

Identification of a Conserved and Functional Iron-responsive Element in the 5'-Untranslated Region of Mammalian Mitochondrial Aconitase*

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Iron-responsive elements (IREs) are RNA stem-loop motifs found in genes of iron metabolism. When cells are iron-depleted, iron regulatory proteins (IRPs) bind to IREs in the transcripts of ferritin, transferrin receptor, and erythroid amino-levulinic acid synthetase. Binding of IRPs to IRE motifs near the 5' end of the transcript results in attenuation of translation while binding to IREs in the 3'-untranslated region of the transferrin receptor results in protection from endonucleolytic cleavage. Iron deprivation results in activation of IRE binding activity, whereas iron replete cells lose IRE binding activation. Here, we report the identification of a conserved IRE in the 5'-untranslated region of the transcript of the citric acid cycle enzyme mitochondrial aconitase from four different mammalian species. The IRE in the transcript of mitochondrial aconitase can mediate *in vitro* translational repression of mitochondrial aconitase by IRPs. Furthermore, levels of mitochondrial aconitase are decreased in mice maintained on a low iron diet, whereas levels of mRNA remain unchanged. The decrease in levels of mitochondrial aconitase is likely due to activation of IRP binding and consequent attenuation of translation. Thus, expression of the iron-sulfur protein mitochondrial aconitase and function of the citric acid cycle may be regulated by iron levels in cells.

IREs are RNA stem loop sequences found in the 5'-UTR¹ of the mRNAs for ferritin and erythroid amino-levulinic acid synthetase and the 3'-UTR of transferrin receptor where they function as a binding site for iron regulatory proteins (IRPs) (reviewed in Refs. 1–3). The two known IRPs, IRP1 and IRP2, bind IREs with equal and high affinity and mediate translational repression of transcripts containing ferritin IREs with equal efficacy (4). IRP1 is a bifunctional protein that also functions as cytosolic aconitase, an enzyme that catalyzes the reversible isomerization of citrate to isocitrate via cis-aconitate in the cytosol (5–8). There are two different aconitases in mammalian cells, cytosolic and mitochondrial, which are encoded by two different genes. Both aconitases contain a [4Fe-4S] cluster that is required for enzymatic activity (9), and active site residues are identical between the two proteins (10). Although IRP1 was initially identified as an IRE binding protein, it was shown after comparison of peptide sequences of IRP1 and pu-

rified cytosolic aconitase that the two activities were derived from the identical protein (9). IRP1 makes a quantitative transition to the iron-sulfur cluster containing cytosolic aconitase when cells are iron-replete and to the IRE binding form when the iron-sulfur cluster is absent from the protein (6). Whereas the role of mitochondrial aconitase in interconversion of citrate and isocitrate in the citric acid cycle is well understood, the reason that aconitase activity is maintained in the cytosol is not clear. Although the two aconitases share approximately 25% sequence identity, mitochondrial aconitase does not bind IREs (5).

Targets of IRP regulation include the transcripts of ferritin, transferrin receptor, and erythroid amino-levulinic acid synthetase. Recently, an IRE has been described in the 5'-UTR of the iron protein subunit of succinate dehydrogenase of *Drosophila melanogaster* (11); the IRE mediates translational regulation by iron of a reporter gene and appears to be responsible for translational regulation of succinate dehydrogenase in *Drosophila* cell lines (12). A consensus IRE has previously been described in the 5'-UTR of porcine aconitase (13, 14), but it has been difficult to determine whether the IRE is functional in cells because antibodies that will immunoprecipitate porcine aconitase have not been available.

In order to determine whether the IRE in porcine aconitase is functional and physiologically relevant, we have evaluated the transcript by three different approaches. The first is to search data bases for other mammalian mitochondrial aconitase transcripts and to evaluate whether the IRE sequence element is conserved when RACE techniques are used to complete the 5'-UTR. The second is to test the efficacy of the 5'-UTR in mediating translational regulation *in vitro*. The third is to measure total levels of mitochondrial aconitase protein in tissues of animals fed on low *versus* high iron diets to evaluate whether there is a change consistent with translational regulation. In this report we demonstrate that an IRE is conserved in the 5'-UTR of mitochondrial aconitase in the transcripts of all four mammalian species that were evaluated. Furthermore, the IRE mediates translational repression *in vitro* and is associated with a significant decrease in levels of mitochondrial aconitase in liver tissue in animals maintained on a low iron diet.

