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Ferritin mRNA: Interactions of iron regulatory element with translational regulator protein P-90 and the effect on base-paired flanking regions

(RNA/structure)

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ABSTRACT **The ferritin iron regulatory element (IRE), a conserved sequence of 28 nucleotides in a hairpin loop, is a conserved mRNA-specific translational regulatory element; flanking the IRE are regions of varying sequence, which form 9–17 base pairs close to the 5′ cap. P-90 is a ferritin mRNA-specific translation regulatory protein purified from animal liver and reticulocytes. To study the P-90–RNA interaction, protein nucleases (RNase S1 and T1) and chemical nucleases FeEDTA and/or 1,10-phenanthroline-Cu were used as probes of an oligonucleotide ($n = 55$), containing the IRE and flanking regions (FL), and natural ferritin mRNA. Footprints and "toeprints" showed that P-90 binding was confined to the stem and loop of the IRE itself. However, P-90 altered the structure of the flanking region by increasing base stacking or helicity (RNase V1 sensitivity). Comparison of the reactivity of the IRE and flanking regions in natural mRNA and the 55-mer showed that long-range interactions included protecting bulges, single-stranded, and stacked regions from protein nucleases as well as stabilizing the P-90–RNA interaction. Structural integration of the IRE with the base-paired flanking regions was indicated by common features of reactivity (periodic hypersensitivity to FeEDTA) and changes in the FL region caused by P-90. The increased secondary structure of the IRE flanking regions caused by P-90 binding to the IRE provides a likely mechanism for blocking initiation of ferritin mRNA translation, since the combined structure (IRE + FL) is so close (8–17 nucleotides) to the cap.**

Ferritin mRNAs, encoding the subunits of a large iron storage protein, contain a regulatory sequence in the 5′ noncoding region that is conserved among all vertebrates studied (reviewed in ref. 1). The conserved mRNA sequence, IRE or iron regulatory element, is a 28-nucleotide hairpin loop, which is required for self-regulation of ferritin synthesis by iron; IRE flanking sequences form a 9- to 17-base-paired stem near the cap (2, 3). Increased cellular iron, which is the best-known regulatory signal, increases ferritin synthesis and iron storage by recruiting ferritin mRNA for polyribosomes, while decreasing transferrin receptor synthesis and iron uptake by degrading the receptor mRNA (4–9). Concerted regulation of the mRNA for the two metabolically related proteins uses the same RNA–protein interactions combined with additional sequences (1, 7, 8, 10–14). Opposite effects of iron on translation of ferritin mRNA and destabilization of the transferrin receptor mRNA coincide with the different locations of the IRE, in either the 5′ noncoding region (ferritin mRNA) or the 3′ noncoding region (transferrin receptor mRNA).

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Cell-free extracts from animal cells (rabbit reticulocytes) reproduce the ferritin mRNA-specific block in translation initiation, independently of added iron (10), indicating the presence of trans-acting factors. Ferritin mRNA-specific trans-acting factors have not been detected in plants (5, 10);§ wheat germ extracts were used to assay a ferritin mRNA-specific translation regulatory protein (P-90) purified from rabbit reticulocytes or liver (18, 19). P-90 (also called FRP) binds oligoribonucleotides that contain the IRE sequence (18, 19); a protein with the molecular mass of P-90 has also been isolated from human liver by using a transferrin receptor IRE sequence as a ligand (20). The effect of iron on IRE–P-90 appears to be indirect; no form of iron has so far been found to specifically reverse the translational inhibition of ferritin synthesis in rabbit reticulocyte extracts (10). To date, neither iron nor hemin has been detected in P-90 (19–21). Interestingly, however, hemin reverses the P-90 inhibition of ferritin synthesis in wheat germ extracts (22); hemin can also alter the P-90–IRE interaction *in vitro* (23, 24).

