The CNC Basic Leucine Zipper Factor, Nrf1, Is Essential for Cell Survival in Response to Oxidative Stress-inducing Agents

ROLE FOR Nrf1 IN \( \gamma \text{gcsL} \) AND gss EXPRESSION IN MOUSE FIBROBLASTS*

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Nrf1 is a member of the CNC-basic leucine zipper (CNC-bZIP) family of transcription factors. CNC bZIP factors, together with small Maf proteins, bind as heterodimers to the NF-E2/AP-1 element. Similarity between the NF-E2/AP-1 element and the antioxidant response element identified in a number of promoters of genes involved in detoxification and antioxidant response raises the possibility that Nrf1 plays a role in mediating the antioxidant response element response. In this study, we exploited the availability of cells from Nrf1 knockout mice to study the role of Nrf1 transcription factor in the regulation of antioxidant gene expression and in cellular antioxidant response. Fibroblast cells derived from Nrf1 null embryos showed lower levels of glutathione and enhanced sensitivity to the toxic effects of oxidant compounds. Our results indicate that Nrf1 plays a role in the regulation of genes involved in glutathione synthesis and suggest a basis for a correspondingly low GSH concentration and reduced stress response.

Oxidants/reactive oxygen species (ROS)³ are by-products of normal metabolic reactions and environmental sources (1, 2). A major source of ROS comes from cellular respiration when molecular oxygen is reduced to water in the mitochondria, from cellular metabolism of fatty acids, and from the respiratory burst pathway exploited by neutrophils and macrophages in the inflammatory response. Exogenous sources of ROS include charity radiation, environmental pollutants, and chemotherapeutic agents. ROS are short-lived and have diverse effects in the cell (1, 2). On the one hand, they serve as key factors in the defense against bacterial infections, can stimulate cell proliferation, and serve as second messengers (3). On the other hand, elevated levels of ROS are highly damaging as they react readily with many cellular components resulting in damage to DNA, lipids, and proteins.

Because of these damaging effects, ROS have been implicated in a wide variety of disease states including cancer, neurodegenerative disease, ischemia-reperfusion injury, atherosclerosis, aging, and immune complex-mediated diseases (4–6).

Cells have evolved a battery of defense mechanisms to protect themselves against damage induced by ROS. Maintenance of intracellular redox balance and metabolism of exogenous toxins are mediated in part by thiol-rich molecules such as glutathione and metallothioneins, and detoxifying enzymes such as NAD(P)H:quinoxidoreductases, glutathione S-transferases, glutathione peroxidases, heme oxygenase, and UDP-glucuronosyltransferases. Essential to constitutive and induced expression of a number of genes that participate in the protection against oxidative stress is the antioxidant response element (ARE)⁴(7). The ARE, also referred to as the electrophile response element, is found in the promoters of a number of these genes (8–11). The ARE has been shown to bind a number of different transcription factors including basic leucine zipper proteins, AP-1, and other novel factors whose identities have yet to be identified (7, 12).

Basic leucine zipper (bZIP) transcription factors play important roles in growth and differentiation. There are several distinct subgroups of bZIP proteins, namely the AP-1, ATF/CREB, Maf, and CNC-basic leucine zipper families (13–15). The CNC-basic leucine zipper (CNC-bZIP) family was identified from independent searches for factors that bind the tandem NF-E2/AP-1 like cis-elements in the \( \beta \)-globin locus control region. Members in this family include p45-NF-E2, Nrf1 (LCRF1, TCF11), Nrf2, ECH, Bach1, and -2 (16–23). Although homologies among the different bZIP proteins are apparent in the basic and leucine zipper regions, the similarity between CNC-bZIP proteins is most remarkable in the basic-DNA binding region, suggestive that they bind the NF2AP-1-like recognition site with similar affinities (14). In addition, a stretch spanning 43 amino acids immediately N-terminal to the basic domain is highly conserved especially between Nrf1, Nrf2, and p45NF-E2. This region is also highly conserved in the Drosophila CNC protein and in the Caenorhabditis elegans Skn protein, but it is not present in Jun, Fos, or other bZIP proteins. This region has been referred to as the “CNC” domain. Whereas CNC-bZIP proteins have been shown to dimerize with the small Maf family of bZIP proteins, it is not known whether they indeed function as obligate heterodimers in vivo (13, 24–27).

