Nrf2 Mediates the Induction of Ferritin H in Response to Xenobiotics and Cancer Chemopreventive Dithiolethiones*

Received for publication, October 18, 2002, and in revised form, November 14, 2002
Published, JBC Papers in Press, November 14, 2002, DOI 10.1074/jbc.M210664200

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Ferritin is a ubiquitous intracellular iron storage protein that consists of 24 subunits of the H and L type. The ability to sequester iron from participation in oxygen free radical formation is consistent with a cytoprotective role for ferritin. Here we demonstrate that ferritins H and L are induced in cells treated with β-naphthalene (β-NF) and chemopreventive dithiolethiones. Induction of ferritin H by β-NF and the dithiolethiones oltipraz and 1,2-dithiole-3-thione (D3T) occurs via a transcriptional mechanism that is mediated by the ferritin H electrophile/antioxidant-responsive element (EpRE/ARE). The murine ferritin H gene contains five potential xenobiotic-responsive element (XRE) sequences in its 5′-promoter region. However, deletion analysis demonstrates that these XRE sequences are not functional in inducing ferritin H in response to β-NF. Electrophoretic mobility shift assays demonstrate that the ferritin H EpRE/ARE binds Nrf2. Transfection of chimeric ferritin H reporter genes with Nrf2 expression vectors and Nrf2 dominant-negative mutants indicate that Nrf2 functions at the EpRE/ARE to mediate transcriptional activation of ferritin H. Induction of ferritin H and L was not seen in Nrf2 knockout cells, demonstrating that this transcription factor is required for the induction of ferritin H in response to polyacrylamide xenobiotics and chemopreventive agents. Nrf2 may also play a role in basal transcription of both ferritin H and L. These results provide a mechanistic link between regulation of the iron storage protein ferritin and the cancer chemopreventive response.

Ferritin is a 480-kDa intracellular protein that can store up to 4500 atoms of iron (1). The protein consists of 24 subunits of the H and L chain type (2). The ratio of subunits within the ferritin protein varies widely by tissue type; the ratio can be further modulated by environmental signals, including cytokine stimulation, stress signals, and disease state (3, 4). The H chain has ferroxidase activity (5), whereas the L subunit is tetrameric and has putative ferroxidase activity (6). The ferritin H subunit can contain two distinct ferroxidase domains (7). The ferritin L subunit contains one ferroxidase domain (8). The ferritin H subunit is responsible for iron nucleation and protein stabilization (6). Because iron functions as a catalyst in the formation of oxygen free radicals, storage of iron by ferritin may serve a cytoprotective function (2). Several laboratories have demonstrated an induction of ferritin by oxidants and pro-oxidant xenobiotics, including agents such as hydrogen peroxide (7), t-BHQ (1), sodium arsenite (8), phorone (9), carbon tetrachloride (10), and aqueous extracts of cigarette smoke (11). Activation of ferritin by these agents is thought to be a stress response mechanism that contributes to limiting cellular and organismal damage from these xenobiotics.

The induction of antioxidant and detoxification cytoprotective enzymes has been proposed as a potential strategy in cancer chemoprevention (reviewed in Ref. 12). Chemopreventives are natural or synthetic compounds that have the ability to block or suppress carcinogenesis (13). Dithiolethiones, including oltipraz (5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione), a synthetic compound currently in clinical trials, and D3T (1,2-dithiole-3-thione) are prototypical examples of such candidate chemopreventive agents (14). The induction of ferritin in rats treated with the dithiolethione D3T represented an initial indication that ferritin may be among the cytoprotective proteins induced by chemopreventives (15).

