Combinatorial mRNA Regulation: Iron Regulatory Proteins and Iso-iron-responsive Elements (Iso-IREs)*

Published, JBC Papers in Press, November 2, 2000, DOI 10.1074/jbc.2000019200

Elizabeth C. Theil‡§ and Richard S. Eisenstein¶
From the 3Children’s Hospital Oakland Research Institute, Oakland, California 94609-1673 and Department of Nutritional Sciences, University of Wisconsin, Madison, Wisconsin 53706-1571

Combinations of RNA elements (mRNA specific) with binding proteins give a wide range of responses to biological signals from iron, oxygen, NO, or growth factors. Combinatorial regulation of transcription to coordinate synthesis of groups of proteins is well known and is exemplified by steroid hormone-responsive genes (1). Combinatorial regulation of mRNA utilization to coordinate synthesis of groups of proteins is unique currently to iron and oxygen metabolism in animals (2–15) (see Fig. 1). The RNA elements are called iso-iron-responsive elements (iso-IREs), 2 and the binding proteins, called iso-iron regulatory proteins (iso-IRPs), are aconitase homologues. Examples of iso-IRE mRNAs are ferritin to concentrate iron, Tfr and DMT-1 for iron uptake, and ferroportin (Fpn1/IREG1/MTP1) for iron efflux. Several proteins for oxygen metabolism are also encoded in iso-IRE mRNAs, exemplified by aminolevulinate synthase (eALAS) in heme synthesis and mt-aconitase in the trichloroacetic acid cycle. Signals that control iso-IRE/iso-IRP binding include iron, oxygen, hydrogen peroxide, NO, and activators of protein kinase C.

When iron regulation of mRNA function was last described in a Minireview (1990) only two IRE-mRNAs (ferritin and Tfr) were known (2), in contrast to the many IRE mRNAs currently known. Iron was the only known signal, and knowledge of structure was limited to RNA sequence and secondary structure determined by prediction and enzymatic/chemical probes (2). Annotation of the literature in the intervening period is in Refs. 4–7. Now much is known about IRE tertiary structure (7). Multiple signaling pathways are known to converge on the IRE/IRP interaction (4, 6, 8–11). The combinatorial RNA/protein family and the effects of the RNA protein complex on protein synthesis are illustrated in Fig. 1. The result of the RNA-binding protein specificity, for the different iso-IRE-containing mRNAs, is quantitative differences in the expression of proteins that are finely tuned over a wide range. Such precise control over the synthesis of each of the proteins relates to the central role of the proteins in normal cell biology: iron trafficking, heme synthesis, and cellular ATP production.

The flexibility of regulation using the IRE/IRP mRNA/protein interactions is illustrated by the liver where the same amount of iron induces ferritin synthesis up to 100-fold (2), but mitochondrial aconitase is only induced 2–3-fold (3); the difference likely relates to a narrow tolerance of cells to concentration changes in trichloroacetic acid cycle enzymes. Differences in the iso-IRE binding in each mRNA suggest a higher percentage of ferritin mRNA will be bound to IRPs than mt-aconitate mRNA (see Fig. 3), allowing quantitative variations in the response of protein synthesis to signals. An alternate mechanism for IRE/IRP control of protein synthesis is regulated mRNA turnover, illustrated by the TFR IRE.

Iso-IRE Structure

IRE-containing mRNAs have been identified in vertebrates, invertebrates, and bacteria. They encode proteins that function in iron uptake, storage, and export (mammals, birds, amphibia, insects, and bacteria) (4, 10–13, 15) and for heme synthesis or the trichloroacetic acid cycle/ATP production (mammals, amphibia, fish, insects, and bacteria). No IREs have been detected in plants, although an IRE-hybridizable, nonferritin sequence in soybean has been observed. 2 IREs present in the 5′ or 3′ noncoding regions of mRNA were originally thought to be structurally the same, based on the predicted secondary structure of the stem loop and the similarity of IRP1 binding. However, based on comparisons among larger numbers of IRE sequences from different mRNAs coupled with additional studies of structure and binding with purified IRPs it is now apparent that the mRNA-specific divergences in IRE sequence and structure define isoforms of the IREs. Variations in IRE structure selectively influence the interactions with iso-IRPs.

