REVIEW ARTICLE

Iron regulatory elements (IREs): a family of mRNA non-coding sequences

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INTRODUCTION: THE IRON REGULATORY ELEMENTS FAMILY

Families of related *cis* regulatory elements in DNA create a recurring theme that is illustrated by the glucocorticoid receptor elements (e.g. Diamond et al., 1990; O'Malley, 1990; Cato et al., 1992) and metal regulatory elements (Silver and Walderhaugh, 1992). In eukaryotic mRNAs, IREs (Iron Regulatory Elements) are the only characterized family of *cis* (non-coding) regulatory elements (reviewed in Theil, 1990,1993; Klausner et al., 1993). IREs appear to be composites of an iron-specific regulatory element fused to either a generic translation control element or mRNA stability element (Figure 1a).

When cellular iron concentrations are low, ferritin mRNA is

not translated (Zahring et al., 1976; Schaefer and Theil, 1981; Shull and Theil, 1982). At the same time, the transferrin receptor (TfR) mRNA is stabilized and translated (Mullner and Kuhn, 1988). Thus when no iron is available to concentrate or to store, the cell is spared the expense of synthesizing ferritin and TfR synthesis allows iron uptake. When iron levels are high, ferritin mRNA is translated and TfR mRNA is degraded, allowing iron storage and preventing iron uptake.

Co-ordinated regulation of iron storage and iron uptake depends on the interaction of a protein regulator, the iron regulatory protein (IRP), and the IRE family of mRNA sequences (Aziz and Munro, 1987; Hentze et al., 1987a,b; Casey et al., 1988; Walden et al., 1989; Rouault et al., 1990; Theil,

Figure 1 Primary and secondary structure of the IRE family

(a) The seven members of the IRE family [ferritin (Fr), transferrin receptor (TfR) and erythroid aminolevulinate synthase (eALAS)] are compared; upper-case letters designate base-paired residues and lower-case letters represent the internal loop/bulge or the hairpin hexaloop in the centre of the linear sequence (taken from Theil, 1993). The full phylogenetic comparison is shown only for the ferritin IREs, but the level of phylogenetic conservation among the other sets of IREs is equally high. In contrast, the conservation between members of the IRE family is much lower. Thus, the human TfR-a, for example, is only 39% identical with the human ferritin IRE, 64% identical with human TfR-c IRE, and 36% identical with human eALAS IRE, but 97% identical with TfR-a in mouse and chick. Red lettering illustrates the conserved hairpin hexaloop. The internal loop or bulge is shown within the tinted box. (b) Secondary structure prediction for IREs. The IRE + FL sequence from bullfrog ferritin H chain (Didsbury et al., 1986) is displayed with the IRE numbered 1–28; vertical lines denote the triplet of base pairs in the FL conserved in all ferritin IREs (see Figure 2).

Abbreviations used: CAT, chloramphenicol acetyltransferase; CuOP, 1,10-phenanthroline-Cu; DMS, dimethylsulphate; eALAS, erythroid aminolevulinate synthase; eIF, eukaryotic initiation factor; FL, flanking regions; hGH, human growth hormone; IRE, iron regulatory elements; IRP, iron regulatory protein; IVT, *in vitro* transcript; luc, luciferase; NB/S, nuclease binding/stability; RB/Ts, ribosome binding/translation; TfR, transferrin receptor; UTR, untranslated region.

	5' FL			IRE	3' FL		
Human H	CGGCGaGGCU	GAG	uCGUCGGGguU	UCCugcUUAACagugcUUGGAcGGAAC	CCGGCG-	CUC	gGGCCCGCCG
Mouse H		GAG	UCGCCGcGGUU	UCCugcUUAACagugcUUGAACGGAAC	CCGGUG-	CUC	
Rat H		GAG	UCGCCGcGGUU	UCCugcUUAACagugcUUGAACGGAAC	CCGGUG-	CUC	
Chick H		GAG	-CGgaGCGGU	UCCuGcgUCAAacagugcUUGGaCGGAAC	CGCcgCG	CUC	
Frog H		GA	-uuAGuAGaGU	UCUugcUUAACaguguUUGAACGGAAC	cCUCUcu	GAG	
Frog H' (M)		GA	-uGAGuAGaGU	UCUugcUUAACaguguUUGAACGGAAC	cCUCUcu	GAG	
Human L	GGcGGU	CCC	gCGGGUCUGUC	UCUugcuUCAAacaguguUUGAcGAACAG	AUCCG--	GGG	ACuUC
Rabbit L	GGGAGa	CCC	GcGGaCCUGUg	UCUugcUUAACaguguUUGAACGGAAC	AGGCCc-	GGG	gaCUCUC
Rat L	GGAGaU	CCC	GaGGaUCUGUa	UCUugcUUAACaguguUUGGAcGGAAC	AGACCCG	GGG	aCUCC

Figure 2 Phylogenetic comparison of the ferritin mRNA base-paired flanking regions (FL) contiguous with the IREs

FL vary in length in rough proportion to the distance of ferritin IREs from the cap (Wang et al., 1990,1991) shortening the distance from 26–33 nucleotides to 7–17 nucleotides. Note the triplet of base pairs 10 or 11 nucleotides from the end of the IRE which are phylogenetically conserved by compensatory mutation. Mutation of the conserved triplet diminishes the effect of IRP binding (taken from Dix et al., 1993).

1993). When iron concentrations are low, the IRE binds the IRP, blocking ribosome binding to the translation-regulated mRNAs and blocking nuclease interactions with the stability-regulated mRNAs (Dickey et al., 1988). An additional feature of the ferritin IRE region is enhanced initiation factor binding (positive control) when iron is high or the IRP is absent (Shull and Theil, 1982,1983; Dix et al., 1992). In erythroid cells, where amplified rates of haem synthesis require unusually large amounts of iron, the aminolevulinic synthase mRNA expressed in erythrocytes also has an IRE (Cox et al., 1991; Dandekar et al., 1991), which binds the IRP to regulate translation, albeit less efficiently than for ferritin mRNA (Bhasker et al., 1993; Melefors et al., 1993).

The relatively unique nature of the IRE family could reflect the central importance to life of regulating iron metabolism (the ferricentric world) or, alternatively, the fact that the highly conserved primary and secondary structure in IREs facilitated identification (Figure 1a). IREs are among the longest conserved primary sequences ($n = 28-30$) currently known, rivalling in length, for example, the mRNA-binding-site sequence in rRNA (Noller, 1991). Families of regulatory RNA elements are well-known in viruses (e.g. Cullen, 1992; Leathers et al., 1993; Houser-Scott et al., 1994). Since deletion and genetic analysis have usually been used for identification of *cis* RNA regulatory elements when conserved primary structure is absent, the results are skewed toward regulatory elements that are constructed from sequences that function in a linear array. Other families of RNA regulatory elements could remain unidentified because they share only tertiary structural motifs, analogous to the four-helix bundle, β barrel, α/β saddle and β - β sandwich motifs in proteins. At the current level of knowledge, conserved higher-order folding motifs in RNA are not well defined and are impossible to identify from linear sequences.