Mechanisms responsible for the induction of cytoprotective proteins in response to challenge with xenobiotics, including certain chemopreventive agents, are beginning to be clarified. To date, two mechanisms responsible for the coordinate induction of antioxidant enzymes and phase II detoxification enzymes have been identified (16). The first mechanism is dependent on an interaction between the inducer and the cytosolic aromatic hydrocarbon (Ah) receptor (17). Upon binding of the ligand to the receptor, the receptor-ligand complex translocates to the nucleus, where it binds to a DNA enhancer element, termed xenobiotic responsive element (XRE). Most ligands capable of binding to the Ah receptor are polyaromatic hydrocarbons, such as β-NF and 2,3,7,8-tetrachlorodibenzo-p-dioxid (16). Genes regulated by this mechanism include phase I activating enzymes, such as the cytochromes P450 1A1 and 1A2 (18, 19), conjugating enzymes such as glutathione S-transferase (GST) Ya (20), and UDP-glucuronosyltransferases 1A1 and 1A6 (21, 22), and antioxidant enzymes such as NADP/H-quinoxidoreductase 1 (NQO1) (23), and Cu/Zn-superoxide dismutase (SOD) (24, 25). A second mechanism of gene activation is Ah receptor-independent and is mediated by an electro-
Regulation of transcription at the EpRE/ARE is incompletely understood. Early studies focused on members of the AP1 transcription factor family (38) as regulators of EpRE/ARE-dependent transcription (39–42). More recently, compelling evidence for the involvement of members of the cap and collar family of transcription factors (43), particularly Nrf2, has been presented. For example, induction of cytoprotective enzymes, such as GST and NQO1, by the phenolic antioxidant butylated hydroxyanisole was lost in cells isolated from Nrf2 knockout mice (44). In addition, forced expression of cap and collar family members, such as Nrf1 and Nrf2, resulted in the induction of EpRE/ARE-dependent reporter gene expression (45–48). Activation of Nrf2 following stimulation of cells with an inducer requires its dissociation from a cytosolic actin binding protein, Keap-1, and subsequent translocation to the nucleus (49, 50). Release of Nrf2 from Keap-1 may be triggered by modification of reactive cysteine residues in Keap-1 (51) and/or post-translational modification of Nrf2 by protein kinases (52).

Our laboratory has identified an EpRE/ARE in the ferritin H gene that mediates the induction of ferritin H transcription in response to H\(_2\)O\(_2\) and t-BHQ (7). The ferritin H EpRE/ARE is 75 bp in length and is located -4.1 kb from the transcription start site (7). It is comprised of the ferritin H basal enhancer (53), FER-1, and an AP1/NF-E2 consensus sequence located 8 bp 3’ of FER-1. The basal enhancer, FER-1, is in turn composed of an element with close sequence similarity to both AP1 and NF-E2 consensus sequences (previously termed AP1-like element (53, 54) and referred to in this report as AP1/NF-E2-like element), and a recognition sequence for the SP1/3 transcription factors (53, 54). The AP1/NF-E2-like and the AP1/NF-E2 consensus sequence of the ferritin H EpRE/ARE are arranged in inverse repeat, and both of these elements are necessary for full induction of ferritin H by H\(_2\)O\(_2\) and t-BHQ (7). An EpRE/ARE has also been identified in the murine ferritin L promoter (55). Ligation of this element to a luciferase reporter gene demonstrated that the ferritin L EpRE is functional as an enhancer element in HepG2 cells treated with t-BHQ (55). Collectively, these results suggest that ferritin may constitute a component of the cytoprotective response induced by xenobiotics (electrophiles or polycyclic aromatic hydrocarbons) and candidate chemopreventive agents. However, the mechanism of ferritin induction by these agents is unknown. Here, we demonstrate that ferritins H and L are induced by oltipraz, D3T, and \(\beta\)-NF in fibroblasts and hepatic cells. Furthermore, we show that induction of ferritin occurs via an EpRE/ARE-dependent mechanism that requires Nrf2. These results link ferritin induction mechanistically to the chemopreventive response.

EXPERIMENTAL PROCEDURES

Cell Culture—NIH3T3, Hepa1-6, and HepG2 cell lines and Nrf2 wild type (+/+) and Nrf2 knockout (-/-) primary mouse embryo fibroblasts were maintained at 37°C in a humidified atmosphere containing 5% CO\(_2\). NIH3T3 (ATCC) cells were grown in Dulbecco’s modified eagle medium (DMEM, Invitrogen) supplemented with 10% bovine calf serum (HyClone), 100 units/ml penicillin G sodium, and 100 \(\mu\)g/ml streptomycin sulfate. Hepa1-6 (ATCC) and HepG2 (ATCC) cells were grown in DMEM supplemented with 10% fetal bovine serum (Gemini Bioproducts), 100 units/ml penicillin G sodium, and 100 \(\mu\)g/ml streptomycin sulfate. The Nrf2+/+ and Nrf2−/− primary mouse embryo fibroblasts were grown in DMEM/F-12 (Invitrogen) supplemented with 15% fetal bovine serum, 2 mM glutamine, 0.1 mM non-essential amino acids, 150 \(\mu\)M \(\beta\)-mercaptoethanol, and 100 units/ml penicillin G sodium, and 100 \(\mu\)g/ml streptomycin sulfate.

Chemicals—Oltipraz and D3T were provided by the Division of Cancer Prevention Repository of NCI, National Institutes of Health, and \(\beta\)-NF was obtained from Sigma. All compounds were dissolved in Me\(_2\)SO and final Me\(_2\)SO concentration in all treatment conditions was 0.01%.

