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CLONING, OVEREXPRESSION AND CHARACTERIZATION OF IRON REGULATORY PROTEINS FROM INSECTS

by

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Dianzheng Zhang

entitled Cloning, Overexpression and Characterization of Iron Regulatory Proteins from Insects

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

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In memory of my father

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ABSTRACT

Iron is essential for life and iron homeostasis is important for all species. Compared to the understanding of iron metabolisms in vertebrates, we know much less about insect intracellular iron homeostasis. The iron regulatory proteins (IRPs) play central roles in this process by interaction with iron responsive elements (IREs). Here, I report the cloning, sequencing, overexpression, purification and characterization of IRP1s from two insect species, *Manduca sexta* and *Aedes aegypti*. Electrophoretic mobility shift assays demonstrated that both IRP1s specifically bind IREs s not only from the same species, but also from human ferritin IRE. Another ferritin subunit also was cloned from *Manduca sexta* and an IRE was identified in the 5'-untranslated region of the mRNA, and the IRE reacted with *Manduca* IRP1 specifically. Transcription/translation assays demonstrated that both IRP1s repress ferritin synthesis *in vitro,* and the repression is IRE dependent. Iron administration to *Manduca sexta* increased hemolymph ferritin levels and decreased fat body IRP1/IRE binding activities without affecting either the IRP1 mRNA or protein levels. These data indicates that translational control of ferritin synthesis by IRP1/IRE interaction could occur in insects in a manner similar to that of mammals. To our knowledge this is the first report of the control of insect ferritin synthesis by IRP1/IRE interaction. The different responses to reducing agent of *Manduca sexta* and mammalian IRP1s could provide a potential future strategy for designing pesticides in insect control.

CHAPTER 1 INTRODUCTION

1. Iron is essential for life and high levels of iron are toxic

Iron is one of the most important micronutrients and plays a central role in the metabolism of all cells. This is evident from its major contribution to many diverse functions, including oxygen and electron transport, DNA replication, bacterial pathogenicity, photosynthesis, oxidative stress control and cell proliferation (Cairo and Pietrangelo, 2000). The importance of iron in health and disease has been reviewed recently (Lieu *et al.*, 2001). Iron also can generate reactive free radicals through Fenton chemistry. Free radicals can cause severe damage to variety of molecules including fatty acids, proteins and nucleic acids (Gutteridge *et al.*, 1983; Yoshie and Ohshima, 1997; Lloyd and Phillips, 1999; Mohamadin, 2001). This can lead to cell aging, and if not corrected, could cause cell death (Aisen, 1998). Although copper is a much stronger catalyst of free radical formation, iron is responsible for free radical damage because of its higher intracellular abundance.

In order to meet body and cellular needs for iron and eliminate damage from iron-overloading, iron homeostasis is tightly controlled (Beard *et al.*, 1996, Bothwell, 1995, Kuhn, 1999). Control of iron balance can be divided into two levels: systemic iron homeostasis and cellular iron homeostasis (Eisenstein, 2000). Several molecules involved in the systemic iron homeostasis including divalent metal transporter 1 (DMT1, Gunshin *et al.*, 1997), Nramp2 (Fleming *et al.*, 1997), hephaestin (Vulpe *et al.*, 1999), IREG1 (McKie *et al.*, 2000, Donovan *et al.*, 2000) and the hemochromatosis gene product (HFE,

Feder *et al.*, 1996) have been characterized recently. Much less is known about the systemic level of iron metabolism control than that at cellular levels.

2. Specific binding of iron by proteins provides a means to keep iron available but prevents iron toxicity

Because of its toxicity, iron is constantly sequestered by proteins in all processes including absorption, transport, storage and function. Once iron is absorbed from the intestine, it is transported by transferrin (Tf). Tf in the blood (Huang *et al.*, 1999 and Ponka *et al.*, 1998). Tfs have been cloned from many species, and the deduced amino acid sequences show that they are very well conserved (Kvingedal *et al.*, 1993; Denovan-Wright *et al.*, 1996; Hoshino *et al.*, 1996). All Tf molecules from mammals have two iron binding pockets. The affinity of Tf for iron is so great that there is almost no free iron in blood (Ponka *et al.*, 1998). Tf delivers iron to the tissues by binding to the transferrin receptor 1 (TfR1). TfR1 binding is followed by receptor-mediated endocytosis and iron transport from the endosome by DMT1 (Fleming *et al.*, 1998). DMT1 homologues have been reported from different mammalian species and given different names such as Nramp2 and DCT. Two TfRs, TfR1 and TfR2 (Kawabata *et al.*, 1999), have been cloned from humans. It is also clear that other proteins, including stimulator of ferrous transport (SFT) and HFE can affect TfR1 function (Gutierrez *et al.*, 1997, Bennett *et al.*, 2000; Lebron *et al.*, 1998). Once the iron enters the cell, it can be used for synthesis of iron-containing molecules or the extra iron can be stored for future

use. The mitochondrion is one of the major intracellular locations where iron is used for synthesis of heme and other iron proteins (Eisenstein, 2000).

Ferritin is involved in iron storage, and stored iron is available when needed. Two ferritin subunits, heavy- (H, Costanzo *et al.*, 1984) and light-chain ferritin (L, Santoro *et al.*, 1986), have been reported in humans and other mammals (Theil, 1990, Harrison and Arosio, 1996). The ferritin subunit homologues have been characterized from other vertebrates, insects (Winzerling and Law, 1997), plants (Theil *et al*, 1997) and some fungi (Howard, 1999). In mammals, the expression of H- and L-subunit mRNAs are tissue specific, and this is likely regulated at the transcriptional level (Bevilacqua *et al.*, 1998, Tsuji *et al.*, 1999). The H-subunit has a ferroxidase center and promotes oxidation of Fe(II) to Fe(III). The L-subunit is more responsible for the formation of the iron core in ferritin (Harrison and Arosio, 1996). The H-subunits tend to be higher in high-iron-uptake tissues such as the heart, brain and muscle, whereas the L-subunits are mainly found in iron-storage tissues such as liver and spleen (Theil, 1998).

