THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 51, Issue of December 22, pp. 30781–30786, 1995
Printed in U.S.A.

Succinate Dehydrogenase b mRNA of *Drosophila melanogaster* Has a Functional Iron-responsive Element in Its 5′-Untranslated Region*

(Received for publication, August 28, 1995)

Stefan A. Kohler, Beric R. Henderson‡, and Lukas C. Kühn§

From the Swiss Institute for Experimental Cancer Research, CH-1066 Epalinges s/Lausanne, Switzerland

Iron-responsive elements (IREs) are *cis*-acting mRNA stem-loop structures that specifically bind cytoplasmic iron regulatory proteins (IRPs). IRP-IRE interactions mediate the coordinate post-transcriptional regulation of key proteins in iron metabolism, such as ferritin, transferrin receptor, and erythroid 5-aminolevulinic acid synthase. Depending on whether the IRE is located in the 5′- or 3′-untranslated region (UTR), binding of IRP will inhibit mRNA translation or degradation, respectively. Here we describe a new IRE in the 5′-UTR of succinate dehydrogenase subunit b (SDHb) mRNA of *Drosophila melanogaster*. The SDHb IRE binds *in vitro* to vertebrate and insect IRPs with a high affinity equal to that of human ferritin H chain IRE. Under conditions of iron deprivation, SDHb mRNA of *Drosophila* SL-2 cells shifts to a non-polysome-bound pool. Moreover, translation of a human growth hormone mRNA with the SDHb IRE in its 5′-UTR is iron-dependent in stably transfected L cells. We conclude that the SDHb IRE mediates translational inhibition both in insect and vertebrate cells. This constitutes the first identification of a functional IRE in insects. Furthermore, *Drosophila* SDHb represents the second example, after porcine mitochondrial aconitase, of an enzyme of the citric acid cycle whose mRNA possesses all necessary features for translational regulation by cellular iron levels.

Cytoplasmic control of mRNA translation and stability is well documented for transcripts that encode proteins in iron metabolism (1–3). As a shared feature, the regulated mRNAs contain specific RNA stem-loop structures, the iron-responsive elements (IREs),[1] which are binding sites for iron regulatory proteins (IRP-1 and IRP-2) (4–9). The RNA binding activity of these trans-acting proteins is induced in the cytoplasm under conditions of iron deprivation. Ferritin H and L chain mRNA, erythroid 5-aminolevulinic acid synthase mRNA and mitochondrial aconitase mRNA, have a single IRE in their 5′-untranslated region (UTR) (10–12). As documented for the first three of these mRNAs, interaction with an IRP prevents ribosomes from initiating translation (13–15). Unlike the translationally regulated mRNAs, transferrin receptor (TfR) mRNA contains five IREs located in the 3′-UTR (5, 6). Here, IRP binding causes an inhibition of TfR mRNA degradation and, consequently, an increase rather than a decrease in protein expression. This coordinate regulation of ferritins and TfR is thought to act as a feedback mechanism to maintain cellular iron homeostasis, since iron scarcity diminishes cellular iron storage but enhances the potential of iron uptake. In contrast, under the inverse conditions of ample iron supply, IRPs are inactivated, permitting ferritin mRNA translation and TfR mRNA degradation. These conditions are likely to prevent iron overload. Similarly, translational inhibition of the key enzyme in erythroid porphyrin biosynthesis, 5-aminolevulinic acid synthase, has been viewed as a mechanism to match this pathway with iron availability (11, 12, 16).

The functional importance of the IRP-IRE interactions is also supported by their phylogenetic conservation (17, 18). IRE-binding proteins have been identified in vertebrates, insects, and annelids but seem to be missing in yeasts, plants, and bacteria. IREs are similarly conserved in the respective mRNAs of vertebrate and some invertebrate species (19, 20). However, it remains unknown how many more hitherto unidentified mRNAs are regulated through IRP-IRE interactions. In fact, a certain variability in the IRE sequences does not permit the isolation of new members of the family by hybridization or PCR methods, and their discovery relies at present mainly on the analysis of newly acquired sequences in data bases. For this purpose, Dandekar *et al.*(12, 21) have designed a computer-aided screening program.

On the basis of phylogenetic comparisons and *in vitro* mutagenesis, the IRE has been defined as a strongly conserved mRNA structure (19, 22). Invariably, it consists of an upper and a lower stem of paired ribonucleotides with a ΔG value for base interactions of approximately -5 kcal/mol. The upper stem comprises always 5 complementary bases, which need to be paired for IRP binding. The sequences of these stems vary among mRNAs from different genes and do not seem to be crucial in the RNA-protein interaction. However, they are well conserved during evolution for any given mRNA species (19). Below the upper stem there is invariably a small 5′-bulge consisting either of a single unpaired C or a C preceded by two additional nucleotides opposite to a single unpaired nucleotide (ferritin IRE). The loop at the tip consists of 6 nucleotides with a consensus CAGUGN sequence. This sequence together with the bulge C confers the binding specificity (23). The consensus loop sequence exhibits the highest affinity for either IRP-1 or IRP-2, but we have recently defined a spectrum of suboptimal sequences with slightly lower affinities that are still able to bind to IRPs *in vitro*. Because of the existence of such alternative sequences, we have carried out computer searches of the EMBL data base to identify possible new candidate IREs in mRNAs. In the course of this search, the recently entered succinate dehydrogenase subunit b (SDHb) gene of *Drosophila*

* This work was supported by the Swiss National Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: MRC Laboratory of Molecular Biology, Hills Rd., Cambridge CB2 2QH, UK.

§ To whom correspondence should be addressed: Swiss Institute for Experimental Cancer Research, Genetics Unit, 155, Ch. des Boveresses, CH-1066 Epalinges s/Lausanne, Switzerland. Tel.: 41-21-692-58-36; Fax: 41-21-652-69-33; E-mail: lukas.kuehn@isrec.unil.ch.