Plasmids—The following ferritin human growth hormone reporter gene constructs have been previously described (7): −4.8kbFH-hGH, −4.13kbFH-hGH (referred to as −4.0kbAP1+ FH-hGH in Ref. 7), −0.6kbFH-hGH, −0.32kbFH-hGH, and 107bpEpREFH-hGH (referred to as 107bpFER-1+ AP1 FH-Igh in Ref. 7). To construct ferritin H-luciferase reporter gene constructs −4.8kbFH-CAT (53) was digested with HindIII to release a fragment that contains the 5’-promoter region of the ferritin H gene, from nucleotide −4819 to +24. The HindIII fragment was ligated into the HindIII multiple cloning site of pGL3 (Promega) to create −4.8kbFH-Luc. To construct −4.2kbFH-Luc, −4.0kbFH-Luc, and −0.25kbFH-Luc, −4.8kbFH-Luc was digested with SacI, BglII, and SmalI to release 616-, 789-, and 4618-bp fragments, respectively. The resulting 9.0-, 8.9-, and 7.1-kb fragments were gel-purified and religated to form −4.2kbFH-Luc, −4.0kbFH-Luc, and −0.225kbFH-Luc, respectively. The −8.4kbh3.5kbFH-Luc internal deletion construct was made by removing an internal EcoRI fragment from −4477 to −941 from −4.8kb FH-Luc by digestion with EcoRI and religation. The 75bpEpREFH-Luc insertion construct was made as follows. Sense and antisense oligonucleotides corresponding to the 75 bp ferritin H EpRE/ARE were synthesized by the Wake Forest University School of Medicine Comprehensive Cancer Center DNA synthesis center laboratory, PAGE-purified, phosphorylated using T4 Polynucleotide kinase, annealed, and ligated into the SmalI site of 0.225kbFH-Luc. The Nrf2 dominant negative mutant expression plasmid (pEF/Nrf2dn) as well as the empty effector (pEF) were kind gifts of Dr. Jawed Alam (56). The Nrf2 expression plasmid (pEF/Nrf2) has been described previously (57).

Northern Blot Analysis—Total RNA was isolated from cells treated for 24 h with vehicle, oltipraz, D3T, and/or \(\beta\)-NF as described by Chirgwin et al. (58) or utilizing the TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. 10–15 \(\mu\)g of RNA were size-fractionated on 1.1% agarose/6.6% formaldehyde gels and transferred to an Immobilon Ny+ nylon membrane (Millipore) by capillary transfer. DNA probes for both ferritin H (59) and L (60) were generated by random prime labeling and subsequently hybridized to the UV-cross-linked RNA blot in Quick Hyb solution (Stratagene) according to the manufacturer’s protocol. Western blots were subsequently exposed to autoradiography; quantitation was performed using a PhosphorImager analyzer (model 445SI, Amersham Biosciences).

Western Blotting of Ferritin Induction—To assess ferritin H and \(\beta\)-actin protein levels, cytosolic extracts were prepared as previously described by Schreiber et al. (61). 50 \(\mu\)g of protein was fractionated on 12% SDS-polyacrylamide gels, transferred to nitrocellulose, blocked with 5% nonfat dry milk in phosphate-buffered saline, washed, and incubated with a 1:1000 dilution of polyclonal rabbit anti-ferritin H peptide antibody (BIOSOURCE International) followed by a 1:200 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad). The blots were developed using the Enhanced Chemiluminescence System (Amersham). To demonstrate \(\beta\)-actin protein levels, cytosolic extracts were fractionated on 12% SDS-polyacrylamide gels, transferred to nitrocellulose, blocked with 5% nonfat dry milk in phosphate-buffered saline, washed, and incubated with a 1:1000 dilution of polyclonal rabbit anti-\(\beta\)-actin antibody (Sigma) followed by a 1:5000 dilution of goat anti-mouse IgG conjugated to horseradish peroxidase (Calbiochem).

Transfection of Ferritin H-human Growth Hormone Reporter Gene Constructs and RNase Protection Assay—NIH3T3 cells were transfected in duplicate with 2 or 3 \(\mu\)g of FH-hGH reporter gene constructs using LipofectAMINE Reagent (Invitrogen) according to the manufacturer’s protocol. Cells were allowed to recover for 20–24 h and treated with 70 \(\mu\)g Oltipraz, 70 \(\mu\)g D3T, or vehicle (Me\(_2\)SO). DNA was isolated after 24 h, and RNase protection analysis (RPA) was performed as described previously (5). The fold induction was calculated based on means and standard errors of three to eight independent experiments.

