The EMBO Journal vol.10 no.7 pp.1903-1909, 1991

Identification of a novel iron-responsive element in murine and human erythroid δ-aminolevulinic acid synthase mRNA

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Communicated by I.W.Mattaj

Iron-responsive elements (IREs) are regulatory RNA elements which are characterized by a phylogenetically defined sequence-structure motif. Their biological function is to provide a specific binding site for the IRE-binding protein (IRE-BP). Iron starvation of cells induces high affinity binding of the cytoplasmic IRE-BP to an IRE which has at least two different known biological consequences, repression of ferritin mRNA translation and stabilization of the transferrin receptor transcript. We report the identification of a novel, evolutionarily conserved IRE motif in the 5′ UTR of murine and human erythroid-specific δ-aminolevulinic acid synthase (eALAS) mRNA which encodes the first, and possibly rate limiting, enzyme of the heme biosynthetic pathway. We demonstrate the function of the eALAS IRE as a specific binding site for the IRE-BP by gel retardation analyses and by *in vitro* translation experiments. In addition, we show that the 5′ UTR of eALAS mRNA is sufficient to mediate iron-dependent translational regulation *in vivo*. These findings strongly suggest involvement of the IRE-IRE-BP system in the control of heme biosynthesis during erythroid differentiation.

Key words: aconitase/erythroid differentiation/heme synthesis/post-transcriptional gene regulation/RNA-protein interactions/translation

Introduction

Two of the best characterized examples of post-transcriptional gene regulation in mammalian cells are involved in the control of cellular iron homeostasis. The expression of the iron storage protein ferritin and of the transferrin receptor (TfR), which is involved in iron uptake, is regulated post-transcriptionally by iron-responsive elements (IREs) that are located in the 5′ UTR of ferritin mRNA or the 3′ UTR of the TfR transcript (reviewed in Klausner and Harford, 1989; Theil, 1990). The IRE serves as the binding site for a specific cytoplasmic binding protein of ~90 kd which is referred to as IRE-binding protein (IRE-BP) (Rouault *et al.*, 1988), ferritin repressor protein (Brown *et al.*, 1989) or iron-regulatory factor (Neupert *et al.*, 1990). High affinity binding of the IRE-BP to ferritin and TfR IREs is induced by iron starvation of cells and results in repression of ferritin mRNA translation and in stabilization of the TfR transcript (Klausner and Harford, 1989; Theil, 1990). The dual effect of IRE-BP binding to IREs thus provides an elegant physiological mechanism for the co-ordinated regulation of cellular iron uptake and storage. Transfection experiments have shown that over-expression of ferritin mRNA in murine fibroblasts does not affect iron regulation of ferritin biosynthesis (Rouault *et al.*, 1987). This observation indicated that the *trans*-acting regulatory factor, the IRE-BP, is in substantial excess over the number of binding sites on ferritin and TfR mRNAs and indirectly implied that the role of the IRE-IRE-BP regulatory system may well extend beyond the regulation of cellular iron uptake and storage.

To address the question of additional mRNAs which could be controlled post-transcriptionally by the IRE-IRE-BP system, we devised a specific database search program for the IRE motif. From a total of 39 hits, we selected one particularly interesting candidate for experimental *in vitro* and *in vivo* analyses. We demonstrate that the mRNAs encoding murine and human erythroid-specific δ-aminolevulinic acid synthase (eALAS) contain a functional IRE and discuss potential implications of these findings for the regulation of heme biosynthesis during erythroid differentiation.

Results

Computer-assisted identification of putative IREs

To screen for mRNAs which may be regulated by the IRE-IRE-BP system, we searched the EMBL nucleotide sequence database [release 25.0 (Kahn and Cameron, 1990)] for sequences that resemble IREs. Figure 1a depicts a combined sequence-structure RNA motif which is largely based on phylogenetic comparisons of ferritin and transferrin receptor IREs (Hentze *et al.*, 1988). This 'consensus IRE' is also consistent with the limited number of IRE mutations tested so far (Hentze *et al.*, 1988; Leibold *et al.*, 1990). IREs share the following features: (i) a six-membered loop with the sequence 5′ CAGUGN 3′ (N = C, U or A); (ii) a top helix of five (or occasionally four) paired bases; (iii) an unpaired 5′ C residue separated by five bases from the loop; and (iv) a bottom helix of somewhat variable length and position.

