Detection of intracellular iron by its regulatory effect

Jau-Yi Li, Gita Ram, Katherine Gast, Xia Chen, Kimberly Barasch, Kiyoshi Mori, Kai Schmidt-Ott, Jianjun Wang, Hung-Chieh Kuo, Cathy Savage-Dunn, Michael D. Garrick, and Jonathan Barasch. Detection of intracellular iron by its regulatory effect. Am J Physiol Cell Physiol 287: C1547–C1559, 2004. First published July 28, 2004; doi:10.1152/ajpcell.00260.2004.—Intracellular iron regulates gene expression by inhibiting the interaction of iron regulatory proteins (IRPs) with RNA motifs called iron-responsive elements (IREs). To assay this interaction in living cells we have developed two fluorescent IRE-based reporters that rapidly, reversibly, and specifically respond to changes in cellular iron status as well as signaling that modifies IRP activity. The reporters were also sufficiently sensitive to distinguish apo- from holotransferrin in the medium, to detect the effect of modifiers of the transferrin pathway such as HFE, and to detect the donation or chelation of iron by siderophores bound to the lipocalin neutrophil gelatinase-associated lipocalin (Ngal). In addition, alternative configurations of the IRE motif either enhanced or repressed fluorescence, permitting a ratio analysis of the iron-dependent response. These characteristics make it possible to visualize iron-IRP-IRE interactions in vivo.

Iron regulatory proteins; iron-responsive element; labile iron pool; transferrin; HFE; neutrophil gelatinase-associated lipocalin; siderophore

Iron serves a number of functions in cells. It is the catalytic site of many enzymes and the binding site for gases in transport proteins. In addition, iron regulates mammalian gene expression. Iron regulates cell cycle activities (such as p21, cdk2, cyclins), signaling [inducible nitric oxide synthase (iNOS), PKC-β], and developmental genes at the transcriptional level (27, 89), but the mechanism of regulation is not yet established (for yeast see Ref. 86). Iron also regulates the posttranscriptional expression of genes that contain stem loop structures in their untranslated regions (UTRs) called iron-responsive elements (IREs; Refs. 2, 44, 67, 70). Cytosolic proteins called iron regulatory proteins (IRP; Refs. 18, 32, 35, 46, 48, 69) bind the stem loops and determine the translation of the message. If a gene contains an IRE in the 5′-untranslated region (UTR) interaction with IRP inhibits translation. Conversely, when the IRE is located in the 3′-UTR the IRE-IRP interaction stabilizes the message by inhibiting access to an endonuclease site. Iron negatively regulates the formation of the IRP-IRE complex by inhibiting the association of IRP-1 with IRE and by triggering the degradation of IRP-2 (16, 18, 34, 35, 39, 41, 82, 88).

Hence, iron promotes the translation of messages containing a 5′-IRE, but it suppresses the translation of messages containing a 3′-IRE. Conversely, low iron levels have the opposite effect (diagrammed in Fig. 1). IREs provide a critical mechanism for the regulation of many proteins involved in iron metabolism, as well as other factors (13, 45).

The regulation of gene expression by iron is thought to depend on the concentration of iron in the cell cytoplasm, which is called the “labile,” “exchangeable,” or “transient” pool of iron (20, 71). Although the chemical nature of this pool is not yet clear and its regulation is complex (18, 33, 85), the activity of the labile pool has been estimated by a variety of methods. For example, the expression level of ferritin (which has a 5′-IRE) and transferrin receptor 1 (which has a 3′-IRE) proteins may reflect the labile iron pool, because ferritin expression is enhanced and transferrin receptor 1 expression is suppressed by iron loading of cells. This relationship, however, may not hold for all mammalian cells (62) or apply to all responses of ferritin and transferrin receptor 1. For example, ferritin (67) and transferrin receptor 1 (12, 38, 50, 63, 79, 84, 90) are not only regulated at the posttranscriptional level by iron but also by stimuli, such as cytokines, that activate iron-independent promoters. Indeed, in some lineages, such as the hematopoietic series (1, 10, 11, 23, 51, 53, 73, 80), transferrin receptor 1 is regulated predominantly by transcriptional rather than posttranscriptional mechanisms. Hence, although ferritin and the transferrin receptor proteins are useful measures of the response to iron in vitro, the endogenous expression of these genes could reflect a myriad of signals. Direct measurements of the IRP-IRE interaction could better reflect the labile pool of iron.

Direct measurement of cytoplasmic iron is possible with calcine and phen-green. These dyes are very important reagents because they detect changes in iron over seconds to minutes and their responses can be quantified (20, 21). One must assume, however, that they chelate iron from the same pool that interacts with the IRPs. Moreover, the dyes are used at concentrations that could perturb these pools. In addition, reversal of the quenched signal can require changing pH or the addition of large doses of iron chelators (21), protocols that are difficult to use in complex tissues. Measurement of iron over hours to days is also not possible with calcine or phen-green because of bleaching and leakage of the dye. Hence, although the dyes are invaluable to detect initial changes in iron that, for example, might occur with acute modulation of iron transport.
The best approach to studying iron metabolism in complex organs is to use methods that can assay single cells, because mechanisms of iron acquisition may vary among adjacent cells. For example, we found a gradient of transferrin uptake and transferrin receptor 1 expression that correlated with the appearance of developmental milestones in the developing kidney (Refs. 19, 24, 87; Barasch J, unpublished observations). Similarly, stage-specific expression and a functional requirement for transferrin receptor 1 were found in the hematopoietic and lymphoid lineages (Refs. 8, 40, 49, 59, 76; reviewed in Ref. 64), but other cell types were indifferent to the absence of transferrin receptor 1 and hence must have obtained iron by an alternative pathway (59). Cellular iron content might also vary by stage and by lineage because different regions of the brain have a different content of ferritin (68, 74) and the IRPs are developmentally regulated (47, 74) and differentially expressed in different cells of an organ (56). These studies indicate that examination of tissue iron requires methods to analyze the IRP-IRE interaction and the labile iron pool at the level of single cells.

