Nrf1 and Nrf2 Play Distinct Roles in Activation of Antioxidant Response Element-dependent Genes

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Nrf1 is a member of the vertebrate Cap’n’Collar (CNC) transcription factor family that commonly contains a unique basic-leucine zipper domain. Among CNC family members, Nrf2 is known to regulate a battery of antioxidant and xenobiotic-metabolizing enzyme genes through the antioxidant response element (ARE). Although Nrf1 has also been shown to bind the ARE, it is unclear whether it plays a distinct role from Nrf2 in regulating genes with this element. To address this issue in vivo, we generated mice bearing a hepatocyte-specific disruption of the Nrf1 gene. Although Nrf2 knockout mice did not exhibit liver damage when they were maintained in an unstressed condition, hepatocyte-specific deletion of Nrf1 caused liver damage resembling the human disease non-alcoholic steatohepatitis. Gene expression analysis revealed that the disruption of Nrf1 causes stress that activates a number of ARE-driven genes in an Nrf2-dependent manner, indicating that Nrf2 cannot compensate completely for loss of Nrf1 function in the liver. In contrast, expression of metallothionein-1 and -2 (MT1 and MT2) genes, each of which harbors at least one ARE in its regulatory region, was decreased in the Nrf1-null mutant mice. Whereas Nrf1 and Nrf2 bound the MT1 ARE with comparable affinity, Nrf1 preferentially activated the reporter gene expression through the MT1 ARE. This study has, thus, identified the first ARE-dependent gene that relies exclusively on Nrf1, suggesting that it plays a distinct functional role in regulating ARE-driven genes.

One of the unique features of transcription factors is that they can be categorized into structurally related groups (or families) based on their DNA binding motifs. It appears that in addition to their common basic functions each member within a specific transcription factor family is likely to have acquired novel properties during its molecular evolution. Consequently, it is probable that a family of transcription factors collectively possesses abilities that enable individual members to fine-tune the expression of target genes during disparate biological processes through a common cis-acting element.

The Cap’n’Collar (CNC) family of transcription factors has been well described. It comprises four closely related factors, Nrf1, Nrf2, Nrf3, and p45 NF-E2 (1, 2). These CNC members contain a CNC domain juxtaposed with a conserved basic region-leucine zipper (bZip) domain. The CNC domain is represented in an evolutionally distant homologue, SKN1 in the nematode Caenorhabditis elegans (3). Although SKN1 binds to DNA as a monomer, all vertebrate CNC members each heterodimerize with one of three small Maf proteins, and the resulting heterodimers bind to the Maf recognition element (TGCTGA(G/C)TCAGCA) or related sequences. Because the vertebrate CNC members cannot bind to Maf recognition element sequences as monomers, small Maf proteins are dispensable partners of CNC bZip transcription factors (1, 2).

The Maf recognition element-related sequence that is referred to as an antioxidant or electrophile response element (ARE/EpRE; hereafter designated ARE for simplicity) has been identified in the transcriptional regulatory regions of antioxidant and xenobiotic-metabolizing enzyme genes (1, 2) and is thought to account for the coordinated up-regulation of these genes during oxidative stress. The ARE has been shown to recruit Nrf2 and small Maf proteins (1, 2). In Nrf2-null mutant mice the up-regulation of many antioxidant and detoxification enzyme genes has been found to be severely impaired (4). Nrf1 has also been shown to contribute to the regulation of ARE-dependent genes (5, 6). Unlike Nrf2-null mice, Nrf1-null mice die by embryonic day 13.5 (E13.5) by anemia (7). Furthermore, Nrf1:Nrf2 compound mutant mice die by E11.5, suggesting that Nrf2 can compensate, albeit partially, for the absence of Nrf1 in embryonic mice (8). Based on the observation that an evolutionally distant CNC homolog SKN1 also contributes to the induction of antioxidant genes (9), it can be hypothesized that...
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the regulation of antioxidant genes is one of the original functions descended from a common ancestral CNC protein.

It is clear that Nrf1 and Nrf2 have acquired specific functions during their molecular evolution. It has been reported that heptatocty-specific conditional targeting of the Nrf1 gene (Nrf1 cKO) causes a liver pathology resembling human non-alcoholic steatohepatitis (NASH) (10); this previous study also reported that the expression of several ARE-dependent genes was decreased in the livers of Nrf1 cKO mice, suggesting that Nrf1 has an overlapping function with Nrf2 in the regulation of antioxidant and detoxifying genes. In stark contrast, however, Nrf2-null mutant mice do not show any liver damage when housed in an unstressed environment (11). Thus, perturbations in the expression of antioxidant genes in Nrf1 cKO mice appear unlikely to account for the NASH-like disorder. Collectively, these observations suggest that Nrf1 possesses unique functions that are not exhibited by Nrf2. To execute its unique activity, Nrf1 protein possesses several distinct domains that are conserved among cross-species Nrf1 homologues (12). For instance, Nrf1 has an endoplasmic reticulum (ER) targeting sequence in its N-terminal domain that is responsible for it being anchored to the ER membrane and an Asn/Ser/Thr-rich (NST) domain through which it is glycosylated within the ER (13). It has also been reported that Nrf1 is cleaved and translocated from the ER to the nucleus in response to ER stress (14), although the functional contribution of Nrf1 to the ER stress response has not been well described.