MATERIALS AND METHODS

Reconstruction of Full-length Porcine Mitochondrial Aconitase—The plasmids utilized for *in vitro* transcription by T7 RNA polymerase were constructed as follows. A porcine aconitase cDNA (kindly provided by Dr. H. Zalkin) that contained the entire coding region and portions of the 5'-UTR was subcloned into *EcoRI* and *BamHI* sites of pGEM3Z (Promega, Madison, WI). The 5' end was completed by incorporation in a PCR product of the first 9 nucleotides of the transcript (AAGC-GACCT), which were absent from the cDNA but known to be present through primer extension analysis (15). The 5'-UTR modified aconitase (sequence TCATATTTGT**ACGTGCACAAAATGG** with mutations from the consensus sequence denoted in bold) was constructed by PCR mu-

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¹ The abbreviations used are: UTR, untranslated region; IRP, iron regulatory protein; IRE, iron-responsive element; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; EST, expressed sequence tag.

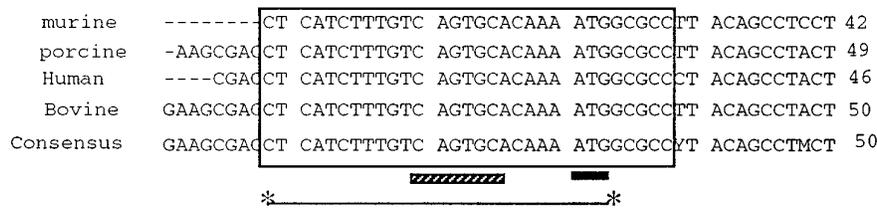


FIG. 1. **Alignment of 5'-UTR sequences of mitochondrial aconitases.** The nucleotide sequences of human and murine *m*-aconitases were obtained from 5' RACE experiments. The sequences of bovine and porcine aconitase were from data base searches. A *solid bar* underlines the AUG start codon, a *hatched bar* underlines the CAGUGC loop sequence of the IRE, and the first and last nucleotides of an IRE containing a four nucleotide lower stem are denoted by *asterisks*.

tagenesis and subcloned into *EcoRI* restriction sites flanking the 5'-UTR sequence. The plasmids containing ferritin + IRE and ferritin - IRE were described previously (4). Full-length transcripts were synthesized as described below.

In Vitro Synthesis of Transcripts of Porcine Mitochondrial Aconitase and Ferritin mRNA—The aconitase and ferritin RNA fragments were prepared by *in vitro* transcription of the aconitase or ferritin cDNA templates described above. The mixture contained 10 μ g of linearized DNA template, 10 mM dithiothreitol, 0.5 mM NTP, 40 units of RNasin (Promega), and 40 units of T7 RNA polymerase (Promega). After transcription at 37 °C for 2 h, templates were digested with 10 units of DNase (Promega) for 10 min at 37 °C, extracted with phenol-chloroform three times, and precipitated with ethanol. Transcription products were quantified by absorbance at 260 nm. The integrity of the transcripts was verified on a formaldehyde-agarose gel, and the lengths of the ferritin and aconitase transcripts were 0.94 and 2.6 kb, respectively.

Purification and Western Blotting of IRP1 and IRP2—Purifications of IRP1 ($M_r = 98,000$) and IRP2 ($M_r = 105,000$) were performed as described previously (16, 17) and were used in the amounts indicated. Western blotting of IRP1 and IRP2 was performed as described previously (17).

