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## Cloning and molecular characterization of two mosquito iron regulatory proteins

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### Abstract

Iron regulatory proteins (IRPs) control the synthesis of various proteins at the translational level by binding to iron responsive elements (IREs) in the mRNAs. Iron, infection, and stress can alter IRP/IRE binding activity. Insect messenger RNAs for ferritin and succinate dehydrogenase subunit b have IREs that are active translational control sites. We have cloned and sequenced cDNAs encoding proteins from the IRP1 family for the mosquitoes, *Aedes aegypti* and *Anopheles gambiae*. Both deduced amino acid sequences show substantial similarity to human IRP1 and *Drosophila* IRP1A and IRP1B, and all of the residues thought to be involved in aconitase activity and iron–sulfur cluster formation are conserved. Recombinant *A. aegypti* IRP1 binds to transcripts of the IREs of mosquito or human ferritin subunit mRNAs. No significant change in *A. gambiae* IRP1 messenger RNA could be detected during the various developmental stages of the life cycle, following iron loading by blood feeding, or after bacterial or parasitic infections. These data suggest that there is no change in gene transcription. Furthermore, bacterial challenge of *A. gambiae* cells did not change IRP1 protein levels. In contrast, IRP1 binding activity for the IRE was elevated following immune induction. These data show that changes in IRP1/IRE binding activity occur as part of the insect immune response. © 2002 Elsevier Science Ltd. All rights reserved.

**Keywords:** Iron; Iron regulatory protein; Mosquitoes; Infection

### 1. Introduction

Diseases transmitted by mosquitoes result in the deaths of more than two million people a year. Among the most devastating of these diseases are malaria, the parasitic infection transmitted by *Anopheles gambiae*, and the viral fevers, dengue and yellow fever, delivered by *Aedes aegypti*. The females of these species blood-feed to complete their life cycles. Viral or parasitic exchange between the mammalian host and the mosquito vector occurs during blood feeding.

Neither do all blood-feeding mosquitoes support the development of a given pathogen, nor will a pathogen that invades a disease vector necessarily be transmitted. Infection of *A. gambiae* with *Plasmodium berghei*, a malarial parasite, activates an innate immune response

(Dimopoulos et al. 1996, 1997; Richman et al., 1997). Nonetheless, parasitic development occurs in most strains of this mosquito, and sporozoites are transmitted. These facts suggest that complex relationships exist between the pathogen and the vector, and that multiple mechanisms allow vector–pathogen compatibility and pathogen delivery.

Iron is important to pathogen growth in mammals (Fry, 1989; Gordeuk et al., 1993; Weinberg, 1993), where changes in host iron metabolism occur as a result of infection (Brock, 1994; Weiss et al., 1995). It is not known whether the iron consumed by the vector in the blood meal is available to support pathogen development, or whether iron influences vector response to an invading pathogen. Furthermore, how the mosquito transports and deals with the iron load from a blood meal has not been studied.

In mammals, iron loading or infection results in enhanced synthesis of the iron storage protein, ferritin (Hentze et al., 1987; Brock, 1994; Schalinske et al., 1998), that is controlled at the translational level by iron

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regulatory protein 1 (IRP1). IRP1 binds to an iron responsive element (IRE) found in the 5'-untranslated region (UTR) of ferritin mRNA (Caughman et al., 1988; Jaffrey et al., 1993; Theil, 1994). When iron levels are low, IRP1 binding to the IRE prevents translation (Gray and Hentze, 1994). When intracellular iron is increased, an iron-sulfur cluster [4Fe-4S] forms in the core of the IRP1; IRP1/IRE interaction declines and ferritin is synthesized (Haile et al., 1992; Gray et al., 1993; Beinert et al., 1996; Kennedy et al., 1997). When the [4Fe-4S] cluster is present the protein functions as cytoplasmic aconitase. Reciprocally, enhanced transferrin receptor synthesis under conditions of low intracellular iron (Casey et al., 1988; Klausner and Rouault, 1993) results from binding of the IRP1 to IREs found in the 3'-UTR of the receptor messenger RNA. Thus, IRP1 effectively controls both iron uptake by cells via the transferrin receptor and iron storage within cells as holoferritin. In contrast to the direct translational response to iron stimulation, infection provokes complex tissue specific changes in the synthesis of ferritin that are subject to both transcriptional and translational control (Brock, 1994; Weiss et al., 1995; Tran et al., 1997; Elia et al., 1999). One component of the response to infection is enhanced IRP1/IRE binding activity resulting from disassembly of the iron-sulfur cluster by nitric oxide (NO) (Pantopoulos and Hentze, 1995; Weiss et al., 1995; Pantopoulos et al., 1996; Wardrop et al., 2000). Ferritin synthesis in activated macrophage cells reflects both a fluctuation in IRP1/IRE interaction in response to NO, as well as enhanced transcription mediated by other immune factors (Weiss et al., 1997).

Proteins from the IRP1 family have been sequenced from *Drosophila* (Muckenthaler et al., 1998) and *Manduca sexta* (Zhang et al., 2001b). IREs are found in the 5'-UTR of mRNAs encoding ferritin subunits from several insects including *Drosophila melanogaster* (Lind et al., 1998; Georgieva et al., 1999), *A. aegypti* (Dunkov et al., 1995), *A. gambiae* (Law and Kafatos, unpublished data), *M. sexta* (Pham et al., 1996; Zhang et al., 2001a) and *Calpodes ethlius* (Nichol and Locke, 1999). Earlier reports showed that ferritin in Lepidopterans is increased in response to iron (Nichol and Locke 1989, 1990). More recently, we reported that *M. sexta* IRP1 interacts with transcripts of the *M. sexta* ferritin mRNA IRE, and in larvae, ferritin is increased in response to iron, while IRP1 binding activity declines (Zhang et al., 2001b). Ferritin synthesis is also upregulated in response to iron stimulation in *A. aegypti* Aag2 cells (Pham et al., 1999) and in whole animals (Dunkov et al., 1995). This regulation appears to be subject to both translational and transcriptional control, and a protein in the cytoplasmic extracts of Aag2 cells binds to transcripts of the *A. aegypti* ferritin subunit IRE (Pham et al., 1999). An IRE found in the *Drosophila* ferritin subunit mRNA is spliced from some ferritin mRNAs under conditions of

high iron availability (Lind et al., 1998; Georgieva et al., 1999). In addition, a functional IRE was identified in the 5'-UTR of the mRNA for *D. melanogaster* succinate dehydrogenase subunit b (SDhb), Au and Scheffler, 1994; Gray et al., 1996; Melefors, 1996). Insect vectors also have nitric oxide synthase (NOS) (Ribeiro and Nussenzveig, 1993; Yuda et al., 1996; Muller, 1997), and others (Luckhart et al., 1998) have shown that the levels of NOS activity in *Anopheles* are increased following infection.

Available information from mammals and insects suggested to us that mosquitoes have an IRP1 that could be involved in the insect immune response. We have cloned and sequenced mosquito IRP1 cDNAs from two vectors, *A. gambiae* and *A. aegypti*, and confirmed in *A. aegypti* that the corresponding protein binds to IREs. We report IRP1 messenger RNA expression in response to blood feeding and infection of these vectors, and provide evidence that changes in protein function are a component of the insect immune response.