Because the distinct phenotypes of Nrf1 and Nrf2 knock-out mice are highly likely to be caused by differences in the expression of their relevant target genes, it is necessary to examine gene expression profiles in mutant mice to understand the molecular basis for the severe consequences after disruption of Nrf1. In this study we have, therefore, independently generated hepatocyte-specific Nrf1 cKO mice and carried out comprehensive gene expression analyses. To our surprise, expression of canonical Nrf2 target genes in the liver was induced by the loss of Nrf1 in Nrf2-dependent manner, indicating that dysregulation of Nrf1 target genes causes an activation of the Nrf2 stress response pathway. We also found that several ARE-driven genes were down-regulated in the Nrf1 cKO mice compared with control mice. Among them, we found that expression of metallothionein-1 (MT1) and -2 (MT2) genes is intimately dependent on Nrf1 but not on Nrf2. A reporter co-transfection assay showed that Nrf1 preferentially activates the MT1 ARE. Moreover, in livers from Keap1 knockout mutant mice (Keap1<sup>1<sub>b/a</sub></sup>), where Nrf2 activates its target genes without any stimuli (15), no significant induction of MT1 expression was observed. These results, thus, demonstrate that Nrf1 has acquired specific roles distinct from those of the other CNC family members.

**EXPERIMENTAL PROCEDURES**

*Hepatocyte-specific Disruption of the Nrf1 Gene—*129Sv mouse Nrf1 genomic DNA was isolated from a BAC clone containing the murine Nrf1 gene from the BACPAC Resources Center at Children’s Hospital Oakland Research Institute (Oakland, CA). We constructed a triple-loxP Nrf1 targeting vector to generate a floxed mouse Nrf1-targeted locus consisting of a loxP site inserted into the third Nrf1 intron and a floxed Neomycin cassette downstream of exon IV (Fig. 1A). A splicing acceptor-fused internal ribosomal entry site-enhanced green fluorescence protein (Invitrogen) cassette was also incorporated into the construct at a site 3′ of the Neomycin cassette. A diphtheria toxin A cassette was placed outside of the Nrf1 gene homology region to allow negative selection. From PCR screening, we identified one clone that carried a homozygously recombinated allele.

The existence of ES cells with the floxed Nrf1-targeted locus was confirmed by Southern blot analysis of NsiI- and SpeI-digested genomic DNA using a 5′ probe located outside the targeting vector. The clone showed positive 19.4- and 13.1-kilobase fragments. The targeted ES cells were microinjected into blastocysts, and chimeric mice were crossed with wild-type animals to generate Nrf1<sup>1<sub>b/a</sub></sup> mice. Hepatocyte-specific deletion of the floxed Nrf1 allele was accomplished by breeding with the albumin-Cre transgenic mice (Alb-Cre, purchased from The Jackson Laboratory (Bar Harbor, ME)). The Alb-Cre mice were crossed with Nrf1<sup>1<sub>b/a</sub></sup> mice to generate Nrf1<sup>1<sub>b/a</sub>-Alb-Cre</sup> mice, which were then crossed with each other to generate hepatocyte-specific Nrf1-deficient (Nrf1<sup>1<sub>b/a</sub>-Alb-Cre</sup>) mice.

**Generation of Compound Mutant Mice—**After mating Nrf1<sup>1<sub>b/a</sub>-Alb-Cre</sup> mice with either Nrf2 homozygous (Nrf2<sup>1<sub>b/a</sub></sup>) mutant mice (4) or Keap1 knockout mutant mice (15), Nrf1<sup>1<sub>b/a</sub>-Alb-Cre</sup> mice or Nrf1<sup>1<sub>b/a</sub>-Alb-Cre</sup> mice were obtained. These offspring were intercrossed to generate the following compound mutant mice: Nrf1<sup>1<sub>b/a</sub>-Alb-Cre</sup> and Nrf1<sup>1<sub>b/a</sub>-Keap1<sup>1<sub>b/a</sub></sup>Alb-Cre</sup> mice.

**RNA Extraction, RT-PCR, and Quantitative RT-PCR—**Total RNA was extracted from livers of three 8-week-old Nrf1<sup>1<sub>b/a</sub>-Alb-Cre</sup> mice and Nrf1<sup>1<sub>b/a</sub>-Alb-Cre</sup> mice using ISOGEN (Nippon Gene), and cDNA was synthesized using random hexamer oligonucleotide primers in reactions that were performed according to protocols recommended by the manufacturer. PCR primers for Nrf1 were as follows: Nrf1-F (5′-GGT GCC TAG TGA GAG TGA GTC CCC C-3′) and Nrf1-R (5′-TGG CTG AAG AGG GAG AAG TC-3′). The primer and probe sequences are described earlier (16). Quantitative RT-PCR was performed using an ABI Prism 7300 or 7700 (Applied Biosystems) instrument as previously reported (17). The primer and probe sequences used to detect NAD(P)H dehydrogenase quinone 1 (NQO1), the glutamate-cysteine ligase catalytic subunit (GCLC), heme oxygenase-1 (HO-1), and glutathione S-transferase π1 (GSTP1) mRNA have been described previously (18, 29). Nrf1<sup>1<sub>b/a</sub></sup>, and MT2 mRNAs were quantified using the following primer and probe sequences: Nrf1-sense (5′-TTG GAA CAG CAG TGG CAA GA-3′), Nrf1-antisense (5′-CTC ACT TGC TGA TGT ATT TAC TTC CAT-3′), Nrf1-probe (5′-FAM CTC ATG TCC ATC ATG GAA AGT CAG GC TAMRA-3′), MT1 sense (5′-AAC TGC TCC TGG TCC AC-3′), MT1 antisense (5′-GCC CGT GGC ACA TTT GG-3′), MT1 probe (5′-FAM CTC CTG CAA GAA GAG GCT CTG CTC CT TAMRA-3′), MT2 sense (5′-TCC TGT GCC TCC GAT GGA TC-3′), MT2 antisense (5′-GTC GGA AGC CTC TTT GCA GA-3′), MT2 probe (5′-FAM AAA GCT GCT GCT GTT GCA-3′).
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CCT GCT GCC CC TAMRA-3'). The rRNA primers and probe were purchased from Applied Biosystems.