[1] The abbreviations used are: IRE, iron-responsive element; IRP, iron regulatory protein; rpA1, ribosomal protein A1; SDH, succinate dehydrogenase; UTR, untranslated region; TfR, transferrin receptor; PCR, polymerase chain reaction.

melanogaster (24) revealed a classical IRE in the 5′-UTR. This enzyme subunit of the citric acid cycle is translated in the cytoplasm prior to its import into the mitochondrial matrix. It contains three different iron-sulfur clusters (25), which are essential for the transfer of electrons to the respiratory chain (26). In the present study we show the functional importance of this SDHb IRE.

MATERIALS AND METHODS

Cell Culture—Insect SL-2 and SL-3 cells were cultured in revised Schneider's *Drosophila* medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum. Murine B16.F1 cells were cultured in Dulbecco's modified Eagle's medium, 10% fetal calf serum, and Ltk$^-$ cells in α-minimal essential medium (Life Technologies, Inc.), 10% fetal calf serum. The α-minimal essential medium contained 100 μM hypoxanthine, 0.4 μM thymidine, and 16 μM aminopterin for stably transfected L cells.

RNA Extraction—Cytoplasmic RNA was prepared from SL-3 cells as described (27). Cells were lysed in 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2.5 mM dithiothreitol, 0.6% Nonidet-P40. The extract was centrifuged at 13,000 × *g* for 30 s, the supernatant was phenol-extracted, and RNA was ethanol-precipitated. RNA was resuspended in water and stored at −80 °C.

cDNA Synthesis and Subcloning—Approximately 2 μg of cytoplasmic RNA from SL-3 cells were used to synthesize a cDNA fragment by reverse transcription. The reaction was initiated with the SDHb-specific primer RP1 (5′-GCAGATCTCGCGTCTTGTTCCCTTTAC-3′), overlapping with nucleotides 2393–2411 (24) and 15 units of avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) according to the supplier's instructions. RNA was degraded by the addition of 1 mg/ml RNase A. In order to amplify an almost complete cDNA, one-fifth of the reverse transcription reaction was PCR-amplified in 30 cycles with Ampli-*Taq* (Perkin-Elmer), RP1 as the 3′-primer and FP1 (5′-AGGGATCCTGTACGCAAATAAGAAAAAC-3′, overlapping with nucleotides 924–943 (24)) as the 5′-primer. Amplification of the 5′-UTR was performed with another reverse primer, RP2 (5′-GTAGATCTTTTCGTTGCGGTTTGTG-3′, overlapping with nucleotides 1001–1018 (24)) in combination with FP1. The annealing temperature was 48 °C for both reactions. Products were analyzed on agarose gels, and the predominant bands were purified and cloned into the *Sma*I site of pGEM-3Zf(−) (Promega). The presence of the correct inserts in the resulting plasmids pG3-SDHb and pG3-SDHb5′ was verified by DNA sequencing. In order to generate SDH-GH for transfection into L cells, the SDHb 5′-UTR was excised from pG3-SDH5′ by *Bam*HI and *Xba*I-digestion and ligated into *Bam*HI- and *Xba*I-cut L5-GH (28). The parent vector L5-GH is a pUC18 derivative that contains the human ferritin H chain promoter fused to the human growth hormone cDNA. The *Bam*HI-site is 9 nucleotides downstream of the transcription start site. To construct 213-GH, a double-stranded oligonucleotide (*Bam*HI-TGCTTCAAGCTCCGTTGGACGGATCT-*Xba*I) coding for a mutated IRE, which binds neither mouse IRP-1 nor IRP-2 *in vitro*,[2] was inserted between the *Bam*HI and *Xba*I sites of L5-GH.

Primer Extension—75 ng of RP2 was 5′-end-labeled using T4-polynucleotide kinase (Boehringer Mannheim) in the presence of 30 μCi of [γ-^{32}P]ATP (Amersham Corp.). After ethanol precipitation with 20 μg of glycogen as carrier, the labeled primer was resuspended in 15 μl of water. 3.5 μl (corresponding to 2.25 × 10^6 cpm) were denatured for 3 min at 75 °C together with approximately 10 μg of SL-3 cytoplasmic RNA, 1.5 μl of 10 × hybridization buffer (1.5 M KCl, 100 mM Tris-HCl, pH 8.3, 10 mM EDTA) in 10 μl final volume and slowly cooled to 42 °C. Reverse transcription was carried out for 1 h by adding 5 units of avian myeloblastosis virus reverse transcriptase (Promega) following degradation of RNA with 15 μg/ml RNase A. After phenol extraction and ethanol precipitation the pellet was directly resuspended in sequencing dye and analyzed on a 6% sequencing gel.

Preparation of Cytoplasmic Extracts—Cellular extracts were prepared as described (7). In brief, 2.4 × 10^6 SL-3 cells were washed once with phosphate-buffered saline and then lysed by resuspension in 10 mM HEPES, pH 7.5, 40 mM KCl, 3 mM $MgCl_2$, 5% glycerol, 0.3% Nonidet-P40, 1 mM phenylmethylsulfonyl fluoride. After 5 min of centrifugation at 4 °C in a microfuge the supernatant was stored in aliquots at −80 °C. The protein concentration was determined using the Bio-Rad protein assay.

[2] B. R. Henderson, E. Menotti, and L. C. Kühn, submitted for publication.

Purification of Recombinant Human IRP-1—Recombinant human IRP-1 carrying an N-terminal histidine-tag was expressed in bacteria and purified on a Ni^{2+}-NTA-agarose column (Qiagen Inc., Chatsworth, CA) as described (29). The eluate was diluted 10-fold in buffer A (20 mM Tris-HCl, pH 8.0, 5% glycerol, 8 mM 2-mercaptoethanol) and applied to a Mono Q column (Pharmacia Biotech Inc.). Bound protein was eluted with a gradient of 0–300 mM KCl in buffer A. Two protein peaks, one at 80 mM and one at 100 mM, both contained IRP as verified by immunoblotting and band shift assays. Only fractions corresponding to the first peak were pooled and used for further experiments.

