Perspective

Cis-Trans Models for Post-Transcriptional Gene Regulation

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THE CONTROLLED REGULATION OF THE EXPRESSION OF genetic information is central to all forms of life. Currently, most available information relates to the regulation at early (initiation of DNA transcription) and late (post-translational) stages in gene expression. Comparatively little is known about the regulation of the complex life of the RNA molecules that mediate the conversion of the genetic code into protein products. These events can be divided into the post-transcriptional production of mature cytoplasmic mRNA and the fate of that mature mRNA. The former includes the splicing of transcripts, post-transcriptional covalent modifications (capping, polyadenylation, and sequence editing), and nuclear-cytoplasmic transport. Although it is clear that some, if not all, of these processes can be regulated, little is known about the molecular mechanisms underlying such regulation. The fate of mature mRNA can be regulated by controlling either the stability of a message or its translation. Work on the regulated expression of the proteins of cellular iron metabolism in higher eukaryotes has provided a relatively detailed picture of the control of the fates of mRNA molecules that encode ferritin and the transferrin receptor (TfR).

The introduction of oxygen into the earth's atmosphere created challenging problems related to the dependency of cells on elemental iron. Whereas the oxidation of ferrous iron to the more insoluble ferric form demanded new mechanisms to obtain environmental iron, the ability of iron to catalyze the generation of dangerous hydroxide radicals required new mechanisms to control detoxification of iron. In higher eukaryotes, the two proteins that mediate the uptake and detoxification of iron are the TfR and ferritin, respectively. Iron is delivered to most cells via endocytosis of diferric-transferrin bound to the TfR (*1*). In the endosome, iron is released from the transferrin and transferred to the cytosol. Once in the cytoplasm, the iron is either used for processes that require this element or it is sequestered in ferritin, a hollow spherical molecule composed of 24 subunits encoded by two highly homologous genes (H and L) (*2*).

The expression of both the TfR and ferritin is highly regulated by the amount of available iron. Limiting iron results in an increase in the number of TfRs, whereas when iron is plentiful, the number of TfRs is decreased. The level of ferritin is regulated in the opposite direction, increasing in the presence of iron and decreasing in its absence. Ferritin regulation allows for adequate sequestration of excess iron and minimizes sequestration when iron is limiting.

We are beginning to understand the molecular basis for the coordinate but opposite regulation of these two genes. Ferritin and TfR mRNAs contain a similar cis-acting RNA element that we have termed the iron-responsive element (IRE) (*3, 4*). These elements interact with a common IRE-binding protein (IRE-BP) (*5, 6*). A model to explain how the IRE and its binding protein can account for the coordinate iron-dependent regulation of both ferritin and TfR expression (*4, 7*) is schematically shown in Fig. 1. As cellular iron becomes limiting, a greater fraction of the cell's IRE-BP is recruited into a high-affinity binding state. The high-affinity interaction between the IRE-BP and an IRE acts as a repressor of translation if the IRE is located within the 5′ untranslated region (UTR) of a mRNA. Within the regulatory region of the 3′ UTR of the TfR mRNA, this same high-affinity interaction represses the degradation of the transcript. In this way, the regulated binding of

