Ferritin plays an important role in the storage and release of iron, an element utilized in cellular processes such as respiration, gene regulation, and DNA replication and repair. Ferritin in animals is composed of 24 ferritin L (FTL) and ferritin H (FTH) subunits in ratios that vary in different cell types. Because the subunits are not functionally interchangeable, both L and H units are critical for maintaining iron homeostasis and protecting against iron overload. FTL and FTH are regulated primarily at a post-transcriptional level in response to cellular iron concentrations. Individual regulation of FTL and FTH is of much interest, and although transcriptional differences between FTL and FTH have been shown, differences in their post-transcriptional regulation have not been evaluated. We report here that FTL and FTH are differentially regulated in 1% oxygen on a post-transcriptional level. We have designed a quantitative assay system sensitive enough to detect differences between FTL and FTH iron regulatory elements (IREs) that a standard electrophoretic mobility shift assay does not. The FTL IRE is the primary responder in the presence of an iron donor in hypoxic conditions, and this response is reflected in endogenous FTL protein levels. These results provide evidence that FTL and FTH subunits respond independently to cellular iron concentrations and underscore the importance of evaluating FTL and FTH IREs separately.

Iron, although central to human health, is the keystone in many human diseases. Excess iron can contribute to the formation of reactive oxygen species, leading to protein, lipid, and DNA damage (1). Iron accumulation is a hallmark symptom of both aging and several neurodegenerative diseases; therefore, understanding the dynamic mechanisms of iron regulation may be the key to treatments and cures. The iron storage protein ferritin is pivotal to coordinating iron metabolism. Ferritin is composed of L and H subunits that, although highly conserved, are genetically separate (2, 3) and maintain distinct functions (for review see Refs. 4 and 5). Ferritin H (FTH)² subunits exhibit ferroxidase activity, converting Fe⁴⁺ to Fe³⁺ so that iron may be stored in the ferritin mineral core (6). Storage and protection of the iron prevents undesirable reactions of Fe³⁺ with oxygen, which may result in the production of damaging reactive oxygen species (7, 8). Storage of the iron by ferritin also protects the cell against the formation of insoluble ferric oxide; blocking the oxidation of Fe⁴⁺ to Fe¹⁺, a reaction otherwise favored at physiological pH and oxygen tension. Ferritin L (FTL) subunits, although void of catalytic activity, are thought to facilitate nucleation and mineralization of the iron center (9). H and L subunits spontaneously assemble in a 24-subunit protein with a flexible H:L ratio (10). The H:L ratio can vary between different cell types (11) but is usually consistent within a cell, except in the case of chronic iron overload (12–14).

Effectively regulating subunit ratio as well as total ferritin is critical to cell survival. FTL and FTH subunits are not interchangeable, and FTL is unable to compensate for the function of FTH in knock-out mice (15, 16). Mutations in ferritin H and L subunits are associated with several human diseases correlating with iron overload, such as neuroferritinopathy (17–19). Several studies have also uncovered a relationship between FTH and cancer (20), linking ferritin expression and cell proliferation (21). The mechanisms associated with differential regulation of FTL and FTH in disease are not well understood, highlighting the need for both focused research in the area and for more refined molecular tools to complete the task.

Because iron must be both readily accessible for cell metabolism and easily stored to prevent cell damage, a tight hierarchy of ferritin transcriptional and translational regulation mechanisms exists (for review see Refs. 20 and 22). Although differential regulation of FTH and FTL subunits has been shown on a transcriptional level (13, 22–24), it has not been shown in a cell line on a post-transcriptional or translational level. Post-transcriptional control of both FTL and FTH is exerted by iron regulatory proteins (IRP1 and IRP2). IRP binding interactions with an iron regulatory element (IRE), a stem-loop structure in the 5′-untranslated region of the ferritin transcript, prevent translation of the transcript, and binding is in direct response to primary signals in the cellular environment, such as oxygen and iron concentration. IRE structures control the translational regulation of ferritin L and H. IREs are stem-loop structures 20–30 nucleotides in length with a conserved CAGUG apical loop sequence (25). In addition, an optional C-bulge is often found in the IRE sequence. This structure within the stem may be needed for efficient IRP binding (26, 27). Ferritin L and H

*This work was supported in part by a grant from the Friedreich Ataxia Research Alliance. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: FTH, ferritin H; FTL, ferritin L; IRE, iron regulatory element; IRP, iron regulatory protein; hRLuc, humanized Renilla luciferase; FLuc, firefly luciferase; FAC, ferric ammonium citrate; DFO, desferrioxamine; PBS, phosphate-buffered saline; NT, no treatment; cAcon, cytosolicaconitase.
IREs have only 49.5% identity, and because small changes in the nucleotide sequence in both stem and loop translate to larger changes in IRP binding ability (28–31), differences in IRE structures may be the basis for differential translational regulation of L and H subunits. In turn, IRP1 and IRP2 are differentially sensitive to IRE structure (32–34). IRP1 binding to the IRE encompasses the stem-loop (35, 36) and has been shown to be more tolerant of base pair changes (37). IRP2 is more responsive to changes in the stem section of the IRE (30). The result is that IRP1 and IRP2 bind overlapping but distinct sets of IRE targets (37); this, combined with sequence variation in the known IREs, suggests a physiological graded response to iron.