## 2. Materials and methods

### 2.1. Cloning and sequencing

Degenerate oligonucleotide primers (a kind gift from Dr M. Hentze, EMBL, Heidelberg, Germany) designed from conserved sequences of mammalian aconitases [*A. aegypti*: 5'-GIGCIGGI(C/T)TI(C/T)TIGCIAA(G/A)AA(G/A)GT-3', and 5'-CIGCIGGI(C/G)(A/T)IAT(A/G)TG(A/G)TCIGT-3'; *A. gambiae*: 5'-CGAATTCGGICC(C/T)TT(C/I)GCC/IGCCCA(A/G)TC-3', and 5'-GAGA TCTGG(C/I)GA(T/C)I(G/C)(C/I)GT(C/I)AC(C/I)AC(C/I)GA(T/C)CA-3'] were used to amplify IRP fragments by PCR. RT-PCR was conducted with *A. aegypti* (Rockefeller strain) larval mRNA, and PCR with *A. gambiae* (Suakoko strain) adult female cDNA using the Superscript II Kit (Life Technologies, Gaithersburg, MD). The amplified *A. aegypti* (710 bp) or *A. gambiae* (342 bp) PCR products were cloned by T/A Cloning Kit (Invitrogen, La Jolla, CA), and sequenced using the United States Biochemical Sequenase Kit (Cleveland, OH). The amino acid sequences showed high identity to a portion of the human IRP1 protein and the fragments were subsequently used as probes to screen respective adult  $\lambda$ -ZAP expression cDNA libraries (Stratagene, La Jolla, CA) (*A. aegypti* library was a kind gift from Dr J. Law, Tucson, AZ). In vivo excision of positive clones obtained from either cDNA library was conducted according to the instructions provided in a  $\lambda$ -Zap cDNA synthesis kit (Stratagene). Double stranded sequencing in both directions was done by automated cycle sequencing using Terminator Kit (Applied Biosystems, Inc., Foster City, CA) and by manual Sanger sequencing (USB) and the results were compared. The cDNA and

deduced amino acid sequences were analyzed using Genetics Computer Group (Devereux et al., 1984) and database searches were done using BLAST programs (Altschul et al., 1990).

## 2.2. Mosquito colonies

*A. gambiae* (Suakoko, 4a r/r) were raised at 28°C (75% humidity, 12-h light/dark cycle) in mosquito culture medium. All adults had daily continuous access to a 10% sucrose solution, while females also were fed on anesthetized BALB/c mice.

## 2.3. Bacterial and *P. berghei* infections

Third and fourth instar larvae and adult mosquitoes were infected with bacteria by pricking the animal with a fine needle coated with a concentrated solution of *Escherichia coli* 1160 and *Micrococcus luteus* A270. Following infection, the mosquitoes were allowed to recover for 24 h prior to dissection and RNA extraction. The rodent malaria parasite, *P. berghei*, was used as a model system of *Plasmodium*–*A. gambiae* interaction. Infected BALB/c mice were evaluated for high levels of parasitemia and the presence of gametocyte-stage parasites (exflagellation) as described previously (Sinden, 1997). Four-day old female mosquitoes were fed on the infected, anesthetized mice, and then maintained at 19°C for 24 h (75% humidity, with a 12-h light/dark cycle) before dissection and RNA extraction. The *A. gambiae* cell line Sua1B was challenged with various elicitors for 8 h prior to the RT-PCR analysis as previously described (Dimopoulos et al., 1996). Total RNA was obtained from larvae, adult female *A. gambiae*, or cultured cells using the RNaid PLUS kit (Bio 101) according to the manufacturer's instructions.

## 2.4. Expression analysis by quantitative RT-PCR

This method was performed as previously described (Dimopoulos et al., 1996). Samples were radio-labeled by adding 0.05 µl α-[<sup>32</sup>P]dATP to each PCR and resolved on a 6% acrylamide gel prior to visualization on X-ray film (Kodak) by autoradiography. The ribosomal protein S7 gene (Salazar et al., 1993) sequence was used as positive internal control. The Gram negative bacteria-binding protein (GNBP) gene sequence was used as a positive control for samples from infected adults; this message is increased in the mosquito immune response (Dimopoulos et al., 1996). PCR cycle numbers were constant for a particular sequence in the multiple samples analyzed in a given experiment and chosen empirically to attain comparable band intensities for the different markers in each experiment while avoiding saturation (except when the abundance of the sequence was very disparate between biological samples). The pri-

mers used were as follows: S7A, 5'-GGCGATCATCATCTACGT-3'; S7B, 5'-GTAGCTGCTGCAAACCTCGG-3'; GNBPA, 5'-GCAACGAGAATCTGTACC-3'; GNBPB, 5'-TAACCACCAGCAACGAGG-3'; *A. gambiae* IRPA, 5'-GAAAGCTTGGGACTGACG-3'; *A. gambiae* IRPB, 5'-CCCAAATACCTCTTTATTGC-3'.

## 2.5. Expression of *A. aegypti* IRP1 in *E. coli*

The open reading frame (ORF) from the 3.2 kb *A. aegypti* IRP1 cDNA clone was amplified by PCR with *Pfu* DNA polymerase (Stratagene). Specific primers were designed with Bam HI and Hind III restriction sites on the sense and anti-sense primers, respectively. The amplified ORF was subcloned into the BamHI/HindIII sites of PQE30 expression vector (Qiagen, Valencia, CA), such that directional insertion resulted in an expressed recombinant protein with a His-6-tag followed by a glycine and a serine residue, then the N-terminus. The plasmid was named as aIRP1/PQE30. Sequencing was done to verify the correct orientation of the ORF, the expected junctions between the vector and the ORF, and to confirm that no mistakes were present in the ORF. The aIRP1/PQE30 clone was transformed into the *E. coli* strain, M15[pREP14] and expressed as described in Gray et al. (1993). The recombinant *A. aegypti* IRP1 was purified to >95% homogeneity by Ni<sup>2+</sup>-NTA-agarose chromatography according to the protocol of Gray et al. (1993) with the following changes. Briefly, culture of the aIRP1/PQE30 clone was grown overnight, and 20 ml of aIRP1/PQE30 culture was used to inoculate one liter of Luria Broth, grown 2 h, and exposed to 1.0 mM IPTG for 5 h. The cells were collected by centrifuge (1800g), washed and stored at –20°C until the protein purification was conducted. Following lysis (Gray et al., 1993), the sample was loaded onto Ni<sup>2+</sup>-NTA agarose (Qiagen) and eluted with 100 mM imidazole in 24 mM HEPES, pH 7.6, 150 mM KOAc, 1.5 mM MgCl<sub>2</sub>, 0.5% glycerol. The fraction containing the recombinant IRP1 was adjusted to 10 mM imidazole with the same buffer and loaded onto Ni<sup>2+</sup>-NTA agarose (1 ml bed volume). Recombinant IRP1 was eluted in a 10–100 mM imidazole gradient (40 ml bed volumes) in the same buffer. Fractions from 20 to 35 mM imidazole contained homogeneous IRP1. The purity of the recombinant *A. aegypti* IRP1 was confirmed by Phast gel (8–25% gradient); the SDS-PAGE was conducted according to manufacturer's instructions (Pharmacia Biotech, Piscataway, NJ). Assessment of the binding activity of the recombinant *A. aegypti* IRP1 for human ferritin IRE and the putative mosquito ferritin IRE was done by electrophoretic mobility shift assay (EMSA). Proteins were determined using BSA as a standard (Bradford, 1976).