Microarray Analysis—Total RNA samples were purified using ISOGEN before being processed and hybridized to the Affymetrix Mouse Genome 430 2.0 Array (Affymetrix). Experimental procedures for the Gene Chip analyses were performed according to the Affymetrix Technical Manual.

Histochemical Analysis—Livers were fixed with Mildform (Wako) and embedded in paraffin using standard procedures. Sections (4 μm) were cut and stained with hematoxylin and eosin. To visualize hepatic lipid content, livers were fixed with 4% paraformaldehyde and embedded in OCT (Tissue Tek). The frozen sections (5 μm) were stained with Oil Red O (Muto Pure Chemicals) and counterstained with hematoxylin.

Clinical Biochemistry—Plasma was obtained by centrifugation at 4 °C and either analyzed immediately or stored at −20 °C. Plasma alanine aminotransferase and aspartate transaminase activities were measured with an automated biochemical analyzer, DRI-CHEM 7000V (Fuji Film, Japan).

Electrophoretic Mobility Shift Assay—Nrf1-CT, Nrf2-CT (19), and MafG (1–123) (20) were tagged with six histidine residues at their N termini and purified by using nickel-chelating affinity chromatography. The oligonucleotide MT1-ARE-F (5'-CGC GGC GCT CTA CAGG AGC CCG GGC TGA CTA TGC GTG GGC T-3') was radiolabeled with 32P and annealed with the complementary strand oligonucleotide MT1-ARE-R (5'-CTA GAG C CC ACC AGT CAC GGC CCC CGC G TC CTT GGC AGA GCC G-3'). To generate unlabeled competitors, a pair of oligonucleotides, MT1-ARE-F and MT1-ARE-R, (above; wild type competitor) and their corresponding mutant oligonucleotides, MT1-mARE-F (5'-CGC GGC GCT CTA CAGG AGC CCG GGC TGA CTA TAA GTG GGC T-3') and MT1-mARE-R (5'-CTA GAG CCC TGG CAT AGT CAC GGC CCC CGC GTC CTT GGC AGA GCC G-3') were annealed. Where required, a 100-fold excess of competitor oligonucleotide was added to individual reaction mixtures. Incubation of the probe and recombinant proteins with or without unlabeled competitor was carried out as described previously (21). Thereafter, the protein-DNA complexes and free probe were resolved by electrophoresis on a 5% polyacrylamide (29:1) gel in 1 X Tris borate EDTA buffer.

Co-transfection Transactivation Assay—The mouse MT1 gene promoter along with its ARE-containing 153-bp upstream region (a kind gift from Dr. G. K. Andrews, University of Kansas Medical Center, Kansas City) (22) was inserted into the pGL2-basic vector (pMT1 ARE-Luc). A reporter construct lacking the putative ARE was generated by deleting sequences CGTGACTATGCG (99 to 88, relative to the transcriptional start site) from the pMT1 ARE-Luc reporter plasmid. A cDNA encoding mouse Nrf1 lacking amino acids 1–30 was inserted into pcDNA3.1 (Promega), generating pcDNA3.1FLAG-Nrf1Δ30 (i.e. Nrf1 lacking its ER localization signal (14)). Hepalc1c17 (Hepal) cells were co-transfected with pMT1 ARE-Luc or pNQO1 ARE-Luc together with or without 100 or 200 ng of each pcDNA3.1FLAG-Nrf1Δ30, and pcDNA3.1FLAG-Nrf2, respectively, using the Lipofectamine Plus (Invitrogen) according to the manufacturer’s protocol. All transfection mixtures included 0.001 μg of the Renilla luciferase reporter, pRL-EF.

Transfected cells were incubated for 24 h before the expression of both firefly and Renilla luciferase was quantified using a dual luciferase reporter assay (Promega); firefly luciferase activity was normalized to co-transfected Renilla luciferase activity for transfection efficiency.

Western Blot Analysis—The preparation of nuclear lysates (23) from Hepa1 cells that had been transfected with pcDNA3.1FLAG-Nrf1Δ30 or pcDNA3.1FLAG-Nrf2 were subjected to immunoblot analysis as described previously. Membranes were probed with antibodies directed against FLAG-M2 (Sigma-Aldrich). To ensure equal loading, membranes were stripped and re-probed with anti-lamin antibodies (Santa Cruz). A horseradish peroxidase-conjugated secondary antibody (Zymed Laboratories Inc.) was used. Blots were developed by enhanced chemiluminescence.