Comparison of primary structure among currently identified IREs indicates that there are seven members of the IRE family, based on phylogenetic conservation (Figure 1a). IREs are only found in animals (Ragland et al., 1990; Lescure et al., 1991; Kimata and Theil, 1994). Depending upon the location in the mRNA [5'- or 3'-untranslated region (UTR)] and the specific structure, IREs control either ribosome binding/translation (RB/Ts) or nuclease binding/stability (NB/S). When IRE family members bind *trans* factors such as the IRP (the IRP was formerly designated IRE-BP or IRF or FRP, or P-90), cells are able to co-ordinately regulate both iron storage (ferritin mRNA) and iron uptake (TfR mRNA), during physiological variations in iron availability; in erythroid cells, an IRE in aminolevulinic

synthase (eALAS) mRNA also controls translation in response to iron (reviewed in Theil, 1990,1993; Klausner et al., 1993).

The two RB/Ts-IRE family members that control iron storage (ferritin mRNA) or eALAS mRNA are located near the cap of the 5'-UTR. The position of RB/Ts-IREs is crucial to function; a distance of < 10–17 nucleotides from the 5' cap appears to be required (Wang et al., 1990,1991; Goossen et al., 1990; Dix et al., 1992). Correct IRE position is achieved in ferritin mRNAs with flanking regions (FL) of variable length that base pair (Wang et al., 1990,1991; Dix et al., 1993) (Figure 2). No FL are required in eALAS mRNAs because the IRE is very close to the cap (Cox et al., 1991; Dandekar, 1991).

A set of five NB/S-IRE family members in the 3'-UTR form the iron-regulated stability element in TfR mRNAs; the IREs are designated TfR-IRE-a, -b, -c, -d or -e. Phylogenetic primary sequence conservation among mammals and birds for each type of TfR-IRE appears to be 100%, in contrast with the conservation between TfR-IRE types a, b, c, d and e in a single species (Figure 1). For example, human TfR-IRE-a is 36% identical with ferritin IREs and only 64% identical with human TfR-IRE-c (Figure 1a), but 100% identical with TfR-IRE-a in other mammals or birds; TfR-IRE-a also has the AUUUA sequence observed in the stability element of *c-fos* mRNA. TfR-IRE-b and -c have a particularly critical role in regulation of TfR mRNA stability (Casey et al., 1988; Mullner and Kuhn, 1988). The special requirements for TfR-IRE-b and -c may relate to the presence of a repeated set of palindromic sequences (AUUAU/UAAUA) in each IRE; the two sets of palindromic sequences can participate in two different predicted secondary structures of similar stability (Scheme 1).

Conserved secondary structure, among the seven members of the IRE family, is a hairpin with a terminal hexaloop of the sequence CAGUGU/C (Figure 1b). Interruptions which occur in the hairpin stem of all IREs have some specificity. For example, all five TfR IREs contain a single nucleotide bulge (C) on the 5' side of the stem. In contrast, the stem of ferritin IREs is interrupted by an internal loop with UGC on the 5' side and C on the 3' side of the stem. Stem structure of IREs also varies. Thus the stems of all TfR IREs are AU-rich (Figure 1a), which is also a characteristic of 3'-UTR mRNA stability elements (Brawerman, 1989; Leeds et al., 1992; Green, 1993). In contrast, the eALAS IREs have a relatively equal distribution of A/U and G/C pairs in the stem. Ferritin IREs have G/C-rich stems as well as an additional G/C base pair at the base of the stem (Figures 1a and 1b). The distinctive features of ferritin IREs may relate to

Figure 3 Schematic diagram of the composite IRE regulatory elements that control either mRNA ribosome-binding and translation (RB/Ts) or mRNA nuclease-binding/stability (NB/S)

the combination of both positive and negative control of translation encoded in ferritin IREs (Shull and Theil, 1982,1983; Dix et al., 1992) and the quantitatively more effective negative regulation (Bhasker et al., 1993; Melefors et al., 1993). Base-paired FL, specific to ferritin IREs, also modulate negative control (Theil, 1993; Dix et al., 1993).

As a family, IREs combine both specificity, to regulate mRNA encoding proteins of iron metabolism (iron storage, iron uptake and erythrocyte haem synthesis), with generic features associated with control of either ribosome binding or mRNA turnover. IREs thus appear to be composite RNA regulatory elements formed by a specific mRNA hairpin hexaloop, recognized in iron regulation, combined with hairpins recognized either during ribosome binding/translation or nuclease binding/stability (Figure 3).

IREs appear to be absent from plant ferritin mRNAs (Ragland et al., 1990; Lescure et al., 1991; Theil, 1993; Theil and Hase, 1993; Kimata and Theil, 1994). The apparent absence of IRE-dependent regulation of ferritin in plants is puzzling in light of the extraordinary sophistication of the IREs, and the high phylogenetic conservation among animals. Downstream sequences in plant ferritin mRNA actually inactivate the animal IRE in chimeric mRNAs (Dix et al., 1992; Kimata and Theil, 1994). In plants, ferritin mRNA has been shown to be regulated either during transcription (Lescure et al., 1991) or post-translationally (Kimata and Theil, 1994). In contrast with the evolutionary divergence of genetic regulatory mechanisms for ferritin, the coding sequence for ferritin is highly conserved between plants and animals; the ferritin sequence is also conserved in micro-organisms (Ragland et al., 1990; Grossman et al., 1992; Theil and Hase, 1993). Animals either acquired IREs after plants and animals diverged, or the IRE is ancient and was

lost in plants after plants and animals diverged. It is noteworthy, when considering such alternative ideas, that an IRE regulator, the IRP, is a member of an ancient protein family, the aconitases (Kennedy et al., 1992; Haile et al., 1992). Sequences encoding the transit peptide, which targets plant ferritin to plastids, would have been acquired by plants after plants and animals diverged (Ragland et al., 1990). Thus, IREs in animal mRNAs could easily be attributed to retention of an ancient mRNA feature which was lost during co-evolution of higher plants and plastids. The question of IRE distribution in contemporary organisms thought to be related to ancient eukaryotes has yet to be answered.