Transfection of Ferritin H-luciferase Reporter Gene Constructs and Luciferase Assay—Hepa1-6 cells were transfected for 4 h with a total of 500 ng of DNA (FH-Luc reporter gene constructs, \(\beta\)-galactosidase trans-
fection control plasmid, and pUC18 as detailed in the respective figure legend) using the LipofectAMINE reagent (Invitrogen) according to the manufacturer’s procedures. Cells were allowed to recover for 20–24 h and subsequently were treated with 25 μM β-NF or vehicle (Me2SO) for 24 h. Cells were harvested, lysed in 1× reporter lysis buffer (Promega), and assayed for luciferase activity. Luciferase activity was assessed using the Luciferase Assay kit (Promega) according to the manufacturer’s protocol. Expression of the β-galactosidase transfection control was measured as previously described (62).

**Isolation of Nuclear Extracts and Electrophoretic Mobility Shift Assay**—Nuclear extracts were isolated as described previously (53). The oligonucleotides used in electrophoretic mobility shift assays correspond to the ferritin H AP1/NF-κB-like element (5’-CTCCATGCAAAAGGATT-3’) present in the ferritin H basal enhancer, FER-1, and the ferritin H API/NF-κB consensus element (5’-AGAATGCTGAGT-CACGGTG-3’). The double-stranded oligonucleotides were end-labeled with [γ-32P]ATP (ICN) using T4 Polynucleotide kinase. Competition experiments were performed using 100-fold molar excess of unlabeled oligonucleotides. Normal rabbit serum or Nrf2 antibody (Santa Cruz Biotechnology, sc-722x) were used in supershift experiments. DNA-protein complexes were isolated on a native 4% polyacrylamide gel (80:1, acrylamide:bisacrylamide). Gels were dried, and DNA-protein complexes were visualized by autoradiography.

**RESULTS**

**Xenobiotics and Chemopreventive Agents Induce Ferritin mRNA—β-NF is a polycyclic aromatic hydrocarbon that has been used to study activation of phase 2 enzymes by both XRE- and EpRE/ARE-dependent mechanisms (23, 24, 32, 63). Oltipraz is an electrophilic dithiolethione that represents a widely studied class of candidate chemopreventive agents. To create a model system in which to explore the mechanism of ferritin induction by these agents, we treated cultured liver cells for varying periods of time with β-NF or oltipraz. As shown in Fig. 1, both β-NF and oltipraz induced ferritin H and L mRNA in Hepa-1–6 cells in a time-dependent manner. We also performed Northern blot analysis of NIH3T3 fibroblasts treated with oltipraz and a second dithiolethione, D3T. As shown in Fig. 2, both these agents induced ferritin H and L mRNA in NIH3T3 cells. Induction of ferritin H as well as ferritin L mRNA by oltipraz in NIH3T3 cells was time-dependent and occurred as early as 3 h after treatment, with induction peaking at 24 h. mRNA induction was accompanied by an increase in ferritin protein of similar magnitude (Fig. 3). Induction of ferritin mRNA by oltipraz, D3T, and β-NF was also seen in HepG2 cells (data not shown). Taken together, these results demonstrate that β-NF, oltipraz, and D3T induce both ferritin H and L in a variety of cells, including murine and human hepatocytes and fibroblasts.

**Induction of Ferritin Is Mediated by the EpRE/ARE**—To assess the mechanism of ferritin induction, we transiently transfected NIH3T3 cells with a chimeric ferritin H-human growth hormone (FH-hGH) gene construct that spans 4.8 kb of the murine ferritin H promoter region fused to the human growth hormone reporter gene (−4.8kbFH-hGH). Subsequently, cells were treated with 70 μM oltipraz or 70 μM D3T. After 24 h, RNA was isolated and RNase protection analysis was performed to assess the induction of the reporter gene as well as the endogenous ferritin H gene. Fig. 4 demonstrates that both oltipraz and D3T induce the endogenous ferritin H mRNA by dithiolethiones. NIH3T3 cells were treated with 70 μM D3T for 24 h or 70 μM oltipraz for 0, 3, 6, 9, 12, and 24 h. Total RNA was isolated from cells, size-fractionated on denaturing agarose gels, transferred to nylon membranes, and allowed to hybridize with cDNA probes specific for ferritin H and L. Ethidium bromide staining was done to assure equal RNA loading. Shown are the average ± S.E. values for four experiments.
gene, confirming the Northern blot analysis shown in Fig. 2. In addition, induction of the −4.8kbFH-hGH reporter gene construct by oltipraz and D3T was observed. These results indicate that induction of ferritin H by oltipraz and D3T is mediated by a transcriptional mechanism.

