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The interaction between the iron-responsive element binding protein and its cognate RNA is highly dependent upon both RNA sequence and structure

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ABSTRACT

To assess the influence of RNA sequence/structure on the interaction RNAs with the iron-responsive element binding protein (IRE-BP), twenty eight altered RNAs were tested as competitors for an RNA corresponding to the ferritin H chain IRE. All changes in the loop of the predicted IRE hairpin and in the unpaired cytosine residue characteristically found in IRE stems significantly decreased the apparent affinity of the RNA for the IRE-BP. Similarly, alteration in the spacing and/or orientation of the loop and the unpaired cytosine of the stem by either increasing or decreasing the number of base pairs separating them significantly reduced efficacy as a competitor. It is inferred that the IRE-BP forms multiple contacts with its cognate RNA, and that these contacts, acting in concert, provide the basis for the high affinity of this interaction.

INTRODUCTION

The expression of two proteins responsible for cellular iron homeostasis, ferritin and the transferrin receptor (TfR), are coordinately regulated at the post-transcriptional level by the intracellular iron (see 1 and references therein). When iron is abundant, ferritin synthesis rises thereby promoting the sequestration of excess intracellular iron, while TfR expression falls resulting in less iron acquisition from the cell's exterior. When the cell is deprived of iron, the opposite occurs—TfR synthesis increases and ferritin synthesis decreases.

The iron-dependent regulation of these two proteins is mediated by structurally related RNA elements found in their mRNAs. These elements, termed iron-responsive elements (IREs) are moderately stable stem-loop structures of approximately 30 nucleotides. In ferritin transcripts, a single IRE is found in the 5′ untranslated region (UTR) whereas in the TfR transcript, five IREs are found in the 3′UTR. The IREs of both transcripts interact with a cytosolic IRE-binding protein (IRE-BP). In the case of ferritin regulation, the interaction of the IRE-BP with the transcript inhibits translation, most likely through an inhibition of translation initiation (2). In the case of TfR regulation, interaction(s) with the IRE-BP results in specific inhibition of the degradation of the TfR mRNA by functionally inhibiting a rapid turnover determinant located within the 3′UTR of the mRNA in the region of the transcript's five IREs (3).

The IRE-BP displays high affinity for the IRE of ferritin mRNA (4–6). All five of the individual IREs of the TfR mRNA are capable of interacting with the IRE-BP although with differing apparent affinities (7). A consensus IRE structure has been inferred from naturally occurring IREs in known ferritin and TfR mRNAs (see Figure 1A). Previous mutational data suggest that the hairpin stem-loop conformation of the IRE is necessary for function, since disrupting base pairing along the upper stem reduces binding of the IRE-BP to the IRE in gel retardation assays whereas restoring basepairing to a non-native sequence restores binding (5, 6). Sensitivity of IRE containing RNAs to chemical and enzymatic nucleases in vitro also support the stem-loop conformation of ferritin IREs (8–10) and of TfR IREs (11).

In the current study, we focus on the sequence of the IRE and expand upon previous insights regarding structure-function requirements for the motif. To determine sequence and structural requirements for the IRE/IRE-BP interaction, we synthesized twenty eight individual RNAs and tested each as a competitor for RNA corresponding to the native ferritin H chain IRE in a gel retardation assay. RNAs with substitutions for and deletions of the nucletides in the loop of the IRE and at the unpaired cytosine residue characteristic of the IRE stems were examined as were RNAs having changes in the size of the hairpin·s loop and the length of the stem.

