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A cytosolic protein binds to structural elements within the iron regulatory region of the transferrin receptor mRNA

(posttranscriptional gene regulation/RNA stability/RNA structure/iron metabolism)

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ABSTRACT **The level of mRNA encoding the transferrin receptor (TfR) is regulated by iron, and this regulation is mediated by a portion of the 3′ untranslated region (UTR) of the TfR transcript. This portion of 3′ UTR of the human TfR mRNA contains five RNA elements that have structural similarity to the iron-responsive element (IRE) found as a single copy in the 5′ UTR of the mRNA for ferritin, whose translation is regulated by iron. Moreover, five very similar elements are also contained in the 3′ UTR of the chicken TfR mRNA. Cytosolic extracts of human cell lines are shown by a gel shift assay involving RNase T1 protection to contain an IRE-binding protein capable of specific interaction with the human TfR 3′ UTR. When the protecting protein is removed, the protected RNA can be digested with RNase T1 to yield oligoribonucleotide fragments characteristic of two of the IREs contained in the TfR 3′ UTR. As judged by cross-competition experiments, the same IRE-binding protein interacts with the ferritin IRE. The apparent affinity of RNA sequence elements for the IRE-binding protein is shown to depend upon the sequence of the RNA. A comprehensive secondary structure for the regulatory region of the TfR mRNA is proposed based on the experimentally demonstrated presence of at least two IRE-like structural elements.**

Vertebrate cells acquire iron via endocytosis of transferrin mediated by the transferrin receptor (TfR). Iron availability modulates the level of TfR mRNA such that higher transcript levels are present when cells are treated with an iron chelator (1). The regulation of TfR expression by iron has been shown to involve two regions of the gene (2), with the major locus of regulation residing in sequences corresponding to the 3′ untranslated region (UTR) of the TfR mRNA (2–4). Evidence has been presented suggesting that the regulation of the TfR by iron involves modulation of mRNA stability (5). Within the 3′ UTR of the TfR mRNA, the region critical for iron regulation has been mapped to a 678-nucleotide (nt) fragment that has potential for a high degree of secondary structure. This portion of the TfR 3′ UTR includes five sequence elements that each resemble the single iron-responsive element (IRE) found in the 5′ UTR of ferritin mRNAs (4). Ferritin expression is also regulated by iron, but this regulation occurs without alterations in ferritin mRNA levels and involves a modulation of ferritin translation (6). The human ferritin heavy-chain IRE has been demonstrated to be necessary and sufficient for iron regulation of ferritin translation (7). Two of the sequence elements from the TfR 3′ UTR have been shown to function as IREs mediating ferritin-like translational regulation when individually inserted into a 5′ UTR (4). On the basis of their structural similarity with the ferritin IRE, their presence in the critical region in the 3′ UTR of the TfR mRNA, and the experimentally demonstrated IRE function of the TfR elements, we have proposed that an mRNA structure containing IREs participates in iron regulation of TfR mRNA levels (2, 8).

The IRE region of the ferritin 5′ UTR has been shown to interact specifically with a cytosolic protein(s) in an *in vitro* gel shift assay (9, 10). To assess experimentally the ability of the 3′ UTR of the TfR mRNA to form IRE stem–loops, we have investigated the interaction of the critical region of the TfR transcript with the cytosolic protein that binds the ferritin IRE. Here we demonstrate that an ≈1-kilobase (kb) RNA fragment derived from the 3′ UTR of the TfR mRNA competes with the ferritin IRE for interaction with a cytosolic protein of human cells. The interaction of the protein with the TfR RNA is inhibited by short synthetic oligoribonucleotides corresponding to individual TfR IREs. In addition, we show that at least two of the IREs of the TfR 3′ UTR are specifically protected from RNase T1 digestion by the cytosolic IRE-binding protein. These results indicate that the TfR RNA forms a structure containing the characteristic IRE stem–loops and support models whereby TfR and ferritin regulation by iron availability may be mediated by a common cytosolic protein.