Traditional searching for known *sequence* homologies between the IRE and entries in the EMBL database identified many 'non-specific' sequences (see Table I) [44 975 for the sequence CAGTGN (N = C, T or A) and 11 347 for the sequence CXXXXXCAGTGN (X = C, T, G or A)]. In contrast, modification of the search command to screen for the combined sequence-structure motif depicted in Figure 1a reduced the number of identified IRE motifs to 86. As expected, none of these 86 entries violated the consensus depicted in Figure 1a. As a second screen, we sorted these 86 entries into three categories: (i) those which correspond to regions of DNA which are not known to be transcribed

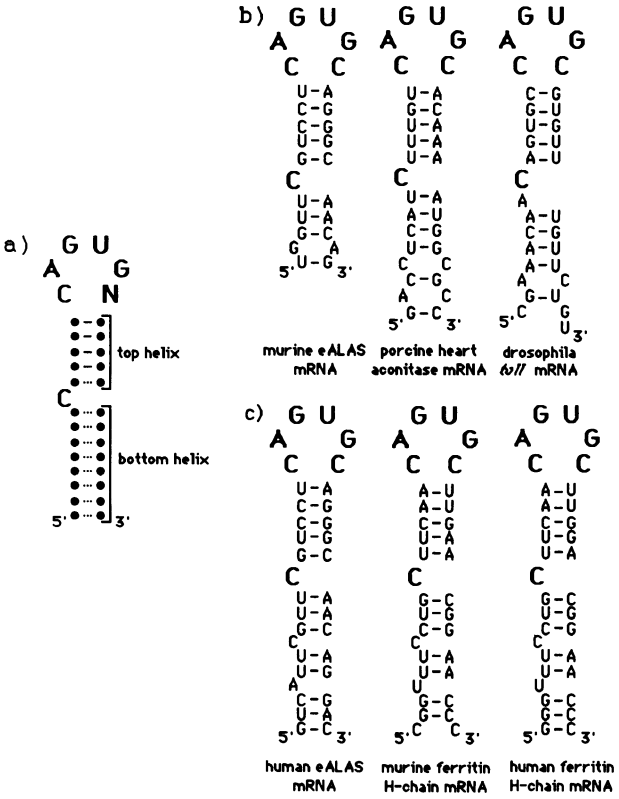

Fig. 1. Computer-assisted identification of novel IREs. (a) The phylogenetically derived IRE consensus motif is shown essentially as previously suggested (Hentze *et al.*, 1988). N represents any ribonucleotide except G, black dots connected with solid bars indicate Watson–Crick base-pairing (allowing for G–U or U–G interactions), black dots connected with broken bars symbolize optional hydrogen bonding. (**b** and **c**) Comparison between the IREs from *Drosophila melanogaster toll*, porcine heart aconitase, erythroid δ-aminolevulinic acid synthase (eALAS) and ferritin H-chain mRNAs. (b) Secondary structure prediction of the motifs drawn as IREs using the program 'fold' of the GCG software package (Devereux *et al.*, 1984) for the three putative IREs identified by the computer screen; the 5′ U of the murine eALAS motif is the 5′ terminal end of the published cDNA sequence (Schoenhaut and Curtis, 1986). (c) Predicted secondary structure for the corresponding regions of the human eALAS cDNA and the IREs identified in murine (Yachou *et al.*, 1989) and human (Costanzo *et al.*, 1986; Hentze *et al.*, 1986) ferritin H-chain mRNAs.

Table I. Identification of consensus motifs from the EMBL database

Searching motif	Total sequences identified	
CAGUGN (N ≠ G)	44 975	
CXXXXXCAGUGN (N ≠ G)	11 347	1st screen
IRE motif (Figure 1a)	86	
non-transcribed DNA	12	
transcribed, non-mRNA	10	2nd screen
mRNA	64	
ferritin/transferrin receptor	25	
in the translated region	30	
in the 3′ UTR	6	3rd screen
in the 5′ UTR	3	

into RNA; (ii) those which are transcribed, but not contained in mature mRNA (such as introns, tRNA, or rRNA sequences); and (iii) sequences which correspond to IRE motifs contained within mature mRNAs. This second screen reduced the score to 64. Importantly, these 64 entries include all 25 different known IRE motifs (from ferritin and TfR mRNAs) which correspond to the consensus IRE shown in Figure 1a and which are contained in the EMBL database. As a third and final screen, the remaining 39 newly identified IRE motifs were ordered by their location within the mRNAs. Thirty entries were found to be localized within the major open reading frame (the 'translated region') of the mRNAs, six entries were localized in the 3′ UTR, and three new IRE motifs were contained in the 5′ UTR of an mRNA (Table I).