Synthesis of RNA Transcripts in Vitro—Human ferritin H chain IRE was transcribed from 1 μg of pSPT-fer plasmid linearized with *Bam*HI as described (6). Similarly, the SDHb core IRE was synthesized using 200 ng of a synthetic double-stranded oligonucleotide containing the T7 RNA polymerase promoter sequence as a template (TAATACGACTCACTATAGGAATTCTAATTGCAAACGCAGTGCCGTTTCAATTGTCTAGACA). Transcripts were separated from the template on 3% NuSieve agarose gel (FMC Corp., Rockland, ME), recovered by migration onto ion exchange membranes (NA45 DEAE, Schleicher & Schüll) as described previously (7), and carefully quantitated. Radiolabeled IREs were synthesized using the same templates as above (for SDHb5′-UTR: 300 ng of *Xba*I-cleaved pG3-SDHb5′) and gel-purified as described (23).

RNA-Protein Gel Retardation Assays—Analysis of RNA-protein complexes was carried out as described previously (6). Briefly, aliquots of cytoplasmic extracts (~2 μg of protein) or purified recombinant IRP-1 (1 ng) were incubated for 10 min with an excess of probe (10^5 cpm; ~0.2 ng) at 25 °C. 2-mercaptoethanol was present at 2% (v/v) in all binding reactions. Where indicated, unbound probe was degraded following a 5-min incubation with 1 unit of RNase T1 (Calbiochem, San Diego, CA). Except for the competition experiments with purified recombinant IRP-1, nonspecific RNA-protein interactions were displaced by the addition of heparin at 5 mg/ml for 10 min. For competition assays, 2.5 × 10^4 cpm (~50 pg) of radiolabeled probe and varying molar excess of unlabeled competitor RNAs were mixed and denatured for 4 min at 70 °C prior to addition of a reaction premix containing buffer and protein. RNA-protein complexes were resolved on a 6% nondenaturing polyacrylamide gel and processed for autoradiography. Competition assays were quantified on a PhosphorImager.

RNA-Protein Cross-linking—Mouse B16.F1 cells were deprived of iron by incubation with 100 μM desferrioxamine for 24 h to induce IRP-1 and IRP-2. Extracts from these cells and from untreated *Drosophila* SL-3 cells (15 μg) were UV-cross-linked to 8×10^5 cpm (1.6 ng) gel-purified radiolabeled SDHb IRE as described previously (7). [^{32}P]RNA-protein complexes were separated on an 8% SDS-polyacrylamide gel, blotted onto a nitrocellulose membrane and detected by autoradiography.

Transfection of L Cells—Ltk$^-$ cells were stably co-transfected with the thymidine kinase gene using the calcium phosphate method with subsequent HAT selection (31). The positive control construct called Fer-GH (12) contains the human ferritin H chain promoter and IRE fused to the human growth hormone cDNA. The RNA of this construct is regulated by iron levels *in vivo* (12). Two more lines were established: one with the construct SDH-GH and another with 213-GH (see "cDNA Synthesis and Subcloning").

Metabolic Labeling and Immunoprecipitation—^{35}S-Labeling and quantitative immunoprecipitation were carried out in a variation of a described protocol (32). About 2 × 10^6 stably transfected, subconfluent L cells were incubated for 16 h with either 60 μg/ml ferric ammonium citrate or 100 μM desferrioxamine (Desferal, gift from Ciba Geigy, Basel). Cells were washed once in warm phosphate-buffered saline and once in methionine-free α-minimal essential medium (Life Technologies, Inc.) and then labeled for 2.5 h in the same medium supplemented with ferric ammonium citrate or desferrioxamine and 40 μCi/ml [^{35}S]methionine (>1000 Ci/mmol, Amersham). Radioactivity incorporated into secreted proteins was determined by precipitation of culture medium with trichloroacetic acid and scintillation counting. For immunoprecipitation, 5 × 10^5 cpm of trichloroacetic acid-precipitable material was diluted to 1 ml with precipitation buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1% Triton X-100), and 5 μl of rabbit anti-human growth hormone antibody (Dako, Carpinteria, CA) was added. The mixture was tumbled for 1 h at 4 °C and then transferred to 50 μl of Protein A-Sepharose beads that had been washed three times with precipitation buffer. After 1 h of tumbling at 4 °C, the beads were washed three times with precipitation buffer prior to boiling for 5 min with 50 μl of 2 × sample buffer. Samples were electrophoresed on a 15% SDS-polyacrylamide gel and processed for autoradiography.

Polysome Gradient and mRNA Detection—Two batches of 2.5 × 10^7

SL-2 cells were treated for 48 h with 150 μM desferrioxamine. One of them was washed with phosphate-buffered saline, and fresh medium containing 60 μg/ml ferric ammonium citrate was added to the cells for additional 4 h. Cell extracts were prepared by lysis at 4 °C in extraction buffer (10 mM Tris-HCl pH 7.5, 1 mM potassium acetate, 2 mM magnesium acetate, 2 mM dithiothreitol, 100 μg/ml cycloheximide, 0.2% Nonidet-P40) supplemented with 1 mM phenylmethylsulfonyl fluoride and 1000 units/ml RNasin. Nuclei and mitochondria were removed by centrifugation for 10 min at 13,000 × *g* at 4 °C. The supernatant was loaded on a 10-ml sucrose gradient (15–60% sucrose in extraction buffer) and centrifuged at 38,000 rpm in a TST41.14 rotor at 4 °C. Fractions of 500 μl were extracted with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated, and analyzed by electrophoresis on a 1.5% agarose/formaldehyde gel and Northern blotting. The membrane was stained with methylene blue and hybridized with a [α-^{32}P]CTP-labeled antisense riboprobe for SDHb (transcribed from *Cla*I-cut pG3-SDHb). After stripping, the membrane was rehybridized with a random-primed, [α-^{32}P]dCTP-labeled DNA probe specific for the *Drosophila* ribosomal protein A1 (33).