Primary—Comparisons of animal IRE sequences reveal that the conservation of sequence identity is much higher (>90% identity) between species for the same mRNA than between different mRNAs in the same species (36–85% identity) (16) (Fig. 2). IREs have 26–30 nucleotides (based on sequence conservation and protein footprint), with a central CAGUG sequence and a C residue five bases upstream. Complementary base pairs flank the C and CAGUGX. In ferritin IREs an additional set of conserved bases, U/C G-C, upstream from C create a pocket related iso-IRP2 binding (Fig. 3). Conserved non-IRE sequences occur in both the ferritin and TIR-IRE regulatory elements and influence function (17, 18).

Secondary—Iso-IREs fold with the CAGUGX in a terminal hexa-loop (Fig. 2) containing a C-G base pair required for IRP binding, because substitution of A for G prevents binding of either iso-IRP1 or iso-IRP2 (2, 9, 19). Substitution of U-A for C-G selectively inhibits binding of iso-IRP2 (19). IRE stems have 9–10 base pairs and form an A-helix (20, 21) with a small distortion caused by the conserved, unpaired C or the internal loop bulge, created by the conserved G-C base pair. Structure appears to be modulated by the base pair closing the hexa-loop (18). In addition, the sequence of base pairs in the upper helix between the stem distortion and the hexa-loop contributes to protein binding, exemplified by the 30-fold change in IRP1 binding for CAA/UUG → UUG/CAA (22). Melting cooperativity of the entire IRE was also influenced by engineering the substitution of one natural helix sequence for another between the bulge/loop and the hexa-loop 3 or when natural iso-IREs were compared.

Tertiary—NMR spectroscopy and nuclease (protein/chemical) probing define IRE structures. The C-G base pair across the CAGUGX hexa-loop pushes AGU into the solvent (18, 20, 23). At the middle of the helix of the ferritin IRE, a G-C base pair forms the internal loop bulge into a pocket of the large groove that selectively enhances IRP2 binding and binds metals (8). Protonation in the physiological range alters the IRE structure at either the IL/B or C-bulge of IREs (20, 21), but the proton acceptor (cytosine phosphate?) is yet not known. The large groove of the IRE stem is enlarged by distortions at the C-bulge or IL/B (20, 21) creating specific base and ribose contact sites for protein (18).

Taken together the studies show the impact of the IRE primary
include ferritin, TfR, DMT-1, eALAS, and hypoxia or minus oxygen. Iso-IRE mRNAs include ferritin, TfR, DMT-1, eALAS, and mt-aconitase. Iso-IRPs are IRP1 and IRP2.

**Fig. 1. Combinatorial regulation of mRNA.** Left, the two known mechanisms of IRE/IRP effects: inhibiting ribosome binding or degradation (nuclease binding) to control mRNA translation or turnover, respectively. Right, the physiology of IRE/IRP effects: an array of cell-specific variables that modify IRE/IRP interactions permit a wide range of responses to iron, oxygen, and other biological signals. R, ribosome; N, nuclease; O2, hypoxia or minus oxygen. Iso-IRE mRNAs participate in the translational regulation of ferritin mRNA was first suggested by data obtained in the 1970s and early 1980s (2, 6, 7). The two proteins identified since then, IRP1 and IRP2 (4, 6), specifically inhibit the translation or turnover of IRE-containing mRNAs. It has become clear that the IRPs also have distinct binding properties (8), sensitivity to environmental iron and oxygen signals, and mechanisms of response to the signals and to phosphorylation (6, 24).

**Iso-IRP Structure**

Iso-IRPs are aconitase homologues and (as for iso-IREs) are each more highly conserved between species (>90% identity) than for IRP1 and IRP2 in the same species (61% identity). The first IRP identified, IRP1, cycles between the RNA binding form (apo-aconitase) and cytoplasmic aconitase (c-aconitase), which has an [4Fe-4S] iron-sulfur cofactor.