Binding sites of the regulatory protein P-90 in the region of the IRE are explored in this report. The effect of P-90 on the cleavage patterns for both chemical and enzymatic nucleases, as well as on primer extension of native ferritin mRNA, showed that P-90 binding sites appear to be confined to the 28-nucleotide conserved sequence; similar results were obtained with a 55-mer containing the IRE and base-paired flanking regions (FL). The data suggest an integrated structure of IRE + FL involving a combined total of 19 base pairs. P-90 binding to the IRE increased the secondary structure in the FL (increased RNase V1 sensitivity), which may explain the inhibition of initiation of ferritin mRNA translation when P-90 is bound.

MATERIAL AND METHODS

RNA. Poly(A)$^+$ RNA from tadpole reticulocytes [ferritin mRNA is ≈10% measured by translation (5) or by hybridization with cDNAs] was analyzed with a primer that hybridized 25 nucleotides downstream from the IRE (2). A 55-mer, corresponding to the bullfrog erythrocyte ferritin H subunit, IRE + base-paired FL (2), was synthesized (25), purified by urea gel (12%) electrophoresis, stored with RNase inhibitor (5 Prime → 3 Prime, Inc.), and analyzed after ^{32}P-labeling with T4 RNase; homogeneity was 95%.

P-90 Binding and RNA Analysis. Rabbit liver P-90 (19) was bound to RNA by incubation in 5 mM Tris·HCl, pH 7.4/50

Abbreviations: IRE, iron regulatory element; FL, flanking region(s).

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§Ferritin in plants is a plastid protein encoded in a nuclear gene with a transit peptide (15), which may explain the difference in regulation of ferritin synthesis by iron (16, 17).

mM KCl/1.75 mM 2-mercaptoethanol for 20 min at 30°C; RNA was heated to 65°C and cooled slowly to 30°C before adding P-90. Previous samples were heated to 65°C and cooled to 4°C (2); a larger fraction of molecules had "closed" conformations after slow cooling. RNA concentrations were 33 μg/ml for poly(A)$^+$ RNA (n = 900 nucleotides) and 20–100 ng/ml for the ferritin IRE oligoribonucleotide (n = 55). The molar ratio of protein to RNA was 130–660:1 for the 55-mer and 70:1 for ferritin mRNA; molar ratios were calculated assuming a molecular weight of 3.5×10^5 for ferritin mRNA (26) and a concentration of 10% of the total poly(A)$^+$ RNA (5, 26). Cleavage with 1,10-phenanthroline-Cu and RNases T1 and V1 followed, in general, procedures previously described (2). RNase S1 was used at a concentration of 1.4×10^8 units/ml and FeEDTA (0.01, 0.1, or 1 mM) was prepared from solutions of ferrous ammonium sulfate and a 2-fold molar excess of EDTA (27). FeEDTA reactions were initiated by adding ascorbate to 1 mM and H_2O_2 to 0.06% and incubating at 30°C for 5 min; magnesium concentrations varied from 0 to 5 mM with no detectable effect. A "toeprint" (28) was obtained after primer annealing followed by P-90 binding, as described above, with primer extension at the usual temperature of 50°C. Calibration of gels for resolving cleavage fragments used dideoxynucleotide triphosphate sequencing reactions (mRNA) (2) or partial NaOH digestion (26 mM NaOH; 30 min at 90°C) and RNase T1 (2–10 units of RNA per ng; 50°C for 30 min) (55-mer). All experiments were repeated at least three times. Computer predictions used the Zucker programs (version 5.0) (29).

Mobility Shift Assays of P-90–RNA Interactions. P-90 was bound as described for footprinting except that 59 mM 2-mercaptoethanol was included in the incubation for the last 10 min with heparin (5 mg/ml). Bound and unbound RNA was separated in 4% acrylamide gels [125–140 V; 100 min (55-mer) or 8 hr (mRNA)]. Detection was by autoradiography (55-mer) or Northern blotting (mRNA) (10, 26).

