Nrf2 Transcriptionally Activates the mafG Gene through an Antioxidant Response Element*

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Nrf2 accumulates in nuclei upon exposure to oxidative stress, heterodimerizes with a small Maf protein, and activates the transcription of stress target genes through antioxidant response elements (AREs). We found that diethyl maleate (DEM), a well known activator of Nrf2, induces one of the small Maf genes, mafG. To elucidate roles MafG might play in the oxidative stress response, we examined transcriptional regulation of the mouse mafG gene. MafG utilizes three independent first exons that are each spliced to second and third coding exons. Among the small maf genes, mafG showed the strongest response to DEM, and of the three first exons, the highest -fold induction was seen with the proximal first exon (Ic). Importantly, one ARE (Ic-ARE) is conserved in the promoter flanking exon 1c of the human and mouse mafG genes. The Nrf2/MafG heterodimer bound the Ic-ARE and activated transcription, whereas DEM failed to activate mafG in nrf2-null mutant cells. Chromatin immunoprecipitation further revealed that both Nrf2 and small Maf proteins associate with the Ic-ARE in vivo. These results demonstrate that mafG is itself an ARE-dependent gene that is regulated by an Nrf2/small Maf heterodimer and suggest the presence of an autoregulatory feedback pathway for mafG transcriptional regulation.

Cells have the ability to adapt to oxidative stress or to exposure to xenobiotics. By inducing a battery of antioxidant and xenobiotic metabolizing enzymes, such as glutathione S-transferase, heme oxygenase-1 (HO-1), and NAD(P)H:quinone oxidoreductase 1 (NQO1), cells protect themselves from oxidative stress and xenobiotics (1, 2). It has long been known that the genes encoding these enzymes are often coordinately regulated through AREs in their gene-regulatory regions (3). The ARE is a Maf (musculo-aponeurotic fibrosarcoma) recognition element (MARE)-related sequence, with strong similarity to the MARE (3) (see Fig. 4A). Basic region-leucine zipper transcription factors, including CNC (C/EBPα/Collar protein) family (Nrf1, Nrf2, Nrf3, and p45 NF-E2) and Bach (BTB and CNC homology) proteins (Bach1 and Bach2), form heterodimeric partners with a small Maf protein (MafG, MafK, and MafF), and then associate with various MARE-related sequences (including AREs).

CNC and Bach proteins possess transcriptional activation and repression domains, respectively. Therefore, ARE-mediated regulation is either positively or negatively regulated, depending on which basic region-leucine zipper factors bind to the AREs. Whereas small Maf proteins lack any recognizable transcriptional effector domains (other than the basic region-leucine zipper motif), they are nonetheless regarded as critical regulators of ARE-mediated transcription, since CNC and Bach proteins require them as obligatory partners for site-specific association with the ARE. Moreover, it is well documented that homodimers and heterodimers composed exclusively of small Maf proteins can act as repressive competitors to small Maf/CNC and small Maf/Bach heterodimers. One implication of these data is that small Maf proteins can participate in both positive and negative regulation of MARE-dependent genes. Additionally, the amount of small Maf proteins could be an important determinant of the transcriptional activity of target genes (for reviews, see Refs. 4 and 5).

The positive contributions of CNC proteins and the negative contributions of Bach1 to ARE-mediated transcription have been validated by gene targeting strategies. ARE-dependent gene induction was greatly impaired in nrf2-null mutant mice (6), and Nrf1 is also reported to contribute to the activation of ARE-dependent genes (7, 8). The ARE-dependent HO-1 gene was significantly derepressed in bach1-null mutant mice, suggesting that HO-1 is negatively regulated by Bach1 (9). Although nrf3-null and bach2-null mutant mice have been reported (10, 11), the contribution of these factors to ARE-mediated regulation is still to be determined.

In comparison with the cnc and bach family-targeted mutant mice, the phenotypes of small maf mutant mice were expected to be more complicated, since small Maf proteins can participate in both positive and negative regulation. The functions of small Maf proteins are compensatory and provide partially overlapping redundancy, since small maf single mutant mice (mafG-, mafK-, and mafF-) showed mild or subtle phenotypes (12–14), whereas small maf compound mutant mice (mafG-mafF- and mafG-mafK-) displayed a greater number of and more profound deficiencies (15, 16).

