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Targeting mRNA to Regulate Iron and Oxygen Metabolism

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ABSTRACT. A family of non-coding sequences in the mRNA (iso-IREs [iron-responsive elements]) regulate synthesis of key proteins in animal iron and oxidative metabolism such as ferritin and mitochondrial aconitase. Differential recognition between iso-IREs and iso-IRPs (iron regulatory proteins) regulates the translation or degradation of the IRE-containing mRNAs. IREs are hairpin loop structures with an internal loop/bulge or bulge that influence the binding of the iso-IRPs. The iso-IRPs have sequence homology to the aconitases and at least one IRP can be converted to an aconitase. Signals that target the iso-IRE/iso-IRP interactions in mRNA include environmental iron, O_2, nitric oxide, H_2O_2, ascorbate, growth factors, and protein kinase C-dependent IRP phosphorylation. Iso-IRE structural specificity suggests a means of pharmacologically targeting mRNA function with chemicals such as Fe–bleomycin and other transition metal complexes that could be extended to other mRNAs with specific structures. With the iso-IRE/iso-IRP system, nature has evolved coordinated combinatorial control of iron and oxygen metabolism that may exemplify control of mRNAs in other metabolic pathways, viral reproduction, and oncogenesis. BIOCHEM PHARMACOL **59**;1:87–93, 2000. © 1999 Elsevier Science Inc.

KEY WORDS. IRE; IRP; iron; ferritin; mRNA regulation; translation; mRNA turnover; mRNA targeting

GENERAL MECHANISM OF mRNA REGULATION—THE IRE FAMILY

Iron and oxygen are at a metabolic crossroad where mismanagement leads to inflammation, toxicity, and tissue damage. Many genes encode proteins to provide efficient and safe use of iron and oxygen for crucial reactions in cell division, respiration, regulation, and detoxification. A subset of such genes contains non-coding sequences that are transcribed to the mature mRNA to regulate protein synthesis in response to environmental changes in iron, free radicals, and metabolic signals. Examples of such genes are ferritin [1–7] m-aconitase [8, 9], the enzyme catalyzing the first step in erythroid heme synthesis (5-aminolevulinate synthase; ELAS) [10–12], and iron uptake proteins such as the transferrin receptor [13, 14] and DCT-1/DMT-1 [15–20]. The non-coding, mRNA sequences are called IREs† and are a family of closely related iso-RNA structures which recognize several binding proteins (iso-IRPs) to permit combinatorial coordination of expression (for reviews, see recent reports by Eisenstein, Hanson and Leibold, Hentze and Kuhn, Rouault and Klausner, and Theil [21–25]).

IREs are sequences of 28–30 nucleotides in the 5′ or 3′ non-coding regions of mRNAs encoding proteins of iron metabolism and oxidative metabolism. The center of the IRE is a conserved sequence, CAGUGX, which forms a hexaloop in the hairpin secondary structure (Fig. 1, right). The base-paired stem varies in an mRNA-specific fashion. Thus, the conservation of IRE sequence among different species of vertebrates is >95% for the same mRNA but only 36–55% for different IRE-containing mRNAs in the same species, showing that the sequences are mRNA-specific isoforms, an iso-IRE family [24, 26]. IREs bind IRPs to block ribosome binding (mRNA translation) or nuclease activity (mRNA turnover) (Fig. 1). Iron, growth factors, heme, and free radicals change the IRE/IRP interaction.

Since one of the IRPs (IRP-1) reversibly binds a radical-sensitive Fe–S complex, many of the physiological studies have been interpretated in terms of direct interactions between iron–protein–RNA. However, the fact that another IRP (IRP2) does not bind the Fe–S complex [27–32], the fact that both IRPs can be phosphorylated by protein kinase C [33, 34], and the fact that hormones and growth factors also influence IRE/IRP interactions [35–37] all suggest that more classical cell signaling pathways can integrate the iron, free radical signals and IRE/IRP interactions with other regulatory pathways (Fig. 2).

TARGETING mRNA

The advantages of targeting mRNA to regulate gene expression, particularly by using small TMCs, has been recently reviewed [38]: mRNA combines cell specificity with low cell copy number, which contrasts with either DNA (low copy number) or proteins (high specificity) as regulatory (or drug) targets (Table 1).

