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Structure and Function of the Iron-Responsive Element from Human Ferritin L Chain mRNA[1]

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We report the cloning and functional characterization of the iron responsive element (IRE) of human ferritin light (L) chain mRNA from a cDNA library of primary human T lymphocytes. Comparison of this palindromic cDNA element to the IRE predicted from the reported genomic sequence revealed significant differences, resulting in a stem-loop structure with lower stability than the IRE of the heavy (H) chain mRNA. Nevertheless, the L subunit IRE mediated efficient binding of the iron regulatory protein (IRP) in a manner comparable to that of human ferritin H chain mRNA *in vitro*. In accordance with previous observations on H form transcripts, the *cis*-acting regulatory IRE motif of human ferritin L chain mRNA was capable of repressing translation under iron deprivation but permitted mobilization of the transcripts into polysomes following iron repletion *in vivo*.

The ubiquitously expressed iron storage protein ferritin forms a hetero-oligomeric protein shell composed of 24 ferritin L and H chain subunits which serves as an iron storage repository that avoids any toxic effects of the element on cells (1). Intracellular iron levels regulate the expression of ferritin chains at the step of translation initiation (2,3) by means of an iron responsive element (IRE), a stem-loop structure located in the 5′ untranslated region (UTR) of ferritin mRNAs (4). In iron depleted cells, initiation of ferritin biosynthesis is blocked by the interaction of the iron regulatory protein (IRP) with this *cis*-acting element. In contrast, the formation of the IRE-IRP complex is diminished at high iron concentrations, allowing recruitment of ferritin mRNA onto ribosomes, which in turn leads to efficient ferritin production (5-7). As determined by numerous mutagenesis experiments (for review see 7 and references therein), the IRE "consensus" structure comprises a bipartite stem, the bottom half of which can be of variable length and the top helix consisting of five base pairs. These two helices are interrupted by an invariant unpaired cytidine residue (and sometimes additional bulged nucleotides) 5′ to the 6 member loop which has the sequence 5′-CAGUGN-3′. With respect to this critical sequence element, a single point mutation in the ferritin L chain IRE has been shown to provide the molecular basis for the upregulation of ferritin synthesis in patients affected by the "hyperferritinemia-cataract syndrome", a hereditary disorder unrelated to iron overload (8).

Up to now, the sequence and predicted structure of the complete IRE in human ferritin L subunit mRNA had only been inferred from analysis of a genomic clone, no *in vitro* or *in vivo* experiments had been reported with this element despite its significance in human physiology (5). Therefore, in the current study we functionally characterized a newly isolated cDNA sequence containing the full length human ferritin L chain IRE, which deviates from the previously reported sequence and structure.

MATERIALS AND METHODS

cDNA library preparation, screening, and identification of human ferritin L chain cDNA. Non-stimulated human T lymphocytes were obtained from the peripheral blood of healthy volunteers by Ficoll-Paque and Percoll (Pharmacia) gradient centrifugation. Poly(A)$^+$ mRNA was isolated with Oligotex-dT beads (Qiagen) according to the specifications of the manufacturer. First and second strand cDNA synthesis starting from 5 μg of mRNA, ligation of primer adapters,

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Abbreviations: cDNA, complementary DNA; IRE, iron-responsive element; IRP, iron regulatory protein; mRNP, messenger ribonucleoprotein; UTR, untranslated region.

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reconstitution of phage lambda-ZAPII XR and packaging (Stratagene) were carried out basically as described in the vendors manual. 9×10^5 independent phages with an average insert length of 2 kb were obtained and amplified to a titer of 3.3×10^{10} plaque forming units. This library was subjected to a differential screening procedure that facilitates the detection of translationally controlled mRNAs upon T cell stimulation (Mikulits et al., unpublished). Among the clones characterized by this protocol, a 0.83 kb cDNA representing the human ferritin L chain mRNA was isolated. A 264 nucleotide long *Sma*I-*Stu*I fragment comprising the 5′ UTR (185 bp) with the putative IRE and part of the N-terminal coding sequence (79 bp) was subcloned into the *Sma*I site of pGEM3Zf(−) (Promega). The integrity of this construct, termed hferL-264, was verified by sequencing (9).