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‖ The abbreviations used are: HO-1, heme oxygenase-1; ARE, antioxidant response element; ChIP, chromatin immunoprecipitation; DEM, diethyl maleate; EMDS, electrophoretic mobility shift assay; LDH, lactate dehydrogenase; MARE, maf recognition element; MEF, mouse embryonic fibroblast; NQO1, NAD(P)H:quinone oxidoreductase 1; PRDX1, peroxiredoxin 1; RACE, rapid amplification of cDNA ends; RT, reverse transcription.

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We recently observed that a fraction of ARE-dependent genes (e.g. NQO1 and thioredoxin reductase 1) are not normally induced in either small maf compound mutant mice or in nrf2-null mutant mice. Therefore, we concluded that small Maf proteins act cooperatively with Nrf2 to activate the transcription of these genes. In contrast, HO-1 transcription is depressed in small maf compound mutant mice, similarly to the bach1-null mutant (16), suggesting that small Maf proteins are also important for collaboratively repressing HO-1. In this way, we genetically confirmed that small Maf proteins act as required cofactors in both positive and negative MARE-dependent regulation of gene transcription.

In the course of previous gene expression analyses, we found that diethyl maleate (DEM), a well known inducer of Nrf2/ARE-dependent genes, induced the expression of mafG. This observation suggested that mafG is an Nrf2/ARE-dependent gene. However, the mechanisms that regulate mafG transcription are only poorly understood. Here, we show that a functional promoter ARE controls mafG induction. By electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) analyses, we demonstrate that Nrf2 and small Maf proteins bind specifically to the mafG ARE. We also confirm that induction of mafG is severely impaired in nrf2-null mutant cells. Thus, mafG is indeed an Nrf2/ARE-dependent gene.

**EXPERIMENTAL PROCEDURES**

*Animals*—Germ line mutagenesis of mouse nrf2 was described previously (6). The mice used in this study were on a mixed genetic background of 129SvJ and ICR.

*Mouse Embryonic Fibroblasts (MEF)—*MEFs were prepared from individual embryos at embryonic day 13.5. The head and internal organs were removed, and the torso was minced and dispersed in 0.25% trypsin/EDTA. MEFs were maintained in Dulbecco's modified Eagle's medium (Sigma) containing 10% fetal bovine serum and antibiotics. To induce ARE-dependent genes, MEFs were treated with 50 μM DEM (Wako Pure Chemicals, Osaka, Japan) for 12 h.

*Quantitative RT-PCR—*Total RNA was prepared from mouse liver or from mouse embryonic fibroblasts using Isogen (Nippon Gene, Japan) following the recommended protocol. Random cDNA was synthesized from the isolated RNAs, and real-time PCR (ABI PRISM 7700) was performed to examine the expressions of mafG and Nrf2 mRNAs. The expression of each ARE-dependent gene at 0 h was set to 1. The error bars represent the S.D. values (n = 3). B, RNA blot analysis was performed to examine the expressions of HO-1, NQO1, and PRDX1 genes. rRNA was used as a loading control.

*Electrophoretic Mobility Shift Assay (EMSA)—*Nrf2CT (18) and Maf1-123 (19) were tagged with 6 histidine residues at the N termini and purified by nickel chelation affinity chromatography. The oligonucleotides NQO1-ARE-F (5'-CGC GTC TGA ACT TTC AGT CTA GAG GCT-3') and MafG-ARE-F (5'-CGC GTC TGA ACT TTC AGT CTA GAG CCG TGA TCC TTG CTT GCT GTT G-3') were radio-labeled with 32P and annealed with the complementary strand oligonucleotides NQO1-ARE-R (5'-CTA GAA ATT TTG CCG ACT CAC TGT GAC TCT AGA CTG AAA GTT CAG A-3') and MafG-ARE-R (5'-CTA GAG CCG CCT GTT CCT GCC GAG TCA TGC TGA CTC AGC GGA TCG-3'), respectively. To generate unlabeled competitors, a pair of oligonucleotides MafG-ARE-F and MafG-ARE-R (above) with their competitor and their corresponding mutant oligonucleotides with base substitutions shown in Fig. 5C (m1, m2, and m3 competitors) were annealed. An increasing amount of competitors, from 10- to 100-fold or a 300-fold excess of competitors was added. Incubation of the probe and recombinant proteins with or without unlabeled competitors was carried out as described previously (3). The protein-DNA complexes and free probe were resolved by electrophoresis on a 5% polyacrylamide (7:1) gel in 1x TBE buffer.