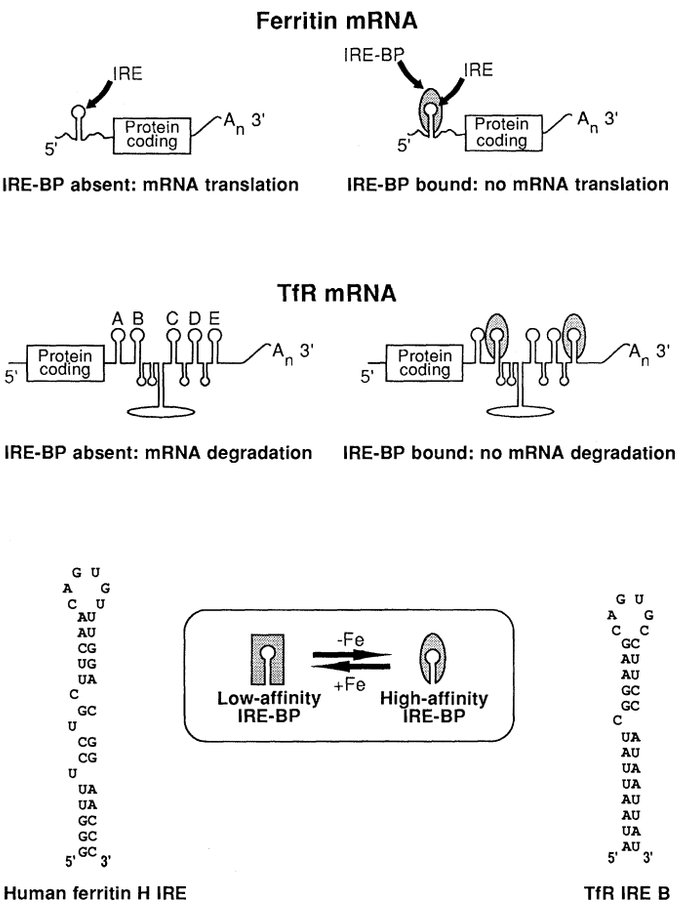

Fig. 1. Coordinate regulation of the expression of ferritin and the transferrin receptor via common cis-acting RNA elements and a common trans-acting RNA binding protein. The 5′ UTR of ferritin mRNA contains a single IRE and the 3′ UTR of the TfR mRNA contains five IREs (*4*) (the latter designated A to E, left to right, and shown schematically above the horizontal). All of these IREs are capable of interacting with the IRE-BP (*5*). The sequence and proposed secondary structure of the human ferritin H chain IRE and one of the TfR IREs are shown. For the TfR IREs, elements B and E have been shown to be the preferred binding sites (*5*). As many as four molecules of IRE-BP may be bound per mRNA (*6*). Treatment of cells with an iron chelator results in high-affinity IRE-BP, whereas treatment of cells with an iron source results in low-affinity IRE-BP (*29, 30*) (inset). When the IRE-BP is bound to the ferritin IRE, translation of ferritin is inhibited. When the IRE-BP is bound to the TfR IREs, degradation of the TfR mRNA is inhibited. The 3′ UTR of the TfR mRNA also contains other RNA structural elements (*5*) (shown schematically below the horizontal), some of which are critical for iron regulation of TfR expression (*26, 28*).

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the IRE-BP can coordinately regulate a decrease in the biosynthesis of ferritin (by translational repression) and an increase in the biosynthesis of the TfR (by inhibition of mRNA degradation).

The Iron-Responsive Element: The Cis-Acting RNA Element

It had been known since the 1940s that the level of ferritin in tissues varies directly with changes in iron (*8*). By the 1970s, there was evidence that regulation of mammalian ferritin synthesis in response to iron occurred at the level of translation (*9*). A similar conclusion was also reached regarding ferritin biosynthetic regulation in amphibia (*10*). The molecular cloning of cDNAs corresponding to ferritin subunits (*11*) allowed the direct measurement of ferritin mRNA levels to confirm the translational control of ferritin (*12, 13*) and identification of the sequences within the ferritin mRNA responsible for iron regulation (*13, 14*). Differences of up to two orders of magnitude in ferritin biosynthesis occurred in the absence of change in the level of ferritin mRNA (*12, 13*). The ferritin mRNA within the cytosol shifted to polysomes as the synthesis of ferritin increased (*13, 15*). The mRNA for human ferritin H chain (approximately 1 kb) contains a 212-nucleotide 5′ UTR. When the 5′ UTR was deleted, all short-term iron regulation was lost (*14*). Further deletion analysis and the cloning of the sequence into the 5′ UTR of two different reporter genes identified a region approximately 35 bases in length that is necesssary and sufficient for translational regulation by iron (*3, 16*). Examination of all ferritin genes cloned to date (human, rat, mouse, rabbit, chicken, and bullfrog) reveals the presence of this highly covered sequence in the 5′ UTR (*7*). All of these sequences are capable of forming moderately stable stem-loop structures with a loop sequence of CAGUGN (Fig. 1).

The IRE-Binding Protein Regulates Translation and mRNA Stability

The location of the IRE in the 5′ UTR of ferritin suggested that it functioned to control translation initiation. Removal of the IRE resulted in a constitutively high level of mRNA translation, and addition of an IRE to a 5′ UTR depressed the rate of translation, an indication that the IRE was functioning to repress translation. The presence of an IRE in the 5′ UTR of ferritin mRNA decreases its in vitro translation (*17*). The first direct evidence for an IRE-BP came as a result of an electrophoretic mobility shift assay in which IRE-containing RNAs were used (*18, 19*). The IRE-BP is a 90-kD cytosolic protein, and the human protein was purified to homogeneity by RNA affinity chromatography (*20*). The analogous protein (or proteins) from rodent cells appears similar in mass (*6, 18*). The ferritin translational repressor protein was purified from rabbit liver by more conventional protein purification methods, and the size of this protein (90 kD) and its IRE binding activity indicate identity with the IRE-BP (*21*). The gene encoding the IRE-BP was localized to human chromosome 9 (*22*).

As with ferritin, the rapid regulation of the expression of the TfR is the result of altered rates of protein synthesis, but this alteration is directly reflected in changes in the levels of cytoplasmic TfR mRNA (*23*). The major locus of iron-dependent regulation mapped to within the 3′ UTR of the TfR message (*24, 25*), which mediates the iron-dependent regulation of the half-life of the TfR message (*26*). Deletion analysis of the 2.5-kb 3′ UTR of the TfR mRNA with the use of convenient restriction sites demonstrated that a 680-nucleotide fragment contained all of the information for this regulation (*4*). Visual examination and computer-assisted analysis suggested that this 680-nucleotide region includes five stem-loop structures (A to E) with striking resemblance to ferritin IREs (*4, 7*) (Fig. 1). When inserted into the 5′ UTR of an indicator gene, stem-loops B or C from the 3′ UTR of the TfR mRNA each functioned as a ferritin-like translational control element (that is, translational repression in response to iron deprivation). Moreover, each of the TfR IREs specifically bound the same cytosolic protein as the ferritin IRE (*5, 6*).