Tissue culture. Human HeLa cervical carcinoma cells were grown in Dulbeccos Modification of Eagles Medium plus 10 % fetal calf serum. Logarithmically growing cells were either harvested directly (L) or incubated for 24 hours with 100 μM desferrioxamine (Des), a specific iron chelator, or supplemented with 50 μg/ml ferric ammonium citrate (Fe) for an additional 4 hours after washing twice with phosphate buffered saline to induce iron overload.

Assay for mRNA binding activity of iron regulatory protein (IRP). RNA-protein interactions were essentially analyzed as described (10). Briefly, cytoplasmic extracts from HeLa cells grown under conditions of iron depletion or excess were obtained by lysis with an isotonic buffer containing the non-ionic detergent NP40. Labeled and unlabelled *in vitro* transcripts of human ferritin L chain templates were produced with T7 RNA polymerase after linearization of the corresponding plasmid, hferL-264. The plasmid pSPT-fer (11) was used for the transcription of labeled human H chain IRE. 5 μg of protein was incubated with 1.3×10^6 cpm ($\sim$1.0 ng) of [α-^{32}P]CTP radiolabeled IRE probes, RNA-protein complexes were separated in non-denaturing polyacrylamide gels, fixed by drying onto a DEAE ion exchange paper and subjected to autoradiography. For competition assays, unlabelled *in vitro* synthesized L subunit cDNA (hferL-264) was added prior to the labeled probe.

Polysome gradient analysis. Translational activation of ferritin L chain mRNA was determined by separating cytoplasmic extracts from 1.5×10^7 iron deprived or iron loaded HeLa cells in linear 15-40 % sucrose gradients as described (12). The RNA extracted from 18 harvested fractions was analyzed by electrophoresis on denaturing 1% formaldehyde agarose gels and subsequent Northern blotting. The nylon membranes were hybridized with a random primed [α-^{32}P]dCTP-labeled probe specific for hferL-264, washed under high stringency and exposed to X-ray film with intensifying screens. Stripped filters were rehybridized with a (γ-^{32}P)-endlabeled oligodeoxynucleotide specific for the human 28S rRNA (5′-ACG GGA GGT TTC TGT CCT CCC-3′).

RESULTS AND DISCUSSION

Cloning and sequence of human ferritin L chain cDNA. Screening a cDNA library prepared from quiescent primary human T cells for translationally controlled mRNAs (details to be published) yielded among other clones a 0.83 kb cDNA encoding the ferritin L chain mRNA subunit. Although the isolated clone showed a nearly identical sequence to exon I and highest homology to exon II-IV of the genomic DNA (data not shown; 13), there were significant differences in comparison to published data in the region comprising the iron responsive element. This discrepancy motivated us to study the biological activity of this element in some detail. To facilitate molecular analysis of the leader sequence, a 264 nucleotide long fragment representing the 5′ UTR (185 bp) and part of the N-terminal coding sequence (79 bp), termed hferL-264, was subcloned into a vector downstream of a promoter for T7 RNA polymerase.

An alignment of the 5′ UTR from hferL-264 with the corresponding reported genomic region (HSAFL12, 13) and a known partial cDNA sequence (14) is depicted in Fig. 1. Differences between hferL-264 cDNA and exon I sequences of the genomic DNA were observed in the 5′ portion containing the IRE, where two guanosine residues critical for the stability of the hairpin were lacking in the reported genomic sequence. Since only a single functional gene has been identified to express human L apoferritin in different tissues (13), an explanation for the deletions might be an error in the analysis of the genomic sequence due to unresolved guanosine residues rather than a ferritin L mRNA variation in T cells. This interpretation was strengthened by comparison of hferL-264 with the partial cDNA clone HSFERL isolated from liver (14), which perfectly matched to our sequence, including one of the G residues missing in the genomic DNA data, but failed to comprise the complete IRE motif.