RESULTS

Generation of Conditional Nrf1 Knock-out Mice—To help further define the in vivo functions of Nrf1, we generated conditional Nrf1 mutant mice using the Cre-loxP system. A targeting vector was constructed in which the coding region of exon IV was flanked with loxP sequences (Fig. 1A). Because this exon encodes the bZip DNA binding domain, we expected that the Cre-loxP recombination would generate a truncated form of Nrf1 that was incapable of binding DNA and transactivating gene expression. We also inserted an internal ribosomal entry site-enhanced green fluorescence protein cassette in the 3′ region of Nrf1 so that its expression could be evaluated by monitoring green fluorescent protein.

We confirmed homologous recombination between the targeting vector and the Nrf1 locus in mouse ES cells by Southern blotting (Fig. 1B). Nsil-digested genomic DNA was hybridized to a probe complementary to the 5′ region of the Nrf1 gene. A homologous recombinant clone was identified by the presence of an additional short DNA fragment created by the presence of a SpeI site in the flanking region of the loxP element.

Chimeric mice were obtained by microinjecting Nrf1floxed/+ ES cells into mouse blastocysts, and the resulting animals were crossed with wild-type C57BL/6J mice to achieve germ line transmission of the floxed Nrf1 allele. In preliminary experiments Nrf1floxed/+ mice were crossed with AYU1-Cre mice, which expressed Cre recombinase ubiquitously and displayed embryonic lethality by E13.5 (data not shown), consistent with a previous Nrf1 gene knock-out analysis (7). We also confirmed that insertion of loxP sites into the wild-type allele did not alter significantly the expression of Nrf1 in livers of adult mice (Fig. 1C).

Hepatocyte-specific Nrf1 Knock-out Mice Show Liver Damage—To generate a hepatocyte-specific deletion of Nrf1 in mice, we crossed Nrf1floxed/+ mice with Alb-Cre transgenic mice, yielding Nrf1floxed+Alb-Cre mice. PCR genotyping results of the Nrf1-floxed allele and Nrf1-deleted allele with mouse liver genomic DNA are shown in Fig. 2A. The Alb-Cre-mediated excision of the floxed allele appeared to be essentially complete, and RT-PCR analysis revealed that Nrf1 mRNA levels were severely compromised in livers of Nrf1floxed+Alb-Cre mutant mice (Fig. 2B).
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FIGURE 1. Generation of Nrf1flox/flox mice. A, the targeting strategy is illustrated. Structures of Nrf1 allele (wild type (WT) allele), Nrf1flox allele, and Nrf1-deleted allele after Cre-mediated recombination are shown. Coding exons of the mouse Nrf1 gene are shown with filled boxes, untranslated regions are shown with a gray box, and loxP sites are indicated with open arrows. B, Southern blot analysis of the Nrf1flox/flox and Nrf1flox/flox and Cre-mediated hepatocyte-specific disruption of Nrf1. Nrf1flox allele, Nrf1flox allele, and Nrf1-deleted allele after Cre-mediated recombination are shown. Coding exons of the mouse Nrf1 gene are shown with filled boxes, untranslated regions are shown with a gray box, and loxP sites are indicated with open arrows. C, Nrf1 mRNA expression level in the liver. Quantitative RT-PCR was performed to examine the expression of Nrf1 mRNA in wild-type and Nrf1flox/flox mice. The expression of Nrf1 mRNA in wild-type mouse was set as 1. The error bars represent S.D.

Histological examination of liver sections from 8-week-old Nrf1flox/flox:Alb-Cre mutant mice stained with hematoxylin and eosin revealed that they contained many lipid vacuoles (Fig. 2C, upper panels). To confirm the lipid accumulation, we also stained frozen sections with Oil Red O (Fig. 2C, lower panels). The results clearly demonstrated that the hepatocytes of Nrf1flox/flox:Alb-Cre mice accumulated a massive amount of lipid when compared with control mice. In clinical biochemistry analyses of mice at 8 weeks of age, plasma alanine aminotransferase and aspartate transaminase levels in Nrf1flox/flox:Alb-Cre mice were significantly higher than those in control mice (Fig. 2D). These results support the previous report that liver-specific knock-out of Nrf1 gene causes pathologic changes in the liver that resemble human NASH (10).

Inducible Expression of Cytoprotective Genes in Nrf1flox/flox:Alb-Cre Mouse Liver in a Nrf2-dependent Manner—We performed microarray analyses of RNA from livers of Nrf1flox/flox:Alb-Cre mice using livers from Nrf1flox/flox animals as controls. Because it has been reported that Nrf1 contributes to the expression of cytoprotective enzyme genes that are known to be regulated by Nrf2, we examined whether the expression of Nrf2 target genes was affected in Nrf1flox/flox:Alb-Cre mouse livers. The results revealed that the expression of a set of Nrf2/ARE-regulated genes is increased rather than decreased even in the absence of Nrf1 (Table 1).

To verify these microarray data, we selected four ARE-dependent genes (NQO1, GSTP1, GCLC, and HO-1) and performed quantitative RT-PCR analysis. We found that the basal expression level of these genes was increased in Nrf1flox/flox:Alb-Cre mouse livers when compared with control (Nrf1flox/flox) mouse livers (Fig. 3). Thus, Nrf1-deficiency resulted in up-regulation of at least some Nrf2-target genes.

