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Ferritin mRNA probed, near the iron regulatory region, with protein and chemical (1,10-phenanthroline-Cu) nucleases

A possible role for base-paired flanking regions

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Summary. Iron stimulates ferritin synthesis in whole cells and animals, by increasing the entry of ferritin mRNA into polyribosomes. Dissection of the regulation at the molecular level has identified a 28-nucleotide, conserved, regulatory sequence (IRE = iron regulatory element) in the 5′ non-coding region of ferritin mRNAs, plus *trans*-acting factor(s), one of which is a 90-kDa protein. The site of iron action is not entirely characterized but may involve heme; sequences in the 3′ non-coding region of ferritin mRNA can modulate regulation. Ferritin mRNA is the first eukaryotic mRNA for which a conserved regulatory sequence and regulator protein have been identified. The same RNA-protein motif is used, through iron-dependent degradation of transferrin receptor mRNA, to decrease synthesis of the receptor and cellular iron uptake. The regulatory structure of the transferrin receptor mRNA is composed, in part, of five copies of the IRE in the 3′ non-coding region. IRE structure, probed by cleavage with RNases T_1, V_1, 1,10-phenanthroline-Cu or modification with dimethyl sulfate, is a hairpin loop with conformational variations dependent on magnesium; a base-paired region flanking the IRE is also structurally sensitive to magnesium. Similar results were obtained with a synthetic 55-mer containing the IRE and with a full-length in vitro transcript with a G→A substitution in the loop. However, in both cases, the IRE structure was closer to the computer-predicted structure and was less affected by magnesium than in native ferritin mRNA, indicating the importance of the loop sequence and RNA interactions outside the IRE structure. The combined IRE + flanking regions in six different ferritin mRNAs form a structure very close to the cap where interference with translational initiation is likely.

Key words: Ferritin mRNA - Translational control - mRNA regulatory element - 1,10-Phenanthroline-Cu nuclease

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Introduction

Protein synthesis can be regulated by changing the amount of mRNA or by changing the utilization of mRNA. The best characterized examples involve activation or inactivation of mRNA synthesis, although changes in mRNA stability include the transferrin receptor (Owen and Kuhn 1987) and proto-oncogene proteins (Shaw and Kamen 1986). Examples of regulation of protein synthesis through specific storage of mRNA are fewer in number but represent fundamental cellular processes such as DNA synthesis (ribonucleotide reductase; Standart et al. 1985), cell division (cyclin; Swenson et al. 1987), stress (housekeeping proteins and heat shock; Lindquist 1987) and photosynthesis (chlorophyll-binding protein; Berry et al. 1988).

Iron induces iron storage (ferritin synthesis) and inhibits iron uptake (transferrin receptor synthesis) in concert through novel RNA structures (reviewed by Theil 1990). For example, iron induces ferritin synthesis as much as 30–50-fold with no change in the amount of ferritin mRNA; during the process stored, inactive ferritin mRNA is translated (Zahringer et al. 1976; Shull and Theil 1982). At the same time, iron decreases transferrin receptor synthesis (and iron uptake) by a process which leads to enhanced degradation of the transferrin receptor mRNA (Owen and Kuhn 1987). The effect of iron requires the same structural motif in each mRNA but the opposite effects of iron (increased or decreased synthesis of the two proteins involved in iron metaoblism) apparently occur because the common structures are in different regions of the mRNAs. All known ferritin mRNAs contain the highly conserved 28-nucleotide sequence, called the iron regulatory element (IRE), in the 5′ non-coding region; the IRE is generally nearer the cap than the initiator AUG. The presence of five more variable copies of the IRE in the 3′ non-coding region of the transferrin receptor mRNA was noticed in 1989 (Koeller et al.) and ample evidence shows that IREs in ferritin and transferrin receptor mRNAs are required for the effects of iron (reviewed in Theil 1990). Both ferritin and transferrin receptor mRNAs

have regulatory sequences in addition to the IREs that are involved in regulation (Dickey et al. 1988; Mullner et al. 1989). Evidence for iron-induced changes in ferritin mRNA synthesis and/or stability has been obtained for frogs and rats (Dickey et al. 1987; White and Munro 1988).