*Transfection—*293T cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics and seeded in a 12-well plate 24 h before transfection. Mouse Nrf2 cDNA was inserted into pEF-BaseIII (3) and used for transient expression of Nrf2 (pEF-Nrf2). pLM-MafG (20) was used for transient expression of mouse MaG. To make pNQO1-ARE-Luc, the pair of oligonucleotides NQO1-ARE-F and NQO1-ARE-R (above) were annealed and insert-d into pRBGP3 (21). To generate pMafG-ARE-Luc and their mutant derivatives, a pair of oligonucleotides MafG-ARE-F and MafG-ARE-R (above), either bearing or without any introduced mutation (Fig. 5C), were annealed and inserted into pRBGP3. These expression vectors and the luciferase reporter vectors were transfected into the cells using FuGENE6 (Roche Applied Science). 12 h after transfection, cells were harvested, and the lysates were used for luciferase assays. The expressions of both firefly and sea pansy luciferase were quantified using a dual luciferase reporter assay (Promega); firefly luciferase activity was normalized to co-transfected sea pansy luciferase activity for transfection efficiency.

**ChiP Analysis—*ChIP analysis was performed essentially as described previously (22). Immunoprecipitations were performed using control rabbit IgG, anti-Nrf2, or anti-p18/MaFk antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunoprecipitated material or a 1:50 dilution of input was used for PCR with the following primers: NQO1 ARE (22); lactate dehydrogenase (LDH) gene promoter (22); and mafG ex promoter, (5'-GCC TGA TCC TTT CCT GCT G-3' and 5'-GCA AGC CTA GAA AGA AGC TGA G-3').

**RESULTS**

*MaFg Gene Expression Is Induced by DEM—*In an earlier study, we found that DEM, a well known inducer of Nrf2/ARE-

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**Table 1**

| Name of oligonucleotide | 5' to 3' sequence |
|-------------------------|------------------|
| MafG-Ia | CCGGACTTTCCCGGGGGTGAAG |
| MafG-Ib | CAGGGCAGTTCCGGTCGCTTGGAAAG |
| MafG-Ic | TTTCGAGAAGCAGGCTCAAGGTTG |
| MafG-int | TCCGTACCGCTCTCGGTTCCT |
| MafG-III-H | CCTGCGCTCCCCCTTCTCCTTATTAG |
| MafG-P (TaqMan probe) | FAM-ACGCTGTCCCGGAGGTATGACGG-TAMRA |

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2 F. Katsuoka, H. Motohashi, J. D. Engel, and M. Yamamoto, unpublished observations.

3 F. Katsuoka, H. Motohashi, J. D. Engel, and M. Yamamoto, submitted for publication.
FIG. 2. Multiple promoters are used for transcription of mouse mafG. A, summary of 5′-RACE analysis. The sequences in boldface, capital letters represent exons, and those in lowercase letters represent introns or interexonal regions. The 5′-ends of the RACE clones are indicated by solid circles. The translational initiation ATG codon of mafG is boxed. B, structure of the mouse mafG gene and the four MafG mRNA splice isoforms identified by RACE. C, tissue distribution of the four MafG mRNA splice isoforms. cDNAs were synthesized from total RNAs prepared from the various tissues of adult animals, as indicated. The relative copy numbers of the MafG mRNA splice isoforms Ia, Ib, Ic, and Ic-int were determined by quantitative RT-PCR using a plasmid containing the cDNA for each splice isoform as the abundance standard. The relative copy number of the Ia splice isoform in brain was used as a reference and set to 1. The error bars represent the S.D. values (n = 3).
dependent genes, induced the expression of \textit{mafG} in MEFs.\textsuperscript{3} To confirm this observation, we treated wild type MEFs with DEM and examined the expression of small \textit{maf} genes by quantitative RT-PCR. Among the small \textit{maf} genes, \textit{mafG} was the most significantly induced by DEM (Fig. 1A). Induction of \textit{mafG} peaked 3 h after DEM treatment before gradually decreasing (Fig. 1B). Thus, \textit{mafG} is a DEM-responsive gene induced at a relatively early stage in the course of the response. The \textit{HO-1} gene showed a similar induction profile (Fig. 1B). Notably, the induction of other ARE-dependent genes, such as \textit{NQO1} and \textit{PRDX1}, peaked only after 12 h following DEM treatment (Fig. 1B).