MATERIALS AND METHODS

Lysate preparation

Cytosolic lysates were prepared from frozen pig liver as previously described (12). The protein concentration of the lysate was 13.6 μg/ml in a buffer consisting of 150 mM KCl, 1.5 mM $MgCl_2$, 20 mM tris hydrochloride (pH 7.4), 0.5 mM dithiothreitol, 10 μg of leupeptin per ml, and 25 μM

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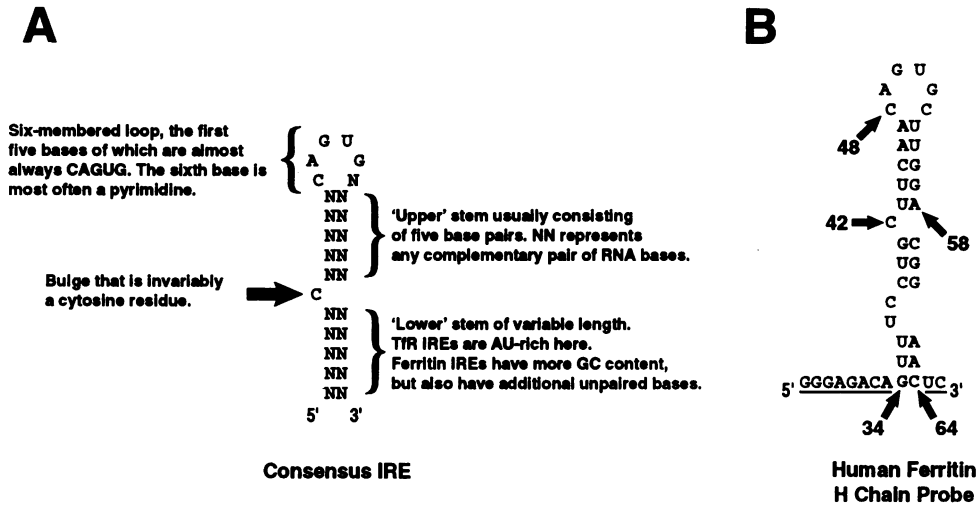

Figure 1. The structure of IREs. **A.** Consensus IRE based on the IREs of known ferritin and TfR mRNAs (see Klausner et al., 1993 and references therein). **B.** Sequence of the the ferritin H chain probe used in gel retardation assays. The underlined nucleotides are not in the native ferritin IRE; numbering of bases is from the transcription start site of the human ferritin H chain mRNA (29).

p-nitrophenyl-*p'*-guanidinobenzoate. Protein determination was by the BCA method (Pierce). Aliquots of lysate were stored at −80°C, and prior to use as a source of IRE-BP in gel retardation assays, lysates were pretreated for approximately 20 minutes at room temperature with Inhibit-Ace (5 Prime → 3 Prime) by adding 20 μl of lysate to 160 μl of Inhibit-Ace (0.5 U/μl) and 220 μl of binding buffer consisting of 40 mM KCl, 40 mM tris (pH 7.4), 5% glycerol, and 0.01% bromphenol blue. To this mixture were added 40 μg of yeast tRNA Gibco-BRL) in a volume of 5 μl.

RNA preparation

RNA transcripts representing the native human ferritin heavy chain IRE and twenty-eight altered RNAs were synthesized by in vitro transcription of synthetic DNA templates according to the method of Milligan et al. (13). The sequence of the DNA molecule used to synthesize the ferritin IRE probe was 5'G-AGTTCCGTCCAA***GCACTG***TTGAAGCAGGAAACTCTCT-CCCTATAGTGAGTCGTATTA3'. This oligonucleotide and the related oligonucleotides used as templates to produce altered RNAs were synthesized on an Applied Biosystems oligonucleotide synthesizer and purified on a urea 20% polyacrylamide gel. Full length oligonucleotides were visualized by UV shadowing and then excised and eluted in 0.1% sodium dodecyl sulfate overnight. The eluates were extracted with phenol-chloroform and ethanol precipitated to recover the DNA. The sequence in boldface type above corresponds to the region expected to form the loop of the IRE hairpin (in the RNA, 5'-CAGUGC-3'). The underlined sequence represents the T7 RNA polymerase promoter and was double stranded in the transcription reaction which contained an oligonucleotide complementary to this region (13). The RNA sequence of the IRE probe is shown in Figure 1B in its hairpin structure.