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† *Abbreviations:* IRE, iron-responsive element; IRP, iron regulatory protein; and TMC, transition metal complex.

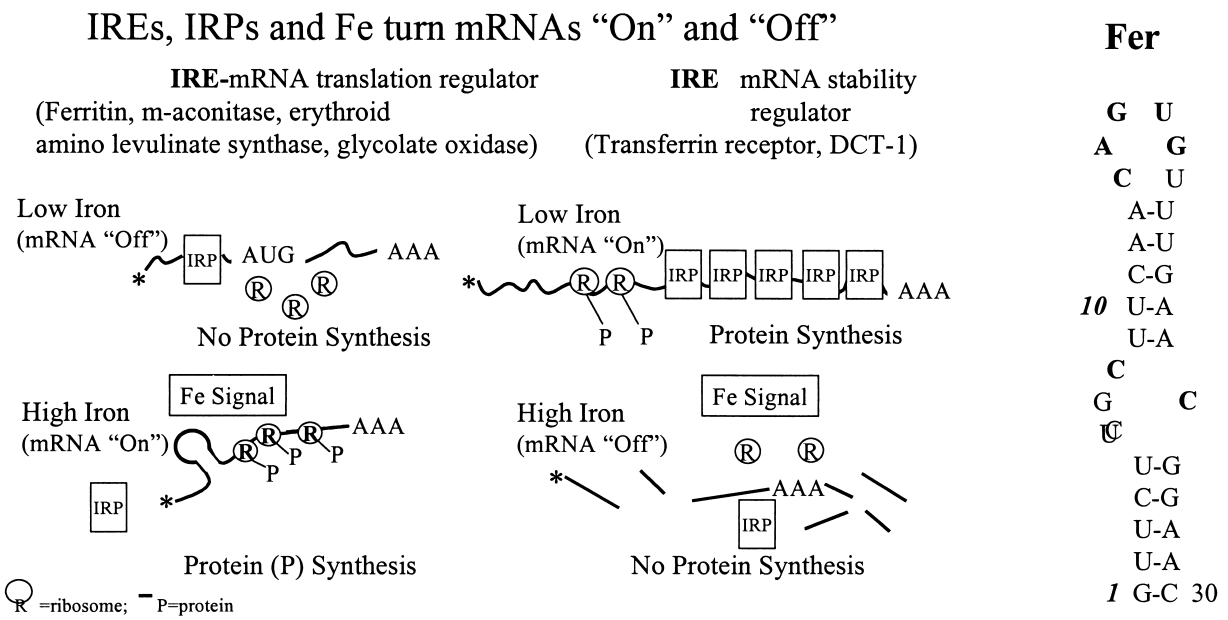

FIG. 1. IREs, IRPs, and Fe turn mRNAs "On" and "Off". *Left*. IRE/IRP regulation. The effect of IRP binding on mRNA function. (figure modified from [69]. *Right*. Secondary structure of the ferritin IRE. U6, G7, G25 (underlined) are specific to the ferritin IRE. IRP footprints bases 1–30, and cross-links to U5 and U 19 next to the G-C base pairs that cross the loops [28, 40, 48].

High sensitivity to small changes in cellular concentrations of regulatory signals is possible with DNA and RNA because of the low copy number. High selectivity of regulatory signals is possible with RNA or protein targets because of the cell specificity of mRNA and proteins. Only RNA combines both features of high sensitivity and high selectivity. Targets for pharmacologic compounds, as well as for natural regulatory signals, are the specific three-dimensional structures of protein and mRNA. Currently, however, the number of known RNA structures is so small compared to protein that from a pharmacologic point of view the main feature of the mRNA targets used is secondary structure ("antisense" RNA). An entire dimension for selectivity and specificity is underutilized. How nature is using the IRE and IRP structures to produce cell-specific, differential responses to regulatory signals will be outlined. The details are being added daily at an explosive rate. How humans might use the IRE/IRP as a model for designing drugs to regulate other metabolic phenomena will be "sketched" at the end of the discussion.