Trans-acting factors have been shown to inhibit ferritin mRNA translation (Dickey et al. 1988) and to bind to the IRE region (Leibold and Munro 1988; Casey et al. 1988; Mullner et al. 1989). One factor, a 90-kDa protein (P-90 or IRE-BP) has been purified (Rouault et al. 1989; Walden et al. 1989) and partly characterized; hemin, affects P-90 activity in heterologous cell extracts (Lin et al. 1990). The combination of the RNA sequence and the regulator protein in vivo allow cells to transmit the iron signal to mRNA and regulate in concert the synthesis of the two metabolically related proteins.

To understand the structure and function of the IRE sequence, we have begun a series of studies of normal and altered IRE sequences, in different contexts, comparing, for example, native ferritin mRNAs for H and (H′) M subunits and an in vitro transcript for the H′(M) subunit with a G→A replacement in the conserved sequence (Wang et al. 1990). In addition to classical protein nucleases, the novel metal-organic nuclease 1,10-phenanthroline-Cu (phenCu) has been used. PhenCu cleaves single-stranded regions of mRNA (Sigman and Chen 1990). The advantages of phenCu include accessibility to single-stranded regions too small for bulky enzymes and the ability to cleave nucleic acids under a wider range of experimental conditions than the protein nucleases. The mechanism of action depends upon specific binding of the phenanthroline followed by generation of an active oxygen species, through reduction of Cu in the presence of dioxygen; the active oxygen species cleaves the RNA at the binding site. Analysis of the structure of the IRE in solution reveals a hairpin loop as predicted by computer, but there are variations in the base-paired regions which are magnesium-dependent. The conformational variation extends to base-paired flanking regions in which primary structure is not conserved but which lengthen the stem of the IRE and bring the structure closer to the cap.

Methods

RNA. Poly(A)-containing RNA was isolated from bullfrog reticulocytes as previously described (Shull and Theil 1982). Ferritin mRNA constitutes about 10% of the total mRNA in such cells. Uncapped in vitro transcripts of the H′(M) subunit intronless pseudogene were made as described by Dickey et al. (1988). The gene is 97% identical to the H′(M) subunit mRNA but has a G→A substitution in the conserved CAGUG sequence of the regulatory region. An oligoribonucleotide ($n=55$) corresponding to the IRE and flanking region of the bullfrog red cell ferritin H chain was synthesized using T_7 RNA polymerase and, as a template, a complementary synthetic oligodeoxyribonucleotide containing the polymerase promotor sequence at the 5′ end (Milligan et al. 1987).

Cleavage or modification reactions and analysis. RNA was alkylated with dimethyl sulfate or cleaved with 1,10-phenanthroline-Cu, or RNase T_1, or V_1, using the conditions described in Wang et al. (1990). Magnesium concentrations used for phenCu or dimethyl sulfate were 0, 0.5 and 5 mM and for the protein nucleases 0 and 0.1 mM; magnesium had no effect on the results with protein nucleases. Briefly, RNA was annealed to a ^{32}P-labelled primer that bound 20 nucleotides downstream from the IRE. Cleavage or modification sites were determined as described by Wang et al. (1990) by primer extension analysis. Reverse transcriptase was used to synthesize fragments of labelled DNA terminated one nucleotide 3′ to the site of RNA cleavage or of methylated C or A. The mixture of fragments was separated by electrophoresis in acrylamide gels that were calibrated by transcribing native RNA in reactions containing one each of the four dideoxynucleotides. Cleavage sites were identified, after autoradiography, as radioactive sites at least three times as intense as the pause sites during reverse transcription of native RNA. Each experiment was repeated at least three times. Fragments of the 55-mer could be analyzed directly by labelling the 5′ end with ^{32}P before cleavage.

Computer predictions. Predictions of secondary structure used the University of Wisconsin Genetics Computer Group Fold program, version 5.0, as developed by Zucker and Steigler (1981) and Freier et al. (1986).

