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IRON-DEPENDENT REGULATION OF FERRITIN AND TRANSFERRIN RECEPTOR EXPRESSION BY THE IRON-RESPONSIVE ELEMENT BINDING PROTEIN

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INTRODUCTION

Gene expression can be controlled at multiple levels including transcription, posttranscription, and posttranslation. In recent years, the mechanisms

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regulating transcription have been studied intensely. Less is known about controls of posttranscriptional processes. Posttranscriptional control can be regulated by altering transcript processing, including capping, splicing, and polyadenylation, and by influencing the rate of transport into the cytoplasm. Once the mRNA is in the cytoplasm, the amount of protein synthesized depends on both the efficiency of translation and mRNA turnover. Translational control can be defined as a change in the efficiency of mRNA translation (37). This regulation can occur globally for all mRNAs, in order to increase total protein synthesis, or specifically for a particular mRNA. The regulation of mRNA turnover can be used to regulate the levels of specific mRNAs by altering the rates of degradation or stabilization (38, 65). In contrast to transcriptional regulation, which requires a delay due to RNA transcription and processing, posttranscriptional regulation occurs rapidly owing to the presence of preexisting mRNA in the cytoplasm. Thus, posttranscriptional regulation of specific genes is advantageous to cells that need to respond rapidly to biological stimuli.

Two examples of proteins regulated at the level of translation and mRNA stability are ferritin and transferrin receptor (TfR), respectively. Ferritin and TfR are the major proteins involved in the regulation of iron homeostasis (Figure 1). TfR is an integral membrane protein that brings iron into the cell via endocytosis of diferric transferrin. Following delivery to the endosome, iron is released and transferred to the iron-storage protein ferritin or to other cellular proteins requiring iron. The need for a posttranscriptional mechanism to regulate ferritin and TfR expression can be inferred by the toxicity of free iron in the cell. Although iron is an essential element and plays a role in many biological processes including oxygen and electron transfer, nitrogen fixation, and DNA synthesis, free iron is toxic to cells. This toxicity is due to the ability of free iron to form reactive hydroxyls that can cause peroxidation of lipid membranes and other cellular constituents. Most iron in cells is complexed. Ferritin is the molecule commonly used for sequestration. Cells regulate the concentration of free iron both by regulating its uptake via the TfR and by the sequestration of iron into ferritin.

Ferritin synthesis is increased by iron and is controlled primarily at the level of translation. Ferritin mRNAs are found in the cytoplasm in either inactive ribonucleoprotein (RNP) particles or in actively translating polysomes. The rate of ferritin protein synthesis is determined by the distribution of ferritin mRNAs between polysomes and RNP particles. The concentration of free iron in the cell regulates this distribution; high iron levels mobilize ferritin mRNAs onto polysomes for translation, whereas low iron levels cause ferritin mRNAs to be in RNP particles. In contrast, TfR synthesis is decreased by iron and is controlled at the level of mRNA stability. Cells respond to an increase

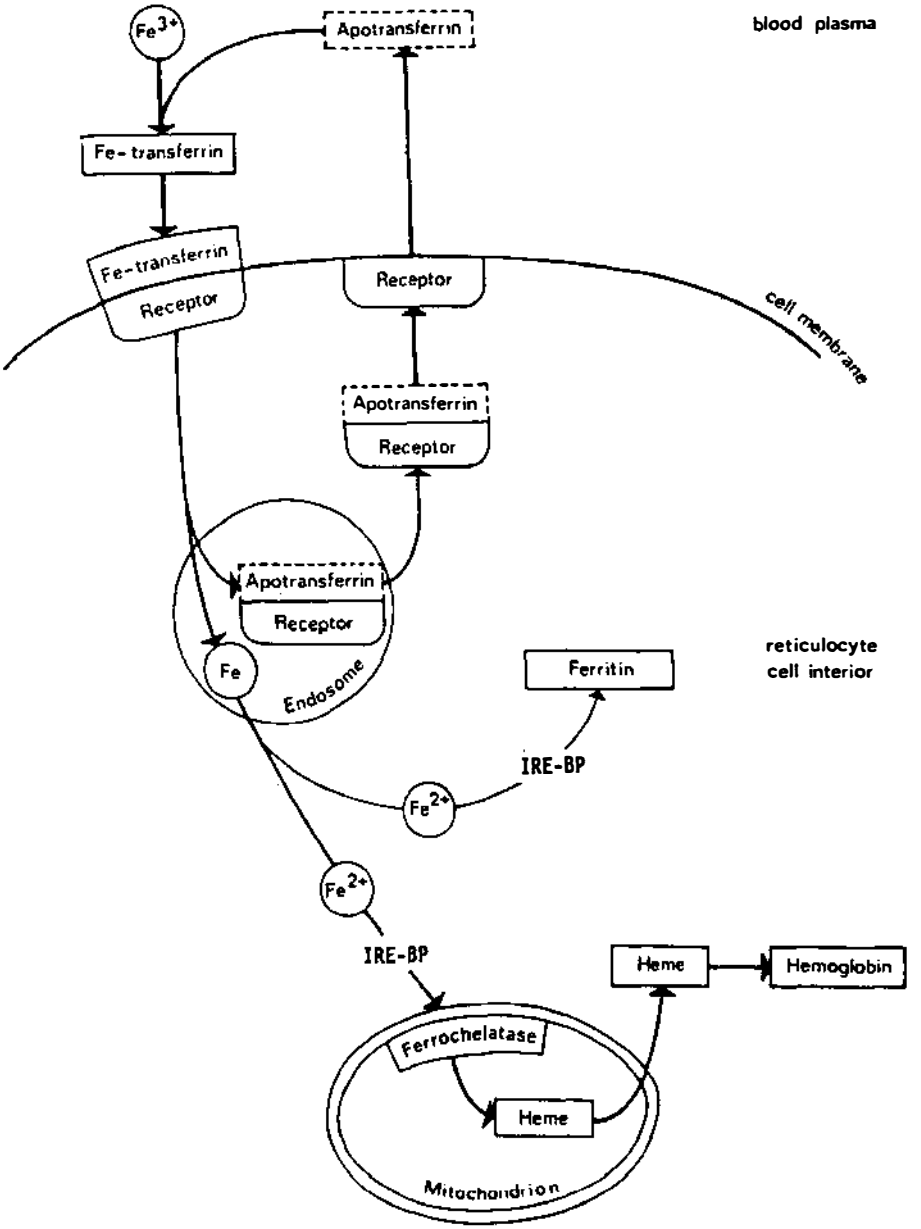

Figure 1 Scheme of iron uptake in the reticulocyte by the transferrin receptor and sequestration into ferritin. Reproduced with permission (68).

in iron concentration by altering the levels of TfR mRNA via an increase in the specific degradation of TfR mRNAs. The net result of ferritin and TfR regulation by iron is a decrease in the free iron levels in the cell, thereby preventing iron toxicity.