Structure of the ferritin L chain IRE. To gain insight into the structural properties of the IRE located in the 5′ UTR of hferL-264, portions of the leader sequence were subjected to extended computer analysis using the algorithm of Zuker (15). The modeling resulted in a palindromic structure of 26 nucleotides with a consensus CAGUGU loop sequence, a conserved C at the bulge position and a folding with a predicted free energy of -2.3 kcal/mol (Fig. 1 and Fig 2.). Hence, the identified ferritin L subunit IRE fulfills the minimal sequence requirements common to all functional IREs (16, 17). On the contrary, using the same software for the calculation of secondary structures, no stable hairpin could be formed with the predicted IRE sequence of the genomic DNA HSAFL12 (13, 18) (data not shown). Direct comparison of the computer modeled IREs of ferritin L and H chain shows the smaller bottom stem of the L subunit IRE to result in lower stability than the H chain IRE, bearing -6.4 kcal/mol free energy of folding (Fig. 2). Therefore, we proceeded to test the ability of the IRE element from hferL-264 to interact with its regulatory factor, IRP, *in vitro* and *in vivo.*

IRP binding activity of hferL-264 in vitro. To examine the functionality of the predicted ferritin L chain IRE, *in vitro* transcribed labeled hferL-264 RNA was incubated with cytosolic HeLa cell extracts. Native gel electrophoresis of the resulting RNA-protein complexes followed by autoradiography indicated a high affinity of hferL-264 transcripts to IRP, which was greater with extracts from iron deprived than with iron loaded cells, indicating proper iron-dependent modulation of binding activity. Complex formation was indistinguishable

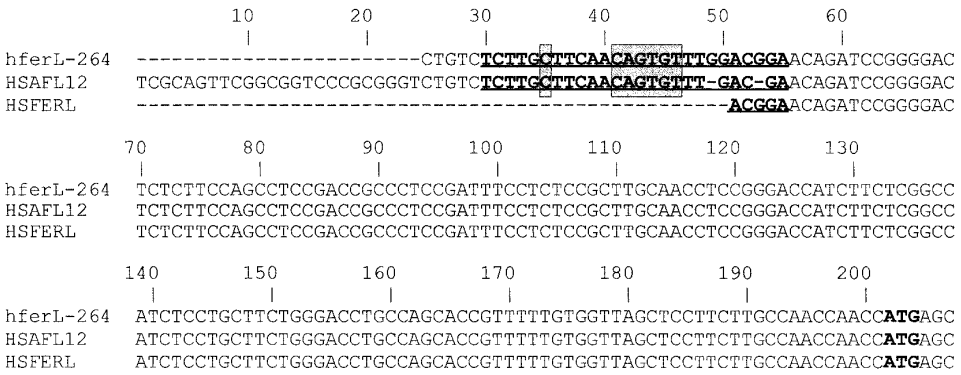

FIG. 1. Comparison of human ferritin L chain leader sequences. The 5′ UTR of the hferL-264 cDNA was aligned with exon I of the genomic sequence HSFL12 (13) and the cDNA leader region of HSFERL (14). Underlined boldface letters correspond to the IRE sequences, the shaded boxes represent the invariable cytosine residue and the consensus loop region of the regulatory motif. Boldface letters indicate the translation start codon. Missing nucleotides are designated by breaks (-) and numbering refers to the genomic DNA. The program "GeneJockey II" was used for this analysis.

from that of the control human ferritin H subunit IRE (Fig. 3). The same results were obtained in a heterologous approach employing extracts from iron depleted and iron rich murine L and 3T6 fibroblasts (data not shown). Further evidence for a direct interaction of IRP and the predicted stem-loop structure in the ferritin L chain mRNA leader were provided by cross-competition experiments. A fixed amount of labeled human ferritin H chain IRE, which contained exclusively the RNA hairpin (11), was incubated with an increasing molar excess of unlabelled hferL-264 RNA. The results revealed that the ferritin L chain IRE indeed acted as a specific competitor for the binding of IRP to the H subunit regulatory element. The extent of cross-competition was comparable to the self-competition of labeled L chain IRE probe with its unlabelled counterpart, suggesting similar affinities of IRP to both types of elements *in vitro*, despite their differences in calculated free energy (Fig. 3; compare also Fig. 2). Unrelated RNAs or single stranded DNAs did not compete IRP binding to either H or L chain IRE elements, even when used at more than 100-fold molar excess (data not shown). Thus, these *in vitro* studies indicate that the hferL-264 sequence contains a functional IRE motif.