RESULTS

Binding of the P-90 Regulator Protein to the 5′ Regulatory Region (IRE) in Natural Ferritin mRNA, Probed with Protein and Chemical Nucleases. The interaction of the ferritin mRNA-specific regulator protein P-90 (19) with natural ferritin mRNA was investigated. A "toeprint" (28) of P-90 shows that P-90 blocked reversed transcription beginning at the first residues in the IRE—i.e., C^{28}, A^{27}, A^{26}, and $G^{25,24}$ (Fig. 1*B*). The four sites observed suggest weak binding at the border of the IRE or the result of performing primer extension analyses at 50°C to minimize "stop" sites in the RNA.

Protein Nucleases. The IRE is a hairpin loop with several bulges and loops (2). Protein nucleases, such as RNases T1 and S1, only cleave sites readily in the hairpin loop at residues G^{14} and A^{13}. [G residues with low accessibility to RNase T1 (residues 5, 16, 20, and 25) are accessible to the smaller chemical nuclease 1,10-phenanthroline-Cu (2).] Both S1 and T1 sites were blocked by P-90 (Fig. 1*A*). P-90 reduced cutting of RNase V1 at U^7, a site within the footprint of the protein (Fig. 2*A*) and increased cleavage at sites in the FL at positions 35–38 and −11 (Fig. 1*A*) outside the footprint; RNase V1 cut helix/stacked regions (30).

Chemical Nucleases. Coordination complexes of redox-active metals have nucleolytic activity that complements protein nucleases as probes of structure (reviewed in ref. 31). The complementarity is particularly important in the case of the IRE ferritin mRNA because the complex secondary/tertiary structure (2) has few sites accessible to the bulky protein nucleases. The two chemical nucleases used to probe P-90 binding to ferritin mRNA were 1,10-phenanthroline-Cu (2) and FeEDTA (27, 32).

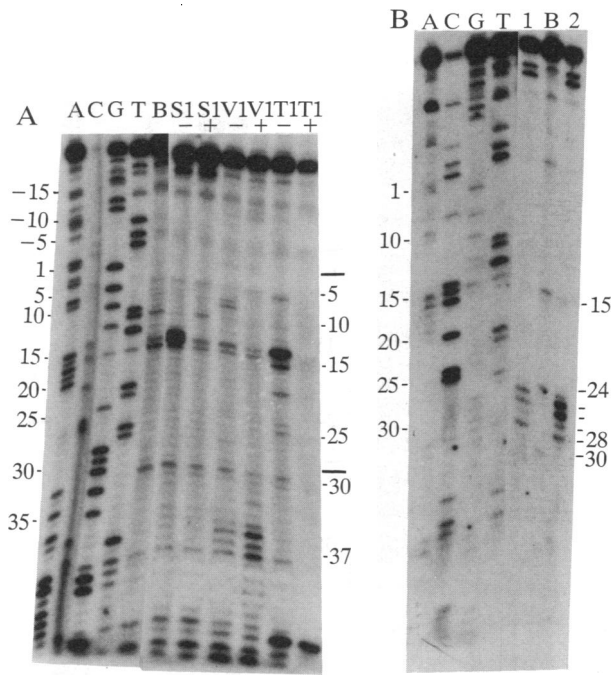

FIG. 1. Sites blocked by P-90 in the region of the conserved regulatory sequence (IRE) of the bullfrog ferritin H-chain mRNA, probed with protein nucleases, RNase V1 and T1, and reverse transcriptase (toeprinting). Fragments of natural ferritin mRNA [bullfrog reticulocyte poly(A)$^+$ mRNA where ferritin mRNA is ≈10%], produced by digestion in the presence or absence of P-90, were identified by primer-extension analysis using a labeled primer that bound 9 nucleotides downstream from the base-paired flanking regions of the IRE (21 nucleotides downstream of the IRE). Hybridizing of the primer to native mRNA before binding of P-90 and primer extension produced a toeprint (28) of P-90. Gels were calibrated by primer extension of natural ferritin mRNA in the presence of dideoxynucleotides and labeled as follows: lanes A, C, G, and T are the sequencing lanes and lane B is primer extension of native mRNA. Numbers 1–28 are the IRE, numbering from the 5′ end, with heavy bars indicating the limits (12–17 represent the hairpin loop); the base-paired flanking region between the IRE and the cap has negative numbers (the cap is −25). Note the sites blocked by P-90 at residues 13, 14, the top of the hairpin loop, and the increased cutting by V1 of the flanking region in the presence of P-90. The toeprint of P-90 is the beginning of the IRE. (*A*) RNases S1 (single-strand), V1 (helix or double strand), and T1 (single strand G); + indicates the presence of P-90. (*B*) Lanes 1 and 2, toeprint of P-90 in the presence of 0.5 and 0 mM magnesium; lane B contains no P-90.

