Bach1 Repression of Ferritin and Thioredoxin Reductase1 Is Heme-sensitive in Cells and in Vitro and Coordinates Expression with Heme Oxygenase1, β-Globin, and NADP(H) Quinone (Oxido) Reductase1*§

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Ferritin gene transcription is regulated by heme as is ferritin mRNA translation, which is mediated by the well studied mRNA-IRE/IRP protein complex. The heme-sensitive DNA sequence in ferritin genes is the maf recognition/antioxidant response element present in several other genes that are induced by heme and repressed by Bach1. We now report that chromatin immunoprecipitated with Bach1 antisemur contains ferritin DNA sequences. In addition, overexpression of Bach1 protein in the transfected cells decreased ferritin expression, indicating insufficient endogenous Bach1 for full repression; decreasing Bach1 with antisense RNA increased ferritin expression. Thioredoxin reductase1, a gene that also contains a maf recognition/antioxidant response element but is less studied, responded similarly to ferritin, as did the positive controls heme oxygenase1 and NADP(H) quinone (oxido) reductase1. Bach1-DNA promoter interactions in cells were confirmed in vitro with soluble, recombinant Bach1 protein and revealed a quantitative range of Bach1/DNA stabilities: ferritin L ~ ferritin H ~ β-globin, β-globin ~ 2-fold > heme oxygenase1 = quinone reductase β-globin ~ 4-fold > thioredoxin reductase1. Such results indicate the possibility that modulation of cellular Bach1 concentrations will have variable effects among the genes coordinately regulated by maf recognition/antioxidant response elements in iron/oxygen/antioxidant metabolism.

Genes encoding proteins that manage proteins of iron and oxygen traffic and metabolism are at the nexus of chemical reactions that are both critical and dangerous to life. The iron porphyrin complex, heme, has emerged as a key signal for iron and oxygen metabolism genes, including ferritin L (ftl)3 (1) and ferritin H (fth) (2). Transcriptional regulation of NADP(H) quinone (oxido) reductase (qr) (3), heme oxygenase1 (ho1) (4), and β-globin (5) by heme requires the maf recognition/antioxidant response element (MARE/ARE), a conserved regulatory sequence found in the promoter or enhancer, and the heme binding transcriptional repressor Bach1.

Both fth and fth contain heme-responsive canonical MARE/ARE promoter sequences (1, 2). The role of Bach1 in heme-regulated ferritin transcription is not known. By contrast, the role of IRP1 and IRP2 in heme-regulated fth and fth mRNA translation is known (6–9). The translational mechanism uses IRP1 and IRP2 to coordinate fth and fth mRNA regulation with that of several other mRNAs important in iron and oxygen homeostasis by binding to iron-responsive elements (IRE) in each of the mRNAs. The IRE is a specific three-dimensional loop-helix-loop-helix structure (10) in the noncoding regions of the mRNAs (11–16). Specific interactions between the iron regulatory proteins IRP1 and IRP2 and the different IREs in the mRNAs create a natural, combinatorial array of RNA-protein complexes (17). In the case of ferritin, when IRP1 or 2 are bound to the mRNA the ability of eukaryotic initiation factor 4F to recruit translational machinery to the mRNA is compromised and translation is repressed (18). Heme is known to repress IRP1 and IRP2 binding to the IRE, thus increasing ferritin expression (9, 19, 20). To our knowledge, fth and fth are the only genes that have mechanistically distinct heme-responsive elements: the MARE/ARE in the DNA promoter and the IRE in the mRNA 5′-untranslated region (21). Together, they respond synergistically to cytoplasmic heme (1). Bach1 is a leucine zipper-basic region transcription repressor protein that regulates MARE/ARE genes β-globin, ho1, and qr (3, 4, 22). Heme reverses the repression by binding to Bach1 to prevent DNA binding, which facilitates binding of NrF2, the transcriptional activator (23). Heme also inactivates Bach1 by inducing nuclear export (24) and polyubiquitination followed by proteasome-mediated degradation (25). Manipulating

3 The abbreviations used are: fth, ferritin L; fth, ferritin H; ho1, heme oxygenase1; IRE, iron-responsive element; IRP1, IRP2, iron regulatory proteins 1 and 2; MARE/ARE, Maf recognition/antioxidant response element; qr, NADP(H) quinone (oxido) reductase; trr, thioredoxin reductase1; Ni-NTA, nickel-nitrilotriacetic acid; siRNA, small interfering RNA.