To delineate the element responsible for transcriptional activation of ferritin H, deletion analysis was performed. FH-hGH 5' deletion constructs containing 4.8, 4.13, or 4.0 kb of the murine ferritin H 5’ flanking region were transiently transfected into NIH3T3 cells, treated with 70 μM oltipraz, and analyzed by RNase protection assay. As shown in Fig. 5, a region located between −4.13 and −4.0 kb of the ferritin H promoter is responsible for activation of ferritin H transcription. Thus, both the −4.8kbFH-hGH and −4.13kbFH-hGH constructs were induced by oltipraz, whereas the −4.0kbFH-hGH construct was not. Because the 75-bp ferritin H EpRE/ARE is located between −4.13 kb and −4.0 kb of the 5’ ferritin H promoter region, this result suggested that the ferritin H EpRE/ARE mediates induction of ferritin H in response to oltipraz. To test this, we used a chimeric gene in which a 107-bp region containing the ferritin H EpRE/ARE was inserted into a minimal ferritin H promoter construct (−0.32kbFH-hGH). As shown in Fig. 6, activity of this promoter was enhanced by oltipraz, demonstrating that the ferritin H EpRE/ARE is sufficient to mediate induction of ferritin H in response to oltipraz. Thus, ferritin H contains a functional EpRE/ARE that mediates responsiveness to oltipraz.

To determine whether the induction of ferritin H by β-NF utilized a similar mechanism, we first considered the potential role of XRE sequences. β-NF can activate transcription via both XRE- and EpRE/ARE-dependent mechanisms (16). The XRE consensus sequence has been defined as 5'-T(A/T)GCGTG-3’ (18), and functional XRE sequences have previously been genetically identified in the cytochromes P450 1A1 and 1A2 (18, 19, 64), GST Ya (20), UDP-glucoronyl transferase 1A1 and 1A6 (21, 22), NQO1 (23), and Cu/Zn-SOD gene (24, 25). Inspection of the ferritin H 5’ promoter sequence revealed five potential consensus XRE sequences, one on the sense strand and four on the antisense strand. Their specific locations and sequences are as follows. The XRE sequence present in the sense strand is located between −3089 and −3083 (5’-CTGCCTG-3’). The XRE sequences in the antisense strand are located between −4579 and −4573 (5’-CAGGAC-3’), −2817 and −2811 (5’-CAGGAC-3’), −413 and −407 (5’-CAGGTT-3’), and −351 and −345 (5’-CAGGAC-3’). To determine if these elements were functional, we prepared ferritin H-luciferase constructs (luciferase was used to simplify assessment of reporter gene expression) (Fig. 7). These constructs spanned −4.8, −4.2, and −4.0 kb of the murine ferritin H 5’ promoter region. In addition, to determine the involvement of the XRE sequence located from −4579 to −4573 independent of the ferritin H EpRE/ARE sequence (−4117 to −4043), an internal deletion construct was made in which 3.5 kb of the ferritin H promoter were removed from the full-length −4.8kbFH-Luc construct spanning a region from −4477 to −941. The ferritin H-luciferase 5’ deletion and internal deletion constructs were transiently transfected into Hepa1–6 cells. Subsequently, cells were treated with 25 μM β-NF for 24 h, and luciferase activity was determined. The constructs −4.8kbFH-Luc and −4.2kbFH-Luc demonstrated luciferase induction in response to β-NF, whereas −4.8kbΔ3.5kbFH-Luc and −4.0kbFH-Luc did not (Fig. 7).
Hence, induction of ferritin H by β-NF is mediated by an enhancer element located between 4.2 and 4.0 kb, a region that contains the ferritin H EpRE/ARE but none of the XRE sequences. To confirm that activation of ferritin H by β-NF occurred via the EpRE/ARE, the 75-bp EpRE/ARE element was inserted into a minimal promoter construct, which contains 225 bp of the ferritin H promoter (~0.225kbFH-Luc). As shown in Fig. 7, β-NF activates ferritin H transcriptionally via an EpRE/ARE- and not XRE-dependent mechanism.

Elements in the Ferritin H EpRE/ARE Bind Nrf2—Nrf2 is a member of the NF-E2 family of transcription factors that has been shown to mediate EpRE/ARE-dependent transcription of a variety of cytoprotective genes (36, 47, 56, 65). The ferritin H EpRE/ARE contains two elements with sequence similarity to the NF-E2 consensus sequence. The AP1 consensus sequence of the ferritin H EpRE/ARE is embedded in a canonical NF-E2 site; the AP1-like sequence in the ferritin H EpRE/ARE also possesses considerable sequence similarity to the NF-E2 consensus (9/11 residues) (Fig. 8). To examine the ability of Nrf2 to bind to the ferritin H EpRE/ARE, we performed electrophoretic mobility shift assays. As shown in Fig. 8, 75bpEpREFH-Luc mediated induction of luciferase activity, whereas ~0.225kbFH-Luc did not. Thus, ferritin H is transcriptionally activated by β-NF via a mechanism that depends on the EpRE/ARE and not the XRE sequences.