A simple method to follow IRP-IRE interactions was suggested by earlier studies that demonstrated that the 5′-IRE can regulate the expression of reporters in vitro (see, e.g., Refs. 29, 37, 52) and that a number of different promoters can be used to drive reporter constructs in vivo (see, e.g., Ref. 77). Analysis of an iron reporter in tissues, however, requires the simultaneous expression of two reporters that are differentially sensitive to iron, to permit ratio imaging of the fluorescent signal. Ratio imaging allows the measurement of iron to be normalized for an iron-insensitive component of reporter expression and for iron-insensitive variations in the detection of fluorescence. To produce two iron-dependent reporters that are differentially sensitive to iron, we ligated the 5′-UTR domain of the ferritin gene and the 3′-UTR domain of the transferrin receptor protein to destabilized forms of green fluorescent protein (GFP)-type proteins. We tested these reporters in cell cultures to learn whether they respond in a manner predicted by a variety of published experiments that have manipulated iron, transferrin, and the IRP system. We found that these reporters rapidly and reversibly respond to changes in cell iron or changes in the IRP-IRE interaction (Fig. 1). When we used both the 5′ and the 3′ configurations of the IREs with different fluoros, we found that we could measure reciprocal responses to iron in the same cell. Because these reporters capitalize on endogenous mechanisms of iron-mediated gene expression, they are likely to assay the same pool of iron that regulates gene expression. We have used these reporters to monitor iron uptake from different sources, including a novel iron transporter called siderocalin, a lipocalin containing a bacterial siderophore (28). Some of these data have been presented in brief abstract form in 2002 (4) and 2003 (5) at the annual meeting of the American Society of Nephrology and in 2003 (3) at the annual meeting of the American Society of Hematology. An abstract by Henderson et al. (36) is also relevant.

MATERIALS AND METHODS

Iron reporters. We used PCR (forward: 5′-gcgcatgacgacgcctggag-gttctg-3′ and reverse: 5′-gcgctggagcgtcagaggtcggagac-3′) to amplify a 206-base pair fragment upstream of the ferritin ATG from a mouse embryo E15 CDNA library (Clontech). The fragment, which contained one IRE, was ligated to a yellow fluorescent protein (YFP) with a 2-h half-life (Clontech) at the BamHI and XhoI sites in the CMV-D2EYFP-N1 vector. The final product was resequenced across the ligation site. The distance between the 5′-IRE and the start codon of ferritin and the 5′-IRE and the start codon of YFP differed by 19 base pairs, which were introduced by the multiple cloning site of the YFP vector. To create a probe with a 3′-IRE, we cloned 900 base pairs of the 3′-UTR of transferrin receptor 1 with the primers 5′-gcggcg-agcctgagctgacctg-3′ and 5′-gcgcgctgctctctggtcgtgat-3′ and mouse embryo E15 cDNA library (Clontech). We chose this sequence because it was conserved in human, chicken, and mouse transferrin receptor 1 and it contained multiple IREs and an endonuclease site. The fragment was ligated at the NotI site in the CMV-D2EYFP-N1 vector. A control probe was also made with the same YFP but without an upstream or downstream IRE. Reporters expressing destabilized cyan fluorescent protein (CFP) were generated by similar methods.

Expression of iron reporters in 293 cells. The IRE and control reporters were introduced into 293 cell lines with Lipofectamine 2000 (Invitrogen), and plasmid-containing lines were then selected by neomycin (400 μg/ml) in DME medium (high glucose) with 10% FCS at 37°C in 5% CO2. Eight colonies with the 5′-IRE-YFP probe, six colonies with 3′-IRE-YFP, and eight colonies with non-IRE-YFP were expanded in neomycin and analyzed by a fluorescence-activated cell sorter (FACS; Excitlab) with a 488-nm laser. Before each experiment, the cells were rinsed and recultured in serum-free DME (0.2 μM iron) overnight to remove most of the serum. The response to iron loading was determined by culture with holotransferrin (1–100 μg/ml; Sigma), iron-containing or iron-free siderocalin/neutrophil gelatinase-associated lipocalin (Ngal, 50 μg/ml; see below), or ferric ammonium citrate or ferric chloride (1–25 μM) in serum-free DME medium. The response to iron chelation was examined with deferoxamine (DFO) mesylate (10 or 20 μM). Single 293 cells expressing IRE-YFP were also followed by time-lapse cinematography. Cells were seeded in 25-mm dishes in DME medium with Hepes (10 mM, pH 7.4), and YFP fluorescence was detected in individual cells with a Nikon inverted microscope, a Hamamatsu digital camera and controller, a Prior Proscan positioner, and a Sage Aircurtain (Watanabe T and Costantini F, unpublished observations). Cells were observed for 3 h to establish baseline fluorescence and then were treated with ferric ammonium citrate (25 μM). Images were collected at 30-min intervals and quantified with the ImageJ program (http://rsb.info.nih.gov/ij/) (a relative standard deviation was derived for analysis of ratio imaging (57)). The response to iron was also detected by YFP immunoblot with