To examine whether the increased expression of these cytoprotective genes depends on Nrf2, we generated a compound mutant mouse line bearing a global deletion of Nrf2 concomitant with hepatocyte-specific deletion of Nrf1. The increase in expression of these four Nrf2 target genes was completely abrogated in the Nrf1:Nrf2 compound knock-out mouse liver. This result suggested that the expression of antioxidant and xenobiotic-metabolizing enzyme genes is up-regulated in an Nrf2-dependent manner in response to endogenous stress caused by the lack of Nrf1. An important observation is that Nrf1 does not contribute to the basal expression of these four genes even in the absence of Nrf2, thereby suggesting that, at least under steady state conditions, Nrf1 does not contribute to the regulation of Nrf2 target genes (Fig. 3).

Identification of Nrf1-dependent Genes in Liver—In the microarray analyses we examined genes that were down-regulated in the Nrf1flox/flox:Alb-Cre mouse livers. We found that 52
genes were decreased more than 3-fold in the Nrf1flox/flox; Alb-Cre mouse when compared with control mouse livers. We categorized these genes into 11 groups according to their function (Table 2). To our surprise, only three of the 52 genes could be categorized as belonging to the xenobiotic metabolism group (i.e. MT1, MT2, and sulfotransferase family 3A member 1). On the other hand, we found that many of the hepatic genes that were down-regulated in Nrf1-null livers contributed to the maintenance of normal homeostatic processes. This group of genes includes glycosylation-related proteins, a chaperone, and various metabolic enzymes. These results, thus, indicate that Nrf1 is involved in the regulation of a number of important biological functions in the liver, which are distinct from those of Nrf2.

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### TABLE 1

**Effect of Nrf1 deficiency on expression levels of typical Nrf2-dependent genes**

| Description of genes | Accession number | -Fold increase |
|----------------------|------------------|----------------|
| Glutathione S-transferase, α1/2 | NM_008182 | 2.90 |
| Glutathione S-transferase, μ2 | NM_008183 | 2.46 |
| Glutathione S-transferase, γ1 | AB041613 | 2.27 |
| Glutathione S-transferase, or4 | NM_010357 | 2.05 |
| Glutathione S-transferase, μ3 | J03953 | 2.01 |
| Glutathione S-transferase, μ6 | NM_008184 | 1.95 |
| Glutathione S-transferase, μ5 | NM_010360 | 1.88 |
| Glutathione S-transferase, ν1 | AK002661 | 1.76 |
| Glutathione peroxidase 2 | NM_003077 | 1.75 |
| Glutathione S-transferase, α3 | AI172943 | 1.72 |
| Glutathione S-transferase, μ1 | NM_010358 | 1.53 |
| Glutathione peroxidase 1 | NM_013541 | 1.41 |
| Peroxiredoxin 1 | AV124700 | 1.34 |
| NADF/H dehydrogenase, quinone 1 | AV158882 | 1.11 |
| Glutathione peroxidase 1 | BI219063 | 1.10 |
| Thioredoxin 1 | NM_011660 | 1.07 |
| Ferritin light chain 1 | NM_010240 | 0.89 |
| Heme oxygenase 1 | NM_010442 | 0.74 |
| Glutamate-cysteine ligase, modifier subunit | NM_008129 | 0.73 |
| Glutamate-cysteine ligase, catalytic subunit | BC019374 | 0.65 |