Iso-IRP1—When peptides from c-aconitase are compared with the sequence of IRP1 predicted from the cDNA, the identity is >98%. In crystals of mt-aconitase the Fe-S cluster is in a solvent-filled cleft (25). By analogy, assuming the IRP-specific insertions are only in the surface loops that do not affect folding (4, 5, 26), the IRE binding site has been suggested to be close to or the same as the binding cleft of the Fe-S cluster. Some residues predicted to reside in the putative cleft of iso-IRP1/c-aconitase are required for both aconitase function and iron regulation of mRNA function/RNA binding, based on site-directed mutagenesis and cross-linking studies (4, 5, 26). It has been hypothesized that the presence or absence of the Fe-S cluster modulates the extent to which the cleft is open and able to bind the IRE complex, and evidence supporting this model has been obtained (26, 42, 43). However, the detailed structural analyses required to prove such a notion have yet to be completed.

Iso-IRP2—The high sequence identity between IRP1 and IRP2, 61%, excludes a 73-amino acid insertion unique to IRP2 near the amino terminus of the protein. IRP2 does not form an Fe-S cluster; iron regulates RNA binding by targeted proteasomal degradation (24). The 73-amino acid loop is required for iron-induced, IRP2 degradation because a chimera of IRP1 with the IRP2-specific loop inserted at the analogous site displayed enhanced degradation in cells with excess iron. The redox state of cysteine residues in both IRPs and complexation with the Fe-S cluster in IRP1 can influence RNA binding and provide a potential site for regulation by oxygen, NO, and oxyradicals (26).

**Multifactorial Regulation of IRP Function**

The ratio of IRP1/IRP2 varies in a cell-specific fashion, although exploration of cell-specific control of the ratio has been studied only briefly (24). Iron regulates IRP expression post-transcriptionally (27) and post-translationally (6, 28). In addition to differences in steady state concentrations of iso-IRPs, the iron signal acts post-translationally on two different IRP activity parameters: direct RNA binding (IRP1) or protein turnover (IRP2) with differing sensitivities. When the iron signal is heme, protein turnover is enhanced for both IRP1 and IRP2 (28). The oxygen signals also have differential effects on the iso-IRPs. In sum, the relative contributions of IRP1 and IRP2 to IRE binding vary over a broad range including the complete absence of IRP1 (29). Ablation of the gene for IRP1 in mice has no detectable phenotype to date, but loss of the IRP2 gene has very severe consequences (30). In addition to the differences in IRP/IRE binding, selective regulation of IRP function sequence and of the base pairs in the IRE hairpin on iso-IRE structure that is the foundation for the selective iso-IRP binding and regulation of the use of different iso-IRE mRNAs. trans-Factor participation in the translational regulation of ferritin mRNA was

**Fig. 2. Iso-IRE structure.** Top, primary structure of an iso-IRE (TfR-IRE); there are five copies of IREs in the TfR turnover element (a, b, c, d, e). Note the high interspecies conservation (>95%), which contrasts with the iso-IRE mRNA-specific variation in the same species (15–65%) (GenBank accession numbers M11507, X01060, M38040, X13753, X55348, and AW454691. Bottom left, hairpin secondary structure. IL/B (ferritin) and C-bulge (eALAS) IREs illustrate a conserved terminal loop with variable midstem distortion and helix base pairs. Bottom right, three-dimensional structure MC-SYM/NMR model of IL/B IRE (ferritin) viewed from the major groove (21). Yellow, IL/B cavity; blue, docked metal (Co(III) hexammine). (See Ref. 20 to compare a C-bulge IRE structure.)

**Fig. 3. IRE/IRP complexes: IRP1 phosphorylation and the Fe-S apo cycle; IRP2 sensitivity to the internal loop/bulge.** The differential behavior of IRP1 and IRP2 in IRE recognition is illustrated in two ways. Left, indirect activation of IRP1 by altering Fe-S cluster stability is enhanced by phosphorylation. For IRP2, in contrast, phosphorylation appears to modulate the redox state of the protein. Red, wild type; yellow, S138A. Phosphomimetic mutants S138D (orange) and S138E (blue) are shown. Data are taken from Refs. 37 and 39. Right, differential binding of iso-IRPs to iso-IREs. IRP1 binds all iso-IREs, whereas IRP2 binds well only when the internal loop/bulge is present (Fig. 2). Lanes 1–5 are iso-IREs of ferritin, Fer-U6, TfR, eALAS, and mt-aconitase, respectively, from Ref. 8.
by different biological signals expands the versatility of this regulatory and sensory network required for maintaining iron homeostasis.

