit (gene (p < 0.03) (Fig. 6E) and from −11- to −2-fold for GCS message levels (p < 0.02) (Fig. 6F) when MafDN mutant was expressed. As expected, examination of RNA from the TetB clone demonstrated that both PDTC (lanes 2 versus 4) and β-NF (lanes 6 versus 8) inducibility were maintained in the presence and
absence of doxycycline (Fig. 6A).

The MafK DN mutant used in these studies has been shown to sequester Nrf2, thereby reducing its binding to its cognate recognition sequence (46). Therefore, we hypothesized that the inhibitory effect of the MafK DN mutant on GCS reporter and endogenous gene expression was the result of decreased Nrf2 binding to the GCS EpRE sequences. To test this hypothesis,

FIG. 5. Selection of a HepG2 clone stably expressing the MafK DN mutant by Northern and Western blot analysis. A, Northern blot analysis was used to evaluate total RNA harvested from multiple HepG2 clones generated by the stepwise transfection of a transactivator plasmid followed by the transfection with the pUHD10–3/MafKDN vector and a hygromycin-expressing plasmid. Following clonal selection, cells were grown in either the absence or presence of 0.1 mg/ml doxycycline which positively or negatively regulates expression of the MafK DN mutant, respectively. A random prime-labeled probe corresponding to the MafK coding region was hybridized to membranes following transfer from agarose formaldehyde gels. Subsequently, membranes were hybridized to a probe corresponding to the loading control, 36B4. B, Western blot analysis of cellular protein isolated from the HepG2 clones mdnL or TetB (expressing the empty pUHD10–3 vector) grown in the absence or presence of 0.1 mg/ml doxycycline. Forty micrograms of total protein was size-fractionated by SDS–polyacrylamide gel electrophoresis using a 15% acrylamide separating gel, followed by transfer to nitrocellulose. The membranes were probed with the NF-E2 p18/MafK antibody (Santa Cruz Biotechnology, Inc.) which was detected with a secondary goat anti-rabbit antisera using the Tropix, Inc. Western-Star Protein Detection Kit.

FIG. 6. RNase protection analysis of endogenous GCS₁ and GCS₆ mRNA following expression of the MafK DN mutant. Twenty micrograms of total RNA, isolated from either the TetB or mdnL cell lines were analyzed by the ribonuclease protection assay. The HepG2 clones were exposed to 25 µM β-NF for 12 h or 100 µM PDTC for 6 h, in the presence or absence of 0.1 µg/ml doxycycline. Representative RPA gels for TetB mRNA (A) or mdnL mRNA (B) are depicted. Lane 4 of the gel depicted in B contains labeled DNA size markers. Plots designated C–F display quantitation of the GCS₁ and GCS₆ message levels. For these experiments, n = 3–4 determinations.
we examined Nrf2 binding to GCS EpRE sequences following expression of the MafK DN mutant. Nuclear extracts were prepared from the HepG2 mdnL clone grown either in the presence or absence of doxycycline. Gel shifts using nuclear extracts prepared from mdnL cells not expressing the MafK DN mutant are shown in Fig. 7A, lanes 1–4. The gel shift reactions in lanes 3 and 4 reveal the presence of Nrf2 bound to the GCS, EpRE4 wild-type sequence and the expected increase in Nrf2 binding induced by exposure to PDTC (lane 4). Nrf2 binding was greatly diminished, however, using extracts harvested from cells expressing the MafK DN mutant (lanes 5–8, compare lanes 3 and 4 versus 7 and 8). A similar reduction in the intensity of the Nrf2 supershift was noted when extracts from MafK DN-expressing mdnL cells treated with β-NF were used (Fig. 7B, lane 12 versus 16) and in the reactions utilizing the GCS, EpRE probe (Fig. 7, C and D).