MATERIALS AND METHODS

Plasmid Constructs and *in Vitro* RNA Synthesis. The 85-base-pair (bp) *Hae* III–*Eag* I fragment from the 5′ leader of the human ferritin heavy chain was cloned into pGEM-3-blue plasmid (Promega) as described (10). Following linearization of the plasmid with *Eco*RI, RNA was synthesized with SP6 RNA polymerase (Promega). The 150-nt β-globin transcript was made with SP6 polymerase after *Acc* I linearization of a β-globin cDNA cloned into pSP64 (11). The 1059-bp *Bgl* II–*Hin*dIII fragment from the 3′ UTR of the human TfR cDNA was cloned into the *Bam*HI and *Hin*dIII sites of pGEM-blue (Promega) and RNA was synthesized with T7 polymerase (Promega) after linearization with *Eco*RI (sense), or with SP6 polymerase after linearization with *Bam*HI (antisense). Transcription reactions were performed according to the Promega protocol. Labeled RNA transcripts were made by inclusion of [α-^{32}P]CTP at 150 Ci/mmol (1 Ci = 37 GBq).

RNAs corresponding to IRE loops A, B, C, D, and E of the human TfR were made from oligodeoxyribonucleotide templates by using T7 polymerase as described (12). Template oligodeoxyribonucleotides were made on an Applied Biosystems 381A DNA synthesizer. Labeled RNAs were made by including [α-^{32}P]CTP at 3.6 Ci/mmol.

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Abbreviations: IRE, iron-responsive element; TfR, transferrin receptor; UTR, untranslated region; nt, nucleotide(s).

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Cell Lines and Cytoplasmic Extract Preparation. Human K562 erythroleukemia and ARH-77 plasmacytoma cells were cultured in RPMI 1640 with 10% fetal bovine serum. S-100 cytoplasmic extracts (13) were made from cells growing at a density of 3–5 × 10^5 per ml. Protein concentrations in extracts were determined by the bicinchoninic acid method (Pierce).

Gel Shift Assay. Gel shift assays were performed as described (9, 10). Labeled RNAs (0.35–10 ng) were incubated with S-100 cytoplasmic extracts (10–40 μg of protein) for 30 min at 25°C. RNase T1 (1 unit, Calbiochem) was then added for 10 min followed by the addition of heparin (Fisher Scientific) to 5 mg/ml for 10 min. Unlabeled competitor RNAs were mixed with labeled probes prior to the addition of S-100 extract. Samples were electrophoresed in nondenaturing acrylamide gels (9) and visualized by autoradiography.

RNase T1 Mapping of Protein Binding Sites. For elution of the protein–RNA complex, incubations were performed with 100 ng of labeled RNA and 400 μg of S-100 extract. A gel slice containing the gel shift complex was cut out after visualization by autoradiography, and the complex was eluted overnight at 37°C (14). After phenol/chloroform extraction and ethanol precipitation the eluted RNAs were separated in a denaturing 8 M urea/12% acrylamide gel and visualized by autoradiography. Gel slices containing individual RNA species were cut out, and the RNAs were eluted overnight and then extracted and ethanol-precipitated. The labeled RNAs