RESULTS

SDHb mRNA from Drosophila melanogaster Contains an IRE in Its 5′-Untranslated Region—Recently, we have identified a large number of IRE sequences capable of binding to IRP-1 and IRP-2 with suboptimal affinities *in vitro* (Ref. 23).[2] These mutants differ in their 6-base loop sequence from the ferritin wild-type IRE (CAGUGN) usually by only one or a few nucleotides. This raised the question whether such IREs might exist in naturally occurring mRNAs. Therefore, we regularly searched new entries to the EMBL nucleic acid data base for potential IREs with either a wild-type or mutant loop sequence. For this we used the computer search program by Dandekar *et al.* (12, 21). In the course of one of these searches, it became evident that the sequence of the recently cloned gene for succinate dehydrogenase, subunit b (SDHb) of *Drosophila melanogaster* (24) contains a typical wild-type IRE apparently close to the 5′-end of the predicted mRNA (Fig. 1*A*). To verify this observation, we resequenced the area in question after amplification of *Drosophila* cDNA made from SL-3 cell mRNA. Appropriate PCR primers were chosen based on the published sequence of Au (24) to obtain either the 5′-end region or the almost entire cDNA as described under "Materials and Methods." The PCR products reflected accurately the sizes of the cDNA predicted without introns (24), excluding the possibility that genomic DNA was amplified. Both the product covering nucleotides 20–112 of the cDNA as well as that with the almost entire cDNA, contained an IRE toward the 5′-end that matched the genomic sequence. In order to verify that this IRE was close to the 5′-end of the mRNA, we carried out a primer extension analysis of SL-3 mRNA (Fig. 1*C*). By comparison to a sequencing reaction of pGEM-3Zf(−), the length of the reverse transcription product was determined to be 121 nucleotides. Thus, the bulge C of the IRE is located in position 71 of the mRNA, which is in good agreement with the previously published data (24). From these results we concluded that *Drosophila melanogaster* SDHb mRNA indeed contains an IRE which is located in the 5′-untranslated region at 71 nucleotides (position of the bulge C) from its 5′-end. This IRE has a consensus CAGUGC sequence in its loop, a single unpaired C at the bulge position, and a predicted free energy of folding of $\Delta G = -5.9$ kcal/mol (Fig. 1*B*).

Drosophila SDHb IRE Binds in Vitro with High Affinity to IRP—In order to verify that the predicted IRE was able to bind IRP from various sources, we performed RNA-protein gel retardation assays. The PCR amplification product corresponding to the almost entire 5′-translated region (nucleotides 20–112) was subcloned into the pGEM-3Zf(−) vector, and radiolabeled sense RNA was transcribed from the T7 promoter. Radiolabeled SDHb core IRE was synthesized from a synthetic double-stranded oligonucleotide containing the T7 RNA polymerase promoter sequence. These RNAs were then incubated in the presence of 2% 2-mercaptoethanol with either insect SL-3 cell extract or purified human recombinant IRP-1. Where the 5′-UTR was used as a probe, RNA-protein complexes were treated with RNase T1, and nonspecific binding was displaced by heparin in all samples prior to the analysis on nondenaturing 6% polyacrylamide gels. Either probe showed a specific band shift with both IRP preparations that was indistinguishable from that of the control human ferritin H chain IRE (Fig. 2). This indicates that the SDHb 5′-untranslated region contains an authentic IRE.