The human Nrf1 gene was isolated as a result of our efforts to clone cDNAs, using a yeast expression system, that encode proteins that bind the tandem NF-E2/AP-1 like cis-elements in the \( \beta \)-globin locus control region (18). Nrf1 is widely expressed with high levels of the transcript found in liver, lung, heart, muscle, and kidney. To determine its physiologic role, we have used gene targeting in embryonic stem cells to generate mice with disruption in the \( nrf1 \) gene (28). \( nrf1 \) is an essential gene
as homozygous disruption of it results in death in utero (28, 29). Homozygous mutant embryos have decreased definitive nucleated red cells, and as a result, these mice suffer from anemia and die in utero (28). The red cell defect, however, does not appear to be cell-autonomous as Nrf1−/− embryonic stem cells contribute efficiently to blood formation in chimeric animals (28). Whether Nrf1 regulates the expression of cytokine and/or other extracellular factors that play a role in erythroid maturation or red cell survival remains to be determined.

The NF-E2/AP-1 element, also referred to as the Maf recognition element, has been shown to be similar to the antioxidant response element (ARE) (30). Based on the similarity of the NF/E2/AP-1 and the ARE sequence, one potential role of CNC-bZIP proteins is in the regulation of antioxidant expression. Whereas regulation of basal and induced expression of antioxidant genes are mediated in part by the ARE sequence, the identity of the ARE-binding protein(s) remains unclear. Members of the CNC-bZIP family and small Maf family of proteins have been implicated recently in mediating ARE function. By transfection experiments, Nrf1 and Nrf2 have been shown to up-regulate the human NQO1 gene promoter in HepG2 liver cells (30). Whereas Nrf2 knockout mice are viable (31–33), they were found to be impaired in the induction of glutathione S-transferase and human NQO1 gene expression (32). Thus, the Nrf2 knockout data provide further genetic evidence for a role of CNC-bZIP proteins in the regulation of ARE-controlled genes.

As Nrf1 and Nrf2 share striking similarities in their DNA-binding domains as well as in their expression patterns, we hypothesize that Nrf1 also plays a role in the regulation of antioxidant gene expression. In this study, we examined this proposed function using mouse fibroblasts derived from Nrf1 null mutant embryos. We found that Nrf1−/− fibroblasts have decreased glutathione levels and are hypersensitive to the toxic effects of oxidants. Glutamylycisteine-light chain synthetase and glutathione synthetase, genes in the GSH biosynthetic pathway, are down-regulated in Nrf1-deficient fibroblasts. The identification of glutamylycisteine light chain synthetase and glutathione synthetase as downstream targets provides important clues to the role of Nrf1 in cellular function and potential relevance to disease states.

**EXPERIMENTAL PROCEDURES**

**Cytotoxicity and Terminal Deoxynucleotidyltransferase-mediated dUTP Nick-end Labeling Assays—**Isolation of mouse fibroblast from embryos was done using standard protocols (34). Mutant embryos were derived from matings of Nrf1−/− mice described previously (28) and from another knockout line bearing the gcsL promoter (28, 29).

Cytotoxic effects of various compounds were determined by either trypan blue dye exclusion or using a microtiter assay for cell survival. For cell survival determination, approximately 1–5 × 105 cells were trypsinized to generate a single cell suspension and preloaded with 1 × PBS with 2% fetal calf serum (FCS). Cells then were treated with 80 μM paraquat. After 4 h of incubation, propidium iodide was added to a final concentration of 1 μg/ml prior to analysis by flow cytometry. The oxidative conversion of DCFHDA to its fluorescent analog was assessed by the amount of fluorescent signal in live cells by flow cytometry.