Iso-IRP1—IRP1/c-aconitate is a bifunctional protein. Iron regulates IRP1 by building an Fe-S cluster on the apoprotein, which blocks RNA binding activity. The Fe-S form of IRP1 is c-aconitate, which is the cytosolic isofrom of the mitochondrial, [4Fe-4S] iron-sulfur enzyme, aconitate (mt-aconitate) (4, 5). The Fe-S cluster is a key determinant in selecting one of the two possible protein functions. Although the detailed structural changes induced upon formation of the Fe-S cluster remain unknown, it is clear that the two functions of the protein are mutually exclusive and that changes in cellular iron status can drive the protein to its functional extremes (4, 6). The shifts in IRP1 function associated with the Fe-S cluster assembly emphasize the emerging role of iron-sulfur proteins as biosensors (25).

Likely a number of gene products will be required to deliver the iron and sulfur, based on studies of Fe-S cluster assembly in nitrogenase and copper trafficking proteins in yeast and humans (25, 33). Assembly of Fe-S clusters in nitrogenase in bacteria involves genes for localized generation of sulfide and delivery of iron for cluster formation (31); analogous genes occur in eucaryotes (34, 48). Understanding the accessory proteins and factors such as NO and H₂O₂ (35, 36), which regulate Fe-S cluster formation, are problems for the future.

Iso-IRP2—Iron decreases the cytoplasmic pool of IRP2 through protein oxidation, followed by its ubiquitination and proteasomal degradation. Agents capable of generating "NO or NO⁺" promote loss of IRP2 protein in cultured cells although the exact details through which these agents act remain to be defined (6, 36). The specific sites and mechanisms of iron-induced oxidation of IRP2 are currently undefined. Cell-specific variations in the activity of the protein oxidation and ubiquitin degradation pathways are likely to contribute to qualitative differences in the effect of iron and oxygen signals on IRP2 activity. Apparent inconsistencies in the literature about hypoxia-induced changes of IRP2 activity, for example, may be attributed to part in such cell-specific variations in IRP2.

The two mechanisms that IRP function will be superimposed upon are cell-specific variations in the ratio of expression of IRP1: IRP2 and in the concentration of iso-IREs. When combined with the differential interaction of the iso-IRPs with iso-IREs (Fig. 3), an enormous range of responses can be predicted and the exquisite sensitivity of the iso-IRE/iso-IRP response is illuminated.

Phosphorylation, an Iron-independent Means for Modulating IRP Function

Iron and oxygen homeostasis are central to cell biology. The cytokine regulation ofIRE-mRNAs, which encode proteins of both iron and oxygen metabolism (2, 7), and the phosphorylation of IRP1 or IRP2 indicate that the iso-IRE/IRP regulatory system is integrated with more general signal transduction pathways.

Phosphorylation of both IRP1 and IRP2 has been demonstrated in phorhodin 12-myristate-treated HL-60 cells in which the RNA binding activity of IRP1 and IRP2 was also stimulated (37). Purified rat liver IRP1 is an efficient substrate for bovine brain protein kinase C (PKC) (Kₐ, ~0.5 μM), accommodating up to 2 mol of phosphate/mol of protein (reviewed in Ref. 32). Ser-138 and Ser-711 were substrates for PKC. Ser-138 is near Asp-125 and is in the region critical for high sensitivity of the iso-IRE/iso-IRP response is illuminated. The shifts in IRP1 function associated with the Fe-S cluster assembly emphasize the emerging role of iron-sulfur proteins as biosensors (25).