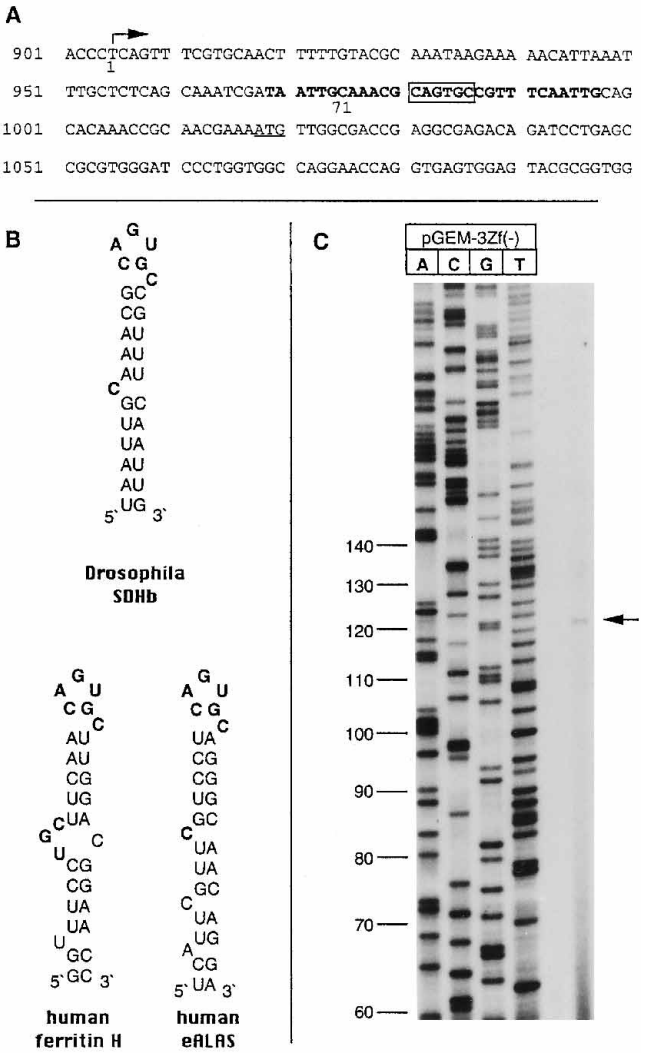

FIG. 1. **Drosophila SDHb mRNA contains an IRE-like sequence.** *A*, the sequence and position of the IRE in the SDHb mRNA of *D. melanogaster* is depicted. The IRE is printed in *boldface letters* with the loop sequence *framed*, and the transcription start-codon is indicated by an *arrow*. The structure of the insect SDHb IRE, as predicted by the GCG "Fold" program (43), is shown in *B* (*top*) in comparison with the IREs of human ferritin H chain and erythroid 5-aminolevulinic acid synthase (*eALAS*) mRNAs (*bottom*). In these structures we have included the recent finding of a C^1–G^5 base pairing in the loop (23, 30). *C*, the transcription start site of the SDHb gene was mapped by primer extension as described under "Materials and Methods," and the product is indicated by an *arrow*. A sequencing reaction of the plasmid pGEM-3Zf(−) (Promega) using an oligonucleotide complementary to the T7 promoter is shown as a size marker.

Further evidence for a direct interaction between *Drosophila* SDHb IRE and IRP was obtained by a UV-cross-linking experiment. Extracts from either SL-3 cells or iron-deprived murine B16.F1 cells were UV-irradiated in the presence of an excess gel-purified radiolabeled SDHb IRE (Fig. 3). Where indicated,

FIG. 2. **The SDHb IRE forms distinct complexes with a protein in *Drosophila* cell extracts and with purified recombinant human IRP-1.** Radiolabeled human ferritin H chain IRE (*Fer*), SDHb IRE (*SDH*), and the entire 5′-UTR of SDHb mRNA (*5′*) form RNA-protein complexes with *Drosophila* and human IRPs. The gel retardation assay was carried out with 2 μg of protein from *Drosophila* SL-3 cell extract or 250 ng of purified recombinant human IRP-1 as described under "Materials and Methods." 2×10^4 cpm of the IREs or 3×10^4 cpm of 5′-UTR were used as probes. After binding, RNase T1 was added to samples containing the 5′-UTR-probe and, after 5 min of incubation at room temperature, heparin was added to all samples at 5 mg/ml. The incubation was allowed to proceed for 10 min before loading of samples on a 6% nondenaturing polyacrylamide gel.

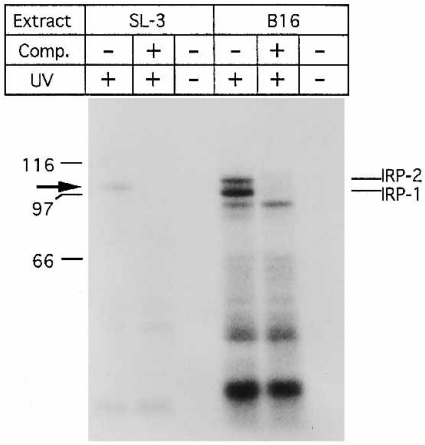

FIG. 3. **Drosophila cytoplasmic extracts contain a specific IRE-binding protein of approximately 100 kDa.** Cytoplasmic protein extracts were prepared from untreated *Drosophila* SL-3 cells or mouse B16.F1 cells that had been deprived of iron by incubation with 100 μM desferrioxamine for 24 h to induce IRP-1 and IRP-2. Extracts (15 μg) were incubated with 8×10^5 cpm (~1.6 ng) of radiolabeled SDHb IRE and UV-irradiated. Where indicated, a 100-fold molar excess of unlabeled ferritin H chain competitor IRE was premixed with the [^{32}P]RNA probe (*Comp.*). Samples were treated with RNase T1 prior to addition of heparin to 5 mg/ml. Proteins were separated on a 8% SDS-polyacrylamide gel, blotted onto a nitrocellulose membrane, and autoradiographed.

unlabeled ferritin H chain IRE was mixed at 100-fold molar excess with the [^{32}P]RNA probe before the addition of extract. Two specifically cross-linked proteins could be detected in B16.F1 cells, presumably IRP-1 (97 kDa) and IRP-2 (105 kDa). One specific band of about 100-kDa size is visible in *Drosophila* extracts. These results confirm the phylogenetic conservation of the IRP-IRE interaction as observed previously (17, 18).

Since functional IREs show a strong affinity for IRPs, we measured the relative affinity of the SDHb probe compared with the human ferritin H chain probe. A fixed amount of radiolabeled IRE (about 50 pg) was analyzed in cross-competition assays with various concentrations of unlabeled RNA for the binding to IRP from man, fruit fly, or mouse (Fig. 4). The results revealed that the *Drosophila* SDHb IRE is an almost as good a competitor as the ferritin H chain IRE in any of the settings, whereas tRNA was unable to compete even at 200-fold molar concentration. Noteworthy, both IREs showed similarly high affinity for IRP-2 in extracts of iron-deprived mouse B16.F1 cells (data not shown).

The SDHb IRE Mediates Translation Inhibition of SDHb

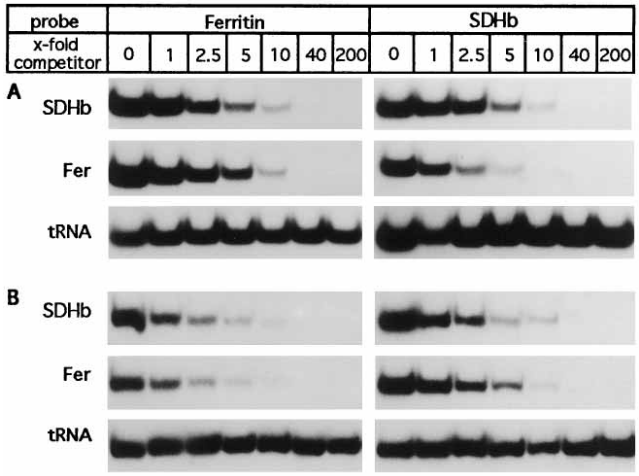

FIG. 4. **The SDHb IRE has a similar affinity for *Drosophila* IRP and human IRP-1 as the ferritin H chain IRE.** Radiolabeled ferritin H chain IRE (*left panels*) or labeled SDHb IRE (*right panels*) was incubated with IRPs in the presence of various concentrations of unlabeled competitor RNA (SDHb IRE, ferritin H chain IRE, or yeast tRNA (expressed as mol of unlabeled/mol of labeled RNA)). Premixed radiolabeled probe (2.5×10^4 cpm 50 pg) and x-fold competitor were incubated with a constant amount (750 ng of protein) of *Drosophila* SL-3 cell extract (*panel A*) or 1 ng of purified recombinant human IRP-1 (*panel B*). To the reactions containing the SL-3 cell extract, heparin was added at 5 mg/ml after binding. To allow direct comparison for each set of competitors, the gels were exposed for the same period of time.