Results and discussion

Properties of the ferritin mRNA IRE region probed by 1,10-phenanthroline-Cu, protein nucleases and dimethyl sulfate

The conserved regulatory sequence of ferritin mRNA, the IRE, can be predicted to fold back on itself, through complementary base pairs to form a hairpin loop. Such a structure would have few sites available for reaction with either single-strand nucleases or PhenCu or dimethyl sulfate (Me_2SO_4). Such, in fact is the case (Fig. 1), particularly at 5 mM magnesium. However, the three single-strand probing reagents used all have different sizes with RNase T_1 the largest, followed by phenCu and Me_2OS_4. Only a single G nucleotide was cleaved by RNase T_1 (position 14 in the predicted hairpin loop) of a total of 13 in the 73-nucleotide region probed in ferritin mRNA (Wang et al. 1990). [Note that the IRE is numbered 1–28, starting at the 5′ end (Figs. 1 and 2).] Such results suggest that the entire IRE and flanking region is base-paired except for the hairpin loop. However, at least five G residues (positions 5, 14, 20, 24, 25) were cleaved by phenCu (Fig. 1), suggesting that much more of the stem has bulges or loops than suggested by the RNase T_1 data. Apparently, the size of the perturbations in the stem determine the accessibility of the probe with the bulky protein nuclease yielding an overestimate of the extent of secondary structure in the IRE region. A similar conclusion can be reached for the IRE flanking region where no T_1 sites were available in contrast to sites for phenCu. The poly(A)-containing RNA analyzed contained ferritin mRNA encoding two subunits the H and H′(M) which share 85% sequence identity (Dickey et al. 1987). The results of analyzing the IRE region were very similar for the two ferritin mRNAs, even though the expression of the two mRNA is tissue-specific (Dickey et al. 1987).