3. Iron regulatory proteins (IRP) play central roles in iron homeostasis as iron bio-sensors

IRPs are considered central regulators of iron metabolism because they control the synthesis of proteins required for the uptake, storage, and usage of iron (Eisenstein, 2000). Two IRPs, IRP1 (Leibold and Munro, 1988, Rouault *et al.*, 1988, Rouault *et al.*,

1990) and IRP2 (Mullner *et al.*, 1989, Henderson *et al.*, 1993) have been cloned from human and other vertebrates. Two IRP1 homologues, IRP1A and IRP1B (Muckenthaler *et al.*, 1998), have been cloned from *Drosophila melanogaster*. IRP1 and IRP2 share a high identity with each other (~70%, Guo *et al.*, 1995), but the IRP2 contains a 73-amino acid fragment near its N-terminal that is absent from IRP1. This results in differences in iron response to intracellular iron availability for these twoo proteins. IRP2 is rapidly degraded in response to high iron levels by proteasome-mediated degradation with the involvement of the 73-amino acid fragment (Iwai *et al.*, 1995, Samaniego *et al.*, 1994). In contrast, IRP1 protein levels are not affected by iron (Tang *et al.*, 1992, Pantopoulos *et al.*, 1995). IRP1 acts as a mRNA binding protein when iron levels are low, but a 4Fe-4S cluster forms in the protein core at high iron levels; this (1) prevents the protein from binding to mRNA and (2) turns it into cytoplasmic aconitase (Constable *et al.*, 1992). In this situation, ferritin synthesis is enhanced and iron storage, in turn, is increased. Meanwhile, TfR1 expression is reduced and iron uptake is inhibited. The net effect of reduced iron uptake and increased storage is to lower the intracellular labile iron pool (LIP). When iron levels are low, both IRP1 and IRP2 interact with ferritin and TfR1 mRNAs (Muckenthaler *et al.*, 1998). The outcomes are different: blocking translation of ferritin, and increasing the stability of TfR1 mRNA, and thus, enhancing the expression of TfR1. The net effect is increasing iron levels by increasing iron uptake and reducing iron storage. The mechanisms responsible for the conversion between holoprotein (with 4Fe-4S cluster) and apoprotein (without 4Fe-4S cluster) are under investigation. Iron is the major factor for the conversion, although some other factors such as nitric oxide (NO)

and reactive oxygen species (ROS) also affect cluster stability (Hentze and Kuhn, 1996 and Caltagirone *et al.*, 2001).

4. Iron responsive elements (IRE) are well conserved in ferritin and TfR1 mRNAs

The IRE is a conserved sequence and structure found in both the 5'-untranslated region (UTR) of ferritin mRNA and the 3'-UTR of the TfR1 message (Leibold and Munro, 1987, Addess *et al.*, 1997, Butt *et al.*, 1996 and Theil, 1998). The IRE is a RNA helix, containing a six-nucleotide (CAGUGN) loop and a minimum ten-base-pair stem with a bulged C or G (Theil, 2000). The bulge separates the stem into upper- and lower-portions of five basepairs each (Hentze *et al.*, 1987, Murray *et al.*, 1987 and Henderson *et al.*, 1994). The bulged C is well conserved except for a G is found in crayfish ferritin IRE (Huang *et al.*, 1999). A single IRE has been found in the 5'-UTR of both human H and L ferritin mRNAs (Hentze *et al.*, 1987), and five IREs were found in the 3'-UTR of TfR1 mRNA (Casey *et al.*, 1988, Mullner and Kuhn, 1988 and Casey *et al.*, 1988).

In addition to ferritin and transferrin receptor mRNAs, IREs have been reported in other mRNAs encoding proteins involved in iron metabolism directly or indirectly. The mRNAs encoding red blood cell aminolevulinate synthase (eALAS, Dandekar *et al.*, 1991 and Cox *et al.*, 1991), *Drosophila* succinate dehydrogenase B (DsdB), and mitochondrial aconitase (Kim *et al.*, 1996) have an IRE in their 5'-UTRs. The mRNAs encoding DMT1 isoform I and glycolate oxidase (Kohler *et al.*, 1999) also have an IRE-

like sequence in their 3'-UTRs. More interestingly, IREs of *Drosophila* ferritin (Lind *et al.*, 1998 and Georgieva *et al.*, 1999) and human DMT1 can be alternatively spliced under certain situations (Lee *et al.*, 1998).

5. IRPs regulate iron metabolism pathways by interaction with IREs

It is well established that IRPs monitor and adjust iron balance at cellular levels by interacting with IREs specifically (Henderson *et al.*, 1996, Hentze and Kuhn, 1996, Muckenthaler *et al.*, 1998). When cellular iron levels are low, IRPs bind to the IRE in the 5'-UTR of ferritin mRNA, and block translation. Under these conditions, the cap binding complex, eIF4F, can bind the mRNA, but cannot make functional contact with the 43S ribosome (Muckenthaler *et al.*, 1998). However, when the IRP dissociates from the IRE, translation proceeds. This ensures that little, if any, ferritin is synthesized when iron is depleted, but the translation of this protein occurs when it is needed. On the other hand, the interactions between IRPs and the IREs in the 3'-UTR of TfR1 mRNA prevent the message from degradation. Of the five IREs in the 3'-UTR of the TfR1 message, three IRP/IRE complexes are sufficient for function, although all five IREs can bind IRP. It is now known that the sequences between these IREs are also involved in the IRP/IRE interaction (Theil, 1998). The interactions between IRPs and IREs can extend the TfR1 mRNA half-life up to six hours (from less than 1 hour, Eisenstein, 2000) and enhance the translation of the TfR1. Synthesized TfR1 will be translocated to the cytoplasmic membrane for enhanced iron uptake. When cellular iron levels are high, TfR1 mRNA

will be degraded after the dissociation of the IRP/IRE complex from its 3'-UTR, and TfR protein synthesis will be reduced. It has reported that mutation in the IRE of ferritin mRNA disturbs the systemic iron balance (Kato *et al.*, 2001, Allerson *et al.*, 1999).

IRP/IRE interaction not only regulates iron uptake and iron storage, it also affects iron usage. Binding of IRP to the IRE at the 5'-UTR of eALAS mRNA blocks translation (Melefors *et al.*, 1993 and Bhasker *et al.*, 1993). eALAS is the rate-limiting enzyme for heme synthesis, and heme synthesis is the major usage of iron. When iron levels are low, IRP/IRE interaction will block translation of eALAS. This will reduce heme synthesis and lower iron usage. When iron levels are high, formation of the iron-sulfur cluster in IRP1 and degradation of IRP2 prevents IRP/IRE interaction and eALAS is translated. The enhanced synthesis of heme will increase the usage of iron and the net effect will be a lowering of the iron level.

Since both *Drosophila* DsdB (Gray *et al.*, 1996, Kohler *et al.*, 1995 and Melefors, 1996) and mammalian mitochondrial aconitase (m-acon, Zheng *et al.*, 1992 and Kim *et al.*, 1996) are enzymes involved in the Kreb's Cycle, IRP/IRE interaction links iron metabolism to energy balance and redox status through these proteins. Studies have also revealed that IRP1 and IRP2 have distinct preference for different IREs (Henderson *et al.*, 1996 and Narahari *et al*, 2000) and this provides additional fine tuning to the regulation of iron-mediated protein synthesis. *In vitro* experiments have demonstrated that IPR1 binds the m-acon IRE with high affinity. Iron regulates both DsdB and m-acon

abundance and activities *in vivo*. The exact physiological significance of this regulation is not clear (Eisenstein, 2000).

