Agric. Chem. Biotechnol. 42(2), 62- 65(1999)

Selection of Putative Iron-responsive Elements by Iron Regulatory Protein-2

Hae-Yeong Kim

Department of Food Science and Institute of Genetic Engineering, Kyung Hee University, Suwon 449-701, Korea

Received April 19, 1999

Iron regulatory proteins (IRPs) 1 and 2 bind with equally high affinity to specific RNA stem-loop sequences known as iron-responsive elements (IRE) which mediate the post-transcriptional regulation of many genes of iron metabolism. To study putative IRE-like sequences in RNA transcripts using the IRP-IRE interaction, Eight known genes from database were selected and the RNA binding activity of IRE-like sequences were compared to IRP-2. Among them, the IRE-like sequence in 3'-untranslational region (UTR) of divalent cation transporter-1 (DCT-1) shows a significant RNA binding affinity. This finding predicts that IRE consensus sequence present within 3'-UTR of DCT-1 might confer the regulation by IRP-2.

Key words : *iron responsive element, iron regulatory protein, iron metabolism, ferritin, untranslated region.*

Mammalian cells appear to regulate cellular iron metabolism post-transcriptionally by means of the interaction of iron responsive elements (IREs) and iron regulatory proteins (IRPs).[1,2] RNA stem-loop known as IRE is found in transcripts of genes of iron metabolism, including ferritin,[3] the transferrin receptor,[4] erythroid 5-aminolevulinate synthease[5] and aconitase.[6] IREs function as the binding sites for a well characterized regulatory protein, the IRE-binding protein.[1,3] Two distinct proteins known as iron regulatory proteins, IRP-1 and IRP-2, function as IRE-binding proteins when cells are depleted of iron. Although the RNA binding activity of each of the two proteins decreases when cells are iron-replete, the mode of regulation differs, and this feature may be of importance in specialized circumstances. IRP-2 is rapidly degraded in cells that are iron-replete, whereas IRP-1 is stable and its function is determined by the presence or absence of iron-sulfur cluster.[1] In addition recent researches showed that peroxynitrite[7] and cytokines[8] modulate IRP-1 and IRP-2 in opposite directions.

Specific IRE nucleotides are phylogenetically conserved as the 6-base loop, a bulge and 8 base-paired sequences.[9,10] In ferritin transcripts, the IRE is located near the 5' end of the transcript, close to the cap site where translational factors initially bind. When IRPs are bound to the IRE, binding of translation inhibition factors to the 5' end of the transcripts is not actively translated. Simple steric hinderance resulting from binding of IRPs to the IRE is the mostly likely cause of inhibition of translation in the case of the ferritin transcript.[11]

The studies on IRE base substitution and deletion,[12] and systematic evolution of ligands by exponential enrichment (SELEX)[13,14] had been done to find endogenous target sequences without sucess.

In this study, putative IRE-like sequences in endogenous RNA transcripts were screened from the database search and the RNA binding affinities by IRP-2 were measured using RNA gel shift assay. The list of potential genes that are regulated by IRP-2 continues to grow, and much remains to be learned about the regulation of mammalian iron metabolism.

Materials and Methods

Purification of IRP-2. Purification of IRP-2 (Mr=105,000) was performed as described previously.[15,16] Approximately 100 mg of cytoplasmic lysate was applied to a Mono-Q HR column (Pharmacia) equilibrated with 40 mM KCl, 25 mM Tris (pH 8.3), and 1 mM DTT. After extensive washing, the IRP-2 was eluted from the column with a linear salt gradient from 40~1000 mM KCl. The proteins were quantitated by BioRad protein assay. The purity of these proteins was verified on SDS-polyacrylamide gel electrophoresis.[17]

Preparation of DNA oligonucleotides for RNA transcripts. The DNA oligonucleotides used as a template of endogenous IRE-like transcripts were constructed by DNA synthesizer (Applied Biosystems, model 391). The 54-nt template contained the antisense sequence of the T7 promoter primer, to which a 20-mer was annealed to create a promoter that results in transcription of RNA complementary to the remaining sequence. The sequences of sense are as follows:

Human H-ferritin, 5'-TTCCTGCTTCAACAGTGCTTG-GACGGAA-3'; Human aconitase, 5'-CCTCATCTTTGTCA

*Corresponding author
Phone: 82-331-201-2660 ; Fax: 82-331-204-8116
E-mail: hykim@nms.kyunghee.ac.kr

Abbreviations: DCT-1, divalent cation transporter-1; IRE, iron responsive element; IRP-2, iron regulatory protein-2; SELEX, systematic evolution of ligands by exponential enrichment; UTR, untranslated region.