ISO-IRE

IRE binding to IRPs involves recognition of the motifs in the hairpin loop and the bulge/loop in the stem [28, 39–46] (Fig. 1, right). IRP footprints cover the entire IRE [40], and contacts which can be cross-linked are at the junction of the hairpin and internal loops or bulges with the stems [28]. Constant sequences in the IREs are CAGUGX in the terminal hairpin loop and C in an internal bulge or bulge loop. The C residue is much less ordered than the other bases in the IRE stems [47, 48]. Mutation of either sequence such as G18 in the hairpin loop (HL1) or the internal loop C8 in the internal loop or bulge (C8A) prevents IRP binding (illustrated in Fig. 2) and has been observed by many workers stem [28, 39–46]. Variable sequences in the IREs also influence IRP binding. The ferritin iso-IRE binds IRP2 much better than the other iso-IREs as illustrated in Fig. 2. Physiological consequences of the difference are discussed below. A decrease in IRP2 binding by the ferritin iso-IRE is achieved by simply deleting a single nucleotide (ΔU6) to convert the internal loop/bulge of the ferritin iso-IRE to the C bulge of the other iso-IREs (Fig. 2). The structure of the ferritin IRE internal loop/bulge is a pocket in the middle of the RNA stem that includes the conserved C, has a pH-dependent conformation, and binds a metal ion [48]. The coincidence of IRP2-binding specificity with RNA conformational flexibility and metal binding in the ferritin IRE internal loop/bulge suggests a contribution of induced fit during RNA–protein binding.

ISO-IRPS

IRP proteins are a family (IRP-1, IRP-2) which are members of the aconitase superfamily. IRP-1 and IRP-2 are each conserved at ~90% sequence identity within the organisms in which they have been characterized and share ~60–70 identity with each other, but 30–50% identity with aconitases. IRP-1 can be converted to an aconitase by forming an Fe_4S_4 sulfur cluster *in vitro*. *In vitro* IPR-1 can actually rescue organisms with an aconitase deletion in yeast [49]. However, IRP-2 cannot be converted into an aconitase because one of the active site residues is absent [27, 28, 50, 51]. In addition, IRP-2 contains a 70 amino acid insertion absent [27, 28, 50]. How the IRP-2-specific sequence affects the structure of IRPs cannot be known with certainty since,

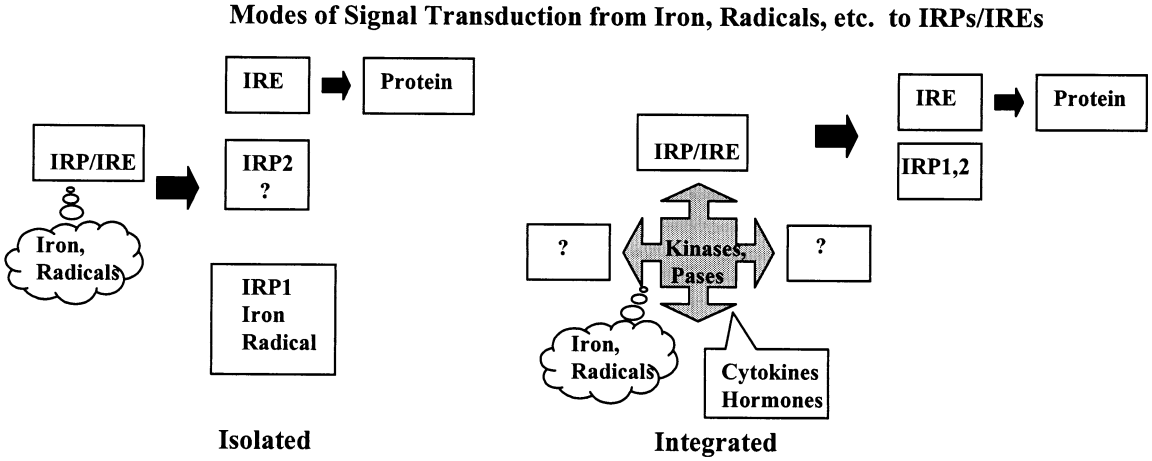

FIG. 2. Modes of signal transduction from iron, radicals etc. to IRPs/IREs. Cartoon of current, alternate ideas for effects of cytoplasmic signals on IRE/IRP interactions.

at this time, only the three-dimensional structure of aconitase, an IRP homologue, has been determined.