\textbf{The mafG Gene Has Three Alternative First Exons—}To decipher the mechanisms controlling \textit{mafG} induction, we examined transcriptional regulation of the \textit{mafG} gene. We previously isolated phage clones containing the mouse \textit{mafG} locus and mapped coding exons II and III but did not determine the nature of the noncoding first exon(s) (13). Therefore, we first performed 5\textsuperscript{-}RACE to determine first exon(s) utilized by the \textit{mafG} gene and identified three (designated Ia, Ib, and Ic) (Fig. 2A). We also identified one splice isoform, designated “Ic-int” (Fig. 2B), which has an Ic first exon and intron sequence lying between the Ic and second exon but lacks the intron between exons II and III, demonstrating that this sequence was not due to genomic DNA contamination. We asked whether these splice isoforms (Ia, Ib, Ic, and Ic-int) are utilized differentially in response to stress. We performed quantitative RT-PCR using cDNA recovered from cells treated or untreated with DEM. The Ia, Ib, and Ic isoforms were all significantly induced by DEM, whereas the Ic-int isoform was not (Fig. 3A). After DEM treatment, the Ib isoform was still the most abundant species of the \textit{mafG} transcripts (Fig. 3A). Notably, upon comparison of the induction ratios for each transcript, we found that isoform Ic was most markedly induced (Fig. 3B).

While searching through the \textit{mafG} genomic locus \textit{in silico}, we discovered a potential ARE sequence 5\textsuperscript{'} to exon Ic (Ic-ARE) (Fig. 4A). The Ic-ARE partially matches the ideal ARE consensus sequence and is similar to the ARE identified in the \textit{NQO1} regulatory element (Fig. 4A). An NCBI data base search led to the identification of a putative first exon for human \textit{mafG} corresponding nicely to the mouse Ic exon (Fig. 4B). The promoter-proximal region of exon Ic is highly conserved between mice and humans, including the putative \textit{mafG} promoter Ic-ARE (Fig. 4B).

\textbf{An Nrf2/MafG Heterodimer Binds to, and Activates Transcription from, the mafG Ic-ARE—}The potential binding interaction of the \textit{Nrf2}/small Maf heterodimer to the Ic-ARE was examined by EMSA. To assess the binding affinity of the \textit{mafG} Ic-ARE, we compared it with the high affinity ARE site identified in the mouse \textit{NQO1} gene as a positive control. The result clearly demonstrated that the \textit{Nrf2}/MafG heterodimer binds well to the \textit{mafG} promoter ARE (Fig. 5A). The binding affinity of the heterodimer for the Ic-ARE appeared to be somewhat weaker than for the \textit{NQO1}-ARE (Fig. 5A); in contrast, MaG homodimers seemed to prefer the \textit{mafG}-ARE over the \textit{NQO1}-ARE (Fig. 5A). The binding activities to the \textit{mafG}-ARE were competed efficiently by a 300-fold molar excess of unlabeled wild type \textit{mafG}-ARE, but not by the mutant \textit{MaG}-ARE, possessing mutated nucleotides at both ends of the TPA response element contained within the \textit{MaG}-ARE (m1) (Fig. 5B). These results showed that DNA binding of Nrf2 and MaG depends on the integrity of the TPA response element core sequence of the \textit{MaG}-ARE. To the contrary, these binding activities were eliminated by the addition of a 300-fold molar excess of the mutated \textit{MaG}-ARE possessing a mutation in the sequence lying just outside of the short ARE consensus (m2). The mutated \textit{MaG}-ARE possessing both of the mutations within a single ARE (m3) behaved in a similar way with m1 mutant oligonucleotides (Fig. 5B). Further study using a lower molar excess of competitors showed that m2-type \textit{MaG}-ARE was almost comparable with the wild type \textit{MaG}-ARE in the ability to suppress the Nrf2/MafG-shifted complexes (Fig. 5C). These results showed that the mutated nucleotides in m2-type \textit{MaG}-ARE are not

