An mRNA Loop/Bulge in the Ferritin Iron-responsive Element Forms in Vivo and Was Detected by Radical Probing with Cu-1,10-phenanthroline and Iron Regulatory Protein Footprinting*

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Messenger RNA (mRNA) regulatory elements often form helices specifically distorted by loops or bulges, which control protein synthesis rates in vitro. Do such three-dimensional RNA structures form in vivo? We now observe formation of the internal loop/bulge (IL/B structure) in the IRE (iron-responsive element) of ferritin mRNA expressed in HeLa cells, using radical cleavage with Cu-phen (Cu-1,10-phenanthroline), and protection of the loop/bulge by the regulatory protein (IRP), expressed by cotransfection. Cu-phen, a metal coordination complex (MC) selected because of binding and cleavage at the IL/B in solution, recognized the same site in mRNA in HeLa cells. Endogenous reductants apparently substituted for the sulphydryl activation of Cu-phen cleavage in solution. Selective RNA IL/B recognition by Cu-phen in vitro is emphasized by resistance to cleavage of a mutated, IL/B IRE in ferritin mRNA. Development of small MCs even more selective than Cu-phen can exploit three-dimensional mRNA or viral RNA structures in vivo to manipulate RNA function. Formation in vivo of the IL/B in the ferritin IRE, which is associated in vitro with greater repression than single IRE structures in other mRNAs, likely contributes to larger derepression of ferritin synthesis in vivo triggered by signals for the IRE/IRP system.

Specific messenger RNA (mRNA) structures, identified in solution and exemplified by a set of noncoding mRNA regulatory elements (IRE)† and proteins (IRP) (1), control rates of protein synthesis (mRNA translation) or mRNA stability. The iso-IRE family, and the two related phosphorylatable regulatory proteins IRP1 and IRP2, are found in mRNAs encoding proteins of iron and oxygen homeostasis and constitute a natural set of combinatorial mRNA/protein interactions that give quantitatively different responses to cellular iron and oxygen signals (1, 2).

Do mRNA tertiary structures such as the ferritin mRNA IRE form in vivo? Metal coordination complexes (MCs), protein nuclease, and alkylating agents have all been used to analyze mRNA solution structure, but MCs are particularly sensitive to the tertiary structure or shape of the RNA binding site (3–7). MCs have specific geometry and relatively rigid shapes contributed by small organic molecules, coordinated to a metal ion (Fig. 1). If the metal is redox active at physiological conditions, as it is for copper, radical cleavage should occur at the MC/RNA binding site to report on the RNA shape. In solution, Cu-phen binds at RNA loops, bulges, and helix distortions in tRNA, mRNA, and rRNA that are indistinguishable for most alkylating agents and too small for access by protein nucleases (3–7). We now show that Cu-phen, an MC, detects the tertiary structure in ferritin mRNA in HeLa cells selectively distinguishing the wild type IL/B from a mutant IRE. Such results link the IRE loop/bulge structure observed directly in solution (6, 8–12) with the predictions from physiological effects of iron on ferritin and other mRNAs that use IRE/IRP regulation (1, 2) and lay a foundation for studying the behavior of other mRNA structures at the redox conditions in living cells (13).

EXPERIMENTAL PROCEDURES

Cloning

Ferritin mRNA Expression Plasmids—Plasmid pcDNA3.1-Del-1DV Myc-His (+) (Invitrogen) with sequences deleted between the vector transcription start and HindIII, encoded full-length frog H ferritin mRNA and was derived from 1DV (14) using the HindIII site at the 5′-end and the EcoRI site at the 3′ end. pcDNA3.1-Del-1DV/Myc-His (+)ΔU6 has the same ferritin mRNA insert with deletion of U6 in the IRE, to convert the internal loop/bulge of the Fer-IRE to a C bulge (11).

IRP1 Expression Plasmid—IRP1 was encoded in pcDNA3.1-Del-IRP1/Myc-His (+), which contained the 5′-untranslated region and the coding region of human IRP1 DNA from pGEM-hIRF (from the ATCC) (15), amplified by PCR, and inserted into the Xhol and HindIII sites.

IRP Activity (RNA Binding) and Immunodetection in Cell Extracts

RNA Binding—RNA (1 ng, 30 nucleotides encoding the ferritin IRE regulatory sequence, 5′-32P-labeled, was heated in 40 mM Hepes Na, pH 7.2, 100 mM KCl to 85 °C for 5 min and slowly cooled to 25 °C after incubation with protein (15 μg of cell protein in the extract in 60 μl KCl, 24 μM Hepes Na, pH 7.2, 4 mM MgCl2, 5% glycerol) for 30 min at 10 °C, with or without 2% mercaptoethanol (10); the RNA-protein complex formed with each extract is shown.