IRP-1 shares very high sequence identity with cytoplasmic aconitase [52, 53]. When a subset of peptides, accounting for 10% of the protein of c-aconitase, was sequenced, it was found to be virtually identical to the cDNA sequence of IRP-1 [54]. It was conjectured that Fe regulation of RNA binding by IRP-1 occurred through the interconversion of the Fe_4S_4 aconitase cluster to the Fe_3S_4 cluster [52]. However, it is now known that heme also modifies the IRP interaction with RNA [55–57] and that neither the Fe_3S_4 nor the Fe_4S_4 form of IRP1 binds RNA [58, 59]. Thus, the significance of the IRP-1 and c-aconitase relationship remains obscure and could represent an evolutionary vestige.

RNA binding by IRPs is regulated by changes in the cytoplasmic levels of iron (Figs. 1 and 3). When iron concentrations in cells are constitutive or deficient, or when IRPs are deficient or absent, much of the IRE-mRNA is found in polyribosomes, whereas much less of the IRE-mRNA is associated with polyribosomes in the absence of iron or the presence of IRPs [60, 5, 11, 8]. The quantitative effect of iron on the IRE/IRE interaction in cells appears to reflect, in part, quantitative variations in the iso-IRP/iso-IRE interaction (see Figs. 2 and 3).

The activity of the iso-IRPs is largely regulated posttranscriptionally either by modifying the proteins, degrading the proteins, or both. Transcriptional regulation is mostly set during cell differentiation to create cell-specific differences in the total amount of the IRPs and the IRP1/IRP2 ratio [27, 28, 61].

Phosphorylation is a shared posttranscriptional modification of iso-IRPs [33, 34, 62], but the mechanistic effects are just beginning to be explored. For example, the presence of phophorylatable sites in IRP-1 appears to influence the formation of the Fe–S cluster *in vivo* [34], but detailed mechanisms are not known.

The Fe–S cluster is an IRP-1-specific posttranslational modification related to regulation. Both an Fe_3S_4 and an Fe_4S_4 form of cluster can be made in IRP1 but neither form binds RNA [22, 58]. Sensitivity of the Fe–S cluster to O2/hypoxia, nitric oxide, ascorbate, Fe, hydrogen peroxide, and many other active oxygen species (ROS) led some investigators to relate the physiological effects of ROS to IRE-dependent changes in protein synthesis [22, 25]. However, iron regulation in the absence of an IRP–FeS cluster [27, 28, 61], the influence of phosphorylation on IRP function [33, 62], and the inability of hydrogen peroxide to convert the Fe–S form of IRP1 to the RNA-binding form [59] all point to a much more complex pathway between cellular signals (Fe, O2/hypoxia, ROS) and IRE-dependent mRNA regulation (Fig. 2).

Proteosomal degradation appears to be the IRP2-specific, since regulated degradation has not been detected for IRP-1. The IRP2-specific amino acid insertion is required

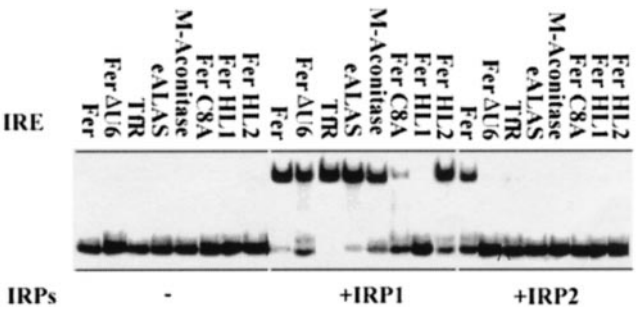

FIG. 3. Differential binding of iso-IRP2 by iso-IREs. RNA sequences corresponding to several iso-IREs (29–30 nucleotides) were transcribed, labeled, annealed to assure high conformational homogeneity, and mixed with purified recombinant IRPs. Bound and unbound RNA was separated by electrophoresis in polyacrylamide gels. Note that while all the natural and some of the mutated iso-IREs bind IRP1, only the ferritin iso-IRE binds IRP2 well. Deletion of a single nucleotide in the internal loop/bulge of the ferritin-IRE (ΔU6) conferred the poor IRP2 binding of C bulge iso-IRE. (Data from [46]). Note that chemical probing studies of the IRE in natural ferritin mRNA (polyA+RNA) and full-length transcripts of ferritin mRNA show that the short IRE has the native conformation [48, 74, 75].