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Bach1 expression changed expression of MARE/ARE genes: (i) Mouse Bach1 knock-outs displayed significant increases in ho1 RNA in several tissues, which were diminished by concomitant knock out of Nrf2 (4); and (ii) Bach1 siRNA treatment of liver cells increased ho1 expression 7-fold (26).

To determine if Bach1 is the heme target in MARE/ARE-dependent regulation of ferritin and thioredoxin reductase1 (trr), in analogy to ho1, β-globin, and gr promoters, we compared effects of Bach1 siRNA and Bach1 overexpression in HepG2 cells on heme-regulated fil, fil, and trr expression and demonstrated that Bach1 was the heme-reversible repressor. In addition, using soluble, recombinant Bach1 and MARE/ARE-DNA duplexes (40 base pairs) in vitro, we showed heme-reversible binding of Bach1 to the fil MARE/ARE sequence. Furthermore, we demonstrated that Bach1 binding to fil, fil, trr, ho1, qr, and β-globin MARE/ARE sequences falls into three stability groups that may relate to differential regulation in vivo.

EXPERIMENTAL PROCEDURES

Cloning and Constructs

The fil promoter and MARE/ARE mutation constructs, cell culture experiments, and luminescence assay have been described previously (1). trr and qr promoter constructs known to contain a functional ARE (27) were used as positive controls.

Cell Culture

Hepa1c1c7, a mouse hepatoma cell line, was used for chromatin immunoprecipitation experiments and was maintained in Dulbecco’s modified Eagle’s medium (4500 mg/liter glucose; Sigma) supplemented with 10% fetal bovine serum and 100 unit/ml penicillin/streptomycin (Invitrogen). HepG2 cells, a human hepatoma line, were used for overexpression and siRNA studies and were seeded in collagen-coated, 12-well plates (160,000 cells/well) using minimal essential medium with 10% fetal bovine serum (Atlanta Biologicals) and 100 unit/ml penicillin/streptomycin (Invitrogen) and cultured in 95% ambient air, 5% CO2, as previously described (1). The concentration of heme (80 μg/ml) was selected for maximum induction of fil expression in HepG2 cells (1); preliminary data on fil induction showed similar effects with either hemin or rabbit hemoglobin. Microscopic observation of the HepG2 cells showed no evidence of toxicity under any of the conditions used.

RNA Analysis

Total RNA was isolated (RNasy; Qiagen) 24 h later. RNA concentrations of all genes were determined by reverse transcription PCR using TaqMan Gene Expression Assay kits (Applied Bioscience) and an ABI 7900 Sequence Detection System (Applied Bioscience). Differences in RNA concentrations were quantified by the cycles to fluorescence midpoint cycle threshold calculation \((2^{-\Delta\Delta C_t}})\) (where \(\Delta C_t\) = experimental gene – housekeeping gene), using β-actin as the housekeeping gene.

Chromatin Immunoprecipitation

The procedures were used as we have previously described them (28).

Chromatin Fixation and Purification—In brief, Hepa1c1c7 cells were fixed by adding formaldehyde to 1% final concentra-

tion for 5 min at 25 °C. Cells were then sonicated to prepare chromatin suspensions of 300 –1000-bp DNA in length. Immunoprecipitations were carried out as previously described (28). Antibodies used were anti-Bach1 (A1–6) (23) and normal rabbit serum (Jackson ImmunoResearch). Normal rabbit serum was used as a negative control. PCR reactions were carried out by using EX Taq DNA polymerase (Takara Shuzo). The primers were as follows: FTH (a gift from Dr. Atsushi Maruyama, Hirosaki University, Japan), 5′-CCTACCCCTCCATGAC- AAAG-3′ and 5′-AGGCAGGCCCCTCTGTTCGTTCT-3′; and fil, 5′-GCCCTTTAGGGAAGGGTAG-3′ and 5′-TAGGCTA-GCCACTCCAGGC-3′. Primers and chromatin immunoprecipitation conditions for mouse β-globin (HS-2), ho1 (E1), and Mcm5 were the same as before (29, 30).