Detection of intracellular glutathione levels was done using GSH assay kit from Oxis International or according to the method described by Griffith (35) from supernatant of approximately 1–2 × 107 cells.

**Plasmids, DNA Transfections, and Luciferase Assays—**A fragment of 834 base pairs corresponding to −1096 to −253 nucleotides (36) of the human GCS light chain synthetase gene promoter was amplified using Pfu polymerase and then cloned into PCRBior (Invitrogen). The promoter fragment was then directionally cloned into the KpnI and XhoI sites of pG535 Basic (Promega) resulting in pG535-Luc. Transfection of Nrf1 expression plasmid was constructed by cloning a 2.2-kilobase pair KpnI/XhoI fragment containing the human Nrf1 cDNA into pEF1a-V5 vector (Invitrogen).

Mouse embryonic fibroblast cells were plated at 50% confluency a day prior to transfection. For cotransfections, equal amounts of the luciferase reporter plasmid and pEF1α-Nrf1 expression plasmid were transfected by LipofectAMINE (Life Technologies, Inc.) according to manufacturer’s protocol. Following transfections, cells were incubated at 37 °C for 48–72 h prior to harvest for luciferase assays. Lysates were prepared according to manufacturer’s protocol and assayed for luciferase activity using the Dual Reporter system (Promega). Activity obtained from each sample was normalized for transfection efficiency by measuring Renilla luciferase activity derived from the pRL-TK plasmid included as internal control in the transfection and protein concentration by the Bradford method (Bio-Rad).

**RT-PCR and Northern Blot Analysis—**RNA extractions were carried out using Ultraspec RNA extraction solution (Biotex). For reverse-transcribed-polymerase chain reaction (RT-PCR), first-strand cDNA was synthesized using random hexamer primers according to manufacturer (Amersham Pharmacia Biotech). PCR were amplified in 10 μl Tris-HCl, pH 8.3, 50 μl KCl, 1.5 μl MgCl2, 0.2 μl dNTP, 0.1 μCi of [α-32P]dCTP (300 Ci/mmol; Amersham Pharmacia Biotech), 10 pmol of each of the primers, and 2.5 units of AmpliTaq polymerase (Perkin-Elmer). PCR primer sequences are as follows: actin, 5′-ATCT- GGCGACACACTTTCAATAGAGCCTG-3′, and 5′-CTGCACTACATCTTCCG-3′; human MAF (25) were done using TnT reticulocyte lysate system from Promega as described previously (28). Binding reactions were carried out in a mixture containing extracts, 32P-labeled double-stranded oligonucleotide probes corresponding to the antioxidant response element of the mouse GCS promoter (26) (wild-type promoter) and GCTATTGTCACTGGATTTGGTGATCCCTCC, 20 μmol Hepes-KOH, pH 7.9, 1 μmol EDTA, 20 μmol KCl, 5 μmol DTT, 4 μmol MgCl2, 1 μg poly(dI-dC), 4% glycerol. Mixtures were incubated at 37 °C for 20 min, and the DNA-protein complexes were resolved on non-denaturing 4% acrylamide gels. For supershift experiments, antibodies were incubated with the extracts for 10 min at 37 °C prior to addition of ARE probe to the binding reaction mix. Antibodies to Nrf1,...
Nrf2, p45-NF-E2, and p18-NF-E2 were purchased from Santa Cruz Biotechnology.

Cell extracts from mouse Hepa1–6 cells were prepared using the protocol described by Jackson (37). Briefly, cells were washed twice with 1× PBS prior to harvest by scraping. Cells were pelleted by a brief centrifugation and lysed by Dounce homogenization in a hypotonic buffer (10 mM Hepes-KOH, pH 7.6, 10 mM KCl, 1.5 mM MgCl₂, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride). Nuclei were collected by centrifugation, followed by extraction in a high salt buffer (20 mM Hepes-KOH, pH 7.6, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 6.25% glycerol). The cytoplasmic fraction was adjusted with 10% volume of buffer containing 300 mM Hepes-KOH, pH 7.6, 1.4 M KCl, 30 mM MgCl₂, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride.