**Examination of the Steady-State mRNA Levels of Transcription Factors Potentially Involved in GCS Subunit Gene Regulation**—Despite increased Nrf2 binding to GCS EpRE sequences following exposure of HepG2 cells to PDTC or β-NF (Fig. 1), Nrf2 mRNA levels in HepG2 cells were unchanged following treatment with 25 μM β-NF (Fig. 8A) or with 100 μM PDTC for 3 or 6 h (B), was size-fractionated in agarose formaldehyde gels, transferred to Hybond N membranes, and hybridized with a labeled probe generated using a 1.3-kb EcoRI fragment of the human Nrf2 cDNA. Subsequently, the membranes were hybridized to a 36B4 loading control probe. For the C–E blots, 2 μg of messenger RNA isolated from HepG2 total RNA were loaded onto agarose formaldehyde gels for evaluation of small Maf expression. Random prime-labeled probes used in these experiments included the Smal/EcoRI fragment of the MafG cDNA, corresponding to the MafG 3'-untranslated region (C and D) and a 36B4 loading control. The blot in E was probed with an in vitro transcribed RNA probe corresponding to the 0.4-kb Smal fragment coding region of human MafG. Northern blot analyses of RNA from β-NF- and PDTC-treated HepG2 cells were performed at least three times in the case of both the Nrf2 probe and the small Maf probes. Blots shown are representative of at least three independent determinations.

**FIG. 7.** Electromobility supershift analysis of GCS EpRE sequences using extracts from the MafK DN-expressing mdnL cell line. Nuclear extracts were prepared from the HepG2 clone mdnL following treatment with 100 μM PDTC for 3 h (A and C) or 0.1% Me2SO or 25 μM β-NF for 6 h (B and D) in the absence or presence of 0.1 μg/ml doxycycline. Probes were prepared from synthetic oligonucleotides corresponding to the wild-type GCS, EpRE4 or GCS, EpRE sequences and were incubated with 10 μg of nuclear extract protein prior to the addition of Nrf2 antibody, where indicated. DNA-protein complexes were immediately analyzed by gel electrophoresis and were visualized using a Molecular Dynamics PhosphorImager.

**FIG. 8.** Northern blot analyses evaluating expression of Nrf2 and small MAF genes following β-NF and PDTC treatment. Ten micrograms of total RNA, isolated from HepG2 cells treated with 25 μM β-NF for 6 or 12 h (A) or with 100 μM PDTC for 3 or 6 h (B), was

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**Nrf2 Activation of Human GCS Gene Expression via EpREs**

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Despite increased Nrf2 binding to GCS EpRE sequences following exposure to PDTC or β-NF (Fig. 1), Nrf2 mRNA levels in HepG2 cells were unchanged following treatment with 25 μM β-NF (Fig. 8A) or with 100 μM PDTC (Fig. 8B). Changes in the expression of the MafG transcription factor were also evaluated, using probes generated from either the 3'-untranslated region of the MafG cDNA sequence or the MafG coding region. Whereas the MafG 3′ probe identified a single transcript, utilization of the MafG coding region probe, capable of hybridizing to other small Maf family member transcripts due to the high sequence homology through the coding regions, resulted in the recognition of multiple transcripts. Regardless of which probe was used, steady-state mRNA levels corresponding to small Maf transcripts were shown to increase following exposure of HepG2 cells to 25 μM β-NF (Fig. 8C). Increases in the small Maf transcripts were also observed following PDTC treatment (Fig. 8, D and E).

**The Requirement of New Protein Synthesis for GCS Subunit Gene Induction**—In order to determine whether induction of the GCSδ, and GCSγ subunit genes by PDTC and β-NF required protein synthesis, we utilized the protein synthesis inhibitor, cycloheximide. Cycloheximide (2 μg/ml) was added to cell cultures at 0, 6, and 12 h during the course of the experiment. This dose and timing regimen reduced the incorporation of [35S]methionine into trichloroacetic acid-precipitable counts to 12% of control at 3 h, 19% at 6 h, 23% at 12 h, and 27% at 16 h. The addition of cycloheximide reduced PDTC induction of the GCS subunit gene expression at 3 and 6 h. Induction of GCSδ message levels by PDTC at 6 h was decreased from ~10- to ~4-fold in the presence of cycloheximide (Fig. 8B), and GCSγ message levels were reduced from ~11- to ~5-fold (Fig. 8C). Cycloheximide exerted a comparable effect on β-NF induction of the GCS subunit genes at 6 and 12 h (Fig. 9, D and E). However, the β-NF-dependent increases in GCSγ message levels in cells treated with and without cycloheximide are similar at 12 h.

GCSδ and GCSγ mRNA levels return to base line within 12 h of PDTC exposure. Interestingly, cycloheximide treatment prevented this predicted decrease in GCS gene expression. At 12 h, GCSδ and GCSγ mRNA levels in cycloheximide-treated cells were ~10- and ~11-fold higher, respectively, than in cells treated with PDTC in the absence of cycloheximide (GCSδ,
RESULTS are means ± S.E. for four determinations.