mRNA under Conditions of Iron Deprivation—Induction of the RNA-binding activity of IRP by iron deprivation and subsequent interaction of IRP with IREs in mRNA 5′-untranslated regions is known to block translation. This inhibition can be measured either in a net decrease of the protein biosynthesis rate (28, 35) or alternatively by a shift of the mRNA from a polysome-bound pool to a free cytoplasmic pool. This shift can be observed upon sucrose gradient centrifugation combined with Northern blotting and specific hybridization (6, 15, 36). In order to test whether the SDHb IRE can confer translational regulation upon a reporter mRNA, we inserted a 5′-fragment of the SDHb cDNA (nucleotides 20–112) into the 5′-untranslated region of a human growth hormone expression vector (L5-GH (28)). This plasmid (SDH-GH) was stably transfected into mouse L cells in parallel with control plasmid constructs containing instead either the wild-type ferritin H chain IRE (Fer-GH (12)) or a mutant IRE that does not bind to IRPs *in vitro* (213-GH).[2] Each of the cell populations was exposed to high iron or iron-deprived medium for 24 h and then labeled for 4 h with [^{35}S]methionine. Radioactivity incorporated into proteins was determined by trichloroacetic acid precipitation, and secreted human growth hormone was quantitatively immunoprecipitated from the medium. *De novo* biosynthesis of growth hormone was clearly inhibited under iron deprivation if translated from an SDHb IRE or a ferritin-IRE containing mRNA, but not from the mRNA with the mutant IRE (Fig. 5). This indicates that the SDHb IRE is functional as a *cis*-acting regulatory element in mouse cells.

Finally, we wanted to obtain direct evidence that iron deprivation regulates SDHb mRNA translation in insect cells. This was analyzed by the mRNA distribution on polysome gradients. *Drosophila* SL-2 cells were incubated in the presence of 150 μM desferrioxamine for 48 h and then either harvested or further incubated for another 4 h with 60 μg/ml ferric amonium citrate. Cytoplasmic extracts were prepared and separated on a 15–60% sucrose gradient as described under "Materials and Methods." RNA from every odd gradient fraction was extracted and analyzed by Northern blotting. Methylene blue staining of

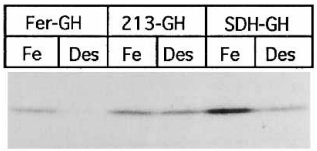

FIG. 5. ***Drosophila* SDHb IRE is an active translational regulatory element in mouse L cells.** The 5′-UTR of the insect SDHb cDNA was cloned into the 5′-UTR of the human growth hormone expression vector L5-GH (28). This plasmid DNA (*SDH-GH*) was stably co-transfected into Ltk$^-$ cells by the calcium phosphate method (31). Control plasmids had either a human ferritin H chain IRE (*Fer-GH*) or a mutated H chain IRE with a more than 100-fold lower IRP binding affinity (*213-GH*). Pools of transfected cells were cultured for 24 h in medium supplemented with either 100 μM desferrioxamine (*Des*) or 60 μg/ml ferric ammonium citrate (*Fe*). Cells were then labeled in methionine-free medium with 40 μCi/ml [^{35}S]methionine, and secreted human growth hormone was quantitatively immunoprecipitated from the culture medium.

the membrane revealed the distribution of ribosomal RNAs (Fig. 6). The membrane was then successively hybridized with probes specific for SDHb and the *Drosophila* ribosomal protein A1 (rpA1). Whereas the rpA1 mRNA could be detected in the same gradient fractions regardless of cellular iron status, SDHb mRNA redistributed from a free RNA-pool (fractions 5–7) under low iron conditions to heavier polysomes (fractions 9–17) upon addition of iron. We conclude that in insect cells translation of the SDHb mRNA is regulated by cellular iron levels.

DISCUSSION

We have identified a new IRE in the 5′-UTR of the *D. melanogaster* SDHb mRNA. This IRE is single-C bulge type and contains the consensus loop sequence CAGUGN, thus resembling the IREs present in TfR and erythroid 5-aminolevulinic acid synthase (5, 34). Its energy of folding ($\Delta G = -5.9$ kcal/mol) lies in the normal range of functional IREs. Using gel retardation assays and UV-cross-linking, we demonstrate that *in vitro* the new IRE binds with an equally high affinity as a human ferritin H chain IRE to IRP in *Drosophila* cell extracts, mouse IRP-1 and IRP-2, and recombinant human IRP-1. These results confirm the earlier notion that the IRP-IRE interaction is phylogenetically conserved between human and fly (17, 18). The SDHb IRE represents a functional *cis*-acting translational regulatory element *in vivo* since it confers iron regulation to an hGH reporter construct transfected into L cells. However, regulation appeared to be slightly less efficient than with the Fer-GH construct (Fig. 5). This might be due to the higher base line of expression in the SDH-GH-transfected cell line. Another reason could be the relatively great distance of the SDHb IRE from the 5′-cap structure (71 nucleotides). The efficiency of IREs as translational control elements was shown to depend on their position with respect to the cap site (15, 35). In mouse B6 fibroblasts, regulation was lost with an IRE located 67 or more nucleotides from the cap (35). However, the authors noted that the relative effect of distance on the efficiency of regulation varied between different cell types.