Both sides of the stem and the hairpin loop of the IRE are blocked by P-90 (Fig. 2). No sites of P-90 binding were observed outside the IRE. The decrease in cutting by FeEDTA at residues 32–35 corresponds to a P-90-induced change in structure (see also results with RNase V1; Fig. 1*A*). Sites blocked by P-90, indicated by reduced cleavage with 1,10-phenanthroline-Cu, occurred at positions $G^{5,14,16,20,24,25}$ and at U^{15} (Fig. 2*B*).

Both the IRE and FL displayed an unusual feature in the FeEDTA reaction: hypersensitivity positions in both the IRE and FL (Fig. 2*A*). The reaction was carried out at 30°C, and the effect was the same at 0.01, 0.1, or 1 mM FeEDTA.

Typical single- and double-stranded regions of RNA do not show hypersensitivity to FeEDTA (32). In ferritin many of the mRNA hypersensitive sites are regularly spaced 10–11 nucleotides apart (Fig. 2) at IRE nucleotides numbered 5, 6, 7, 14, 15, 23, 24, and 25 and at FL nucleotides −6, −7, 35, and 36. Additional hypersensitive sites are U^{19}, G^{20}, and C^{30}, which is also a primer-extension pause site (Fig. 2). Such results suggest that the IRE + FL may have helical features or regions of tertiary structures.