Immunodetection—Protein in cell extracts (150 μl) NaCl, 5 mM; EDTA, 10 mM Tris, pH = 7.4, 1% Triton X-100, and protease arrest (Genetotechnology) resolved in SDS gels (15 μg of protein/lane), were transferred to nitrocellulose and detected with c-Myc antibodies (Invitrogen) and horseradish peroxidase-IgG (Pierce).

Transient Transfections

HeLa cells (8 × 10⁶ cells/well) cultured in Dulbecco’s modified Eagle’s medium + 10% fetal bovine serum for 16–18 h at 37 °C, 5% CO2 were incubated with plasmid DNA (1 μg/well), PLUS (Invitrogen) reagent (6 μl PLUS/well, and 4 μl of LipofectAMINE™/well (Invitrogen) LipofectAMINE™/well in six-well plates. Two micrograms of IRP1 for “footprinting” on the IRE, HeLa cells were cotransfected with plasmids encoding ferritin and IRP1 as described for RNA expression, except the cells (1 × 10⁶ cells/plate) were plated in a 10-cm plate the day before transfection and cotransfected with 1.5 μg of pcDNA3.1-Del-1DV/Myc-His (+) ferritin
mRNA and 5.0 μg of pcDNA3.1-Del-IRP1/Myc-His(+) or with pcDNA3.1/Myc-His/LacZ plasmid DNA encoding β-galactosidase in the LacZ sequence, as a control for cotransfection (cotransfection frequency (β-galactosidase) ~20%).

**Cu-phen Targeting in Cells**

48 h after transfection and 1 h of culture in prewarmed Opti-MEM (Invitrogen), Cu-phen was added (to 225 μM) for 30 min. Cells were rinsed with phosphate-buffered saline, collected by scraping, and washed one time with phosphate-buffered saline; cell pellets were frozen at ~80 °C. Cell survival (trypan blue exclusion), ~60% with Cu-phen, was likely diminished because of cutting tRNA. (Survivals should increase in the future with identification and use of more specific MCs.)

**RNA Cleavage Analysis**

Sites of Cu-phen binding/cleavage were identified by the increase in shorter cDNA fragments (11, 12) and quantitated by a PhosphorImager (Molecular Dynamics) with ImageQuant software.

RNA dissolved in water, and stored at ~80 °C until use, was heated at 65 °C for 3 min, quickly cooled to 25 °C, and reacted in 25 mM Hepes-Na, pH 7.2, 40 mM KCl, 18 μM Cu-phen, and 0.05% mercapto-propionic acid (to reduce copper); after 5 min at 25 °C, 2,9-dimethyl-1,10-phenanthroline was added to 7 mM as a “quench,” with the same cDNA analysis used with RNA isolated from HeLa cells. Cu-phen sites in the IRE of ferritin mRNA in HeLa cells are the same as an IRE template for32P-labeled cDNA synthesized with ethanol, was the template for32P-labeled cDNA synthesized with a labeled primer; bound 40 nucleotides downstream from the ferritin IRE. Mixtures of cDNA were resolved on calibrated urea-acrylamide gels as described previously (16).

**RESULTS**

The ferritin IRE (Fer-IRE) structure was selected for the study of mRNA structure in vivo (in cultured HeLa cells), because the IL/B of the Fer-IRE contributes to selectivity of regulatory protein binding in solution (10). In addition, Cu-phen binds selectively in solution, discriminating between the IL/B and a C-bulge (11). High IRE structural specificity in solution is also indicated by the specific MC site that is blocked by magnesium and is conformationally sensitive to protons (9, 11, 12). Cu-phen has the added advantage of being relatively hydrophobic and should cross cell membranes. HeLa cells were chosen for the study because they display the IRE-mRNA regulation typical of many cell types (17).

When Cu-phen was added to cultures of HeLa cells in which ferritin mRNA was expressed at high levels from a transfected plasmid in HeLa cells, the IRE was recognized and cleaved by Cu-phen (Figs. 1B and 2A and C). Cu-phen cut the RNA at the end of the IRE-flanking region helix. Cu-phen recognizes the IL/B region of the ferritin IRE, modified from Fig. 3 in Ref. 9.

Sites of Cu-phen binding (mRNA cleavage) in the IRE were determined by reverse transcriptase run-off using cleaved mRNA recovered from the treated cells as the template, and a primer binding near the IRE (Fig. 2). cDNA fragments were separated in DNA sequencing gels calibrated with cDNAs synthesized from intact mRNA and mixtures of deoxy- and dideoxynucleotide triphosphates (see Refs. 11 and 12 as examples). The background reaction, due to reverse transcriptase pausing, is ~4 times lower than reverse transcriptase run-off from RNA fragments produced by Cu-phen cleavage.