Immunoprecipitation with Bach1 Antiserum—48 h after transfection with Bach1 siRNA, as described above, nuclear proteins were extracted from the cells using the NE-PER nuclear extraction kit (Pierce) and Halt Protease Inhibitor Mixture (Pierce). Equal amounts of nuclear protein (80 μg) from each treatment were run on a Tris-HCl, 12% SDS-PAGE gel (Bio-Rad). Proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad) using Towbin’s buffer (25 mM Tris, 192 mM glycine, 0.5% SDS, 20% v/v methanol) containing 20% methanol and a Mini Trans-Blot cell (Bio-Rad) run at 350 mA for 1 h. Membranes were incubated overnight at 4 °C in a solution of T-TBS containing 1% nonfat milk and 1,000-fold dilution of anti-Bach1 antiserum A1–6 raised in rabbit (24). Blots were washed in T-TBS (TWEEN 20-Tris-buffered saline, pH 7.6, 0.1% Tween 20, 20 mM Tris-Cl, 137 mM NaCl) and then incubated for 1 h with a 30,000-fold dilution of alkaline phosphatase-conjugated, anti-rabbit IgG antibody (Sigma), followed by washing in T-TBS. Blots were developed with incubation with 0.56 mM 5-bromo-4-chloro-3-indoyl phosphate (Sigma) and 0.48 mM nitroblue tetrazolium (Sigma) in 10 mM Tris/HCl and quantified by densitometry.

Bach1 Overexpression

The Bach1 overexpression plasmid was obtained from Origene (catalogue number SC107917); cells were transfected with 1 μg of the plasmid DNA 24 h after seeding, using FuGENE 6 transfection reagent (Roche Applied Science). Control cells were transfected with empty vector pCMV6XL-6. RNA was extracted and analyzed as described above.

Decreasing Bach1 mRNA

Targeting Bach1 RNA—A complex of four unique siRNA duplexes (obtained from a commercial vendor) (Dharmacon) that were developed and described by Shan et al. (26) was used to decrease Bach1 RNA. A titration showed that 50 nM siRNA decreased Bach1 RNA more than either 25 or 100 nM siRNA. The cells were transfected using Dharmafect 2 transfection reagent according to the manufacturer’s protocol (Dharmacon) 24 h after seeding. Control cells were transfected with 50 nM siControl Non-targeting Duplex 1 (Dharmacon), consisting of four siRNA duplexes designed to avoid inhibiting any known eukaryotic genes. Bach1 siRNA-treated cells and controls were treated with Me2SO (0.2%) or Me2SO (0.2%) with hemin (80
μM) 24 h after transfection. The final concentration of Me₂SO was 0.2% for all treatments.

**RNA Analysis**—Total RNA was analyzed by reverse transcription PCR as described above.

**Luciferase Reporter Experiments**—4 h after siRNA transfection, the cells were co-transfected with pRLSV40 Renilla luciferase, a control for transfection, and the experimental luciferase constructs (test promoter + firefly luciferase). siRNA-treated cells and controls were treated with Me₂SO (0.2%) or Me₂SO (0.2%) control for transfection, and the experimental luciferase constructs.

**MARE/ARE DNA Gel Retardation Assay**—32P-labeled single-stranded DNA (40 bp) labeled with 32P on one strand. Recombinant, soluble Bach1 from E. coli was used with double-stranded DNA (40 bp) labeled with 32P on one strand. Bach1/DNA binding was used to assess transactivation potential of protein-DNA binding compared to Bach1/DNA complexes purified with MicroSpin G-50 columns (Amersham Biosciences) followed by annealing to the complementary strand to make 32P-labeled, double-stranded probes. Mouse β-globin containing the tandem MARE of HS2 (22) and human ho1 (35) MARE sequences were used as positive controls because they are known to be regulated by Bach1. Gene sequences and accession numbers are shown in Table 1. DNA and DNA-protein complexes were resolved by 5% polyacrylamide gel, dried, and analyzed using phosphoimager. To quantify the results, the intensities of bound or unbound [32P]DNA were determined using ImageQuant software. The percent shift was calculated as follows: ([32P]DNA:protein intensity/[32P]DNA intensity + [32P]DNA:protein intensity) × 100. To compare Bach1-DNA interactions among the different MARE/ARE sequences, the % bound DNA (1.6 pm) was plotted versus the protein:DNA ratio over the range zero, 9:1, 18:1, 36:1, and 72:1. The apparent Kd (protein concentration required for 50% DNA binding) for each DNA duplex was calculated as previously described (36). The data (Table 1) are presented as the mean (n = 3) with the error as the standard deviation.