IRE FUNCTION IN REGULATING mRNA TRANSLATION AND STABILITY

Co-ordination of iron uptake and storage in animal cells exploits the same RNA-protein interaction (IRE+IRP) in two different mRNA contexts (reviewed in Theil, 1990,1993; Klausner et al., 1993). When environmental and cellular iron concentrations are high, changes in the interaction of the IRE and IRP lead to positive control of iron storage (and erythroid haem synthesis) (Figure 3). Conversely, when environmental iron levels are low, changes in the IRE-IRP interaction yield negative control of iron storage and haem synthesis combined with positive control of iron uptake to enhance iron flow into the cell (TfR increase) and within the cell. A recent observation indicates that even changes in the intracellular iron distribution, such as that required in liver regeneration following partial hepatectomy, enhance IRE-IRP interactions. For example, TfR mRNA increases and ferritin decreases with no net change in extracellular or intracellular iron (Cairo and Pietrangelo, 1994). The molecular

identity of the cellular iron signal is not fully known, but models currently under study will be described in the section on the IRP.

The original identification of the IRE sequences resulted from comparing the non-coding sequences of bullfrog and rat ferritin cDNAs (Leibold and Munro, 1987). Shortly after, the observation of the similarity of the IRE sequence in ferritin mRNAs to sequences in the TfR mRNA stability element led to the awareness that IREs participated in the co-ordinated regulation of mRNAs encoding the two proteins of iron metabolism (Koeller et al., 1989). Transfection experiments, in which the conserved non-coding ferritin sequence was linked to chloramphenicol acetyltransferase (CAT) or human growth hormone (hGH) 'reporters', demonstrated the importance of the IRE sequence for iron-regulated protein synthesis (Hentze et al., 1987a,b; Aziz and Munro, 1988), and led to the designation of the sequence as the IRE (iron regulatory element). The ability of the IRE to regulate mRNA translation is now known to be context-dependent, however, as the IRE regulated poorly when fused to plant ferritin mRNA (Dix et al., 1992; Kimata and Theil, 1994) or tubulin mRNA (Coulson and Cleveland, 1993).

Negative control of ferritin and erythroid aminolevulinate synthesis: IRE-IRP binding

Negative control of mRNA is inhibition of protein synthesis. Evidence that interactions between cytoplasmic proteins and mRNAs in eukaryotic cells would negatively regulate translation was first obtained by Spirin in 1969; the stored, inactive mRNAs were called informosomes. However, it was not until many years later, during animal studies of the induction of ferritin synthesis by iron, that evidence for a specific ferritin mRNA-protein (IRP) interaction regulating translation was obtained (Shull and Theil, 1982,1983; Dickey et al., 1988; Walden et al., 1988; Harrell et al., 1991). Earlier studies by Munro and colleagues had indicated that induction of ferritin synthesis was associated with a change in the distribution of ferritin mRNA between the polyribosomal supernatant fraction and polyribosomes themselves (Zahringer et al., 1976).

Initiation is the site of protein synthesis affected by the RB/Ts-IRE-IRP interaction in ferritin mRNAs and in eALAS mRNAs. For example, when polyA⁺ RNA from erythroid cells of the embryonic cell line (naturally rich in ferritin mRNA), was mixed with reticulocyte extracts, containing endogenous IRP, the ferritin mRNA could not form polyribosomes, in contrast with globin mRNA (Dickey et al., 1988). On the other hand, in wheat germ extracts, where the IRP was absent, both ferritin and globin mRNAs formed polyribosomes (Dickey et al., 1988). Thus rabbit reticulocyte extracts mimic whole cells low in iron (IRP-IRE interactions prevent ribosome binding), while wheat germ extracts mimic iron-treated cells (no IRE-IRP interaction). When the ferritin or eALAS mRNA distribution is examined in extracts of control and iron-treated cells that contain endogenous IRP, the ferritin mRNA distribution shifts to polyribosomes (Zahringer et al., 1976; Bhasker et al., 1993; Melefors et al., 1993).

Purified IRP can be added to wheat germ extracts to negatively regulate translation of ferritin mRNA, eALAS mRNA and chimeras of the IRE + hGH (Walden et al., 1988,1989; Bhasker et al., 1993; Dix et al., 1993). In fact, the effect of hepatocyte fractions on ferritin mRNA translation in wheat germ extracts was used to purify the IRP based on function (Walden et al., 1988,1989). Co-transfection into yeast of one plasmid encoding hIRP with another chimeric plasmid of the IRE + luc (luciferase) or the IRE + CAT, reproduced the negative regulation of animal cells in an IRP-dependent manner (Oliveira et al., 1993).

Although the IRE-IRP interaction has been shown to negatively regulate translation of mRNAs encoding animal ferritin, hGH, CAT and luc (Dickey et al., 1988; Walden et al., 1989; Dix et al., 1992; Bhasker et al., 1993; Melefors et al., 1993), mRNAs encoding plant ferritin or animal tubulin are not well-regulated by the IRE (Dix et al., 1992; Coulson and Cleveland, 1993; Kimata and Theil, 1994). No pattern for the context-dependent structure/function relationships of the IRE has yet emerged. However, secondary structure predictions show that in the IRE + soybean ferritin chimera, the IRE hairpin loop structure does not form because of downstream secondary structure involving the IRE (Kimata and Theil, 1994).

The IRE, in its native context, folds into the IRP-binding site. In the 5'-UTR of ferritin mRNA, for example, only the IRE appears to be protected by the IRP, although conformational changes occur in the FL when the IRP is bound to the IRE (Harrell et al., 1991). In addition, a 'toeprint' of the IRP (termination of the reverse transcription reaction on ferritin mRNA complexed with the IRP) occurs at the 3'-end of the IRE (Harrell et al., 1991).

Positive control of TfR synthesis: IRE-IRP binding

Positive control of mRNA is enhancement of protein synthesis. The IRP-NB/S-IRE interaction controls TfR expression positively, since stabilization of the mRNA leads to increased TfR synthesis. Stabilization of TfR mRNA has been observed in normal or transfected cells during iron deficiency or cell proliferation (Casey et al., 1988; Mullner and Kuhn, 1988; Koeller et al., 1989; Rothenberger et al., 1990; Cairo and Pietrangelo, 1994). In contrast with the RB/Ts-IREs of ferritin and eALAS mRNAs, there are no cell-free extracts currently available in which to study IRE-dependent mRNA stabilization. However, the conditions recently developed to study plant mRNA stability *in vitro* (Byrne et al., 1993) or *c-myc* mRNA half-life *in vitro* (Herrick and Ross, 1994) may be useful for studying TfR mRNAs as well.

The fragment of the TfR mRNA that includes NB/S-IREs b, c and e in the 3'-UTR is required for TfR mRNA stabilization, determined by deletion analysis of constructs analysed by transfection (Mullner and Kuhn, 1988; Casey et al., 1989). The minimum length of the TfR mRNA 3'-UTR, which contains the full iron-responsive stability element, is approx. 250 nucleotides and includes several AU-rich regions and several single sites between the NB/S-IREs (Mullner and Kuhn, 1988).