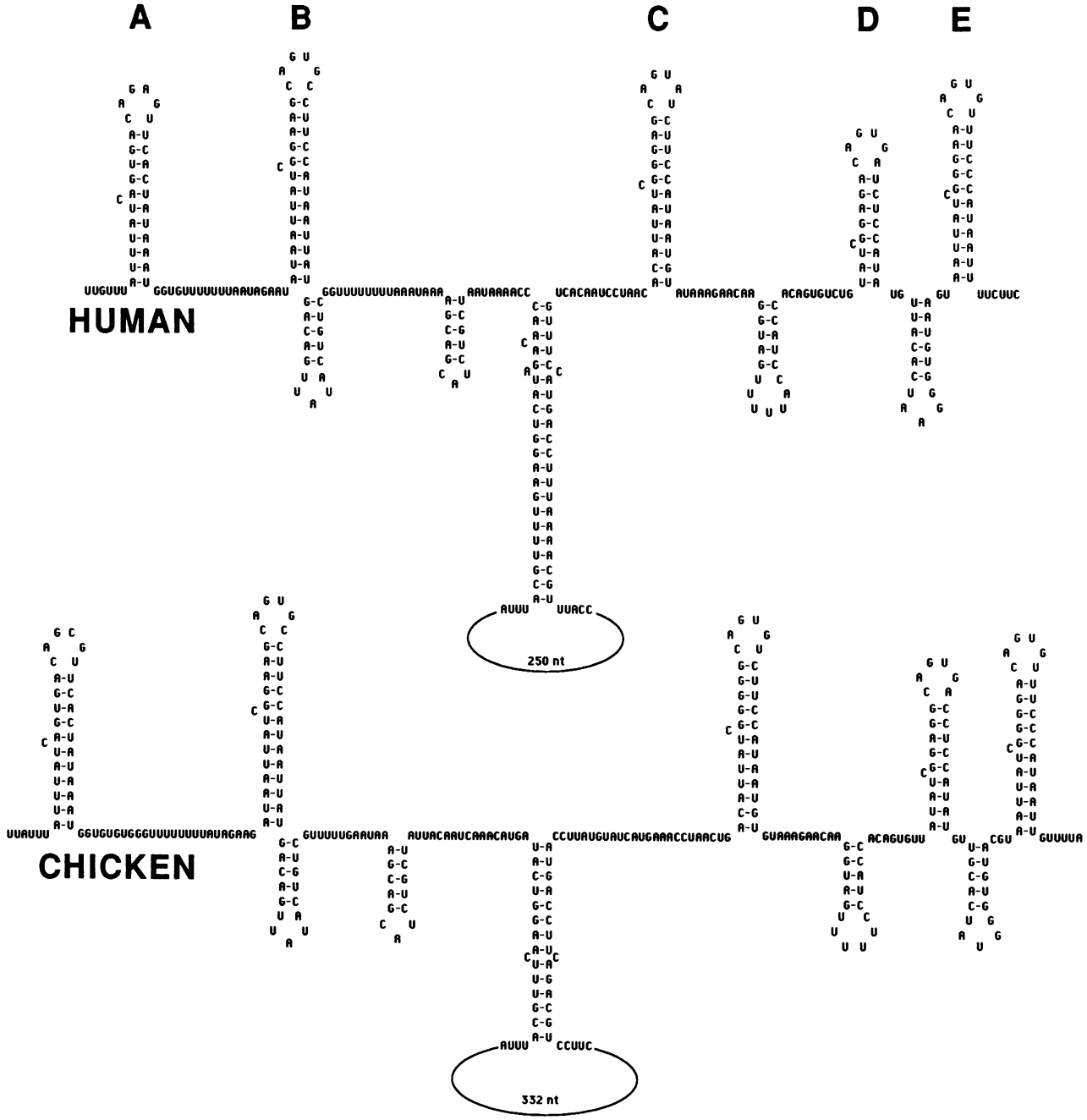

FIG. 1. Potential secondary structures for portions of the 3′ UTR of the human and chicken TfR mRNA. Potential folding of nt 3233–3838 of the human TfR mRNA and of a corresponding segment of the chicken TfR mRNA are shown (see ref. 4 for numbering system). The human sequence was determined from clones derived from plasmid pCDTR1 (see text). The chicken sequence is that of cDNA clones isolated from a λgt10 chicken embryo library (15). Both sequences were determined by the dideoxy chain-termination method (16). The structures are drawn such that the five potential IRE-like structures (A–E) that were previously identified (4) are shown above the horizontal sequence segments. The sequences of the large central loops (250 nt in the human and 332 nt in the chicken) where sequence similarity is <50% are not shown.

were resuspended in 10 mM Tris·HCl/1 mM EDTA, pH 8.0, digested with RNase T1 (10 units), electrophoresed in a denaturing 20% acrylamide gel, and visualized by autoradiography.