GTGCACAAAATGGCG-3'; Mouse LYL, 5'-GTGGAAAC TGCTACAGGGTTAGCATTCTAT-3'; HIVTAR, 5-AGAC CAGATTTGAGAATGGGAGCTCTCTGGCT-3'; Human translationl elongation factor 2 (EF2), 5'-TGCCTCTATGCC AGTGTGCTGACCGCC-3'; Mouse clathrin-associated protein(AP47), 5'-TTTGGCCTTCCCAGTGTGGAAGCTGAA-3'; Human transferrin receptor, 5'-ATTATCGGAAGCAGTGC-CTTCCATAAT-3'; Rat divalent cation transporter (DCT-1), 5'-GCCATCAGAGCCAGTGTGTTTCTATGGT-3'.

***In vitro* synthesis of RNA transcripts.** The RNA fragments were prepared by *in vitro* transcription of cDNA templates.[18] The mixture contained 1 μg of oligonucleotide, I mM DTT, 0.1 mM NTP, 10 units of RNasin, and 10 units of T7 RNA polymerase. After transcription at 37°C for 1 hr, a reaction mixture was extracted with phenol-chloroform and precipitated with ethanol. The transcription products were quantified by absorbance at 260 nm. The integrity of the transcripts was verified on formaldehyde-agarose gel.[19]

RNA gel shift assay. Reaction mixtures of gel shift assay contained increasing concentrations (0, 20, 40, 80, and 150 nM) of recombinant purified IRP-2 with 100 nM ^{32}P-labeled IRE probe[3] and 20 units of RNasin. The reactions were carried out at 25°C for 15 min. The reaction mixture was loaded onto 8% native polyacrylamide gel and electrophoresis was performed at 150 volts for 3 hrs. After electrophoresis, the gels were fixed and subsequently dried. The RNA-protein complex was quantitated for IRP-2 at the indicated protein concentrations on a PhosphorImager (Molecular Dynamics) using Image-Quant software.

Search of GenBank data bases for IRE-like sequence. The GenBank data base[20] was scanned using DNA searching program for IRE-like sequence containing RNA secondary structure. The algorithm used required six loop sequence and eight Watson-Crick base pairs including the bulge C. Sequences that matched the consensus IRE (Fig. 1) or any of the alternative ligands were considered candidates for further evaluation if their position within the transcript was determined to be within the 5'- or 3'-UTR.

Results and Discussion

IRP-2 binds ferritin IRE with strong affinity. Increasing concentrations of purified recombinant IRP-2 were added to 100 nM radiolabeled ferritin IRE probe (Fig. 2). The IRE-IRP-2 complex was determined at the indicated IRP-2 concentration. From the formal binding curves and Scatchard analyses[15] of the interaction of IRP-2 with IRE, the K_d was measured as 25±12 pM. This result showed that IRP-2 binds ferritin IRE with high affinity. The K_d values of binding of the consensus IRE to IRP-1 ranged from 5 to 50 pM.[14]

Some endogenous transcripts contain IRE-like sequence. The database was used to screen for IRE-like sequences maintaining a six-membered loop, bulge and 8 base pairing.

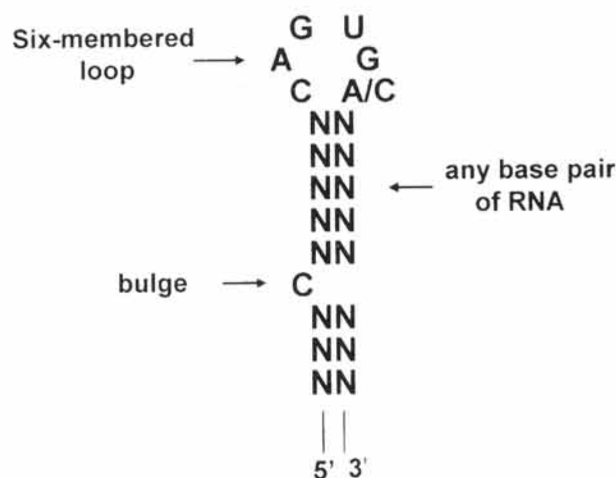

Fig. 1. The sequence and secondary structure of consensus IRE. NN represents any complementary pair of RNA bases.

