Direct Fe\textsuperscript{2+} Sensing by Iron-responsive Messenger RNA•Repressor Complexes Weakens Binding*  

Received for publication, July 3, 2009, and in revised form, August 18, 2009. Published, JBC Papers in Press, August 31, 2009, DOI 10.1074/jbc.M109.041061

Mateen A. Khan\textsuperscript{1}, William E. Walden\textsuperscript{2}, Dixie J. Goss\textsuperscript{3,4}, and Elizabeth C. Theil\textsuperscript{1,5}  

From the \textsuperscript{1}Department of Chemistry, Hunter College, City University of New York, New York, New York 10065, the \textsuperscript{2}Department of Microbiology and Immunology, University of Illinois, Chicago, Illinois 60612-7334, the \textsuperscript{3}Children’s Hospital Oakland Research Institute, Oakland, California 94609, and the \textsuperscript{4}Department of Nutrition Science and Toxicology, University of California, Berkeley, California 94720

Fe\textsuperscript{2+} is now shown to weaken binding between ferritin and mitochondrial aconitase messenger RNA noncoding regulatory structures (\textit{iron-responsive element} (IRE)-RNAs) and the regulatory proteins (IRPs), which adds a direct role of iron to regulation that can complement the well known regulatory protein modification and degradative pathways related to iron-induced mRNA translation. We observe that the \(K_d\) value increases 17-fold in 5’- untranslated region IRE-RNA•repressor complexes; Fe\textsuperscript{2+}, is studied in the absence of \(O_2\). Other metal ions, Mn\textsuperscript{2+} and Mg\textsuperscript{2+} have similar effects to Fe\textsuperscript{2+} but the required Mg\textsuperscript{2+} concentration is 100 times greater than for Fe\textsuperscript{2+} or Mn\textsuperscript{2+}. Metal ions also weaken ethidium bromide binding to IRE-RNA with no effect on IRP fluorescence, using Mn\textsuperscript{2+} as an \(O_2\)-resistant surrogate for Fe\textsuperscript{2+}, indicating that metal ions bound IRE-RNA but not IRP. Fe\textsuperscript{2+} decreases IRP repressor complex stability of ferritin IRE-RNA 5–10 times compared with 2–5 times for mitochondrial aconitase IRE-RNA, over the same concentration range, suggesting that differences among IRE-RNA structures contribute to the differences in the iron responses observed in vivo. The results show the IRE-RNA•repressor complex literally responds to Fe\textsuperscript{2+}, selectively for each IRE-mRNA.

Iron (e.g. ferrous sulfate, ferric citrate, and hemin) added to animal cells changes translation rates of messenger RNAs encoding proteins of iron traffic and oxidative metabolism (1–4). To cross cell membranes, iron ions are transported by membrane proteins such as DMT1 or carried on proteins such as transferrin. Inside the cells, iron is mainly in heme, FeS clusters, non-heme iron cofactors of proteins, and iron oxide minerals coated by protein nanocages (ferritins). Iron in transit is thought to be Fe\textsuperscript{2+} in labile “pools” accessible to small molecular weight chelators, and/or bound loosely by chaperones.

When iron concentrations in the cells increase, a group of mRNAs with three-dimensional, noncoding structures in the 5’-untranslated region (UTR)\textsuperscript{3} are derepressed (Fig. 1A), i.e. the fraction of the mRNAs in mRNA•repressor protein complexes, which inhibit ribosome binding, decreases and the fraction of the mRNAs in polyribosomes increases (5–7). The three-dimensional, noncoding mRNA structure, representing a family of related structures, is called the iron-responsive element, or IRE, and the repressors are called iron regulatory proteins (IRPs). Together they are one of the most extensively studied eukaryotic messenger RNA regulatory systems (1–4). In addition to large numbers of cell studies, structures of IRE-RNAs are known from solution NMR (8–12), and the RNA•protein complex from x-ray crystallography (13). Recent data indicate that demetallation of IRP1 and disruption of the [4Fe-4S] cluster that inhibits IRP1 binding to RNA will be enhanced by phosphorylation and low iron concentrations (1, 2, 14–16). Such results can explain the increased IRP1 binding to IRE-mRNAs and increased translational repression when iron concentrations are abnormally low. However, the mechanism to explain dissociation of IRE-RNA•IRP complexes, thereby allowing ribosome assembly and increased proteosomal degradation of IRPs (1, 2, 14, 15) (Fig. 1A), when high iron concentrations are abnormally high, is currently unknown.