### TABLE 2

**Genes whose expression levels were decreased in Nrf1flox/flox; Alb-Cre mice compared with Nrf1flox/flox mice**

| Gene category and description | Accession number | -Fold decrease |
|------------------------------|------------------|----------------|
| Xenobiotoxic metabolism      |                  |                |
| Metallothionein 2            | AA796766         | 8.03           |
| Metallothionein 1            | BC027262         | 7.76           |
| Sulfotransferase family 3A, member 1 | NM_020565 | 3.07 |
| Metabolic enzyme             |                  |                |
| Pyruvate dehydrogenase kinase, isoenzyme 4 | NM_013743 | 3.68 |
| Acyl-CoA thioesterase 1      | NM_012006 | 3.25 |
| Chaperone                    |                  |                |
| Heat shock protein 8          | BB764222 | 3.07 |
| Glycosylation                |                  |                |
| Glycophosphatidylinositol anchor attachment protein 1 | NM_010331 | 3.32 |
| Phosphatidylinositol glycan, class O | BB546713 | 3.04 |
| Transporter                  |                  |                |
| ATP-binding cassette, sub-family F (GCN20), member 1 | AV309591 | 11.85 |
| Major facilitator superfamily domain containing 3 | BC019171 | 7.57 |
| Sideroflexin 2               | NM_053196 | 3.13 |
| Cell cycle and differentiation |                  |                |
| Growth arrest and DNA-damage-inducible gene 45y | AK007410 | 15.55 |
| Cdk42-binding protein kinase α | BM17074 | 4.01 |
| Bcl-associated death promoter | NM_007522 | 3.91 |
| Cyclin-dependent kinase 5    | NM_007668 | 3.02 |
| Signal transduction          |                  |                |
| Signal peptide phosphate 3  | BC023131 | 7.28 |
| Serine/threonine kinase 24 (STE20, homolog, yeast) | BG066777 | 4.71 |
| Rho guanine nucleotide exchange factor (GEF) 12 | AI481688 | 3.17 |
| Calcium/calmodulin-dependent protein kinase 2B | BI157430 | 3.07 |
| TRAF family member-associated NF-κB activator (TANK)-binding kinase 1 | NM_019786 | 3.07 |
| SCY1-like 2 (Saccharomyces cerevisiae) | BM249802 | 3.05 |
| Cytoskeletal organization    |                  |                |
| Rho GTPase-activating protein 6 | AF177664 | 4.32 |
| Dopachrome tautomerase        | NM_010024 | 3.03 |
| Nuclear proteins             |                  |                |
| Cysteine and glycine-rich protein 1 | BF124540 | 3.78 |
| Ribonuclease P40 subunit (human) | R75260 | 14.53 |
| GTP-binding protein 2        | NM_019581 | 4.85 |
| Coiled-coil domain containing 91 | AK007017 | 3.85 |
| Small nuclear ribonucleoprotein N | NM_03174 | 3.68 |
| Ribonuclease H2, subunit B   | AU017373 | 3.54 |
| Histone 2, H3c1 /histone 2, H2aa1 | BC010564 | 3.00 |
| Immunological proteins       |                  |                |
| Histocompatibility 2, D region locus 1 | L23495 | 4.93 |
| Interleukin 1 receptor, type 1 | NM_008362 | 3.68 |
| Miscellaneous                |                  |                |
| C-type lectin domain family 2, member h | AF130410 | 13.37 |
| Crystallin, μ                 | NM_016669 | 9.92 |
| Zinc finger, A20 domain containing 2 | AA124553 | 3.53 |
| Zinc finger protein 259       | AK016551 | 3.47 |
| B-cell CLL/lymphoma 7B       | BM199789 | 3.20 |
| PRA1, RING-H2 motif-containing | NM_010119 | 3.19 |
| EH-domain containing 1        | NM_010119 | 3.19 |
| Zinc finger, AN1 domain 2B    | BC01195 | 3.17 |
| Rhomboid domain containing 2  | BRZ3005 | 3.14 |
| Amino acid oxidase synthase 1 | AI255644 | 3.11 |
| Myocyte enhancer factor 2D   | BG067166 | 3.09 |

**Figure 3.** Nrf1 deficiency results in up-regulation of Nrf2-target genes, NQO1, GSTP1, GCLC, and HO-1. Expression of xenobiotic metabolizing enzymes and antioxidant enzymes were examined by quantitative RT-PCR. The four genes were selected based on the result of the microarray analysis. RNA samples were prepared from livers of Nrf1flox/flox, Nrf1flox/flox; Alb-Cre, Nrf1flox/flox; Nrf2-/-, and Nrf1flox/flox;Nrf2-/-; Alb-Cre mice. The expression level of each mRNA was normalized to the rRNA abundance. The expression of each gene in Nrf1flox/flox mouse was set to 1. The error bars represent S.E. of mean of three independent experiments.
genes was compromised in Nrf1flox/flox:Alb-Cre mouse livers when compared with their expression levels in control mice (Fig. 4). It is well known that the expression of MT is induced in response to heavy metals and that this process is mediated by the zinc-finger transcription factor MTF1 through the metal response element in gene promoters (24). Our microarray data showed that MTF1 expression level did not change in the Nrf1flox/flox:Alb-Cre mouse livers (results not shown), indicating that the reduction of MT1 in the Nrf1flox/flox:Alb-Cre mouse livers was not caused by down-regulation of MTF1. Because the other ARE-dependent genes were induced in Nrf1flox/flox:Alb-Cre mice in an Nrf2-dependent manner (Fig. 3), these results raised the possibility that in mouse liver the MT1 and MT2 genes are preferentially and specifically regulated by Nrf1, but not by Nrf2, through the ARE in their regulatory regions.

MT1 ARE Is Preferentially Regulated by Nrf1 Not by Nrf2—Transcriptional control of the MT1 gene has been extensively studied, especially with regard to transcription factor MTF1 and its binding to the metal response element sequence (24). In addition to this metal response element, it has been reported that the MT1 gene has a conserved ARE in its regulatory region (25). We also confirmed that AREs are present in the promoter regions of orthologous vertebrate MT genes, such as those from mouse, human, chicken, Xenopus, and zebrafish (Fig. 5A). However, contribution of the CNC family factors to the regulation of MT1 expression has not been well documented.

To examine whether Nrf1 and Nrf2 bind to the mouse MT1 ARE, we performed electrophoretic mobility shift assay analyses. For this purpose, truncated Nrf1, Nrf2, and MafG proteins containing their DNA binding domains (i.e. Nrf1-CT, Nrf2-CT, and MafG1–123) were prepared in a bacterial expression system. We found that both Nrf1-MafG and Nrf2-MafG heterodimers could bind to the MT1 ARE with similar affinity (Fig. 5B). The observed binding activity was specific because it was competed by 100-fold molar excess of an unlabeled wild-type ARE probe but not by a mutant ARE probe (Fig. 5B). We conclude that Nrf1 and Nrf2 exhibit similar binding affinities for the MT1 ARE.