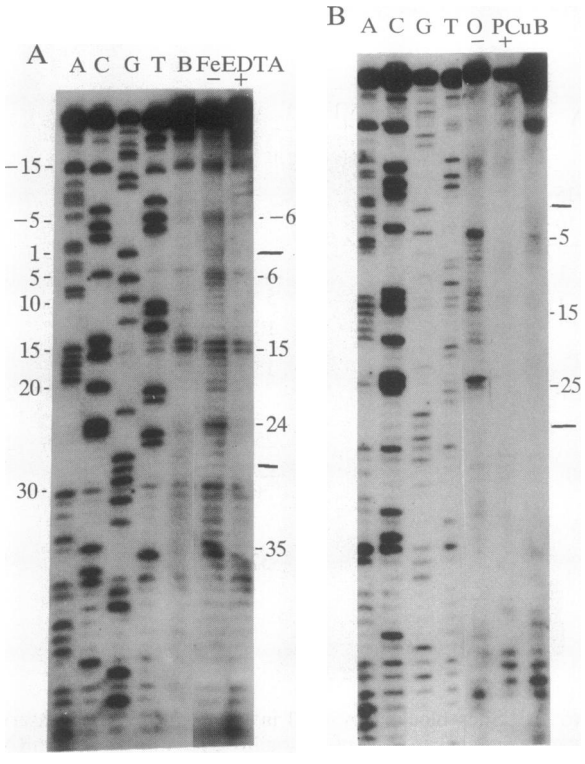

FIG. 2. Sites blocked by P-90 in the region of the conserved regulatory sequence (IRE) of the bullfrog ferritin H chain, probed with chemical nucleases, FeEDTA, and 1,10-phenanthroline-Cu (OPCu). Fragments produced by digestion of natural ferritin mRNA with chemical nucleases at 30°C were analyzed by primer extension as described in Fig. 1. P-90 bound within the conserved IRE sequence and blocked every OPCu site within the sequence (*B*). Similarity of structure in the IRE and FL is indicated by the variable intensity of cleavage (primer-extension stop sites); the results were the same with 0.01, 0.1, or 1 mM FeEDTA. Lanes A, C, G, and T are the sequencing lanes and lane B is primer extension of native mRNA, which often shows a pause site at positions 14 and 15. Numbers 1–28 are the IRE, numbering from the 5′ end, with heavy bars indicating the limits (12–17 represent the hairpin loop); the base-paired flanking region between the IRE and the cap has negative numbers (the cap is −25). + indicates the presence of P-90. (*A*) FeEDTA. (*B*) OPCu.

Effect of Sequence Context on the Structure of the IRE and P-90 Binding for Ferritin mRNA. Functional studies have suggested that sequence context should affect the iron-independent translation of ferritin mRNA (10). The structure of a 55-mer containing the H-chain ferritin mRNA 5′ (IRE + FL) was compared to natural ferritin mRNA, probing with protein and chemical nucleases, with and without P-90.

Long-range interactions with the IRE + FL in natural ferritin mRNA are indicated by the increase in accessibility of RNase V1 and T1 sites in the 55-mer. In the case of RNase V1, 10 additional sites are available in the FL of the 55-mer; only the FL base pairs at the base of the 55-mer were resistant (Fig. 2). Two additional G residues were readily cleaved by both RNase T1 and 1,10-phenanthroline-Cu in the 55-mer; examples are G^{-5} and G^{-7} (Fig. 3). Only 1,10-phenanthroline-Cu cleaved G^{-5} and G^{-7} natural ferritin mRNA (2).

The footprint of P-90 on the 55-mer is in the IRE as in full-length ferritin mRNA, although the footprint was less clear (Fig. 3), probably because of the lower stability of the P-90–55-mer complex (Fig. 4). Enhanced sensitivity of FL to RNase V1 upon P-90 binding was also preserved in the 55-mer (Fig. 3); the slight shift of sites closer to the IRE (nucleotides 30 and 32–35) may indicate "fraying" at the ends of the 55-mer, which are also resistant to RNase V1.

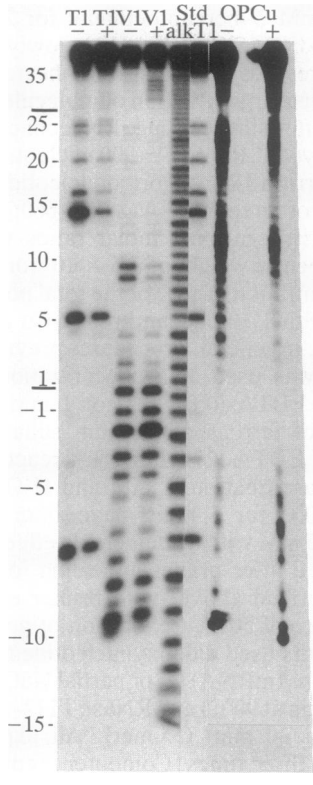

FIG. 3. Structure in solution of an oligonucleotide (n = 55) containing the ferritin mRNA conserved regulatory sequence (IRE) and base-paired flanking regions. The 5′ ^{32}P-labeled RNA was incubated with protein or chemical nucleases as described and the fragments in the partial digests were separated by electrophoresis in denaturing acrylamide gels; ≥95% of ^{32}P migrated with the 55-mer in uncleaved samples. Note the large number of RNase V1 (helical) sites in the flanking region, which is predicted to be base-paired and the additional V1 sites created by binding of P-90. Only G residues predicted to be in bulges or loops were readily accessible to RNase T1; −2 site is marginally enhanced by P-90 binding. The IRE is numbered 1–28, starting from the 5′ end and is indicated by solid bars (12–17 represent the hairpin loop); the flanking sequence upstream from the IRE is indicated by negative numbers. Note that when looking from the top to the bottom of the figure, the numbers are reversed when compared to the results obtained by primer extension in Figs. 1 and 2, because of the labeling of the 5′ end of the oligonucleotide before cleavage. Footprints with the 55-mer have a high background because of the low stability of the P-90–55-mer complex (see Fig. 4). Std alk (NaOH) and Std T1 are digests under denaturing conditions used to calibrate the gel. OPCu, 1,10-phenanthroline-Cu.