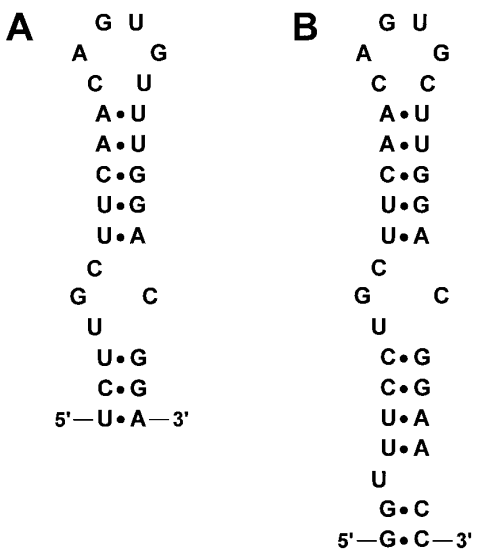

FIG. 2. Proposed stem-loop formation of the human ferritin L chain IRE (A) and comparison with the human ferritin H subunit IRE (B) as depicted by L. Kühn and co-workers (21). The computer modeled secondary structure is located at position 14 to 39 of the hferL-264 mRNA as predicted by the "foldrna" program of the GCG software package (15).

Translation control of human ferritin L chain mRNA in vivo. To monitor translational inhibition of the human ferritin L subunit mRNA at the level of initiation via the IRE stem-loop structure, cytosolic HeLa cell extracts containing free messenger ribonucleoprotein particles (mRNPs), ribosomal subunits as well as polysome bound mRNAs were separated on sucrose gradients (12). While free mRNPs remain in the $<$80S region, ribosome-associated transcripts sediment further into the gradient. RNA was extracted from the fractions and subjected to Northern blotting for analyzing the proportion of ferritin mRNA bound in polysomes. As shown in Fig. 4, under iron deprivation, cellular ferritin L chain mRNA was almost exclusively restricted to the ribosome-free compartment (lanes 2-7), indicating translational repression at the step of peptide-chain initiation. In contrast, at high iron concentrations, a fraction of L chain transcripts switched to the pool of RNA associated with polyribosomes (lanes 9-18). Since this resulted in a more than 20-fold increase in the relative proportion of ferritin L chain mRNA bound to ribosomes as quantitated by laser densitometry (data not shown), a rise of similar magnitude in the rate of protein synthesis must be expected, although under

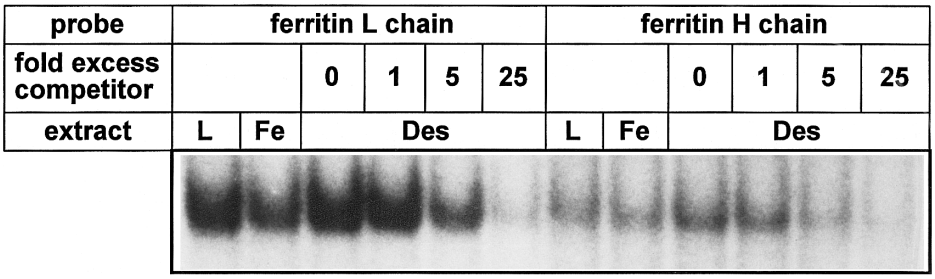

FIG. 3. Complex formation of the human ferritin L and H chain IRE with IRP. 5 μg of cytoplasmic extract from HeLa cells incubated either with the iron chelator desferrioxamine (Des) or with ferric ammonium citrate (Fe) was used in RNA/protein gel retardation assays as described in Materials and Methods. L, control extract from logarithmically growing, untreated cells. Labeled RNA probes either contained the human ferritin L (hferL-264) or H chain IRE (pSPT-fer, 11), unlabelled L subunit RNA was used for competition at the fold molar excess indicated.

both conditions the majority of transcripts remained in the free mRNA fraction. Taken together, in agreement with data obtained from polysome analysis in rodent cells (3), our observations provide *in vivo* evidence that the identified element represents the bona-fide *cis*-acting IRE within the human ferritin L chain mRNA leader sequence and is differentially bound by IRP in response to changes in iron concentration.