To address this issue we designed a novel in vivo reporter system that isolates and quantifies IRE/IRP interaction, improving upon prior systems (38–40), with the advantages of co-expression from a bi-directional promoter in stably transfected cells. We exploited this reporter system to discern distinct differences in FTL and FTH translation in response to iron replete and iron deplete conditions in normoxic and hypoxic environments. We found that in lowered oxygen conditions (1% oxygen) FTL is the primary responder to an increase in iron concentrations on a translational level. This differential response was not seen in typical normoxic (20% oxygen) tissue culture conditions. To our knowledge, this is the first study to quantify differential regulation of FTL and FTH at the translational level.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rabbit polyclonal antibody against FTL and mouse monoclonal antibody against B-actin were from Sigma. The rabbit polyclonal antibody against FTH was from Abcam (Cambridge, MA). The chicken polyclonal antibody against IRP1 was kindly provided by Dr. Betty Leibold (University of Utah, Salt Lake City, UT). The rabbit polyclonal antibody against IRP2 was from Santa Cruz Biotechnology, Inc. (Cambridge, MA). The chicken polyclonal antibody against IRP1 was kindly provided by Dr. Betty Leibold (University of Utah, Salt Lake City, UT). The rabbit polyclonal antibody against IRP2 was kindly provided by Dr. Betty Leibold (University of Utah, Salt Lake City, UT). These antibodies were from Sigma unless otherwise stated. All of the other chemicals were from Sigma unless otherwise stated. All of the tissue culture reagents were from Invitrogen unless otherwise stated.

**Construction of Plasmids/Vectors**—To isolate the post-transcriptional effects of IRP/IRE binding, a series of reporter constructs were assembled under the control of a bidirectional promoter that connected the forward (hRLUC) and reverse (hFLUC) genes (41). Our promoter system was designed to work with the constitutively expressed tet repressor expressed in the Invitrogen Flp-In T-REx system. To generate stable cell lines, Flp-In™ T-REx-293 cells were co-transfected with either pBL-16-FTL or pBL-16-FTH and the Flp™ T-REx recombinase plasmid pOG44 using Lipofectamine 2000. The cells were not passaged more than five times before transfection. Stable integrants were selected by culture in hygromycin B (75 μg/ml) and blasticidin-HCl (15 μg/ml). Individual colonies were isolated and used for assays. All of the cell lines were maintained in Dulbecco’s modified Eagle’s medium high glucose and 10% fetal bovine serum (HyClone, Logan, UT). A minimum of 20 colonies for each construct was evaluated for comparable hRLUC/FLUC expression to determine that the cell lines used in our experiments were a valid representation of the indicated construct. Cell lines were induced with 1 μg/ml doxycycline (Sigma) for 24 h before use in the luciferase experiments. Hypoxic samples were incubated in a Billups-Rothenberg hypoxia chamber (Del Mar, CA), in 1% O2, 5% CO2, balance N2.

**Dual Luciferase Assay**—The dual luciferase reagent kit from Promega was used according to the manufacturer’s directions. The cells were seeded in a 96-well solid-bottomed DYNEX Microlite TCT (tissue culture treated) white luminescence plate and induced with doxycycline for 24 h. After 24 h of induction, each well was washed twice with PBS, pH 7.4 (Invitrogen), and lysed according to the manufacturer’s instructions. All of the luminescence assays were performed in a Turner Biosystems Veritas plate-reader luminometer (Turner Biosystems, Sunnyvale, CA) with an integration time of 10 s according to the Promega dual luciferase reagent protocol.

To make a positive control cell lysate, the BI-16-CON cell line was induced with doxycycline for 24 h, lysed in large quantities, and stored at −80 °C. Two 20-μl aliquots of the positive control were used in each plate for each luminescence assay. In addition, two 20-μl aliquots of a negative control lysate, made from the parental HEK 293 Flp-In™ T-REx cell line lacking luciferases, as well as two 20-μl reagent-only (no cell lysate) controls, were also included in each plate. The plates were kept protected from the light before initiating the luminometer assay.

**Real Time Reverse Transcription-PCR**—HEK 293 cells were grown for 24 h under normoxic (20% O2) and hypoxic (1% O2), with no treatment (NT), 50 μg/ml ferric ammonium citrate (FAC), or 100 μM desferrioxamine (DFO). RNA was extracted from the cells and made into cDNA using SuperScript III™ Celldirect cDNA synthesis system (Invitrogen) as per the manufacturer’s protocol. Quantitative real time PCR
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was carried out in a Stratagene MX3000 sequence detection system (Stratagene, La Jolla, CA), using TaqMan Universal PCR Master Mix according to the manufacturer’s recommendations (Applied Biosystems, Foster City, CA). TaqMan primer sets designed for hRLUC and FLUC were purchased from IDT (Coralville, IA) and used at a final concentration of 400 nM. The primer sets were: FLUC sense, aagattcaaagtgcgctgctggtg; FLUC antisense, ttgcctgatacctggcagatggaa; hRLUC sense, aagattcaaagtgcgctgctggtg; hRLUC antisense, tggacgatgcctgatcttgctgctg; and hRLUC probe, 5'--FAM-ttagtgtagctgctgacactccaaa-TAMRA-sp-3'. The cycling conditions were 10 min at 95 °C and then 40 cycles of 95 °C for 30 s, 55 °C for 1 min and 72 °C for 30 s. Standard curves for each primer set were made with dilutions of pBl-16 CON. The data were analyzed using the Mx3000P software (version 2.0).

**Immunoblots**—HEK 293 Flp-In™ T-REx cells were plated at <50% confluency and treated the next day with complete medium (NT) or medium supplemented with 50 μg/ml FAC or 100 μM DFO. The cells were then grown under normoxic (20%) or hypoxic (1%) conditions for 24 h. The cells were scraped and lysed in 2X Laemml Buffer (20% glycerol, 2% SDS, 100 mM Tris, pH 6.8, fresh 125 mM dithiothreitol). IRP1 and IRP2 Western blots were performed by loading 100 μg of protein on an 8% SDS-PAGE gel (37.5:1). FTL and FTH Western blots were performed by loading 40 μg of protein on a 15% SDS-PAGE gel (37.5:1). The proteins were transferred to Immobilon P™ membrane (Millipore, Billerica, MA) using a Bio-Rad semi-dry transfer apparatus and blocked overnight at 4 °C in 20% evaporated Carnation milk/PBS mixture.