The coordinate regulation of ferritin and TfR is achieved through specific sequences within the mRNAs themselves. These sequences, termed iron-responsive elements (IREs), are located in the 5'- and 3'-untranslated regions of ferritin and TfR mRNAs, respectively. A cytoplasmic protein, the iron-responsive element binding protein (IRE-BP) modulates the posttranscriptional regulation of ferritin and TfR mRNAs through interaction with IREs in a process thought to be mediated by intracellular free iron. The IRE-BP has also been referred to as the ferritin repressor protein (FRP) and the iron regulatory factor (IRF).

Our purpose in this review is to discuss how the IRE-BPs regulate the posttranscriptional expression of ferritin and TfR. Several excellent reviews of the structure and the expression of ferritin (16, 25, 40, 57, 58, 82–85, 92) and TfR (40, 45, 84, 92) have been published in recent years. Thus, these topics are briefly covered here to provide the reader with sufficient background information.

STRUCTURE AND FUNCTION OF FERRITIN AND TRANSFERRIN RECEPTOR

In humans, approximately 25% of the total body iron stores is in the iron-storage protein ferritin and its derivative, hemosiderin (see 16, 58, 92). Hemosiderin is an aggregate of protein and iron found in lysosomes and is thought to result from from the degradation of ferritin. Ferritin is a ubiquitous protein found in vertebrates, invertebrates, plants, fungi, and bacteria. In vertebrates, the highest amount of ferritin is found in tissues that store iron, such as liver and spleen. Ferritin has a molecular weight of about 500,000. Crystallographic studies have shown that ferritin consists of a protein shell of 24 subunits assembled to form a cavity that can accommodate a core of up to 4500 iron atoms as ferric oxyhydroxide with variable amounts of phosphate (see 16, 82, 83, 85). The protein shell contains eight channels through which iron and small molecules can enter and exit. The ferritin shell consists of two types of subunits: heavy (H), 21,000 M_r, and light (L), 19,000 M_r. The primary amino acid sequences for ferritin H and L subunits have been obtained for a variety of organisms. Among mammals, the L subunits are 82–88% identical, whereas the H subunits are 95% identical (see 25, 57). *Rana castesbian* (bullfrog) ferritin contains three subunits (H, M, L) that are similar in size and sequence but differ in cell expression and regulation by

iron (19, 21). To date, there is no evidence for the presence of tissue-specific H and L mRNAs in mammals.

Cells acquire iron via transferrin, a serum glycoprotein that binds to the TfR on the cell surface. TfR is a transmembrane protein; it is a dimer of two 90,000 dalton subunits linked by a disulfide bond. Each subunit has two N-asparagine-linked carbohydrates and binds one molecule of diferric transferrin with high affinity. After binding of diferric transferrin to TfR, the TfR-diferric transferrin complex is internalized by receptor-mediated endocytosis (Figure 1) (18, 39). At about pH 5.5, iron is dissociated from the TfR-transferrin complex, where it is transferred to ferritin and to other cellular proteins. The process by which iron is transferred from the endosome to other proteins is unknown. Apotransferrin bound to the TfR is recycled to the cell membrane, where it is dissociated from the receptor and is available for binding new iron.

THE IRON-RESPONSIVE ELEMENT

The cloning of the rat ferritin L gene (49) and the chicken ferritin H gene (81) led to the identification of the highly conserved IRE motif in their 5'-untranslated regions. This sequence has subsequently been identified in the 5'-untranslated regions of all vertebrate ferritin H and L sequences to date (Figure 2*A*). Functional IREs have been identified in other mRNAs involved in iron metabolism. Five IREs have been identified in the 3'-untranslated region of human (9, 42, 46, 55) and chicken (12, 42) transferrin receptor mRNAs. The ferritin and TfR IREs permit the coordinate regulation of ferritin and TfR synthesis (discussed below). One IRE has been identified in the 5'-untranslated region of the human erythroid 5-aminolevulinate synthase (erythroid ALAS) mRNA (15, 17, 22, 79) and one in the 5'-untranslated region of mitochondrial porcine aconitase mRNA (17). ALAS is the first enzyme in the heme biosynthetic pathway; aconitase is a mitochondrial iron-sulfur (Fe-S) enzyme in the Krebs cycle. IRE-like sequences have been reported in the mRNAs of *Drosophila melanogaster* maternal effect gene *toll* (17) and in *Azotobacter vinelandii* bacterioferritin (E. Stiefel, personal communication). The *toll* IRE does not appear to be functional (17). Whether the *A. vinelandii* IRE-like sequence functions as an IRE is not known.

Ferritin H and L IREs from human, rat, mouse, chicken, bullfrog, and the clawed toad *(Xenopus laevis)* are about 28–35 nucleotides in length and share approximately 90% homology with each other (Figure 2*A*). Although ferritin, TfR, mitochondrial aconitase, and erythroid ALAS IREs share limited sequence homology, secondary structure predictions suggest that all these IREs