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\textbf{Fig. 3. Inducibility of MafG splice isoforms by DEM.} A and B, quantitative RT-PCR was performed to examine the inducibility of the \textit{MaG} mRNA splice isoforms Ia, Ib, Ic, and Ic-int by DEM. cDNAs were synthesized from total RNAs prepared from MEFs treated with 0, 50, and 100 \textmu M DEM. The relative copy numbers of Ia, Ib, Ic, and Ic-int were quantified as described in the legend to Fig. 2. The error bars represent the S.D. values (\textit{n} = 3). For A, the relative copy number of the Ia splice isoform with 0 \textmu M DEM was used as a reference and set to 1. For B, the relative copy number of each splice isoform with 0 \textmu M DEM was used as a reference and set to 1.
critical for the binding of Nrf2/MafG heterodimer and MafG homodimer to Ic-ARE at least in vitro (Fig. 5C).

To examine whether the mafG promoter Ic-ARE is functional, we performed transient transfection assays. Oligonucleotides containing ARE sequences were inserted into luciferase reporter genes and transfected into 293T cells. In order to make a comparison with the activity of a known ARE, we co-transfected luciferase reporter constructs containing either the ARE from the mafG promoter or the ARE derived from the mouse NQO1 gene. The ARE reporter gene was strongly activated by co-transfection with the Nrf2 expression vector alone (Fig. 5D). The ARE/luciferase reporter was activated to even higher levels when co-transfected with expression vectors that simultaneously forced Nrf2 and MafG expression (Fig. 5D). The induction of the reporter gene was comparable using either the mafG promoter or NQO1 promoter ARE sites (Fig. 5D). We further dissected the Ic-ARE activity by investigating the trans-activation potential of mutant AREs tested in EMSA. Strong induction of the reporter gene that is normally elicited by the Nrf2 expression vector was no longer observed with a mutated reporter gene possessing an m1-type mutation (Fig. 5E). Consistent with the result of EMSA, this showed that the TPA response element core sequence of the Ic-ARE is critical. Whereas m2-type mutation did not affect the binding activity of Nrf2/MafG, this mutation abolished Nrf2-mediated induction of the ARE luciferase reporter gene (Fig. 5E). It is noteworthy that both m1-type and m2-type mutated reporter genes were slightly activated when both Nrf2 and MafG expression vectors were co-introduced by transfection. However, no combination of factors was able to activate the reporter gene possessing the

Fig. 4. A putative ARE in the mafG gene Ic promoter. A, alignment of the MARE, the short ARE, the long ARE, the mafG Ic-ARE, and the ARE from the mouse NQO1 gene regulatory region. The MafG genomic locus is depicted at the top. The boxes represent exons, and the circle indicates the position of the mafG Ic-ARE. Each sequence was aligned, with asterisks indicating the nucleotides that are identical between the long ARE and the MafG Ic-ARE or between the MafG Ic-ARE and the NQO1 ARE. B, comparison of the first exons and the 5' upstream regulatory regions of human and mouse mafG genes. For A and B, the arrows indicate nucleotides corresponding to the long ARE. The TPA response element core sequence of the MARE is shaded in dark gray. The GC boxes of the MARE are shaded in light gray. The abbreviations follow standard IUPAC nomenclature (where M represents A or C, R is A or G, Y is C or T, W is A or T, and S is G or C).
m3-type mutation (Fig. 5E). These mutational analyses suggest that the sequences outside of the classical short ARE are important for maximum induction of mafG transcription.

Nrf2 and Small Maf Interact with the Endogenous mafG Ic-ARE—In order to confirm the biological significance of the data obtained from both EMSA and reporter transfections, ChIP experiments were performed using Hepa-1c1c7 cells. To induce nuclear accumulation of Nrf2, Hepa-1c1c7 cells were treated with DEM for 3 h, fixed with formaldehyde, and used for ChIP assays.