The ferritin IL/B (U/C5, G6, C7, C25) binds Mg(II)(H₂O)₆, Co(III)(NH₃)₆, and Cu(II)(Phen)₂ (Fig. 2). Protons in paired bases at the edge of the IL/B pocket have pH-dependent exchange rates in the physiological range. Apparently the size of the helix distortion caused by a single unpaired C is too small to accommodate Cu(II)(Phen)₂ in contrast to the IL/B (9, 21, 41). Mg(II) inhibits Cu(II)(Phen)₂ binding in the IL/B, and in the absence of Cu(II)(Phen)₂, Mg(II) binding affects protons on U10, G18, U21, and G22 (9, 41). Metal complexes with tri- and bipyridyl ligands (Ru(tpy)²/²py) and with the glycophosphate Fe-bleomycin bind to the terminal loop of IRPs at G16 or U/C19, respectively, but are apparently too large or bulky to bind to the IL/B (17, 23).

Proteins—RNAs—RNases (S₁ and T₁) find few sites in IREs except in the terminal loop (G16, U17) (T1 and S1) and upper stem (U10, C11) (V1) whereas IRP1 protects the entire IRE (7, 17) and forms cross-links at U6 and U19 (42). Many schemes of IRP/IRE regulation by iron or more recently by NO assumed that the IRP binding of iron caused the IRE-IRP complex to dissociate, by analogy to simpler repressor-DNA complexes and from the protection that IRPs provide to chemical and enzymatic probes and polyribosome entry. However, no direct information is available on the sequence of events or whether or not the IRE/IRP complex is physically separated in response to the iron or oxygen signals. Cys-437, -503, and -506, required for c-aconitate activity, are in the substrate cleft of the aconitate structure (42–44); Cys-437 is also required for IRP1 to bind RNA. Several active site Arg residues and amino acids in domain 4 are also required for IRE binding (26).

The iso-IRP2/iso-IRE complex is the more critical cell target for control because: 1) IRP2 binding and translation regulation in vitro is more sensitive to IRE structure (8, 9, 19); 2) cells can be regulated with no detectable IRP1 (29); 3) ablation of the IRP2 gene in mice is very severe compared with IRP1, which has no detectable phenotype (30). The full impact of the multiple iso-IRE/iso-IRP complexes (Fig. 1) remains to be determined.

Six types of variables define iso-IREs: IRP2 binding (5), the distortion of the major groove in the IRE helix by the C-bulge or IL/B, the base pair closing the hexa-loop (45, 46), the presence of conserved IRE flanking sequences, an AU-rich lower stem, and the base pair 5’ of the C bulge. In addition, the base sequence of the helix between the stem distortion (C-bulge or IL/B) and the hexa-loop is unique for each iso-IRE and influences protein recognition (22).

Perspective

Combinatorial iron regulation of mRNA encoding proteins of iron homeostasis was obscured by variations in the magnitude of regulatory effects over a range of 50–100-fold (47) and because some of the mRNA targets were not identified until very recently. Although the trans-acting protein factors (IRP) responsible for the iron-dependent regulation had been known for some time, awareness of the impact of multiple IRP proteins, additional signals such as oxygen or c-aconitate, cytokines, and PKC phosphorylation of IRPs, and the structural variations in iron-responsive elements (iso-IREs) is more recent.

The functional effects of mRNA-specific variations in IRE and IRP structure are superimposed on the shared IRE hairpin loop and combinations of helix distortions, stem sequences, and flanking regions to produce IRE isoforms that are mRNA-specific and highly conserved among species (≥95% identity). IRE sequences among the different mRNAs in humans are only 35–85% identical. The effect is differential IRP binding and translation repression that can account for the wide range of responses observed to the
same physiological signal. Phosphorylation of IRPs by PKC and iron/oxygen/cytokine signaling integrates regulation of IRE mRNAs in ways that are only beginning to be explored. Combinatorial approaches to create a range of signal responses are more commonly understood in physics and engineering but have recently been identified in nature through the action of specific networks of transcription factors and their gene targets. Given the theories that life evolved from an RNA world, the existence of a combinatorial mRNA regulation is restricted to coordinating iron and oxygen metabolism or occurs elsewhere remains to be discovered.

Acknowledgment—We are grateful to Dr. Hans Johansson for the tabulation of iso-IRE sequences.