RESULTS

A secondary structure for the region of the human TfR mRNA that is critical for regulation by iron (4) is proposed in Fig. 1. The sequence of the human TfR 3′ UTR contains five sequence elements capable of forming stem–loops (labeled A–E) that resemble the ferritin IRE. The human sequence shown in Fig. 1 is the sequence contained in our clones derived from the plasmid pcDTR1 (17). This sequence differs from the published sequence of a distinct TfR cDNA (18) for the 3′ UTR in that the loop region of element C contains the sequence CAGUAU rather than CAGUGU. Synthetic elements corresponding to human TfR mRNA elements B or C (based on the sequence of ref. 18) have been shown to be capable of individually substituting for a ferritin IRE in translational regulation when placed in the context of a 5′ UTR (4). It can also be seen in Fig. 1 that a strikingly similar structure can be formed with the sequence of a portion of the chicken TfR 3′ UTR. The overall sequence similarity between the two sequences represented in Fig. 1 is 78%. Both structures shown contain five IRE stem–loops and several other similar secondary structure elements. Included in both proposed structures is a long central stem with a large loop whose sequence and potential structure are not shown. The similarity between the human and chicken sequences in this large loop region is <50%. Excluding this loop, 92% of the nucleotides are identical. Within the five structures A–E are six differences (out of 153 nt) between the human and the chicken sequences. None of the changes affect the ability to form the characteristic IRE stem–loops.

Cytosolic extracts of human K562 cells contain a protein that specifically interacts with the single IRE of the 5′ UTR of human ferritin heavy-chain mRNA (10). A single nucleotide deletion in the loop of the ferritin IRE eliminates both IRE function and interaction with the cytosolic protein as judged by a gel shift assay. Moreover, the amount of IRE-binding activity in extracts of K562 cells is increased by treatment of cells with an iron chelator.

The formation of the complex between the ferritin IRE and the IRE-binding protein was inhibited by addition of unlabeled RNA corresponding to an ≈1-kb fragment of the TfR 3′ UTR (termed TfR Bg-H) that includes elements A–E (Fig. 2). No competition was seen with antisense RNA corresponding to the same region of the TfR transcript. When a radiolabeled TfR Bg-H RNA was added to cytosolic extracts of human ARH-77 plasmacytoma cells, treated with RNase T1 and heparin, and examined by nondenaturing gel electrophoresis, a complex was observed. Comparison of the complex formed using the TfR Bg-H RNA with that generated by the ferritin RNA demonstrated that the two comigrated (data not shown). Formation of this complex was inhibited by unlabeled TfR Bg-H and by unlabeled ferritin RNA but not by RNA derived from a β-globin cDNA (Fig. 3). The results of Figs. 2 and 3 indicate that the TfR Bg-H RNA contains at least one element capable of interaction with a specific IRE-binding protein.

To determine which IRE(s) of the TfR Bg-H RNA was involved in the formation of the protein–RNA complex seen on the nondenaturing gels, we isolated the RNA from the complex and analyzed this RNA in a denaturing gel (Fig. 4, gel II). Three major species of RNA were separated electrophoretically and each was individually eluted from this gel and digested with RNase T1 prior to electrophoresis in a denaturing 20% acrylamide gel in order to fingerprint the protected RNA regions (Fig. 4, gel III). The sizes of the

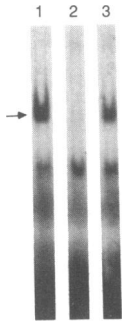

FIG. 2. The iron regulatory region of the 3′ UTR of the human TfR mRNA competes with the ferritin IRE for specific binding of a cytoplasmic protein. A labeled 85-nt RNA (0.35 ng) from the 5′ leader of the human ferritin heavy chain containing a single IRE was incubated with S-100 cytoplasmic extract (20 μg of protein) from human K562 cells in the presence of no unlabeled competitor RNA (lane 1) or a 500-fold excess of unlabeled sense (lane 2) or antisense (lane 3) RNA corresponding to the 3′ UTR of the human TfR mRNA. Samples were electrophoresed in a nondenaturing 4% acrylamide gel and visualized by autoradiography. The specific complex is indicated by the arrow.