The native ferritin IRE was labeled with [α-^{32}P]-CTP (Amersham) to a specific activity of approximately 24,000 cpm/ng. The RNAs used to compete with the [^{32}P]IRE probe in gel retardation experiments were trace-labeled with [5,6-^{3}H]-UTP (Amersham) to a specific activity of

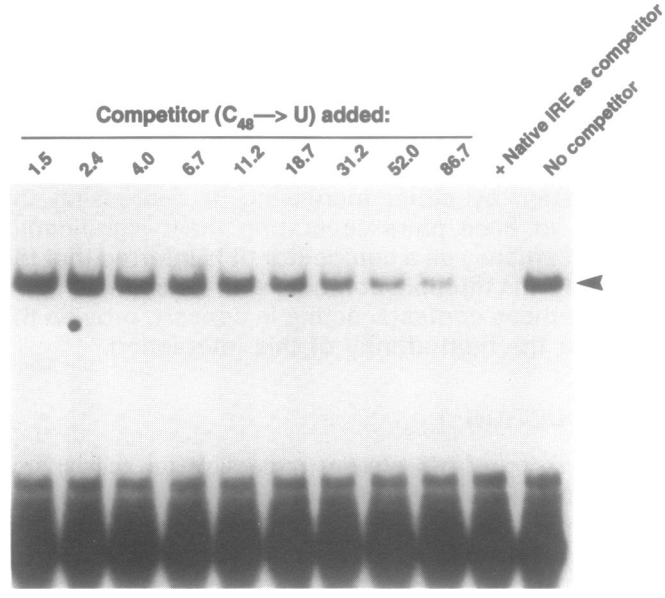

Figure 2. Gel retardation assay and competition with altered RNA. Gel retardation assay with 1 ng ^{32}P-labeled ferritin H chain IRE probe was performed as described under Materials and Methods. The indicated amounts (ng) of ^{3}H-labeled competitor (C_{48}→U) were added. Unbound probe migrates at the bottom of the gel and the IRE/IRE-BP complex is retarded in the gel (arrow). The inclusion of ^{3}H-labeled native IRE (240 ng) completely eliminated the ^{32}P-labeled complex.

6.0−8.5×10^{3} cpm/ng (depending on the number of uridine residues in the particular RNA). Labeled RNAs were purified essentially as described (4). RNA was visualized by autoradiography in the case of ^{32}P-labeled IRE and by UV shadowing in the case of ^{3}H-labeled competitor RNAs.

Following purification, RNAs were resuspended in DEPC-treated water and RNA concentrations were measured by scintillation counting. The ^{32}P-labeled RNA was stored at 4°C, and the ^{3}H-labeled RNA was stored at −20°C. To dissociate

potential intramolecular interactions formed during storage, RNAs were heated to 65°C for five minutes and then chilled in an ice bath prior to use in gel retardation assays. Following each use of ^{3}H-labeled RNAs in competition experiments, these RNAs were analyzed by nondenaturing 12% polyacrylamide gel electrophoresis and ethidium bromide staining to ensure that the RNAs had not been degraded in storage.

Competition experiments

Binding affinities of altered RNAs were determined by measuring their efficacy in competing for the formation of the ^{32}P-labeled IRE/IRE-BP complex. Gel retardation assays were used to determine the quantity of bound ^{32}P-labeled complex in each competition. Gel retardation assay mixtures were prepared in 20 μl reaction volumes. The ^{3}H-labeled competitor RNAs were serially diluted in 10 μl of DEPC-treated water. Approximately 1 ng ^{32}P-labeled native IRE diluted in 5 μl binding buffer was added. Subsequently, 5 μl of the lysate preparation (3.2 μg total protein) was added. These conditions were chosen based on preliminary experiments that demonstrated that 1 ng ^{32}P-labeled IRE was in sufficient excess such that larger quantites of ^{32}P-labeled IRE did not increase the intensity of the bound fraction of RNA in gel retardation experiments. The quantities of ^{32}P-labeled native IRE and ^{3}H-labeled competitor used in the competition experiments were determined immediately prior to each experiment by scintillation counting.