RNA Gel Retardation Assay—Reaction mixtures of gel retardation assay contained 40–160 nM of purified IRP1 or IRP2 with 30 ng of 32 P-labeled IRE probe (18). The reactions were carried out at 25 °C for 15 min. The reaction mixture was loaded onto a 8% native polyacrylamide gel and electrophoresed at 150 V for 3 h. After electrophoresis, the gels were fixed and subsequently dried. The amount of 32 P-labeled complex was determined on a PhosphorImager (Molecular Dynamics) using ImageQuant software. Competition assays were performed by adding *in vitro* the ferritin H chain IRE or porcine mitochondrial aconitase IRE (native sequence UCAUCUUUGUCAGUGCACAAAUGG), purified, and quantitated as described previously (4).

In Vitro Translation—Cell-free translation was performed with rabbit reticulocyte extract (Promega) as described by the manufacturer's protocols. The final concentrations of mRNA and [35 S]methionine were 20 μ g/ml and 0.8 mCi/ml, respectively. Either the mRNA for porcine aconitase reconstructed to contain the full 5' end or a reconstruction that contained three mutations of the 5'IRE (described under reconstruction of mitochondrial aconitase) was added to the *in vitro* translation mix. The mRNA encoding ferritin H chain or an mRNA containing a non-IRE stem-loop in the 5'-UTR (ferritin - IRE) was also added to the *in vitro* translation mix. Purified IRP1 and IRP2 were titrated into the reaction as indicated. The reaction volume was 25 μ l, and the translation was carried out at 30 °C for 60 min. Quantitation of 35 S-labeled complex was done by PhosphorImager as described previously (4).

Use of the 5'-RACE Reaction to Obtain the 5'-UTRs of Human and Murine Mitochondrial Aconitase—Amplification of the human mitochondrial aconitase transcript was performed with anchor-ligated human heart cDNA (Clontech, Palo Alto, CA) used as template. The anchor primer (5'-CTGGTTTCGCCACCTCTGAAGGTTCCAGAATCGA-TAG-3') was used in combination with two degenerate aconitase-specific 5' primers (GSP1, 5'-GCAARCGNCTNAAYCGNCCKCTNACNCT-NT-3', and GSP2, 5'-GAYCTNCTNGARAARAAYATHGACATYGTNC-G-3') to amplify the 5' end of human mitochondrial aconitase. The sequence of these primers was based on the peptide sequence near the N terminus of porcine mitochondrial aconitase and the expectation that the sequence of human aconitase would be similar. The primary PCR reaction was performed with anchor primer and aconitase-specific primer (GSP1) and a 1:10 dilution of primary PCR reaction was then used in the secondary PCR reaction with a nested aconitase primer (GSP2) and an anchor primer. The thermocycle profile for the PCR reaction was 1 min at 94 °C (denaturation), 1 min at 60 °C (annealing),

and 1 min at 72 °C (extension) for 30 cycles. The PCR product was isolated from agarose gel and cloned into *SrfI* site of pCR-SK(+) vector (Stratagene, La Jolla, CA) and inserts were sequenced with Sequenase (U. S. Biochemical Corp.).

The 5' end of murine mitochondrial aconitase was determined using anchor ligated murine heart mRNA (Clontech) with primers derived from a murine cDNA found in the GenBank[®] EST data base (accession number R74804). The sequence of primer 1 was 5'-CAACCGTTTACG-GACAATGTTAATGTTCTTC-3', and the sequence of the nested primer was 5'-GGTGTCCATATAACAATCTTCTCTGAGAG-3'.

Dietary Iron Manipulations of Mice—After weaning, littermates of B6 \times 129 F1 mice were divided into three groups, which were maintained on a low iron diet (Harlan Teklad, Madison, WI) containing 3.447 μ g of iron/gm of formula, a normal diet containing 54.5 μ g/gm, or a high iron diet (1,750 μ g/g ferrous sulfate). After a period of between 9 and 16 weeks, the animals were sacrificed, and lysates of liver, spleen, and heart were made using small slices of tissue in a Dounce AA homogenizer in lysis buffer (40 mM KCl, 25 mM Tris, pH 7.4, 1 mM dithiothreitol, 10 μ g/ml aprotinin (ICN), 10 μ g/ml leupeptin (Boehringer Mannheim), 5 μ g/ml AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; ICN).