## 2.6. Electrophoretic mobility shift assay (EMSA)

Transcripts were designed from the putative IREs found in the 5'-UTRs of the ferritin subunit mRNAs for *A. aegypti* (*Aedes* IRE, Dunkov et al., 1995) and *A. gambiae* (*Anopheles* IRE, unpublished). Double stranded DNA templates with the following sequences were synthesized (Gibco BRL, Grand Island, NY): *Aedes* IRE template: 5'-GAAAGCTTCGAGTCACCTTCTGTGCC AGTGTGTATAAAGGTTGACAACGGATCCCC-3', 3'-CTTTCGAAGCTCAGTGGGACACGGTCACA CATATTTCCAAGTGTGCCTAGGGG-5'; *Anopheles* IRE template: 5'-GAAAGCTTAAGCTGTGACCTTC TGTGCCAGTGCATATAAAGGCCGACAACCTTGA TCCCC-3', 3'-CTTTCGAATTTCGACAGCTGGAAGA CACGGTCACGCATATTTCCGGCCTGTTGAACTA GGGG-5'. These fragments were cloned into the Hind III (5'-end; AAGCTT) and BamHI (3'-end; GGATCC) sites of the pTZ19R vectors provided by the RNA Gel Shift Kit (MBI Fermentas, Amherst, NY). Human heavy chain ferritin subunit IRE (human IRE) template was provided by the kit in the same vector. The respective clones for each template were made linear by EcoRI digestion, and transcription was conducted according to the manufacturer's instructions. A non-specific stem loop transcript (NSL) was prepared (Milligan et al., 1987), and synthesized using RNA Transcription Kit (Stratagene). Transcripts were labeled with  $\alpha$ -[<sup>32</sup>P]-CTP (56  $\mu$ Ci,  $>3 \times 10^6$  mCi/mmol; Amersham). Following transcription, transcripts were ethanol precipitated, suspended in DEPC-treated water, and quantified from  $\alpha$ -[<sup>32</sup>P]-CTP incorporation. In order to quantify transcripts for competition assays, the transcripts were labeled with trace levels of  $\alpha$ -[<sup>35</sup>S]-CTP (0.01  $\mu$ Ci, 10 mCi/ml; Amersham).  $\alpha$ -[<sup>35</sup>S]-CTP incorporated at this concentration was not detectable by autoradiography for film exposed at  $-80^\circ\text{C}$  for several days. The sequences of the four RNA transcripts were: human IRE, 5'-GGGAU CCUGCUUCAACAGUGCUUGGACGGAACC-3'; *Aedes* IRE, 5'-CGAGUCACCUUCUGUGCCAGUGUG UAUAAAGGUGACAACG-3'; *Anopheles* IRE, 5'-GAA AGCUUAAGCUGUCGACCUUCUGUGCCAGUGCG UAUAAAGGCCGACAACUUGAUC-3'; and NSL, 5'-GAAAGUCGCCUUCUGUGCCAGUGUGUAUA AAGGCCACUUUC-3'. The expression clone for recombinant human IRP1 was a kind gift from Dr M. Hentze (EMBL, Heidelberg, Germany). Recombinant human IRP1 (human IRP1) was expressed and purified according to the procedures of Gray et al. (1993). The *A. gambiae* cell line Sua1B was challenged with LPS for 6 h prior to harvest; control cells were unchallenged. Cytoplasmic extracts from either naive (unchallenged control) or challenged cells were prepared by adding 10 mM HEPES (pH 7.9) containing protease inhibitor cocktail (Complete, EDTA-free, (Boehringer Mannheim)) to  $10.0\text{--}50.0 \times 10^6$  cells. In the presence of

this solution, the cells were scraped from the plates, transferred to a corex tube and flash frozen in liquid nitrogen. The cell solution was thawed and centrifuged at  $18,500g$  for 30 min at  $4^\circ\text{C}$ . The cytoplasmic extract (supernatant) was stored separately at  $-80^\circ\text{C}$  until analyses by western blotting and EMSA were conducted. EMSA (Leibold and Munro, 1988) was done as a 20  $\mu$ l reaction. Recombinant *A. aegypti* IRP1 (50 ng), recombinant human IRP1 (50 ng), or *A. gambiae* cytoplasmic extract (26 mg) was added to 16.9 mM HEPES, 0.84 mM  $\text{MgCl}_2$ , 16.9 mM KCl, 5.6 mM DTT, 2.8% glycerol, 0.28 units RNasin, then unlabeled transcript as designated together with labeled transcript (50 fmol) was added and the mixture was incubated for 30 min at room temperature (RT). Following incubation, 6 units of RNase T1 (Stratagene) was added and the reaction held at RT, for 10 min. Heparin (Sigma, St. Louis, MO) was added to a final concentration of 3 mg/ml, and the reaction mixture incubated for another 10 min. The bound transcripts were separated on 6.5% acrylamide gel at 100 V for 2 h and visualized after autoradiography on X-ray film (Kodak). Human IRP1/human IRE interaction served as positive controls. NSL was used to evaluate non-specific binding.

## 2.7. Immunoblot

Cytoplasmic extracts were prepared as described above from either naive (unchallenged control) or cells challenged with bacteria. Samples and standards were loaded onto a 10% homogeneous SDS-polyacrylamide gel and electrophoresis was conducted at 200 V for 1 h. The proteins were transferred to nitrocellulose membrane (BioRad, Hercules, CA) using the BioRad Mini Blot System according to the manufacturer's instructions. Staining with Ponceau Red confirmed equal transfer of protein in all lanes. Blots were blocked for 2 h using BLOTTO and probed for 1.5 h using rabbit anti-rat IRP1 anti-serum (antibody #3245, a gracious gift from Dr R. Eisenstein, University of Wisconsin, Madison, WI, (Eisenstein et al., 1993)). Following exposure to the primary anti-serum, the blot was treated for 1 h with goat anti-rabbit IgG linked to alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA) and developed for 5 min. Cytoplasmic extract prepared from *A. aegypti* Aag2 cells and recombinant human IRP1 from the RNA Gel Shift Kit (MBI Fermentas) were used as controls.

## 3. Results

### 3.1. Mosquito IRP1 sequences and comparisons

The *A. aegypti* and *A. gambiae* cDNA clones each encoded a polypeptide of 901 amino acids with a pre-

dicted mass of approximately 98 kDa and 88% identity to each other (Fig. 1). The deduced amino acid sequences reveal that all residues involved in aconitase activity (Beinert et al., 1996; Frishman and Hentze, 1996) are fully conserved, as are the cysteine residues that could participate in the formation of an iron–sulfur cluster (Fig. 1, underlined). Multiple sequence alignment analysis (Fig. 1) shows that the mosquito sequences have a high degree of identity to *Drosophila* IRP1A (74%) and IRP1B (73%), and to human IRP1 (67%). The mosquito sequences are only 57% identical to human IRP2, and lack a 73-amino acid sequence found near the N-terminus that is characteristic of IRP2 (Rouault et al., 1990). The mosquito IRP1s have about 30% identity with porcine mitochondrial aconitase. Thus, it is clear that the cloned sequences represent mosquito IRP1s.

3.2. EMSA of recombinant *A. aegypti* IRP1

In order to evaluate whether our cDNA encodes a potentially functional IRP1, we overexpressed *A. aegypti* IRP1 in *E. coli* and purified the recombinant protein (Fig.