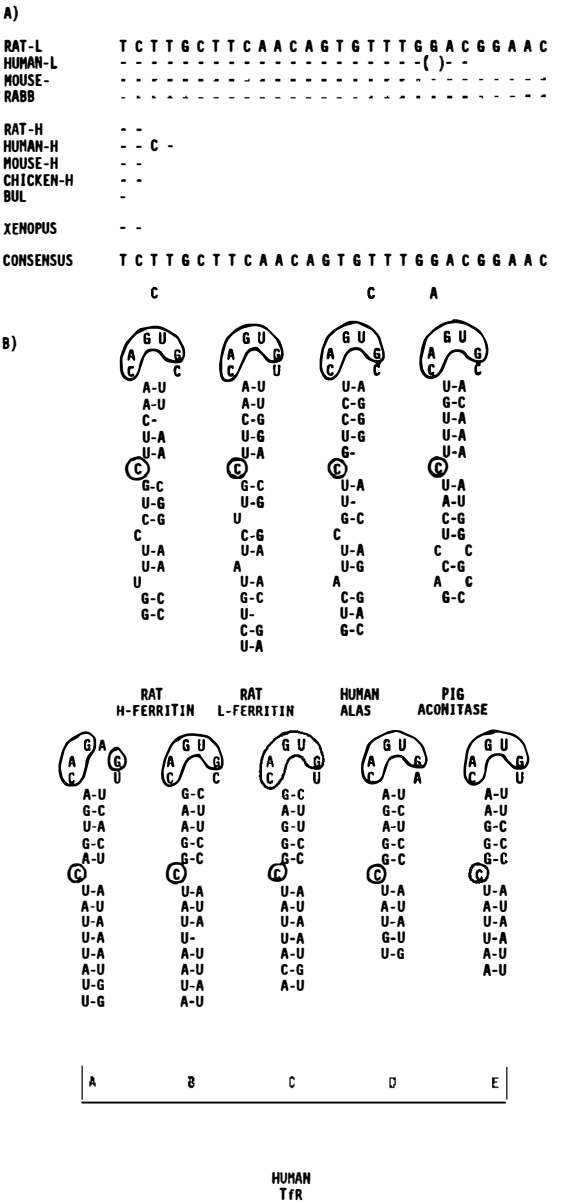

Figure 2 Sequence and structure of the iron-responsive element. (*A*) Sequences of ferritin L IREs from rat (49), human (78), mouse (25), and rabbit (25), and ferritin H IREs from rat (59), human (14), mouse (4, 47), chicken (81) bullfrog (19, 21), and *X. laevis* (54). A consensus sequence for the ferritin IRE is located below the sequences. Ambiguous sequences, ( ). (*B*) Computer-predicted IRE stem-loop structures. IRE stem-loop structures from the mRNAs for rat ferritin H (59) and L (2), human erythroid ALAS (15, 17, 22, 79), and TfR (9) and pig mitochondrial aconitase (17). Conserved nucleotides in the loop and the bulge cytosine in the stem are marked.

can be folded into a characteristic stem-loop structure (Figure 2*B*). This structure contains a six-membered loop and a base-paired stem with a bulge cytosine located five nucleotides away from the loop. The loop sequence is conserved in the IREs and has a consensus of C–A–G–U/A–G–U/C/A. The bulge cytosine in the stem and its location five nucleotides from the loop are conserved in all IREs. The sequences in the stem are conserved between ferritin H and L IREs but not between erythroid ALAS, aconitase, and TfR IREs. The negative free energy values for the IREs range between –20 and –30 kcal/mol (33, 96). As mentioned above, *A. vinelandii* was reported to contain an IRE-like sequence; however, the structure of this sequence is not similar to the consensus IRE. Possibly during evolution the prokaryotic IRE diverged from the eukaryotic IRE. The conformation of the IRE is important in binding to the IRE-BP and is discussed below.

STRUCTURE AND FUNCTION OF THE IRON-RESPONSIVE ELEMENT BINDING PROTEINS (IRE-BPs)

Conservation of IRE-BPs

IRE-BPs were first identified by RNA-protein gel shift and RNA-protein UV cross-linking assays (50). A ferritin IRE RNA was radiolabeled and incubated with a rat liver cytoplasmic protein extract, followed by resolution of the RNA-protein complexes on native polyacrylamide gels. UV-cross-linking of these complexes and analysis by SDS-polyacrylamide gel electrophoresis demonstrated that one of the complexes contained a protein of about 90,000 M_r. Subsequently, IRE-BPs have been identified in human (73), mouse (56), and rabbit (87) tissues and cells. IRE-BP binding activity has also been identified in protein extracts from *Xenopus,* fish, and *D. melanogaster* (72). No RNA-binding activity was found in extracts of budding or fission yeast or in bacteria using a human IRE (72). It is likely that either the IRE-BPs are not present in lower eukaryotes and prokaryotes or that IREs have diverged during evolution and no longer recognize mammalian IRE-BPs.

Purification, Cloning, and Characterization of IRE-BPs

IRE-BPs have been purified from human (74), rabbit (87), and rat (E. Leibold, unpublished data) livers and human placenta (60). Human placenta and liver IRE-BPs were purified by RNA-affinity chromatography, whereas rabbit liver and rat liver IRE-BPs were purified by conventional chromatography. The molecular weights determined by gel filtration and SDS polyacrylamide gels are similar for the IRE-BPs (90,000–100,000 daltons). Two IRE-

BPs similar in size were purified from human placenta by RNA-affinity chromatography (60). Whether the placental proteins are proteolytic degradation products of one another, posttranslationally modified forms, or distinct proteins is not known.

IRE-BP cDNAs have been isolated from human (76) and rat liver (E. Leibold, unpublished data). The rat liver IRE-BP encodes a protein of 889 amino acids with a predicted molecular weight of 97,946. The amino terminus of rat liver IRE-BP was confirmed by direct sequence analysis and was determined to be 100 amino acids longer at the amino terminus than the reported human liver IRE-BP (76).

Comparison of the rat IRE-BP sequence with that of the human sequence demonstrated that they are 92% identical and 95% similar when conservative amino acid changes are considered. IRE-BPs share homology with mitochondrial pig (95) and yeast (28) aconitases (~ 32–36% identity and 53–56% similarity, respectively) (75). Aconitase is a Fe-S enzyme in the Krebs cycle that catalyzes the interconversion of citrate to isocitrate via the intermediate *cis*-aconitate. Mitochondrial aconitase exists in two states: an active state that contains a [4Fe–4S] cluster and an inactive state that contains a [3Fe-4S] cluster (5, 27, 70) (Figure 5). The highest degree of homology between the IRE-BP and aconitase was observed for amino acids, which form the active sites of aconitase, including three cysteines that are ligands for three iron-atoms. In mitochondrial aconitase, the fourth ligand on iron is a hydroxyl group that is protonated on addition of citrate (5). The structural similarities between the IRE-BP and mitochondrial aconitase suggest that the IRE-BP may have evolved from a common metal binding motif by the addition of a RNA-binding domain.

In addition to mitochondrial aconitase, a cytoplasmic form of aconitase has been characterized from pig heart (24) and bovine liver (C. Kennedy and H. Beinert, personal communication). The function of cytoplasmic aconitase is not known. The mitochondrial and cytoplasmic aconitases are similar in that they have a Fe-S cluster and aconitase activity, but they differ in their isoelectric points, stabilities, and molecular weights, which suggests that they are distinct proteins (24, 27). Interestingly, cytoplasmic aconitases and the human IRE-BP have similar molecular weights and have been localized to chromosome nine (36, 75, 80). Recent data indicate that bovine cytoplasmic aconitase binds the mitochondrial aconitase IRE, which would suggest that the IRE-BP and cytoplasmic aconitase are the same protein (C. Kennedy, H. Beinert, H. Zalkin, personal communication). What role aconitase activity plays in IRE-BP regulation is not clear. The amino acid sequence of cytoplasmic aconitase will be needed in order to obtain definitive proof of its identity.