We focused our attention on the latter three mRNAs, because previous work suggested that the presence of an IRE in the correct position within the 5′ UTR of an mRNA is sufficient for its translational regulation (Aziz and Munro, 1987; Hentze *et al.*, 1987a,b; Caughman *et al.*, 1988; Goossen *et al.*, 1990). Thus, the three newly identified mRNAs appeared to be prime candidates for IRE-mediated translational regulation, if the function of the putative IREs as binding sites for the IRE-BP could be demonstrated. In contrast to the involvement of IREs in translational control, regulation of the stability of TfR mRNA requires multiple IREs and an additional 'high turnover determinant' in the 3′ UTR (Casey *et al.*, 1988, 1989; Mullner *et al.*, 1989), and the six newly identified entries only contain single IRE motifs within their 3′ UTRs; nothing is currently known about biological roles for IREs that are located within the coding region of an mRNA. Figure 1b depicts the three identified mRNAs which encode porcine heart aconitase (Zheng *et al.*, 1990), the *Drosophila melanogaster* maternal effect gene *toll* (Hashimoto *et al.*, 1988), and the erythroid-specific form of murine δ-aminolevulinic acid synthase (Schoenhaut and Curtis, 1986). The 5′ UTR of the hepatic 'house-keeping' form of δ-aminolevulinic acid synthase (hALAS) (Bawden *et al.*, 1987; Yamamoto *et al.*, 1988) did not reveal any IRE-like motifs. Comparison of the putative IRE in murine eALAS mRNA with the human eALAS mRNA homolog (May *et al.*, 1990) reveals its high degree of conservation (Figure 1c). We reasoned that the conservation of a long RNA motif in the untranslated region of eALAS mRNAs further argued in favor of a biological role and evaluated the function of the putative eALAS IREs.

The IRE motif contained in eALAS mRNA, but not in toll mRNA, specifically binds the IRE-BP

First, we examined the ability of the eALAS IRE motif to bind specifically to the IRE-BP by gel retardation assays. To this end, two ^{32}P-labeled RNA probes which correspond to the human eALAS or the human ferritin H-chain IRE motifs were synthesized *in vitro* and incubated with cytoplasmic extracts from human placenta, a known source of IRE-BP (Neupert *et al.*, 1990). The formation of specific IRE–IRE-BP complexes was assessed after electrophoresis through non-denaturing polyacrylamide gels. As shown in Figure 2, both the ferritin and the eALAS IRE probes formed co-migrating RNA–protein complexes (compare lane 2 with lane 9). Excess unlabeled competitor RNA transcripts derived from (non-IRE containing) firefly luciferase (lanes 4 and 11) or a human ferritin IRE point mutant which fails to bind the IRE-BP with high affinity (referred to as ΔCfe in lanes 7 and 14), did not affect RNA–protein complex formation with either probe. In contrast, excess unlabeled eALAS IRE transcript fully competed for complex formation between the ferritin IRE and the IRE-BP (lane 6) and excess

unlabeled ferritin IRE transcripts completely abolished complex formation between the IRE-BP and the eALAS probe (lane 12). For equal amounts of placental extract and labeled probe of comparable specific activity, we reproducibly observed a 2- to 3-fold stronger signal with the ferritin than with the eALAS IRE probe. This could be explained by a difference in binding affinities, but the question requires further analysis. We conclude that the eALAS IRE is specifically recognized by the IRE-BP.

Interestingly, the *Drosophila toll* IRE motif (Figure 1b) fails to compete with the eALAS and the ferritin IREs for binding to the IRE-BP (Figure 2, lanes 3 and 10). When used as a ^{32}P-labeled probe, the *toll* IRE motif also does not form a complex with the IRE-BP (data not shown). Thus, the *toll*-derived IRE motif provides the first example of an RNA sequence which matches the 'IRE consensus' described in Figure 1a and yet does not bind the IRE-BP, at least under the conditions employed in our gel retardation assays. We note that the predicted negative free energy for folding as an IRE is substantially lower for the *toll* IRE motif than for the eALAS or ferritin IRE (Table II).

The IRE-like motif in porcine heart aconitase mRNA remains to be evaluated experimentally. However, aconitase contains an iron–sulphur cluster, which appears to be part of the active catalytic site that interconverts citrate, *cis*-aconitate and isocitrate in the Krebs cycle (Kennedy *et al.*, 1987). The inactive form of aconitase containing a [3Fe–4S] cluster can be activated *in vitro* by introduction of a fourth Fe ion under reducing conditions. Moreover, the primary amino acid sequences of porcine heart aconitase and the human IRE-BP are highly similar (Hentze and Argos, 1991), suggesting an unexpected functional linkage between aconitase and the IRE-BP. The identification of an IRE motif in an mRNA that encodes a protein which requires iron ions for its activity and which closely resembles the IRE-BP is—at least—an intriguing coincidence and warrants further exploration.