Metal ion binding changes conformation and function of most RNA classes, e.g. rRNA (17), tRNA (18, 19), ribozymes (20–23), riboswitches (24, 25), possibly hammerhead mRNAs in mammals (26), and proteins. Although the effects of metal ion binding on eukaryotic mRNAs have not been extensively studied, Mg\textsuperscript{2+} is known to cause changes in conformation, shown by changes in radical cleavage sites of IRE-RNA with 1,10-phenanthroline-iron and proton shifts in the one-dimensional NMR spectrum (12, 27). The Mg\textsuperscript{2+} effects are observed at low magnesium concentrations (0.1–0.5 mM) and low molar stoichiometries (1:1 and 2:1 = Mg:RNA).

We hypothesized that Fe\textsuperscript{2+} could directly change the binding of the IRE-mRNA to the iron regulatory protein for several reasons. First, other metal ions influence the IRE-RNA structure (12, 27). Second, in IRE-RNA/IRP cocryosts there are exposed RNA sites in the IRE-RNA/IRP complex that are accessible for interactions (13) (Fig. 1B). Third, regions in the IRE-RNA are hypersensitive to Fe\textsuperscript{2+}-EDTA/ascorbate/H\textsubscript{2}O\textsubscript{2}, suggesting selective interactions with metals and/or solvent (28).

\textsuperscript{1}This work was supported, in whole or in part, by National Institutes of Health Grants DK20251 (to E. C. T.) and DK47281 (to W. E. W.). This work was also supported by National Science Foundation Grant MCB0814051 (to D. J. G. and M. K.), a Professional Staff Congress-City University of New York Faculty Award (to D. J. G.), and the Children’s Hospital Oakland Research Institute Foundation (to E. C. T.).

\textsuperscript{2}To whom correspondence may be addressed: 695 Park Ave., New York, NY 10065. Tel.: 212-772-5383; Fax: 212-772-5332; E-mail: dgoss@hunter.cuny.edu.

\textsuperscript{3}The abbreviations used are: UTR, untranslated region; IRE, iron-responsive element; IRP, iron regulatory protein repressor for IRE mRNA; mt, mitochondrial; EtBr, ethidium bromide.
We now report that Fe$^{2+}$/H$_{11001}$ weakens IRE-RNA/IRP binding, whereas Mg$^{2+}$/H$_{11001}$ requires 100 times the concentration and Mn$^{2+}$/H$_{11001}$ is comparable with Fe$^{2+}$/H$_{11001}$; the Fe$^{2+}$/H$_{11001}$ effect was diminished in mutant IRE-RNA and IRE-RNA selective in wild type sequences: ferritin IRE-RNA > mt-aconitase IRE-RNA.

EXPERIMENTAL PROCEDURES

Preparation of Binding Proteins and RNA

Isolation of recombinant rabbit IRP1 from yeast used previously described methods (29). RNA oligonucleotides for frog ferritin H, frog ferritin H U6, and mt-aconitase (30, 29, and 29 nucleotides, respectively) were purchased from Genelink (Hawthorne, NY). The frog H (FerH) IRE-RNA was used as a model because of extensive structural information, e.g., "footprinting," solution NMR, x-ray diffraction of cocrystals with IRP, and direct comparisons between natural, poly(A), 5'-UTR-IRE mRNA, with full-length transcripts and RNA aptamers (28, 30). After dissolving in 40 mM HEPES/KOH, pH 7.2, RNA was melted and annealed as described (31), by heating to 85 °C for 15 min with slow cooling to 25 °C. Melting and annealing decreased the $K_d$ by ~5-fold for the ferritin-IRE-RNA but had little effect on the mt-aconitase IRE-RNA.