6. Differences and similarities of iron metabolism regulation between mammals and insects

Regulation of iron metabolism and iron homeostasis has been studied in many living organisms (Eisenstein, 2000, Theil, 1998 and Winzerling and Law, 1997). In general, animals differ from plants and lower eukaryotes in maintaining iron homeostasis. Plants and lower eukaryotes maintain iron homeostasis primarily by transcriptional control of iron related proteins (Theil, 1998). In contrast, animal cells appear to do so by both transcriptional and translational control mechanisms. Compared to the understanding of iron homeostasis in mammals and plants, much less is known about insect iron homeostasis (Winzerling and Law, 1997).

Research to date indicates that insects and animals share some similar regulatory pathways with some distinct differences (Nichol and Locke, 1999). Cellular iron is stored in ferritin in mammals. In contrast, most ferritin in insect species found in hemolymph (Winzerling and Law, 1997, and Nichol and Locke, 1999). Lepidopteran hemolymph ferritin is >660kDa and composed of several subunits (Pham *et al.*, 2000). Available evidence to date shows that Lepidopteran fat body is a source of hemolymph ferritin (Renaud *et al.*, 1991 and Huebers *et al.*, 1988). The deduced amino acid sequences of the

ferritin subunits so far sequenced in *Calpodes* and *Manduca* fat body have leader sequences that signal secretion and the N-terminal sequences of the mature proteins match the amino-terminal sequences of hymolymph ferritin subunits.

Although both human H and L ferritin subunits contain an IRE in their 5'-UTRs, the sequences and structures of the IREs are sufficiently different to enable them to bind differently to different IRPs. This could be part of the reason for the different regulation for these two subunits (Theil, 1998). The ferritin H subunit is more abundant in high iron uptake tissues such as heart and muscle, and L subunit more abundant in long-term iron storage tissues (Harrison *et al.*, 1998). However, all mammalian ferritin IREs are near their 5'-cap structure (<60 nt, Theil, 1998). Several insect ferritin subunits have been cloned, and all of them resemble the mammalian H- and L-subunits (homologues of vertebrate H and L chain subunits, HCH and LCH). For Lepidoptera, two ferritin subunits have been cloned from *Calpodes ethlius* (Nichol and Locke, 1999) and one from *Manduca sexta* (Pham *et al.*, 1996), and each of them has an IRE in their 5'-UTRs. Several ferritin subunits have been cloned from Diptera including the HCH ferritin subunit from *Aedes aegypti* (Dunkov *et al.*, 1995) that also has an IRE at the 5'-UTR of the mRNA. In contrast to IREs in human H and L subunits, the IREs of *Calpodes* HCH and LCH are identical (Nichol and Locke, 1999). Also, the insect IREs are relatively far away from their 5'-cap structure (>100nt). Furthermore, the IRE in *Drosophila* ferritin mRNA can be alternatively spliced (Lind *et al.*, 1998). Collectively, the evidence

indicates that regulation of ferritin synthesis in insects may be different from that of human, as well as other mammals.

Two IRP1s (IRP1A and IRP1B) have been cloned from *D. melanogaster*, and they share high identity with mammalian IRP1s (Muckenthaler *et al.*, 1998). *In vitro* experiments demonstrated that *Drosophila* IRP1s react with ferritin IRE specifically. Yet, no IRP2 has been reported from insects. The *Drosophila* SdhB mRNA has an IRE at the 5'-UTR (Melefors, 1996 and Kohler *et al.*, 1995) and *in vivo* experiments shown that the synthesis of SdhB responds to iron administration and is controlled by *D. melanogaster* IRP1 (Gray *et al.*, 1996). Taken together, these data suggest that IRP1/IRE interaction could be one of the control mechanisms of insect cellular iron homeostasis.

7. Why study insect iron regulatory proteins

I propose to clone, over-express and characterize IRP1s from two insect species, *Manduca sexta* and *Aedes aegypti*. The knowledge obtained from *Manduca* IRP1 could be useful in designing of insecticides. Current data indicate that the insect IRP1 message varies during development, and plants do not have an IRP1 (Theil, 1998). The information about insect IRP1 could be useful in the design of compounds to act as insecticides without affecting plant nutrition. *Aedes aegypti* is responsible for transmitting the viral fevers, dengue and yellow fever (Coates *et al.*, 1998). More

knowledge regarding the difference and similarity of iron metabolism regulation between human and mosquito will be useful for the development of new insect control methods.

8. Hypothesis and outcomes of research

Based on the findings that (1) IRP1s have been reported in *Drosophila* (Muckenthaler *et al.*, 1998), (2) insect ferritin mRNAs contain IREs at their 5'-UTR (Dunkov *et al.*, 1995, Nichol and Locke, 1999 and Pham *et al.*, 1996), (3) mRNAs of insect proteins with IREs at their 5'-UTR are subject to iron control *in vivo* (Melefors, 1996), and (4) iron feeding to mosquitoes can change ferritin levels (Dunkov *et al.*, 1995), I hypothesize that insect ferritin synthesis is controlled by IRP1/IRE interaction in a similar way to that of mammals. To study the control of ferritin expression in insects by IRP1, I propose to conduct the following research:

(1) To clone the second (HCH) ferritin subunit from *Manduca sexta* and evaluate whether it has an IRE in the 5'-UTR of its mRNA.

(2) To clone, overexpress and purify IRP1s from both *Manduca sexta* and *Aedes aegypti*.

(3) To conduct electrophoretic mobility shift assays to determine if the IRP1 can bind IREs specifically.

(4) To measure *in vitro* transcription/translation with or without addition of IRP1 to determine if IRP1 can control ferritin translation by binding to IRE.

(5) To demonstrate that IRP1/IRE interaction is one of the mechanisms for maintaining insect iron homeostasis.

CHAPTER 2 PRESENT STUDY

The methods, results, and conclusions of this study are presented in the papers appended to this dissertation. The following is a summary of the most important findings in these papers.

I have successfully cloned and sequenced the IRP1 proteins from both *Manduca sexta* and *Aedes aegypti*. The deduced amino acid sequences show than *M. sexta* IRP1 consists of 891 amino acid residues, and *A. aegypti* IRP1s consists of 901 amino acid residues. Comparative sequence analyses indicate that these clones represent insect IRP1s, not IRP2 or mitochondrial aconitase (Appendix A and C). In order to know whether the insect IRP1s could interact with IREs specifically and whether they could inhibit ferritin synthesis *in vitro*, I overexpressed and purified the two insect IRP1s from *E. coli*.

The electrophoretic mobility shift assay (EMSA) results showed that recombinant insect IRP1s bind specifically not only to transcripts of the ferritin IRE from the native species, but also with transcripts of the human ferritin subunit IRE. In addition, recombinant human IRP1 bound both *Manduca* and *Aedes* ferritin IREs. These experiments provided further evidence that the insect cDNA clones encoded a functional IRP1, and that IREs found in these insect mRNAs could be active translational control elements (Appendix A and C).