Western Blotting of Murine Mitochondrial Aconitase—Western blotting was performed using antibodies raised in rabbits to a high density multiple antigenic peptide system (19) containing the sequence EK-NINIVRKRLNRPLTLSEK, which begins 49 amino acids from the N terminus of human mitochondrial aconitase and is highly conserved among species. The murine peptide was predicted from the EST sequence to be identical to the human peptide sequence to which antibody was raised, although there was one undetermined nucleotide in the EST, and the identity of one amino acid within the murine sequence spanned by the peptide was therefore not known. Use of preimmune sera and competition with *in vitro* translated mitochondrial aconitase verified the specificity of the identified band.

Northern Analysis of Murine Mitochondrial Aconitase—A 480-nucleotide sequence of the 5' end of the porcine aconitase was amplified by PCR and labeled by random priming. Murine liver was extracted using a Triazol reagent (Life Technologies, Inc.), and RNA was separated on a 1.2% formaldehyde agarose gel, transferred to a Gene-Screen Plus (Dupont NEN) membrane, and probed. Signals were quantitated by PhosphorImager.

RESULTS

The IRE Sequence in Aconitase Is Conserved in Human, Porcine, Murine, and Bovine mRNA—To obtain the 5' end of human mitochondrial aconitase cDNA, a human heart cDNA library was used as the template in 5' RACE experiments. Inserts of 161 bp containing 5' end sequences of human aconitase cDNA were identified using gene specific primers and were sequenced. The 5' end of murine aconitase was also obtained using EST sequences and 5' RACE. In addition, the sequence of bovine aconitase was obtained in a search of the GenBank[®]. The alignment of nucleotide and protein sequences from human (this study), murine (this study), porcine (15), and bovine (GenBank[®], accession number Z49931) mitochondrial aconitase is shown in Fig. 1. The 5'-UTR sequences of *m*-aconitase from all four species are identical in the region spanned by the IRE (nucleotides 5–34). The end of the porcine mitochondrial aconitase has been mapped (15) and contains 8 nucleotides 5' of the IRE. Although nucleotides 5' of the IRE were not identified in RACE of the murine transcript, it is

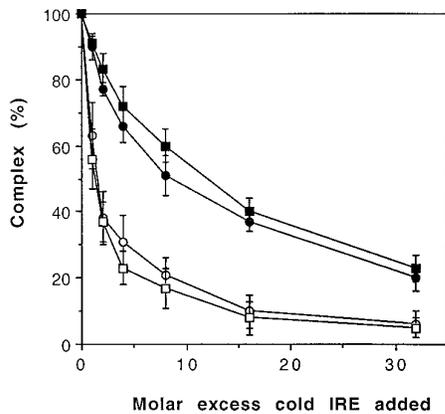


FIG. 2. Competition assays for binding of ligands to IRP1 or IRP2. Increasing amounts of unlabeled ferritin H chain IRE or the human *m*-aconitase IRE 1 (1–32-fold molar excess of labeled ligand) were added as competitors in the indicated amounts to a gel retardation assay in which radiolabeled ferritin H-chain IRE (30 ng) was incubated with purified IRP1 (circles) or IRP2 (squares) as indicated under "Materials and Methods." Unlabeled ligands included the ferritin IRE (open symbols) and aconitase IRE (closed symbols).

possible that the endogenous transcript contains more nucleotides at the 5' end of the transcript that were not identified in the RACE experiment.

The IRE Motif in Mitochondrial Aconitase mRNA Specifically Binds IRP1 and IRP2—Competition studies revealed that binding affinities of IRP1 and IRP2 were equivalent for the IRE of mitochondrial aconitase (data not shown). Competition studies were performed to compare the binding affinity of IRPs for the ferritin IRE versus the *m*-aconitase IRE. Unlabeled ferritin or mitochondrial aconitase IRE and radiolabeled ferritin IRE were mixed and added to IRP(s). Quantitation of gel shift complexes yielded the curves depicted in Fig. 2. The concentration required for 50% inhibition of IRE binding (IC_{50}) for ferritin IRE was 160 ± 40 nM, whereas that of mitochondrial aconitase IRE was 920 ± 90 nM.