2(A)). Under our assay conditions, EMSA showed specific binding activity of the recombinant *Aedes* IRP1 for transcripts containing human or *Aedes* ferritin IRE; recombinant human IRP1 interaction with both of these transcripts served as a positive control (Fig. 2(B) and (C)). Thus, IRP1/IRE recognition occurred between insect and mammalian RNAs and proteins. The specificity of binding was demonstrated by competition assays (Fig. 2(C)). Binding of the recombinant IRP1 to IRE-containing transcripts was reduced effectively by 25 to 100-fold excess of unlabeled transcript, but was unaffected by a 100-fold excess of NSL transcript. Extracts from *E. coli* without IRP1 encoding plasmids showed no binding activity for the ferritin IRE (data not shown). Assays using a 500-fold excess of NSL showed no competition, and the recombinant mosquito IRP1 did not bind radio-labeled NSL transcript under our assay conditions (data not shown). These data support the conclusion that our *A. aegypti* cDNA encodes a functional IRP1 that binds specifically to ferritin subunit IRE transcript.

A



B

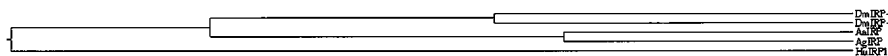


Fig. 1. Alignment and dendrogram of deduced amino acid sequences. Protein sequences of the mosquito and other IRP1s were aligned using the BLAST program (GCG). (A) Alignment of human (Hu) IRP1, *Drosophila* (Dm) IRP1a, *Drosophila* (Dm) IRP1b, *A. aegypti* (Aa) IRP1, and *A. gambiae* (Ag) IRP1. The amino acid residues involved in aconitase activity and iron–sulfur cluster formation are underlined. Aconitase active-site residues that are substituted in the mammalian IRP2 are marked with asterisks. (B) Neighbor-joining tree of five IRP1s drawn by CLUSTALW.

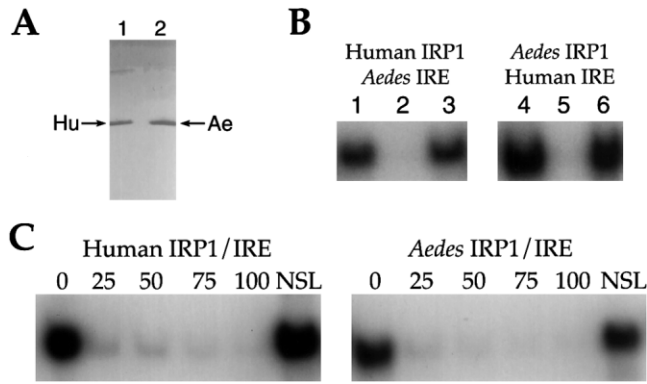


Fig. 2. Electrophoretic mobility shift assay of recombinant *A. aegypti* iron regulatory protein 1 (IRP1). Recombinant *A. aegypti* IRP1 or human IRP1 was over-expressed in *E. coli*, purified from cytoplasmic extracts and the purity evaluated by SDS-PAGE as described in Section 2. EMSA was conducted according to Leibold and Munro (1988) as adapted for the insect transcripts and described in Section 2. (A) The purity of the recombinant proteins: lane 1, human IRP1; lane 2, *Aedes* IRP1. (B) Cross reactivity assays of the IRP1 from one species with ferritin subunit IRE transcript of the other species. Lanes 1–3: human IRP1/[<sup>32</sup>P]-*Aedes* IRE transcript analyzed alone (lane 1), with 100-fold excess trace labeled [<sup>35</sup>S]-*Aedes* IRE transcript (lane 2), or 100-fold excess trace labeled [<sup>35</sup>S]-NSL transcript (lane 3). Lanes 4–6: *Aedes* IRP1/[<sup>32</sup>P]-human IRE transcript analyzed alone (lane 4), with 100-fold excess trace labeled [<sup>35</sup>S]-human IRE transcript (lane 5), or with 100-fold excess trace labeled [<sup>35</sup>S]-NSL transcript (lane 6). (C) *A. aegypti* IRP1/IRE interaction. Human or *A. aegypti* recombinant protein (50 ng) and [<sup>32</sup>P]-IRE transcripts (50 fmol) together with [<sup>35</sup>S]-trace-labeled transcript (0–100-fold excess) as designated were added to samples containing binding buffer. The human IRP1/IRE reactions were used as a positive control. NSL: nonspecific stem loop transcript. *Aedes* IRP1: *A. aegypti* IRP1; *Aedes* IRE: transcript of the *A. aegypti* ferritin subunit putative IRE sequence (Dunkov et al., 1995); Human IRE: transcript of the human heavy chain ferritin subunit IRE sequence.

### 3.3. Expression of the *Anopheles* IRP1

Once we determined that our cDNAs encoded mosquito IRP1s, we wanted to evaluate changes in IRP1 messenger RNA following iron loading by blood feeding, during infection and throughout the life cycle of the vector. We used quantitative RT-PCR to assess mRNA expression of the *Anopheles* IRP1 gene. These results indicated that the mRNA is constitutively expressed, and is present at all developmental stages without significant variations (Fig. 3(A)). Either undiluted blood meal or a blood meal diluted 50% with plasma did not alter the messenger RNA levels by 12 or 24 h post-feeding (Fig. 3(B)). When *A. gambiae* cells, larvae or adults were challenged with heat-killed bacteria or malarial parasites, the *A. gambiae* IRP1 RNA level was not significantly changed, in contrast to an immune-inducible (Dimopoulos et al., 1997) GNB marker (Fig. 3(C)). Because mammalian blood cells contain IRP1, to assure negative control, we conducted RT-PCR using our specific mosquito primers on mouse RNA. We were unable

to detect a PCR product on mouse RNA in repeated assays under these conditions (data not shown).

### 3.4. *Anopheles* IRP1 and IRP1 binding activity following infection

Since no differences in mRNA levels were observed following infection for either animals or cells, we evaluated changes in both IRP1 protein and IRP1/IRE binding activity in *A. gambiae* Sua1B cells following immune challenge with LPS. LPS is a very potent inducer of an immune response in these cells and upregulates a variety of immune markers in this cell line. Immunoblotting showed that the protein levels of IRP1 did not change for these cells following infection (Fig. 4(B)). In contrast, EMSA results revealed that the binding activity of the *A. gambiae* IRP1 for *A. gambiae* IRE was upregulated following bacterial challenge (Fig. 4(A)).

## 4. Discussion

The mammalian IRP1 plays important roles in regulating the translation of messenger RNAs with IREs in response to iron (Klausner et al., 1993; Theil, 1994; Hentze and Kuhn, 1996). The binding activity of mammalian IRP1 for the IRE is also responsive to oxidative stress (Cairo et al., 1995; Oria et al., 1995; Hentze and Kuhn, 1996; Wardrop et al., 2000) and infection (Weiss et al., 1995). We are interested in the potential relationship between the mosquito IRP1 and immunity in disease vectors, and as a first step we have cloned and sequenced IRP1 homologues from two distantly related mosquitoes, *A. aegypti* and *A. gambiae*, and evaluated the IRP1 following an induced immune response.