The association of Nrf2 with the NQO1 ARE was first examined as a positive control (22). As reported, Nrf2 and small Mafs associate strongly with the NQO1 ARE in DEM-treated cells but only weakly in mock-treated cells (Fig. 6). In the absence of IgG or in the presence of preimmune rabbit IgG, immunoprecipitations failed to select the NQO1 ARE (as well as other nonspecific genomic regions), demonstrating that the sites were not enriched in nonspecific fashion (Fig. 6). The LDH gene promoter region was not immunoprecipitated with either Nrf2 or small Mafs, suggesting that the antibodies used enriched DNA in a sequence-specific manner (Fig. 6). Under such conditions, these experiments showed that both Nrf2 and small Mafs associated with the mafG Ic-ARE in DEM-treated cells (Fig. 6). These results clearly demonstrated that Nrf2 and small Mafs can associate with an endogenous genomic region containing the mafG promoter Ic-ARE. Interestingly, small Mafs also strongly associated with the mafG Ic-ARE in vehicle-treated cells, suggesting occupation of the Ic-ARE by either inactive or repressive binding complexes prior to DEM induction.

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seems to be a stress-inducible gene in many species. 

Induction of the mafG Gene Is Dependent on Nrf2—Finally, we sought genetic confirmation to support the dependence of mafG transcription on Nrf2. To this end, we prepared nrf2-null embryonic fibroblasts, treated the cells with DEM, and examined mafG mRNA accumulation. Before DEM treatment, the expression of mafG was slightly elevated in nrf2-null mutant cells (in comparison with wild type controls) by ~1.5-fold. However, mafG was no longer significantly activated in nrf2-null mutant cells after adding DEM, whereas a dose-dependent induction of the mafG was observed in wild type cells (Fig. 7). These results demonstrate that mafG induction is dependent on Nrf2.

DISCUSSION

In this study, we examined the transcriptional regulation of the mouse mafG gene and concluded that mafG is itself an ARE-dependent gene controlled by Nrf2. In agreement with the present data, other Nrf2 inducers have also been reported to induce the mafG gene. A hamster mafG homolog was identified as a hydroxen peroxide-inducible gene in a differential display analysis of gene expression (23). Another group reported that pyrrolidine dithiocarbamate, a thiol compound, induces human MAFG (24). Furthermore, it has been reported that the zebrafish mafG homolog is inducible by DEM (25). Thus, mafG seems to be a stress-inducible gene in many species.

When we initially cloned the first exons of mafG by 5’-RACE analysis, we found that the mouse mafG gene has at least three alternative first exons (Ia, Ib, and Ic). We previously reported that mafK and mafF also have multiple untranslated first exons (14, 26). Taken together, it seems clear that all three small maf genes utilize multiple first exons. MafG does not appear to have tissue-specific first exons, such as the mafK In exon that is employed exclusively in neurons (27). Nonetheless, expression analysis revealed that mafG first exons are utilized differentially in various tissues.

Based on the sequences derived from the 5’-RACE clones, we concluded that none of the alternative splice isoforms of mafG produce a change in the amino acid composition of the protein. We inferred that utilizing multiple first exons presents itself as a solution by which small maf genes can confer distinct, broad (but not ubiquitous) expression profiles. Although we cloned a splice isoform of mafG (Ic-int) that contains the intron between exon Ic and exon II and is expressed at a significant level, its biological significance is yet to be determined.

All three first exons of mafG were induced upon oxidative stress. Whereas the Ib transcript was the most abundant before and after DEM treatment, the Ic transcript was the most strongly activated by DEM relative to its constitutive expression level. These observations show that the alternative first promoters vary in their inducible response to DEM. The critical transcription factor-binding site that could be responsible for DEM-mediated induction was anticipated to be an ARE(s). We searched for AREs in the mafG locus and identified one in the proximal promoter of exon Ic. Although the short ARE consensus sequence was originally defined as 5’-RGTGACnnnGC-3’, subsequent studies revealed that functional AREs, such as the one in the NQO1 gene, are often represented by a long ARE consensus sequence, 5’-TMAnnRGTAYnnnGCRwwww-3’ (22, 28). Indeed, the mafG Ic-ARE is similar to the longer consensus sequence and is also similar to the ARE found in the NQO1 gene regulatory region. Since the Ic-ARE is well conserved in the human MAFG gene, we suspect that these AREs are evolutionarily conserved for mafG regulation.