IRON-DEPENDENT POSTTRANSCRIPTIONAL REGULATION BY IRE-BPs

Translational Regulation by IRE-BPs

The discovery of the IREs and the IRE-BPs has generated intense interest in the translational regulation of ferritin. For many years investigators have known that administration of iron to animals and cells resulted in an increase in ferritin synthesis (see 1, 25, 25a, 58, 71, 82, 83, 85, 90). This induction was not due to the production of new mRNA, since pretreatment of rats with actinomycin D, an inhibitor of RNA transcription, failed to suppress ferritin synthesis (13, 77, 94). It was demonstrated, however, that the increase in ferritin synthesis in rats treated with iron was due to an increase in the amount of ferritin mRNAs on polysomes (93). These experiments led Munro and co-workers (94) to postulate that a preexisting pool of ferritin mRNA that is present in the cytoplasm can be shifted by iron to actively translating polysomes.

The translational shift of ferritin mRNA from RNP particles to polysomes by iron was demonstrated in rat liver (1, 90) and human hepatoma cells (71) using ferritin H and L cDNAs to determine cytoplasmic location and levels of ferritin mRNA. Figure 3 shows an example of the redistribution of ferritin mRNA to polysomes after iron injection. Male rats were injected intraperitoneally with ferric ammonium citrate. Four hours later, livers were isolated and RNP particles and the polysomes were fractionated on sucrose gradients. Total RNA was isolated from each fraction and hybridized to radiolabeled ferritin H cDNA on Northern blots. After an injection of iron, liver ferritin H mRNAs are shifted from the inactive RNP pool (Figure 3*B*, lanes 1–5) to actively translating polysomes (Figure 3*B*, lanes 6–10). Ferritin L mRNA is shifted to a similar extent (data not shown). Approximately 75% of the ferritin H and L mRNAs shifted to polysomes within 30 min after an intraperitoneal injection of iron and showed a maximal shift by 4 hr (90). By 16 hr, ferritin mRNA returned to the RNP pool and was no longer translated at an increased rate. Metabolic labeling studies in rats demonstrated that ferritin synthesis in liver begins to increase within 1 hr, with maximal induction at 5 hr after iron injection (23, 58).

In contrast to the acute iron administration described above, a recent study showed that in female rats chronically fed a high iron diet, only ferritin L mRNA shifted to the polysomes, whereas ferritin H mRNA remained in the RNP pool (8). Other studies carried out with male rats fed a high iron diet showed that ferritin H and L mRNAs were unaffected by the high iron diet and did not shift to the polysomes (K. White, MIT dissertation, 1987). The

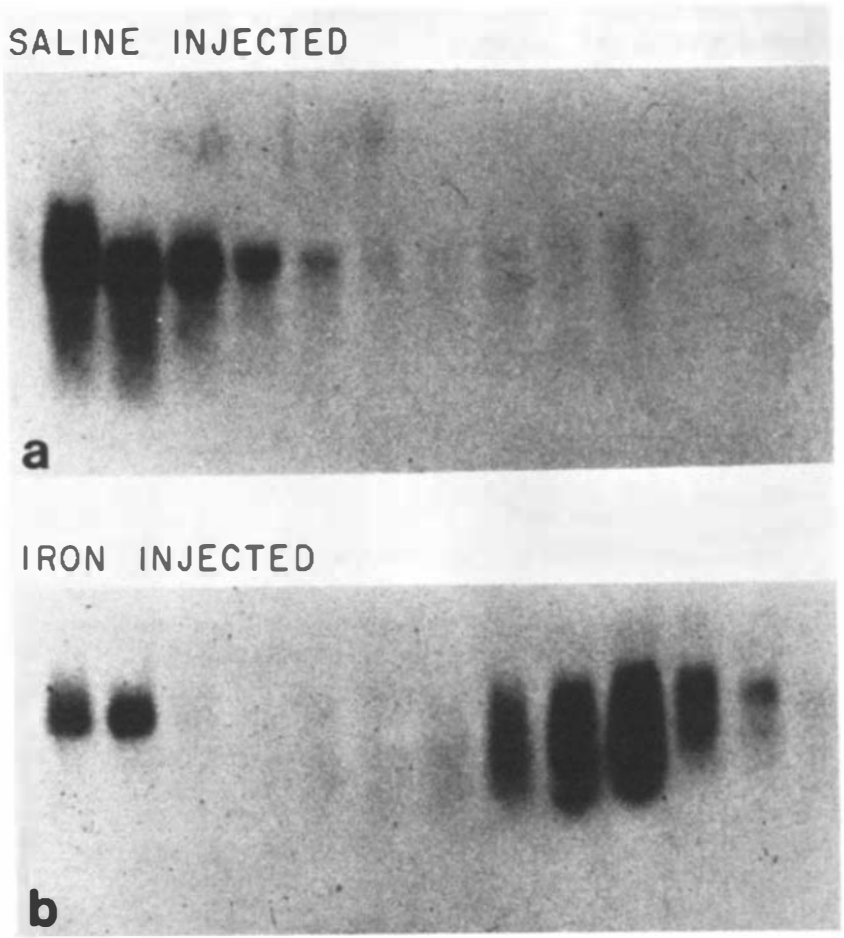

Figure 3 Translational shift of ferritin mRNA from RNP particles to polysomes by iron. Rats were injected intraperitoneally with saline (*a*) or ferric ammonium citrate (*b*). Four hours later, livers were isolated and RNP particles and polysomes were fractionated on sucrose gradients. Total RNA was isolated from each fraction and hybridized to radiolabeled ferritin H cDNA on Northern blots. RNP fractions are in lanes 1–5; polysomal fractions are in lanes 8–12 (K. White, unpublished data).

final iron content of the livers differed in the two studies, suggesting that sex differences in iron metabolism may exist.

REGULATION BY IREs The first studies to demonstrate a role for the IRE in the translational regulation of ferritin by iron were carried out using plasmids in which the 5'-untranslated region of rat ferritin L (2) or human ferritin H (34) genes was fused to heterologous reporter genes. When cells were trans-

fected with these constructs and treated with iron, an increase in the activity or levels of the reporter proteins was observed. Deletions of the sequence containing the IRE abolished the response to iron (2, 34). A region of approximately 35 nucleotides containing the IRE was shown to be necessary and sufficient for iron-dependent translation regulation (11). Theil and co-workers (20) using a wheat germ in vitro translation lysate reported that a 70-nucleotide region in the 3'-untranslated region of bullfrog ferritin mRNA was involved in iron-mediated translational regulation of ferritin synthesis (20). This work was not substantiated by others, who have shown both in vitro (7) and in vivo (11) that the 3'-untranslated region does not contribute to the iron-dependent translational regulation of ferritin. The 3'-untranslated region may be required for translation, but may not be important for iron modulation.