![Diagram of iron-dependent posttranscriptional regulation of gene expression. The 5′-IRE determines whether ferritin or a substitute such as yellow fluorescent protein (YFP) is translated. Conversely, the 3′-IRE determines the stability of mRNA for transferrin receptor 1.](http://ajpcell.physiology.org/lookup/suppl/doi:10.1152/ajpcell.00285.2003/-/DC1//C1548.F1.png)
rabbit anti-GFP/YFP antibodies (Clontech) and by fluorescent microscopy. Anti-GAPDH (Chemicon) and anti-transferrin receptor 1 (Zymed) antibodies were used in control experiments.

**Retroviral constructs.** The 5′-IRE-d2EYFP fragment was isolated by digestion with XhoI and NotI and then ligated into pMIG, a retroviral vector [based on MSCV; kindly provided by Dr. Luk van Parijs (14)], that was modified by inserting a 30-base pair linker containing NotI. The d2EYFP fragment was cloned into the modified MIG vector at the XhoI and NotI sites. For the 3′-IRE-d2EYFP fragment, the 3′-IRE was isolated from pLVX-IRES-puro (Stratagene) by digestion with XhoI and NotI and then ligated into pMIG at the XhoI and NotI sites. To produce viral particles, we cotransfected these constructs with retroviral packaging constructs (pCL-Eco) (58) and pSVS-G (Clontech) with Lipofectamine 2000 (Invitrogen) into 293 cells. Virus was collected from 48- to 72-h media.

Expression of iron reporters in TRvb-1 cells. TRvb-1 cells [a kind gift of T. McGraw (54)] were grown in 24-well plates to 80% confluence under continuous selection with genetin (200 μg/ml). TRvb-1/HFE and TRvb-1/Hβ2-α-microglobulin cells [kind gifts of W. Sly (81)] were grown with geneticin (200 μg/ml) and puromycin (10 μg/ml). The iron reporters were introduced by viral infection with polybrene (16 μg/ml) and centrifugation (1,800 rpm, 90 min). Cells were analyzed after two passages.

**Siderocalnin/Ngal.** Recombinant, purified Ngal from BL-21 bacteria (87) was obtained by glutathione S-transferase (GST)-reduced glutathione (GSH) chromatography (Pharmacia) followed by gel filtration to remove impurities (Superdex75, SMART System, Pharmacia). Ngal was loaded with enterochelin in either its iron-free or in its iron-saturated form (EMC Microcollection), using a 2-to-1 molar ratio of siderophore:siderocalnin/Ngal, washed in a 10-kDa microcon, and added to the reporter lines. Alternatively, 293 cells or TRvb-1 cells stably expressing 5′- and 3′-IRE-YFP reporters were transfected with 24p3/Ngal containing a signal sequence (pcDNAIII vector), and the cells were analyzed after 48 h by FACS.

**IRP.** IRP-1 was analyzed by immunoblot (Abcam, MA). IRP-1 and IRP-2 were analyzed by real-time PCR from 293 cell RNA prepared by on-column DNase digestion (Qiagen). Total RNA (1.3 μg in 50 μl) was reverse transcribed with an oligo(dT) primer (1 μM), 10 μl of RNase inhibitor (Invitrogen), and 4 μl of Omniscript reverse transcriptase for 60 min at 37°C. Human IRP-1 (GenBank accession no. M58510.1) was amplified with forward primer AGAGCGTGATCGGGAGT (positions 1101–1082), and 200 nM of each primer with dNTP (200 nM), Taq reverse primer AGCCACTCCTACTTGCCTGA (positions 1101–1082), and 200 nM of each primer with 1 μl of RNase inhibitor (Invitrogen), and 4U of Omniscript reverse transcriptase (Qiagen) for 40–60 cycles at 95°C (30 s) and 60°C (30 s) and the MyiQ iQ SYBR green super mix (Bio-Rad) for 40–60 cycles at 95°C (30 s), 60°C (30 s), and 72°C (30 s). The products were analyzed by 2% agarose gel and then sequenced. Real-time PCR used 0.2 μl of cDNA and 200 nM of each primer with 1× iQ SYBR green super mix (Bio-Rad) for 40–60 cycles at 95°C (30 s) and 60°C (30 s) and 72°C (30 s). The products were analyzed by 2% agarose gel and then sequenced. Real-time PCR used 0.2 μl of cDNA and 200 nM of each primer with 1× iQ SYBR green super mix (Bio-Rad). The product was quantified by comparison with β-actin mRNA (GenBank accession no. BC0414861) with forward primer CTCCTGCTTGGCGATGC (positions 13–30) and reverse primer GTGTCAGGGTGTAGTGCATG (positions 651–629). Comparison of 293 cDNA and human liver cDNA (Clontech, BD Biosciences) was calculated according to the comparative threshold cycle (Ct) method, where the amount of target, normalized to endogenous β-actin, and relative to a calibrator, is given by 2−ΔΔCt. The specificity of the amplifications was checked by melting curves analysis. Electrophoretic mobility shift assay also evaluated IRP binding, but, as previously reported (6), IRP-1 dominates the gel shift in 293 cells.