Stability of the P-90–RNA complex ("mobility shift") was much greater for natural ferritin mRNA than the 55-mer (Fig. 4). A 3-fold molar excess of P-90 was sufficient to shift 100% of the full-length ferritin mRNA. A 3-fold molar excess shifted only 25% of the 55-mer. 2-Mercaptoethanol at 100 mM, known to enhance binding (33), or magnesium at 3 mM did not change the results detectably. In addition to the specificity of P-90 for the IRE shown previously (19), P-90 did not complex an IRE + FL oligodeoxyribonucleotide (n = 55), an RNA oligomer of the hairpin loop of murine 5S RNA (E. S. Maxwell, personal communication) or globin mRNA in the same poly(A)$^+$ RNA 30.

DISCUSSION

Regulation of ferritin synthesis by control of mRNA function is conserved among all vertebrates studied (reviewed in ref. 1). Ferritin mRNA provides a unique opportunity to understand structure–function relationships in eukaryotic mRNA

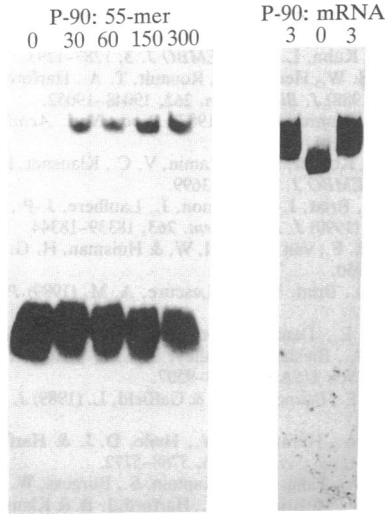

FIG. 4. The relative stability of complexes of P-90 with oligonucleotides and natural mRNA. The oligonucleotide, a 55-mer with the sequence of IRE and base-paired flanking region of tadpole ferritin mRNA H chain, was labeled with ^{32}P at the 5′ end. Natural ferritin mRNA, ≈900 nucleotides long (26), was detected after binding and electrophoresis by transfer to nylon membranes and hybridization to a ^{32}P-labeled ferritin H-chain cDNA (pJD5F12) (26). The alteration of electrophoretic mobility of RNA by P-90, analyzed in 4% acrylamide gels, was measured after binding in 50 mM KCl/5 mM Tris·HCl, pH 7.4/1.75 mM 2-mercaptoethanol at 30°C; various magnesium concentrations up to 3 mM or 2-mercaptoethanol up to 100 mM had no effect on the P-90–55-mer interaction. Examination of P-90 specificity, beyond that used during isolation and characterization (19), included absence of P-90 binding to an RNA oligomer of a hairpin in murine 5S RNA, to an oligodeoxyribonucleotide with the IRE sequence and to globin mRNA. Note the small fraction of oligonucleotide that remains bound to P-90, even in a 300-fold molar excess of protein, compared to the natural mRNA, which remained completely bound with only a 3-fold molar excess. (*A*) Oligonucleotide. The molar ratio of protein to RNA is indicated above each lane. (*B*) Natural ferritin mRNA. The molar ratio of protein to RNA is indicated above each lane.