Analysis of Fluorescence Data

Protein fluorescence intensities (332 nm (280 nm excitation)) were corrected for dilution, as needed, and for inner filter effects; maximum dilutions were <7%. Nonlinear least squares fitting of the data used KaleidaGraph software (version 2.1.3; Abelbeck Software). Ethidium bromide fluorescence was measured at 595 nm, with excitation at 510 nm. Scatchard analysis of EtBr binding used KaleidaGraph software.

Effect of Metals on RNA-Protein Complexes, RNA, and Protein

Solution—Solutions of Fe$^{2+}$-O$_2$, Mg$^{2+}$, or Mn$^{2+}$ were added to both RNA and protein solutions at the same concentrations, and the solutions were incubated separately for 15 min before adding to binding buffer that contained the same metal ion concentration as the RNA and protein solutions. RNA and protein mixtures were incubated in binding buffer (29), 40 mM HEPES/K$_{11001}$, pH 7.2, 100 mM KCl, 5% glycerol, and 2% 2-mercaptoethanol, for 15 min at 25 °C, before making fluorescence measurements. When Fe$^{2+}$ was used, all incubations were anaerobic. The protein concentration was 0.1 M and the RNA concentration varied from 0 to 1.0 M; because of tight binding, 0.05 M protein was also analyzed for ferritin RNA titrations to determine $K_d$. The order of addition to the binding buffer had no effect on the results. In the case of Fe$^{2+}$, nitrogen-purged solutions of 0.1 M HCl used to dissolve FeSO$_4$ and inhibit oxidation were diluted to 0.001 M H$_{11001}$ and further diluted 1:100 into the RNA or protein solutions.

Gels—Solutions of RNA and protein ± Fe$^{2+}$-O$_2$ were prepared as in the solution studies. Free and bound RNA was resolved by electrophoresis (EMSA) in 1% agarose gels followed...
Fe$^{2+}$ Destabilizes IRE-RNA/IRP

Ferrous Ions (-O$_2$) Weaken Ferritin and mt-Aconitase IRE-RNA-IRP Repressor Complexes—We selected two IRE-mRNAs to study, ferritin and mt-aconitase, because iron induces the synthesis of both proteins in whole animals and cells and because the iron responses are different: iron induces ferritin synthesis in the liver more than ~150-fold and in mt-aconitase ~4-fold (32). The two IRE-RNA structures are different (Fig. 2) as is the binding of IRP2 and IRP1 in the electrophoretic mobility shift assay (31). When the $K_d$ for IRP1 binding to the two IRE-RNAs was determined by protein fluorescence quenching, the values differed 10-fold (Figs. 2 and 3).

Fe$^{2+}$, 5 µM, increased the $K_d$ for ferritin IRE-RNA 6-fold and mt-aconitase RNA 2-fold (Fig. 3A), showing that Fe$^{2+}$ weakens IRE-RNA/IRP1 binding and suggesting that excess cellular Fe$^{2+}$ can derepress, i.e. facilitate formation of repressor-free, IRE-mRNA. Stabilities of IRE-RNA/IRP1 complexes were decreased 17- and 6-fold for ferritin and mt-aconitase IRE-RNA/IRP complexes, respectively, at 50 µM Fe$^{2+}$. Thus, Fe$^{2+}$, as well as the IRP1 repressor itself, recognize differences in IRE-RNA structure. The result is that Fe$^{2+}$ weakens the RNA/protein repressor interaction differentially for the IRE-mRNAs that regulate ferritin and mt-aconitase synthesis.