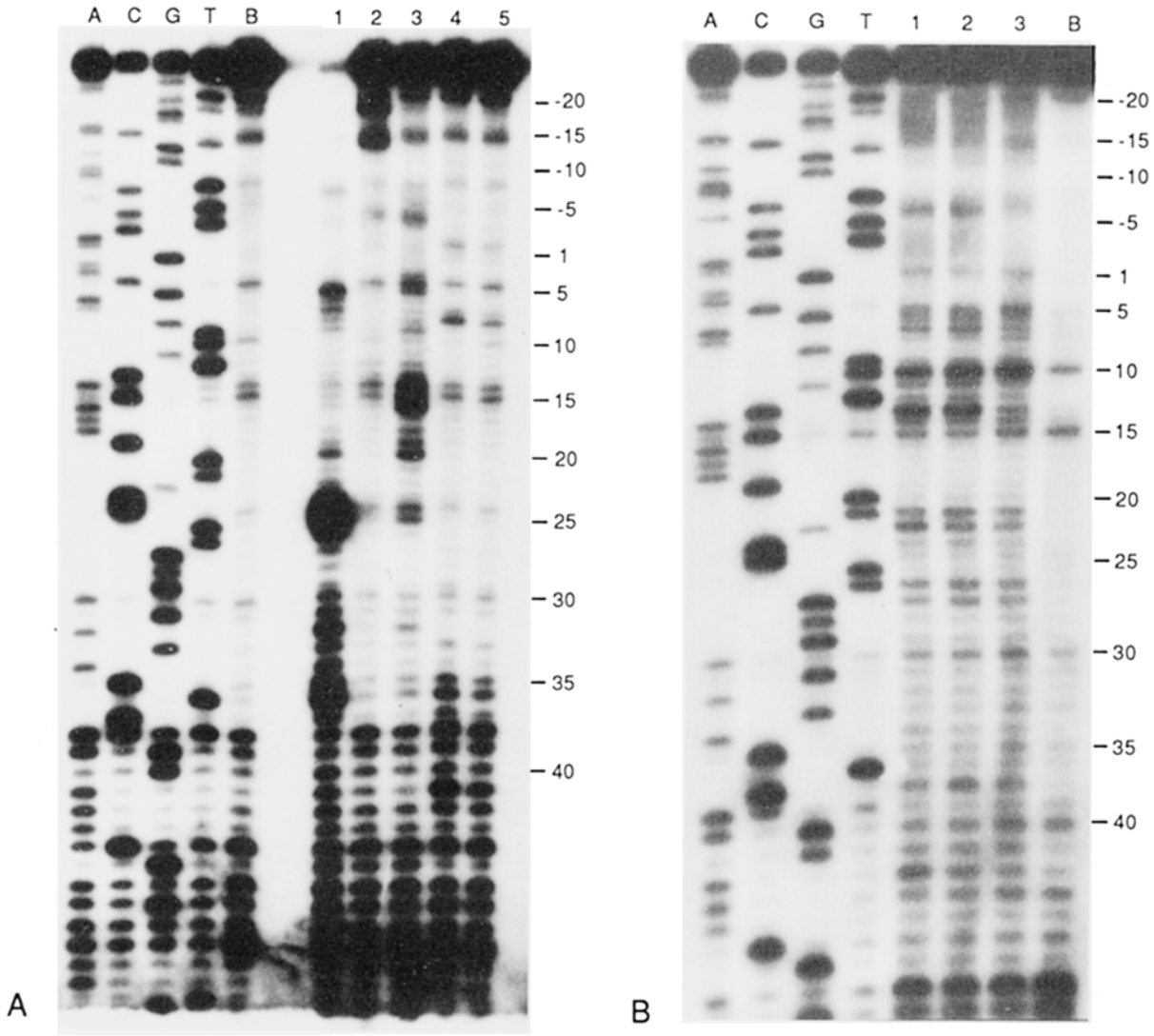

Fig. 1. The effect of magnesium on the structure of the ferritin RNA regulatory element, the IRE. (**A, B**) Modification or cleavage sites were determined by primer extension and analysis of the products on gel; calibrated reactions contain one of each of the four dideoxynucleotide triphosphates (See Methods). (A) Cleavage pattern of bullfrog red cell ferritin H chain mRNA cleaved by phenCu (lanes 1–3) and V_1 (lanes 4 and 5); reproduced from Wang et al. (1990) with permission. (B) Results of methylation of bullfrog red cell ferritin H chain mRNA. The magnesium concentration was zero, 5.0, and 0.5 mM during the reactions analyzed in lanes 1, 2 and 3 (A and B), respectively. Lanes A, C, G, T are the sequencing lanes and B is primer extension of unmodified mRNA. Note the sensitivity to conformational change of phenCu compared to Me_2SO_4. The IRE is numbered 1–28 from the 5′ end. Nucleotides at positions 14, 15, 16, 18 and 19 are cleaved by phenCu at 0.5 mM magnesium but not at zero or 5 mM magnesium. Similar results were obtained when the H′(M) ferritin subunit mRNA was examined

Interestingly, the 3′ non-coding regions had different structures when probed with phenCu (Wang and Theil, unpublished results) which may relate the differential expression.

Conformational flexibility was also displayed by the IRE region in addition to small perturbations along the predicted stem. For example, the conformation assumed by ferritin mRNA made only five sites accessible to phenCu in 5 mM magnesium while 13 were accessible in 0.5 mM magnesium and 10 in the absence of magnesium (Fig. 1). Magnesium had a much smaller effect on the conformation of the pseudogene IVT, particularly in the hairpin loop where most of the residues were accessible; the G/A switch may have interrupted secondary interactions involving the loop residues.