Gel retardation assays were performed on 0.5×TBE, nondenaturing, 8% polyacrylamide gels with electrophoresis for 90 minutes at 180 volts. The gels were subsequently dried and the amount of ^{32}P-labeled RNA-protein complex in each competition was determined on a Molecular Dynamics Phosphorimager using ImageQuant™ software. Backgrounds were determined by including a lane in which 240 ng ^{3}H-labeled native IRE was used as a competitor. Inhibitory constants were calculated by dividing the amount of altered RNA competitor needed to give a specific level of competition by the amount of ^{3}H-labeled native IRE observed to give the same level of competition. Because the amount of unbound ^{32}P-labeled IRE is almost constant (changing by less than 5%) throughout each competition, the inhibitory constant calculated in this manner is also equal to the IC_{50}, the quantity of competitor needed to inhibit the ^{32}P-labeled RNA-protein complex to an amount half that seen without competitor present (14). For ease of comparison the IC_{50} values are expressed in units of ng/20μl reaction volume since 1ng/20μl of ^{32}P-labeled IRE was used. Thus the IC50 values of each competitor is a direct indicator of how well the affinity of the competitor for the IRE-BP as compared to the native IRE (i.e. a competitor with an IC50=20 interacts with the the IRE-BP with a 20-fold lower affinity than does the native IRE). Each competition experiment involved a titration of a competitor RNA over several points so that competition in the range of 30−70% were attained. Typically the three points closest to 50% inhibition from each competition were used to calculate the IC_{50} for that competition. The IC_{50} for each mutant IRE was calculated as the average of the IC_{50} obtained from two to four different competition experiments.

RESULTS AND DISCUSSION

To determine the effect of alteration in the sequence/structure of an IRE on interaction with the IRE-BP, twenty eight RNAs were synthesized and each was independently assessed for its ability to compete with a ^{32}P-labeled IRE probe in a gel retardation assay. Data were analyzed using the method of van Zoelen (14) based on an equivalent competition principle for comparison of nonidentical ligands in interaction with a common receptor. Competition analysis is more accurate in assessing relative affinities than direct binding curves with individual ligands particularly with those of lower affinity where it may be difficult to attain conditions of staturation (15). A gel retardation competition experiment with the porcine IRE-BP is shown as Figure 2. The amount of native ^{32}P-labeled IRE probe bound was quantitated at each concentration of competing RNA. The data from four such competition curves are shown as Figure 3. In our calculations, the native IRE and altered competitor RNAs were considered as nonidentical 'ligands' for the same IRE-BP binding site. The concentration required for 50% inhibition of IRE binding (referred to as IC_{50}) was computed (14). Competition of a ^{3}H-labeled IRE probe with the ^{32}P-labeled IRE probe produced an IC_{50} value of 1 as would be predicted for self competition (Figure 3, upper panel). A summary of the IC_{50} values of the twenty eight altered RNAs is given in Figure 4.

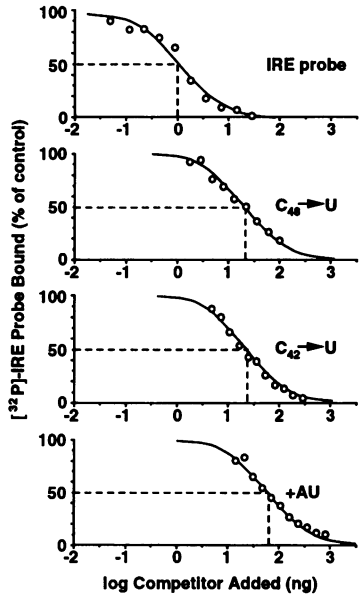

Figure 3. Representative competition curves for altered RNAs. Gel retardation assays with 1 ng ^{32}P-labeled ferritin H chain IRE probe were performed as described under Materials and Methods. The indicated ^{3}H-labeled RNAs at various levels were added as competitors and the amount of ^{32}P-labeled probe bound determined. Dashed lines indicate the IC_{50} for each competitor (expressed in units of ng/20μl reaction volume) and is defined as the level of competitor that produces 50% inhibition. Note that the IC_{50}=1 (0 on the log scale shown) for the self competition (upper panel) and that the IC_{50}=63 for the competitor termed +AU (lower panel).