**RESULTS**

Bach1, FTL, and FTH MARE/ARE DNA Sequences Are Co-precipitated from Chromatin of Cultured Cells—The similarity of the MARE/ARE promoter sequences and regulation of ferritin genes (1, 2) to that of other MARE/ARE genes that are regulated by Bach1 (3, 4, 30, 37) suggests that Bach1 will be present in chromatin complexes that contain ftl and fth DNA sequences. To test the hypothesis, we examined the DNA immunoprecipitated from Hepa1c1c7 cell chromatin with antibodies to Bach1. The data show (Fig. 2) that both fil and fth DNA sequences were contained in the Bach1 antiserum precipitate. β-globin and ho1 sequences were also precipitated by the same antiserum as previously observed; qr sequences have also been found in Bach1 immunoprecipitates of chromatin (3, 4,
Bach1 Regulation: Ferritin and Other MARE/ARE Genes

Bach1 in chromatin from Hepa1c1c7 cells was analyzed by immunoprecipitation with Bach1 antibodies or normal rabbit serum (NRS). Gel images are shown of PCR products of ftl (ferritin H) and ftl (ferritin L) with β-G (β-globin H5-2) and ho1 (HO1 E1) as positive controls and Mcm5 promoter as a negative control. The results are representative of three independent experiments.

FIGURE 2. Bach1 binds to FTL and FTH MARE/ARE DNA in chromatin of cultured cells. Bach1 in chromatin from Hepa1c1c7 cells was analyzed by immunoprecipitation with Bach1 antibodies or normal rabbit serum (NRS). Gel images are shown of PCR products of ftl (ferritin H) and ftl (ferritin L) with β-G (β-globin H5-2) and ho1 (HO1 E1) as positive controls and Mcm5 promoter as a negative control. The results are representative of three independent experiments.

30, 37). Thus, based on chromatin precipitation, ftl and ftl are members of the Bach1-regulated gene family.

Effects of Changing Bach1 Concentrations on MARE/ARE Gene Expression—We reasoned that the turnover of Bach1-DNA interactions indicated in earlier studies (23, 30) could mean that MARE/ARE DNA were not saturated even under steady state conditions. If so, increased expression of Bach1 might further repress MARE/ARE genes. To test whether Bach1 regulates ftl and ftl genes, we examined effects of Bach1 overexpression. The concentrations of ftl, ho1, trr, and qr RNA decreased when HepG2 cells were transfected with a Bach1 cDNA expression plasmid (Fig. 3); Bach1 mRNA increased significantly. Hemin reversed the repression caused by increased Bach1 expression (Fig. 3), confirming earlier results for ho1 (4). The results also indicate that constitutive levels of Bach1 are insufficient to repress fully all the MARE/ARE genes. In addition, endogenous heme may decrease Bach1 concentrations in the HepG2, as recently indicated in murine embryonic fibroblasts when heme synthesis was inhibited (25). The similar contrasts with some previous observations, e.g. Ref. 22. In one case where small Maf protein was required for Bach1 binding, high concentrations of urea used in the isolation may have led to misfolding of Bach1, which was reversed in the presence of small maf protein (21). In another case (3), the V5 conjugate of Bach1, and residual reticulocyte proteins that bound Bach1, may have altered Bach1-DNA interactions that were also rescued by small maf proteins (3). Determination of the stoichiometry of binding of Bach1 to each DNA sequence (32) is outside the scope of this report where the focus is on comparing and enlarging the size of the Bach1/MARE-ARE family of genes and providing the first insight to differences in the Bach1 inter-