Fig. 9. The effect of cycloheximide treatment on GCS\textsubscript{h} and GCS\textsubscript{l} steady-state mRNA levels in cells treated with β-NF or PDTC. A, a representative RNase protection assay examining 20 μg of total RNA isolated from HepG2 cells treated with 0.1% Me\textsubscript{3}SO, 25 μm β-NF, or 100 μm PDTC for 0–16 h, in the absence or presence of 2 μg/ml cycloheximide (added at 6, 9, and 12 h). B–E, quantitation of multiple RNase protection assay experiments. For each time point, GCS\textsubscript{h} or GCS\textsubscript{l} mRNA levels were normalized to levels measured in untreated cells and are plotted as RNA fold increase versus time (in hours).

mRNA, p < 0.04 and GCS\textsubscript{l} mRNA, p < 0.02; PDTC treatment versus PDTC/cycloheximide co-treatment). Similar observations were made at the later time points for GCS mRNA in β-NF-treated cells. In fact, co-treatment of HepG2 cells with cycloheximide and β-NF resulted in -12- and -14-fold increases in GCS\textsubscript{h} and GCS\textsubscript{l} mRNA, respectively, at 16 h compared to GCS mRNA levels in untreated cells. These increases in GCS message levels were significantly higher than levels found in cells treated with β-NF alone, at any time point (β-NF treatment versus β-NF/cycloheximide co-treatment at 16 h, GCS\textsubscript{h} p < 0.03, GCS\textsubscript{l} p < 0.05).

DISCUSSION

Binding of the transcription factor Nrf2 to GCS EpRE promoter sequences significantly increased following exposure to β-NF or PDTC (Fig. 1). This Nrf2 binding occurred only when a functionally intact EpRE was used, establishing an association between Nrf2 binding and EpRE-dependent expression of the GCS genes (Fig. 1C (53)). Although others have shown that in vitro translated Nrf2 proteins are capable of binding EpRE sequences when dimerized to other bZip proteins, to our knowledge this is the first demonstration of Nrf2 binding to EpRE sequences using nuclear extracts prepared from treated cells.

The increased Nrf2 binding was not related to an increased expression of the transcription factor following xenobiotic exposure; Northern analyses (Fig. 8) and preliminary Western analyses (46) have shown that total cellular levels of Nrf2 mRNA and protein, respectively, do not change following β-NF or PDTC treatment. Therefore, it is likely that Nrf2 is post-translationally activated following β-NF or PDTC exposure, resulting in the increased binding of Nrf2 to GCS EpRE sequences. This suggestion is consistent with the essential elements of a model for Nrf2 activation recently proposed by Itoh and colleagues (54), who similarly noted that total Nrf2 protein levels did not change following xenobiotic exposure in their system. Their data suggest that Nrf2 is normally sequestered in the cytoplasm in association with Keap1, which itself is thought to be anchored to the cytoskeleton via a double glycine repeat domain. Following exposure to electrophilic or oxidative stress, an alteration in the Nrf2-Keap1 complex results in liberation of Nrf2, permitting its translocation to the nucleus. The authors speculate that the alteration resulting in dissociation of Nrf2 from the cytoskeleton may result from either electron transfer reactions generated within the double glycine repeat moiety of Keap1 or by the oxidation of key cysteine residues in Nrf2 or Keap1. Interestingly, our previous studies suggested that thiol oxidation is an important signal for GCS subunit gene induction by PDTC (7), although a specific target was not identified. Therefore, the increased binding of Nrf2 to GCS EpREs following β-NF or PDTC treatment could conceivably be a consequence of the enhanced nuclear localization of the transcription factor following changes in the cellular redox status.

The functional relevance of Nrf2 binding to the GCS EpRE sequences with regard to GCS gene expression was confirmed by altering Nrf2 expression and monitoring resultant changes in GCS gene expression. The overexpression of Nrf2 significantly increased GCS\textsubscript{h} and GCS\textsubscript{l} promoter/reporter expression (Fig. 2). Furthermore, expression of a MafK dominant negative mutant, which has been demonstrated to reduce Nrf2 binding to its cognate recognition sequence (46), not only effectively decreased GCS reporter transgene activation by β-NF and PDTC (Fig. 3) but also significantly reduced the corresponding expression of the endogenous GCS genes in response to the two agents (Fig. 6). Expression of this mutant also reduced the binding of Nrf2 to GCS EpRE sequences incubated with nuclear extracts from β-NF- or PDTC-treated HepG2 cells (Fig. 7), thereby implicating the involvement of Nrf2 binding in GCS gene inducibility.