The availability and functional validation of the IREs of both human ferritin L and H chain mRNAs may be helpful in determining whether these elements play a role in governing the tissue specific subunit ratio of the two protein isoforms. While L-rich ferritin oligomers are found predominantly in tissues with a specific role in iron storage like liver, tissues which are not normally storing large amounts of iron such as heart have a preponderance of H chain-rich variants (19). This distribution is most likely controlled via transcriptional mechanisms since the gene promoter regions of the

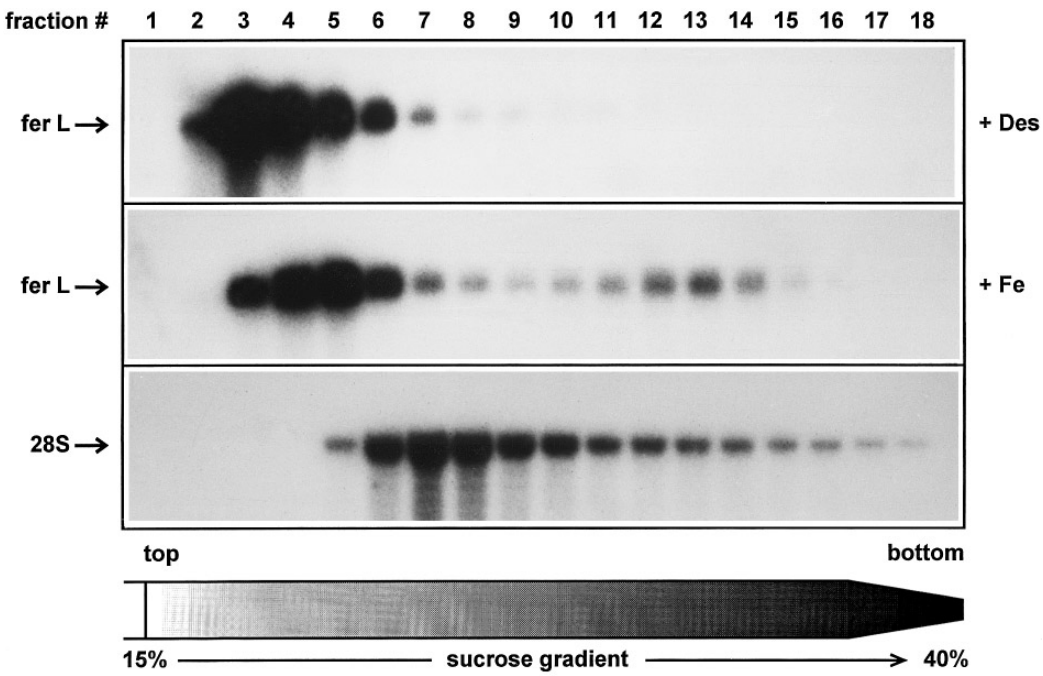

FIG. 4. Mobilization of the human ferritin L chain mRNA into polyribosomes upon iron administration. Cytoplasmic extracts of iron deprived or iron loaded HeLa cells were separated in linear sucrose gradients as described under Materials and Methods. Northern blots from the 18 RNA fractions were sequentially hybridized with a random primed [α-^{32}P]dCTP-labeled probe specific for human ferritin L chain mRNA (hferL-264) and with a [γ-^{32}P]-endlabeled oligo-deoxynucleotide specific for the human 28S rRNA as a control for the distribution between ribosome-free and ribosome-bound mRNA fractions within the gradient. The dominant 28S rRNA signals in lanes 7 and 8 indicate the formation of the 80S initiation complex and mark the border between unbound and polysome-associated ferritin mRNA. Lanes 1 and 18 represent the top and the bottom of the gradient, respectively.

same ferritin isoform from different species bear more resemblance to each other than the 5′ flanking sequences of H and L chain within the same organism (20). However, a variation in translational efficiency arising from structural and stability differences in the IREs may further modulate the final level of ferritin L chain subunits produced in response to iron administration: although our competition experiments indicated similar IRP binding affinities to both elements they were not sufficiently precise to rule out some additional contribution from this level of regulation.

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