FIGURE 3. Increasing Bach1 expression increases repression of MARE/ARE genes. HepG2 cells were transfected with DNA encoding Bach1 in plasmid in pCMV6-XL6 (Origene) (1 μg DNA/well). Control cells were transfected with pCMV6-XL6. Me2SO (0.2%), with or without hemin (80 μM) was added 20 h after transfection; after 24 h RNA concentrations of Bach1, ftl, qr, trr, and ho1 were determined by real-time PCR. The data were normalized to the control values shown as the dashed line. The experiments were carried out at the same time for both promoters, allowing the use of the same control within each of three independent experiments. Data are presented as averages (n = 3) ± S.D. *, significantly different from control (p < 0.05).
actions among them. Sorbital and betaine, known to improve
the solubility of recombinant proteins expressed in E. coli (31),
were used in this study to obtain soluble Bach1 protein that
could be isolated without urea. All or any of these factors, sol-
ubility, absence of urea, and/or a long V5 tag or competing
protein may relate to the binding of Bach1 to MARE/ARE DNA
in the absence of small Maf proteins.

Hemin reversed the Bach1-
ftl MARE/ARE DNA interactions
in vitro (Fig. 4, right), supporting conclusions that the effects of
hemin on MARE/ARE genes observed in vivo reflect heme-
Bach1 interactions (1–4). To determine whether the heme
effect on Bach1/DNA binding was iron-dependent, cobalt pro-
toporphyrin IX was tested under the same conditions as hemin
and also blocked Bach1-DNA interactions in vitro (Fig. 4, right).
The in vitro results complement in vivo studies that have dem-
onstrated ho1 induction by cobalt-protoporphyrin (38–40).
Protoporphyrin IX alone had no effect on ftl MARE/ARE-
Bach1/DNA interactions in vitro (data not shown), contrasting
with ftl studies in HepG2 cells (1). Thus, a metalloporphyrin
appears to be required to reverse Bach1/DNA binding in vitro.

Bach1 Concentrations Required for Full Binding in Vitro Vary
among Different MARE/ARE Gene Sequences—The limited
endogenous concentrations of Bach1 suggested by the
increased repression when Bach1 was overexpressed in HepG2
cells (Fig. 3) could mean that under conditions where Bach1 is
limiting, some MARE/ARE DNA sequences form more stable
complexes with Bach1 than others do. To determine if there are
variations in the stability of Bach1 complexes with different
MARE/ARE DNA sequences, we compared the percentage of
DNA bound by equal concentrations of DNA with ftl, ftth, qr,
ho1, or trr MARE/ARE DNA sequences at different stoichiom-
etries of soluble, recombinant Bach1 protein. The range of pro-
tein/DNA ratios was 0–72:1, and the DNA concentrations were
1.6 pm (Fig. 5). A protein:DNA ratio of 72:1 caused a complete
or nearly complete binding of ftl, ftth, qr, β-globin, and ho1
MARE/ARE DNA (Fig. 5). However, at lower Bach1 concentra-
tions, each DNA displayed quantitative differences in the frac-
tion of DNA complexed to Bach1 (Table 1, Fig. 5).

trr MARE/ARE DNA–Bach1 complexes were less stable
than the other MARE/ARE sequences tested. Only ~40% of the
trr DNA was bound at the protein:DNA ratio (72:1) sufficient
to bind all the DNA of the other MARE/ARE sequences (Fig. 5).
The sequence of the trr MARE/ARE deviates the most from
the other MARE/ARE genes (Table 1). Three groups of
DNA-protein complex stabilities were observed (Table 1, Fig.
5): (i) The same as β-globin (ftl and ftth); (ii) stability signifi-
cantly different from β-globin (ho1, qr, trr); and (iii) significantly
(p < 0.05) different from trr (β-globin, ftl, ho1, and qr).