Mouse anti-human IRP2 and chicken anti-rat IRP1 antibody were used at 1:500 and 1:1,000 dilutions, respectively. FTL and FTH rabbit polyclonal antibodies were used at 1:10,000 and 1:100,000 dilutions. Mouse anti-actin antibody was used at a 1:5000 dilution. Appropriate horseradish peroxidase-conjugated secondary antibodies were used as directed by the manufacturers, followed by visualization using ECL Advance™ (Amersham Biosciences). The images were obtained with the Kodak Gel Logic 440 imaging system (Rochester, NY) and analyzed with Kodak molecular imaging software (version 4.0).

To compare the intensity of the bands under different treatment conditions, a reference lane was used. This reference lane was FAC (normoxia) for the FTL and FTH1A slots and no treatment (hypoxia) for IRP1 and IRP2 Western slots. The selection of the reference lane was based on the low (almost background) protein levels of the no treatment (normoxic) samples.

**Bandshift Assays**—Unmodified Flp-In™ T-REx-293 cells were used for bandshift assays. The cells were washed with PBS and lysed in 60 μl of lysis buffer (20 mM HEPES, pH 7.5, 25 mM MgCl₂, 0.5% Nonidet P-40) and 1:100 EDTA-free protease inhibitor in a 60-mm plate. The cells were lysed the day of the assay and were not subject to freezing to avoid destabilizing the cytosolic aconitase iron sulfur cluster. 12 μg of protein was used in each sample.

[32P]RNA FTL and FTH transcripts were synthesized using XbaI-linearized pSP72/FTL/T7 or pSP72/FTH/T7 plasmid. Transcription reactions were performed using 5 μl (50 μCi) of [α-32P]UTP (800 Ci/mmol) (MP Biomedicals, Solon, OH), and 1 μl of T7 RNAP containing an RNase inhibitor (Ambion) was added to the probe mixture. The contents were incubated for 1 h at 25 °C. Following the incubation the reaction was filtered through a Sephadex G-50 fine exclusion column. Both FTL and FTH probes were made to the same specific activity. Bandshift assays were performed as described (42) with the following modification: a 4% acrylamide stacking gel was added to the 8% resolving gel. The gel was dried and exposed for autoradiography.

A portion of the probe sample amounting to 80,000 cpm was used in the bandshifts. 2.8% 2-mercaptoethanol (2ME) was added to the 2ME control sample and incubated 10 min prior to adding the radioactive probe. 2 μl of the probe, diluted in lysis buffer, was added to each sample and incubating at room temperature for 10 min. 2 μl of RNase T1 (1 unit/μl) and 5 μl of RNase A (0.004 μg/μl) was added, and the sample was incubated at room temperature for an additional 10 min. 2 μl of heparin (50 μg/ml) was then added and followed by another 10-min room temperature incubation.

Band intensity was quantified using a phosphorimaging device (Bio-Rad). Bandshift analysis was also performed with extracts from cell lines BI-16 CON, BI-16-FTL, and BI-16-FTH to confirm binding uniformity within the construct lines.

**Data Analysis**—Upon testing our cell lines we found that there was variation between lots in the Promega dual luciferase reagent kit. To accommodate this variation, each individual hRLUC/FLUC data point was scaled according to the BI-16 positive control cell lysate included in each plate. Statistical significance between the means of different conditions was determined by applying an unpaired t test with no assumption of equal variance in the data.

**Aconitase Assay**—In-gel aconitase activities were assayed as described (42). Briefly, HEK 293 cells were treated for 24 h in either normoxic (20% O₂) or hypoxic (1% O₂) conditions. The cell culture medium was supplemented with either 50 μg/ml FAC or 100 μM DFO or was left untreated. The cells were washed in ice-cold PBS and then lysed in 1% Triton X-100 lysis buffer (20.5 mM Tris, pH 8.3, 192 mM glycine, 3.6 mM citrate) at 4 °C. Aconitase activity was measured by loading 40 μg of protein on an 8% resolving gel. The gel was dried and exposed for 3 days. The gel image was analyzed with a phosphorimager and the area of interest was quantified using software developed in the Lab. The area of interest was determined by visualizing the gel and selecting the aconitase band, and the band intensity was measured. The data were analyzed using the Mx3000P software (version 2.0).

Bandshift analysis was performed with extracts from cell lines BI-16 CON, BI-16-FTL, and BI-16-FTH to confirm binding uniformity within the construct lines. Statistical significance between the means of different conditions was determined by applying an unpaired t test with no assumption of equal variance in the data.

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RESULTS

A Bidirectional Reporter System Accurately Reflects IRE/IRP Interaction—To isolate IRE/IRP regulation, we employed a dual reporter system using a control firefly luciferase (FLUC), co-expressed with a Renilla luciferase (hRLUC), linked to a 5′ H11032 IRE (Fig. 1A). Transcription of the reporters was controlled by a bidirectional, doxycycline-inducible promoter system of our own design (41). The system was devised so that an IRP binds the IRE in the hRLUC mRNA and prevents translation but has no effect on the translation of the co-expressed FLUC RNA. The ratio of hRLUC:FLUC luminescence can be used to quantify collective IRE/IRP binding.