Despite sequence differences in the stems of ferritin, erythroid ALAS, mitochondrial aconitase, and TfR IREs, all are predicted to form very similar, stem-loop structures, which suggests that the stem-loop structure may be important for IRE-BP binding. First, chemical and enzymatic nuclease footprinting experiments demonstrated that binding of the IRE-BP to the IRE was confined to the stem-loop (32). Second, mutant ferritin IRE RNAs, which contain base substitutions disrupting base-pairing of the stem, prevented IRE-BP binding (3, 48), whereas compensating substitutions, which restored base-pairing, restored IRE-BP binding (48). Third, deletion of a nucleotide in either the stem or the loop abolished protein binding to the IRE in vitro and iron-dependent regulation in vivo (10, 33, 73). These studies demonstrated that conformation of the IRE RNA is important for binding to the IRE-BP.

Because a particular RNA conformation appears to be important in IRE-BP binding, a computer search using an IRE motif was used to scan sequence databases to identify other IRE-containing mRNAs that may be regulated by the IRE-BPs (17). Two genes, *D. melanogaster toll* and porcine mitochondrial aconitase, contained IRE-like sequences consistent with the IRE consensus structure (Figure 2*B*). The *toll* IRE failed, however, to compete with either ferritin or erythroid ALAS IRE for binding to the IRE-BP (17). The predicted negative free energy of the *toll* IRE was lower than that of erythroid ALAS or ferritin IREs. These data suggested either that the *toll* IRE did not form a stable structure capable of binding the IRE-BP or that specific nucleotides not present in the *toll* sequence may be needed for IRE-BP binding. The IRE in mitochondrial aconitase mRNA appears to be functional, since it forms a RNA-protein complex when incubated with porcine lysates (C. Kennedy, H. Beinert, H. Zalkin, personal communication). These data suggested that mitochondrial aconitase may be translationally regulated by iron via the IRE-BPs.

POSITIONAL REQUIREMENTS OF THE IRE The location of the IRE in ferritin mRNAs is important for translational regulation. Placement of either the B or C TfR IREs (Figure 2*B*) into the 5'-untranslated region of a reporter gene conferred iron-dependent regulation of translation similar to that observed for the ferritin mRNA (9). Thus, IREs located in the 5'-untranslated region of an mRNA regulate mRNA translation. Other studies demonstrated that translational repression of ferritin mRNA is dependent on the position of the IRE within the 5'-untranslated region (29). Ferritin IREs are typically located 30–39 nucleotides from the cap site of the mRNA (84), except in *X. laevis*, where the IRE is located 174 nucleotides from the cap site (54). When the IRE is moved 67 or more nucleotides downstream from the cap site, the IRE-BP/IRE complex does not interfere with translation (29). Secondary structure in the 5'-untranslated region, especially near the cap site, has been shown to limit accessibility of the cap to initiation factors and to inhibit translation (43, 44, 63). In contrast, secondary structure either in the coding region or in the 5'-untranslated region of mRNAs near the AUG initiation site is not as inhibitory (91). It is thought that the translational machinery can melt through RNA secondary structures during scanning. Thus, the data suggest that IRE-BPs repress translation either by physically blocking the cap binding protein complex from binding to the cap site or by interacting with one of the initiation factors. The location of the *X. laevis* IRE is difficult to reconcile with these data and requires further investigation.

TRANSLATIONAL REPRESSION BY IREs Ferritin mRNAs were shown to be poorly translated in a rabbit reticulocyte lysate compared to translation in a wheat germ lysate, thus suggesting that a translational repressor specific for ferritin mRNA was present in the reticulocyte lysate (20, 86). The addition of purified IRE-BP to a wheat germ lysate programmed with mouse liver mRNA (86) or with a ferritin RNA synthesized in vitro (7, 86) repressed ferritin synthesis. This repression was relieved by the addition of excess ferritin IRE transcript, indicating that the IRE was required for regulation. These experiments showed a direct role for the IRE-BP in translational repression of ferritin synthesis.

As mentioned above, erythroid ALAS mRNA contains an IRE in its 5'-untranslated region (15, 17, 22, 79). ALAS is the first enzyme in heme biosynthesis and catalyzes the formation of 5-aminolevulinic acid by the condensation of glycine and succinyl CoA (see 22). ALAS activity is highest in hepatocytes and in erythroid cells, where heme is used in the synthesis of heme-containing proteins, such as cytochrome P450, and hemoglobin, respectively (see 22). ALAS is encoded by two separate genes: a ubiquitously expressed gene, termed the hepatic gene, and an erythroid-specific gene (69, 79). Only the erythroid ALAS mRNA contains an IRE. In hepatic tissues,

ALAS expression is feedback inhibited by heme. The repression of ALAS expression by heme is thought to be due to the transcriptional repression of the ALAS gene (80a) and/or to a decrease in the stability of the ALAS mRNA (31a). In contrast to hepatic tissues, the expression of ALAS in erythroid cells is stimulated in the presence of heme (27a). Evidence suggests that erythroid ALAS is regulated at the translational level by heme (26). The erythroid ALAS IRE is functional, as measured by binding to the IRE-BP in RNA-protein gel shift assays (15, 17), and is capable of regulating iron-dependent translation in transfected murine fibroblasts (17). Thus, the presence of an IRE in erythroid ALAS suggests that a translational mechanism is used to couple porphyrin synthesis with iron availability. Whether iron or heme is the direct inducer of erythroid ALAS synthesis via the IRE-BP remains unclear (discussed below).

Whether iron interacts with the IRE-BP as heme or in a nonheme chelatable form is a matter of controversy. Hemin has been shown to relieve the repression of ferritin translation by the IRE-BP in wheat germ lysates (51). Other forms of iron (Fe^{3+} plus H_2O_2, Fe^{3+}, and Fe^{2+} chelated with EDTA, Zn^{2+}-protoporphyrin IX, and protoporphyrin IX) did not relieve ferritin repression. Co^{3+}-protoporphyrin IX, however, was effective in relieving ferritin repression. Hemin was also shown to be cross-linked to a specific site on the IRE-BP in vitro (51a). Whether hemin is cross-linked to the IRE-BP in vivo is unknown. In other studies, heme inhibited binding of the IRE-BP to the IRE when measured by RNA-protein gel shift assays (31). Hemin appears to inhibit other nucleic acid protein interactions (31), which suggests that the inhibitory effect of hemin observed in vitro may not be specific for the IRE-BP/IRE interaction.