RESULTS

Nrf1 in gcsL Promoter Function and Oxidative Stress

![Graph A](image1)

**Fig. 1. Enhanced sensitivity of Nrf1−/− cells to oxidants.** Representative survival experiments of fibroblasts treated with oxidants paraquat (A) and cadmium chloride (B) are shown. Black bars represent Nrf1−/− cells, and open bars represent Nrf1+/− cells. Each data point represents average values ± S.D. sampled at least 4 times. Viabilities of untreated cells were set as 100%. Mean values were analyzed using Student’s unpaired t test. p values were <0.001.

Nrf1−/− Fibroblasts Are Sensitive to the Toxic Effects of Oxidant Compounds—The survival of MEF in different concentrations of oxidants was tested to examine whether a deficiency in Nrf1 would result in increased sensitivity of cells to oxidative stress. Fibroblast cells derived from wild type and Nrf1 null embryos were cultured in the redox cycling herbicide paraquat.
A dose-dependent loss of cell viability was seen in both wild type and Nrf1−/− cells examined. However, wild type MEF cells were less sensitive to the toxic effects of paraquat compared with Nrf1−/− cells (Fig. 1A). The concentration of paraquat required for 50% killing of +/+ and −/− cells were 0.6 and 0.2 mM, respectively. We also observed a considerable alteration in morphology of Nrf1−/− cells exposed to paraquat. Whereas wild type cells remained flat and adherent, mutant cells showed rounding and numerous cell-surface blebbings (data not shown) in addition to increased cell death (Fig. 2A). To determine whether the cells are undergoing apoptosis, terminal transferase assays to detect nuclear DNA fragmentation were done. The number of fluorescent labeled cells before and after paraquat treatment was not appreciably increased in wild type MEF cells (Fig. 2A). In contrast, the percentage of labeled cells increased considerably in paraquat-treated Nrf1−/− MEF cells. Labeled cells were detected at even 0.6 mM concentration of paraquat tested. Nrf1 null cells were also tested with other oxidant compounds to determine whether enhanced toxicity was restricted only to paraquat. An increased sensitivity was also seen when Nrf1 null cells were treated with cadmium chloride (Fig. 1B), and the decrease in viability was very pronounced with diamide treatment (Fig. 2B). These results demonstrate that Nrf1 null cells are more susceptible to various compounds known to cause oxidative stress and that cell death appears to occur via apoptosis.

Nrf1-deficient Cells Show Elevated Levels of Free Radicals—Because cytotoxicity of paraquat is known to be associated with redox cycling and superoxide generation (36), it seemed reasonable to determine whether Nrf1−/− MEF cells accumulate higher levels of free radicals. To test this idea, intracellular oxidation of the reporter DCFHDA fluorescent dye was measured by flow cytometry. Basal levels of fluorescent DCFHDA formation in Nrf1−/− MEF cells were similar to that seen in wild type control cells (Fig. 3). This suggests that Nrf1−/− MEF cells were not under increased free radical burden compared with wild type cells under normal conditions. With paraquat treatment, there was approximately a 3–4-fold increase in fluorescence detected in Nrf1−/− MEF cells. In contrast, no increase in fluorescence was detected in wild type control cells. This result indicates that wild type cells were able to cope with the increased intracellular oxidative burden as a result of superoxide generation from paraquat treatment, whereas Nrf1−/− MEF cells were diminished in this capacity.