Direct proof for the iron-dependent translation of SDHb in insect cells would require a specific antibody that recognizes the native protein for immunoprecipitation. Since no such antibody was available to us, we analyzed the association with ribosomes of SDHb mRNA from iron-depleted and iron-replenished *Drosophila* cells on a polysome gradient. In the case of ferritin mRNA, translational inhibition in iron-deprived rodent cells promotes a substantial shift of the mRNA from the polysome-bound mRNA to lighter, nonbound mRNA fraction (15, 36). Here we show that association of the SDHb mRNA with polysomes could only be detected when iron was abundant, indicating that under conditions of low iron, translation of the

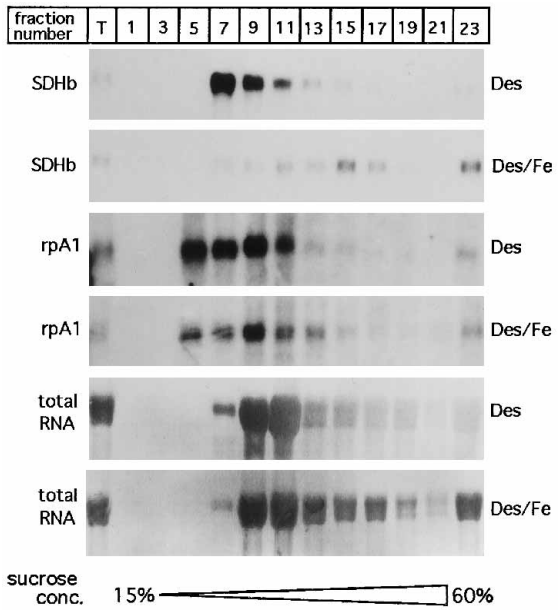

FIG. 6. **Redistribution of insect SDHb mRNA between a polysome-bound and a nontranslated pool as a function of cellular iron.** SL-2 cells of *D. melanogaster* were either cultured for 48 h in medium with 150 μM desferrioxamine (*Des*) or after the desferrioxamine treatment for an additional 4 h in medium with 60 μg/ml ferric ammonium citrate (*Des/Fe*). Cells (2.5×10^7) were then lysed and centrifuged at $13{,}000 \times g$ for 10 min. The supernatant was separated on a 15–60% sucrose gradient. RNA from sucrose gradient fractions was analyzed by electrophoresis on a 1.5% agarose/formaldehyde gel and Northern blotting. Blots were hybridized with ^{32}P-labeled probes of the *Drosophila* SDHb cDNA and the rpA1 cDNA. A sample corresponding to the total RNA that was loaded onto the gradient is shown in the first lane (*T*).

mRNA was inhibited. This pattern is indicative of a block in the ribosome association with the SDHb mRNA in iron-deprived *Drosophila* cells.

Besides porcine mitochondrial aconitase (12, 37), *Drosophila* SDHb is the second enzyme of the citric acid cycle, which seems to be subject to translational regulation by iron. This finding is presently difficult to interpret in terms of its physiological meaning. Whereas regulation of ferritins and transferrin receptor corresponds to a compensatory feedback loop in the control of iron homeostasis adjusting iron storage and uptake according to iron levels (4–9), it is less evident why enzymes in cellular energy production should be coupled to iron availability. One possible explanation relates to the fact that both enzymes are iron-sulfur proteins. Mitochondrial aconitase contains one [4Fe-4S] cluster, which is essential for aconitase activity (38), and the SDHb subunit appears to contain three different clusters: one [4Fe-4S], one [3Fe-4S], and one [2Fe-2S] cluster (25), which are needed to deliver electrons to the electron transport chain (26). It seems possible in the case of such vital enzymes that the synthesis of apo-protein lacking the iron-sulfur cluster might be detrimental to enzyme subunit assembly and mitochondrial function. Such a hypothesis implies that expression of other mitochondrial iron-sulfur proteins should also be subject to iron regulation. It will therefore be of interest to learn whether this is indeed the case. Another connection previously proposed for the putative translational control of mitochondrial aconitase is the idea that cytoplasmic citrate might be needed for transport of iron into mitochondria and that under conditions of iron scarcity a certain level of citrate needs to be preserved to ensure this transport (39). However, it remains unexplained why SDHb subunit should be controlled in the same way.

Thus far mRNAs that contain an IRE were found to be

regulated in all vertebrates, indicating the functional importance of a regulated expression of these proteins. However, despite the strong phylogenetic conservation of the IRP-IRE regulatory system between insects and mammals, the SDHb IRE does not seem to be conserved in humans; IRE-like sequences are absent from the recently published human genomic SDHb sequence (40). Consistent with this we found that, in contrast to ferritin H chain and transferrin receptor mRNA, SDHb mRNA of human HL-60 cells is not retained by an IRE affinity column consisting of immobilized IRP.[3] However, down-regulation of SDH activity in skeletal and heart muscle of iron-deprived rats has been reported (41, 42), but the level at which regulation occurs has not been determined. These notions suggest that SDHb synthesis, for a specific reason, may be regulated in insects but not in humans. Thus, the present study is the first one to identify a functional IRE in insects and demonstrates that iron-dependent translational control by IRP-IRE interaction is conserved in insects. This opens the possibility of applying genetic approaches to the further investigation of this post-transcriptional regulatory system.

Acknowledgements—We thank Drs. Thomas Dandekar and Matthias Hentze for making their computer search program and the plasmids L5-GH and Fer-GH available to us.