Affinity for the IRP was found to be NB/S-IRE-b > NB/S-IRE-e > NB/S-IRE-a = NB/S-IRE-d > NB/S-IRE-c, using *in vitro* gel-retardation studies with individual IREs (Koeller et al., 1989). However, both NB/S-IRE-b (high-IRP affinity) and NB/S-IRE-c (low-IRP affinity) are required for regulation (Muller and Kuhn, 1988). The apparent differences between the IREs required for regulation and the relative affinity of the NB/S-IREs for the IRP indicates that IRP binding is not the only factor in NB/S-IRE function. When the IRP interaction with NB/S-IREs in the 3'-UTR is disrupted by excess cellular iron, TfR mRNA is degraded, indicating that resistance to nuclease degradation was IRP dependent (Figure 4).

Since the RB/Ts-IREs in ferritin mRNAs can assume several, alternative conformations (Wang et al., 1990,1991; Dix et al., 1993), it is possible that the NB/S-IREs can also assume alternate conformations. The requirement for NB/S-IRE-b, and -c, in spite of the relatively low IRP/IRE-c affinity (Koeller et al., 1989), may be explained by the ability of NB/S-IREs b and c to form alternate conformations based on the palindromic repeat (AUUAU/UAAUA). Unfortunately, there have as yet been no

Figure 4 Schematic diagram of the different protein-IRE interactions

Left: RB/Ts (ribosome binding/translation)-IREs binding initiation factors. Centre: either RB/Ts or NB/S binding IRP. Right: NB/S (nuclease binding/stability)-IREs binding nuclease.

Scheme 1 Predicted secondary structures of the 3'-UTR stability element in TfR mRNA

In the structure predicted on the left, the hairpin loop sequences of IRE, the CAGUGU/C sequences (indicated by italics) are actually part of a stem; the two sets of palindromic, AU-rich (AUUAU/AUAAU) sequences (indicated by overlines) of each IRE are paired between IREs

structural studies of the NB/S-IREs comparable with RB/Ts-IREs to test such a hypothesis. There is, however, a secondary structure prediction of the 3'-UTR stability element in TfR mRNA, published before the recognition of the IRE structures in the 3'-UTR (see left-hand side of Scheme 1) which could explain the requirement for NB/S-IREs b and c.

Phylogenetic conservation in the 3'-UTR of mammalian and avian TfR mRNAs of the two sets of palindromic sequences able to form two short hairpins or one long hairpin seems unlikely to be a coincidence. In fact, the non-IRE (long hairpin) structure is the secondary structure predicted by default parameters for the folding programs (Mullner and Kuhn, 1988); the five IREs are only formed when the program is set to keep the CAGUG single-stranded.

It is easy to imagine that a fairly mobile equilibrium exists between the two structures, the five short hairpins and the long hairpin. Binding of the IRP by any IRE such as high-

affinity NB/S-IRE-a or -b, could, for example, then shift the equilibrium toward the five IRE, short hairpin structures.

Positive control of ferritin synthesis: IRE-eIF binding

IRP binding to IREs regulates protein synthesis negatively, if the IRE is an RB/Ts type (ferritin and eALAS mRNA), or positively, if the IRE is an NB/S type (TfR-IREs a, b, c, d and e) (Figure 3). However, even before the identification of the IRE, positive control of ferritin synthesis had been demonstrated (Schaefer and Theil, 1981; Shull and Theil, 1982).

The first observation of positive control of ferritin synthesis was the effect of elongation inhibitors on ferritin synthesis in iron-treated erythrocytes (Schaefer and Theil, 1981). In the experiments, iron induced ferritin synthesis approx. 50-fold in erythrocytes, relative to other proteins; under such conditions it is now known that the IRP inhibition of translation is prevented. Addition of low concentrations of the elongation inhibitors cycloheximide or emetine reversed the iron-induction of ferritin synthesis relative to other proteins. The explanation of the effect of elongation inhibitors on iron-induced ferritin synthesis relates to the fact that in the absence of the IRP ferritin mRNA appears to bind initiation factors and ribosomes more efficiently than other mRNAs [as later demonstrated in cell-free extracts (Shull and Theil, 1982, 1983)]. Slowing elongation of all mRNAs by the inhibitors compensated for the faster rate of initiation of translation by ferritin mRNA, which made the relative rate of ferritin synthesis decrease.

To demonstrate that ferritin mRNA actually is a more efficient mRNA than other mRNAs in erythroid cells, polyA⁺ RNA (protein-free) isolated from either iron-treated or normal erythrocytes, was added to wheat germ extracts (no IRP) at increasing concentrations (Shull and Theil, 1982). Ferritin synthesis continued to increase in proportion to RNA added at concentrations above those in which the other mRNAs had saturated the translation extract. The effect was the same whether or not the RNA came from iron-treated cells. Thus, at high mRNA

Table 1 Effect of concentration of eIF relative to mRNA on translation of ferritin mRNA relative to globin mRNA

A functional increase in $[eIF]/[mRNA]$ was achieved for whole cells, by incubating erythrocytes of the bullfrog embryonic cell line in medium containing elongation inhibitors (cycloheximide, emetine); the cells were isolated from animals treated with iron which eliminated the effect of IRP binding. Elongation inhibitors increase the relative rate of initiation of less efficient mRNAs (in this case mostly globin) compared with more efficient mRNAs (ferritin). With polyA⁺ and *in vitro* transcripts (IVT) a change in the $[eIF]/[mRNA]$ ratio was achieved by adding more mRNA to the protein-synthesizing cell-free extracts. Data for whole cells is from Schaefer and Theil (1981), for polyA⁺ RNA from Shull and Theil (1982), and for the IVT from Dix et al. (1992).

Type of mRNA	Ratio of ferritin/globin synthesis	
	$[eIF] > [mRNA]$ (no competition in mRNA mixtures)	$[eIF] < [mRNA]$ (no competition in mRNA mixtures)
Relative efficiency of mRNA translation in natural mRNA mixtures		
Endogenous (suspensions of embryonic erythrocytes) [†]	0.70	1.6
Poly A ⁺ RNA (from embryonic erythrocytes) ^{*†}	0.66	1.5
Relative efficiency of mRNA translation in separate reactions		
<i>In vitro</i> transcripts of ferritin mRNA capped [‡]		
+IRE	1.6	1.2
-IRE	0.2	0.2

* PolyA⁺ RNA from reticulocytes of the bullfrog embryonic line (tadpoles) is 65% globin mRNA and 5% ferritin mRNA (Shull and Theil, 1982).