To determine whether our constructs quantified the IRE/IRP response, as designed, we established stable HEK 293 cell lines that contained a single copy of a bidirectional construct (Fig. 1A) in a single chromosomal location. Flp-In cell lines with constitutive expression of the tetracycline repressor are readily available from Invitrogen as Flp-InTM T-REx 293 cells. In addition, IRP regulation in varying oxygen conditions cells had previously been assessed in HEK 293 (43, 44), facilitating data interpretation. Stable cell lines were made containing the FTL IRE (BI-16-FTL), the FTH IRE (BI-16-FTH), as well as a control cell line with no functional IRE (BI-16-CON) linked to the hRLUC reporter. The IRE bearing constructs differed by only a few base pairs (Fig. 1B), and each IRE was predicted to fold (45) into similar stem-loop structures (Fig. 1C). Transcription of the reporters was induced with doxycycline, and the cells were treated with either 50 μg/ml of the iron donor, FAC, or 100 μM of the iron chelator, DFO, before incubation in typical tissue culture oxygen conditions (atmospheric 20% O2/5% CO2).

The addition of 50 μg/ml FAC increased hRLUC translation, and the addition of 100 μM DFO decreased hRLUC translation in both BI-16-FTL and BI-16-FTH cell lines (Fig. 2A, normoxic samples) in accordance with the predicted IRE/IRP interaction. Relative hRLUC expression in the presence of DFO is higher in the BI-16-FTL cell line than in the BI-16-FTH cell line (p < 0.05). There was no difference between FTL and FTH cell lines with regard to the addition of FAC, or with no treatment at all. A slight decline in the luciferase expression ratio was observed in the DFO- and FAC-treated BI-16-CON samples compared with no treatment (p < 0.05). In the case of the DFO-treated samples, the slight decrease (11%) in relative hRLUC seen in BI-16-CON is eclipsed by the marked decrease seen in the BI-16-FTL and BI-16-FTH samples. This result is also consistent with previous findings that DFO treatment is associated with a small decline in other investigated protein levels (43, 46).

In addition, although the BI-16-CON FAC-treated sample also shows a slight decrease in relative hRLUC expression, both the FTL and FTH FAC-treated samples show increases rather than decreases in relative hRLUC expression (Fig. 2A). Taking all of
these data into consideration, we find FTL and FTH cell lines generate similar and predictable responses to FAC and DFO treatment under normoxic conditions.

We verified that this regulation of our bidirectional constructs was post-transcriptional by analysis of FLUC and hRLUC mRNA using real time reverse transcription-PCR. We treated BI-16-CON, BI-16-FTL, and BI-16-FTH cell lines with either NT, 50 \( \mu \text{g/ml} \) FAC, or 100 \( \mu \text{M} \) DFO and incubated the cells in either 20\% or 1\% oxygen. We measured levels of both FLUC and hRLUC mRNA after 24 h of treatment and calculated the hRLUC/FLUC mRNA ratios. The addition or chelation of iron both caused a general decline in the ratio of hRLUC to FLUC mRNA (Fig. 2B); however, these declines did not reach significance in either normoxic or hypoxic conditions (\( p > 0.05 \) in all cases). Furthermore, whereas the hRLUC/FLUC enzyme activity of the BI-16-CON control generally followed the same trend as the corresponding mRNA ratios, the activities of the IRE-bearing reporters did not (compare A with B in Fig. 2). Thus, changes in the expression ratio of our bidirectional IRE-bearing reporters reflect post-transcriptional regulation.

To verify that changes in IRE-binding reporter activity correlated with responses in the opposite direction by IRP binding activity, we conducted standard bandshift assays to quantify cellular IRP binding activity in our HEK 293 cell lines. Bandshift analysis was executed using a \( ^{32} \text{P} \)-labeled ferritin L chain IRE RNA as described (42) with minor modifications. To avoid artificially increasing IRP1 binding, bandshift assays were conducted the same day as extract preparation, without any freezing or thawing. We determined that IRP binding patterns were identical in all three reporter HEK 293-derived cell lines (data not shown); therefore, the Flp-In HEK 293 cell line was used for subsequent bandshift assays (Fig. 2C). Fig. 2C shows that overall IRP binding parallels the response of our reporter system. The addition of FAC decreases IRE/IRP binding, whereas DFO increases binding. These data indicate that expression from the construct does reflect overall IRP binding in normoxic conditions, in a cell line.

Translation of FTL and FTH Transcripts Is Differentially Regulated in Hypoxic Conditions—The contrast between commonly used lab tissue culture oxygen tension and native tissue oxygen tension is great (47, 48). Furthermore, oxygen levels have been shown to have an impact on IRP stability and binding (43, 44, 49). The constant presence of oxygen, reactive oxygen species, hypoxia, or oxidative stress induced by ischemia/reperfusion is known to perturb the iron sulfur cluster found in cytosolic aconitase, promoting increased RNA binding activity or inactivation of the protein completely (for review see Ref. 50). In addition, Hanson et al. have shown that IRP2 is stabilized in hypoxic conditions (43, 44, 51) and has been shown to be the predominant binding IRP at physiologic oxygen tensions when IRP1 remains in cytosolic aconitase form (49). Therefore, we evaluated the IRP response of our cell lines in 1\% oxygen, which may more closely correspond to the oxygen tension of native tissue (52). The cells were treated with 50 \( \mu \text{g/ml} \) FAC or 100 \( \mu \text{M} \) DFO and incubated in hypoxic (1\%) and normoxic (20\%) oxygen conditions, and then IRE/IRP binding was quantified by measuring the ratio of hRLUC to FLUC expression.