We previously reported cloning and characterization of *Manduca sexta* LCH ferritin subunit cDNA and identified an IRE in the 5'-UTR of the mRNA (Pham *et al*, 1996). N-terminal amino acid sequencing indicated that at least another ferritin subunit,

presumably the HCH subunit, exists in *Manduca sexta*. In order to know (1) whether the *Manduca* HCH ferritin subunit mRNA has an IRE, and (2) if it is identical to that of LCH ferritin subunit mRNA, and (3) would it serve as a binding site for IRP1, I cloned the second ferritin subunit from *M. sexta*. Analysis of the deduced amino acid sequence showed that all the residues necessary for the ferroxidase activity were conserved. An IRE also was identified from the HCH mRNA and it was similar, but not identical, to that of the LCH subunit mRNA (Appendix B).

In order to know whether the translation of *M. sexta* ferritin subunits could be repressed by recombinant IRP1, I evaluated the transcription/translation of the ferritin subunit cDNA in the presence of recombinant IRP1 *in vitro* using a TNT-coupled wheat germ lysate system. The assays consistently demonstrated that the *M. sexta* IRP1 repressed synthesis of the HCH and LCH ferritin subunits *in vitro* by inhibiting translation without affecting transcription (Appendix A). Repression occurred for both subunits assayed either separately or together. Removal of either the IRE or the total 5'-UTR from *Manduca* HCH ferritin mRNA abolished the IRP1 repression. The *Aedes* IRP1 also inhibited translation of *Aedes* ferritin. Addition of *Aedes* ferritin IRE transcripts to the *in vitro* transcription/translation system partially inhibited IRP1 repression. Based on these data, I concluded that insect IRP1s repress ferritin synthesis *in vitro* by binding to the IRE on the 5'-UTR of the mRNA and preventing translation (Appendix B).

Mammalian IRP1s serve as iron biosensors and control iron homeostasis. In order to learn if insect IRP1s serve the same function, different iron concentration in the form of ferric ammonium citrate (FAC) were administrated to late fifth-instar *M. sexta* larvae. The responsive ferritin levels, levels of IRP1 mRNA and protein, and IRP1 binding activity were analyzed. IRP1 mRNA and protein levels were not significantly changed by iron injection relative to controls. In contrast, IRP1/IRE binding activity was responsive to iron injection. Iron administration resulted in a progressive decline in IRP1 binding activity relative to water injection in dose-responsive manner that was significant at 20 μM FAC. However, above this FAC concentration, IRP1 binding activity increased slightly and then stabilized. Hemolymph ferritin levels increased significantly in response to iron treatment at 10 μM and 20 μM FAC relative to insects injected with water. This increase coincided with decline in IRP1 binding activity. At higher FAC concentrations, the hemolymph ferritin levels dropped and then stabilized. These results show that iron administration altered expression of ferritin in hemolymph correlated inversely with fat body IRP1 binding activity without affecting either IRP1 mRNA or protein levels. This indicates that IRP1s in insect could serve the same purpose as that in mammals when iron levels are elevated (Appendix A).

It is well established that mammalian IRP1s become cytoplasmic aconitase when iron levels are elevated by forming an iron-sulfur clusters in the protein core. The iron-sulfur cluster can be constituted *in vitro*. All the residues necessary for iron-sulfur cluster formation and for aconitase enzymatic activity are fully conserved. Therefore, it would be

interesting to conduct *in vitro* cluster formation for both insect IRP1s and to measure their aconitase activities. It has been shown that an iron-chelating agent (EDTA) abolished human IRP1 aconitase activity *in vitro*. To know whether iron is necessary for insect IRP1 aconitase activity, enzymatic activities could be measured in the presence or absence of EDTA.

In the iron administration experiments, we noticed that compared to the no-injection group, water injection increase IRP1/IRE binding activity and reduced the hemolymph ferritin levels. These changes indicate that IRP1/IRE binding activity, as well as ferritin levels are affected by either wounding caused by injection or stimulation of immune response. The IRP1 binding activity of *M. sexta* fat body lysate is dramatically reduced when treated with reducing agent (β-mercaptoethanol) *in vitro*, although the human IRP1 binding activity is increased under the same conditions. These differences indicate that IRP1/IRE interaction *in vivo* in insects could differ from that of mammals. Since the IRP/IRE binding activity of recombinant *M. sexta* IRP1was not significantly affected by β-mercaptoethanol, some factor(s) other than IRP1 itself in the insect fat body are likely involved in the response of the lysate IRP1/IRE binding activity to reducing agent. In addition, bacterial challenge of *A. gambiae* cell did not change IRP1 level, but IRP1 binding activity for the IRE was elevated following immune induction (Appendix C). Understanding of the differences between insect and mammalian iron metabolism pathway will open a new field for both basic and applied sciences. The findings from this research could provide strategic designing of insecticides for insect control.

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APPENDIX A. *MANDUCA SEXTA* IRP1: MOLECULAR CHARACTERIZATION AND *IN VIVO* RESPONSE TO IRON

(Accepted by ***Insect Biochemistry and Molecular Biology*** for publication)

Manduca sexta IRP1: Molecular characterization and *in vivo* response to iron

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Abstract

Manduca sexta IRP1 was cloned and sequenced. The deduced amino acid sequence of *Manduca* IRP1 shows high similarity to other IRP1 proteins. The Cys residues required as ligands for the iron sulfur cluster, as well as all residues necessary for aconitase activity are conserved in the insect protein. Purified recombinant *Manduca* IRP1 binds specifically to transcripts of the iron responsive element (IRE) of *Manduca* or human ferritin subunit mRNA. Binding activity of the recombinant protein was not influenced by the presence of β-mercaptoethanol. However, IRP/IRE binding activity of cytoplasmic extracts from fat body was decreased by reducing agents in a dose-responsive manner. Fat body IRP1/IRE binding activity was reduced for *M. sexta* larvae injected with low doses of iron, while IRP1 mRNA and protein levels remained stable. At higher iron doses, binding activity increased and stabilized. Hemolymph ferritin levels showed an inverse relationship to IRP1/IRE binding activity. These data suggest that the *Manduca* IRP1 is likely involved in translational control of ferritin synthesis in a manner similar to that found in vertebrates. However, factors other than iron can influence IRP/IRE interaction and hemolymph ferritin levels in insects.

1. Introduction

Iron is a required nutrient for all living organisms. Yet, iron can promote the formation of toxic free radicals by the Fenton reaction. Many organisms prevent toxicity, but maintain iron availability, by storing excess iron in ferritin (Theil, 1990, Andrews et al., 1992). Mammalian ferritins have been extensively studied. These proteins consist of 24 subunits configured as a hollow sphere wherein iron is stored.

Lepidopteran ferritin is ~660 kDa, composed of several subunits and appears structurally similar to the mammalian ferritins (Nichol and Locke, 1989, Pham et al., 1996). Lepidopteran ferritin is abundant in hemolymph, and several lines of evidence imply that fat body is a source of hemolymph ferritin. Early work showed that transferrin-bound iron is taken up by the fat body and reappears in hemolymph associated with a high mass protein (Huebers et al., 1988). The deduced amino acid sequences of ferritin subunits expressed by fat body of *Calpodes ethlius* (S and G subunits, Nichol and Locke, 1999) and *Manduca sexta* [ferritin light chain homologue (LCH), Pham et al., 1996] have a leader sequences signaling secretion. The predicted N-terminal sequences of the mature proteins correspond to the amino-terminal residues of hemolymph ferritin subunits. Finally, ferritin can be visualized in the fat body endoplasmic reticulum and secretory system. Iron loading of larvae does not result in an increase in iron-loaded ferritin in fat body, but does increase ferritin in hemolymph (Locke et al., 1991).