Binding of the IRE-BP to the eALAS IRE is sensitive to redox perturbations

Having established that the eALAS IRE is specifically recognized by the IRE-BP, we tested whether binding is sensitive to redox perturbations. This sensitivity has previously been shown to be a biochemical characteristic of the ferritin IRE–IRE-BP complex (Haile *et al.*, 1989; Hentze *et al.*, 1989a). Cytoplasmic extracts from murine erythroleukemia (MEL) cells or B6 fibroblasts were treated with 2% 2-mercaptoethanol (2-ME) or remained untreated before addition of ferritin (Figure 3A and B) or eALAS (Figure 3C) IRE probes. Figure 3 shows that both IREs form defined complexes with murine IRE-BP in the absence of 2-ME. Competition studies revealed that the upper complex (marked by arrowheads) represents the specific IRE–IRE-BP interaction, whereas the lower complex can be competed for by non-IRE containing RNA molecules (Figure 3A). Addition of the reducing agent increases formation of the specific complex with both probes to a similar extent in both extracts, but 2-ME hardly affects the formation of the lower non-specific complex. The slightly faster migration of the eALAS IRE–murine IRE-BP complex is reproducible and awaits further characterization. Similar to the ferritin IRE, binding of the eALAS IRE to the IRE-BP is also increased in extracts from iron-starved human and murine cells and can be abolished by the oxidizing agent diamide (data not shown). Thus, binding of the eALAS IRE to the IRE-BP displays the previously recognized biochemical characteristics of the ferritin IRE–IRE-BP interaction.

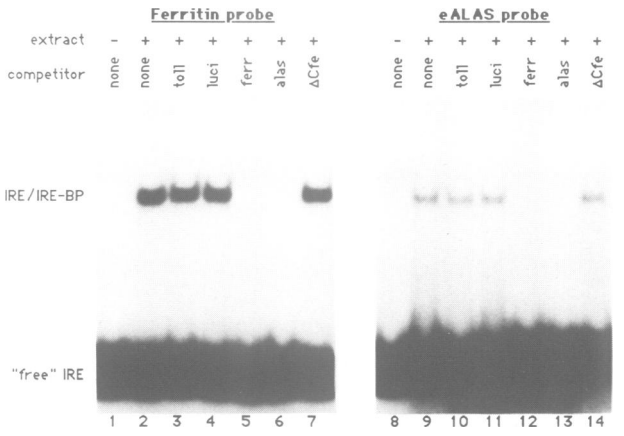

Fig. 2. Specific binding of the human eALAS IRE to the IRE-binding protein. 15 μg of cytoplasmic extract from human term placenta (lanes 2–7 and 9–14) were analyzed in the presence (lanes 3–7 and 10–14) or absence (lanes 2 and 9) of unlabeled competitor RNA transcripts with 3.000 c.p.m. of ^{32}P-labeled RNA transcripts corresponding to the human ferritin H-chain (lanes 1–7) or human eALAS (lanes 8–14) IRE motifs. The positions of the 'free' labeled RNA probes and the IRE–IRE-BP complexes are marked.

Table II. mRNAs containing IRE motifs in their 5′ untranslated region

mRNA	Negative free energy of IRE motif
Porcine heart aconitase	−3.2 kcal/mol
Murine eALAS	−5.1 kcal/mol
Human eALAS	−5.2 kcal/mol
Drosophila toll	−0.4 kcal/mol
Ferritin from different sources	−2.1 kcal/mol – −6.7 kcal/mol

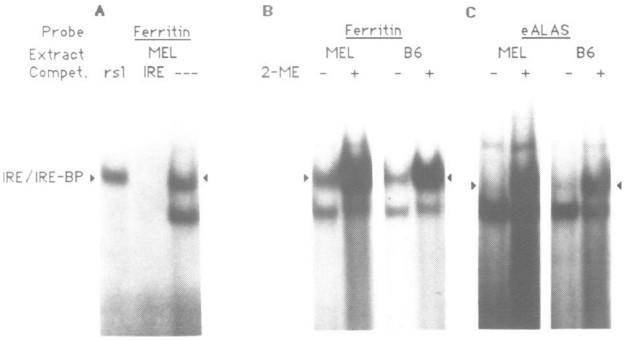

Fig. 3. Binding of the murine IRE-BP to the eALAS IRE is sensitive to chemical reduction. **A**. Detergent extracts from MEL cells were analyzed with a ferritin IRE probe in the presence or absence of unlabeled rsl or human ferritin IRE competitor RNA transcripts. **B** and **C**. Detergent extracts from MEL cells or B6 fibroblasts were treated with 2% 2-mercaptoethanol (2-ME) (+) or remained untreated (−) prior to addition of ferritin (B) or human eALAS (C) IRE probes and analyzed by native gel electrophoresis. The position of specific IRE–IRE-BP complexes is indicated by arrowheads, the position of excess 'free' probe is not shown.