**Nematode strains and manipulations.** To create iron reporters in Caenorhabditis elegans, we inserted the sma-3 promoter (83) into pPD117.01-GFP between Salt and Clat. 5′-IRE and 3′-IRE sequences were then amplified from the mammalian IRE-YFPs with primers 5′-IRE reverse (5′-CCGGTACCCGTCATGCTATCCGAGGT-3′), 5′-IRE forward (5′-CCATCGATCCGCGGATCCG-3′), 3′-IRE reverse (5′-CCGGCCCCTCCACAACTATATA-3′), and 3′-IRE forward (5′-CCACTAGTACTCTCAATTCTGCAGCTT-3′) and inserted into sma-3::GFP. Worms (N2 strain) were then microinjected with these plasmids (20 ng/μl) (55) together with 100 ng/μl pRF4 (rol-6 plasmid). Lines carrying these constructs had the roller phenotype. Worms were grown at 20°C on EZ plates (550 mg Tris-Cl, 240 mg Tris-H, 3.1 g Bactopeptone, 8 mg cholesterol, 2.0 g NaCl, 200 mg streptomycin sulfate, and 20 g agar per liter) seeded with OP50 bacteria (9) and ferric ammonium citrate (100 μM) or DFO (20 μM) to vary iron.

**RESULTS**

**Single-wavelength reporters of cytoplasmic iron.** We used 293 cells to analyze the response of two different iron reporters and a control construct. Real-time PCR demonstrated that 293 cells contain both IRP-1 and IRP-2, expressed 0.1- and 20.60-fold in 293 cells relative to a human liver cDNA library, respectively (normalizing these samples for β-actin). The Ct was 27.3 for IRP-1, 24.2 for IRP-2, and 25.4 for β-actin, indicating that the IRP transcripts were expressed at high copy number. Because both IRP-1 and IRP-2 can regulate iron-dependent messages in vitro, and IRP-2 dominates iron metabolism in vivo (56), these data indicate that 293 cells are useful to examine the expression of an IRE-based reporter.

We produced an iron reporter by ligating the 5′-UTR of the mouse ferritin light chain, which contains a single IRE, with a destabilized form of YFP to produce a 5′-IRE-YFP reporter (Fig. 1). The 5′-IRE-YFP was expressed by a cytomegalovirus (CMV) promoter rather than the endogenous ferritin promoter because the ferritin promoter demonstrates both iron-dependent and iron-independent regulation.

When the 5′-IRE-YFP reporter was expressed in 293 cells, YFP was expressed in an iron-responsive fashion (Fig. 2A). As little as 1 μM ferric ammonium citrate (test doses ranged from 1 to 50 μM; Ref. 78) produced a small but often detectable increase in fluorescence, whereas 5 μM ferric ammonium citrate reproducibly increased fluorescence 2.41 ± 0.3-fold and the higher doses of iron increased the signal further. Other iron donors such as ferric and ferrous chloride and ferric sulfate had a similar effect. Clones expressing the 5′-UTR reporter (Fig. 2B) demonstrated reciprocal responses to iron (ferric ammonium citrate, 25 μM) and iron chelation by DFO (20 μM). Mean fluorescence varied sevenfold on average with these treatments [210 ± 26 vs. 33.3 ± 3.3 (mean ± SE) fluorescence units with iron and DFO, respectively; n = 8 independent clones; P < 0.001]. All cells responded to iron and DFO, suggesting that the 5′-IRE-YFP was not expressed in excess of the IRPs. In addition, the expression of ferritin and transferrin receptor 1 proteins was similar in transfected and parental 293 cells, indicating that the reporters did not squelch endogenous IRP-IRE interactions (not shown).

The response to iron was likely at the posttranscriptional level, because changes in 5′-IRE-YFP message were not detected by real-time PCR (not shown) and electrophoretic mobility assays revealed that the addition of iron blocked the binding of IRP to an IRE probe (supplemental data for this article may be found at http://ajpcell.physiology.org/cgi/content/full/00260.2004/DC1). It remained possible, however, that changes in 5′-IRE-YFP fluorescence might be due to
global changes in protein synthesis or degradation. To rule out this possibility, we examined cells transfected with the YFP vector lacking the IRE. Ferric ammonium citrate (1–50 μM; Fig. 2C) failed to increase the fluorescence of these cells, and none of the clones responded to iron or to DFO [331 ± 73 fluorescence units with iron (25 μM) vs. 331 ± 58 fluorescence units with DFO (20 μM); n = 8 clones, P = 0.99; Fig. 2C], suggesting that the IRE was the critical component of the iron response. It is also notable that, after the addition of iron to 5'-IRE cells or DFO to 3'-IRE cells increased fluorescence almost to the level of the non-IRE 293 cells.

To demonstrate further the specificity of the response to iron, we created a reporter with 3'-IREs and an endonuclease site by using the 3'-UTR of mouse transferrin receptor 1 DNA and the destabilized YFP vector (Fig. 1). The 3'-IRE-YFP cells had the opposite set of responses to iron and DFO as the 5'-IRE-YFP clones. Iron repressed fluorescence of 3'-IRE-YFP cells (Fig. 2, E and F), whereas DFO enhanced fluorescence (93 ± 27 with 25 μM iron vs. 287 ± 66 with 20 μM DFO; n = 8 clones, P = 0.03). All cells in the 3'-IRE-YFP clones responded to iron or DFO, and DFO raised the 3'-IRE-YFP fluorescence to levels similar to those found in the non-IRE-YFP clones. These data demonstrate reversible regulation of 5'- and 3'-IRE YFP reporters by an iron-sensitive mechanism.