To date, the mRNA sequences of all ferritin subunits from Lepidopterans have an iron responsive element (IRE) in the 5'-untranslated region (UTR) (Pham et al., 1996, Nichol and Locke, 1999). The mammalian ferritin mRNA IRE is a well known translational regulatory control site. In mammals, when intracellular iron concentrations are low, iron regulatory proteins, IRP1 and IRP2, bind to the IRE. IRP/IRE interaction blocks recruitment of the small ribosomal subunit to the cap-binding complex preventing ferritin synthesis (Muckenthaler et al., 1998a). When iron becomes available, IRP/IRE interaction declines and ferritin is translated (Hentze and Kuhn, 1996, Eisenstein and Blemings, 1998). IRP2 is rapidly degraded in the presence of increased iron (Iwai et al., 1998, Hanson and Leibold, 1999). However, IRP1 is unique in that when intracellular iron increases, an iron sulfur cluster (4Fe-4S) forms in the protein core that prevents IRP1/IRE interaction (Haile, 1999, Beinert et al., 1996). In mammals, the formation of the iron sulfur cluster allows IRP1 to function as an iron biosensor (Constable et al., 1992). In addition, it permits IRP1 to respond to other cell factors, such as nitric oxide (NO) and hydrogen peroxide. These compounds alter iron sulfur cluster formation and retention, and thereby up-regulate IRP1/IRE interaction and down regulate ferritin synthesis (Hanson and Leibold, 1999, Hentze and Kuhn, 1996). When the 4Fe-4S cluster is present, the protein becomes cytoplasmic aconitase. Recent work indicates that cytoplasmic aconitase is important for energy metabolism (Narahari et al., 2000).

Available evidence suggests that the ferritin mRNA IRE of insects could be a functional site for translational control of ferritin synthesis. Lepidopteran hemolymph ferritin

subunit messages have a 5'-UTR IRE and the protein increases in response to iron loading (Winzerling *et al.*, 1995). IRP1 proteins are conserved in invertebrates (Huang et al., 1996, Rothenberger et al., 1990, Muckenthaler et al., 1998b), and two IRP1s have been sequenced from Dipterans, *Drosophila IRP1A* and *IRP1B* (Muckenthaler et al., 1998b). The deduced amino acid sequences of these proteins display 86% identity, high similarity to the human IRP1, are expressed in all embryonic stages. In addition, studies of the IRE identified in *Drosophila* succinate dehydrogenase subunit b (Muckenthaler et al., 1998b, Kohler et al., 1995, Melefors, 1996) shows that translational control of protein synthesis by IRP/IRE interaction occurs in insects.

We are studying the properties, expression and activity of Lepidopteran IRP1. We report the cloning and sequencing of the *M. sexta* IRP1. We show that the recombinant *M. sexta* IRP1 binds specifically to transcripts of the IRE. We found that IRP1 is expressed by *M. sexta* fat body, and that fat body IRP1 binding activity declines in the presence of β-mercaptoethanol. Neither IRP1 message or protein expression responds to iron administration. In contrast, IRP1/IRE binding activity of fat body declines in a dose-responsive manner at low iron concentrations. However, at higher iron concentrations, binding activity increases. Hemolymph ferritin levels correlate inversely with fat body IRP1/IRE binding activity.

2. Materials And Methods

2.1. Cloning and Sequencing

Degenerate oligonucleotide primers (a kind gift from Dr. M. Hentze, EMBL, Heidelberg, Germany) [5'-GGIGCIGGI(C/T)TI(C/T)TIGCIAA(G/A)AA(G/A)GT-3', and 5'-CCIGCIGGI(C/G)(A/T)IAT(A/G)TG(A/G)TCIGT-3'] were used to amplify IRP fragments from *M. sexta* 5th instar larval mRNA by RT-PCR with the Superscript II Kit (Life Technologies, Gaithersburg, MD). A 710bp PCR product was obtained, cloned using T/A Cloning Kit (Invitrogen, La Jolla, CA) and sequenced using the United States Biochemical Sequinase Kit (Cleveland, OH). The deduced amino acid sequence of the fragment showed high identity to the human IRP1 protein and the fragment was subsequently used as probe to screen a *M. sexta* 5th instar fat body Lambda-ZAP II cDNA expression library (Stratagene, La Jolla, CA). *In vivo* excision of positive clones obtained from the cDNA library was conducted according to the instructions provided in the Lambda Zap II kit. Double stranded sequencing of a full length positive clone (3.2 kb) in both directions was done by automated cycle sequencing using Terminator Kit (Applied Biosystems, Inc.; Foster City, CA), and by manual Sanger sequencing (USB Kit) and the results were compared. The cDNA and deduced amino acid sequences were analyzed using Genetics Computer Group (Devereux et al., 1984, GCG, Madision, WI) and database searches were done using BLAST programs (GCG, Altschul et al., 1990).

2.2. Expression of M. sexta IRP1 and human IRP1 in Escherichia coli

The open reading frame (ORF) from the 3.2 kb *M. sexta* IRP1 cDNA clone was amplified by PCR with *Pfu* DNA polymerase (Stratagene) with two specific primers, sense primer, 5'-AG**CCATGG**CGGCCAAATCAAAT-3' and anti-sense primer, 5'-TT**TCTAGA**TAGAGCATTTTCCTAATCAT-3' (*NcoI* and *XbaI* sites are in bold). The amplified ORF was subcloned into the *NcoI/XbaI* sites of the pTrcHis2B vector (Invitrogen, California) such that directional insertion resulted in an expressed recombinant protein with two extra amino acids (Tyr and Leu) added to the C-terminus followed by a c-myc epitope and polyhistidine (6xHis) tag. The construct was named as IRP1/pTrcHis2B. Sequencing was conducted to verify the correct orientation of the ORF, the expected junctions between the vector and the ORF, and to confirm that no mistake was present in the amplified ORF. The expression clone for the recombinant human IRP1 was a kind gift from Dr. M. Hentze (EMBL, Heidelberg, Germany). The IRP1/pTrcHis2B or the human IRP1 clone were transformed into the *E. coli* strain BL21-CodonPlus(DE3)-RIL competent cells (Stratagene) and grown in phosphate buffered saline (PBS)/Luria Broth containing 100μg/ml ampicillin (adjusted to pH 7.2 with 4.0 M NaOH) at 30°C for 20 hours. Pellets (1L of broth = 1 pellet) were obtained by centrifugation and stored at –80°C.