**FIGURE 2.** A, FTL and FTH IREs are differentially regulated by cellular iron status in tissue culture. The cells were treated with FAC, DFO, or NT, grown in either 20\% or 1\% oxygen, and harvested after 24 h. The y axis values are hRLUC/FLUC expression ratios normalized to a positive cell lysate run in each plate. All of the changes are significant (\( p < 0.05 \)) when compared with the corresponding control, except for FTL HYP/NT and FTL HYP/DFO; there was no significant difference between these two samples. *\( p < 0.05 \) for FAC- and DFO-treated samples when compared with the no treatment control. \( ^{\circ} \), \( p < 0.05 \) for hypoxic samples when compared with the same sample in normoxic conditions. \( \ast \), \( p < 0.05 \) for FTH samples when compared with the corresponding FTL sample. The error bars indicate the S.E. for a sample number of 30. B and C, IRE/IRP interaction regulates the luciferase constructs. BI-16-CON, BI-16-FTL, and BI-16-FTH cell lines were grown as described previously, and FLUC and hRLUC mRNA was measured by real time reverse transcription-PCR and is expressed in \( B \) as the relative ratio of hRLUC/FLUC mRNA. The error bars indicate the S.E. for a sample number of three. C, bandshift analysis on a 5\% gel, using a \( ^{32} \text{P} \)-labeled FTL IRE RNA and cell extracts prepared from FTL HEK 293 cells grown in normoxic conditions with FAC, DFO, or NT. Both IRP1 and IRP2 bound to the radioactive IRE co-migrate under these conditions. The experiment was performed four times with a representative blot shown.
We observed marked differential regulation of BI-16-FTL and BI-16-FTH cell lines both with the addition of FAC and with DFO in hypoxia. Although both FTL and FTH cell lines showed increased hRLUC expression with the addition of FAC in hypoxia, compared with normoxia, the response of the BI-16-FTL FAC-treated sample in hypoxia was greater than that of BI-16-FTH (10-fold versus 5.5-fold, p < 0.0001; compare Fig. 2A). The response of the BI-16-FTL DFO-treated sample in hypoxia also showed greatly increased relative hRLUC expression than BI-16-FTH (p < 0.0001). This observation was also true in normoxic conditions (p < 0.0001). Hypoxic conditions increased hRLUC expression (p < 0.05) when compared with the corresponding normoxic sample (BI-16-FTL or BI-16-FTH), except in the case of no treatment, where low oxygen concentrations decrease relative hRLUC expression.

As shown in Fig. 2A, hypoxic conditions generally increased relative hRLUC expression compared with normoxic conditions. Because the increase was largely consistent across all BI-16-CON samples, the overall expression pattern for BI-16-CON is the same in hypoxia as in normoxia. However, when the BI-16-FTL and FTH cell lines were incubated in hypoxia with no treatment, the sample showed decreased relative hRLUC expression, when compared with normoxic samples with no treatment (p < 0.0001) (Fig. 2A), rather than the increase seen in the control cell line.

Differential Regulation of Endogenous FTL and FTH Proteins in Hypoxia—We anticipated that the changes in IRP binding measured by our constructs would be reflected in overall endogenous FTL and FTH protein levels. To determine this, we examined FTL and FTH protein levels by immunoblot analysis. We found that the increase seen in FTL-IRE- and FTH-IRE-mediated hRLUC expression, in the luciferase assay, with the addition of 50 μg/ml FAC, directly coincides with an increase in both FTL and FTH protein (Fig. 3). In addition, the intensified response seen in HYP/FAC with the BI-16-FTL cell line is supported by the fact that FTL protein levels show a greater increase (40.5-fold, p < 0.05) than that seen in FTH protein (4.4-fold, p < 0.05) when FAC treatment is coupled with hypoxia (Fig. 3, A and B).

Differential Regulation of FTL and FTH by IRP1 and IRP2—We then asked which IRP was responsible for the exacerbated hRLUC response of BI-16-FTL in HYP/FAC and HYP/DFO. Because our reporter system reflects the combined binding effects of both IRP1 and IRP2, we examined IRP1 and IRP2 RNA binding activity independently using RNA bandshift assays. To investigate IRP1 and IRP2 participation in the regulation of FTL and FTH, separate FTL and FTH 32P-labeled IREs (Fig. 1C) were used in the bandshift assays. We used the gel shift assay described by Tong and Rouault (42), with minor modifications, to separate IRE-bound human IRP1 and IRP2, which usually co-migrate. The cells were exposed to conditions identical to those in the luciferase assay at 1 and 20% oxygen for 24 h, to make sure that binding was not subject to the biphasic IRP response in hypoxia (43). In addition, the cells were lysed, and the protein concentration was determined immediately before incorporation in the bandshift sample to avoid artificially increased binding of IRP1 or decreased binding of IRP2 through oxygen-mediated degradation (43, 44, 49, 51). Finally, we included a HYP/NT sample treated with 2.7% 2-mercaptoethanol, a reducing agent used to selectively increase IRP1 binding, to identify the IRP1 band on the gel shift.