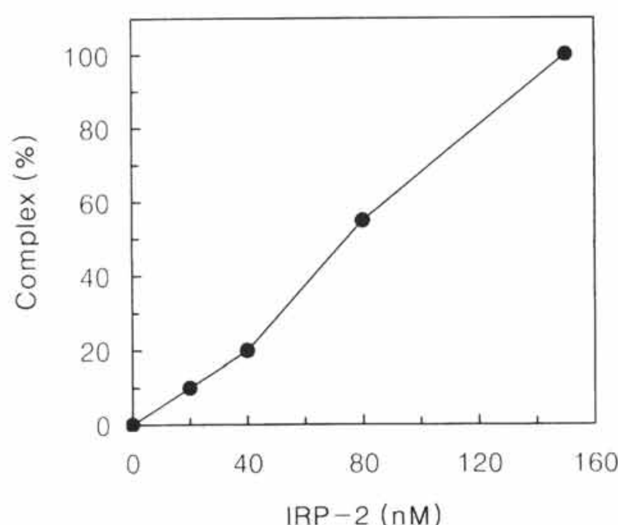

Fig. 2. Graphic representation of quantitated gel shift assays of IRP-2. The RNA-protein complex was quantitated for IRP-2 at the indicated protein concentrations, and 100% of complex was determined at 150 nM protein.

From this database search, many potential IREs were found within mRNAs. Out of 72 mRNAs identified in the database search, eight genes which contained potential IREs sequence were selected to study *in vitro*. The IRE-like sequence of human ferritin, human aconitase, mouse LYL or HIV TAR was found in 5'-UTR, mouse AP-47 or human elongation factor EF II in coding sequence and human transferrin receptor or mouse divalent cation transporter-1 (DCT-1)[21] in 3'-UTR. These genes contained 6 loop bases, bulge, and at least 8 base-pairs in RNA transcripts.

IRP binds IRE-like sequences with significant affinity. The IRE binding affinity of IRP-2 was assessed by gel shift assay and labeled complexes were quantitated by PhosphorImager. As shown in Fig. 3, transferrin receptor (lane 8) and aconitase (lane 6) show similar affinity to ferritin (lane 5). However, iron metabolism of these genes

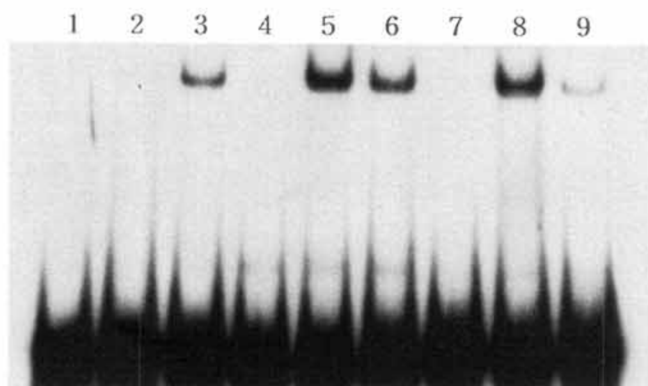

Fig. 3. RNA binding activities of endogenous IRE-like transcripts. (lane 1. control; lane 2. mouse LYL; lane 3. mouse DCT-1; lane 4. HIV TAR; lane 5. human H-ferritin; lane 6. human aconitase; lane 7. human EF II; lane 8. human transferrin receptor; lane 9. mouse AP-47).

Table 1. Ability of selected IRE-like sequences to bind IRP *in vitro*.

Gene	Sequences bulge/six-membered loop	Binding ratio
5'-UTR		
Human ferritin	C/CAGUGC	100
Human aconitase	C/CAGUGC	60
Mouse LYL	C/CAGGGU	3
HIV TAR	U/CUGGGA	< 1
Coding sequence		
Mouse AP-47	C/CAGUGU	
Human EF II	C/CAGUGC	< 1
3'-UTR		
Human transferrin receptor	C/CAGUGC	100
Mouse DCT-1	C/CAGUGU	30

were already studied. Surprisingly, the binding activity of the RNA fragment in DCT-1 to IRP was measured as 30% (Table 1, lane 3 in Fig. 3). This IRE-like sequence in 3'-UTR of DCT-1 shows significant RNA binding affinity. This gene was recently identified as metal-ion transporter gene.[21] Taken together, DCT-1 would be the putative gene which might be involved in iron metabolism. Important questions on the scope of this regulatory system and the interplay between the two IRPs remain to be answered. An exciting possibility is that more targets of IRP binding will be identified, and that these additional targets will include transcripts which encode proteins involved in the response to iron metabolism in mammalian cells.

Acknowledgments. This research was supported by a Genetic Engineering Research Grant (1997) from the Ministry of Education, Republic of Korea.