2.3. Chromatographic purification of recombinant IRP1

The following procedure was used to purify either the human IRP1 or the *Manduca* IRP1 from bacterial cell lysates. To each cell pellet, we added ice-cold PBS-buffer (20 ml, pH 7.4) and solid K_2SO_4 to a final concentration of 0.5 M. The pellets were then ruptured by ultra-sonication and centrifuged at 2000*g* (15 min, 4°C). The supernatant was collected, centrifuged at 20,400*g* (60 min, 4°C). The supernatants of 8 pellets from the second centrifugation were combined and potassium hydrogen phthalate (PHP) solution, adjusted to pH 7.0 with 4.0 M NaOH), was added to a final concentration of 60 mM PHP. The lysate was then passed through a Whatman No. 1 filter. All chromatographic procedures were carried out in a cold room at 8°C. Freshly regenerated Ni^{2+}-NTA agarose (Qiagen, Germany) was packed into a column (4.0 cm, 1.0 cm i.d.) and equilibrated with 60 ml of buffer A (60 mM PHP, 0.5 M K_2SO_4, pH 6.5) at 0.5 ml/min. Lysate from 8 pellets (approximately 170 ml) was loaded onto the column at the same flow rate. After washing with 100 ml of buffer A and 10 ml of buffer B (30 mM tricine, 0.5 M K_2SO_4, pH 7.0), the protein was eluted with approximately 35 ml of buffer C (60 mM tricine, 0.5 M K_2SO_4, pH 5.0). The eluted fractions containing the recombinant IRP1 were pooled and concentrated (Collodion nitrocellulose membranes 25,000 MW cut-off, Schleicher & Schuell, Germany) against buffer D (30 mM tricine, 40 mM KCl, pH 8.0) to approximately 300 µl. The protein concentrate was loaded onto a Q Sepharose Fast Flow anion-exchange column (12.5 cm, 1.0 cm i.d.) equilibrated with buffer D. Recombinant IRP1 was eluted using a 40 ml linear salt gradient from buffer D to buffer E

(30 mM tricine, 1.0 M KCl, pH 8.0) at 1.0 ml/min using a Pharmacia GP-250 Plus FPLC (Pharmacia Biotech, Uppsala, Sweden). The fractions containing the IRP1 were pooled and concentrated in a Centricon YM-30 concentration tube (Millipore, Bedford, MA, USA). Protein was evaluated using bovine serum albumin as a reference standard (Bradford, 1976). Identification of the IRP1 was done by western blot and the level of purity was assessed by SDS-PAGE as described below.

2.4. Electrophoresis Mobility Shift Assay (EMSA)

Manduca IRE transcript was designed from the IRE found in the 5'-UTR of *M. sexta* ferritin LCH mRNA (Pham et al., 1996) and prepared as follows. Two oligonucleotides were synthesized (Gibco BRL, Grand Island, NY) and annealed to form a DNA fragment.

```
5′-
GAAAGCTTGAAAGTCGCCTTCTGCGCCAGTGTGTGTAAAGGCTGCACTTTGGGATCCCC
-3′
3′-
CTTTCGAACTTTCAGCGGAAGACGCGGTCACACACATTTCCGACGTGAAAGCCTAGGGG
-5′
```

This fragment was cloned into the HindIII (5'-end; AAGCTT) and BamHI (3'-end; GGATCC) sites of the pTZ19R vector provided by the RNA Gel Shift Kit (MBI Fermantas, 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 BamHI digestion and transcription was conducted according to the manufacturer's instructions. Transcripts were labeled with $\alpha[P^{32}]$CTP (56 μCi, > $3x10^6$ mCi/mmol; Amersham). Following transcription, transcripts were

precipitated in ethanol, suspended in DEPC-treated water, and quantified from $\alpha[P^{32}]$CTP incorporation. In order to quantify the transcript for competition assays, transcripts were labeled with trace levels of $\alpha[S^{35}]$CTP (0.01 μCi, 10 mCi/ml; Amersham). Incorporation of $\alpha[S^{35}]$CTP at this concentration was not detectable by autoradiography for film exposed at -80°C for several days. The RNA transcripts were: 5'-GGGAAAGCUUGCAUGCCUGCAGGUCGACUCUAGAGGAUC-3'(human IRE), 5'-

GGAAAGCUUGAAAGUCGCCUUCUGCGCCAGUGUGUGUAAAGGCUGCACUU UCGGAUC-3'(*Manduca* IRE). EMSA was done as described elsewhere (Leibold and Munro, 1988) with the following modifications. Labeled transcripts (50 fmol), recombinant *M. sexta* IRP1 (50 ng) or recombinant human IRP1 (50 ng), plus unlabeled transcript as designated were added to 16.9 mM HEPES, 0.84 mM $MgCl_2$, 16.9 mM KCl, 5.6 mM DTT, 2.8% glycerol and 0.28 units RNasin in a 20 μl reaction and 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, RT. The bound transcripts were separated on 6.5% acrylamide gel at 100 volts for 2 hrs and visualized after autoradiography on X-ray film (Kodak). Human IRP1/ human IRE interaction served as positive controls. RNA Ladder (Stratagene) was used as a nonspecific competitor.

2.5. Iron Treatment of Insects

A solution of ferric ammonium citrate (100 mM FAC) was prepared immediately prior to use by adding 17.6 mg of FAC to1 ml water bubbling with nitrogen. From this 100 mM solution, serial dilutions were made to final concentrations of 10, 20, 40, 60, 80, and 100 mM FAC. Fourth day, 5th instar *M. sexta* larvae (56 insects) were separated into eight groups of seven insects each. Treatment groups were defined as not injected (NI), water injected (WI) or by FAC concentration. Each injected animal received 10 µl of water or 10 µl FAC solution at the desired concentration administered into a proleg. After injection, insects were maintained separately and allowed to continue feeding for 16 hrs at RT.

2.6. Preparation of hemolymph

Hemolymph (~0.3 ml) was collected by bleeding the insect from a proleg. Hemolymph for each treatment group was collected in 80 µl of 4.0% phenylthiolurea (PTU) and immediately centrifuged at 1800*g* (4°C). An aliquot of the supernatant was taken for immediate Bradford assay (Bradford, 1976) using bovine serum albumin as a standard. A second aliquot was taken for SDS-PAGE to which sample treatment buffer was added (v/v). Remaining hemolymph and SDS-PAGE aliquots were flash frozen in liquid nitrogen and stored at –80°C.

2.7. RNA Purification and quantitative RT-PCR

Fat body was dissected from insects, the samples pooled by treatment group, and total RNA was purified using RNeasy Mini Kit (Qiagen Inc., Valencia, CA) according manufacturer's instruction. Two μg of total RNA were used in a 20 μl reaction for first strand cDNA synthesis by random priming in the presence of reverse transcriptase SUPERSCRIPT II RT (Gibco BRL) . A 20 μl-PCR reaction was done with 0.4 μl of cDNA using *M. sexta* IRP1- or actin-specific primers (35 cycles for actin and 45 cycles for IRP1: 95°C for 1 min, 59°C for 1 min and 72°C for 1min). Primers for *Manduca* IRP1 were 5'-TCCGCCCTGAGTATCGCG-3' and 5'-ATCACGCTCGGAT TCGAA-3'. Primers for *Manduca* actin were 5'-ATGTCGGACGACGATGTTGCT-3' and 5'-GAGCCTCGGTGAGCAGGAC-3'. These primer pairs each resulted in one band of product. Samples (10 μl) from the PCR reaction were run on 1.0 % agarose gel and the PCR products were scanned by laser densitometer (Molecular Dynamics, Sunnyvale, CA) and quantified by ImageQuaNT (Molecular Dynamics).