The regulatory region required for iron-dependent TfR mRNA stability regulation appears to be complex. The availability of TfR sequence from two distant species (human and chicken) allows prediction of structure based on phylogenetic conservation. Of 160 nucleotides that encode the five IREs, only 6 differ in the two species, and all six changes are conservative of the IRE sequence-structure motif (*4, 5, 27*). Nonetheless, this regulatory segment can be reduced to 250 nucleotides containing only three IREs without change in iron regulation (*28*). Two types of deletions within the smaller regulatory fragment can be distinguished: those that specifically affect IRE function and those that affect non-IRE structures. The alteration that best illustrates an IRE effect results from the removal of the first C residue from each of the IRE loops, resulting in five-membered loops. We showed earlier that this altered IRE is incapable of functioning as a translational control element (*7*) and that it cannot bind to the IRE-BP with high affinity (*19*). The altered fragment (with 3 of the 250 nucleotides deleted) gives no iron-dependent mRNA stability regulation and no longer binds with high affinity to the IRE-BP. Other deletions, particularly of the small non-IRE stem-loops, also eliminate regulation but have no effect on the binding of the RNA by the IRE-BP (*28*). Although both IRE and non-IRE changes can abrogate regulation, a careful examination of their "regulatory phenotypes" yielded a revealing difference. For a given level of transcription, the IRE alterations resulted in very low levels of TfR mRNA. In contrast, the non-IRE mutants resulted in high levels of mRNA, comparable to that seen with the regulated constructs after treatment of cells with the iron chelator desferrioxamine. This difference was interpreted as reflecting two distinct functions within the 3′ regulatory domain, both of which are required for iron regulation. One must be related to an RNA instability determinant that gives this mRNA a short half-life (a requirement for rapid regulation of mRNA levels), and the other is the IRE that can regulate the use of this instability element.

A Novel Regulatory Mechanism: The Sulfhydryl Switch

The mechanism by which any regulatory nucleic acid binding protein responds to physiologic signals represents one of the great unknowns in molecular biology. Initial studies in which either gel shift or ultraviolet cross-linking were used demonstrated that the apparent amount of IRE-BP increased in lysates of cells that had been starved of iron and decreased when the cells had been loaded with iron (*6, 18, 19*). A more quantitative analysis of the interaction between the IRE-BP and its cognate RNA has helped to clarify these observations. The cytosol contains binding activity with two distinct affinities, one with a dissociation constant (K_d) of 10 to 30 p*M* and the other with a K_d of 2 to 5 n*M* (*29*). All available evidence suggests that the two affinities represent the same protein. The fraction of total sites displaying picomolar affinity varied from less that 1% in iron-fed cells to more that 50% in iron-starved cells. This shift did not require protein synthesis (*6, 30*), suggesting that the same population of IRE-BP had been interconverted by changes in the cell's iron status.

The biochemical basis for the affinity change of the IRE-BP appears to be the reversible oxidation-reduction of one or more disulfides in the protein (*30*). The low-affinity IRE-BP was converted to the high-affinity form in vitro by the addition of reducing agents to cell lysates, and the high-affinity form was switched to the low-affinity state by treatment with agents that catalyze disulfide formation. In cell in which the IRE-BP had been maximally activated by treatment with an iron chelator, little (if any) IRE-BP isolated from the cell was in an oxidized form, whereas in cells treated with an iron source, virtually none of the IRE-BP was in a reduced form. Thus, the iron status of the cell appears to set the redox state of the IRE-BP (*29, 30*). We termed this novel biochemical regulatory mechanism a sulfhydryl switch. To date, we have been unable to reproduce the switch in cell lysates by the addition or chelation of iron.

Perspectives for Future Research

Much concerning the molecular details of post-transcriptional gene regulation via IREs and the IRE-BP remains to be determined. First, a clearer picture of the sequence-structure that defines an IRE must be elucidated. Only detailed quantitative analysis will allow determination of the exact contributions of any nucleotide sequence and structure to affinity, specificity, and the ability to be responsive to the redox state of the IRE-BP. Second, the biochemical basis of the iron-dependent redox shift of the IRE-BP must be understood. Does the IRE-BP itself bind iron or an intracellular iron compound? Alternatively, iron may indirectly determine the affinity of the protein, perhaps via an oxidoreductase. Only a complete description of the sequence and eventually the structure of the IRE-BP will define the nature of its RNA interactions and of its sulfhydryl switch. Third, the mechanism by which binding of the IRE-BP actually alters translation initiation and mRNA stability needs to be defined. Does the IRE-BP act sterically or by specific interaction with or modification of components of the translation or degradative machinery? Fourth, the nature of the control of TfR mRNA stability must be examined in more detail. What are the actual sequence and structure of the instability determinant and what is the enzymology of the RNA turnover? Finally, there is a need to assess whether the information derived from the study of this RNA regulatory system can guide us to a better understanding of other post-transcriptionally regulated genes.

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