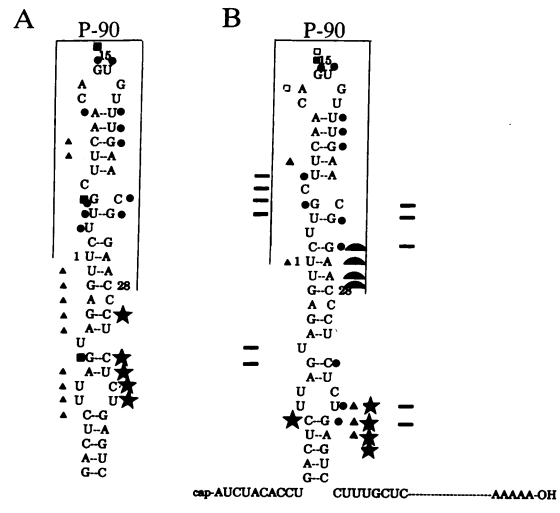

FIG. 5. Structure of the ferritin mRNA 5′ regulatory region (IRE + base-paired FL) with P-90: the effect of context. The IRE is shown stacked on a base-paired flanking region; secondary structure, but not sequence, is conserved in the flanking region. Sites blocked by P-90, indicated by a reduction in accessibility to chemical or protein nucleases (see Figs. 1–3) upon P-90 binding, are restricted to the IRE and are similar for both the oligonucleotide (n = 55) and natural ferritin mRNA (n = 900). Structural integration of the IRE and FL was indicated by the change in base stacking or helicity (RNase V1 sensitivity) in the flanking region when P-90 bound to the IRE (Figs. 1*A* and 3) and by the regularity of FeEDTA hypersensitivity in both regions (Fig. 2*A*). The structure of the regulatory region in the 55-mer (IRE + FL) was much closer to that predicted by computer analysis than the same sequence in natural mRNA. Long-range mRNA interactions with the IRE + FL are shown by a greater deviation from predictions for mRNA vs. 55-mer (compare RNase V1 and T1) and by greater P-90/mRNA stability (see Fig. 4); such observations support the functional effects previously observed for the 3′ untranslated region (10, 12). (*A*) The 55-mer. (*B*) Natural ferritin mRNA. P-90 binding (footprint) is indicated by a solid line around the IRE. □, RNase S1; ■, RNase T1; ●, 1,10-phenanthroline-Cu; ▲, stacking or helix-specific nuclease RNase V1; ★, RNase V1 sites enhanced by P-90 binding; , toeprint of P-90; ▬, regularly spaced sites of hypersensitivity to FeEDTA (increased solvent accessibility) in the absence of P-90.

because of identification of a conserved mRNA regulatory element, the IRE, and purification of a specific translational regulator protein, P-90 (19). IRE-binding proteins also occur in a variety of vertebrates and invertebrates (34). Studies of RNA–regulator protein interactions have included 5S rRNA (35), prokaryotic ribosomal protein mRNAs and tRNAs (36–38), phages T4 and R17, and human immunodeficiency virus (39–44). Certain RNA regulator proteins bind specific sites in DNA as well—e.g., TAT (43, 44) and TfIIIA (35)—but the role of P-90 in gene transcription is unexplored.

P-90 binding to the 5′ regulatory region of ferritin mRNA appeared limited to residues in the IRE (Figs. 1–3 and 5), although the protein is large (90 kDa). The "toeprint" began at the 3′ end of the IRE, while the footprint extended along both sides of the stem and included the entire conserved sequence; reactivity of 14 of the 28 nucleotides in the IRE could be analyzed and is illustrated in Fig. 5. The predicted amino acid sequence of an 87-kDa IRE-binding protein had none of the common structural motifs of nucleic acid binding proteins (21), suggesting unusual protein–mRNA interactions.

The computer-predicted hairpin loop and base-paired FL are generally compatible with mapping studies in solution for ferritin mRNA (2) and the 55-mer (Fig. 5). However, the structure of the base pairs in the IRE between residues 7 and 22 next to the hairpin loop is particularly deviant because residues $A^{22,23}$ are always accessible to dimethyl sulfate even at high magnesium concentrations (5 mM) when 1,10-phenanthroline-Cu cleaves little. Bulges in A^{-3} and C^{29} are always inaccessible and "stem" residues U^{19} and G^{20} display variable sensitivity to cleavage by FeEDTA (Fig. 2*A*) and are cut by 1,10-phenanthroline-Cu at low magnesium concentrations (2). Reversing the potential base pairs C^9·G^{20}, A^{10}·U^{19}, and A^{11}·U^{18} decreases binding of regulatory protein(s) by 10-fold (45). The importance of primary sequence to secondary/tertiary structure is clear not only for the IRE but is also exemplified for T4 DNA polymerase (gp43) binding to RNA (46). The apparent contrast for the IRE compared, e.g., to R17 (41) or QB (42) could be differences in RNA–protein interaction or the analyses used.