### Fig. 6
The ferritin H EpRE/ARE mediates transcriptional activation of ferritin H in response to dithiolethiones. A, schematic of the insertion constructs. 0.32kbFH-hGH contains 320 bp of the ferritin H promoter region fused to the human growth hormone reporter gene. 107bpEpEFRE-hGH is the same as ~0.32kbFH-hGH with the exception that a 107-bp region containing the 75-bp ferritin H EpRE/ARE was inserted in front of the 320-bp ferritin H minimal promoter region. B, FH-hGH reporter gene constructs were transiently transfected into NIH3T3 cells. 20–24 h after transfection, cells were treated with vehicle (Me2SO) or 70 μM oltipraz for 24 h. Total RNA was isolated, and 10 μg of RNA was used for RNase protection analysis. C, average ± S.E. of four independent experiments for induction of the transfected hGH gene.

### Fig. 7
β-NF activates ferritin H transcriptionally via an EpRE/ARE- and not XRE-dependent mechanism. A, schematic of the ferritin H-luciferase (FH-Luc) reporter gene constructs used. The internal deletion in the 4.8kb3.5kbFH-Luc construct is signified by a dotted line. The location of the EpRE/ARE and XRE sequences are indicated by black and gray rectangles, respectively. B, 50 ng of reporter gene plasmids and 10 ng of β-galactosidase transfection control plasmid were transfected together with 440 ng of pUC18 to generate a total of 500 ng of DNA. 20–24 h after transfection cells were treated with vehicle (Me2SO) or 70 μM β-NF for 24 h. Cell lysates were obtained to perform luciferase and β-galactosidase assays as described under “Experimental Procedures.” Luciferase values were normalized to β-galactosidase expression, and -fold induction was calculated with ~4.8 kb FH-Luc treated with vehicle defined as 1. Shown are the average ± S.E. values of three independent experiments.

### Fig. 8
Schematic of the 75-bp ferritin H EpRE/ARE. A, sequences above and below the schematic indicate the presence of AP1/NF-E2-like and AP1/NF-E2 consensus elements in this region. Arrows above and below the sequence denote the orientation of the transcription factor binding sites. B, listing of the EpRE/ARE, AP1, and NF-E2 consensus sequence.
To demonstrate that Nrf2 mediates transcriptional activation of ferritin H via an EpRE/ARE-dependent mechanism, nuclear extracts were isolated from HepG2 cells that had been treated with vehicle (MeSO), 70 μM oltipraz, or 25 μM β-NF for 6 h. 10–20 μg of extract was incubated with 32P-labeled AP1/NF-E2-like or AP1/NF-E2 consensus oligonucleotide (100,000–150,000 cpm), and 100-fold molar excess of specific (SC) and nonspecific (NC) competitors where indicated. A NFκB binding element (Promega) was used as a nonspecific competitor. Following a 20-min incubation at room temperature, an antibody specific for Nrf2 or normal rabbit serum (c) was added to the indicated samples for a 30-min room temperature incubation, an antibody specific for Nrf2 or normal rabbit serum was included. Bands are pointed out by the large arrows, and supershifted Nrf2 is marked by the small arrows.

Role for Nrf2 in Induction of Ferritin by Oltipraz, D3T, and β-NF—To test involvement of Nrf2 in ferritin induction, we compared the ability of Nrf2+/+ and Nrf2−/− primary mouse embryo fibroblasts to induce ferritin H and L in response to oltipraz, D3T, and β-NF. As shown in Fig. 10, induction of ferritin H and L mRNA was blocked in Nrf2−/− cells, whereas both ferritin H and L were induced in the Nrf2+/+ cells. Basal levels of ferritin H and L mRNA were also reduced in Nrf2 knockout cells, suggesting a role for Nrf2 in both basal and induced ferritin transcription.