We then tested the possibility that Nrf1 and Nrf2 differentially activate transcription through the MT1 ARE. We performed a reporter co-transfection/transactivation assay. The MT1 gene regulatory region containing the ARE was linked to a luciferase gene, and the reporter construct was transfected into Hepa1 cells simultaneously with either FLAG-Nrf1Δ30 or FLAG-Nrf2 expression plasmids. Although the abundance of FLAG-Nrf1Δ30 and FLAG-Nrf2 was found to be comparable (Fig. 6A), the MT1 reporter gene was strongly activated by FLAG-Nrf1Δ30 but was only weakly activated by FLAG-Nrf2 (Fig. 6B). Both Nrf1- and Nrf2-mediated activations were severely compromised when the MT1 ARE sequence was deleted from the reporter gene, demonstrating that transactivation activity is strictly dependent on the ARE. In stark contrast, FLAG-Nrf2 activated a luciferase reporter gene driven by NQO1 ARE much more strongly than did FLAG-Nrf1Δ30 (Fig. 6C). These results, thus, indicate that the MT1 ARE

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**FIGURE 4.** Expression of MT1 and MT2 genes depends on Nrf1. The expression of MT1 and MT2 genes was examined by quantitative RT-PCR. RNA samples are prepared from Nrf1flox/flox and Nrf1flox/flox:Alb-Cre mice. The expression level of each mRNA was normalized to the rRNA abundance. The expression of each gene in Nrf1flox/flox mouse was set to 1. The error bars represent S.D.

**FIGURE 5.** Binding of Nrf1 and Nrf2 to MT1 ARE. A, an alignment of AREs found in the promoter regions of the mouse, human (1E), chicken, Xenopus, and zebrafish MT1 genes. Numbers indicate the position relative to the transcriptional initiation site. ARE consensus sequences are shaded. B, electrophoretic mobility shift assay was performed with MT1 ARE probe. DNA binding ability of the heterodimers consisting of Nrf1-CT/MafG1–123 or Nrf2-CT/MafG1–123 was examined. Constant amounts of MafG1–123 and increasing amounts of Nrf1-CT (lanes 3–7) and Nrf2-CT (lanes 8–12) were incubated with MT1 ARE probe. The black arrowhead indicates the DNA binding activity of heterodimeric proteins. A 100-fold molar excess of unlabeled wild-type MT1 ARE probe (Cp wt) (lanes 6 and 11) or mutant MT1 ARE probe (Cp mt) (lanes 7 and 12) was added for competition reactions.
gene is an ARE-dependent gene regulated by Nrf1 but not by Nrf2.

**MT1 Is Not induced by Constitutive Activation of Nrf2 in Keap1 Knockdown Mutant Mice Liver**—To further support our contention that Nrf2 does not regulate MT1, we explored whether or not MT1 is induced in the livers of Keap1 knockdown mutant mice (Keap1kd/kd) where Nrf2 can constitutively activate its target genes. Indeed, Nrf2 target genes were induced in Nrf1flox/flox;Keap1kd/kd mice, as has been reported (Fig. 7) (15). No significant difference was observed in the MT1 gene expression between Nrf1flox/flox;Keap1kd/kd and control Nrf1flox/flox mice, conclusively demonstrating that the MT1 gene is not induced by Nrf2 in vivo (Fig. 7).

**DISCUSSION**

Because Nrf1 and Nrf2 show similar binding specificity and expression profile, these two factors have been suggested to share mutually overlapping target genes and overlapping activities (1, 2). However, gene-targeting experiments in mice revealed that Nrf1-null mice (7) exhibit a different phenotype from that of the Nrf2-null mice (4), suggesting that these two transcription factors perform distinct functions. However, the analysis of Nrf1 has been hampered because simple knock-out of the Nrf1 gene in mice provokes embryonic lethality. Therefore, to identify specific roles that Nrf1 plays, we have undertaken a comprehensive exploration of its function in vivo. To this end, we generated hepatocyte-specific Nrf1 gene knock-out mice and examined the pathological and biochemical changes in the livers of the mutant mice. Surprisingly, microarray analyses revealed that loss of Nrf1 did not result in the down-regulation of well known Nrf2-target genes, but rather, Nrf2-target genes were up-regulated in Nrf1 cKO mice. Furthermore, we found that Nrf1 governs stress response genes, such as growth arrest and DNA damage-inducible genes as well as genes involved in glycosylation and the transmembrane transport of xenobiotics. These results, thus, demonstrate that Nrf1 has acquired unique functions that cannot be compensated by the
Other CNC family transcription factors during its molecular evolution.

An unexpected finding during the present study was that expression levels of Nrf2-target genes were up-regulated in Nrf1flox/flox Alb-Cre mice when compared with their expression in control mice. This induction is apparently dependent on Nrf2 as the induction of these genes in Nrf1flox/flox Alb-Cre mice was reduced upon simultaneous deletion of Nrf2. We envisage that in the absence of Nrf1, Nrf2 can be activated by certain endogenous stresses (Fig. 8). It is well known that Nrf2 is activated by reactive oxygen species, inflammatory cytokines, and ER stresses (26–28). Therefore, a fascinating hypothesis is that the disruption of Nrf1 may provoke ER stress, which eventually leads to the activation of Nrf2 (13, 14).