Dimethyl sulfate (ME_2SO_4) is a traditional probe for single-stranded A and C residues measured by primer extension. There are 20 C and 15 A residues in the ferritin mRNA sequence probed. Only six were alkylated, four in the IRE and one in the base-paired flanking region (Fig. 2). However, the sites alkylated were not affected by the magnesium concentration over the range which has a large effect on phenCu cleavage (Fig. 1). Apparently, even in the most 'closed' conformation for a reagent the size of phenCu, dimethyl sulfate accessibility is still possible. Similar results were

obtained with the IVT of the ferritin subunit pseudogene except that the A of the G→A replacement in the hairpin loop was also alkylated. Two of the Me_2SO_4 sites are in a region at the base of the loop predicted to be base-paired and accessible to phenCu only at low magnesium concentrations. In contrast to the availability of certain A and C residues in the IRE stem to Me_2SO_4, the A and C residues just outside the IRE (Fig. 1) are predicted to be in a bulge but are not readily accessible to Me_2SO_4 or phenCu under any conditions. Such results confirm that Me_2SO_4 and phenCu are acting nonrandomly at specific sites and suggest that the unreactive nucleotides may be stacked in a helix formed by the bottom of the IRE and the flanking region even, if they are not always base-paired.

RNase V_1, an enzyme which recognizes base-paired or stacked structures had few sites of reactivity, although sites were seen in each of the regions of predicted base-pairing (Figs. 1A, 2). The overall stem structure of the IRE and the flanking regions is interrupted by two areas accessible to small reagents such as phenCu or Me_2SO_4; one is five base pairs from the hairpin loop (nculeotides 5, 6, 24, 25; Fig. 2) and the other is in the middle of the base-paired flanking regions (nucleotides 32, 35, 36).

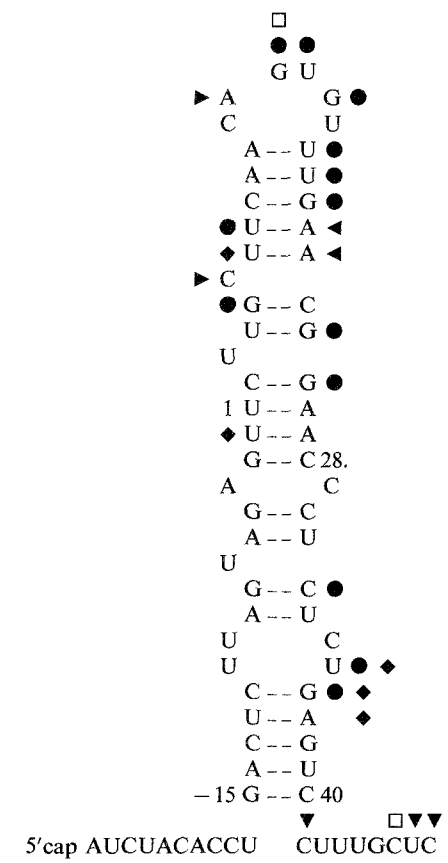

Fig. 2. Structure of the IRE. The figure is reproduced from Wang et al. (1990) with permission. The IRE is numbered 1-28 beginning at the 5′ end. Major cleavage or modification sites are indicated as follows: RNase V_1 (◆); RNase T_1 (□); phenCu (●); Me_2SO_4 (▼). Note that a secondary structure prediction with no hydrogen bond between G5 and C23 has essentially the same stability as with the hydrogen bond and explains the phenCu accessibility to G5. A55-mer containing the IRE and flanking region of the ferritin H chain in RNA has similar reactivity with the exception of a cluster of RNase V_1 sites in the flanking region around −10, indicating altered stacking or secondary interactions

The 55-mer, which in general displayed the same overall structure as the corresponding sequence in the context of native ferritin mRNA, had a notable difference in RNase V_1 reactivity. Although the 55-mer has some V_1 sites in the same location as ferritin mRNA in the region of the IRE sequence (nucleotides −1 and 8), the cleavage sites in ferritin mRNA in the flanking region (nucleotides 32, 35, 36) are absent from the 55-mer. Moreover, clustered RNase V_1 sites occur around nucleotide −10 which is opposite the sites in the full-length mRNA. Such an observation suggests that long-range interactions in the full-length mRNA cause an alteration in stacking or twisting which cannot form when the sequence is examined in isolation.