IRP1 and IRP2 Repress *In Vitro* Translation of Mitochondrial Aconitase mRNA—Rabbit reticulocyte lysates were used to study the *in vitro* translation of ferritin and *m*-aconitase transcripts. It has been previously shown that equal amounts of IRP1 or IRP2 repress ferritin mRNA translation to the same extent (4). When equal molar amounts of IRP1 or IRP2 were added to the reaction mixtures containing the reconstructed porcine aconitase mRNA template, equal translational repression of porcine aconitase was observed (Fig. 3, A and B). However, the translational repression of mitochondrial aconitase by IRPs *in vitro* was less than that of ferritin (Fig. 3, A and B), a finding that is consistent with the apparently lower binding affinity (Fig. 2).

In order to assess the *in vivo* relevance of translational regulation of mitochondrial aconitase, mice were placed on diets that were either low in iron, normal, or high in iron (see "Materials and Methods"). After periods of 9–16 weeks on the diets, the animals were sacrificed, and the levels of mitochondrial aconitase in liver tissue were quantitatively assessed by Western blot analysis in liver tissue. Although levels of mitochondrial aconitase mRNA were equal between animals on low iron versus high iron diets (Fig. 4B), significant differences were measured in the levels of mitochondrial aconitase in murine liver. Mitochondrial aconitase from animals on a high iron diet increased by a factor of 1.8 ± 0.2 compared with animals on a low iron diet (average of three independent studies). Quantitation of mRNA levels of mitochondrial aconitase was performed on three separate occasions using RNA derived from the same tissue samples that were used in quantitation of

levels of mitochondrial aconitase. The greatest increase in mitochondrial aconitase mRNA levels in any sample was 9% over the low iron control (not shown), and in the example shown (Fig. 4B), the levels of mitochondrial aconitase decreased by 6% in the tissue from animals on a high iron diet, whereas in the third sample (not shown) the levels of the two were virtually identical. In the same mice, ferritin varied over a much larger range, as is indicated in Fig. 4A. Levels of IRP1 were identical in tissues from animals maintained on the low, normal, and high iron diets, a finding that is consistent with previous observations on the effect of manipulations of iron levels on levels of immunodetectable IRP1 (20, 21). IRP2 levels were increased in animals maintained on the low iron diet, as is indicated in Fig. 4A, a finding that is consistent with immunoblot data previously obtained for changes of IRP2 levels upon iron treatment in tissue culture cell lines (17, 22). IRE binding activity of IRP2 was increased on the low iron diet, whereas binding activity of IRP1 was not observably increased in these lysates (data not shown). Levels of mitochondrial aconitase mRNA were unchanged by the change in dietary iron in murine tissues that showed significant changes in levels of mitochondrial aconitase protein, indicating that the change in levels of the protein are not attributable to changes in the transcription rate. In contrast, levels of ferritin H chain mRNA increased approximately 2-fold, a change that has previously been observed on several occasions (23, 24). The range of ferritin regulation seen here was 13-fold, and therefore most of the range of regulation can be attributed to translational regulation, as has been reported in the past (reviewed in Ref. 1), although there is clearly a small transcriptional contribution to the range of regulation in this setting. The level of regulation accounted for by translational regulation would be approximately 5-fold, higher than that of mitochondrial aconitase and comparable with the range of regulation seen in the *in vitro* translation system. None of the mice used in the study had developed iron-deficiency anemia,² indicating that the level of iron deprivation induced by the dietary manipulation was not sufficient to affect hematopoiesis.