[†] Differences between $[eIF] < [mRNA]$ and $[eIF] > [mRNA]$ are significant; $P > 99\%$.

[‡] Equimolar concentrations of ferritin *in vitro* transcripts with and without the IRE were compared with natural globin (rabbit reticulocyte polyA⁺ RNA = 90% globin) using individual reaction mixtures for each RNA; thus, competition will not be observed. However, ferritin (+IRE) is always translated significantly faster than globin (Dix et al., 1992). Note also the effect of the IRE deletion on translation rate of ferritin mRNA (data from Dix et al., 1992).

concentrations, when eIFs and ribosomes became limiting, ferritin mRNA bound a disproportionately higher amount of eukaryotic initiation factors (eIFs) and ribosomes than the other mRNAs.

The combination of release from negative control plus positive control appears to be necessary to explain the levels of ferritin synthesis observed in iron-treated erythroid cells (Shull and Theil, 1983). However, in cells with rapid rates of protein synthesis (excess eIFs and ribosomes), such as hepatocytes and fibroblasts, rates of iron-induced ferritin synthesis reflect only the change in the IRE-IRP interaction and do not appear to depend on the competitiveness of ferritin mRNA (Shull and Theil, 1983; Walden and Thach, 1986). Table 1 compares, using the same parameters, the results of erythrocyte ferritin synthesis at various effective concentrations of eIF relative to mRNA in erythroid cell suspensions, wheat germ extracts directed by erythroid polyA⁺ RNA and in wheat germ extracts directed by capped *in vitro* transcripts (IVT) of DNA encoding wild-type and mutant erythrocyte ferritin mRNAs.

The IRE is the sequence of ferritin mRNA responsible for high translational efficiency (Table 1; Dix et al., 1992). Thus, in analogy to DNA elements such as the glucocorticoid response element (Diamond et al., 1990), the ferritin IRE encodes both negative control, expressed by IRP binding, and positive control, expressed by efficiency of eIF binding. The identity of the IRE region as the positive control element was demonstrated by comparing translation of IVTs encoding the wild-type ferritin mRNA with that of ferritin mRNA with the first 60 nucleotides deleted (the IRE + the 5'-FL + the 10-nucleotide leader between the FL and the cap) (Dix et al., 1992). Each mRNA was translated at equal concentrations in individual reactions with wheat germ extract and the proteins synthesized were compared. Globin was used as a control because it was the predominant mRNA in the intact erythroid cells and in the polyA⁺ RNA studied. The full-length ferritin IVT was translated faster than globin except when the IRP was present (Dix et al., 1992), reproducing the observations in cell suspensions and with polyA⁺ mRNA from erythroid cells in wheat germ extracts. However,

deletion of the IRE region decreased the rates of translation to one-fifth of that of globin, independent of the presence or absence of the IRP and independent of the ratio of eIF/mRNA. Whether the IRE in eALAS mRNA will also exhibit positive control has yet to be tested (Bhasker et al., 1993).

PROTEINS WHICH INTERACT WITH THE IRE

IRPs

IRPs have been characterized from humans, rabbits and rats (Rouault et al., 1990; Patino and Walden, 1992; Yu et al., 1992). The phylogenetic sequence conservation among the cloned cDNAs encoding the RNA-binding proteins is very high (approx. 93%); two members of the IRP family are known, IRP-1 and IRP-2, based on the existence of similar but distinct cDNAs (Rouault et al., 1990). In addition, heterogenous protein-RNA complexes form with cell extracts and, after u.v.-cross-linking, are attributed to IRP-1 and IRP-2 (Leibold et al., 1990). Finally, IRPs can be phosphorylated under physiological conditions; the peptide phosphorylated in IRP-1 is completely conserved in IRP-2, suggesting that both proteins can be phosphorylated (Eisenstein et al., 1993). IRP proteins which recognize the IRE have been observed in both vertebrates and invertebrates (Rothenberger et al., 1990).

Expression of the IRP appears to be independent of cellular iron concentrations (Yu et al., 1992). However, the levels of the IRP mRNA or mRNA-binding activity vary depending on the tissue and the state of differentiation (Testa et al., 1991; Yu et al., 1992; Patino and Walden, 1992). Tissue-specific differences in IRP expression could be related to concentration of TfR mRNA, ferritin mRNA, or both. The fact that the most abundant levels of IRP mRNA are in kidney and liver, two tissues relatively rich in ferritin mRNA, suggests that IRP mRNA concentrations reflect more closely ferritin mRNA concentrations than TfR mRNA concentrations.

If iron does not regulate the levels of IRP mRNA and protein, what is the signal which transduces cellular iron concentrations to change ferritin and TfR synthesis? A definitive answer is not

Table 2 Relief of translational competition by ferritin mRNA at saturating mRNA concentrations in wheat germ extracts, by adding partially purified initiation factors

Conditions for protein synthesis and specific immunoprecipitation of ferritin are described in Shull and Theil (1982). Partially purified initiation factors from wheat germ were kindly provided by J. Ravel. Wheat germ extracts were selected for the analysis, since rabbit reticulocytes (RR) contained a *trans* factor which specifically inhibited ribosome binding to ferritin mRNA (Dickey et al., 1988); the factor is now called the IRP and is a cytoplasmic apoconitase (Haile et al., 1992). The high ferritin mRNA concentrations required to saturate endogenous IRP in RR, cannot be achieved effectively by adding increasing amounts of polyA⁺ mRNA since the ferritin mRNA concentration is only 5% of the total; saturation of endogenous IRP can be observed using *in vitro* transcripts at > 4 nM in RR extracts (Dix et al., 1992).

Ratio of [eIF]/[mRNA]	Total protein synthesis (65% globin)* (10 ⁴ × d.p.m./30 min)	Ferritin synthesis (immunoprecipitable) (10 ⁴ × d.p.m./30 min)
[eIF] < [mRNA]	11.4 ± 2.1	1.35 ± 0.25
[eIF] > [mRNA] (added eIF-4B 69 µg/ml)	18.3 ± 2.4†	1.74 ± 0.56

* Functional polyA⁺ RNA from reticulocytes of the bullfrog embryonic line (tadpoles), composed mainly of globin mRNA (60%) and ferritin mRNA (5%) (Shull and Theil, 1982), was translated under conditions saturating for globin mRNA but not ferritin mRNA (40 µg/ml). The conditions have been described previously (Shull and Theil, 1982).

† Significantly different from the value with endogenous initiation factors at the 99% confidence level. The results are the average of four experiments with two different polyA⁺ RNA preparations.

yet available. However, an intriguing interrelationship between available iron and IRP binding has been observed. The IRP is an apoconitase in the cytoplasm (Rouault et al., 1991; Kennedy et al., 1992). Aconitases are a family of proteins which catalyse the interconversion of isocitrate and citrate and which require an Fe-S cluster for catalytic activity (Beinert and Kennedy, 1989); among the peptides analysed, the homology between cytoplasmic aconitase and IRP-1 is essentially 100% (Kennedy et al., 1992).