DISCUSSION

Bach1 binding to the chromatin region of ftl and ftth genes
observed in this study indicates that Bach1 is the heme-sensi-
tive repressor of these genes and can explain the molecular
basis of the previously observed heme-mediated regulation of
ftl and ftth genes (1, 2). Ferritin genes expand the family of
Bach1-regulated genes from those involved in oxygen, heme,
and quinone metabolism (3, 4, 26, 41) to genes for iron metab-
olism. The increased repression observed when Bach1 was
overexpressed (Fig. 3) indicates that endogenous concentra-
tions of Bach1 were too low to fully repress either ftl, ftth, trr,
or the positive control MARE/ARE genes ho1, β-globin, and qr.

The observation that hemin reversed the repression by high
concentrations of Bach1 (Fig. 3) emphasizes that Bach1 repres-
sion reflects both Bach1 protein concentrations and other cel-
ular factors such as heme. Down-regulation of Bach1 was also
observed in cells expressing hepatitis C proteins (42).

Comparison of the different MARE/ARE gene sequences
(Table 1) that share Bach1 binding and heme regulation (4, 26,
Bach1 Regulation: Ferritin and Other MARE/ARE Genes

TABLE 1
Homology of MARE/ARE sequences and recombinant Bach1 binding characteristics

| Gene | MARE/ARE probe sequence | % DNA shifted<sup>a</sup> | K<sub>p</sub> <sup>b</sup> |
|------|--------------------------|-------------------------|--------|
| β-G  | TGGAGCAAGACATTCTGACATGCTGGCAGATCATGGCTGAG | 38.3 ± 22.9 | 45.9 ± 7.3 |
| fil  | GGCGCTGACGACATTCTGACATGCTGGCAGATCATGGCTGAG | 34.8 ± 21.6 | 56.3 ± 11.9 |
| hfb  | CACCCCGAGATTGCTGACATGCTGGCAGATCATGGCTGAG | 32.2 ± 19.3 | 53.9 ± 17.6 |
| qr   | CAAATCCAGACATTCTGACATGCTGGCAGATCATGGCTGAG | 15.4 ± 11.4 | 69.4 ± 11.9 |
| hoi  | TTTTCTGATATTCTGACATGCTGGCAGATCATGGCTGAG | 9.9 ± 12.6 | 82.5 ± 18.8 |
| ttr  | ATCCAGCAGAGATTCTGACATGCTGGCAGATCATGGCTGAG | 2.5 ± 0.2 | 165.2 ± 22.1 |
| h  | ND<sup>c</sup> | 11.1 ± 4.9 | NA<sup>d</sup> |

<sup>a</sup> Gene names, location from the transcription start site or locus control region, and GenBank<sup>TM</sup> accession numbers: human ferritin L (β-G, −34370, AF139813), human heme oxygenase1 (ho1, −3947, D17675), human NADP(H) quinone (oxido) reductase1 (qr, −457, M81596), mouse β-globin (β-G, HS2 locus control region, M57463), human thioredoxin reductase1 (trr, −57 AF247671), human ferritin L MARE/ARE mutation (fil). 20-bp core sequences (1) are underlined.

<sup>b</sup> DNA sequence used in gel shift assays. Underlined portions are core sequences; bolded indicate mutation.

<sup>c</sup> Molar ratio soluble recombinant Bach1:<sup>32</sup>P DNA probe.

<sup>d</sup> Apparent K<sub>p</sub>, Picomoles of Bach1 protein needed to shift 50% of the<sup>32</sup>P probe.

<sup>e</sup> Statistically different from β-globin, the best characterized MARE/ARE, p < 0.05 (Student’s t-test).

<sup>f</sup> ND, not detected; NA, not applicable (binding abrogated).

41) and the different stabilities of the DNA-repressor complexes in vitro suggest the possibility that differential MARE/ARE promoter binding to Bach1 in vivo will amplify effects of changes in Bach1 protein concentrations. Exploration of effects of combinations of small Maf and Bach1 protein that have been observed in vivo or with insoluble recombinant or V5-tagged Bach1 (3, 22) are outside the scope of this report and are also technically daunting since, to date, small Bach1 without any tag cannot be isolated as a soluble protein. Analysis of Bach1 binding stoichiometry for the different MARE/ARE is a study for the future.