GSH Levels and Transcripts Encoding GSH Biosynthetic Genes Are Decreased in Nrf1−/− Fibroblasts—Glutathione (GSH) is a major redox molecule found at high concentrations in all cells. GSH has numerous functions, an important one is to protect cells against ROS by means of its nucleophilic and reducing capacity. A decrease in GSH levels could result in a diminished reserve in intracellular reducing capacity resulting in an enhanced sensitivity to oxidants. Indeed, it has been shown that drug-induced GSH depletion in mice results in enhanced paraquat toxicity (39). To test whether Nrf1 deficiency has an effect on glutathione levels, GSH contents of wild type and Nrf1−/− cells were measured. GSH levels of Nrf1−/− cells were approximately half of control wild type cells (Table 1), whereas oxidized GSH (GSSG) levels were not found to be different between control wild type cells and Nrf1−/− cells (data not shown). These findings imply that lowered GSH levels in Nrf1−/− cells were not from increased GSSG formation as a result of GSH oxidation under conditions of oxidative stress. One possibility for lowered GSH levels is that the synthesis of GSH is diminished in Nrf1−/− cells.

The formation of GSH occurs by a two-step process catalyzed by the sequential action of γ-glutamylcysteine synthetase (GCS) and glutathione synthetase (GSS). The GCS holoenzyme is composed of two subunits as follows: a light chain, designated GCSL, bears a regulatory function, and a heavy chain, designated GCSH, is responsible for the catalytic function. As antioxidant response elements have been described in the promoter regions of both the human GCS heavy and light chain genes, we examined the expression of these genes in wild type and Nrf1−/− fibroblasts. By RT-PCR analysis, the gcs light chain gene transcript showed a 3–4-fold reduction in Nrf1−/− fibroblasts. This result indicates that wild type cells were able to cope with the increased intracellular oxidative burden as a result of superoxide generation from paraquat treatment, whereas Nrf1−/− cells were diminished in this capacity.
shown. Percent of control are in parentheses.

fected in Nrf1 mutant fibroblasts. Although exposure of MEF were used as loading controls.

termined whether induced levels of these genes are also af-

2 cycles. Amplification of GCSL, GSS, and GCSH was am-

3 fold in Nrf1

2 fibroblasts. Levels of

t İstanbul 18, and 21 cycles.

2 cycles. Actin and ARPO were amplified for 15, 18, and 21 cycles.

3rd lanes

1st lane

2nd lane

4th lane

5th lane

A double-stranded oligonucleotide probe with a mutated ARE

indicates the Nrf1

MAF complex; double arrows indicate supershifted complexes; bracket indicates p45NFE2-MAF complexes, and asterisks indicates nonspecific complex.

fibroblasts compared with wild type fibroblasts (Fig. 4A). Similar results were obtained by Northern blot analysis (Fig. 4B). In contrast, no significant difference in expression of the heavy chain gcs gene was detected between wild type and Nrf1

fibroblasts (Fig. 4A and B). As expression of human GCSH and GCSL genes has been shown to be inducible after treatment with oxidants such as β-naphthoflavone (36, 40), we next determined whether induced levels of these genes are also affected in Nrf1 mutant fibroblasts. Although exposure of MEF cells to paraquat resulted in an increase of gcsL gene transcript levels, expression levels were reduced in Nrf1

fibroblasts compared with wild type cells treated with paraquat (Fig. 4, A and B). Thus, the absence of Nrf1 did not abolish induction of gcsL gene expression. Levels of gss gene transcript were also examined and were found to be down-regulated approximately 3-fold in Nrf1

cells (Fig. 4A). Induction of GSH synthetase gene expression was blunted but not abolished in Nrf1

cells treated with paraquat (data not shown). Whether ARE-like elements play a role in the expression of gss is currently not known. Whereas the changes in expression levels for gcsL and gss are modest, these results were consistently reproducible and have been confirmed in another lot of independently derived Nrf1

fibroblasts.