REFERENCES

1. Darimont, B. D., Wagner, R. L., Apriletti, J. W., Stallcup, M. R., Kushner, P. J., Baxter, J. D., Fletterick, R. J., and Yamamoto, K. R. (1998) Genes Dev. 12, 3343–3356
2. Theil, E. C. (1990) J. Biol. Chem. 265, 4771–4774
3. Eisenstein, R. S., and Blemings, K. P. (1998) Mol. Cell. Biol. 18, 238–248
4. Rouault, T. A., and Klausner, R. D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 8175–8182
5. Eisenstein, R. S., Kennedy, M. C., Walden, W. E., and Eisenstein, R. S. (1998) Mol. Cell. Biol. 18, 2319–2326
6. Eisenstein, R. S. (2000) Annu. Rev. Nutr. 19, 623–642
7. Theil, E. C. (1998) J. Biol. Chem. 273, 23637–23640
8. Ke, Y., Wu, J., Leibold, E. A., Walden, W. E., and Eisenstein, R. S. (1997) J. Biol. Chem. 272, 16529–11653
9. Roth, R. D., and Eisenstein, R. S. (1997) Gene Regulation: New Development Strategies (Badman, D. G., Bergeron, R. J., and Brittenham, G. M., eds) pp. 133–144, The Saratoga Group, Ponte Vedra Beach, FL
10. Goessling, L. S., Mascetti, D. P., and Thach, R. E. (1998) J. Biol. Chem. 273, 12555–12557
11. Schalinske, K. L., Blemings, K. P., Steffen, D. W., Chen, O. S., and Eisenstein, R. S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10681–10686
12. Rouault, T. (2000) in Iron Chelators: New Development Strategies (Badman, D. G., Bergeron, R. J., and Brittenham, G. M., eds) pp. 133–144, The Saratoga Group, Ponte Vedra Beach, FL
13. Zheng, L., Cash, V. L., Flint, D. H., and Dean, D. R. (1998) J. Biol. Chem. 273, 13264–13272
14. Eisenstein, R. S., Kennedy, M. C., and Beinert, H. (1997) in Metal Ions and Gene Regulation (Silver, S., and Walden, W. E., eds) pp. 157–216, Chapman and Hall, New York
15. Pena, M. M. O., Lee, J., and Thiele, D. J. (1999) J. Nutr. 129, 1251–1260
16. Voisine, C., Schilke, B., Ohlson, M., Beinert, H., Marszalek, J., and Craig, E. A. (2000) Mol. Cell. Biol. 20, 3677–3684
17. Oliveira, L., and Drapier, J. C. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 6550–6555
18. Kim, S., and Pinka, P. (2000) J. Biol. Chem. 275, 6220–6226
19. Schalinske, K. L., and Eisenstein, R. S. (1996) J. Biol. Chem. 271, 7168–7176
20. Narahari, J., Ma, R., Wang, M., and Walden, W. E. (2000) J. Biol. Chem. 275, 16227–16234
21. Brown, N. M., Anderson, S. A., Steffen, D. W., Carpenter, T. B., Kennedy, M. C., Walden, W. E., and Eisenstein, R. S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15255–15250
22. Bates, D. M., Popescu, C. V., Khoshshila, N., Vogt, K., Beinert, H., Munck, E., and Kiley, P. J. (2000) J. Biol. Chem. 275, 6234–6240
23. Wang, Y.-H., Sezken, S. R., and Theil, E. C. (1999) Nucleic Acids Res. 18, 4463–4468
24. Basilion, J. P., Rouault, T. A., Massinople, C. M., Klausner, R. D., and Burgess, W. H. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 574–578
25. Schalinske, K. L., Anderson, S. A., Tuazon, P. T., Chen, O. S., and Eisenstein, R. S. (1997) Biochemistry 36, 3950–3958
26. Swenson, G. R., Patino, M. M., Beck, M. M., Gaffield, L., and Walden, W. E. (1991) Biol. Metals 4, 48–55
27. Mullner, E. W., and Kuhn, L. C. (1988) Cell 53, 815–825
28. Blissler, C. R., Burgiel, G., Neupert, B., Emery-Goodman, A., Kuhn, L. C., and May, B. K. (1993) J. Biol. Chem. 268, 12899–12905
29. Chen, O. S., Schalinske, K. L., and Eisenstein, R. S. (1997) J. Nutr. 127, 238–248
30. Lamp, T., and Rouault, T. A. (1998) Mol. Cell. Biol. 18, 807–815