DISCUSSION

In this paper, we demonstrate that the 5'-UTR of porcine mitochondrial aconitase contains a functional and conserved IRE that binds IRPs and mediates translational repression of full-length mitochondrial aconitase *in vitro*. The IRE of mitochondrial aconitase is contained within a short 5'-UTR and is a likely target for binding by one or both IRPs in cells. The 2-fold regulation of mitochondrial aconitase that we observe in animals is not nearly as impressive as the range of regulation of ferritin observed in these animals. However, we speculate that the smaller degree of regulation of mitochondrial aconitase may more precisely meet the needs of the cell. Ferritin is needed when cells are iron replete to detoxify and store iron, but it is not needed in cells that are iron-depleted. Although iron supplies may vary in cells, the need of cells to maintain systems that are devoted to transformation and capture of energy such as the citric acid cycle should remain high despite changes in iron availability. It is possible that some repression of biosynthesis of mitochondrial aconitase is advantageous in iron-depleted cells, because incorporation of an iron-sulfur cluster into mitochondrial aconitase is required for enzymatic function, and lack of sufficient iron could lead to synthesis of non-functional apoprotein. However, full repression of biosynthesis of mitochondrial aconitase, unlike ferritin, could be detrimental because of the central role that the citric acid cycle plays in intermediary metabolism.

² K. Iwai, unpublished data.

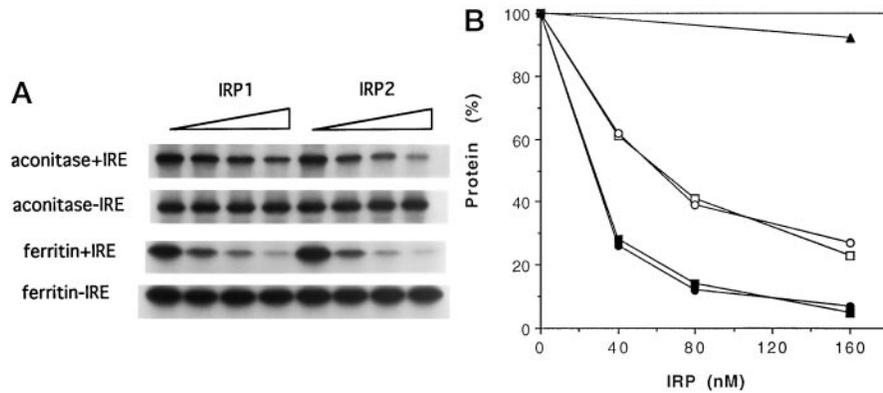


FIG. 3. **The IRE of mitochondrial aconitase mediates translational repression by IRP1 and IRP2.** *A*, binding to IREs quantitatively predicts translational repressor activity. Equal amounts of IRP1 versus IRP2 were added to reactions in which IRE containing RNA transcripts were present at a 64 nM concentration. Amounts of added IRP1 (first four lanes) or IRP2 (second four lanes) were 0, 40, 80, and 160 nM. Biosynthetically labeled mitochondrial aconitase or ferritin was resolved on either an 8 or 15% SDS-polyacrylamide gel, respectively, and the relevant translation product is depicted. *B*, graphic representation of translational inhibition produced by IRP1 versus IRP2 on equal amounts of ferritin or aconitase transcript as depicted in *A*.

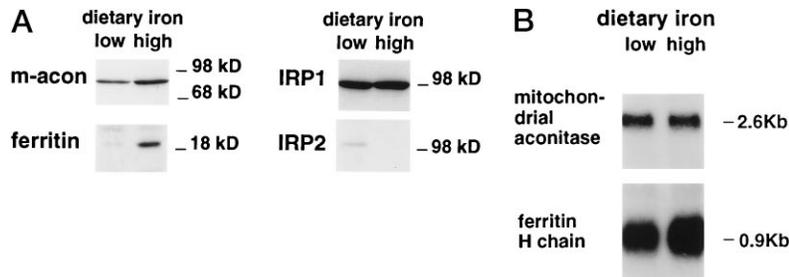


FIG. 4. *A*, the effect of dietary iron manipulations on levels of mitochondrial aconitase and ferritin. Levels of mitochondrial aconitase and ferritin increase in mice on a high iron diet, whereas IRP1 levels do not change, and IRP2 levels increase in animals on a low iron diet. Amounts of proteins were assessed in Western blot assays using antibodies specific for each protein. *B*, levels of mitochondrial aconitase mRNA are unchanged by dietary iron manipulation, whereas ferritin levels are increased 2-fold on a high iron diet. Northern analyses of mRNA levels were performed and quantitated as described under "Materials and Methods."