REFERENCES

1. Klausner, R. D., Rouault, T. A., and Harford, J. B. (1993) *Cell* **72,** 19–28
2. Melefors, O., and Hentze, M. W. (1993) *Blood Rev.* **7,** 251–258
3. Kühn, L. C. (1994) in *Baillière's Clinical Haematology*, Vol. 7, (Hershko, C., ed) pp. 763–785, Bailliere Tindall, London, United Kingdom
4. Leibold, E. A., and Munro, H. N. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 2171–2175
5. Casey, J. L., Hentze, M. W., Koeller, D. M., Caughman, S. W., Rouault, T. A., Klausner, R. D., and Harford, J. B. (1988) *Science* **240,** 924–928
6. Müllner, E. W., Neupert, B., and Kühn, L. C. (1989) *Cell* **58,** 373–382
7. Henderson, B. R., Seiser, C., and Kühn, L. C. (1993) *J. Biol. Chem.* **268,** 27327–27334
8. Guo, B., Yu, Y., and Leibold, E. A. (1994) *J. Biol. Chem.* **269,** 24252–24260
9. Samaniego, F., Chin, J., Iwai, K., Rouault, T. A., and Klausner, R. D. (1994) *J. Biol. Chem.* **269,** 30904–30910
10. Leibold, E. A., and Munro, H. N. (1987) *J. Biol. Chem.* **262,** 7335–7341
11. Cox, T. C., Bawden, M. J., Martin, A., and May, B. K. (1991) *EMBO J.* **10,** 1891–1902
12. Dandekar, T., Stripecke, R., Gray, N. K., Goossen, B., Constable, A., Johansson, H. E., and Hentze, M. W. (1991) *EMBO J.* **10,** 1903–1909
13. Aziz, N., and Munro, H. N. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 8478–8482
14. Walden, W. E., Daniels-McQueen, S., Brown, P. H., Gaffield, L., Russell, D. A., Bielser, D., Bailey, L. C., and Thach, R. E. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 9503–9507
15. Gray, N. K., and Hentze, M. W. (1994) *EMBO J.* **13,** 3882–3891
16. Melefors, O., Goossen, B., Johansson, H. E., Stripecke, R., Gray, N. K., and Hentze, M. W. (1993) *J. Biol. Chem.* **268,** 5974–5978
17. Rothenberger, S., Müllner, E. W., and Kühn, L. C. (1990) *Nucleic Acids Res.* **18,** 1175–1179
18. Guo, B., Brown, F. M., Phillips, J. D., Yu, Y., and Leibold, E. A. (1995) *J. Biol. Chem.* **270,** 16529–16535
19. Theil, E. C. (1994) *Biochem. J.* **304,** 1–11
20. Von Darl, M., Harrison, P. M., and Bottke, W. (1994) *Eur. J. Biochem.* **222,** 353–366
21. Dandekar, T., and Hentze, M. W. (1995) *Trends Genet.* **11,** 45–50
22. Kühn, L. C., and Hentze, M. W. (1992) *J. Inorg. Biochem.* **47,** 183–195
23. Henderson, B. R., Menotti, E., Bonnard, C., and Kühn, L. C. (1994) *J. Biol. Chem.* **269,** 17481–17489
24. Au, H. C., and Scheffler, I. E. (1994) *Gene* (*Amst.*) **149,** 261–265
25. Singer, T. P., and Johnson, M. K. (1985) *FEBS Lett.* **190,** 189–198
26. Sucheta, A., Cammack, R., Weiner, J., and Armstrong, F. A. (1993) *Biochemistry* **32,** 5455–5465
27. Gough, N. M. (1988) *Anal. Biochem.* **173,** 93–95
28. Hentze, M. W., Caughman, S. W., Rouault, T. A., Barriocanal, J. G., Dancis, A., Harford, J. B., and Klausner, R. D. (1987) *Science* **238,** 1570–1572
29. Gray, N. K., Quick, S., Goossen, B., Constable, A., Hirling, H., and Kühn, L. C. (1993) *Eur. J. Biochem.* **218,** 657–667
30. Sierzputowska-Gracz, H., McKenzie, R. A., and Theil, E. C. (1995) *Nucleic Acids Res.* **23,** 146–153
31. Graham, F. L., and Van der Eb, A. J. (1973) *Virology* **52,** 456–467
32. Caughman, S. W., Hentze, M. W., Rouault, T. A., Harford, J. B., and Klausner, R. D. (1988) *J. Biol. Chem.* **263,** 19048–19052
33. Qian, S., Zhang, J. -Y., Kay, M. A., and Jacobs-Lorena, M. (1987) *Nucleic Acids Res.* **15,** 987-1003
34. May, B. K., Bhasker, C. R., Bawden, M. J., and Cox, T. C. (1990) *Mol. Biol. & Med.* **7,** 405–421
35. Goossen, B., and Hentze, M. W. (1992) *Mol. Cell. Biol.* **12,** 1959–1966
36. Aziz, N., and Munro, H. N. (1986) *Nucleic Acids Res.* **14,** 915–927
37. Zheng, L., Kennedy, M. C., Blondin, G. A., Beinert, H., and Zalkin, H. (1992) *Arch. Biochem. Biophys.* **299,** 356–360
38. Beinert, H., and Kennedy, M. C. (1989) *Eur. J. Biochem.* **186,** 5–15
39. Melefors, O., and Hentze, M. W. (1993) *BioEssays* **15,** 85–90
40. Au, H. C., Ream-Robinson, D., Bellew, L. A., Broomfield, P. L. E., Saghbini, M., and Scheffler, I. E. (1995) *Gene* (*Amst.*) **159,** 249–253
41. Ackrell, B. A. C., Maguire, J. J., Dallman, P. R., and Kearney, E. B. (1984) *J. Biol. Chem.* **259,** 10053–10059
42. Tanne, Z., Coleman, R., Nahir, M., Shomrat, D., Finberg, J. P. M., and Youdim, M. B. H. (1994) *Biochem. Pharmacol.* **47,** 1759–1766
43. Devereux, J., Haeberli, P., and Smithies, O. (1984) *Nucleic Acids Res.* **12,** 387–395

[3] S. Kohler, unpublished results.