We used immunoblots to confirm that the IREs regulate the translation of YFP proteins and showed that, although the non-IRE-YFP protein was unresponsive to iron, the 5'- and 3'-IRE-YFP proteins varied with iron loading and iron chelation, in a manner similar to endogenous transferrin receptor 1.
These reciprocal changes could also be detected by microscopy. Ligation of the 5′-IRE sequence to the YFP reporter markedly diminished the fluorescence (compare Fig. 4, A vs. C), but the addition of iron reactivated the signal (compare Fig. 4, C vs. D). Conversely, iron reduced the fluorescence of the 3′-IRE-YFP reporter (compare Fig. 4, E vs. F). These reciprocal responses cannot be explained simply by global changes in protein synthesis or degradation, but rather they depend on predicted functions of 5′- and 3′-IREs in reciprocally regulating the posttranscriptional expression of proteins.

We next examined whether IRE-based reporters are specific for iron. We found that the addition of gallium, copper, and zinc had only modest effects on the fluorescence of 5′-IRE-YFP and 3′-IRE-YFP and that these effects were similar to changes in the non-IRE-YFP (Fig. 5). The lack of response to gallium was particularly notable because gallium can occupy iron-binding sites, although much higher doses were used to block iron transport in prior studies (15, 60). Nonetheless, assuming that the metals entered the cells, the data indicate that the IRE reporters have a favorable specificity profile compared with calcein (which, in solution, is sensitive to 1 μM metals; Molecular Probes).

Finally, it should be noted that the IRE reporters were effective in mammalian cells but were not responsive to iron when introduced into C. elegans (using the sma-3 promoter, which drives GFP expression in the pharynx, hypodermis, and intestine; Ref. 83). This finding is consistent with gel shift analyses that show that the C. elegans ortholog of IRP-1 (Aco1) does not bind mammalian IREs (30), and it further demonstrates the specificity of our reporters.

Rapid responses to iron. Because translation of the 5′-IRE-YFP protein and degradation of 3′-IRE-YFP RNA after the addition of iron are time-consuming events, we examined how fast the reporters could respond to iron. Using FACS, we detected a response within 60 min of the addition of iron. Similarly, time-lapse cinematography of single cells detected a response to iron by 60 min and a plateau of the signal after 5 h (Fig. 6). 3′-IRE-YFP cells exhibited the reciprocal response (not shown), and non-IRE-YFP cells were not responsive to iron at all.

The destabilized YFP proteins have a 2-h half-life. Consequently, changes in fluorescence should be rapidly reversible. Using FACS analysis, we found that reversion to baseline fluorescence occurred over 8–12 h (Fig. 7) after removal of iron and the rate of reversion was enhanced when DFO (20 μM, not shown) was included in the medium. The return to baseline occurred at approximately equal rates for both reporters, indicating either that the regeneration of IRP-1 binding activity or the de novo synthesis of IRP-2 was rate limiting, rather than the resynthesis of the 3′-IRE-YFP RNA. These data show that the IRE-iron reporters are useful to detect rapid changes in cellular iron, even within the lifespan of rapidly cycling cells.

Manipulation of transferrin pathway. In the preceding studies we used “non-transferrin-bound iron” (ferric ammonium citrate) to test the reporters. Because transferrin is the major iron transporter of the adult (64), we compared transferrin to non-transferrin-bound iron. We found a dose-dependent, saturable increase in 5′-IRE-YFP fluorescence and a dose-dependent, saturable decrease in 3′-IRE-YFP fluorescence (Fig. 8) after exposure to holotransferrin. The response to transferrin differed, as one might expect, from the response to non-transferrin-bound iron, because only 20 μg/ml of transferrin (at maximum 500 nM iron) had a maximal effect on fluorescence (a 3-fold increase in 5′-IRE fluorescence and a 2-fold decrease in 3′-IRE fluorescence), suggesting saturation of high-affinity receptors, whereas much larger doses of non-transferrin-bound iron (>25 μM) still did not saturate the capacity of the reporters to increase in fluorescence (see Fig. 2). In contrast, apotransferrin (not shown) had only a slight effect on the reporters, demonstrating that apotransferrin fails to reoxidize with iron from the culture medium (which contains at minimum 0.25 μM Fe). These data show that the behavior of the reporters can distinguish iron-loaded from unloaded carriers, as well as different mechanisms of iron acquisition.

Import of iron from transferrin is regulated by HFE, the protein defective in hemochromatosis. In one model using cell lines, overexpression of HFE decreased iron delivery (66, 72), as a result of an interaction with transferrin receptors (22, 31), whereas the coexpression of HFE with the interacting protein β2-microglobulin stimulated transferrin delivery (81). We tested whether the reporters could distinguish the differential effect of these proteins by virally introducing IRE-YFP constructs into TRvb-1 (transferrin receptor 1-deficient CHO cells that carry human transferrin receptor 1; Ref. 54) expressing either HFE or HFE plus β2-microglobulin (Ref. 81; Fig. 9). In the HFE-expressing cells, ferric ammonium citrate increased 5′-IRE-YFP fluorescence and decreased 3′-IRE-YFP fluorescence (the ratio of 5′- to 3′-YFP fluorescence = 3.1-fold compared with untreated cells in basal medium; n = 6); however, there was no change in fluorescence after culture with human holotransferrin (ratio of 5′ to 3′ fluorescence = 1.1 compared with untreated cells in basal medium; n = 6). In contrast, cells expressing HFE and β2-microglobulin demonstrated responses to both non-transferrin-bound iron (ratio of 5′ to 3′ fluorescence = 3.3; n = 6) and holotransferrin (ratio of 5′