In at least two previous reports, investigators failed to observe cooperative activation of AREs by Nrf2 and small Maf proteins (29, 30). Although we also failed to observe this synergistic transcriptional activation in 3T3 or COS1 cells, we did detect cooperativity in 293T cells. In the reporter analysis using 293T cells, we observed that the transcription mediated by the mafG Ic-ARE was cooperatively activated by Nrf2 and MafG. In this assay, a mutation outside the short ARE consensus of the Ic-ARE (Fig. 5C; m2) greatly inhibited induction of the reporter gene by Nrf2 and MafG. Since this mutation did not affect the binding of the Nrf2/Maf heterodimer in EMSA, unidentified factor(s), which recognize the sequence outside of ARE, might play critical cooperative roles with the Nrf2/Maf heterodimer in the transcriptional activation (31). Taken together, our observations provided support for the longer ARE consensus sequence as important for strong Nrf2-mediated induction.

ChIP analysis showed that Nrf2 actually binds to the mafG Ic-ARE in a chromosomal context. Moreover, DEM treatment of nrf2-null mutant cells led to no mafG induction. That is not to say that these data refute a possible contribution of Nrf1 or Nrf3 to mafG regulation, but in MEFs, neither Nrf1 nor Nrf3 is able to compensate for the absence of Nrf2. In fact, Nrf2 and Nrf1 have been shown to play important roles in ARE-dependent gene induction in an overlapping manner (8). ChIP analysis also showed that small Mafs bind to the mafG Ic-ARE. Although the small Maf antibody used in the ChIP assay recognizes primarily MaF, we suspect that all three small Maf proteins can bind to the Ic-ARE, since this DNA binding motif is conserved among the small Maf proteins (5).

ChIP analysis showed that small Mafs bind to the mafG Ic-ARE even in untreated cells (Fig. 6), indicating that there is the small Maf binding activity independent of Nrf2. Since MaF homodimer is able to bind to the mafG Ic-ARE in EMSA, it is possible that small Maf proteins form inactive homodimers by themselves and occupy the mafG Ic-ARE in untreated cells. However, it is also possible that small Maf proteins heterodimerize with Bach1 to form a transcriptional repressor. The Bach1-MafG heterodimer may repress mafG gene transcription through mafG Ic-ARE in the same way as it represses HO-1 gene expression (9, 16). The contribution of Bach1 proteins to the regulation of mafG is under investigation.
This study also showed that ARE-dependent genes are not induced uniformly by DEM. Indeed, mafG and HO-1 were rapidly induced, whereas NQO1 and PRDX1 were slowly induced. These facts may help us to understand the biological significance of the induction of mafG upon exposure to oxidative stress. It is possible that rapidly induced genes may affect the expression of other ARE-dependent genes. Induced MafG could be utilized as a heterodimeric partner for Nrf2 to further induce ARE-dependent genes at a later stage of differentiation. On the other hand, induced MafG might heterodimerize with Bach1 or homodimerize with itself to repress ARE-dependent genes. These two possibilities are not mutually exclusive. We recently found that ARE-dependent genes are regulated differentially by small Maf proteins. MaF induction may exert distinct influences on the expression levels of target genes, depending on their dimeric context.

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4. MafG induction may exert a significant of the induction of mafG upon exposure to oxidative stress. It is possible that rapidly induced genes may affect the expression of other ARE-dependent genes. Induced MafG could be utilized as a heterodimeric partner for Nrf2 to further induce ARE-dependent genes at a later stage of differentiation. On the other hand, induced MafG might heterodimerize with Bach1 or homodimerize with itself to repress ARE-dependent genes. These two possibilities are not mutually exclusive. We recently found that ARE-dependent genes are regulated differentially by small Maf proteins. MafG induction may exert distinct influences on the expression levels of target genes, depending on their dimeric context.

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