Nrf1 Binds the ARE in the GCSL Gene Promoter—To test whether Nrf1 binds the putative ARE in the human GCSL promoter, gel-shift assays were done. Nrf1 and human MAF proteins derived from rabbit reticulocyte lysate programmed with Nrf1, and human MAF expression plasmids were tested for binding to double-stranded oligonucleotide probes corresponding to the ARE element of the human GCSL promoter. Whereas binding was observed using rabbit reticulocyte lysate programmed with Nrf1 expression plasmid alone, an increase in binding was observed using lysates programmed with both Nrf1 and human MAF expression plasmids (Fig. 5A, compare 2nd and 3rd lanes). Binding in the presence of anti-Nrf1, as well as anti-Maf (mouse p18NFE2) antibodies, resulted in partial loss of the slow complex (Fig. 5B, compare 2nd and 3rd lanes with 1st lane), and no loss was seen when antibodies against Nrf2 and p45-NF-E2 were used (Fig. 5B, 3rd and 4th lanes). Hence, we conclude that the slow migrating band most likely represents a complex made up of Nrf1 and Maf proteins. A double-stranded oligonucleotide probe with a mutated ARE showed decreased binding (Fig. 5C, compare 1st and 2nd lanes), thus demonstrating binding specificity to the ARE sequence. Gel-shift assays using extracts from wild type and Nrf1 mutant mouse embryonic fibroblasts did not demonstrate with clarity conclusive differences between wild type and mutant extracts due to formation of numerous diffuse complexes (data not shown). Thus, to determine whether the binding data using in vitro derived peptides have any relevance in vivo, we used extracts derived from a mouse liver cell line. As shown in Fig. 5D, a similar slow migrating complex was detected that was largely abolished by anti-Nrf1 antibody (compare 1st and 2nd lanes).
Interestingly, two additional faster migrating bands were also detected in lysates programmed with a combination of Nrf1 and human MAF plasmids (Fig. 5A, 3rd lane), as well as human MAF plasmid alone (data not shown). Both of these complexes were also specific to the ARE sequence as they bind less efficiently to the mutated ARE probe (Fig. 5C, compare 1st and 2nd lanes). Binding in the presence of anti-p45NF-E2 antibody supershifted the two fast complexes (Fig. 5B, 4th lane). Thus, the data suggest that these complexes contain endogenous p45-NF-E2 and in vitro derived human MAF-G proteins. However, binding in the presence of anti-p18NF-E2 antibody did not abolish the two fast complexes (Fig. 5B, 5th lane). Presumably, there is no cross-reactivity of this antibody, which is directed against the mouse Maf-K protein, to the human MAF-G protein. The basis for two p45-NF-E2/MAF bands is currently not clear.

**Nrf1 Regulates Expression of GSH Biosynthetic Genes**—We then tested an 834-bp insert of the human GCSL gene promoter containing a putative ARE to drive luciferase reporter gene expression in mouse embryonic fibroblasts derived from wild type or Nrf1 mutant embryos. If our hypothesis is correct, expression of the reporter should be significantly reduced in Nrf1−/− cells. Indeed, expression of the 834-bp GCSL promoter reporter gene was approximately 3-fold higher in wild type cells compared with expression in Nrf1−/− cells (Fig. 6). Co-transfection with human Nrf1 expression vectors enhanced luciferase expression from the GCSL gene promoter-reporter plasmid by approximately 2-fold in wild type cells. More importantly, cotransfection of Nrf1 expression plasmid into Nrf1−/− cells resulted in expression of the reporter plasmid that was equivalent in magnitude to that in wild type cells (Fig. 6).

To corroborate our findings with reporter gene constructs, we examined whether diminished expression of endogenous gcsL and gss genes can be rescued by ectopically expressed Nrf1. The human Nrf1 cDNA was introduced back into Nrf1−/− cells by transient transfection. Nrf1−/− cells transfected with Nrf1 expression plasmid showed an increase in gcsL and gss gene transcripts by RT-PCR compared with cells transfected with control plasmid (Fig. 7). Thus, reconstitution of Nrf1 function restores higher expression levels of endogenous gcsL and gss genes. These findings correlate well with the differential reporter plasmid expression observed in wild type and Nrf1−/− cells. Hence, the data taken together support the idea that Nrf1 is important for expression of gcsL and gss genes.