Binding to the FTL IRE—Both IRP1 and IRP2 binding patterns in normoxia were as expected, with a decrease in binding with the addition of 100 μM DFO (p < 0.05) and a decrease in binding with the addition of 50 μg/ml of FAC (p < 0.05). There was also increased IRP1 binding in hypoxic conditions with DFO, when compared with no treatment in hypoxic conditions (p < 0.05) (Fig. 4A). As expected from the BI-16-FTL data, there was significantly more IRP1 binding in the NORM/DFO than in HYP/DFO sample (p < 0.05). On the other hand, IRP2 does not show a statistically significant difference in binding when HYP treatments are compared with NORM treatments with either no treatment or the addition of chelation of iron. Although IRP2 binding showed the expected trends, with respect to iron concentrations, it did not reach significance in our assays (Fig. 4A).
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**A**

|          | Normoxia | Hypoxia  |
|----------|----------|----------|
|          | NT/FAC/DFO | NT/FAC/DFO |
| FTL IRE  | IRP1     | IRP2     |
| FTH IRE  | IRP1     | IRP1     |

**B**

|          | Normoxia | Hypoxia  |
|----------|----------|----------|
|          | NT/FAC/DFO | NT/FAC/DFO |
| IRP2     | b-actin  | b-actin  |
| IRP1     | b-actin  | b-actin  |

**C**

Protein (%)

|          | Normoxia | Hypoxia  |
|----------|----------|----------|
|          | NT/FAC/DFO | NT/FAC/DFO |
| c-Aconitase activity | 0 | 0 |

**D**

|          | Normoxia | Hypoxia  |
|----------|----------|----------|
|          | NT/FAC/DFO | NT/FAC/DFO |
| c-Aconitase activity | 0 | 0 |

**FIGURE 4.** A, IRP binding activities of FTL and FTH IREs. HEK 293 cells were grown for 24 h, as detailed under “Experimental Procedures.” RNA bandshift analysis on an 8% gel was done using 32P-labeled FTL and FTH IRE RNAs. The experiment was performed six times with a representative blot shown. B and C, IRP protein levels in HEK 293 cells. HEK 293 cells were grown and treated as before. Cells containing whole cell protein were blotted and probed for IRP1 and IRP2 expression. Experiments were carried out five and four times for IRP2 and IRP1, respectively, and representative blots are shown. The blots were stripped and reprobed for β-actin using mouse anti-actin. Statistical analysis was done by comparing the ratios of each treatment, with normoxic NT for IRP1 and hypoxic NT for IRP2. IRP2 levels were close to background for normoxic NT; therefore, hypoxic NT was used, as a reference for analysis. *, p < 0.05 for FAC and DFO treated samples when compared with the no treatment control. D, in-gel activity of cytosolic aconitase from HEK 293 cell lysates. The cells were grown and treated as before. Chromogenic stains of the gel for aconitase activity using thiazolyl blue tetrazolium bromide was performed as detailed under “Experimental Procedures.” The assay was done in triplicate with a representative gel shown.

**Binding to the FTH IRE**—IRP binding patterns with the FTH probe differed from those of the FTL probe. There was no significant difference in IRP1 binding between normoxic NT and NORM/FAC, but there was a difference between NORM/NT and NORM/DFO (p < 0.05). As with FTL, the FTH probe showed increased IRP1 binding in NORM/DFO when compared with HYP/DFO (p < 0.05), supporting stabilization of the nonbinding aconitase form of IRP1 in hypoxia (52), but there was no change in IRP1 binding when NORM/FAC is compared with HYP/FAC, a sample comparison that showed an obvious difference in the luciferase assay. IRP2 did not show a significant change in binding to the FTH IRE under any treatment conditions when normoxic conditions are compared with hypoxic conditions (Fig. 4A).

Evidence of low IRP1 and IRP2 binding levels in the bandshift assay prompted us to investigate overall IRP1 and IRP2 levels in our HEK 293 cell line. Analysis of IRP2 immunoblots showed low but detectable levels of IRP2 protein with no treatment in both 1 and 20% oxygen when compared with β-actin protein levels and a detectable decrease in protein levels with the addition of FAC in both oxygen conditions (Fig. 4, B and C, p < 0.05). The normoxic DFO-treated samples show an expected up-regulation of IRP2 protein levels, when compared with no treatment (p < 0.05). As expected, hypoxic conditions increased IRP2 protein levels, and a detectable decrease is seen with the addition of FAC in hypoxia (p < 0.05). This agrees with the recent literature stating that hypoxia stabilizes IRP2 (43, 44, 49, 51) (Fig. 4, B and C).

Unlike IRP2, immunoblot analysis using an IRP1 antibody demonstrated that IRP1 protein levels are robustly detectable when compared with β-actin and remain unchanged with the addition of either FAC or DFO in either normoxic or in hypoxic conditions after 24 h (Fig. 4, B and C). The lack of change seen in IRP1 protein levels in HEK 293 cells under these different conditions is consistent with the fact that IRP1 binding is based on the status of the iron sulfur cluster in the protein and not on overall protein levels. These findings are in agreement with previous data showing that IRP1 protein levels remain largely static (51, 53).

**Changes in Aconitase Activity**—Based on the facts that 1) the decrease in IRP1 binding may approach the lower detection threshold of the bandshift assay, 2) immunoblot analysis is unable to discern between the mutually exclusive aconitase and RNA-binding forms of IRP1, and 3) the aconitase form of IRP1 is stabilized in hypoxia, possibly accounting for the exacerbated decrease in binding seen with FTL HYP/FAC, we examined cytosolic aconitase (cAcon) activity to gain further insight into IRP1 binding. Cytosolic aconitase activity is defined by the presence of a 4Fe-4S cluster in the IRP1 protein (54). To test cytosolic aconitase activity, we employed a native PAGE analysis previously described by Tong and Rouault (42), with minor modifications, that separates mitochondrial and cytosolic aconitases. The separated enzymes are then assayed for aconitase activity in the gel. The cells were exposed to no treatment, 50 μg/ml FAC, or 100 μM DFO and incubated in either normoxic or hypoxic conditions, as described in previous experiments. As shown in Fig. 4D, cytosolic aconitase activity remains unchanged with the addition of FAC in both normoxia and hypoxia and shows a significant decrease with the addition of DFO in both normoxia and hypoxia (p < 0.05). When normoxic DFO and hypoxic DFO treatments are compared with each
other, there is increased aconitase activity in hypoxic conditions with DFO treatment ($p < 0.05$). This increase in cytosolic aconitase activity in hypoxic conditions with the addition of DFO, when compared with the same treatment in normoxic conditions, coincides with decreased IRP1 binding under like conditions for both FTL and FTH IREs.