predicted fragments after RNase T1 digestion of the TfR IREs are as follows: A, 4, 12, and 13 nt; B, 2, 3, 13, and 15 nt; C, 2, 14, and 22 nt; D, 4, 5, and 11 nt; E, 5, 9, and 21 nt. The 13/15 doublet unique to IRE loop B was observed in the RNase T1 digests of two of the three RNAs isolated from the specific protein–RNA complex. The size difference between these two protected RNAs can be accounted for by inclusion in the larger RNA of the 12 nt just 3′ of element B; RNase T1 fragments corresponding to those predicted for these nucleotides were observed. The third RNA isolated from the protein–RNA complex yielded an RNase T1 digestion pattern consistent with it being element E. Examination of the entire sequence of the TfR Bg-H probe indicated that RNase T1 fragments of 15 and 21 nt are uniquely found in element B and element E, respectively. We did not observe RNA in the protein–RNA complex that could be ascribed to IRE element A, C, or D. Increasing the lysate/probe ratio in the initial incubation by a factor of 10 over that used in Fig. 4 did not result in any alteration in the pattern of protected RNAs isolated from the complex. This finding suggests that the absence of other IREs in the mapping experiment was not due to a limiting amount of IRE-binding protein.

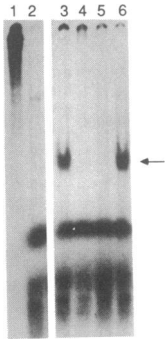

FIG. 3. Specific binding of a cytoplasmic protein to the iron regulatory region of the 3′ UTR of the human TfR mRNA. Labeled RNA (0.5 ng) corresponding to the *Bgl* II–*Hin*dIII portion (1059 nt) of the human TfR 3′ UTR was incubated with S-100 cytoplasmic extract (40 μg of protein) from human ARH-77 cells (lane 3). Competition with a 500-fold excess of unlabeled TfR (lane 4), ferritin (lane 5), or β-globin (lane 6) RNA is shown. Samples were electrophoresed in a nondenaturing 5% acrylamide gel. The specific complex is indicated by the arrow. Lanes 1 and 2 show probe in the absence of S-100 extract without or with RNase T1 digestion, respectively.

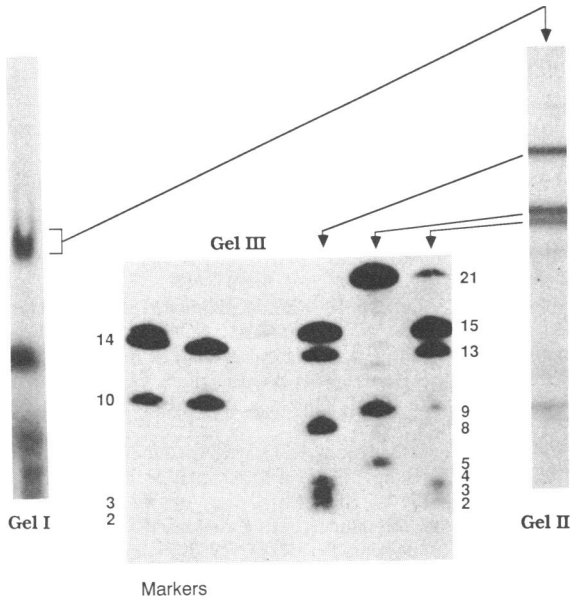

FIG. 4. RNase T1 mapping of protein binding sites in the iron regulatory region of the TfR mRNA. The gel shift complex indicated in gel I was generated by incubation of labeled TfR Bg-H probe with S-100 lysate as in Fig. 3. Labeled RNAs were eluted from the RNA–protein complex generated by incubation of 100 ng of labeled RNA from the TfR Bg-H fragment with S-100 extract (400 μg of protein) from ARH-77 cells (not shown) and were separated in a denaturing 12% acrylamide gel (gel II). After visualization by autoradiography three labeled RNAs were eluted from gel slices and digested with RNase T1. The RNase T1 digestion products from these RNAs were separated in a denaturing 20% acrylamide gel (gel III). Size markers were generated by RNase T1 digestion of oligoribonucleotides of known sequence. Numbers indicate lengths (nt).