**DISCUSSION**

Nrf1 is a widely expressed CNC-bZIP factor and together with small MAF proteins bind as heterodimers to the NF-E2/AP-1 site (18). We have previously shown that nrf1 is an essential gene in mice, the targeted disruption of which results in embryonic lethality at around mid-gestation period (28). Because of its ability to activate gene expression through the NF-E2/AP-1 site, Nrf1 has been implicated in regulating globin gene expression (18). However, this role has not borne out. Whereas mutant animals suffer from anemia, the cause appears to be from defective erythropoiesis and not globin expression (28). The extent in which anemia contributes to lethality is unclear, and target genes for Nrf1 have not previously been identified yet.
Glutathione is an important molecule found at high concentrations inside cells. It protects against damage by direct scavenging of free radicals that are made by cells during normal and xenobiotic metabolism, and it also functions as an important substrate in enzyme-catalyzed detoxification reactions (41, 42). GSH synthesis is catalyzed by the sequential action of two cytoplasmic enzymes, GCS and GSS (41, 42). GCS is composed of a heavy and a light chain subunit, and GSS is a monomeric protein. How GCS and GSS levels are regulated and how they in turn regulate intracellular GSH levels are not yet understood. Studies on regulation of GCS \(_L\) and GCS \(_H\) gene expression have revealed putative antioxidant response elements in 5′-flanking sequences of these genes (36, 43, 44). The ARE has been shown to be important in basal and induced expression of a number of antioxidant genes (7). Whereas the ARE sequence has been studied in detail and has been found to be very similar to the NF-2/2/AP-1-binding site, the nature of the ARE-binding proteins is not clear. In the GCS heavy chain gene, a distal ARE located 3.1 kilobase pairs upstream of the transcriptional start site is required for both constitutive and \(\beta\)-naphthoflavone-induced expression (40). Similarly, an ARE sequence located approximately 300 bp upstream from the transcriptional start site of the GCS \(_L\) gene is required for expression (36). Information regarding gss regulation is lacking currently.

In this study, we found that disruption of the \(nrf1\) gene results in hypersensitivity of fibroblasts to the toxic effects of various oxidizing agents. \(nrf1^{-/-}\) cells have lower levels of GSH, and we found that both basal and induced expression of gcs\(_L\) and gss are reduced correspondingly in \(nrf1\) mutant cells. We have shown that \(nrf1\) binds to the ARE found in the GCS \(_L\) gene promoter and that maximal gcs\(_L\) gene promoter function in MEF cells requires \(nrf1\). More importantly, cDNA rescue experiment clearly implicates gcs\(_L\) and gss as downstream targets for \(nrf1\) regulation. Because of the importance of GSH in detoxification of reactive oxygen species and maintenance of cellular redox balance, it is reasonable to assume that one underlying cause of enhanced sensitivity is reduced GSH levels in \(nrf1\)-deficient cells. The importance of \(de\ novo\) GSH synthesis in maintaining intracellular GSH balance has been demonstrated in experimental GSH deficiency from inhibition of GCS activity by buthionine-sulfoximine treatment (45, 46). Although we cannot rule out increased turnover or transport of GSH in \(nrf1\) mutant cells, we nevertheless believe that reduced synthesis contributes to the low GSH level. Whereas expression of gcs\(_L\) did not appear to be significantly affected in \(nrf1\)-deficient cells, the synthesis of GSH would, however, still be affected with decrease levels of gcs\(_L\) chain expression. In the absence of GCS \(_L\) subunit, it has been shown that the catalytic subunit has a much lower affinity for its substrate, glutamate, and has an increased sensitivity to feedback inhibition from GSH (47). The heavy chain alone then would be less active under physiologic conditions and points to the importance of the light chain in GSH synthesis. As GSH is not taken up efficiently by the cell from the surrounding medium, a decrease in synthesis would eventually lead to its depletion (41, 45, 46).