The mosquito IRP1 sequences have high identity to the IRP1 proteins of *Drosophila* (Muckenthaler et al., 1998), as well as to human IRP1 (Rouault et al., 1990; Hirling et al., 1992). All the residues necessary for iron-sulfur cluster formation and for aconitase enzymatic activity are fully conserved (Rouault et al., 1990; Beinert et al., 1996; Frishman and Hentze, 1996). The mosquito sequences show much less identity to either vertebrate IRP2 or mitochondrial aconitase. In addition, mosquito sequences contain neither the 73-amino acid residue insert (Guo et al., 1994; Muckenthaler et al., 1998) nor the aconitase active-site residue substitutions that characterize IRP2 (*A. aegypti* IRP1: I180, T219, I446, R548, S790; *A. gambiae*: I181, T219, I446, R548, S790). Our sequence analysis indicates that our mosquito clones encode proteins of the IRP1 family, and that IRP1 proteins are as well conserved among insects as they are in other invertebrates (Muckenthaler et al., 1998; Huang et al., 1999) and vertebrates (Hentze and Kuhn, 1996; Eisenstein and Blemings, 1998). Sequence comparisons by the CLUSTALW program indicate that all four IRP1

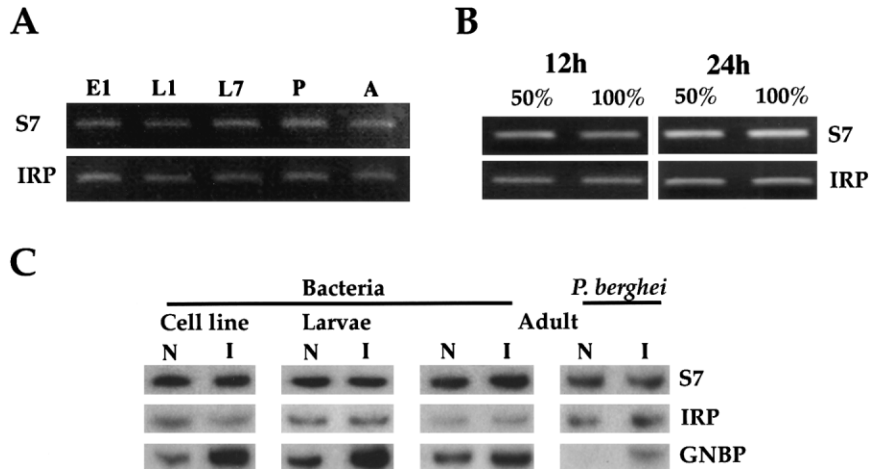


Fig. 3. Expression analysis of *A. gambiae* IRP mRNA levels by RT-PCR. Ribosomal protein S7 gene expression level was used as the normalization standard in the RT-PCR assay and GNBP gene expression level was used as a positive control for immune induction in the assays of both bacterial and parasitic infections. (A) AgIRP message levels in various developmental stages. (E1) egg at day 1; (L1) larvae at day 1; (L7) larvae at day 7; (P) pupae and (A) adult. (B) AgIRP mRNA levels at 12 and 24 h after blood feeding on 50% diluted (50%) and undiluted (100%) blood. (C) AgIRP message levels in naive (N) and bacterially challenged (I) cells, larvae and adults, and in *P. berghei* infected (I) adults 24 h after feeding on an infected mouse.

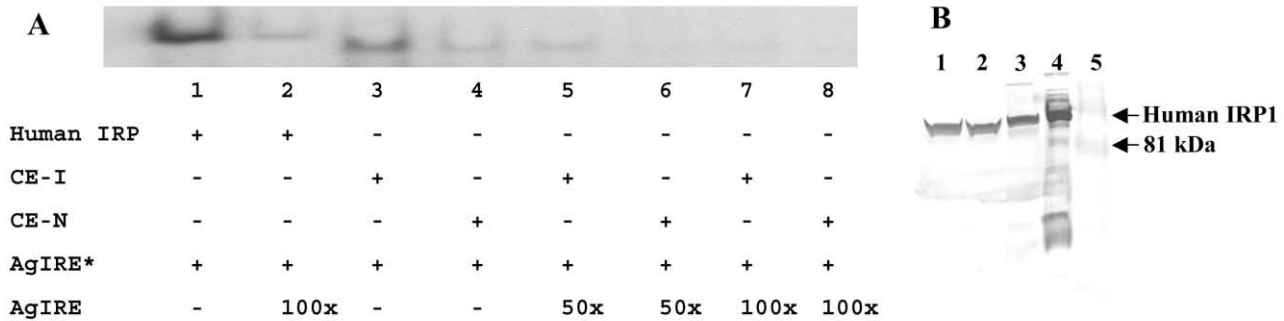


Fig. 4. IRP1 protein and binding activity of cytoplasmic extracts of infected *A. gambiae* Sua1B cells. (A) Infection increases IRP1/IRE binding activity of mosquito cells. EMSA was conducted using cytoplasmic extracts prepared from naive (CE-N) or LPS treated (CE-I) *A. gambiae* Sua1B cells. Recombinant human IRP1 was used as a positive control. Samples were prepared with cytoplasmic extract (26 g) or recombinant human IRP1 (50 ng), labeled *A. gambiae* ferritin IRE (AgIRE\*, 50 fmol) and varying concentrations (2500 fmol (50x) or 5000 fmol (100x)) of excess unlabeled *A. gambiae* ferritin IRE transcript (AgIRE). (B) Infection does not alter IRP1 protein levels of mosquito cells. Immunoblot of *A. gambiae* cell cytoplasmic extracts using as probe anti-IRP1 antiserum. Lane 1, *A. gambiae* Sua1B naive cell extract (30 g); lane 2, extract from *A. gambiae* Sua1B cells treated with LPS (30 g); lane 3, *A. aegypti* Aag2 cell extract (10 g); lane 4, recombinant human IRP1 (0.5 g); and lane 5 molecular weight markers.

sequences from insects cluster apart from human IRP1, as expected from the phylogenetic relationship. Interestingly these two mosquito species, thought to have diverged from each other, have IRP1 sequences that are more closely related to each other than IRP1a and IRP1b sequences of *Drosophila*. Thus, the IRP1 duplication found in *Drosophila* is ancient; it remains to be seen whether additional IRP1 genes exist in mosquitoes.

The EMSA results show that recombinant *A. aegypti* IRP1 binds specifically to transcripts of either mosquito or human ferritin subunit IREs. In addition, recombinant human IRP1 binds mosquito ferritin IRE transcripts. These experiments provide further evidence that the mosquito cDNA clone encodes a functional IRP1, and

that IREs found in these insect mRNAs could be active translational control elements.

*A. gambiae* IRP1 mRNA was uniformly expressed in all stages of the life cycle. Studies in *Drosophila* suggest that IRP1 mRNAs are expressed throughout embryonic development (Muckenthaler et al., 1998). Mosquito IRP1 message levels were unresponsive to blood feeding suggesting that constituents in blood, including iron, do not influence IRP1 transcription. In vertebrates, IRP1 messenger RNA (Yu et al., 1992) and protein (Tang et al., 1992; Yu et al., 1992) levels do not respond to iron stimulation. Instead, iron stimulation results in the formation of an iron-sulfur cluster in the core of IRP1 (Haile et al., 1992; Beinert et al., 1996) that reduces

IRP1/IRE binding interaction and thereby alters the synthesis of proteins that have IREs in their messenger RNA (Chen et al., 1998; Eisenstein, 2000). Since the mosquito IRP1 retains the necessary residues for iron–sulfur cluster formation, iron stimulation could result in functional changes in this protein without alterations in either transcription or translation.