When cytoplasmic aconitase is freed of its Fe-S cluster, the apoconitase binds the IRE (Haile et al., 1992), which has been interpreted to mean that the Fe-S cluster is the iron regulatory signal. The problem with that idea is that little is known about either the biosynthesis or the reversible biosynthesis of Fe-S clusters in any proteins. To confound the problem, determining the nature of low-molecular-mass iron in cells, and determining the pathway iron follows between release from transferrin in the endosome and the appearance of iron in haem or ferritin has eluded several generations of investigators. Thus, while it is clear that an iron-deficient cell will be less likely to synthesize an Fe-S cluster in apoconitase, thereby increasing the RNA-binding activity of the protein, it is not clear whether formation/disruption of the Fe-S cluster in aconitase is the major regulatory signal for IRE-dependent mRNA translation or stability. Since phosphorylation also alters the RNA-protein interaction, apparently independently of the Fe-S cluster (Eisenstein et al., 1993), it is entirely possible that the iron regulatory signal which regulates the IRE-IRP interaction is transduced through a signalling pathway which terminates in altered phosphorylation of the IRP.

Cytoplasmic aconitase has distinctive spectral features, compared with the more extensively characterized mitochondrial aconitase (Kennedy et al., 1992). Further characterization of the cytoplasmic aconitase (Fe-S IRP) may determine whether the distinctive features of the Fe-S cluster have metabolic significance. It is noteworthy that when the IRP/apoconitase was isolated, using inhibition of translation as the analysis (Walden et al., 1990), the amount of protein was only approx. 10% of that of cytoplasmic aconitase and the separation procedure was very different from that required to isolate cytoplasmic aconitase (Patino and Walden, 1989; Kennedy et al., 1992); no iron was found in the IRP preparations. Such observations suggest that the IRP/apoconitase could be separated in the cell from cytoplasmic aconitase and that the potential catalytic activity of the IRP/apoconitase may not relate to its mRNA regulatory activity. The IRP, as with many proteins, appears to have

oxygen-sensitive sulphhydryl groups; the sulphhydryl groups are necessary for IRE binding. Thus the addition of reducing agents to crude extracts can increase the amount of IRE-binding activity (reviewed in Theil, 1990, 1993; Klausner et al., 1993). Only future experiments will clarify the physiological relevance of the unprecedented observation that an RNA-binding protein can bind a cofactor (Fe-S) to lose RNA-binding activity and gain the capacity to catalyse the interconversion of the small, metal-binding organic molecules, citric acid and isocitric acid.

eIFs

The role of eIFs in positive control of ferritin mRNA translation was shown using partially purified eIFs from wheat germ (Table 2). Competition between ferritin and the other mRNAs in the polyA⁺ mRNA from embryonic erythroid cells was relieved by adding exogenous eIFs. When partially purified wheat germ eIF-4B was added to polyA⁺ RNA translated under limiting conditions of eIFs, globin synthesis increased significantly (Table 2). In contrast, ferritin mRNA, which was already maximally engaged with eIFs and ribosomes, showed no significant change in translation when eIF-4B was added. Partially purified eIF-4B from wheat germ is now known to contain large amounts of cap-binding protein. However, earlier studies in cell extracts, using 7-methyl-GMP to bind cap-binding protein, did not differentially affect ferritin mRNA translation (Shull and Theil, 1982). Thus whether it is the cap-binding protein in partially purified eIF-4B which relieved the competition between ferritin and globin mRNAs, or whether it is another factor remains to be investigated in the future.

IRE STRUCTURE

Structure-dependent cleavage by shape-selective metal complexes and proteins as probes of IRE structure

The complex folding and tertiary structure of the IRE makes it relatively inaccessible to bulky protein nucleases. For example, there is only one cleavage site for the single-stranded nucleases RNAases S₁ and T₁ in the 55 nucleotide IRE + FL in spite of the several single-stranded loops predicted from the secondary structure (Harrell et al., 1991). Moreover, there are only a few sites of cleavage in the IRE + FL by the double-stranded nuclease RNAase V₁, in spite of the large number of base pairs predicted in the secondary structure (Harrell et al., 1991). Probes of single-stranded regions of the IRE + FL with small reagents such as

Figure 5 Results of using transition-metal complexes to probe structure/function in ferritin IRE

RNA (either polyA⁺ from embryonic erythroid cells or capped *in vitro* transcript) was mixed with the cleavage reagent, after melting and annealing, and incubated at pH 7 in 40 mM KCl for 5–10 min, usually at 30 °C. Cleavage conditions are established to insure less than one cut per native RNA molecule. Any temperature, ionic strength or pH in the range 4–8 could be used. For Fe-EDTA, CuOP and Fe-bleomycin, free radicals were produced by reducing the metal in the presence of dioxygen. For Rh(phen)₂phi³⁺, free radicals were produced by irradiation with visible light (Chow et al., 1992). With the exception of Fe-EDTA, cleavage reports the binding site of the complex. Since the radical produced by Fe-EDTA is the size of the solvent, cleavage with Fe-EDTA reports on the relative solvent accessibility of different parts of the molecule, defining the 'outside' and 'inside' of the folded RNA structure. Cleavage sites in each reagent are shown on the predicted secondary structure of the ferritin IRE + FL; the IRE is numbered 1–28. Data are taken from: Fe-EDTA (Harrell et al., 1990); OPCu (Wang et al., 1990,1991); Fe-bleomycin (Dix et al., 1993); Rh(phen)₂phi³⁺ (A. R. McKenzie and E. C. Theil, unpublished work). Key to symbols: !, Fe-EDTA; #, OPCu; ##, OPCu and 0.5 mM Mg; +, Fe-bleomycin site induced by mutation of CUC/GAG in FL; *, Rh(phen)₂phi³⁺ site lost by CUC/GAG mutation.

dimethylsulphate (DMS) also gave aberrant patterns. On the one hand DMS skipped bases in the hairpin loop that were adjacent to others which were accessible. On the other hand DMS alkylated certain bases in pairs on the IRE stem, indicating stem distortions at the site. However, DMS was apparently too small to be sensitive to conformational changes caused by changes in the concentration of magnesium (Wang et al., 1990,1991).