When we analyzed IRE-RNA/IRP1 interactions by fluorescence quenching, the titration used a constant protein concentration (0.1 µM) for the ferritin IRE-RNA, additional data were collected with 0.05 µM protein; RNA varied from 0 to 1.0 µM. To evaluate the possible effect of using a constant RNA concentration and titrating with variable protein concentrations, and to obtain data from electrophoretic mobility shift gel electrophoresis as in earlier studies of the IRE-RNA-protein complexes (31, 33, 34), we examined the effect of Fe$^{2+}$ on RNA/protein interactions, in the absence of oxygen, by electrophoretic mobility shift assay. A constant 0.1 µM RNA was used with variable protein concentrations (0 to 2.0 µM) ± 50 µM Fe$^{2+}$-O$_2$. Gels were prepared and analyzed in the absence of air to stabilize Fe$^{2+}$. The fraction of RNA complexed to the protein was diminished by the presence of Fe$^{2+}$ (Fig. 3B). Differences between ferritin and mt-aconitase RNAs in the gels are observed most readily by comparing the unshifted RNA bands (see Fig. 3). Changing the order of addition of RNA and protein to the reaction mixture had no effect. The direct effects of Fe$^{2+}$ observed by the stability of the RNA-protein complex (Fig. 3) complement the indirect effects of cellular iron, as previously observed, on the RNA/protein binding that is mediated by protein degradation and, for IRP1, changes in [4Fe-4S] assembly (1, 2, 15).

Magnesium and Manganese Weaken Ferritin and mt-Aconitase IRE-RNA-IRP Repressor Complexes—Mn$^{2+}$ is chemically similar to Fe$^{2+}$ but can be studied in air (22). Mn$^{2+}$ also weakens IRE-RNA/IRP1 interactions, with differential effects for the two IRE-RNAs as well. For example, for 50 µM ferritin IRE-RNA, IRP1 binding was weakened 8-fold by Mn$^{2+}$ and 17-fold by Fe$^{2+}$. For the mt-aconitase IRE-RNA/IRP complex, the effects of Fe$^{2+}$ and Mn$^{2+}$ were identical (Fig. 3A), and smaller than for the ferritin IRE-RNA/IRP complex.

Mg$^{2+}$ was studied because of the direct metal interactions observed with ferritin IRE-RNA by NMR spectroscopy (11) and because Mg$^{2+}$ influences the structure/function of many classes of RNA (e.g. Refs. 17 and 26). Only when Mg$^{2+}$ concentrations are 100 times higher than Mn$^{2+}$ or Fe$^{2+}$ was comparable weakening of the IRE-RNA/IRP complexes observed (Fig. 3A). The metal selectivity and RNA selectivity for altering IRE-RNA/IRP stability raises the possibility that metal ions played a selective role in the evolution of IRE-RNA/IRP complexes or the possibility that metal ions besides iron contribute to regulation of in vivo 5'-UTR IRE-RNA/IRP interactions.

Divalent Metal Ions Bound to IRE-RNA but Not IRP1—There is no evidence that metal ions bind to IRP1, except for the FeS cluster added to the apoIRP1 that yields c-aconitase. Furthermore, there are no obvious, additional, predicted metal binding sequences in the primary sequence of IRP1 (14). On the other hand there is evidence that metals bind to IRE-RNA from solution NMR and metal nuclease cleavage (27, 28, 35). To obtain more direct evidence that the effect of metal ions on the stability of the IRE-RNA-protein complex reflects metal ions bound to IRE-RNA and not to the IRP repressor, we examined the effect of metal ions on the
Fe²⁺, Mg²⁺, and Mn²⁺ selectively weaken IRE-RNA/IRP-protein interactions.

**A** Intrinsic fluorescence of IRP and the fluorescence of ethidium bromide bound to IRE-RNA. We used Mn²⁺ as a surrogate for Fe²⁺ to facilitate experiments in air, because the effects on stability of the RNA-protein complex are similar to Fe²⁺ (Fig. 3).

Mn²⁺ does not bind to IRP1, based on the absence of changes in intrinsic fluorescence (Fig. 4) when manganese is added; Mg²⁺ does not bind either, using the same analytical approach (Fig. 4). By contrast, addition of IRE-RNA to IRP1 decreases the intrinsic fluorescence of IRP1 (Fig. 2).