The pronounced decrease in efficacy of the IRE in the transcript of porcine mitochondrial aconitase in mediating translational repression when compared with the efficacy of the ferritin IRE in mediating repression (Fig. 3) may be related to the stability of the IRE stem-loop. The IRE of mitochondrial aconitase is noteworthy in that the stems of the four species discussed here are relatively rich in AU base pairs, and the lower stem predicted in the optimal secondary structure predicted for the RNA sequence consists of only four base pairs, one of which is a G-U pair (25). When the IRE of mitochondrial aconitase consisting of a four-base pair lower stem is synthesized and used as a competitor in RNA binding assays, it is less efficacious as a competitor than the ferritin IRE (Fig. 2). Other RNA structures of comparable stability to the consensus IRE are selected in computer folding when additional 5' and 3' sequences that flank the consensus IRE are included in the sequence analyzed (25), and it is possible that the nucleotides that can form the IRE structure also contribute to competing structures, so that the consensus IRE binding site for IRPs may be intermittently unavailable. Although we have not directly demonstrated a change in the rate of biosynthesis of mitochondrial aconitase in cells in tissue culture using biosynthetic labeling and immunoprecipitation, we have demonstrated a clear decrease in total protein in the tissues of animals maintained on a low iron diet, and the range of regulation seen in this setting correlates with that observed in the *in vitro* translational regulation assay. Thus, the lower stability of the IRE in mitochondrial aconitase may account for the decrease in the range of regulation seen in the *in vitro* translation system and the decrease in range of regulation of mitochondrial aconitase *in vivo* relative to that observed with the ferritin IRE.

It is interesting that another citric acid cycle enzyme, the succinate dehydrogenase subunit b of *D. melanogaster*, contains a functional IRE (11), whereas the transcript of the human succinate dehydrogenase homologue has no recognizable IRE (12, 26). We have shown here that relatively unstable IREs are conserved in the mitochondrial aconitases of four mammalian species. Because iron is required for the function of two iron-sulfur proteins of the citric acid cycle, mitochondrial aconitase and succinate dehydrogenase, and numerous enzymes of the respiratory chain, it may be advantageous for the cell to reduce synthesis of these proteins during periods of iron deprivation. It is intriguing that an IRE is found in the transcript of a subunit of one of the two iron-sulfur proteins of the citric acid cycle of *Drosophila*, whereas it is found in the transcript of the other in mammalian cells. It could be an accident of evolution that insects acquired and retained an IRE in one of the two transcripts, whereas mammalian cells acquired and retained an IRE in the other transcript that encodes an iron-sulfur protein. It will be interesting to examine the sequences of the 5'-UTR of succinate dehydrogenase transcripts in other insect species to determine whether IREs are present.

Mitochondrial aconitase and succinate dehydrogenase are additions to a growing list of transcripts that are translationally regulated by IREs. We have previously speculated that IREs may arise by mutation in untranslated regions of transcripts and that they may be retained in transcripts when the presence of the IRE confers a functional advantage (27). In the cases of both mitochondrial aconitase and succinate dehydrogenase, it may be advantageous to decrease synthesis of the protein when the prosthetic group required for function cannot be synthesized. It seems unlikely that functional IREs are

present in the transcripts of two citric acid cycle iron-sulfur proteins by coincidence, and although simple economy in matching production of iron-sulfur proteins to iron supply may be the reason that these genes are translationally regulated, the possibility of a more generalized effect of iron on regulation of the citric acid cycle merits future consideration.

Note Added in Proof—After submission of this article, data on the translational regulation of the iron-sulfur protein subunit of succinate dehydrogenase and on mammalian mitochondrial aconitase was published by Gray *et al.* (Gray, N. K., Pantopoulos, K., Dandekar, T., Ackrell, B. A. C., and Hentze, M. W. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 4925–4930).

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