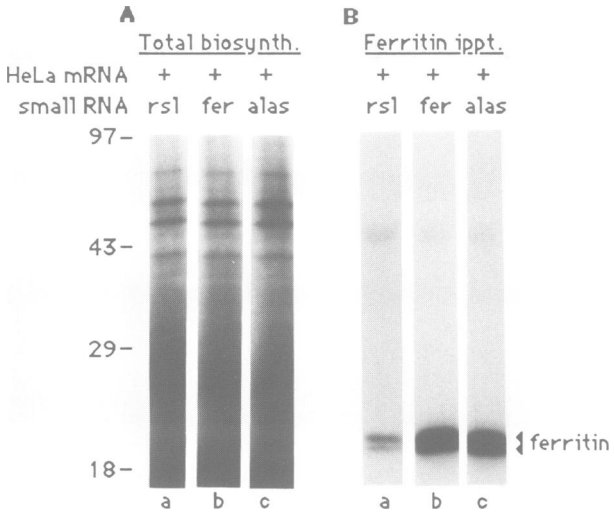

Fig. 4. Derepression of ferritin mRNA translation in cell-free extracts by the human eALAS IRE. Poly(A) enriched HeLa cell mRNA was translated in rabbit reticulocyte lysate (panel **A**). The endogenous rabbit IRE-BP was titrated by addition of 100 ng/15 μl reaction of a 34 nucleotide non-IRE containing stem−loop transcript (rsl), the human H-chain ferritin IRE (fer) or the human eALAS IRE (alas). Translation of ferritin H- (upper arrowhead) and L-chain (lower arrowhead) mRNA was assessed by immunoprecipitation of equal quantities of [^{35}S]methionine labeled polypeptides with a specific polyclonal anti-ferritin antibody (panel **B**). Molecular mass standards are shown on the left.

Functional sequestration of the IRE-BP by the eALAS IRE in cell-free translation

The specific recognition of the eALAS IRE by the IRE-BP in gel retardation assays raised the question of whether the eALAS IRE could also modulate IRE-BP function in cell-free translation extracts. We tested the effect of the eALAS IRE on the *in vitro* translation of ferritin mRNA in rabbit reticulocyte lysates. Total poly(A)-enriched RNA from HeLa cells was translated in the presence of [^{35}S]methionine (Figure 4A), and ferritin was immunoprecipitated from the total translation products (Figure 4B).

Rabbit reticulocyte lysate contains active IRE-BP which (partially) represses the translation of ferritin mRNA in this system (Walden *et al.*, 1988). The repression persists after the addition of small non-IRE containing RNA molecules (Figure 4B, lane a and data not shown), but is relieved when the IRE-BP is titrated by addition of *in vitro* transcribed ferritin IRE (Figure 4B, lane b). Addition of an equal amount of eALAS IRE transcript also effectively derepresses ferritin mRNA translation *in vitro* (Figure 4B, lane c). The three small RNAs do not display non-specific differential effects on total protein biosynthesis (Figure 4A). We conclude that the eALAS IRE mirrors the IRE-BP binding characteristics of the ferritin IRE in three different assay systems.

The 5′ UTR of eALAS mRNA mediates iron-regulation in vivo

The identification of a functional IRE-BP binding site in the 5′ UTR of eALAS mRNA and the presence of the IRE-BP in extracts from MEL cells (Figure 3) strongly predict that eALAS expression is translationally controlled by iron in erythroid cells. While MEL cells have proven to be a suitable model to study various aspects of heme biosynthesis during erythroid differentiation (Friend *et al.*, 1971; reviewed by Ponka *et al.*, 1990), experimental evaluation of this prediction requires a detailed analysis of eALAS expression during different stages along the differentiation pathway. Moreover, direct analysis of eALAS mRNA translation requires immunoprecipitation of metabolically labeled eALAS polypeptides with specific antibodies which are currently not available. Therefore, we decided to evaluate the capacity of the 5′ UTR of eALAS mRNA to confer iron-dependent translational regulation to a human growth hormone (hGH) indicator transcript after transfection into murine B6 cells. This approach allows the direct analysis of eALAS−hGH hybrid mRNA translation in cells where ferritin IRE function has been intensively studied (Hentze *et al.*, 1987a,b; Caughman *et al.*, 1988; Goossen *et al.*, 1990).