Consistent with the notion that endogenous stresses activate Nrf2, we recently observed that Nrf2 target genes are induced in liver specific knock-out of tRNA^{Sec} (29). In this case, during translation, tRNA^{Sec} inserts selenocysteine into a number of Se-dependent antioxidant enzymes including glutathione peroxidase and thioredoxin reductase (29). These data show that the Nrf2 gene battery represents a second line of defense that is principally of an exogenous origin. Thus, Nrf2 is crucial for maintaining cellular homeostasis under severe stress conditions. In contrast, Nrf1 is indispensable for countering steady state stress under normal homeostatic conditions, and the lack of Nrf1 activates Nrf2 as a backup defense against the endogenous stressors that are usually neutralized by Nrf1. We envisage the diversity of CNC members has conferred on higher animals multilayered defenses against the numerous endogenous and environmental stressors they encounter.

In the livers of Nrf1 knock-out mice, two of the genes down-regulated to the greatest extent are MT1 and MT2, whose products are cysteine-rich heavy metal-binding proteins. Both MT1 and MT2 genes have been reported to harbor at least one ARE in their promoter proximal regions. These AREs have also been reported to mediate the regulation by bZip proteins Fos and Fra-1 (30) and Nrf2 (31). In contrast, another team of researchers reported that MT1 expression was not decreased in the livers of Nrf2 knock-out mice (32). In agreement with the latter observation, we found that in the livers of Keap1 knockout mice, MT1 gene expression is not induced in response to the constitutive activation of Nrf2. The microarray analysis further revealed that in the absence of Nrf1, both MT1 and MT2 are down-regulated, whereas canonical Nrf2 target genes are up-regulated in an Nrf2-dependent manner. These results, therefore, indicate that both MT1 and MT2 genes are specifically activated by Nrf1.

The reporter cotransfection/transactivation analyses support our conclusion that Nrf1 and Nrf2 differentially regulate members of the ARE gene battery. Nrf2 but not Nrf1 effectively activates the NQO1-ARE luciferase reporter gene expression, whereas Nrf1 but not Nrf2 activates reporter gene expression through the MT1 ARE. In marked contrast, however, electrophoretic mobility shift assay showed that both Nrf1-small Maf and Nrf2-small Maf heterodimers bind to the MT1 ARE with similar affinity. Therefore, DNA binding specificity seems not to be able to account for their target gene differences. We surmise that whereas both Nrf1 and Nrf2 bind the MT1 ARE, only Nrf1 can form a complex that activates transcription of the MT1 gene. Indeed, in contrast to the high level structural conservation of the DNA binding and dimerization domains, the activation domain is not conserved between Nrf1 and Nrf2. Thus, affinity purification–mass spectrometry analysis of the coactivator complexes of Nrf1 and Nrf2 emerges as a viable strategy to answer this important question.

MTs protect hepatocytes against excess zinc and cadmium, free radicals, and various toxic agents (25). For instance, knock-out of the MT1 and/or MT2 gene renders mice sensitive to liver damage by alcohol (34). Furthermore, MT1 and MT2 double mutant mice have a tendency to suffer from obesity, and although they show increased hepatic lipid accumulation, no hepatitis was observed in these mutant mice (35). At present, a link between MTs and regulation of cellular energy balance is unclear.

Other Nrf1 target genes responsible for the onset of NASH remain to be clarified. We, therefore, searched for ARE sequences within the 400-bp upstream (i.e. promoter proximal region) of those genes listed in Table 2, as many functional AREs have been found in the region. We found 11 genes that have potential ARE sequences in the region but have not been reported to be ARE-dependent. Interestingly, among these genes, pyruvate dehydrogenase kinase isozyme 4 is regulated by peroxisome proliferator-activated receptor α (36). Dehydrogenase kinase isozyme 4 is a key regulatory enzyme involved in the switching of energy source from glucose to fatty acids in response to environmental conditions (33, 37). The expression level of peroxisome proliferator-activated receptor α was comparable in Nrf1flox/flox Alb-Cre mice with the level of expression in wild-type mice (data not shown). We also found that glycosylation-related genes, such as glycosylphosphatidylinositol anchor attachment protein 1 and phosphatidylinositol glycan class O, and stress response gene Gadd45γ harbor one or multiple AREs in their promoter proximal regions. Although it remains to be clarified whether there is a direct link between the reduction of these genes and the NASH-like liver damage in
Distinct Roles of Nrf1 in ARE-dependent Gene Regulation

Nrf1^box/box\_Alb-Cre mice, this mouse model seems to provide an important clue to our understanding of NASH.

As mentioned above, we have generated hepatocyte-specific Nrf1 knock-out mice independently from the earlier work of Chan and co-workers (10). We have found that the NASH-like liver pathology and lipid accumulation in the mutant mice are in good agreement with the previous report. Importantly, gene expression profiling has not been reported previously in Nrf1 knock-out mice. Furthermore, among the genes examined previously, we found a few differences in the two hepatocyte-specific Nrf1 knock-out mice. We surmise that this could be due to a mouse strain difference, or it may be due to different experimental conditions.

In conclusion, we have demonstrated that Nrf1 contributes to the expression of a subtype of ARE-dependent genes that is clearly distinct from those regulated by Nrf2, indicating that each CNC family member has acquired a specific target gene profile. A better understanding of the individual function of CNC members should provide insight into the orchestrated regulation of the ARE gene battery.

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