**B** Mn²⁺ binds to ferritin IRE-RNA, in experiments using EtBr fluorescence as a reporter (Fig. 4A). For example, EtBr fluorescence decreases when Mn²⁺ was added to EtBr and RNA; the \( K_d \) between EtBr and RNA was 41.6 ± 2.3 × 10⁶ liter/mol in the absence of Mn²⁺ and 18.0 ± 0.6 × 10⁶ liter/mol in the presence of Mn²⁺; the decrease in \( K_d \) is significant (p < 0.01), and indicates metal binding to RNA. The stoichiometry of EtBr binding to IRE-RNA was 4.8 ± 0.3, and decreases to 2.6 ± 0.1 (Fig. 4) in the presence of Mn²⁺. Given the general similarities between Fe²⁺ and Mn²⁺ in destabilizing the IRE-RNA/IRP complex, undetectable Mn²⁺ binding to IRP1, and detectable Mn²⁺ binding to IRE-RNA and general similarities of Fe²⁺ and Mn²⁺ chemistry, the results indicate that Fe²⁺ destabilizes the IRE-RNA/IRP complex by binding to the RNA. In vivo when Fe²⁺ accumulates to concentrations that saturate normal Fe²⁺ chaperones and transporters, and fill ferritin, Fe²⁺ becomes available to destabilize IRE-RNA/IRP complexes and increase the synthesis of the encoded proteins.

**FIGURE 3.** Fe²⁺ selectively weakens IRE-RNA/IRP1 interactions. A, IRE-RNA binding to IRP was measured, ± Fe²⁺-O₂, as IRP1 fluorescence quenching in solution (constant protein concentration) as described in the legend to Fig. 2 (see "Experimental Procedures"). The IRE-RNAs used were FerH IRE-RNA, FerH ΔU⁶ IRE-RNA, and mt-aconitase IRE-RNA. Data were analyzed as previously described (45, 46). The \( K_d \) values are the averages of three titrations. Relative stabilities (1/\( K_d \)) of the IRE-RNA/IRP1 complexes are used in the graph, right. B, IRE-RNA binding to IRP ± Fe²⁺-O₂ was measured by electrophoretic mobility shift assay (constant RNA concentration). Anaerobic solutions of RNA and protein were prepared as in A; Fe²⁺ was 50 μM when present (see "Experimental Procedures"). A constant 0.1 μM RNA concentration was used with varying concentrations of protein; RNA was stained with ethidium bromide. Note the larger amount of IRP1 repressor required to bind (shift) an equivalent amount of mt-aconitase IRE-RNA compared with ferritin IRE-RNA ± Fe²⁺-O₂. The data are representative of two independent experiments and the significance of the effect of metal ions on the \( K_d \) values is p < 0.01.
Ferritin IRE-RNA binds IRP1 repressor more stably than the
Ferritin ΔU⁶ Mutant or mt-Aconitase IRE-RNAs—Because ferritin
IRE-RNA differs from mt-aconitase IRE-RNA by a U⁶ bulge in the helix, we analyzed the ferritin ΔU⁶ mutant IRE-RNA. Ferritin ΔU⁶ IRE-RNA should behave more like mt-aconitase than ferritin IRE-RNA, based on the predicted RNA secondary structure (Fig. 2) and the regions of contact in the IRE-RNA/IRP1 crystal structure (Fig. 1B). In fact, stability of the ferritin ΔU⁶ IRE-RNA/IRP1 complex is between that of the mt-aconitase IRE-RNA/IRP complex (3-fold higher) and the ferritin IRE-RNA/IRP complex (3-fold lower). Thus, the U⁶ bulge in ferritin RNA explains only part of the difference in IRP