Transition-metal complexes have proven to be effective probes of RNA structure that complement traditional protein nucleases and alkylating reagents and are becoming particularly useful in identifying sites of higher-order RNA interactions to target for site-directed mutagenesis (Wang et al., 1990,1991; Harrell et al.,

1991; Dix et al., 1993; Theil, 1994). In general, the organic ligands in the transition-metal complexes bind to specific sites on nucleic acids and the metal serves as a reporter for the binding interaction by producing free radicals which cleave the nucleic acid at, or near, the site of binding. The same chemistry has been exploited for many years in the use of Fe-bleomycin, which cleaves DNA and RNA, as an anti-tumour agent (Carter et al., 1990). Since the transition-metal complexes are intermediate in size between the bulky protein nucleases and the small alkylating reagents, they access sites in the IRE and FL for which no information could be obtained with more classical probes. Moreover, transition-metal complexes are active under a variety of conditions, e.g. temperature, pH and ionic strength, which are useful for probing nucleic acid conformation but which also alter the activity of protein nucleases. Finally, the organic moiety of the transition-metal complexes can be varied in shape and size.

Analysis of sites of higher-order RNA structure recognized and cleaved by transition-metal complexes relies on standard sequencing procedures to detect the sites of cleavage. For example, when the RNA is too long to end-label efficiently, or when the RNA of interest is present in a mixture of RNAs, a specific primer and reverse transcriptase are used to generate a family of radioactive cDNA fragments from the RNA fragments; each size class of cDNA fragment represents the distance from the primer to a specific cleavage site. The mixture of radioactive cDNAs is resolved by electrophoresis in a sequencing gel calibrated by separating a standard sequencing set of four families of dideoxynucleotide fragments; cleavage and alcohol precipitation is carried out late in one day and the cDNA synthesis and gel electrophoresis is completed the next day. Uncut RNA is used as a control to monitor cDNA fragments produced by pausing of reverse transcriptase. When the RNA structure of interest is short enough to be efficiently labelled at the 3'- or 5'-end, fragments produced by transition-metal cleavage can be analysed directly by electrophoresis in acrylamide gels. The gel is calibrated with uncleaved, denatured RNA that has been partially hydrolysed with NaOH or with RNA partially hydrolysed with RNAase T₁.

The use of transition-metal complexes to study RNA has just begun. However, the amount of information already obtained [Table 4 and reviewed in (Theil, 1994)] about higher-order structures of the IRE has been enormously helpful (i) in demonstrating the folding of the IRE, (ii) for understanding the functional effects of site-directed mutation, and (iii) for evaluating the structural significance of the ferritin-specific FL (base-paired flanking regions).

Fe-EDTA, which was initially selected for showing the IRP 'footprint' over the entire IRE, produced an unexpected bonus of information when the reaction of the IRE without the IRP was examined (Harrell et al., 1991). Even in the absence of IRP, regions within the IRE and FL appeared to be protected from cleavage compared with other regions (Figure 5). Activated oxygen radicals produced by Fe-EDTA are about the size of solvent water and cleave both single-stranded and A-helical (Watson-Crick base-paired) RNA equally well. In ribozymes, sites poorly cleaved by free radicals generated by Fe-EDTA and O₂ delineated the solvent-inaccessible interior of the molecule (Celander and Cech, 1991). Thus the heterogeneity of the cleavage of the IRE and FL indicates that both the IRE and the FL are folded into a structure with a solvent-inaccessible interior (Harrell et al., 1991).

1,10-Phenanthroline-Cu (CuOP), in contrast with Fe-EDTA, is a larger complex that binds directly to RNA at distortions in the A-helical double-stranded RNA or to single-stranded regions (Murakawa et al., 1989). By annealing ferritin mRNA in solutions

Table 3 Comparison of the structural and functional properties of RNA and proteins

Property	RNA	Proteins
Structure		
Backbone	Single-stranded	Single-stranded
Linkage	Polyester	Polyamide
Side chains	Heteroaromatic	Heteroaromatic, aromatic, aliphatic, ionic
Folding forces	Hydrogen bonds, ionic interactions, hydrophobic interactions	Hydrogen bonds, ionic interactions, hydrophobic interactions
Function	Recognition, structure and information	Recognition and structure
	Catalysis	Catalysis

containing different concentrations of magnesium, before cleaving with CuOP, it was observed that the IRE + FL could fold into three different conformations (Figure 5). In the absence of magnesium, bases in the stem flanking the internal loop bound CuOP. When the magnesium concentration was 0.5 mM, regions in the upper stem and hairpin loop bound CuOP and, at 5 mM Mg, no sites in the IRE or FL were in a sufficiently distorted conformation to bind CuOP (Wang et al., 1990,1991). The relationship of each conformation and the kinetics of switching among conformations to positive and negative control of ferritin mRNA translation is not yet known. However, it is possible to imagine that the IRP regulator binds preferentially to one conformation and eIFs bind preferentially to another conformation, with competition for binding one or other of the proteins being a crucial component of regulation in the cell.

Fe-bleomycin and Rh(phen)₂phi³⁺ are bulky complexes which appear to target higher-order interactions (Carter et al., 1990; Chow et al., 1992). Both have been useful in understanding the effect of FL mutation on IRE function. For example, when the conserved triplet of base pairs in the ferritin IRE (CUC/GAG) (Figure 3) were disrupted by mutation (GAG/GAG), the IRP still bound, but the effect on translation rate was decreased 2.5-fold; restoration of the base pairs by a second set of (GAG/CUC), a combination which also occurs in nature (Figure 2) restored normal regulation (Dix et al., 1993). In the GAG/GAG FL mutation, a new Fe-bleomycin site appeared in the IRE, 20 nucleotides downstream from the mutation (Figure 5), which emphasized the importance of the FL in enhancing the formation of a particular IRE conformation (Dix et al., 1993). Moreover, the results showed that even if the IRP can bind, as it does to the GAG/GAG mutant, the RNA-protein complex is not as effective as the wild type in inhibiting ribosome binding. Rh(phen)₂phi³⁺ cleaved a single site in the IRE + FL, adjacent to the CUC/GAG pairing, which was disrupted in the GAG/GAG mutant but which was restored in the GAG/CUC revertant (A. R. McKenzie and E. C. Theil, unpublished work). The results show that an unusual higher-order structure exists at the site of the triplet of base pairs which enhances the functional efficiency of the IRE-IRP complex and which was disrupted in the GAG/GAG mutation (Figure 5).