It is interesting that mutation of the electrophile response element (ARE) core failed to alter basal expression from the GCS \(_L\) gene promoter in HepG2 cells (36). It is not known whether this is a reflection of differences between human HepG2 cells and mouse embryonic fibroblasts. The fact that basal and induced expression of gcs\(_L\) and gss were not completely abolished suggests that other factors also contribute to the control of expression of these genes. In agreement with our results, analyses of the GCS \(_L\) gene promoter showed that other cis-active elements besides the ARE (electrophile response element) contribute to induced expression (36, 48). In addition to the ARE, a number of AP1 sites are also present in the GCS \(_L\) gene promoter, and the AP-1 site located 33 bp upstream from the ARE is also important for both basal and induced expression of the gcs\(_L\) gene promoter.

Because previous studies indicated that a distal ARE is essential for both basal and \(\beta\)-naphthoflavone-induced expression of human GCS \(_H\) promoter-reporter constructs (40), it is interesting that endogenous gcs\(_H\) expression was not noticeably down-regulated in \(nrf1\)-deficient cells. It is possible that the regulation of the gcs\(_H\) gene is different between mouse and human. It is also possible that ARE of the mouse gcs\(_H\) promoter does not represent a real target for \(nrf1\). Alternatively, binding by other factors may substitute for \(nrf1\) function. The findings that ARE-responsive genes, such as nqo1 and glutathione S-transferase, are down-regulated in \(nrf2\) knockout mice would make a \(nrf2\) likely candidate (32). The overlapping expression patterns of \(nrf1\) and \(nrf2\) suggest that these factors are partially redundant. The extent to which \(nrf2\) contributes to the regulation of gcs\(_H\), gcs\(_L\), and gss via the ARE is currently being examined. Moreover, binding by bZIP proteins other than CNC-bZIP factors to the ARE is also possible and therefore needs testing. Clearly then, the mere presence of an ARE does not necessarily constitute a binding site for \(nrf1\) or \(nrf2\). It is therefore important to evaluate potential target genes in \(nrf1\) null animals. Other putative binding sites present in the 5′-flanking sequence of the human GCS \(_L\) gene include AP-2, MRE, and Sp-1. The zinc finger containing factor, MTF-1, transactivates genes through the MRE. MTF-1 appears to play a role in oxidative stress response by regulating expression of metallothionein genes, and recent knockout experiments in mice showed that MTF-1 regulates expression of the gcs\(_H\) gene (49). Interestingly, gcs\(_L\) and gss gene expression in the MTF-1 knockout mice was not affected. Finally, the AP-2 transcription factor has also been recognized to play a role in stress response in mammalian cells (50, 51).

Based on our findings, we proposed that \(nrf1\) plays a role in the regulation of glutathione synthesis via gcs\(_L\) and gss gene expression. In the absence of \(nrf1\), cellular GSH levels are reduced resulting in enhanced sensitivity to oxidants. Whereas the difference in sensitivity was not dramatic compared with wild type cells, we believe that the heightened response has physiologic significance. Human conditions associated with mutations in \(nrf1\) are not known, but it is notable that inherited disorders affecting enzymes in GSH metabolism have been described (41, 42). Most patients probably represent leaky mutations as absolute deficiencies, and this would not be expected to be compatible with life. In generalized GSS deficiency, patients have metabolic acidosis, 5-oxoprolinuria, and hemolytic anemia. These findings become manifest in the first few days of life and some eventually develop neurologic defects. Patients with GCS deficiency exhibit hemolytic anemia and central nervous system degeneration. It is interesting that hemolytic anemia is a common finding in these deficiencies. Presumably, the failure to maintain GSH levels compromises the membrane integrity and leads to red blood cell destruction. Whether hemolysis plays a role in the anemia phenotype in \(nrf1\) knockout mice is currently being tested.

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