**DISCUSSION**

Our study provides the first evidence that FTL and FTH are differentially regulated on a translational level. We have designed and tested a sensitive dual reporter system that is able to isolate and quantify combined IRP binding to an individual IRE. We confirmed the reliability of the construct and used this reporter system to demonstrate a measurable difference between IRP binding to FTL and FTH IREs in both normoxic and hypoxic conditions. Western blots on both FTL and FTH subunits parallel our luciferase results, confirming that the overall protein levels of FTL and FTH increase with the addition of FAC in hypoxic conditions (versus normoxic conditions) and that the increase is greater with FTL. Surprisingly, the corresponding EMSA bandshift assays were not sensitive enough to detect binding changes in either IRP1 or IRP2 that would account for this difference. Our findings not only confirm a major level of differential regulation of FTL and FTH transcripts but also offer a superior and more sensitive assay to quantify IRE/IRP binding in cell lines.

Ferritin L and ferritin H subunits are differentially regulated on both a transcriptional and post-transcriptional level (22); thus, it is important to isolate and identify which regulatory mechanism is responsible for changes in ferritin levels. FTL and FTH subunits are genetically independent. The gene sequences for the L and H subunits are located on chromosomes 11 and 19, respectively (3), and although the two genes have broad homology, they differ in their noncoding regions (55). These differences in the noncoding regions provide for the differential transcriptional regulation of FTL and FTH and were the subject of a reporter system developed by Hintze and Theil (38). By placing a luciferase reporter under the direction of the FT promoter and 5′-untranslated region, minus an IRE, they were able to investigate transcriptional regulation of FTL, independent of IRE/IRP regulation. Additionally, use of the luciferase protein in lieu of the endogenous FTL protein eliminated confounding effects of iron-dependent FTL protein half-life changes. Here we present a complementary system that isolates the IRE/IRP regulation of both FTL and FTH and eliminates differences in transcriptional regulation. Use of a control reporter with no IRE controls for possible post-transcriptional regulation, and an internal firefly luciferase control, driven by the same bidirectional promoter and not preceded by an IRE, ensures consistent control results for every assay (Fig. 1A). Furthermore, use of the Invitrogen Flp-InTM system allows us to establish stable cell lines with a single copy of the construct, in a reproducible genomic location, and the doxycycline inducible promoter allows us to control the time of induction and level of expression (41).

Because the FTL and FTH IREs differ by only a few nucleotides (Fig. 1B), there has been the overall assumption that there should be little or no IRP binding difference between FTL and FTH. The functional difference we established is supported by previous research specifically targeting nucleotide base changes in the IRE (30, 31, 37, 50, 56). Both Ke et al. (30, 31) and Henderson (37) used species-specific variants of the FTH IRE to investigate variations in IRP binding. Henderson, using both mouse and human FTH IREs, demonstrated that each IRP is able to recognize exclusive subsets of IREs and that this was conserved between the two species (37). These results paralleled those by Ke et al. (30), who demonstrated, using a bullfrog FTH IRE, that changes in key IRE structural components, such as the internal loop-bulge, alters IRP binding. Recent resolution of the crystalline structure of IRP1 complexed with the frog FTH IRE presents several key findings in IRE/IRP binding. These data show that the helix sequence and mid-helix distortion near the C-bulge, as well as the terminal loop and C-bulge itself, are influential in IRP binding (57). This section of sequence differs between FTL and FTH IREs (Fig. 1, B and C). The findings also suggest that IRP1 and possibly IRP2 employ alternate bonding groups to recognize and bind different IREs and that this facilitates a graded response of IRE/IRP binding (57). These data support the idea that small variations in IRE nucleotide sequence alter binding and are consistent with our findings.

Our luciferase data strongly support differential regulation of FTL and FTH mRNA translation through IRE/IRP interaction, and this difference is more evident in hypoxic (1% oxygen) conditions. The small difference seen in response to DFO between BI-16-FTL and BI-16-FTH in normoxia became pronounced in hypoxia. In addition, although there was no difference in response to iron (50 μg/ml FAC) in normoxia, a large difference emerged in hypoxia. In all cases, the luciferase data indicate that the FTH IRE is less responsive to iron changes than the FTL IRE. Thus, FTL appears to be the primary responder to the addition of FAC in hypoxic conditions (Fig. 2A). The Western blot data for FTL and FTH under the same conditions follows the same trend; however, there are some differences between the increase in luciferase levels and the increase in endogenous protein levels. When NORM/FAC is compared with HYP/FAC luciferase levels with the FTL IRE, there is a 2-fold increase in luciferase levels. Western blot data on the endogenous FTL protein show an increase of ~2.5-fold. Similarly, the same comparison of luciferase data with the FTH IRE shows only a 25% increase in hypoxia, whereas there is a 2-fold increase in the endogenous FTH protein level. These differences underscore the importance of our reporter system. As noted earlier, there are extensive differences in the transcriptional control of FTL and FTH (for review see Ref. 5) and differences in half-life between the two proteins (for review see Refs. 5, 58, and 59). Our reporter system circumvents these differences and focuses on the IRE/IRP control component of expression. Although the Western blot data agree with the luciferase data in the sense that FTL is more responsive than FTH to the addition of FAC in hypoxia, endogenous protein levels also reflect these transcriptional and half-life differences, explaining why the Western blot increases are higher in general than the increases seen in the luciferase data.