Base-paired regions which flank the IRE

Curiously, the IRE itself is not in the same position in all ferritin mRNAs (Theil 1990) as might be expected for a specific role in translation. In general, the IRE is closer to the cap than to the initiator AUG suggesting a role in translation initiation. Earlier studies with cycloheximide had shown the importance of initiation in the iron-regulated synthesis of ferritin (Schaefer and Theil 1981; Dickey et al. 1988). However, the IRE is rather far from the cap to inhibit initiaton (23-35 nucleotides, Table 1). If the IRE and the base-paired flanking region are considered together, the combined structure is only 8-17 nculeotides from the cap, close enough to influence translation initiation. The idea that the structure of the IRE and base-paired flanking region may be interdependent is supported by the observation that magnesium affects the conformation of both the IRE and base-paired flanking sequences (Fig. 1, nucleotides −4 and 30-37 in the flanking region and 5-27 in the IRE). Moreover, recent experiments showed that mov-

Table 1. The relation of the IRE and base-paired flanking (FL) region to cap of ferritin mRNA

Ferritin mRNA	$-\Delta G^0$ (kcal/mol)	Base pairs in FL	Nucleotides from cap	
			FL	IRE
IRE	3.7	—	—	—
Human H	19.2	9	17	31
Human L	20.8	15	12	27
Rat H	20.0	16	16	30
Rat L	24.6	14	11	31
Chicken H	36.7	17	10	33
Bullfrog H	12.8	9	10	26
Bullfrog H′(M)	18.3	10	8	23

Secondary structure predictions used the Fold program version 5.0. The references for the sequence data are: human H (Constanzo et al., 1986); human L (Santoro et al., 1986); rat H (Murray et al., 1987); rat L (Leibold and Munro, 1987); chicken H (Stevens et al., 1987); and bullfrog H and H′(M) (Dickey et al. 1987)

ing the functional IRE further away from the cap abolished iron regulation of expression in transfection experiments (Gossen et al. 1991). The secondary structure of the IRE flanking regions for H and H'(M) ferritin subunits from bullfrog was predicted reasonably well by recent computer programs as assessed by reactivity to phenCu, and RNases T_1 and RNase V_1 (Fig. 2).

To explore further the possibility that base-paired regions in other ferritin mRNAs also produce a combined structure near the cap, the predicted secondary structure of the IRE and flanking region was predicted from published sequences for ferritin mRNAs from several mammals and chicks; the distance of the IRE from the cap was 23–33 nucleotides. First, the results in Table 1 show that the combined flanking region and IRE have a much greater predicted stability than the IRE alone. For example, the 28-nucleotide IRE has a predicted ΔG^0 of 3.7 kcal/mol but the stability increases from -12–-37 kcal/mol to -153.4 kcal/mol, depending on the mRNA, simply by including the 18–34 nucleotides base-paired nucleotides of the flanking regions (Table 1). Secondly, the combined structure is only 8–17 nucleotides from the cap for seven different ferritin mRNAs (Table 1). An apparent exception to the idea that the flanking region plus IRE interact to place a stable structure near the cap is a cDNA recently described from *Xenopus* that contains an IRE 167 nucleotides from the cap, with eight base pairs in the flanking region and a $-\Delta G^0 = 11.7$ kcal/mol (Moskaitis et al. 1990). The significance of the *Xenopus* sequence to the argument is difficult to assess since several open reading frames are contained in the 167-nucleotide sequence between the cap and the IRE. Moreover, nothing is known about the mRNA itself, nor about regulation of the mRNA by iron. In contrast, six ferritin subunit mRNAs, two each from human, rat and bullfrog, are all known to be regulated by iron (reviewed in Theil 1990).

In general, the data obtained fit well with the hypothesis that the hairpin loop formed by the conserved IRE region is the site of recognition for the ferritin-mRNA-specific regulator protein, particularly since P-90 binds to the isolated IRE (reviewed in Theil 1990). However, the IRE and the base-paired flanking region could form an interdependent structure, indicated by the similar magnesium sensitivity, which brings the combined IRE and flanking region structure closer to the cap. If true, the flanking region may be needed to allow the IRE to block initiation when the regulator protein is bound.

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