Transition-metal complexes which cleave nucleic acids have mostly been relegated, to date, to the single task of monitoring protein-binding sites ('footprints') on DNA and RNA. The potentially more important exploitation of the transition-metal complexes to identify sites of unusual structure and function as targets for site-directed mutagenesis has been little used, even though only micromolar amounts of RNA are needed; in contrast millimolar amounts of RNA are needed for n.m.r. analysis. Exploring the folding of complex and previously intractable RNA structures, such as conserved leader sequences in viruses (Cullen, 1992), with shape-selective transition-metal complexes

Table 4 Comparison of reactivity of the IRE in the synthetic oligoribonucleotide (*n* = 30) and in ferritin mRNA

Probe	30-mer	Ferritin mRNA
RNAase S ₁ (single-stranded regions)*	G15, U16	G15, U16
RNAase V ₁ (double-stranded regions)†	U9	U9, C10
1,10-phenanthroline-Cu	G25	G25, G26
Fe-EDTA‡	U5, G6, C7, G25, G26	U5, G6, C7, G25, G26

* Potential number of sites based on predicted secondary structure = 10.
† Potential number of sites based on predicted secondary structure = 20.
‡ Potential number of sites based on predicted secondary structure = 30.

could easily identify unusual, higher-order structures; the lure of such data should be irresistible in the future.

N.m.r. spectroscopy as a probe of IRE structure

The similarity of the forces which stabilize three-dimensional folding of both proteins and RNA can produce such specific shapes that whether IRPs recognize and bind IRE or whether IRE recognizes and binds IRPs is a moot point. To emphasize the similarities between RNA and protein structure, the properties are reviewed in Table 3. Magnesium-dependent changes in conformation of the ferritin IRE (Wang et al., 1990,1991) and the effect of protein binding on the conformation of the ferritin FL (Harrell et al., 1991), are probably analogous to allosteric changes in proteins.

Determining the distinctive three-dimensional structure of the IREs, indicated by probing with transition-metal complexes (Table 3), has begun with the n.m.r. spectral analysis of the ferritin IRE in the absence of Mg. Conditions under which the 30-mer corresponding to the IRE sequence assumes a single conformer in solution were selected based on the conditions used for probing full-length mRNA with the transition-metal complexes (Figure 5). The similarity of conformation of the 30-mer to the IRE, in the context of the full-length ferritin mRNA, could be confirmed using CuOP and Fe-EDTA as well as RNAases V₁ and S₁ (Table 4); structural conclusions based on the 30-mer are thus relevant to the IRE in full-length mRNA (Table 4).

The folded structure of the IRE has remarkable temperature stability. For example, the *t_m* of the major helix/coil transition is 52 °C, with a minor transition at 73 °C. The stability is apparently maintained by multiple interactions of imino protons based on the existence of as many as seven nuclear Overhauser effects (nOes) for the resonance of a single imino proton resonance

ranging over field frequencies from 14.3 to 6.59 p.p.m. When a single base substitution is made in the hairpin loop, the temperature stability decreases and the major helix/coil transition occurs at 42 °C (Sierzputowska-Gracz et al., 1994).

PERSPECTIVES: THE IRE AS A MODEL FOR REGULATION, STRUCTURE AND DRUG DESIGN

IREs are the first family of eukaryotic mRNA *cis* elements to be identified. Natural, conserved variations in IRE sequence as well as position in the regulated mRNAs make them a paradigm for RNA structure and function.

The regulatory function of the IRE family in mRNA is coordinating the synthesis of several metabolically related proteins (ferritin for iron storage, TfR for iron uptake, and eALAS for haem biosynthesis in erythrocytes). IREs regulate mRNA translation (RB/Ts-IREs) or stability (NB/S-IREs), in response to changes in environmental iron by binding a variety of proteins; the RNA-binding proteins are generic (nucleases or initiation factors) or specific (the IRP). IRP interactions with RB/Ts-IREs inhibit translation and ribosome binding; when the IRP is not bound, the RB/Ts-IRE in ferritin mRNAs binds eIFs unusually efficiently, making the ferritin IRE both a negative translation control element (IRP binding) and a positive translation control element (enhanced eIF binding). IRP interactions with NB/S-IREs stabilize mRNA and prevent nuclease binding; NB/S-IREs are positive translation control elements, albeit indirectly. Experimental analysis of IRE function *in vitro*, combined with engineering either mRNA, regulator protein (IRP), or both, allow investigation of eukaryotic mRNA structure and function at a level of molecular detail that is so far unique.

IREs are found in animals. During evolution, only the coding sequence of ferritin was conserved in higher plants. The regulation of ferritin in plants is independent of the IRE, which functions poorly even when it is introduced experimentally. Possibly transport of ferritin to plastids had a greater selective advantage for plants than having an IRE.

IRP regulators, the proteins which form complexes with members of the IRE family, appear to be a family themselves. Whether there is a match between isoforms of IREs in RNA and isoforms of IRPs, the RNA-binding protein, remains to be examined in the future. Modification of IRP-1 by an Fe-S cluster or by phosphorylation modulates IRE interactions, indicating the outline, in part, of the iron-induced signal-transduction pathway. When the IRP is bound to an Fe-S cluster, rather than to RNA, aconitase activity appears. Whether the aconitase activity of the IRP-Fe-S relates to the iron-chelating properties of the substrates citrate and isocitrate, or is merely a remnant of IRP evolution is not currently known.

IRE structure is a hairpin with a terminal hexaloop. The hexaloop and the five-base-pair upper stem is common to all IRE family members. However, the internal loop/bulge and lower stem of the hairpin are variable for each type of IRE, suggesting a composite of the iron-specific RNA regulatory structure fused to a generic mRNA regulatory element. For example, RB/Ts-IREs appear to be a composite of the specific hairpin hexaloop fused to an ordinary, double-stranded, 5'-UTR to confer both translation inhibition and specificity of IRP binding. Similarly, NB/S-IREs appear to be a composite of the specific hexaloop hairpin fused to an AU-rich stability element in the 3'-UTR. In the case of the ferritin IRE, which encodes both negative and positive control, base-paired FL with a conserved triplet of base pairs modulate the activity of the IRE.

Extensive folding, long-range interactions and conformational flexibility appear to define the specific three-dimensional structure

in the IRE. In addition to site-directed mutagenesis, n.m.r. spectroscopy and classical structure probes, shape-selective transition-metal complexes which act as nucleases of various sizes (such as CuOP, Fe-bleomycin, and Fe-EDTA) have been enormously helpful in defining regions of the IRE of unusual structure and focusing mutagenesis and n.m.r. studies. Use of transition-metal complexes to probe long, intractable viral sequences should be particularly useful in the future.

Rational drug design, targeted to specific RNA structures, is a potential application of the investigation of IRE structure with transition-metal complexes and n.m.r. Targeting the three-dimensional features of RNA exploits the increased structural specificity of RNA compared with DNA, and the relatively low concentration of RNA compared with protein. Obvious RNAs for future studies are viruses, but mRNAs encoding oncogenic proteins are another group of candidates. The novelty of IRE structure and function has attracted a great deal of attention in fundamental physiology, and structural and molecular biology. But as in all basic studies, new practical possibilities are also being illuminated.

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