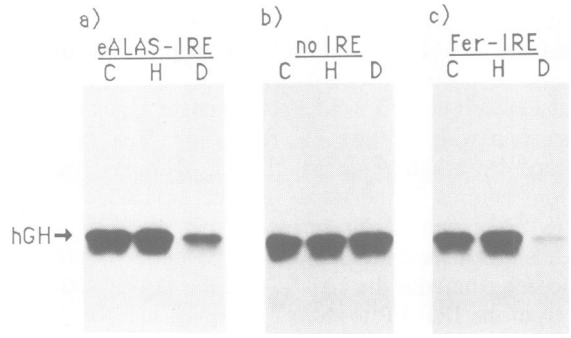

Fig. 5. The 5′ UTR of murine eALAS mRNA mediates translational iron regulation *in vivo*. Stable transformants were analyzed by quantitative immunoprecipitation after treatment with 100 μM hemin (H) or 100 μM desferrioxamine (D) and metabolic labeling with [^{35}S]methionine. Control samples which received neither hemin nor desferrioxamine are marked with a 'C'. Panel **a** shows the analysis of construct eALAS−GH (eALAS−IRE), panel **b** shows the analysis of L5−GH (no IRE), and panel **c** shows the analysis of construct Fer−GH (Fer−IRE). The position of specifically precipitated hGH polypeptides is indicated by an arrow. The reproducibility of the effect of cellular iron perturbations on the three cell lines was confirmed by immunoprecipitation of endogenous murine ferritin (not shown).

Complementary oligonucleotides which encompass the 5′ UTR of the murine eALAS cDNA (Schoenhaut and Curtis, 1986) were synthesized and cloned into the 5′ UTR of an IRE-less hGH indicator construct to create eALAS−GH. Since previous work had demonstrated an effect of the position of an IRE within the 5′ UTR on its ability to serve as a translational regulator (Goossen *et al.*, 1990), the position of the eALAS IRE in eALAS−GH was maintained with regard to its physiological position in eALAS mRNA [the unpaired 5′ C residue of the IRE is located 16 nucleotides downstream from the transcription start site in the authentic eALAS mRNA (Schoenhaut and Curtis, 1989) and 30 nucleotides downstream from the transcription start site in the indicator eALAS−GH mRNA]. Figure 5 shows an analysis of the iron responsiveness of eALAS−GH (panel a) after stable transfection into murine B6 cells. The ferritin IRE containing construct Fer−GH serves as a positive control (panel c) while the previously described (Casey *et al.*, 1988) IRE-less parent vector L5-GH is included as a negative control (panel b). To alter the cellular iron status, transfectants were treated with hemin (H) as a source of iron or with the iron chelator desferrioxamine (D) or remained as untreated controls (C). Cells were then metabolically labeled with [^{35}S]methionine, and quantitative immunoprecipitation of hGH was performed.

The data depicted in Figure 5 show that the 5′ UTR of eALAS mRNA confers iron regulation to a non-regulated parent construct (compare panel a with panel b) and demonstrate the function of the eALAS IRE as a translational regulator. The range of iron regulation of the hGH indicator construct under control of the ferritin IRE was somewhat higher than the range of the eALAS−IRE regulated construct (compare panel c with panel a), whereas iron regulation of endogenous ferritin was similar in the three cell lines (not shown). It is tempting to speculate that this quantitative difference is functionally related to the apparently weaker binding of the IRE-BP to the eALAS IRE (see Figure 2), but this point awaits more detailed investigation. We conclude that the 5′ UTR of eALAS mRNA is sufficient to mediate translational iron regulation *in vivo* in transfected murine fibroblasts.

Discussion

The discovery of IREs in the 5′ UTR of mRNAs which had previously escaped attention, validates the computer-assisted screening approach upon which this study is based. We hope that the success of this rapid screening method will encourage searches for other biologically important, experimentally defined RNA sequence−structure motifs. It is possible that our screen may have missed additional biologically relevant IREs, because (i) the minimal necessary stability of the bottom helix of the IRE (Figure 1a) remains to be established and because (ii) it is currently unknown whether the conserved sequence 5′ CXXXXXCAGUGN 3′ is absolutely required for IRE function. In fact, the 3′ UTR of human TfR mRNA contains an element with the loop sequence 5′ CAGAGU 3′ which appears to bind the IRE-BP (Koeller *et al.*, 1989).

Co-ordinated regulation of iron uptake, storage and utilization in erythroid differentiation?

We have demonstrated that the 5′ UTR of eALAS mRNA contains a functional IRE which specifically binds to the IRE-BP and which mediates translational iron regulation *in vivo*. This demonstration provides a starting point for the exploration of the possibly complex biological mechanisms by which the eALAS IRE influences eALAS expression during the course of erythroid differentiation. Taken at face value, our data suggest that the expression of eALAS mRNA is translationally controlled by iron, or heme as a carrier of iron, during the development of erythroid precursor cells into mature erythrocytes.