A clue to the nature of the iron moiety in IRE-BP comes from sequence analysis of the human IRE-BP cDNA (76). The homology between the amino acid sequences of the IRE-BP and mitochondrial aconitase, and specifically the conservation of the metal-binding sites (75), suggested that IRE-BPs may contain a Fe-S cluster. Furthermore the ability of cytoplasmic aconitase to bind IREs (C. Kennedy, H. Beinert, H. Zalkin, personal communication), in addition to the similarities in molecular weights (24, 27, 75) and chromosomal location (75, 80), suggested that cytoplasmic aconitase and the IRE-BP may be the same protein. To date, however, neither iron or heme has been reported in the IRE-BP.

Stabilization of TfR mRNA by IRE-BPs

TfR synthesis is regulated by intracellular iron and by the proliferative state of the cell. Cells treated with intracellular iron chelators increased TfR synthesis, whereas cells treated with iron salts or hemin decreased TfR synthesis (6,

52, 64, 67, 88, 89). The iron-dependent regulation of TfR is due to changes in TfR mRNA levels (66). The sequences that mediate TfR mRNA turnover are located in the 3'-untranslated region of TfR mRNAs (9, 10, 55, 62). The regulatory domain contains five IREs (A–E) (Figure 2*B*) that are predicted to be part of a larger RNA secondary structure (42, 55). The TfR IREs compete with ferritin IRE for the IRE-BP in RNA-protein gel shift assays (3, 42, 48, 56), thus suggesting that they have similar binding affinities. The TfR mRNA regulatory region was further delineated to a region of 250 nucleotides containing three of the five IREs (10). Deletion of a single nucleotide from each of the three IRE loops abolished RNA-binding activity in vitro and the iron-dependent regulation in mouse fibroblasts (10). Similar results were obtained by other investigators who showed that IRE-BP activity parallels TfR expression in phytohemagglutinin-stimulated lymphocytes and during the maturation of monocytes to macrophages (81a). Sequences other than the five IREs within the 250 nucleotide regulatory region were also shown to be important in regulation of TfR mRNA (10). These data suggest that the ability of the IRE-BP to interact with the TfR is important for regulation and that this interaction may protect the TfR mRNA from degradation by cellular RNases.

A Model for the Coordinate Regulation of Ferritin and TfR by IRE-BPs

A model for the coordinate regulation of ferritin and TfR expression by the IRE-BP has been postulated from data obtained by many laboratories (Figure 4). The function of an IRE is determined by its location within an mRNA. IREs located in the 5'-untranslated region of an mRNA mediate translational repression, whereas IREs located in the 3'- untranslated region mediate mRNA stabilization. The IRE-BP interacts with both IREs. When iron levels are low, IRE-BPs bind with high affinity to the IREs, repress ferritin synthesis, and stabilize TfR mRNA. These interactions result in decreased iron storage and increased iron uptake. When iron levels are high, IRE-BPs bind with low affinity to the IREs, increase ferritin synthesis, and destabilize TfR mRNAs. The result is increased iron storage and decreased iron uptake. The binding of the IRE-BP to the 5'-IRE may mediate translational repression by blocking the accessibility of the cap site to the cap-binding protein complex and the 43S pre-initiation complex. The binding of IRE-BP to 3'-IREs may stabilize TfR mRNAs either by forcing the TfR mRNA to adopt a conformation that makes it less susceptible to RNases or by protecting a specific RNAse cleavage site in the mRNA. The coordinate regulation of ferritin and TfR by the IRE-BPs allows cells to respond rapidly to incoming iron by regulating the amount of iron taken up by the cell via TfR and the amount sequestered by ferritin.

Control of Iron Uptake and Storage Within the Cell: Responses to High and Low Iron Concentrations

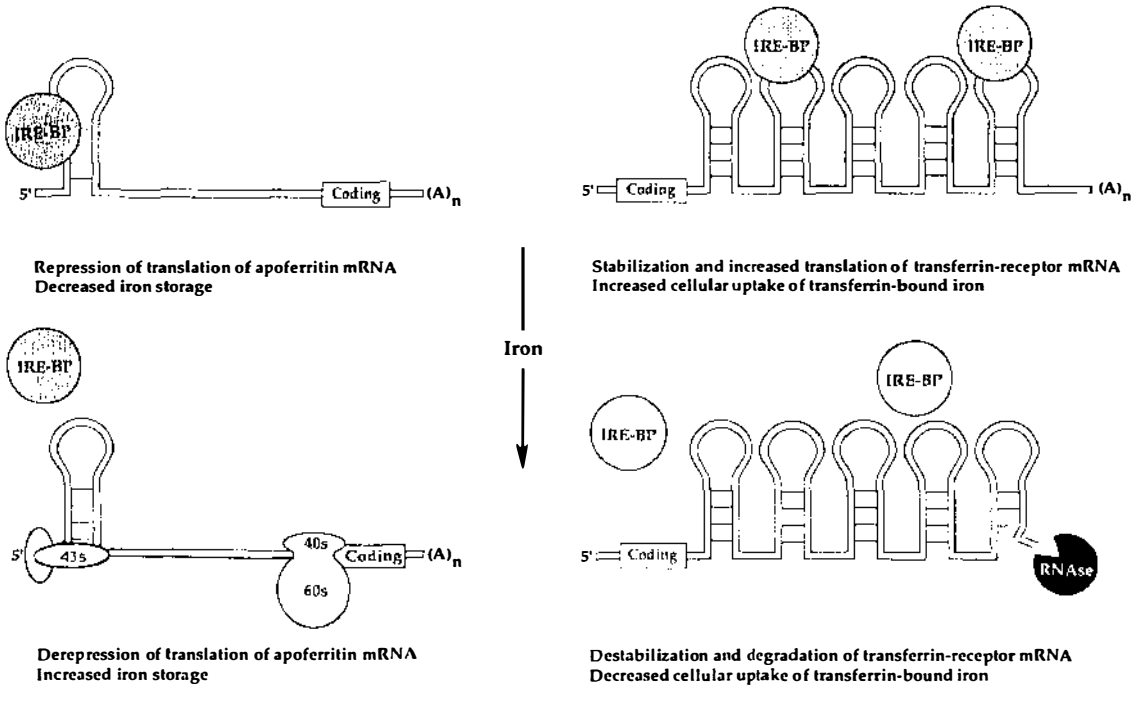

Figure 4 Proposed mechanism for the regulation of ferritin and TfR expression by the IRE-BP via the IREs. See text for detailed description. Reproduced with permission (41).