**FIGURE 4.** Metal ions alter fluorescence of IRE-RNA/EtBr complexes but not of apoIRP1. Experiments with hydrated manganous ions, models for ferrous iron because of the similarity of effects on IRE-RNA/IRP complexes (Fig. 3) and general chemical similarities, are facilitated because of the relative insensitivity of Mn²⁺ to air at neutral pH, contrasting with Fe²⁺. All experiments were in binding buffer (see “Experimental Procedures”). Protein fluorescence was measured as described in the legend to Fig. 2. EtBr-IRE-RNA fluorescence (excitation at 510 nm and emission at 525 nm) was measured in solutions with 0.1 μM ethidium bromide and 2.0 μM IRE-RNA. A, fluorescence spectra of EtBr-FerH IRE RNA complexes ± metal ions (50 μM Mn²⁺ and/or 50 μM Mg²⁺): 1) RNA; 2) RNA + Mn²⁺; 3) EtBr; 4) EtBr + Mn²⁺; 5) EtBr + RNA + Mn²⁺ + Mg²⁺; 6) EtBr + RNA + Mn²⁺; 7) EtBr + RNA + Mg²⁺; and 8) EtBr + RNA. B, stoichiometry of EtBr binding to IRE-RNA in the presence (●) and absence (○) of 2 μM Mn²⁺; C, fluorescence spectra from solutions of IRP1 ± 50 μM Mn²⁺ or 50 μM Mg²⁺; 1) IRP1; 2) IRP1 + Mn²⁺; and 3) IRP1 + Mg²⁺; D, bar graph comparing the effects of manganous ions on IRP1 and IRE-RNA. The data are the results of duplicate experiments and the error is the S.D.; * significantly different from controls, p < 0.01 for Mn²⁺ and <0.02 for Mg²⁺.
binding by ferritin and mt-aconitate IRE-RNAs. Mn\(^{2+}\) or Mg\(^{2+}\) had only small effects on the mutant IRE-RNA-IRP complex, in contrast to the complexes of wild type IRE-RNA with IRP (Fig. 3A), indicating that in the ferritin ΔU\(^{6}\) IRE-RNA-IRP1 complex the metal binding sites that influence stability or the RNA conformational change responsible for decreased IRP binding are blocked by deletion of U\(^{6}\). Because both ferritin and ferritin ΔU\(^{6}\) IRE-RNAs have identical base pairs but differences in IRP binding, because ferritin ΔU\(^{6}\) and mt-aconitate IRE-RNAs have similar secondary structures around the two IRP1 binding sites of the C\(^{8}\) bulge and CAGUG loop (Fig. 2), and because metal effects on the protein-RNA complex differ among ferritin, ferritin ΔU\(^{6}\), and mt-aconitate IRE-RNAs, the phylogenetically conserved differences among each IRE-mRNA clearly contribute to the inherent stability and metal responses of the IRE-RNA-IRP complexes.

DISCUSSION

The translation of 5′-UTR IRE-mRNAs increases when concentrations of environmental iron, such as ferrous salts, ferric chelates, and heme increase as the result of iron-induced changes in the IRE/IRP interactions and by degrading IRP (1–4). However, the chemical identity of the cellular iron signal targeted to IRE-RNA/IRP interactions is unknown, although insertion of a [4Fe-4S] cluster, after cluster synthesis/transport by ISC protein catalysts and chaperones (36), influences IRE-RNA-IRP1 interactions. When cellular iron concentrations are high, a larger fraction of each 5′-UTR IRE-mRNA is in the polyribosomes (5, 6, 37). Iron deficiency, by contrast stabilizes IRP concentrations and destabilizes the FeS cluster in IRP1, possibly mediated by IRP phosphorylation, and a smaller fraction of the 5′-UTR IRE-mRNAs are in polyribosomes (16). (A subset of the IRE-mRNA family, mainly involved in iron absorption and transport, regulates mRNA turnover with the mRNA stabilized by IRP binding. The 3′-UTR IRE-RNAs have specific structural features that place them outside the scope of this study.) IRE-RNA-IRP complexes occur in the cytoplasm, but whether the RNA-protein complex forms during IRE-mRNA processing in the nucleus, or during transport to the cytoplasm for mRNA storage or use remains unknown. The effect of Fe\(^{2+}\) on the stability of the 5′-UTR IRE-RNA-IRP complexes reported here indicates that the complex can sense increases in concentrations of aquated Fe\(^{2+}\) that result in weaker RNA/protein interactions.