Several sites outside the IL/B of the ferritin IRE were Cu-phen binding sites in HeLa cells, but not in the in vitro transcripts of the ferritin mRNA, such as residue ~13 (Fig. 2C) at the end of the IRE-flanking region helix. Cu-phen recognizes the ~13 site not only in ferritin mRNA transcripts in HeLa cells.
cells, but also in native ferritin mRNA (polyA^+) that was synthesized in frog reticulocytes of the embryonic (tadpole) lineage, but analyzed in vitro with specific primers (11, 16). Such mRNAs likely differ from in vitro transcripts in the structure of the cap, which suggests that the cap structure influences the conformation of the ferritin IRE flanking region. Previous structure/function links observed between the mRNA cap and the ferritin IRE are conserved length, conserved sequences, and regulatory effects of altering the distal sequence. Regulation of ferritin synthesis, for example, was much more sensitive in different IRE-mRNAs had quantitatively different responses (3). Selectivity of Cu-phen footprint of a regulatory protein (IRP1) over the IL/B of the ferritin mRNA IRE in HeLa cells. In cells with plasmid encoded IRP1, no Cu-phen binding to the Fer-IRE was observed above background at the IL/B sites in HeLa cells, showing that IRP1 protected the IL/B in vivo. A, cDNA fragments synthesized using RNA from cells cotransfected with vectors encoding ferritin mRNA and either IRP1 or β-galactosidase. B, characterization of IRP1 expressed in HeLa cells transfected with DNA with or without IRP1 coding sequences. Left, immunoblot of Myc-tagged IRP1 protein in HeLa cell extracts (see “Experimental Procedures”). Lane 1, control without plasmid. Lane 2, transfection with pCDNA3.1-Del-IRP1/Myc-His(+). Right, IRP activity (RNA binding, see “Experimental Procedures”). Lane 1, control, without plasmid, showing endogenous IRP activity. Lane 2, protein from transfection with pCDNA3.1-Del-IRP1/Myc-His(+); results are from a single transfection. C, densitometry of the cDNA from cells treated with Cu-phen after cotransfection with DNA encoding ferritin mRNA and IRP1 (G7 and G26, G27 = background level). D, densitometry of the cDNA from cells treated with Cu-phen after cotransfection with DNA encoding and expressing ferritin mRNA and β-galactosidase (G26, G27 cleavage above background). Data are representative of results from three independent transfections and RNA analyses.

DISCUSSION

Observation of the mRNA-specific IL/B in ferritin IRE in vivo (Figs. 2 and 3), using the MC reporter Cu-phen, confirms the tertiary structure of the mRNA element observed in solution (9, 11, 12). Specificity of IRE structure in each mRNA is the basis for the natural combinatorial mRNA regulation displayed by the IRE/IRP system (1). In the future, combinatorial chemical synthesis can improve selectivity and recognition of control structures such as the IRE (1), AU-rich elements (22), and internal ribosome entry sites (23) so that RNA structure may be further studied and possibly manipulated in vivo. (Note that since Cu-phen is an “off-the-shelf” reagent, despite its IRE/IL/B selectivity, Cu-phen also cleaves tRNA (4), and a site at the base of the BIV-tar element, inserted into β-galactosidase mRNA in HeLa cells.)

Targeting three-dimensional mRNA structure in vivo complements the manipulations of three-dimensional protein structure in cells, a common strategy for understanding protein function or for developing medical therapies. RNA targeting shares high cell specificity with proteins, but has the added advantage of lower copy number and target size.

Understanding RNA function in vivo is only one potential use of MCs targeted to specific RNAs. Inactivation of viral mRNAs or prophylactic ferritin synthesis for protection from iron toxicity are other possible uses of MCs. The selectivity of targeting IRE structure with small molecules is matched by the natural selectivity of IRP/IRE interactions in different mRNAs. For example, the iron induction of proteins encoded in different IRE-mRNAs had quantitatively different responses to iron in the same tissue of whole animals (2, 24). Induction of ferritin synthesis, for example, was much greater than m-aconitase synthesis. (Effects of iron on m-aconitase regulation were small enough to have gone unnoticed until the IRE sequence was detected, whereas mRNA regulation of ferritin had been known long before the RNA sequence was determined (25, 26).) The differences between the iron-responses of ferritin and m-aconitase mRNA in vivo were attributed in part to the behavior of the IL/B in the ferritin IRE in solution and cell-free protein synthesis (10, 11). Linking the solution studies on IRE structures (e.g. Refs. 8–12, 14–16) and physiological regulation of proteins encoded in IRE-mRNAs by the observation of the IL/B structure in vivo (Figs. 2 and 3) emphasizes the importance of tertiary structure in mRNA regulatory elements.

REFERENCES

mRNA (IRE) Structure Probed in Vivo with Cu-phen