MECHANISM OF ACTIVATION OF IRE-BPs BY IRON

How do the IRE-BPs sense the iron status of the cell? The data indicate that iron regulates the activity of the IRE-BP. IRE-BP activity is increased in cells pretreated with the iron chelator desferrioxamine and is decreased in cells pretreated with iron (56, 73). The fact that induction was not blocked by actinomycin D, a transcription inhibitor, suggests that new mRNA was not necessary for increased IRE-BP activity. Cycloheximide, a translation inhibitor, delayed the increase in IRE-BP activity by 8 hr, suggesting a post-transcriptional mechanism for the activation of IRE-BP (56). IRE-BP activity was decreased in rats treated with iron over the course of 16 hr; no change in the levels of IRE-BP mRNA was noted (E. Leibold, unpublished data). These data indicate that the IRE-BP is regulated posttranscriptionally by iron.

To account for the differences observed in IRE-BP binding activities with iron and iron chelation treatments, investigators demonstrated that the IRE-BP has two distinct binding affinities for the IRE: a high affinity form (K_d = 20 pM) and a low affinity form (K_d = 3 nM) (30). In iron-depleted cells, which have increased RNA-binding activity, most of the IRE-BP is found in the high affinity form, whereas in iron-treated cells, which have decreased RNA-binding activity, very little of the IRE-BP is in the high affinity form. Thus, one effect of iron is to decrease the number of IRE-BPs in the high affinity form. The low affinity form in iron-treated cells can be activated in vitro by the addition of reducing agents, whereas no further activation of the IRE-BP can be obtained in iron-depleted cells (35). These data indicate that a functional sulfhydryl group in the IRE-BP can be reduced and oxidized, depending on the iron status of the cell. The interconversion of the IRE-BP from a high affinity form to a low affinity form would be a rapid way for IRE-BPs to respond to changes in cellular iron levels without the synthesis of new mRNA or protein.

One obvious model to explain IRE-BP activation by iron predicts that the IRE-BP is a regulatory protein that senses changes in intracellular iron concentration (75) (Figure 5*A*). The model is based on the interconversion of the Fe-S cluster of mitochondrial aconitase from the active [4Fe-4S] form to the inactive [3Fe-4S] form (5, 27). This model predicts that the IRE-BP is found in cells in either a [4Fe-4S] low RNA affinity form or a [3Fe-4S] high RNA affinity form. The proportion of the IRE-BP in these forms depends on the iron status of the cell. When iron levels are high, the IRE-BP is converted to the low RNA affinity form [4Fe-4S] by the addition of the fourth iron to the cluster. The fourth iron may prevent RNA binding either by sequestering a free cysteine or by stabilizing the IRE-BP structure, thereby rendering the RNA-binding site inaccessible. A structural comparison of the sequence of the active sites of mitochondrial aconitase with the human IRE-BP sequence

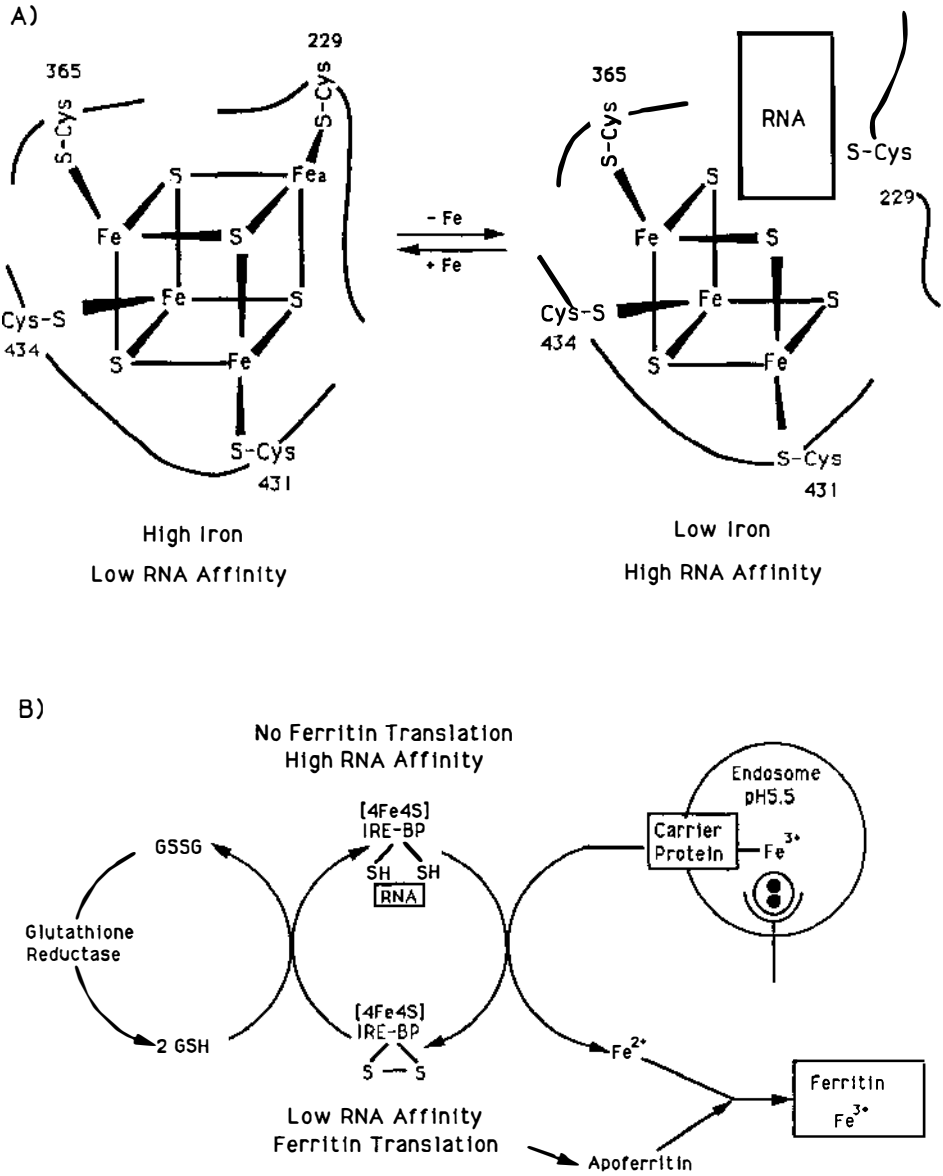

Figure 5 Models for the activation of the IRE-BP by iron. (*A*) Schematic model of the interconversion of the 4Fe and 3Fe cluster of IRE-BP based on the Fe-S cluster of aconitase (5). Fea is the labile fourth iron in aconitase. The numbers identifying the cysteine residues are from the human IRE-BP sequence (75). Adapted from Beinert (5). (*B*) Schematic model of IRE-BP activation by iron assuming the presence of an iron carrier protein. Transferrin receptor, Ψ ; diferric transferrin, ⊙ ; GSH, glutathione reduced; GSSG, glutathione oxidized. See text for detailed description.