The addition of ferritin H and L genes to the Bach1/MARE/ARE transcription family emphasizes the roles of ferritins in minimizing oxidative stress. Ferritin traps both reactants in oxy/radical chemistry in the solid mineral of a protein cage (Fe<sup>2+</sup>/O<sub>2</sub> in eukaryotes or Fe<sup>2+</sup>/O<sub>2</sub> or H<sub>2</sub>O<sub>2</sub> in bacteria) and this fact is sometimes overlooked. The role of ferritin in response to oxidative stress is illustrated by a number of studies and this fact is sometimes overlooked. The role of ferritin in response to oxidative stress is illustrated by a number of studies and this fact is sometimes overlooked. The role of ferritin in response to oxidative stress is illustrated by a number of studies and this fact is sometimes overlooked.

44–46) Throughout the study we have used the term MARE/ARE sequences to group the six genes under study. The MARE sequence, (TGCTGAG/CTCAGCA) (47–49) present in hoi and β-globin genes is similar to the consensus core sequence of the ARE (TGACnnnGC) in Phase II antioxidant response genes qr, ttr, and ferritin (fil and hfb) (Table 1) (1, 3, 27). For that reason, we combined the two terms. Moreover, the genes were all regulated similarly by Bach1 and heme when compared under the same condition as in this study. The shared regulatory mechanisms and sequences between the two cis-element families suggest that they could be merged. However, it remains to be experimentally determined if all members of the MARE/ARE gene family are regulated by Bach1.

Heme, an important signaling molecule in oxidative stress pathways, links regulation of genes encoding the oxidant-protection and antioxidant proteins such as hoi, fil, fl, qr, and ttr (1, 3, 23, 26, 27) with the oxygen-carrying protein, hemoglobin. The proposed regulatory mechanism is heme-induced displacement of Bach1 followed by Nrf2 binding on the MARE/ARE DNA for both qr (3) and hoi (23). However, the effect of increasing Bach1 on repression of the MARE/ARE genes illustrates the opportunities for changing Bach1/DNA binding by direct heme binding as in Fig. 4 or by changing nuclear transport and degradation of Bach1. The identification of a range of Bach1:MARE/ARE DNA stabilities for the different genes in the family may be useful for understanding quantitative variations in responses of Bach1-regulated genes to signals such as heme.

Ferritin MARE/ARE-controlled transcription and IRE-controlled translation use a common pathway for heme-induced degradation of the DNA repressor Bach1 and the RNA repressor IRP2. Recent data show polyubiquitination of Bach1 by the ubiquitin E3 ligase HOIL-1 in response to heme (25), which is the same ligase that modifies IRP2 in response to heme (7, 9).

Thus, the observation of heme regulatory synergy by heme when both the DNA and RNA promoters were combined (1) can be explained by HOIL-1 modification of both Bach1 and IRP2. Sharing regulatory elements in DNA among ferritin and other antioxidant response genes and in mRNA among ferritin and other iron-trafficking genes is complemented by sharing the same regulatory molecule, heme, for protein repressors of the DNA and RNA promoters to coordinate ferritin expression with iron metabolism and oxidative stress protection.

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<sup>a</sup> Gene names, location from the transcription start site or locus control region, and GenBank<sup>TM</sup> accession numbers: human ferritin L (β-G, −34370, AF139813), human heme oxygenase1 (ho1, −3947, D17675), human NADP(H) quinone (oxido) reductase1 (qr, −457, M81596), mouse β-globin (β-G, HS2 locus control region, M57463), human thioredoxin reductase1 (trr, −57 AF247671), human ferritin L MARE/ARE mutation (fil). 20-bp core sequences (1) are underlined.

<sup>b</sup> DNA sequence used in gel shift assays. Underlined portions are core sequences; bolded indicate mutation.

<sup>c</sup> Molar ratio soluble recombinant Bach1:<sup>32</sup>P DNA probe.

<sup>d</sup> Apparent K<sub>p</sub>, Picomoles of Bach1 protein needed to shift 50% of the<sup>32</sup>P probe.

<sup>e</sup> Statistically different from β-globin, the best characterized MARE/ARE, p < 0.05 (Student’s t-test).

<sup>f</sup> ND, not detected; NA, not applicable (binding abrogated).

<sup>4</sup> K. Igarashi, K. Hintze, and E. C. Theil, unpublished observations.