Nrf2 Mediates Transcription of Ferritin H at the EpRE/ARE—To demonstrate that Nrf2 mediates transcriptional activation of ferritin H via an EpRE/ARE-dependent mechanism, we transiently cotransfected a Nrf2 dominant negative mutant expression plasmid and the 75bpEpREFH-Luc reporter gene construct into Hepa1–6 cells. As seen in Fig. 11, cotransfection of the 75bpEpREFH-Luc reporter gene construct with an empty expression vector (pEF) and subsequent treatment with 25 μM β-NF for 24 h resulted in induction of luciferase activity. However, cotransfection of a dominant negative mutant of Nrf2 (pEF/Nrf2dnm) and the 75bpEpREFH-Luc reporter gene construct suppressed β-NF-induced activation of luciferase activity. In addition, pEF/Nrf2dnm decreased basal activity of the 75bpEpREFH-Luc reporter gene construct, supporting the results in Fig. 10 indicating that Nrf2 affects basal as well as inducible ferritin H expression. Neither pEF/Nrf2dnm nor β-NF had any effect on basal or inducible expression of a ferritin H minimal promoter construct lacking the EpRE/ARE (−0.225kbFH-Luc) (Fig. 11). To confirm these results, 75bpEpREFH-Luc was cotransfected with increasing amounts of a 72bp expression plasmid (pEF/Nrf2). As shown in Fig. 12, Nrf2 activates luciferase activity in a dose-dependent manner. This response requires the EpRE/ARE, because −0.225kbFH-Luc was unaffected by increasing amounts of pEF/Nrf2 (Fig. 12).

DISCUSSION

Several laboratories have demonstrated that ferritin H and L are induced in response to oxidants and pro-oxidant xenobiotics (7–11), and a transcriptional mechanism has been impli-
of transcriptional activation of ferritin H in response to structs fused to the human growth hormone gene, the results
evidence for transcriptional activation of ferritin H and L in the
dithiolethiones oltipraz and D3T is the result of a transcrip-
tion in response to polycyclic aromatic hydrocarbons, such as β-NF. However, deletion analysis of the ferritin H 5’-pro-
moter region using ferritin H-luciferase (FH-Luc) reporter gene constructs demonstrated that these elements are non-func-
tional in ferritin induction by β-NF, because reporter con-
structs containing these putative XRE sequences but lacking the EpRE/ARE were not induced in response to β-NF (Fig. 7). Rather, the EpRE/ARE controls ferritin H transcription in response to β-NF (Fig. 7). Because the five XRE sequences closely resemble the consensus XRE sequence (5'-T(A/ T)GGCGTG-3') (18), it was surprising that none of these ele-
ments in the ferritin H promoter region conferred inducibility of the gene in response to β-NF. In particular, comparison of the ferritin H XRE sequences to XRE sequences identified previously in various genes showed that the ferritin H XRE sequences at nucleotides (nt) –4579 to –4573 and nt –351 to –345 are identical to the functional XRE identified in the rat UDP-glucoronol transferase 1A1 (21) and human CYP 1A2 (19) gene. Likewise, the XRE sequence at nt –3089 to –3083 and nt –2817 to –2811 are identical to the XREs present in the rat and human Cu/Zn-SOD genes, respectively (24, 25). It has been
suggested that the XRE core sequence itself is insufficient to confer transcriptional induction, and that additional nucleo-
tides flanking the core sequence exert an important influence on the ability of the Ah receptor to bind to the core XRE sequence (70). These contextual requirements may contribute to the inactivity of the XRE elements in the ferritin H promoter.

The electrophile/antioxidant-responsive element consensus sequence resembles the recognition sequence for transcription factors of the AP1 and NF-E2 family of DNA binding proteins, allowing for a wide variety of transcription factors to bind to this element and mediate basal as well as inducible transcription. Several laboratories have demonstrated the involvement of the transcription factor Nrf2 in inducing cytoprotective proteins in response to a variety of agents, including β-NF and dithiolethiones (47, 65, 71). Some genes require Nrf2 for basal as well as D3T-inducible transcription, whereas others showed induction by D3T independent of Nrf2 status (65). Results
presented here demonstrate that treatment of Nrf2+/+ and
Nrf2−/− primary mouse embryo fibroblasts results in induction of ferritin H and L mRNA in wild type but not Nrf2 knockout cells, indicating that Nrf2 is necessary for dithiothrione- and β-NF-induced transcription of both ferritin H and L. Gel shift and transfection experiments indicate that Nrf2-mediated induction of ferritin H targets the ferritin H EpRE/ARE (Figs. 9, 11, and 12). Although we did not quantitate nuclear Nrf2 following treatment with oltipraz, D3T, or β-NF, the increasing intensity in band shift seen in Fig. 9 is consistent with increased binding of Nrf2 to the ferritin H EpRE/ARE following stimulation with xenobiotics. This would be concordant with demonstrations of nuclear translocation of Nrf2 following treatment with inducing agents (36, 49).