Fig. 3. Response to iron, assayed by immunoblot with anti-YFP. YFP is constitutively expressed in non-IRE-YFP cells, but the addition of 5′- or 3′-IRE results in iron-dependent translation. 5′-IRE suppresses translation in low iron (DFO, 20 μM), whereas the 3′-IRE cassette suppresses translation in the presence of ferric ammonium citrate (25 μM). Transferrin receptor 1 (TIR1) also responded to iron, but GAPDH was unchanged. Extract from 10⁵ cells was loaded on each lane of the immunoblot.
Fig. 4. Response to iron assayed by fluorescence microscopy. The addition of the 5’-IRE sequence suppressed the expression of YFP (compare A with C), but the addition of ferric ammonium citrate (Fe; 25 µM) to the culture medium (12 h) enhanced fluorescence in the same field (compare C and D). Conversely, the addition of iron to 3’-IRE-YFP cells suppressed fluorescence (compare E and F). Non-IRE-YFP cells in contrast were not responsive to iron (compare A and B). Exposure time = 3 s for all panels.

Fig. 5. Specificity for iron. 5’-IRE-YFP (shaded bars), 3’-IRE-YFP (open bars), and non-IRE-YFP (solid bars) 293 clones were treated with different metals (25 µM) for 12 h, and then mean fluorescence was calculated by FACS. The change in cellular fluorescence after the addition of the metal is displayed. Note the specific response to iron in 5’-IRE-YFP and in 3’-IRE-YFP cells but the more limited and nonspecific responses to other metals.
to 3′ fluorescence = 1.9; n = 6). The parental cell line likewise demonstrated (not shown) responses to both non-transferrin-bound iron (ratio of 5′ to 3′ fluorescence = 3.3; n = 6) and holotransferrin (ratio of 5′ to 3′ fluorescence = 2.8; n = 6). Cells infected with the non-IRE-containing reporter did not respond to non-transferrin-bound or to transferrin-bound iron (ratio of 1.09 and 1.05, respectively, compared with cells in basal medium). These findings indicate that the combination of HFE and β2-microglobulin positively regulates the steady-state level of cellular iron by permitting transferrin uptake, whereas HFE alone is not effective. In contrast, the HFE-β2-microglobulin complex does not regulate the acquisition of non-transferrin-bound iron. These results confirm a prior study using these cell lines (81) in showing that HFE plus β2-microglobulin is the physiological regulator for iron uptake from transferrin, and they demonstrate that the IRE reporters can detect manipulations of the transferrin pathway.

Ngal/siderocalin lipocalin. The lipocalins are structurally related proteins that bind and transport low-molecular-weight chemicals (25, 88). Ngal was recently renamed siderocalin because it binds bacterial siderophores with high affinity and these siderophores chelate iron (28). When Ngal/siderocalin was cloned in the XL-1B bacterial strain, it contained a siderophore called enterobactin that is stoichiometrically loaded with iron. When Ngal/siderocalin is cloned in BL-21 strains, in contrast, it is siderophore free and iron poor. We previously found (87) that Ngal induced the conversion of rat kidney mesenchymal cells into epithelial cells that form complete nephrons in vitro. The response of the mesenchyme was enhanced by utilizing Ngal obtained from XL-1B bacteria. This preparation upregulated ferritin and downregulated transferrin receptor 1 proteins in cell lines, suggesting that it was iron loaded and able to donate iron to the cytoplasm (87).

To examine these hypotheses further, we generated matched samples of iron-loaded and iron-free Ngal by combining a single lot of Ngal produced in BL-21 bacteria with either iron-loaded or iron-free enterobactin (EMC Microcollection). Iron-loaded Ngal-enterobactin was red in color, whereas the iron-free Ngal-enterobactin had no color (Fig. 10A).
containing Ngal-enterochelin markedly enhanced the growth of rat kidney mesenchyme, the expression of epithelial E-cadherin, and de novo tubulogenesis, whereas the iron-poor Ngal-enterochelin had much less effect (Fig. 10B), suggesting that Fe-Ngal-enterochelin donated iron. To further test whether iron-loaded Ngal-enterochelin could donate iron, we added these preparations to the YFP reporter cells. We found that the iron-loaded Ngal-enterochelin doubled the fluorescence of the 5'-IRE-YFP cells [no addition: 43 ± 2.8 (n = 6); Fe-Ngal-enterochelin: 85 ± 9.7 (n = 6); P < 0.004] whereas the iron-poor Ngal-enterochelin reduced the fluorescence [Ngal-enterochelin: 33 ± 2.4 (n = 6); P < 0.04; Fig. 10C]. The opposite was found when the two preparations of Ngal were added to the 3'-IRE-YFP cells. Iron-loaded Ngal reduced the fluorescence [no addition: 149 ± 6.9 (n = 5), Fe-Ngal-enterochelin: 95 ± 21.8; P < 0.045] whereas iron-poor Ngal enhanced the fluorescence [Ngal-enterochelin: 191 ± 22 (n = 6); P < 0.004]. Control cells containing the YFP reporter lacking the IRE had no response to either form of Ngal [no addition: 980 ± 84 (n = 6); Fe-Ngal-enterochelin: 921 ± 178 (n = 6, P = 0.78); Ngal-enterochelin: 1,035 ± 86 (n = 6, P = 0.69)]. To determine whether Ngal might downregulate the message for IRP-1 or IRP-2, and hence mimic iron delivery, we measured IRP transcripts by real-time PCR, but we failed to find a difference when cells were treated with either iron-loaded or iron-poor Ngal-enterochelin or were left untreated. These data implicate iron-loaded Ngal-enterochelin as an iron donor and, conversely, iron-free Ngal-enterochelin as an iron chelator. The data also demonstrate that growth and epithelial conversion of the rat mesenchyme in vitro is enhanced by iron donation by this novel transporter.