Our findings also suggest a direct molecular mechanism by which iron availability could regulate heme biosynthesis. ALAS catalyzes the first step in the heme biosynthetic pathway which is completed by the incorporation of ferrous iron into protoporphyrin IX via ferrochelatase. Several reports have suggested that the synthesis of δ-aminolevulinic acid constitutes the rate limiting step in heme biosynthesis in the liver (London *et al.*, 1964; Levere and Granick, 1965; Abraham *et al.*, 1983; reviewed in Ponka *et al.*, 1990). Further, in the hepatic system ALAS activity is inhibited by heme (Levere and Granick, 1965). In contrast, in erythroid progenitor cells heme (or heme as a carrier of iron) stimulates ALAS activity (Abraham *et al.*, 1989, 1990). Other investigators have challenged this view and favored a direct role of iron availability as the rate limiting molecular step (Ponka and Schulman, 1985; Laskey *et al.*, 1986). Several lines of evidence have indicated that eALAS expression is translationally controlled (Elferink *et al.*, 1988; Dierks, 1990), but the available data did not permit a direct demonstration of translational regulation or an identification of the regulatory signal. Dierks (1990) also recently noticed similarities between the element contained in murine eALAS mRNA which is shown in this report to function as an IRE and the IREs contained in ferritin mRNAs. Elucidation of the mechanism by which iron availability controls eALAS expression via the IRE−IRE-BP system may help to reconcile some of the conflicting views in our understanding of the regulation of heme biosynthesis during erythroid differentiation. Thus, the biological roles of the IRE−IRE-BP system may well extend from the coordinated regulation of cellular iron uptake and iron storage to the regulation of a metabolic pathway which involves massive iron utilization.

Materials and methods

Database screen for IRE motifs

The EMBL nucleotide sequence database version 25.0 (Kahn and Cameron, 1990) consisting of 5.3×10^7 nucleotides and 4.15×10^4 entries was screened. Searches of the sense strand were performed on the EMBL VAX cluster using Pascal programs. The search for the IRE consensus motif shown above included four obligatory criteria: (i) presence of the primary sequence 5′ CAGTGN 3′, N = C, A, or T, as a six nucleotide loop; (ii) presence of a top helix consisting of four uninterrupted base pairs followed by either a fifth base pair or an additional nucleotide on the 5′ and 3′ end of a four membered helix; (iii) presence of a bulged C on the 5′ end of the top helix; (iv) ability of the following eight nucleotides on the 5′ and 3′ end to form a bottom helix defined by the following scoring scheme: each GC (or CG) pair scored 3 points, each AU (or UA) scored 2 points, each GU (or UG) scored 1 point, a one nucleotide bulge scored −2, opposing unpaired nucleotides had a score of 0, and bulges >1 nucleotide were not permitted. The combined score for the bottom helix had to exceed 7 to score a hit. The source code of the IRE search program is available upon request.

Generation of in vitro transcripts

^{32}P-labeled RNA probes (sp. act. $\sim 0.5 \times 10^6$ c.p.m./μg) were generated by the method of Milligan *et al.* (1987) using cloned T7 RNA polymerase; the sequence of the DNA template for the transcription of the ferritin IRE was 5′ GGGATCCGTC CAAGCACTGT TGAAGCAGGA TCCCTATAGT GAGTCGTATT A 3′ and 5′ GTCCTGTTGC CCTGCACTGA GGACGAACGA ATGACCCTAT AGTGAGTCGT ATTA 3′ for the eALAS IRE. Unlabeled competitor RNAs for ferritin (ferr), eALAS (alas), *toll* and a 34 nucleotide long non-IRE containing stem−loop transcript (rsl) were transcribed by the same method (template for the *toll* IRE motif: 5′ CGAACAAACA CGCACTGGCA CTGTTGTTTA CCCTATAGTG AGTCGTATTA 3′, template for the rsl transcript: 5′ GGGTACGACC AAGTTCGTGA CAACTTCTCT ACCCTATAGT GAGTCGTATT A 3′). Synthetic DNA oligonucleotides, labeled and unlabeled *in vitro* transcription products were purified by gel electrophoresis [15% polyacrylamide: bisacrylamide (20:1), 8 M urea] and eluted according to standard procedures (Sambrook *et al.*, 1989). The RNA was finally dissolved in DEPC-treated H_2O. A transcript corresponding to the 1.7 kb *Hin*dIII−*Sac*I fragment from firefly luciferase (luci) was generated with T3 RNA polymerase from plasmid pEMBL 131+, the competitor labeled ΔCfe corresponds to a 0.08 kb *Bgl*I−*Bam*HI fragment which includes a mutated ferritin IRE (omission of the 5′ C residue of the IRE loop, derived from construct Δ−165 in Hentze *et al.*, 1988) and was transcribed with T7 RNA polymerase from a pGEM 3zf− plasmid.