IREs are found in 5'-UTR of the messages of several insect ferritin subunits (Dunkov et al., 1995; Pham et al., 1996; Lind et al., 1998; Georgieva et al., 1999; Nichol and Locke, 1999) and in the mRNA of *D. melanogaster* SDhb (Kohler et al., 1995; Gray et al., 1996; Melefors, 1996). Syntheses of both ferritin (Locke and Nichol, 1992; Winzerling and Law, 1997) and SDhb (Gray et al., 1996; Melefors, 1996) are increased in response to iron administration. In *A. aegypti*, ferritin synthesis is increased at all life stages in animals that are exposed to iron supplements (Dunkov et al., 1995), and both ferritin mRNA and protein are upregulated by either an artificial blood meal or a vertebrate blood meal (J.H. Law, personal communication). In addition, ferritin synthesis is upregulated in mosquito embryonic cells (Aag2) exposed to iron-supplemented culture medium, and synthesis regulation appears subject to both translational and transcriptional control mechanisms (Pham et al., 1999). Recently, we reported that recombinant *M. sexta* IRP1 represses the in vitro translation of ferritin subunits (Zhang et al., 2001a), and hemolymph ferritin is increased, while fat body IRP1/IRE binding activity is decreased, in larvae following iron administration (Zhang et al., 2001b). Taken together, these data indicate that the insect IRP1 functions in the translational control of ferritin synthesis in a manner similar to the mammalian IRP1. If this is the case in mosquitoes, regulation of ferritin synthesis by mosquito IRP1 is one probable mechanism whereby female mosquitoes adapt to the iron load of a blood meal. Future studies on iron–sulfur cluster formation in the mosquito IRP1, IRP1 protein levels and IRP1/IRE binding activity in females following blood feeding will test these hypotheses.

We are most interested in the effects of microbial infection on the transcription, translation and binding activity of vector IRP1. Messenger RNAs altered by infection represent potential intervention points for control of disease transmission, and knowledge of vector immune response will add to our understanding of vector–pathogen interactions. We have shown that neither bacterial challenge nor parasitic infection of whole animals or cells results in changes in the IRP1 mRNA level. Further, bacterial challenge of *A. gambiae* cells which upregulates immune markers did not significantly alter cytoplasmic IRP1 concentrations. In contrast, IRP1/IRE binding interaction was upregulated by bacterial induction of these cells. These findings indicate that the mosquito IRP1/IRE interaction is increased as part of the

mosquito immune response without changing either IRP1 messenger RNA or protein levels.

We think it probable that in response to bacterial challenge the *A. gambiae* cells produce NO, and that NO interacts with the IRP1 resulting in the increased binding activity we observed. There are several lines of evidence that lead us to this hypothesis. In other studies, we have found that bacterial challenge of these cells upregulates NOS (Dimopoulos and Kafatos, unpublished). In mammals, infection results in changes in IRP1/IRE binding activity that parallels NO production (Weiss et al., 1997) without a change in IRP1 levels (Phillips et al., 1996). Production of NO results from an enzymatic reaction catalyzed by NOS (Weiss et al., 1993; Lowenstein et al., 1994). NOS message levels in *Anopheles* are increased following a blood meal infected with parasites (Luckhart et al., 1998), and measurements of NOS activity in infected *Anopheles* correspond with NOS message expression. NO appears to increase IRP1/IRE binding activity by disassembly of the iron–sulfur cluster (Pantopoulos et al. 1994, 1996; Hentze and Kuhn, 1996; Wardrop et al., 2000). The high homology of our IRP1 sequence with that of mammals and complete conservation of the cysteine residues involved in iron–sulfur cluster formation support the idea that an iron–sulfur cluster can form in the mosquito IRP1 and prevent IRE binding activity. If this is the case, NO produced following infection of mosquitoes could result in increased IRP1/IRE binding activity by interaction with the iron–sulfur cluster, and thereby alter the translation of messages with IREs during the vector immune response. Alternatively, mammalian IRP1/IRE interaction is also enhanced by phosphorylation of the IRP1 (Eisenstein et al., 1993; Schalinske and Eisenstein, 1996). Ser 138 (Brown et al., 1998) and Ser 711 (Eisenstein et al., 1993) of the mammalian IRP1 can be phosphorylated by phospholipid-dependent protein kinase C (PKC). The mosquito sequences lack the site at Ser138. However, there is a potential PKC phosphorylation site (RXT) at *A. aegypti* residues 157–159, and the site at Ser 711 is also conserved. PKC is activated following immune induction by LPS in insects (Lanz-Mendoza et al., 1996). If immune induction in these insect cells is accompanied by activation of PKC, then increased IRP1/IRE interaction could occur by this mechanism.

In summary, to our knowledge this is the first report of the sequence and expression of IRP1 in mosquitoes. IRP1 message is expressed in various life stages. Immune induction does not alter message expression or protein levels. In contrast, IRP1/IRE binding activity is enhanced. An up regulation of IRP1 binding activity during the immune response could down regulate ferritin and succinate dehydrogenase subunit b expression, and thereby influence iron and energy metabolism in these animals during infection.