[27–29, 31]. For hypoxia, and possibly other cellular signals such as ascorbate and nitric oxide [63–65], changes in IRP2 activity appear larger than for IRP1 [66]. In addition, some cells only have IRP2 and are still able to regulate iron metabolism [61]. Such observations suggest that the proteosomal degradation pathway for IRP2 is integrated more fully with metabolic signaling pathways in cells than the inactivation of RNA/IRP-1 interactions (Fig. 2, right).

COMBINATORIAL COORDINATION OF mRNA REGULATION

The existence of a family of mRNA regulatory sequences, the iso-IREs, which share common sequence and binding properties to coordinate expression in the cytoplasm, is very unusual, at least at the current state of knowledge. Superimposed on the similarity of IRE structure and function are mRNA-specific differences in the IRE structure [46–48] and protein binding that are conserved in evolution [24, 26]. Added to the iso-IRE complexity is the family of RNA-binding proteins, the iso-IRPs, which display iso-IRE-sensitive binding properties [46], different cellular expression [29, 31, 61], different regulatory properties [29–32], and different sensitivity to environmental signals [25].

Cells can exploit the following molecular combinations to co-regulate IRE-containing mRNAs:

- Total IRE concentration
- Ratio of iso-IREs:relative amounts of iso-IREs
- Total IRP concentration
- Ratio of iso-IRPs
- Ratio of unmodified to modified IRP (Fe–S/phosphorylation)
- Differential binding of iso-IREs/iso-IRPs

Nature has evolved a truly combinatorial set of RNA/protein interactions to amplify one or more signals for a series of co-regulated responses. Thus, a single signal such as environmental iron can have different metabolic effects. Such a scheme is illustrated in Fig. 3, in which a cell which has IRP/IRE = 5:7, IRP1/IRP2 = 3:2, IRE-Fer/IRE-m-acon = 5:2. At the constitutive concentration of iron, one half of the m-acon (mitochrondrial aconitase) mRNA molecules are translated and one-fifth of the Fer (ferritin) mRNA molecules are translated. An increase in cellular iron which blocks all IRE/IRP interactions will produce a 2-fold increase in m-aconitase synthesis and a 5-fold increase in ferritin synthesis. Such a differential effect makes good physiological sense, since large differences in ferritin synthesis can be important to concentrate or detoxify a bolus of iron, but large swings in m-aconitase synthesis would seriously disrupt oxidative metabolism, even though, some sensitivity to environmental changes in iron (or other IRE/IRP-responsive signals) is useful. Teleogy aside, the effect of a single dose of iron on the synthesis of aconitase and ferritin in the liver of a rat has many of the quantitative features predicted [67, 68] by the combinatorial model shown in Fig. 4.

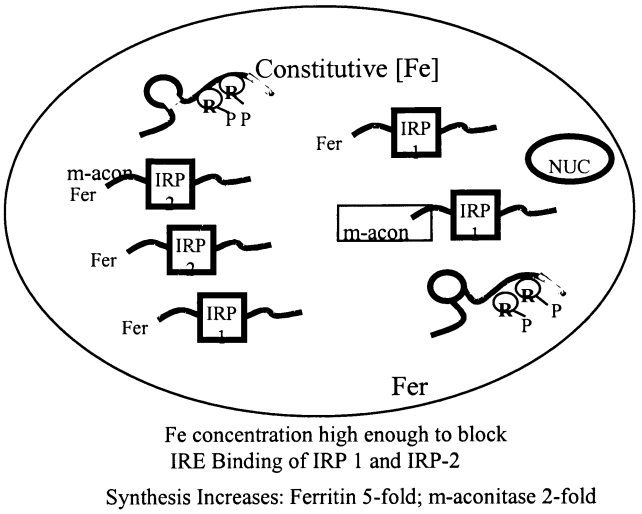

FIG. 4. Combinatorial model for iso-IRE/iso-IRP interactions. Iso-IREs display differential binding to Iso-IRPs (see Fig. 2). The effect creates a wide range of quantitative translational responses to the same signal (cellular iron concentrations) by MRNAs containing different iso-IREs. Thus, a single iron dose will produce different levels of induction of protein synthesis for m-aconitase and ferritin in the same cell. Such differences have been observed in vivo in the liver of rats predicted [67, 68]. The figure is modified from Theil *et al.* [69].