References

1. Klausner, R. D., Rouault, T. A. and Harford, J. B. (1993) Regulating the fate of mRNA: thecontrol of cellular iron metabolism. *Cell* **72**, 19-28.
2. Mascotti, D. P., Rup, D. and Thach, R. E. (1995) Regulation of iron metabolism. *Annu. Rev. Nutr.* **15**, 239-261.
3. Leibold, E. A. and Munro, H. N. (1988) Cytoplasmic protein binds *in vitro* to a highly conserved sequence in the 5'UTR of ferritin H-and L-subunit mRNA. *Proc. Natl. Acad. Sci. USA* **85**, 2171-2175.
4. Bhasker, C. R., Burgiel, G., Neupert, B., Emery-Goodman, A., Kuhn, L. C. and May, B. K. (1993) The putative iron-responsive element in the human erythroid 5-aminolevulinate synthase mRNA mediates translational control. *J. Biol. Chem.* **268**, 12699-12705.
5. Dandekar, T., Stripecke, R., Gray, N. K., Goossen, B., Constable, A., Johansson, H. E. and Hentze, M. W. (1991) Identification of a novel iron-responsive element in murine and human erythroid delta- aminolevulinic acid synthase mRNA. *EMBO J.* **10**, 1903-1909
6. Kim, H. Y., Klausner, R. D. and Rouault, T. A. (1996) Identification of a conserved and functional iron-responsive element in the 5'-Untranslated region of mammalian mitochondrial aconitase. *J. Biol. Chem.* **271**, 24226-24230.
7. Bouton, C., Hirling, H. and Drapier, J. C. (1997) Redox modulation of iron regulatory proteins by peroxynitrite. *J. Biol. Chem.* **272**, 19969-19975.
8. Recalcati, S., Taramelli, D., Conte, D. and Cairo, G. (1998) Nitric oxide mediated induction of ferritin synthesis in J774: Macrophages by inflammatory cytokines. *Blood* **91**, 1059-1066.
9. Haile, D. J., Hentze, M. W., Rouault, T. A., Harford, J. B. and Klausner, R. D. (1989) Regulation of interaction of the iron-responsive element binding protein with iron-responsive RNA elements. *Mol. Cell. Biol.* **9**, 5055-5061.
10. Hentze, M. W., Rouault, T. A., Harford, J. B. and Klausner, R. D. (1989) Oxidation-reduction and the molecular mechanism of a regulatory RNA-protein interaction. *Science* **244**, 357-359.
11. Stripecke, R., Oliveira, C. C., McCarthy, J. E. G. and Hentze, M. W. (1994) Proteins binding to 5'-untranslated region sites: a general mechanism for translational regulation of mRNAs in human and yeast cells. *Mol. Cell. Biol.* **14**, 5898-5909.
12. Jaffrey, S. R., Haile, D. J., Klausner, R. D. and Harford, J. B. (1993) Deletion analysis of promoter region of the human transferrin receptor gene *Nucleic Acids Res.* **21**, 4627-4631.
13. Henderson, B. R., Menotti, E., Bonnard, C. and Kuhn, C. (1994) Optimal sequence and structure of iron-responsive elements. *J. Biol.* Chem. **269**, 17481-17489.
14. Butt, J., Kim, H. Y., Basilion, J. P., Cohen, S., Iwai, K., Philpot, C. C., Altschul, S., Klausner, R. D. and Rouault, T. A. (1996) Differences in the RNA binding sites of iron regulatory proteins and potential target diversity. *Proc. Natl. Acad. Sci. USA* **93**, 4345-4349.
15. Basilion, J. P., Kennedy, M. C., Beinert, H., Massinople, C. M., Klausner, R. D. and Rouault, T. A. (1994) Overexpression of iron-responsive element binding protein and its analytical characterization as the RNA-binding

form, devoid of an iron-sulfur. *Arch. Biochem. Biophys.* **311**, 517-522.

16. Samaniego, F., Chin, J., Iwai, K., Rouault, T. A. and Klausner, R. D. (1994) Molecular characterization of a second iron-responsive element binding protein. *J. Biol. Chem.* **269**, 30904-30910.
17. Philpott, C. C., Haile, D. J., Rouault, T. A. and Klausner, R. D. (1993) Modification of a free Fe-S cluster cysteine residue in the active iron-responsive element-binding protein prevents RNA binding. *J. Biol. Chem.* **268**, 17655-17658.
18. Rouault, T. A., Stout, C. D., Kaptain, S., Harford, J. B. and Klausner, R. D. (1991) Structural relationship between an iron-regulated RNA protein and aconitase. *Cell* **64**, 881-883.
19. Kim, H. Y., Klausner, R. D. and Rouault, T. A. (1995) Translational repressor activity is equivalent and is quantitatively predicted by in vitro RNA binding for IRP1 and IRP2. *J. Biol. Chem.* **270**, 4983-4986.
20. Benson, D., Lipman, D. J. and Ostell, J. (1993) GenBank database. *Nucleic Acids Res.* **21**, 2963-2965.
21. Gunshin, H., Mackenzie, B., Berger, U. V., Gunshin, Y., Romero, M. F., Boron, W. F., Nussberger, S, Gollan, J. L. and Hediger, M. A. (1997) Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* **388**, 482-488.