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## References

- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. *Journal of Medical Biology* 215, 403–410.
- Au, H.C., Scheffler, I.E., 1994. Characterization of the gene encoding the iron-sulfur protein subunit of succinate dehydrogenase from *Drosophila melanogaster*. *Gene* 149, 261–265.
- Beinert, H., Kennedy, M.C., Stout, C.D., 1996. Aconitase as iron-sulfur protein, enzyme, and iron-regulatory protein. *Chemical Reviews* 96, 2335–2373.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72, 248–254.
- Brock, J., 1994. Iron in infection, immunity, inflammation and neoplasia. In: Brock, J., Halliday, J., Pippard, M., Powell, L. (Eds.), *Iron Metabolism in Health and Disease*. WB Saunders, Philadelphia, PA, pp. 354–389.
- Brown, N.M., Anderson, S.A., Steffen, D.W., Carpenter, T.B., Kennedy, M.C., Walden, W.E., Eisenstein, R.S., 1998. Novel role of phosphorylation in Fe-S cluster stability revealed by phosphomimetic mutations at Ser-138 of iron regulatory protein 1. *Proceedings of the National Academy of Sciences of the United States of America* 95, 15235–15240.
- Cairo, G., Tacchini, L., Pogliaghi, G., Anzon, E., Tomasi, A., Bernelli-Zazzera, A., 1995. Induction of ferritin synthesis by oxidative stress. Transcriptional and post-transcriptional regulation by expansion of the 'free' iron pool. *Journal of Biological Chemistry* 270, 700–703.
- Casey, J.L., Hentze, M.W., Koeller, D.M., Caughman, S.W., Rouault, T.A., Klausner, R.D., Harford, J.B., 1988. Iron-responsive elements: regulatory RNA sequences that control mRNA levels and translation. *Science* 240, 924–928.
- Caughman, S.W., Hentze, M.W., Rouault, T.A., Harford, J.B., Klausner, R.D., 1988. The iron-responsive element is the single element responsible for iron-dependent translation regulation of ferritin biosynthesis. *Journal of Biological Chemistry* 263, 19048–19052.
- Chen, O.S., Blemings, K.P., Schalinske, K.L., Eisenstein, R.S., 1998. Dietary iron intake rapidly influences iron regulatory proteins, ferritin subunits and mitochondrial aconitase in rat liver. *Journal of Nutrition* 128, 525–535.
- Devereux, J., Haeberli, P., Smithies, O., 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Research* 12, 387–395.
- Dimopoulos, G., Richman, A., Della Torre, A., Kafatos, F.C., Louis, C., 1996. Identification and characterization of differentially expressed cDNAs of the vector mosquito, *Anopheles gambiae*. *Proceedings of the National Academy of Sciences of the United States of America* 93, 13066–13071.
- Dimopoulos, G., Richman, A., Muller, H.M., Kafatos, F.C., 1997. Molecular immune responses of the mosquito *Anopheles gambiae* to bacteria and malaria parasites [see comments]. *Proceedings of the National Academy of Sciences of the United States of America* 94, 11508–11513.
- Dunkov, B.C., Zhang, D., Choumarov, K., Winzerling, J.J., Law, J.H., 1995. Isolation and characterization of mosquito ferritin and cloning of a cDNA that encodes one subunit. *Archives of Insect Biochemistry and Physiology* 29, 293–307.
- Eisenstein, R.S., Tuazon, P.T., Schalinske, K.L., Anderson, S.A., Traugh, J.A., 1993. Iron-responsive element-binding protein. Phosphorylation by protein kinase C. *Journal of Biological Chemistry* 268, 27363–27370.
- Eisenstein, R.S., Blemings, K.P., 1998. Iron regulatory proteins, iron responsive elements and iron homeostasis. *Journal of Nutrition* 128, 2295–2298.
- Eisenstein, R.S., 2000. Iron regulatory proteins and the molecular control of mammalian iron metabolism. *Annual Review of Nutrition* 20, 627–662.
- Elia, G., Polla, B., Rossi, A., Santoro, M.G., 1999. Induction of ferritin and heat shock proteins by prostaglandin A1 in human monocytes. Evidence for transcriptional and post-transcriptional regulation. *European Journal of Biochemistry* 264, 736–745.
- Frishman, D., Hentze, M.W., 1996. Conservation of aconitase residues revealed by multiple sequence analysis. Implications for structure/function relationships. *European Journal of Biochemistry* 239, 197–200.
- Fry, M., 1989. Diferric transferrin reductase in *Plasmodium falciparum*-infected erythrocytes. *Biochemical and Biophysical Research Communications* 158, 469–473.
- Georgieva, T., Dunkov, B.C., Harazanova, N., Ralchev, K., Law, J.H., 1999. Iron availability dramatically alters the distribution of ferritin subunit messages in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America* 96, 2716–2721.
- Gordeuk, V.R., Thuma, P.E., Brittenham, G.M., Biemba, G., Zulu, S., Simwanza, G., Kalense, P., M'Hango, A., Parry, D., Poltera, A.A., 1993. Iron chelation as a chemotherapeutic strategy for falciparum malaria. *American Journal of Tropical Medicine and Hygiene* 48, 193–197.
- Gray, N.K., Quick, S., Goossen, B., Constable, A., Hirling, H., Kuhn, L.C., Hentze, M.W., 1993. Recombinant iron-regulatory factor functions as an iron-responsive-element-binding protein, a translational repressor and an aconitase. A functional assay for translational repression and direct demonstration of the iron switch. *European Journal of Biochemistry* 218, 657–667.
- Gray, N.K., Hentze, M.W., 1994. Iron regulatory protein prevents binding of the 43S translation pre-initiation complex to ferritin and eALAS mRNAs. *EMBO Journal* 13, 3882–3891.
- Gray, N.K., Pantopoulos, K., Dandekar, T., Ackrell, B.A., Hentze, M.W., 1996. Translational regulation of mammalian and *Drosophila* citric acid cycle enzymes via iron-responsive elements. *Proceedings of the National Academy of Sciences of the United States of America* 93, 4925–4930.
- Guo, B., Yu, Y., Leibold, E.A., 1994. Iron regulates cytoplasmic levels of a novel iron-responsive element-binding protein without aconitase activity. *Journal of Biological Chemistry* 269, 24252–24260.
- Haile, D.J., Rouault, T.A., Harford, J.B., Kennedy, M.C., Blondin, G.A., Beinert, H., Klausner, R.D., 1992. Cellular regulation of the