2.8. Preparation of cytoplasmic extract

Fat body was rinsed exhaustively with dissecting buffer (20 mM HEPES, 150 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.2 mM EDTA, 0.1 mM PMSF, 0.1 mM diisopropyfluorophosphate (DFP), 0.04% PTU and inhibitor cocktail (400 μl/100ml

solution, Zeigler et al., 1995) and dissected to dry ice. The tissues were combined by treatment group in 50-ml conical tubes. Frozen fat body was covered with dissecting buffer, flash frozen using liquid nitrogen and stored at –80°C. To prepare cytoplasmic extracts, fat body was thawed on ice and all steps were carried out on ice or at 4°C. Thawed fat body was homogenized and centrifuged twice at 17,500*g* for 15 and 20 min, and then at 180,000*g* for 1 hr in a Beckman Ti60 rotor. Following each centrifugation, the interface layer was collected. The supernatant was collected from the last centrifugation, frozen in liquid nitrogen and kept at –80°C.

2.9. Immunoblot of M. sexta fat body cytoplasmic extract

A pre-cast 10-20% SDS-polyacrylamide gradient Mini gel (BioRad, Hercules, CA) was run at 100 volts until the dye front passed from the bottom of the gel. The gel was blotted to nitrocellulose membrane (BioRad) in a Mini Trans-Blot Transfer cell (BioRad) at 100 volts for one hour. Ponceau Red S staining confirmed efficient protein transfer. The membrane was blocked overnight at 4°C in a modified BlottO (7% nonfat milk, 3% BSA, 0.05% Triton X-100 in PBS, pH 7.4) followed by one hr at 37°C. The membrane was then incubated in rabbit anti-rat IRP1-specific antiserum (Eisenstein et al., 1993, a generous gift from Dr. Richard Eisenstein, Madison, WI) in dilution buffer (3% BSA, 0.05% Triton X-100, PBS, pH 7.4) at a dilution of 1:2000 for two hr at RT. The membrane was washed three times in wash buffer (0.05% Triton X-100, PBS, pH7.4), and incubated in anti-rabbit alkaline phosphatase conjugate (Jackson ImmunoResearch

laboratories, West Grove, PA) antibody at a dilution of 1:1000 for one hr, RT. The membrane was washed three times in wash buffer, followed by a 10 min. equilibration in development buffer (100 mM Tris, pH 95). The blot was developed in alkaline phosphatase substrate (BioRad) until the bands were visualized. For hemolymph ferritin, the membrane was blocked as described and then incubated with anti-*M. sexta* ferritin antiserum (Winzerling et al., 1995) in dilution buffer at a dilution of 1:2000 for two hrs at RT. The membrane was washed three times in wash buffer and incubated in anti-rabbit horseradish peroxidase conjugate antibody (Jackson ImmunoResearch laboratories, West Grove, PA) at a dilution of 1:100,000 for one hr, RT. The membrane was again washed three times in wash buffer, and subsequently developed in SuperSignal® West Pico chemiluminescent substrate (Pierce, Rockford, IL) and exposed to Blue XB-1 film for 2 min (Eastman Kodak Company, Rochester, NY). Blots were performed in triplicate.

2.10. Statistical Analyses

Differences among groups were evaluated by analysis of variance using Bonferroni's test, and t-tests with significance set at $p<0.05$.

3. Results

The deduced amino acid sequence of the *M. sexta* IRP1 consists of 891 amino acid residues with a predicted mass of 97,308 daltons (Fig. 1, Genebank Assession AY032658). This is similar to the mass of the human IRP1 (98,398 daltons, Rouault et al., 1990) and *Drosophila* IRP1A (98,632 daltons, Muckenthaler et al., 1998b). While the predicated residue sequence shows high identity to *Drosophila* IRP1A and IRP1B (73%, Table 1), crayfish IRP1 (70%) and human IRP1 (68%), identity is lower for either the human IRP2 (58%) or mitochondrial aconitase (33%) (Table 1). In addition, the 73-amino acid sequence that distinguishes the mammalian IRP2 is missing from our insect sequence (Fig 1). These data indicate that we have cloned a member of the IRP1 family, not IRP2 or mitochondrial aconitase.

Comparison of the deduced amino acid sequence of the *M. sexta* IRP1 with sequences of other similar proteins shows that the residues of *M. sexta* IRP1 C441, C507, and C510 (Fig. 1, bolded, uppercased and underlined) that correspond to the cysteines that serve as ligands for the iron sulfur cluster of mitochondrial aconitase (C358, C421 and C424, Beinert et al., 1996) are conserved. Further, the remaining residues that constitute the active site of porcine mitochondrial aconitase (Q72, D100, H101, H147, D165, S166, H167, N258, E262, I425, N446, R447, R452, D568, R580, S642, S643 and R644, Beinert et al., 1996) also are conserved in *M. sexta* IRP1 (bolded and upper case, *MIRP:* Q90, D129, H130, H182, D208, S209, H210, N301, E305, I511, N539, R540, R545,

D682, R703, S782, S783 and R784). From these data, we conclude that *M. sexta* IRP1 likely has properties and functions that are similar to the mammalian IRP1.

M. sexta IRP1 was over-expressed with a C-terminal His_6 tag and isolated to >90% purity from bacterial lysate (Fig. 2A). Western blotting with Penta-HIS monoclonal antibody (Fig. 2B) specifically identified both the recombinant *M. sexta* IRP1 and the recombinant human IRP1 expressed with a His_6-tag. Recombinant *M. sexta* IRP1 also reacted with a rabbit anti-rat IRP1-specific antiserum (Fig. 2C, Eisenstein et al., 1993). This reaction shows that *M. sexta* IRP1 and rat IRP1 share common epitopes.

We evaluated RNA binding activity of recombinant *M. sexta* IRP1 by EMSA (Fig. 3). The *M. sexta* IRP1 bound radio-labeled transcripts of *M. sexta* ferritin LCH IRE (Fig. 3A), and trace-labeled LCH IRE transcripts were able to compete effectively with [^{32}P]-labeled LCH IRE in a dose-responsive manner. Nonspecific RNA did not compete with LCH IRE for IRP1 binding (Fig. 3A, lane 6) and lysate from bacteria without plasmid showed no binding activity for the LCH IRE (Fig. 3A, lane 1). Studies using tRNA as nonspecific RNA showed similar results.