Since Nrf2 homodimers bind inefficiently to DNA (27, 36, 37), we hypothesized that a second bZip transcription factor, such as a small Maf or Jun protein, heterodimerized with Nrf2 to up-regulate GCS gene expression. Unfortunately, the shift data were somewhat ambiguous, providing some evidence of small Maf and JunD proteins in complexes binding the GCS\textsubscript{h} EpRE4 and GCS\textsubscript{l} EpRE sequences (Fig. 1). As an alternative approach to evaluate the potential involvement of JunD in GCS gene expression, we expressed an AP-1 dominant negative mutant in HepG2 cells (Fig. 4). The significant reduction in GCS\textsubscript{h} and GCS\textsubscript{l} constitutive gene expression observed in cells overexpressing the AP-1 dominant negative mutant in HepG2 cells (Fig. 4). The significant reduction in GCS\textsubscript{h} and GCS\textsubscript{l} constitutive gene expression observed in cells overexpressing the AP-1 dominant negative, AFos, suggested a role for Jun family members in supporting the basal expression of the GCS subunit genes (Fig. 4). These findings agree with earlier studies that established the importance of AP-1-binding sites in the constitutive expression of both the GCS\textsubscript{h} and the GCS\textsubscript{l} promoter/reporter transgenes (9, 45). β-NF induction of the GCS\textsubscript{h} and GCS\textsubscript{l} reporter transgenes was inhibited to 70 and 56%, respectively, in cells transfected with the AFos mutant, suggesting that although induction can occur under these conditions, Jun family members may be required to achieve full
β-NF inducibility. This hypothesis has similarities to that of Venugopal and Jaiswal (28), who proposed that heterodimerization of Jun proteins with Nrfl or Nrfr bind to and mediate expression via EpREs. We further demonstrated that AFOS expression did not influence PDTC induction, implying that Jun proteins were not required for the inducible response to this agent.

The presence of small Maf proteins in GCS EpRE-protein complexes was likewise demonstrated in the supershift experiments (Fig. 1). Both small Maf antibodies used for supershift analyses were raised against MafG but in all likelihood interact with other small Maf proteins with varying degrees of affinity (55). Therefore, determination of whether Nrf2-dependent activation of GCS gene expression requires Maf participation and, if so, which of the small Maf proteins are involved, have yet to be firmly established. Other than the increase in GCS reporter expression in Nrf2-expressing cells observed with the lowest level of MafG co-expression (Fig. 2), we have little direct evidence that small Maf proteins are involved in the positive regulation of the GCS subunit genes. On the other hand, the data are not sufficiently strong to preclude small Maf involvement in transactivation of the two genes.

In addition to modest Maf supershifts detected using the GCS EpRE probes, the temporal relationship between increases in mRNA transcripts corresponding to small Maf factors (Fig. 8) and increases in GCS subunit gene expression following β-NF and PDTC treatment is also consistent with a model in which small Maf proteins play a role in regulating the expression of the GCS subunit genes. Up-regulation of MafG and other small Maf family members precedes that of GCS subunit gene induction by both inducers used in these studies, even though the kinetics of GCS induction are quite distinct for the two. Consequently, following treatment with β-NF or PDTC, there is potentially an increased availability of small Maf factors capable of dimerizing with Nrf2 to effect binding to GCS EpRE sequences, resulting in transcriptional activation. The demonstration that full inducibility of the GCS subunit genes requires new protein synthesis (Fig. 9) is also consistent with this possibility.

The PDTC inducibility of MafG, like that of the GCS subunit genes, presumably involves generation of a pro-oxidant signal since induction can be inhibited by pretreatment of cells with N-acetylcysteine.2 Although there was little prior evidence in the literature supporting the up-regulation of MafG message levels in response to pro-oxidants such as β-NF or PDTC, MafG induction was noted following exposure to the prototypical pro-oxidant H2O2 (56), providing precedence for the oxidative activation of small Maf expression suggested by the current studies.

Whereas small Maf proteins have been shown to heterodimerize with Nrf2 to positively regulate gene expression, transfection of the MafG-expressing plasmid resulted in the inhibition of GCS reporter transgene expression (Fig. 2). It has been demonstrated that varying levels of small Maf proteins will influence the relative abundance of Maf-bZip heterodimers and Maf-Maf homodimers, thereby imparting either positive or negative regulatory influences, respectively, via EpRE/T-MARE sequences. The formation of Maf homodimers might therefore provide a mechanism to explain the seemingly contradictory inhibitory effect of MafG overexpression on GCS expression in Nrf2-expressing transfecants.