showed that cysteine 229 in the IRE-BP is predicted to be positioned in the active cleft, where it could serve as the ligand for the fourth iron (75). When iron levels are low, the IRE-BP is converted to a high affinity form [3Fe-4S] containing a high affinity RNA-binding site. This RNA-binding site can be made accessible by a conformational change in the protein. Although this is an attractive model, we do not favor this model for the following reasons. First, cytoplasmic aconitase, in contrast to mitochondrial aconitase, contains four iron atoms when isolated, suggesting that the fourth iron is not labile (C. Kennedy and H. Beinert, personal communication). Second, cytoplasmic aconitase containing either no iron (apoenzyme), [3Fe-4S], or [4Fe-4S] binds the IRE with similar affinities (C. Kennedy, H. Beinert, and H. Zalkin, personal communication). Third, this model does not account for the in vitro activation of the IRE-BP by reducing agents.

An alternative model for IRE-BP activation by iron predicts that a putative iron carrier protein senses iron concentration and facilitates the conversion of the IRE-BP from a high RNA affinity form to a low RNA affinity form (Figure 5*B*). According to this model, the IRE-BP containing a [4Fe-4S] cluster exists in either a high RNA affinity form containing a pair of reduced thiolates or a low RNA affinity form containing an oxidized disulfide bond. In the reduced conformation, the sulfhydryl may interact directly with RNA or juxtaposed amino acids may form a high RNA affinity binding site. The model predicts that when iron enters the cell via receptor-mediated endocytosis, ferric irons dissociate from the TfR-transferrin complex and bind to the putative carrier protein. The Fe^{3+}-carrier protein complex interacts with the IRE-BP, creating a redox couple in which the oxidation of the thiols is coupled to the reduction of ferric ions on the carrier. The Fe-S cluster in the IRE-BP serves as an electron shuttle to the electrophilic Fe^{3+}-carrier. The net charge on the Fe-S cluster before and after the redox coupling remains constant. The oxidized form of IRE-BP is only a transient state and is rapidly reduced by glutathione to the high RNA affinity form. This model couples iron transport with translational regulation; the driving force is the endocytic iron uptake via the TfR. This model provides an explanation for data indicating that IRE-BP can be activated in vitro by reducing agents and is unaffected by iron (35). There is evidence that a oxidoreductase/iron transporter system is present in reticulocyte endocytic vesicles (61). This transporter system could function as the carrier protein in this model. Moreover, this model could account for the data showing that cytoplasmic aconitase containing no iron, [3Fe-4S], or [4Fe-4S] binds the IRE with similar affinities (C. Kennedy, H. Beinert, personal communication). However, this model is speculative: we do not have evidence to support the presence of an endocytic membrane carrier protein that interacts with the IRE-BP/IRE complex. Nonetheless, the presence of an iron carrier protein, which can sense the iron status of the cell,

could couple IRE-BP activation with TfR-mediated iron uptake and translational regulation of ferritin synthesis.

RELATED FAMILIES OF IRE-BPs

Recent data indicate that there may be a family of related IRE-BPs. First, two IRE-BP species similar in size (100,000 M_r and 95,000 M_r) have been isolated from human placenta by IRE affinity chromatography (60). These proteins have different isoelectric points, but similar IRE binding characteristics. Human liver contained only one of the IRE-BPs (60). Second, a cDNA isolated from a human T cell library has a predicted amino acid sequence 57% identical and 75% similar to IRE-BP (76). This cDNA was isolated using oligonucleotide probes derived from peptide sequences from the human IRE-BP. Third, RNA-protein gel shift assays showed that a second RNA-protein complex, in addition to the IRE-BP complex, was formed when a radiolabeled IRE was incubated with protein extracts from rat (50) and mouse (72). The second RNA-protein complex found in rats appears to be a specific complex, since it is resistant to RNase T1 digestion and heparin competition and can be purified by RNA affinity chromatography. The IRE-BP and the related IRE-BP in rat show differences, such as size and charge (B. Guo and E. Leibold, unpublished data). Whether the related IRE-BPs found in rat liver and human placenta are distinct proteins or the same protein altered by posttranslational modifications is unclear. The cloning and characterization of these proteins should allow experimental studies both in vivo and in vitro to determine their function.

CONCLUDING REMARKS

The past several years have witnessed important advances in our understanding of the iron-dependent translational regulation of ferritin expression by the IRE-BPs. In particular, the discovery of IREs in other mRNAs involved in iron metabolism has opened new pathways for research in iron metabolism and posttranscriptional regulation. Many unanswered questions remain. First, the homology of the IRE-BP with aconitase has provided clues to the mechanism by which IRE-BPs function. Does the IRE-BP act as a redox-sensitive regulatory molecule that modulates its activity, depending on the iron status of the cell? Or is IRE-BP activation facilitated by another protein that senses iron status and interacts with the IRE-BP? What are the important domains for RNA binding in the protein and what structural changes take place in the IRE-BP when RNA binds? Direct evidence is needed to demonstrate that the IRE-BP contains Fe-S and/or heme. Second, related IRE-BPs that are similar to the IRE-BPs have been identified. The function of these proteins is

unknown. Are these proteins involved in iron homeostasis? Or if not, what are their functions? Third, the mechanism regulating the stabilization of TfR mRNAs by the IRE-BPs is not understood. How do IRE-BPs protect TfR mRNA from degradation? Where does this regulation occur in the cell? Are specific RNAses involved in this regulation? Finally, we do not know how iron is transferred from the endosome to the IRE-BP. Does the carrier protein physically couple endosomal transfer of iron to the IRE-BP/IRE complex? Over the next few years, as we seek and obtain answers to these questions, we will gain a better understanding of the regulation of iron metabolism as well as the mechanisms regulating posttranscriptional gene regulation.

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