Because cAcon is stabilized in hypoxic conditions, whereas IRP1 binding is decreased (50), we expected the difference in
BI-16-FTL and FTH regulation in HYP/FAC to be due to a decrease in IRP1 binding to FTL. However, when we conducted standard bandshift assays, we were surprised to find that there was no significant difference in either IRP1 or IRP2 binding in hypoxia, for either FTL IRE or FTH IRE probe, when no treatment was compared with FAC (Fig. 4A). Despite the fact that this is not statistically significant, past literature strongly supports that IRP1 shows decreased binding with decreased oxygen tension, because hypoxia stabilizes the cytosolic aconitase form (Ref. 49; for review see Refs. 50 and 60). In addition, IRP1 protein is subject to iron-dependent degradation independent of the iron sulfur cluster (61). Wang et al. (62) also noted a discord between their in vitro bandshift data and in vivo luciferase data and suggested that this could be a function of enhanced IRP binding activity that occurs only in vivo (62) or a result of subcellular localization of the IRPs (62–65).

Additionally, even in hypoxic conditions, which are known to stabilize IRP2 (50), we did not see increased IRP2 binding. Western blot data quantifying IRP2 protein showed barely detectable IRP2 levels in the presence of FAC, (Fig. 4, B and C). Some cell lines have been known to have little or no detectable IRP2 (66); however, Schneider and Leibold (43) have demonstrated not only detectable IRP2 levels in a HEK 293 cell line but also quantified IRP2 IRE binding, although a supershifting technique was used there. Because we took precautions to prevent IRP2 degradation, and were successfully able to detect IRP2 binding in mouse 3T3 cells (data not shown), we can only assume that low IRP2 protein levels, and low IRP2 binding is characteristic of our Invitrogen Flp-In™ HEK 293 cell line. However, this emphasizes the importance of a sensitive reporter system in a living cell.

Because we were unable to account for the exacerbated response of FTL with the use of IRP binding assays, we used aconitase activity to help shed some light on the data seen with our luciferase constructs. Based on the premise that reduced binding of IRP1 is often coupled with an increase in cytosolic aconitase activity (50), we investigated whether the decrease in IRP binding seen in hypoxic conditions overall, and particularly with the FTL IRE when compared with the FTH IRE, is accompanied by increased cAcon activity. Our data show little change in aconitase activity with the addition of FAC in either oxygen condition. The addition of DFO reduces cAcon activity in both oxygen conditions when compared with respective no treatment samples, but the HYP/DFO sample shows significantly more activity when compared with NORM/DFO (Fig. 4D). There was little change between NORM/FAC and HYP/FAC samples (Fig. 4D). The higher amount of cAcon activity seen in hypoxia, with the addition of DFO, when compared with the same conditions in normoxia, parallels the bandshift results that indicate there is a decrease in IRP1 binding with both FTL and FTH IRE probes (Fig. 4A). It should be noted that the entire effect of the difference seen in the luciferase results under these conditions (Fig. 2A) cannot be solely attributed to a decrease in binding of IRP1, because we are not able to rule out the possibility that IRP2 may be contributing but is unable to be detected by bandshift assay.

In a direct comparison of luciferase data, the BI-16-FTL cell line shows a greater fold increase in hypoxia with the addition of FAC than the BI-16-FTH cell line. This translates to less IRP binding to the BI-16-FTL construct IRE with the addition of FAC, or, conversely, the BI-16-FTH construct hRLUC was more “off” than FTL, after the addition of iron (Fig. 2A). It is likely the response seen in 1% oxygen conditions is more representative of results seen in vivo and is attributed to the proximity of the oxygen tension to physiologic tissue oxygen values, widely accepted to be ~3% oxygen (47, 48, 52). The addition of DFO shows a greater fold decrease in hRLUC/FLUC with the BI-16-FTH cell line than with the BI-16-FTL cell line, also indicating that the BI-16-FTH construct is more “off” than FTL in the absence of iron (Fig. 2A).

So, why would FTH be less sensitive to increases in iron concentrations? Cozzi et al. (21) postulate that FTH may act as an iron buffer conditioned to respond to the immediate needs of iron homeostasis, as opposed to chronic overload or chelation. The data of Cozzi et al. support the theory that FTH levels are maintained at a steady threshold to avoid overexpression of FTH, which is known to result in reduced cell growth and iron deficiency (21, 67). This may also explain why FTH levels do not change dramatically in brain samples from iron-fed mice, despite significant changes in the H:L ratio (68).

To conclude, we present a highly sensitive reporter system that isolates and quantifies IRP binding to an IRE, offering the first evidence for differential translation of FTI and FTH subunits. Furthermore, this difference is accentuated in conditions of 1% oxygen tension, which approaches in-tissue oxygen tension. Because ferritin has been implicated in cell proliferation in cancer, our findings offer another avenue for possible therapeutic intervention. These results also influence the interpretation of some previous research concerning IRP binding, which has been conducted using either FTL or FTH IREs. The results obtained with a single ferritin IRE are often used to assess the effects on ferritin as a whole, but because of the differences between the FTL and FTH IREs in our assay, they may not be appropriate for a direct comparison. Further refinement of FTI and FTH regulation independently is likely to offer not only a better understanding of both neurodegenerative diseases and cancer but also more polished molecular tools for investigating iron homeostasis.

Acknowledgments—We thank Dr. Jawed Alam and the Department of Molecular Genetics at the Ochsner Clinic Foundation for generously providing space for us to rebuild and continue this research after Hurricane Katrina. We also thank Robert Kutner and the Louisiana State University Health Sciences Center Vector Core for assistance in real-time reverse transcription-PCR. We are grateful to Dr. Betty Leibold for providing the IRP1 antibody and for critical reading of the manuscript.

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