It has also been demonstrated that EpRE-regulated transgenes can be down-regulated by Fra1 (26, 57), another bZip factor, which like the small Maf proteins lacks a transactivation domain. Interestingly, Fra1 expression is also increased in response to either β-NF (45) or PDTC (58). It is conceivable that the decrease in GCS message levels, observed at the late time points following β-NF or PDTC induction (Fig. 9), is a consequence of increased expression of either the small Maf or Fra1 proteins and the subsequent binding of small Maf homodimers or Fra1-containing heterodimers to the GCS EpRE sequences. Furthermore, the suppression of GCS gene down-regulation by protein synthesis inhibition suggested in Fig. 9 implicates the involvement of newly synthesized negative regulatory factors in this process, for which small Maf and/or Fra1 proteins are prime candidates.

By using the data presented here and evidence from the literature, we have formulated a working model for GCS gene regulation following exposure to pro-oxidants like β-NF and PDTC (Fig. 10). We hypothesize that exposure of HepG2 cells to β-NF or PDTC results in post-transcriptional activation of Nrf2, affecting its release from Keap1 and resulting in increased nuclear translocation of the transcription factor. Exposure to these agents similarly increases the steady-state messenger RNA levels corresponding to small Maf factor(s) and perhaps other potential Nrf2 heterodimerization partners. Release of Nrf2 and the transcriptional activation of genes encoding small Maf proteins are presumably a consequence of a pro-oxidant signal (7, 54), the latter event being blocked by co-treatment of cells with N-acetylcysteine.2 Once released from Keap1, Nrf2 heterodimerizes with bZip transcription factors, forming a complex that binds GCS EpRE/T-MARE sequences. Binding of the Nrf2-bZip complex to GCS regulatory sequences results in transcriptional activation of GCS gene expression. Potential heterodimerization partners of Nrf2 include small Maf proteins, JunD, or other bZip proteins. In fact, depending on the specific GCS EpRE/T-MARE sequence and on the inducing agent, different combinations of Nrf2-bZip complexes may be responsible for GCS gene induction. According to the working hypothesis, transcriptional activation of the small MAF genes and the progressive accumulation of small Maf proteins or other negative regulatory factors, such as Fra1, alter the balance of Nrf2 and other bZip factors in the nucleus in favor of formation of Maf homodimers or Fra1-bZip heterodimers. Small Maf homodimers or perhaps bZip heterodimers including Fra1 may compete with Nrf2-bZip complexes for binding to

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**FIG. 10.** The working model for GCSα and GCSβ up-regulation (A) and down-regulation (B) in response to the xenobiotics β-NF and PDTC.
GCS EpRE-T-MARE sequences, resulting in the time-dependent down-regulation of GCS-inducible gene expression.

In conclusion, we have demonstrated that Nrf2 is a positive regulator of GCS gene expression. Overexpression of Nrf2 significantly increases GCS, and GCS reporter transgene expression. Increased Nrf2 binding to wild-type GCS EpRE regulatory sequences was observed following exposure of HepG2 cells to β-NF or PDTC. The binding of Nrf2 to GCS EpRE sequences was reduced by overexpression of a MafK dominant negative mutant. Expression of this mutant also eliminated GCS reporter transgene activation by β-NF and PDTC and reduced the endogenous GCS mRNA induction. Preliminary studies presented in this paper suggest that both small Maf protein(s) and JunD may constitute Nrf2 binding partners, but definition of the specific Nrf2 heterodimerization partner(s) important for GCS gene induction requires further investigation.

Acknowledgments—The work presented in this manuscript was dependent on reagents generously provided by a number of investigators. The human Nrf2 cDNA was obtained from Dr. Etsuro Ito (Hiroshima University). The human MafG cDNA was provided by Dr. Nancy Andrews (Harvard University). The Andrews’ laboratory also supplied a MafG antibody, prepared by Dr. Volker Blank (51). The second MafG antibody was provided by Dr. Kazuhiko Igarashi (Tohoku University). We utilized two dominant negative mutants, the mouse NF-E2 p18 (MafK) dominant negative mutant from Dr. Stuart Orkin’s laboratory and an MafG antibody, prepared by Dr. Volker Blank (51). The second MafG antibody was made dependent on reagents generously provided by a number of investigators.

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