RNA structural requirements for IRE-BP binding

Based on comparision of IREs from ferritin mRNAs and TfR mRNAs from different species a consensus IRE structure has been inferred (see Figure 1). The IRE-BP has been shown to bind to the IRE derived from the ferritin transcript and to all five IREs derived from a TfR transcript even though there is little similarity in the stem sequence between the IREs of ferritin and TfR mRNAs (7). However, it appears that base pairing of the stem is critical to IRE function since both Liebold *et al.* (5) and

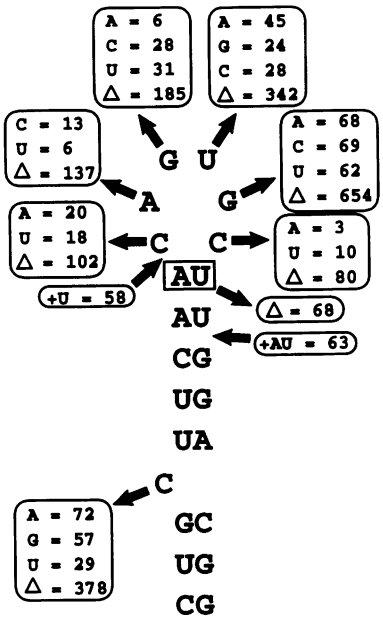

Figure 4. IC_{50} values for 28 altered RNAs. Each RNA was assessed as a competitor in a gel retardation assay and the data analyzed as described in figures 2 and 3. The IC_{50} (expressed in units of ng/20μl reaction volume) for each deletion (Δ) or indicated substitution are indicated. As these assays are designed, the IC50 values of each competitor is a direct indicator of how well the affinity of the competitor for the IRE-BP as compared to the native IRE (i.e. a competitor with an IC50=20 interacts with the the IRE-BP with a 20-fold lower affinity than does the native IRE). No substitution was found to be benign or to produce an RNA with greater apparent affinity for the IRE-BP. Deletions at each position tested reduced efficacy of the RNA as competitior more drastically than did substitutions at that position. Addition of one AU pair to the 'upper stem' or removal of one AU pair resulted in IC50 values of 63 and 68, respectively.

Bettany *et al.* (6) have shown that targeted disruption of the base pairing reduces RNA-protein interaction. Restoration of base-pairing restores interaction with the IRE-BP although it is not clear that such restoration to non-native sequence yields RNAs that have an identical affinity for the protein (5, 6). Deletion of one base within the upper stem sequence also ablates function (16). Other proteins have been described that interact with elements of RNA secondary structure formed by Watson–Crick base-pairing. In several instances, including the coat protein of R17 coliphage (17), Xenopus protein TFIIIA (18), E.coli ribosomal proteins (19), and HIV Tat protein (20, 21), and HIV rev protein (22) it has been demonstrated that the recognition of RNA by RNA binding proteins is highly dependent upon the maintenance of secondary structure. As is the case with the IRE, certain nucleotides in RNA stems in these RNAs can be changed without significant effect on RNA-protein interaction so long as as base pairing is maintained. In contrast, sequence specificity within an RNA duplex has been implicated in the interaction of the HIV rev protein with its cognate RNA (23).

Unpaired bases in an RNA duplex not only destabilize RNA structure but also have the effect of introducing a kink in the otherwise cylindrical run of the helix (24, 25). Such distortion may actually contribute to specific protein recognition. It is noteworthy that several RNA binding proteins other than the IRE-BP (e.g. R17 coat protein and HIV Tat) recognize RNA structures having stems interupted by bulges of unpaired bases. Deletion from the IRE stem of the invarient bulge C (ΔC_{42}) is very deleterious (IC_{50}=378) suggesting that this base is very important in the RNA–protein interaction. Although no base substitution for the bulge C is as good as the native IRE, all base substitutions at this position yielded RNA that is significantly better as a competitor than ΔC_{42}. This finding suggests that the unpaired base of the IRE stem may be playing a structural role in recognition perhaps by distortion of the RNA helix.