Several possibilities might explain the absence of other elements as protected fragments. It is possible that the binding of the cytosolic protein to elements A, C, and D within the Bg-H RNA is excluded for steric reasons by binding to elements B and E, which are the preferred binding sites. Alternatively, the other potential IREs in the TfR Bg-H RNA may be incapable of interaction with the IRE-binding protein even in the absence of steric interference from other elements.

To begin to address some of these issues, synthetic oligoribonucleotides corresponding to elements A–E were prepared and analyzed independently for interaction with the IRE-binding protein (Fig. 5). Using our gel shift assay procedure with a radiolabeled RNA corresponding to the 5′ UTR of ferritin as probe, we assessed the ability of unlabeled elements A–E to compete for complex formation. Each of the synthetic TfR IREs was shown to be capable of eliminating the specific complex. However, a range of effectiveness of inhibition was observed. Elements B and E were the most effective competitors and had similar apparent affinities for the IRE binding protein. Elements A and D had a lower apparent affinity than elements B and E but were more effective as competitors than element C. An unlabeled RNA from a region of the TfR Bg-H containing no IREs did not compete for formation of the specific gel shift complex even at the highest concentration tested (data not shown). When radiolabeled RNAs corresponding to elements B and C were used as probes in a gel shift assay, they were found to bind with the same relative affinities seen in Fig. 5 (i.e., B >> C, data not shown). While it is clear that each of the TfR elements is capable of interacting with the IRE-binding protein, the sequence differences between these elements can be seen to have significant effects on their effectiveness as targets for that protein.

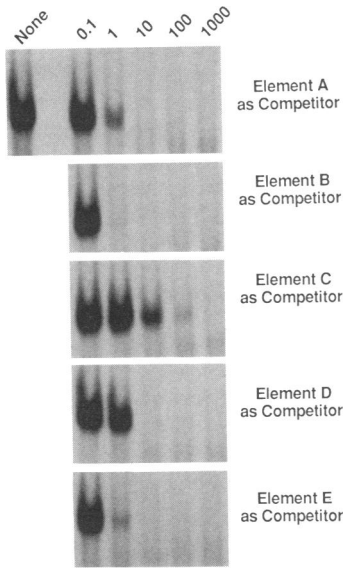

FIG. 5. Isolated elements A–E from the human TfR mRNA can interact specifically with an IRE-binding protein. Labeled RNA (0.5 ng) from the ferritin 5′ UTR was incubated with S-100 extract (10 μg of protein) in the presence of the indicated amounts (0.1–1000 ng) of unlabeled oligoribonucleotide corresponding to elements A–E.

DISCUSSION

Many eukaryotic genes are regulated by modulation of the level of the specific gene transcript. This can be accomplished by altering the rate of transcription or by controlling the half-life of the transcript. Sequence motifs that influence message stability have been identified in a variety of mRNAs (19). Examples of these sequence motifs are found within the transcripts encoding histones, lymphokines, cytokines, and protooncogene products (20, 21). In each of these examples, the region of the mRNA influencing transcript stability has been localized to the 3′ UTR. It has been suggested that the 3′ UTR may contain target sites for an endoribonuclease, the action of which would render the transcript susceptible to destruction by exonucleases (21).