Structural integration of the IRE and FL is indicated by common features of reactivity to FeEDTA (Figs. 2 and 5), which suggest tertiary structure (47). [All ferritin mRNAs currently known have an FL and IRE (1, 3).] P-90 binding to the IRE also alters the structure of the FL, indicated by increased sensitivity to RNase V1 (stacking or helicity) for both the 55-mer and full-length ferritin mRNA (Figs. 1 and 3). Other regulator proteins decrease stacking of cognate mRNA (48), but increases have not been commonly observed.

Subtle features of the structure are dependent on the context of the sequence, although the general structure of the IRE + FL is independent of sequence context (Figs. 1–3 and 5). For example, more sites for RNases V1 and T1 were accessible in the 55-mer, suggesting a sequestering of the IRE + FL or long-range interactions in full-length ferritin mRNA. Interactions between the 3′ and 5′ noncoding regions of

ferritin mRNA had been suggested (10). An additional difference between the 55-mer and full-length ferritin mRNA is the stability of the IRE–P-90 interaction measured by mobility shift during electrophoresis (Fig. 4). Two types of explanations can account for the apparent lower stability of the P-90 interaction with 55-mer compared to full-length mRNA. First, the fraction of the 55-mer in the binding conformation could be smaller or less readily formed from other conformers; conformational heterogeneity of IRE + FL is clear (2). Separation of conformers has been suggested for the R17 RNA–coat protein interaction (49). Alternatively, sequences outside the IRE + FL could also bind P-90 or could stabilize the P-90–IRE complex by caging.

The mechanism by which the IRE–P-90 complex inhibits translation of ferritin mRNA needs to explain the key role of initiation in the regulation of ferritin synthesis (4, 10, 50–52). Proteins that regulate prokaryotic mRNA generally block the Shine–Dalgarno sequence and/or the initiation codon (53); in ferritin mRNA, the IRE + FL is distant (≈100 nucleotides) from the initiator AUG (1). Capture of capped eukaryotic mRNAs for initiation, unnecessary for the tethered transcription complexes of prokaryotes, involves specific cap-binding proteins. If the IRE were close to the cap, the secondary structure in the stem could interfere with cap binding and initiation, as shown for structures ≈15 nucleotides from the cap (e.g., see refs. 54 and 55) and proposed for regulating ferritin synthesis (51). The IRE is fairly far from the cap of ferritin mRNAs [23–35 nucleotides (1)]. However, the combination of the IRE and the base-paired flanking regions is close to the cap (8–16 nucleotides) in seven ferritin mRNAs studied (3). Thus, the functional regulatory structure in the 5′ noncoding region of ferritin mRNA could be the combination of the IRE + FL.

Consider the known features of the IRE and FL. (*i*) The two regions appear to be structurally integrated (magnesium-dependent conformational changes and periodic hypersensitive sites to FeEDTA occurred in both IRE and FL) (ref. 2; Figs. 2 and 5). (*ii*) P-90 binding to the IRE increased helicity (RNase V1 sensitivity) in the FL, close to the cap (ref. 5; Fig. 1*A*). (*iii*) The calculated $-\Delta G^\circ$ is only 3.7 kcal/mol (1 cal = 4.189 J) for the IRE but 13–35 kcal/mol for the IRE + FL (3). (*iv*) The IRE alone, without the flanking region, exhibited iron-regulated expression only when the sequence was 8 nucleotides from the cap (56). Such observations suggest that P-90 binding to the IRE converts the conformationally variable IRE + FL to a more stable structure near the cap, which inhibits translation initiation.

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