Gel retardation assays

Detergent extracts were prepared from murine B6 fibroblasts and MEL cells as previously described (Hentze *et al.*, 1989b). Crude homogenate from human term placenta ('placental extract') was prepared as described by Neupert *et al.* (1990). Equal aliquots (15 μg) of cellular extract were incubated with 3.000 c.p.m. of the appropriate probe at 22°C; where indicated, 2% (v/v) 2-ME or an ~400-fold excess of unlabeled competitor RNA were added to the extract 2 min prior to the addition of probe. After 30 min, 3 mg/ml heparin were added for an additional 10 min. Analysis of RNA−protein complexes by non-denaturing gel electrophoresis and

autoradiography was performed as previously described (Leibold and Munro, 1988). When analyzed on the same gel, extracts from human cells typically gave rise to a single retarded RNA–protein complex, whereas multiple bands (usually two) could be identified from murine extracts.

In vitro translation in rabbit reticulocyte lysates

Total RNA from HeLa cells was isolated by the guanidinium isothiocyanate method and subsequently enriched for poly(A)$^+$ RNA by a batch procedure with oligo(dT)–cellulose from Boehringer Mannheim (Sambrook *et al.*, 1989). Cell-free translations were performed as described by Clemens (1984) in the presence of [^{35}S]methionine (0.5 μCi/μl) and poly(A)$^+$-enriched RNA from HeLa cells (20–25 ng/μl). Rabbit reticulocyte lysate was purchased from Promega and adjusted to 63 mM K^+ and 0.68 mM Mg^{2+}. All samples were maintained on ice before incubation for 1 h at 30°C. Ferritin mRNA translation was assessed by immunoprecipitation of ferritin polypeptides from equal amounts of ^{35}S-labeled translation products, electrophoretic separation on SDS–polyacrylamide gels and autoradiography as described (Goossen *et al.*, 1990; Johansson *et al.*, 1991).

Plasmid constructions

The indicator constructs used in this report are derived from the previously described L5–GH plasmid (Casey *et al.*, 1988) which contains adjacent 5′ *Bam*HI and *Xba*I 3′ sites sandwiched between the well characterized transcription start site of the ferritin promoter (Costanzo *et al.*, 1986; Hentze *et al.*, 1986) and the protein coding region of the human growth hormone gene. Pairs of complementary oligodeoxyribonucleotides were synthesized with an Applied Biosystems DNA synthesizer. They were annealed, phosphorylated and the double-stranded DNA was cloned between the *Bam*HI and *Xba*I sites of L5–GH. The correct nucleotide sequences of the insertions of the resultant plasmids were confirmed by DNA sequencing as previously described (Casey *et al.*, 1988). The sequence of the oligonucleotide insert of construct eALAS—GH was 5′ GGATCCTCACCGTCTTTGGT TCGTCCTCAG TGCAGGGCAA CAGGACTTTG GGCTCAGGTC TAGA 3′ and of construct Fer—GH 5′ GGATCCTGCT TCAACAGTGC TTGGACGGAT CTTCTAGA 3′.

Cell culture and transfections

Stable transformants were generated as previously described (Hentze *et al.*, 1986) by cotransfection of the hGH indicator plasmid with the herpes simplex virus thymidine kinase gene into murine B6 fibroblasts and subsequent selection in medium containing hypoxanthine, aminopterin and thymidine (HAT). All stable cell lines were maintained in HAT medium.

Analysis of mRNA translation in vivo

Stably transformed cells were incubated at 37°C for 4 h with 100 μM hemin or 100 μM desferrioxamine, washed twice with methionine-free medium and labeled with 40 μCi/ml [^{35}S]methionine for 2 h at 37°C. Quantitative immunoprecipitation from equal amounts of ^{35}S-labeled polypeptides with polyclonal anti-hGH antibodies (National Hormone and Pituitary Program, Baltimore, MD, USA), analysis by SDS–PAGE and autoradiography were performed as described (Goossen *et al.*, 1990).

Acknowledgements

We thank Dr Brian K.May for communicating the human eALAS IRE sequence prior to publication, Professor J.Heep for providing human placentae and Dr Enzo Sorrentino for the gift of MEL cells. We acknowledge the National Hormone and Pituitary Program (University of Maryland School of Medicine) and the National Institute of Diabetes and Digestive and Kidney Diseases for the gift of polyclonal antisera against human growth hormone. We are indebted to Drs K.V.Anderson and C.Hashimoto for a *toll* cDNA clone. We acknowledge N.G.Abraham, E.Coccia, T.Graf, A.Kulozik, I.Mattaj, L.Phillipson and H.Stunnenberg for critically reviewing the manuscript and for making helpful suggestions. This work was partially supported by fellowships from Boehringer Ingelheim Fonds to T.D., from the Ministry for Science and Technology of Brazil to R.S., and by a grant from the Deutsche Forschungsgemeinschaft to M.W.H.

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Received on January 18, 1991; revised on February 11, 1991