IRP activation. Because the IRE-based reporters are regulated by the binding of IRP proteins, inhibition of IRP binding activity should mimic treatment of cells with iron. Nitric oxides (NO) have been reported to modify IRP function (17, 43, 85). NO, which is generated by sodium nitroprusside (SNP), inhibits IRP-2 activity by nitrosylation of a cysteine (44), and this effect could not be reversed by iron chelation in some studies (7, 41, 65) but was reversed in other works (6, 42).

We found that addition of SNP (1–100 μM) to the reporter cells increased 5'-IRE fluorescence as much as 30-fold and decreased 3'-IRE fluorescence as much as 6-fold (Fig. 11). In contrast, there was no effect of SNP on the non-IRE-YFP reporters, indicating that these changes could not be attributed to global changes in protein synthesis. In addition, the effect of SNP could not be mimicked by adding the same concentration of iron (Fig. 11) or ferricyanide, a metabolite of SNP, or reversed or diminished by DFO (not shown), suggesting that the effect of SNP cannot be attributed to the delivery of iron alone. These findings are consistent with prior work (7, 41, 65) that showed that NO directly modifies IRP (44). The data indicate that the fluorescent IRE reporters are sensitive to modification of IRP activity.

Ratio imaging analysis. Because the 5'-IRE, 3'-IRE, and non-IRE reporters demonstrated differing responses to iron and NO, it may be possible to use more than one of these constructs in the same cell to measure a ratio of responses to factors that
affect the IRP-IRE interaction. Measurement of a ratio may increase the specificity and perhaps the sensitivity of the iron-dependent signal. To create reporters suitable for ratio imaging, we ligated a 5’-IRE with a destabilized CFP and then introduced this vector into 293 cells. To determine the feasibility of two-color imaging, we analyzed the 5’-IRE-CFP, 3’-IRE-YFP, and untransfected 293 cells by confocal microscopy to measure crossover between the fluorescent channels as well as autofluorescence. Only 4.2% and 3.6% of CFP fluorescence crossed into the YFP channel in transiently (n = 556 cells) and stably (n = 635 cells) transfected 293 cells, respectively. Addition of iron and DFO did not alter this crossover. Conversely, YFP-expressing cells demonstrated little crossover into the CFP channel (n = 460 cells). Moreover, autofluorescence was <2% of the CFP or the YFP signal (n = 225). These values show the feasibility of using two-color reporters in the same cell to measure IRP-IRE interactions.

5’-IRE-CFP was transiently introduced into 3’-IRE-YFP expressing clones. The ratio of 5’-IRE-CFP to 3’-IRE-YFP fluorescence decreased from 2.7 ± 0.03 (n = 2,481 cells) to 0.66 ± 0.007 (n = 2,628 cells) in the presence of DFO and increased in a dose-responsive fashion to 12.3 ± 0.18 (n = 1,588 cells) in the presence of ferric ammonium citrate. DFO (10 μM) and iron (≥10 μM) produced significant changes in mean CFP and YFP fluorescence (P < 0.001; n = 4 independent experiments; 515–668 cells/measurement) (Fig. 12). These data show that the IRE reporters are useful to measure iron-dependent fluorescence with ratio analysis and data collection by confocal microscopy.