THE IRE MODEL FOR THERAPEUTIC mRNA TARGETING

Specific three-dimensional structures in RNA are predictable from knowledge of t-RNA, ribosymes, and viral RNAs, and from differential rates of mRNA translation and turnover *in vivo* and *in vitro*. Knowledge of such structures has been difficult to find. For example, during the last five years there have been ~20 papers on three-dimensional structure analysis of mRNA (Medline); only 1/3 studied eukaryotic mRNA, of which 60% were concerned with IREs. The iso-IRE family of mRNA elements provides a clear currently rare example of specific RNA structures that can serve as a model for development of mRNA as a target for drugs.

Three major reasons for messenger RNA as a useful target for drugs are:

- Cell specificity (like proteins)
- Conformational flexibility (like proteins)
- Small target size (like DNA)

Thus, mRNA combines the cell specificity of proteins with the small cellular target size of DNA (Table 1). Clearly, the potential of mRNA as a target is very high, as the use of antisense RNA has indicated. However, antisense approaches rely on RNA secondary structure only. An entire dimension is ignored!

The sensitivity of the RNA three-dimensional structure is illustrated by reactions of t-RNA and the ferritin IRE with TMCs, stable combinations of metal ions with organic ligands of different three-dimensional shapes (reviewed in Theil [70]. If the metal in the complex is redox-active, the

TABLE 1. Specificity and concentration of cellular regulatory targets

	Cell specificity	Cell concentration	Response potential	
Molecule	(Target size)		Selectivity	Sensitivity
DNA	Low	Low	Low	High
Protein	High	High	High	Low
RNA	High	Low	High	High

binding site of the complex is reported by cleavage of the RNA. RNA-binding sites of the drug Fe–bleomycin are so rare [71, 72] that for many years RNA was thought to be resistant, in contrast to DNA. Site specificity of Fe–bleomycin is restricted to double-stranded regions with sufficient helix distortion and base specificity to permit intercalation of the bleomycin pyrimidine ring into the RNA helix [73]. The ferritin IRE will bind bleomycin at a single site near the junction of the hairpin loop and the stem (Fig. 1, right), presumably because of the C-G base pair across the loop. The site is so sensitive that changes in the ferritin mRNA sequence outside the IRE will alter the Fe–bleomycin binding site [74]. Other complexes which bind to selective sites in IREs include cobalt hexammine, $Cu(phen)_2$ (1,10-phenanthroline-Cu), and Ru tpy(bpy). Cobalt hexammine and $Cu(phen)_2$ bind almost exclusively at or near the internal loop/bulge of the ferritin IRE (G7 or G26,27, respectively—Fig. 1) [75], while Ru tpy (bpy) binds in the hairpin loop (G16—Fig. 1) [43, 76]. Changes in the IRE sequence which change IRP binding [77] also change TMC binding so that TMC selectivity among iso-IREs is possible.

What could be the advantage of targeting an IRE in an mRNA with a TMC? The combinatorial effects possible among the iso-IRE/iso-IRP interactions are regulated so that even for ferritin some of the mRNA remains untranslated when cytoplasmic iron levels are high [11, 12]. Abrogating ferritin IRE regulation further with a TMC could expand ferritin synthesis beyond that possible with natural regulation, for example, creating an expanded ferritin pool to help manage toxicity of iron overload. Moreover, c-myc up-regulation of IRP2 and cell transformation [78] might be abrogated by a TMC. In addition, c-myc mRNA itself, and other oncogene mRNA and viral mRNA such as transactivation response elements contain RNA structures which can be TMC targets to alter function.

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