There are only a few examples in which specific mediators or extracellular signals physiologically regulate the stability of specific target transcripts. These mediators include estrogen, glucocorticoids, sterols, and iron. We (2, 4) and others (3, 5) have recently identified a region of the long 3′ UTR of the TfR mRNA that is responsible for the iron regulation of the level of this message. The sequence of the region is notable for its potential to assume highly stable secondary structures. Included among these possible structures are five stem–loop elements resembling the RNA regulatory structures that we have described as being responsible for the iron-regulated control of the translation of human ferritin heavy-chain mRNA (22). We have termed these structures IREs. In ferritin transcripts, a single IRE is found in the 5′ UTR. That the IRE-like sequences of the TfR 3′ UTR could indeed function as IREs in conferring iron-dependent translational control was demonstrated by insertion of individual TfR sequence elements into the 5′ UTR of a reporter gene. Thus, these studies identified the cis-acting RNA sequences involved in iron-mediated regulation. Until now there has been no evidence for specific trans-acting protein factors that interact with this, or any other, regulatory 3′ UTR and thus mediate the ability of the cell to determine the stability of a particular message. Recently, a cytosolic protein that binds to the IRE involved in translational regulation of ferritin has been reported in rodent (9) and human (10) cells. This binding activity is regulated by the cell in response to iron perturbations. Here, we have demonstrated that an ≈1-kb portion of

the TfR mRNA 3′ UTR containing the five IRE-like elements is capable of interacting with the IRE-binding protein first detected by using RNA from the 5′ UTR of ferritin. We have mapped the interaction with this RNA by RNase T1 protection and provided direct evidence for interaction with two (elements B and E) of the five IREs contained in the structure we propose in Fig. 1. We have shown that elements A, C, and D are also capable of interacting with the IRE-binding protein when synthesized as isolated oligoribonucleotides, albeit with lower apparent affinities than elements B and E. The absence of detectable protein bound to elements A, C, and D may be due to the lower relative affinities for these elements (most notably C), steric constraints, or a combination of these factors.

The implication of the ability to detect interaction of the IRE-binding protein with the TfR message is that the RNA from the TfR 3′ UTR adopts a secondary structure that contains IRE elements. A strikingly similar structure containing five IREs can be formed with the comparable region of the chicken TfR mRNA sequence. Phylogenetic similarities in RNAs have been shown to be useful in predicting the correct structure from alternative RNA secondary structures (23, 24), and thus the striking conservation of the IRE structures supports the importance of these stem–loops. The predicted structure we have proposed for the regulatory portion of the TfR mRNA contains five IRE-like elements (Fig. 1), and we have provided direct experimental evidence for two such elements (B and E) in the mapping of the protected RNA in the RNA–protein complex.

Recently, an alternative secondary structure for a portion of the TfR mRNA 3′ UTR has been proposed (5). This proposed structure encompasses the TfR mRNA sequences beginning 5′ of the element designated by us as IRE B and ending 3′ of IRE C but depicts these two elements as being base-paired to each other rather than as independent stem–loops. Although this alternative has a very similar calculated free energy to an identical region folded as in Fig. 1, no direct experimental evidence for that structure was provided. We have proposed a model in which the same (or a related) IRE-binding protein mediates the regulation of both ferritin and TfR expression (4, 8). In ferritin regulation, the protein is envisioned as determining the distribution of ferritin mRNA between polysome (translated) and nonpolysome pools. Iron-dependent redistribution of ferritin mRNA between these pools has been demonstrated (25). In the case of TfR regulation, the situation appears to be more complex. The interaction(s) with the IRE-binding protein is here envisioned as protecting the TfR mRNA from an endoribonuclease cleavage that serves as the rate-determining event in the turnover of the transcript. Part of the complexity in the case of the regulation of the level of TfR mRNA is evidenced by the presence of five IRE elements in its 3′ UTR. We previously showed that removal of either the 5′ third of the critical region (containing elements A and B) or the 3′ third of this region (containing elements C–E) results in loss of iron regulation (4). A more thorough understanding of the relevant RNA structure(s) and further characterization of the IRE-binding protein should provide insight into the mechanism by which TfR mRNA levels are regulated. These insights may well be applicable to other biological systems in which RNA–protein interactions participate.

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