Destabilization of the IRE-RNA-IRP complex by Fe\(^{2+}\) competes with the stabilization conferred by the very large number of bonds between the protein and the RNA complex in crystals (13); no IRE-RNA/IRP or IRE-RNA crystals have been obtained with metals to date (38). In the IRE-RNA-IRP complex, protein-RNA bonds are clustered at the RNA C\(^{8}\) bulge and the CAGUG terminal loop of the IRE-RNA structure (13). A number of RNA-protein bonds involve RNA sites that are hypersensitive to cleavage by Fe\(^{2+}\)-EDTA/ascorbate/H\(_{2}\)O\(_{2}\) (28), suggesting specific interactions with Fe\(^{2+}\)-EDTA or solvent or both. The observed destabilization of the IRE-RNA-IRP complex is most likely explained by metal binding to exposed sites on the IRE-RNA (Fig. 1B), for several reasons. First, metal ions did not bind to the IRP1 in the current study. Second, there are no predicted metal binding sites in the IRP protein (14) beyond cysteine/[Fe-S] interactions. Third, in this study, metal ions decrease conformation-sensitive EtBr binding to RNA. Finally metal binding to IRE-RNA has been detected by NMR spectroscopy and metalonuclease cleavage (12, 27, 35).

Structural differences between ferritin and mt-aconitate IRE-RNAs are reflected in the stability differences of the RNA-protein complexes observed here, because the same IRP is present in both complexes and only the RNA is different. IRE-RNA structural differences then, explain, at least in part, the 30-fold difference in iron-induced synthesis in vivo for the two proteins and differences in IRP1 and IRP2 binding in vitro (31, 32). Because ferritin is an ancient IRE-RNA and mt-aconitate evolved more recently (39), the lower stability of the mt-aconitate IRE-RNA-protein complex and the smaller iron response may reflect the shorter time for evolutionary fine-tuning of the IRE-mRNA. However, the different iron responses of the two IRE-RNAs could also reflect the physiological function of each encoded protein. For example, large fluctuations in mt-aconitate synthesis could be deleterious to cell oxidative metabolism, which would explain the relatively small changes in synthesis induced by iron. By contrast large changes in ferritin synthesis allow cells to respond to large changes in iron and oxygen stress through ferritin mRNA translation (more sensitive to iron signals) as well as ferritin DNA transcription (more sensitive to oxidant signals) (40, 41).

Fe\(^{2+}\), Mn\(^{2+}\), and 100× Mg\(^{2+}\) destabilize wild type IRE-RNA-IRP complexes, but the IRE-RNA mutation greatly reduced the metal ion effects; a single nucleotide deletion that eliminated the ferritin-selective IRE-RNA U\(^{6}\) bulge also greatly reduced the metal ion effects and weakened IRP binding. Thus, the genetic selective forces acting upon each IRE-mRNA appear to be extremely high. Moreover, the phylogenetic conservation of each IRE-RNA structure is extremely high (>90%) and includes variations among wild type IRE-RNA structures such as the initiator AUG in the mt-aconitate IRE-RNA, and base pairing flanking sequences around the ferritin-IRE RNAs of vertebrates (27, 28, 39, 42). The family of IRE-RNA structures with selective recognition by IRP 1 and 2 (29, 31, 43) yields a combinatorial array of RNA-protein complexes (44) with a range of physical stabilities that are tunable by Fe\(^{2+}\) and other cellular signals.

Acknowledgment—We are grateful to Dr. Takehiko Toshia for the graphics in Fig. 1B.

REFERENCES

1. Wallander, M. L., Leibold, E. A., and Eisenstein, R. S. (2006) Biochim. Biophys. Acta 1763, 668–689
2. Rouault, T. A. (2006) Nat. Chem. Biol. 2, 406–414
3. Leipuviene, R., and Theil, E. C. (2007) Annu. Rev. Nutr. 28, 197–213
4. Zähringer, J., Baliga, B. S., and Munro, H. N. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 857–861
5. Hentze, M. W. (1993) J. Biol. Chem. 268, 5974–5978
6. Hentze, M. W., Muckenthaler, M. U., and Andrews, N. C. (2004) Cell 117, 285–297