DISCUSSION

The interaction of the cytoplasmic pool of iron with the IRP proteins regulates the expression of messages that contain IREs (Fig. 1). To assay the activity of the IRP proteins in living cells, we have developed a series of fluorescent molecular probes whose expression is dependent on the IRP-IRE interaction. These reporters are appropriate reagents for a number of reasons. First, the reporter cells were sensitive to exogenous iron in a range similar to that of cells loaded with calcein (21). Second, the fluorescence response was dependent on the IRE, because constructs lacking the IRE were not responsive to changes in iron (Fig. 2). Moreover, changes in the fluorescent response correlated well with changes in the binding of IRP to IRE (supplemental data). Third, IRE-based reporters utilize an endogenous mechanism of iron sensing and hence are expected to assay the same pool of iron that regulates the expression of endogenous messages that contain IREs. Although high levels
of expression of a reporter construct can render a reporter unresponsive (“squelching”; see, e.g., Ref. 26), we did not find cells that failed to respond to DFO and we did not find changes in endogenous messages that depend on the IRPs. In fact, because only a single copy of YFP or CFP is detectable in transgenic mice (77), it will not be necessary to express high levels of the reporter constructs in vivo. Fourth, because the location of the IRE-IRP generated reciprocal responses to iron, and because these responses could be compared with one another (Fig. 12) as different fluorescent proteins can be independently monitored, IRE-based reporters provide a ratio analysis of iron-dependent gene expression. Comparison of iron-sensitive IRE reporters with iron-insensitive non-IRE reporters provides a second method of ratio analysis. Ratio analysis is very useful to validate subtle changes in IRP-IRE interaction, as well as to exclude changes in reporter expression that reflect non-iron-, non-IRP-based events. For example, treatment with apotransferrin induced a small increase in 5'-IRE, a small decrease in 3'-IRE, and no change in non-IRE fluorescence (not shown), indicating the delivery of a small quantity of iron. In contrast, treatment with copper and gallium induced small changes in the expression of 5'-IRE, 3'-IRE, and the non-IRE constructs, ruling out that these metals mimic the effect of iron (Fig. 5). The reciprocal responses of the IRE reporters compare favorably to the metal-sensing dyes, which respond only by quenching (20, 21). Fifth, the availability of fluorescent proteins with short half-lives permits reversibility of the signal (Fig. 7). This is a necessary precondition to follow IRP activity in real time. We used fluorescent proteins with a 2-h half-life, but even finer resolution may be obtainable with reporters with shorter half-lives because of reduced background and more rapid resolution of the deflections. Indeed, reversibility of our reporters differs from the LacZ or CAT reporters, which are not reversible, and from calcein, which may be difficult to reverse. Sixth, the changes in fluorescence were visible by standard microscopy (Fig. 4) and could be followed by time-lapse cinematography (Fig. 6). Hence, it should be possible to analyze individual cells in a complex tissue. These data indicate that the IRE-IRP system provides the specificity and sensitivity to follow the actions of iron without undue perturbation of the endogenous system. The reporters detect relative changes in the IRP-IRE interaction, whereas calcein is believed to detect absolute values of exchangeable iron. We used many standard models of the IRP-IRE system to test the reporter constructs. For example, the IRE reporters could distinguish the effects of different iron transporters. The effect of holotransferrin rapidly saturated (above 500 nM iron; Fig. 8), but the effect of non-transferrin-
bound iron, given in even larger doses, did not saturate its signal (5–50 μM, Fig. 2). This distinction is consistent with higher affinity mechanisms of transferrin compared with non-transferrin-bound iron delivery (78). In fact, the reporters could distinguish the two pathways in cells transfected with HFE and β2-microglobulin, which regulate transferrin, but not non-transferrin iron traffic (Fig. 9). Iron delivery by the iron-loaded enterochelin-Ngal complex was also detected (Fig. 10). Given the response to different iron carriers, we speculate that a good use of the IRE-based reporters may be to identify new iron delivery pathways by screening fractions of blood or other tissue extracts with transferrin receptor 1-negative cell lines carrying the IRE reporters. This technique may be superior to measuring ferritin levels, which could be misleading because the candidate factor might regulate ferritin at the transcriptional level. The converse of these experiments, the isolation of new compounds that chelate iron, could also be aided by the reporter cells. For example, we found that the iron-free enterochelin-Ngal complex reduced the 5′-IRE-YFP fluorescent signals, and had no effect on non-IRE-YFP cells, indicating that it chelated iron.

The IRE reporters were also useful to evaluate factors that directly effect IRP proteins. For example, we found that SNP, a NO\textsuperscript{−} generator, mimicked the addition of iron. The effect of SNP, however, was many times greater than the effect of the same concentration of iron or ferricyanide (a metabolite), consistent with prior data suggesting that NO\textsuperscript{−} directly inhibits IRP activity (see, e.g., Ref. 44). Curiously, the NO\textsuperscript{−} generator S-nitroso-N-acetyl-penicillamine (SNAP) (61, 65, 75) did not reproduce the effect of nitroprusside, suggesting that the pool of iron that can be affected by NO\textsuperscript{−} was quite small. Support for this explanation comes from the finding that the addition of iron generally had a greater effect on fluorescence than did DFO. In this light, the dramatic effect of SNP is likely attributable to inhibition of IRP binding to IRE, most likely the inhibition of IRP-2. This assignment is based on the dramatic effect of NO\textsuperscript{−} on IRP-2 (6, 41, 43, 44) and not IRP-1 (43) in other cells and the abundance of IRP-2 message in 293 cells. Because 293 cells also express IRP-1, and because they derive from kidney, where, unlike other organs, IRP-1 is required for basal iron sensing (56), an unequivocal assignment of the NO\textsuperscript{−} target, however, should await the introduction of the iron reporters into knockout cells.

We foresee that the IRE reporters will be useful to examine developing organs and to determine the effects of gene deletions. Given that transferrin receptor 1 is expressed in a stage- and cell type-specific manner in kidney, hematopoietic, and lymphoid cells (8, 19, 24, 40, 76, 87) and is absolutely required only at a single stage of development (8, 59) by the latter tissues, mechanisms of iron acquisition may be quite heterogeneous in vivo. This idea is also suggested by the fact that ferritin expression (68, 74) varies among different regions and stages of the developing brain, and the IRPs are themselves developmentally regulated (47, 74), indicating that cellular iron loading may vary from one cell type to another. Because the IRE-based reporters can monitor single cells in a reversible fashion, we propose that these constructs will be useful in vivo to detect changes in iron and IRP activity during organ development.

In sum, we have developed a method of iron sensing that is complementary to the fluorescent dyes. Although calcein and phen-green may be useful to detect the initial rates of iron flux and can be calibrated to yield absolute iron concentrations, our probes can be used to provide long-term, continuous monitoring of relative changes in cellular iron that accompany cell growth and development. Our probes detect the actions of IRP signal transducers in living cells.

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