Our experiments also indicate that the number of base pairs in the upper stem is also a critical feature of the IRE. Removal of a single AU base pair or addition of one AU base pair resulted in IC_{50} values of 68 and 63, respectively. An effect of upper stem length has also been reported by others (6). The stem length constraint may be due to a requirement that the invariant bulge C and the loop be a certain distance apart to make important contacts with the IRE-BP. It is also possible that the helical twist characteristic of five base pairs is critical to maintain a particular spatial orientation between contact points with the IRE-BP. Removal or addition of a base pair would effect both distance between protein contact points and their relative spatial orientation. Similar findings have been reported by Berkhout and Jeang (26) for HIV Tat protein with its cognate TAR RNA where increasing the number of bases in the loop or increasing the number of base pairs between the loop and the bulged nucleotides of the stem adversely affected the interaction.

RNA sequence requirements for IRE-BP binding

We have found that the sequence of the loop and the bulge C are critical for the IRE/IRE-BP interaction. Removal or substitution of any base in the IRE loop significantly decreases interaction with the IRE-BP. Our conclusions in this regard differ somewhat with those of Barton *et al.* (27) who reported that a C >A change in the stem bulge and certain substitutions of loop nucleotides (single base changes at positions corresponding to bases G_{50} and U_{51} of our sequence) had relatively little impact on interaction of the rat IRE-BP with variants of the rat ferritin L chain IRE. One of these changes corresponding to G_{50} >A was relatively benign in our analysis (IC_{50}=6), but others showed much more profound effects in our analysis than seen by Barton *et al.* (27). The study of Barton et al. (27) also used competitive inhibition assays, but employed a different 'parental' IRE (RNA related to rat L chain IRE vs. RNA related human H chain IRE), a different source of IRE-BP (rat vs. pig) and somewhat different reaction conditions. These differences may account for the apparent discrepancies between the two studies.

In previous studies, we have noted that a human ferritin H chain mRNA with a deletion analogous to ΔC_{48} is not translationally regulated in response to iron levels (16). A similar deletion within all of the IREs of a TfR transcript also eliminated iron regulation (3). Although we have not defined the relationship between the IC_{50} values obtained here and physiological regulation, these results would suggest that IC_{50}=102 is above the value compatible with regulation of either ferritin translation or TfR mRNA stability. Many of the other RNA alterations examined have IC_{50} values similar to that of ΔC_{48} and might be predicted to yield unregulated mRNAs if introduced as mutations in native IREs. Some deletions are more deleterious than others, particulary ΔG_{52} and ΔU_{51}. These bases appear to be hypercritical in recognition by the IRE-BP.

The position numbered 53 in our probe is the most variable base in native IREs. It is noteworthy that this is also the position most tolerant of nucleotide substitution in our competition experiments (IC_{50} = 3–10). The fact that naturally occurring ferritin IREs have bases other than cytosine at position 53 suggests

that IC_{50} values in this range are compatible with IRE function in the context of cellular ferritin regulation. The C_{53} position is not not nearly as tolerant of a deletion as it is of substitution. Indeed, substitutions at any position of the IRE loop were always less deleterious than deletions that decrease from six the number of loop bases. Similarily an increase in loop size to seven bases was not well tolerated. Surprisingly, none of the mutations tested were benign. In addition, no sequence alteration was found to increase affinity for the IRE-BP. In this regard the IRE-BP is distinguished from R17 coat protein where several benign RNA sequence changes were noted in a study analogous to this one. Moreover, one substitution that actually increased the apparent affinity of this interaction was noted (28).

In summary, both the structure and the sequence are very important for the interaction of the IRE with the IRE-BP. Previous studies have emphasized the significance of having a base-paired stem. We have shown that the IRE-BP is relatively intolerant of base substituions at virtually any of the positions in the IRE loop or at the unpaired cytosine residue of the IRE stem. Any increase or decrease in the number of bases in either the IRE loop or the IRE upper stem decrease affinity of the interaction. These findings suggest that multiple contacts exist between the IRE-BP and its cognate RNA. These contacts acting together provide the basis for the high affinity that characterizes the IRE/IRE-BP interaction. These results also point out the need to scrutinize carefully RNA sequences that appear to resemble an IRE motif since even what may appear to be subtle sequence changes can have profound functional significance.

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