- iron-responsive element binding protein: disassembly of the cubane iron-sulfur cluster results in high-affinity RNA binding. Proceedings of the National Academy of Sciences of the United States of America 89, 11735–11739.
- Hentze, M.W., Rouault, T.A., Caughman, S.W., Dancis, A., Harford, J.B., Klausner, R.D., 1987. A cis-acting element is necessary and sufficient for translational regulation of human ferritin expression in response to iron. Proceedings of the National Academy of Sciences of the United States of America 84, 6730–6734.
- Hentze, M.W., Kuhn, L.C., 1996. Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress. Proceedings of the National Academy of Sciences of the United States of America 93, 8175–8182.
- Hirling, H., Emery-Goodman, A., Thompson, N., Neupert, B., Seiser, C., Kuhn, L.C., 1992. Expression of active iron regulatory factor from a full-length human cDNA by in vitro transcription/translation. Nucleic Acids Research 20, 33–39.
- Huang, T.S., Melefors, O., Lind, M.I., Soderhall, K., 1999. An atypical iron-responsive element (IRE) within crayfish ferritin mRNA and an iron regulatory protein 1 (IRP1)-like protein from crayfish hepatopancreas. Insect Biochemistry and Molecular Biology 29, 1–9.
- Jaffrey, S.R., Haile, D.J., Klausner, R.D., Harford, J.B., 1993. The interaction between the iron-responsive element binding protein and its cognate RNA is highly dependent upon both RNA sequence and structure. Nucleic Acids Research 21, 4627–4631.
- Kennedy, M.C., Antholine, W.E., Beinert, H., 1997. An EPR investigation of the products of the reaction of cytosolic and mitochondrial aconitases with nitric oxide. Journal of Biological Chemistry 272, 20340–20347.
- Klausner, R.D., Rouault, T.A., 1993. A double life: cytosolic aconitase as a regulatory RNA binding protein. Molecular Biology of the Cell 4, 1–5.
- Klausner, R.D., Rouault, T.A., Harford, J.B., 1993. Regulating the fate of mRNA: the control of cellular iron metabolism. Cell 72, 19–28.
- Kohler, S.A., Henderson, B.R., Kuhn, L.C., 1995. Succinate dehydrogenase b mRNA of *Drosophila melanogaster* has a functional iron-responsive element in its 5'-untranslated region. Journal of Biological Chemistry 270, 30781–30786.
- Lanz-Mendoza, H., Bettencourt, R., Fabbri, M., Faye, I., 1996. Regulation of the insect immune response: the effect of hemolin on cellular immune mechanisms. Cellular Immunology 169, 47–54.
- Leibold, E.A., Munro, H.N., 1988. Cytoplasmic protein binds *in vitro* to a highly conserved sequence in the 5' untranslated region of ferritin heavy- and light-subunit mRNAs. Proceedings of the National Academy of Sciences of the United States of America 85, 2171–2175.
- Lind, M.I., Ekengren, S., Melefors, O., Soderhall, K., 1998. *Drosophila* ferritin mRNA: alternative RNA splicing regulates the presence of the iron-responsive element. FEBS Letters 436, 476–482.
- Locke, M., Nichol, H., 1992. Iron economy in insects. Annual Review of Entomology 37, 195–215.
- Lowenstein, C.J., Dinerman, J.L., Snyder, S.H., 1994. Nitric oxide: a physiologic messenger. Annals of Internal Medicine 120, 227–237.
- Luckhart, S., Vodovotz, Y., Cui, L., Rosenberg, R., 1998. The mosquito *Anopheles stephensi* limits malaria parasite development with inducible synthesis of nitric oxide. Proceedings of the National Academy of Sciences of the United States of America 95, 5700–5705.
- Melefors, O., 1996. Translational regulation in vivo of the *Drosophila melanogaster* mRNA encoding succinate dehydrogenase iron protein via iron responsive elements. Biochemical Biophysical Research Communications 221, 437–441.
- Milligan, J.F., Groebe, D.R., Witherell, G.W., Uhlenbeck, O.C., 1987. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. Nucleic Acids Research 15, 8783–8798.
- Muckenthaler, M., Gunkel, N., Frishman, D., Cyrklaff, A., Tomancak, P., Hentze, M.W., 1998. Iron-regulatory protein-1 (IRP-1) is highly conserved in two invertebrate species — characterization of IRP-1 homologues in *Drosophila melanogaster* and *Caenorhabditis elegans*. European Journal of Biochemistry 254, 230–237.
- Muller, U., 1997. The nitric oxide system in insects. Progress in Neurobiology 51, 363–381.
- Nichol, H., Locke, M., 1999. Secreted ferritin subunits are of two kinds in insects molecular cloning of cDNAs encoding two major subunits of secreted ferritin from *Calpodes ethlius*. Insect Biochemistry and Molecular Biology 29, 999–1013.
- Nichol, H.K., Locke, M., 1989. The characterization of ferritin in an insect. Insect Biochemistry and Molecular Biology 19, 587–602.
- Nichol, H.K., Locke, M., 1990. The localization of ferritin in insects. Tissue and cell 22, 767–777.
- Oria, R., Sanchez, L., Houston, T., Hentze, M.W., Liew, F.Y., Brock, J.H., 1995. Effect of nitric oxide on expression of transferrin receptor and ferritin and on cellular iron metabolism in K562 human erythroleukemia cells. Blood 85, 2962–2966.
- Pantopoulos, K., Weiss, G., Hentze, M., 1994. Nitric oxide and the post-transcriptional control of cellular iron traffic. Trends in Cellular Biology 4, 82–86.
- Pantopoulos, K., Hentze, M.W., 1995. Nitric oxide signaling to iron-regulatory protein: direct control of ferritin mRNA translation and transferrin receptor mRNA stability in transfected fibroblasts. Proceedings of the National Academy of Sciences of the United States of America 92, 1267–1271.
- Pantopoulos, K., Weiss, G., Hentze, M.W., 1996. Nitric oxide and oxidative stress (H<sub>2</sub>O<sub>2</sub>) control mammalian iron metabolism by different pathways. Molecular and Cellular Biology 16, 3781–3788.
- Pham, D.Q.-D., Zhang, D., Hufnagel, D.H., Winzerling, J.J., 1996. *Manduca sexta* hemolymph ferritin: cDNA sequence and mRNA expression. Gene 172, 255–259.
- Pham, D.Q.-D., Winzerling, J.J., Dodson, M.S., Law, J.H., 1999. Transcriptional control is relevant in the modulation of mosquito ferritin synthesis by iron. European Journal of Biochemistry 266, 236–240.
- Phillips, J.D., Kinikini, D.V., Yu, Y., Guo, B., Leibold, E.A., 1996. Differential regulation of Irp1 and Irp2 by nitric oxide in rat hepatoma cells. Blood 87, 2983–2992.
- Ribeiro, J.M., Nussenzveig, R.H., 1993. Nitric oxide synthase activity from a hematophagous insect salivary gland. FEBS Letters 330, 165–168.
- Richman, A.M., Dimopoulos, G., Seelye, D., Kafatos, F.C., 1997. Plasmodium activates the innate immune response of *Anopheles gambiae* mosquitoes. EMBO Journal 16, 6114–6119.
- Rouault, T.A., Tang, C.K., Kaptain, S., Burgess, W.H., Haile, D.J., Samaniego, F., McBride, O.W., Harford, J.B., Klausner, R.D., 1990. Cloning of the cDNA encoding an RNA regulatory protein — the human iron-responsive element-binding protein. Proceedings of the National Academy of Sciences of the United States of America 87, 7958–7962.
- Salazar, C.E., Mills-Hamm, D., Kumar, V., Collins, F.H., 1993. Sequence of a cDNA from the mosquito *Anopheles gambiae* encoding a homologue of human ribosomal protein S7. Nucleic Acids Research 21, 4147.
- Schalinske, K.L., Eisenstein, R.S., 1996. Phosphorylation and activation of both iron regulatory proteins 1 and 2 in HL-60 cells. Journal of Biological Chemistry 271, 7168–7176.
- Schalinske, K.L., Chen, O.S., Eisenstein, R.S., 1998. Iron differentially stimulates translation of mitochondrial aconitase and ferritin mRNAs in mammalian cells. Implications for iron regulatory proteins as regulators of mitochondrial citrate utilization. Journal of Biological Chemistry 273, 3740–3746.
- Sinden, R.E., 1997. Infection of mosquitoes with rodent malaria. In: Crampton, J.M., Beard, C.B., Louis, C. (Eds.), The Molecular Biology of Disease Vectors: A Methods Manual. Chapman & Hall, London, UK, pp. 67–91.
- Tang, C.K., Chin, J., Harford, J.B., Klausner, R.D., Rouault, T.A.,

1992. Iron regulates the activity of the iron-responsive element binding protein without changing its rate of synthesis or degradation. *Journal of Biological Chemistry* 267, 24466–24470.
- Theil, E.C., 1994. Iron regulatory elements (IREs): a family of mRNA non-coding sequences. *Biochemical Journal* 304, 1–11.
- Tran, T.N., Eubanks, S.K., Schaffer, K.J., Zhou, C.Y., Linder, M.C., 1997. Secretion of ferritin by rat hepatoma cells and its regulation by inflammatory cytokines and iron. *Blood* 90, 4979–4986.
- Wardrop, S.L., Watts, R.N., Richardson, D.R., 2000. Nitrogen monoxide activates iron regulatory protein 1 RNA-binding activity by two possible mechanisms: effect on the [4Fe–4S] cluster and iron mobilization from cells. *Biochemistry* 39, 2748–2758.
- Weinberg, E.D., 1993. The development of awareness of iron-withholding defense. *Perspectives in Biology and Medicine* 36, 215–221.
- Weiss, G., Goossen, B., Doppler, W., Fuchs, D., Pantopoulos, K., Werner-Felmayer, G., Wachter, H., Hentze, M.W., 1993. Translational regulation via iron-responsive elements by the nitric oxide/NO-synthase pathway. *EMBO Journal* 12, 3651–3657.
- Weiss, G., Wachter, H., Fuchs, D., 1995. Linkage of cell-mediated immunity to iron metabolism. *Immunology Today* 16, 495–500.
- Weiss, G., Bogdan, C., Hentze, M.W., 1997. Pathways for the regulation of macrophage iron metabolism by the anti-inflammatory cytokines IL-4 and IL-13. *Journal of Immunology* 158, 420–425.
- Winzerling, J.J., Law, J.H., 1997. Comparative nutrition of iron and copper. *Annual Review of Nutrition* 17, 501–526.
- Yu, Y., Radisky, E., Leibold, E.A., 1992. The iron-responsive element binding protein. Purification, cloning, and regulation in rat liver. *Journal of Biological Chemistry* 267, 19005–19010.
- Yuda, M., Hirai, M., Miura, K., Matsumura, H., Ando, K., Chinzei, Y., 1996. cDNA cloning, expression and characterization of nitric oxide synthase from the salivary glands of the blood-sucking insect *Rhodnius prolixus*. *European Journal of Biochemistry* 242, 807–812.
- Zhang, D., Albert, D., Kohlhepp, P., Pham, D.Q.-D., Winzerling, J.J., 2001a. Repression of *Manduca sexta* ferritin synthesis by IRP1/IRE interaction. *Insect Molecular Biology* (in press).
- Zhang, D., Ferris, C., Gailer, J., Kohlhepp, P., Winzerling, J.J., 2001b. *Manduca sexta* IRP1: molecular characterization and *in vivo* response to iron. *Insect Biochemistry and Molecular Biology* (in press).