Our experiments showed not only that Nrf2 is involved in activating transcription of ferritin H and L in response to dithiothriones and β-NF, but also suggested the involvement of Nrf2 in basal transcription of these genes, since expression of ferritin H and L mRNA was decreased in Nrf2−/− cells when compared with the wild type cells (Fig. 10). Effects on ferritin H basal transcription were confirmed by cotransfecting 75bpEpREFH-Luc with a dominant negative mutant of Nrf2, which both suppressed EpRE/ARE-dependent induction of ferritin H in response to an inducer and decreased basal expression. A similar involvement of Nrf2 in basal transcription has been reported for other genes, including glutathione S-transferases (72) and γ-glutamylcysteine synthetase (57).

The ferritin H EpRE/ARE has two sites with which Nrf2 interacts, namely the AP1/NF-E2 consensus sequence and the AP1/NF-E2-like element of FER-1 (Fig. 9). Although we did not examine the interaction of the ferritin L EpRE/ARE with Nrf2, inspection of the ferritin L promoter reveals a NF-E2 consensus sequence embedded in the ferritin L EpRE/ARE, suggesting that Nrf2 may be involved in the coordinate regulation of both ferritin subunits through targeting of the EpRE/ARE.

Nrf2 has been demonstrated to be important in the regulation of several EpRE/ARE-dependent genes. However, Nrf2-independent mechanisms of gene regulation at the EpRE/ARE also exist. For example, it was recently reported that the ability of the model chemopreventive agent sulforaphane to induce some but not all EpRE/ARE-dependent genes was abrogated in the intestine of Nrf2 knockout mice (73). Kwak et al. (65) reported that in the livers of Nrf2 knockout mice treated with D3T, induction of ferritin was enhanced rather than suppressed. These findings differ from results presented here that indicate Nrf2 is required for induction of ferritin by oltipraz, D3T, and β-NF. However, our experiments were performed in knockout cells derived from a different genetic background (74) than those used by Kwak et al. Given the existence of alternative pathways of regulation at the EpRE/ARE, and the potential of AP1 and Maf proteins and other transcription factors to modulate Nrf2 activity both positively and negatively, it is possible that the relative abundance and/or activity of such factors may influence gene inducibility. Thus, cellular context and genotype may determine the contribution of Nrf2 to the regulation of target genes. For example, in the GCSβ gene, higher concentrations of c-Jun repressed expression, presumably due to formation of c-Jun/c-Fos complexes that interfered with binding of the Nrf2/c-Jun complex to the EpRE/ARE (75). Genetic variation, including polymorphisms in Nrf2 itself, may add an additional level of complexity to such interactions (76).

Collectively, these observations may point to the existence of additional regulatory pathways that permit fine-tuning of the cellular response to xenobiotics, perhaps allowing the coordinate induction of subsets of antioxidant and detoxification genes in different cell types dependent on cellular context, genotype, and xenobiotic challenge.

We have previously identified a number of transcription factors that assemble at the FER-1 component of the ferritin H EpRE/ARE. These include members of the AP1 family such as JunD and FosB, and the ATF/CREB family member ATF1 (54). Results presented here implicate Nrf2 as an additional participant in this transcription factor complex, conferring added potential for both positive and negative regulation at this element. Nrf2 has been shown to bind to DNA as a heterodimer with small Maf (MafG and MafK) proteins, an interaction that can be negatively regulated by large Maf proteins such as c-Maf (77). Nrf2 may also interact directly with other transcription factors, such as AP1 (45, 47) and ATF4 (78). Because the DNA recognition sequence for AP1 and Nrf2 family members overlaps considerably, it has also been suggested that under selected circumstances Nrf2 function may be modulated indirectly through displacement of Nrf2 from DNA by AP1 family members (75). Nrf2 can also interact with global adaptor and chromatin remodeling factors such as p300/CAF1 (79). We have previously demonstrated that the p300/CAF1 transcriptional adaptor proteins are involved in mediating basal ferritin H transcription through the FER-1 element, possibly due to an interaction between p300/CAF1 and FER-1 binding proteins (80).

Indirect evidence indicates that p300/CAF1 may be necessary for EpRE/ARE-mediated induction of ferritin H, because NIH3T3 cells stably transfected with E1A lose the ability to induce ferritin H mRNA in response to t-BHQ, a classic inducer of antioxidant and phase II enzymes (67). These results suggest that Nrf2 may mediate some of its effects on basal and inducible ferritin H expression through interaction with p300/CAF1. Further studies will be required to test how the assembly of this complex array of transcription factors, cofactors, and chromatin remodeling factors is regulated at the ferritin EpRE/ARE.

Acknowledgments—We are grateful to Dr. Y. Tsuji for thoughtful advice and guidance and to Dr. J. Wilkinson for many helpful discussions. We thank Rong Ma for expert technical assistance.

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