We also analyzed the primary sequence of the *M. sexta* IRP1 for the predicted RNA-binding site residues (Fig. 1, asterisked of human IRP1) C437, R536, R541, R780 (Philpott et al., 1994), S138 (Schalinske et al., 1997b, Brown et al., 1998), residues 116-140 (Basilion et al., 1994, Gegout et al., 1999, Swenson and Walden, 1994), residues 56-

65, 685-689, and 728-737 (Kaldy et al., 1999, Gegout et al., 1999). All of these regions are well conserved except regions 56-65, 133-137 and 728-737 and S138. Because of these differences, we evaluated whether the recombinant *M. sexta* IRP1 would bind to human ferritin heavy chain IRE transcripts. We found that either the human or insect IRP1 would react with transcripts from the other species (Fig. 3B). We conclude from these analyses that the *M. sexta* IRP1 can be identified by the rat anti-IRP1-specific serum, that the insect and mammalian IRP1s show related immunity, and that residues crucial to IRE binding are sufficiently conserved to allow IRP/IRE interaction across species. Based on these data, we wanted to evaluate *in vivo* fat body IRP1 expression and IRP/IRE binding activity.

Mammalian IRP1 binding activity is increased in the presence of reducing agents as measured by EMSA (Schalinske et al., 1997a). Thus, before conducting *in vivo* studies, we evaluated the effect of β-mercaptoethanol on *M. sexta* IRP1/LCH IRE interaction. As expected, recombinant human IRP/IRE interaction was increased in the presence of reducing agents in a dose-responsive manner (Fig 4). However, the recombinant *M. sexta* IRP1/IRE interaction was not significantly changed by the presence of reducing agents, even at high levels. In contrast to either of the recombinant proteins, *M. sexta* fat body IRP/IRE binding activity declined in the presence of increasing concentrations of reducing agent. These data indicate that, in contrast to the mammalian IRP1, *in vivo* binding activity of *Manduca* IRP1 cannot be evaluated in the presence of high levels of reducing agents.

In mammals, the IRP1 binding activity declines with iron administration (Schumann et al., 1999, Chen et al., 1997); this results in increased ferritin synthesis. The decline in IRP1/IRE interaction occurs without a change in the IRP1 message or protein level (Tang et al., 1992). We evaluated binding activity, mRNA and protein levels of *M. sexta* fat body IRP1 from late fifth instar larvae injected with increasing amounts of iron. IRP1 mRNA levels were not significantly changed by iron injection relative to the water injected control animals as evaluated by quantitative RT-PCR (Fig. 5, upper panel). Iron administration also did not significantly alter *M. sexta* IRP1 levels as shown by western blotting (Fig 5, lower panel). In contrast to IRP1 mRNA and protein, IRP1/IRE binding activity was responsive to iron injection (Fig. 6). Iron administration resulted in a progressive decline in IRP1 binding activity relative to water injected animals (controls) in dose-responsive that was significant at 20 mM FAC. However, above this FAC concentration, IRP1 binding activity increased slightly and then stabilized at high iron concentrations.

Hemolymph ferritin levels increased significantly in response to iron treatment at 10 and 20 mM FAC relative to insects injected with water (Fig. 7). This increase coincided with a decline in IRP1 binding activity (Fig.8). Unexpectedly, ferritin levels at 40 mM FAC were similar to those observed for water injected animals, and ferritin stabilized at this lowered level despite increased iron dosage. Although ferritin was significantly greater at 60 mM than that of water injected insects, it did not differ significantly from ferritin of insects injected with FAC concentrations at 40-100 mM. Ferritin levels at higher

concentrations of iron coincided inversely with IRP1 binding activities (Fig.8). Comparison of means from IRP1/IRE binding activities with hemolymph ferritin revealed a negative correlation of IRP1/IRE binding activity with hemolymph ferritin of -0.647 ($p < 0.06$).

Late fifth instar larvae are in rapid growth; for this reason we allowed the insects to continue feeding following iron injection. Thus, we included a group of non-injected insects to observe the effects of injection. Water injection significantly up-regulated IRP1 mRNA relative to non-injected insects. However, this effect was not reflected in the IRP1 protein levels. Water injection also caused an increase in IRP1/IRE binding activity and a significant fall in hemolymph ferritin relative to non-injected insects. Since the effects of water injection were opposite to those seen for FAC injection at low iron concentrations, iron dominated the effect of water injection on IRP1 message, IRP1/IRE binding activity and hemolymph ferritin. IRP1 mRNA, IRP1 protein levels, IRP1 binding activity, and hemolymph ferritin represent triplicate assays from one experiment. We repeated this experiment, as well as an experiment in a lower dose range of FAC concentration (0-50 mM, data not shown), with similar results.

In summary, we conclude from these data that iron administration at low concentrations decreases IRE binding activity of fat body IRP1 without changing mRNA or protein levels and coincides with an increase of hemolymph ferritin. At higher iron concentrations, a different response is invoked that results in attenuation of IRP1/IRE

binding activity. Hemolymph ferritin levels also do not increase in response to iron administration at higher concentrations. We think it likely this occurs because enhanced IRP1/IRE interaction suppresses ferritin synthesis and attenuates this response. These data also suggest that IRP1/IRE binding activity of *M. sexta* fat body, as well as hemolymph ferritin respond to factors other than iron.

4. Discussion

We are studying iron metabolism in Lepidopterans because they are major insect pests, and *M. sexta* can serve as a model for insects of this order. We have cloned the *M. sexta* IRP1 as supported by the following evidence: 1) the deduced amino acid sequence has high identity to human and invertebrate IRP1s, as well as conserved residues for iron sulfur cluster formation and aconitase activity, 2) the 73 amino acid sequence that characterizes the IRP2 is absent from our sequence, 3) recombinant IRP1 protein specifically binds human and *M. sexta* ferritin subunit IRE transcripts, and 4) recombinant *M. sexta* IRP1 share immune epitopes with rat IRP1.

Recombinant *M. sexta* IRP1 and human IRP1 bind to ferritin IRE transcripts of the other species. This was not surprising because a *Drosophila* IRP was first observed by EMSA of insect cytoplasmic extracts bound to human ferritin IRE transcripts (Rothenberger et al., 1990). A comparison of the residues within the critical regions for RNA binding activity shows that all are reasonably well conserved except S138, and residues in regions 56-65, 133-137 and 720-737. Phosphorylation of S138 provides a mechanism of iron-independent regulation of mammalian IRP1/IRE binding activity (Brown et al., 1998). In *M. sexta* this residue is A141, yet the protein retains binding activity. This finding concurs with Brown et al., (1998) who showed that mutation of the Ser to an Ala in the mammalian IRP1 does not significantly influence the aconitase activity or negatively influence IRE binding activity. This also implies that the control mechanisms

involving phosphorylation of the IRP1 are different for insects. In the case of human IRP1 region 720-737, three pairs of residues are strongly protected from proteolysis, R721-G722, R728-L729 and R732-F733 (Gegout et al., 1999). The *M. sexta* sequence has a conservative substitute of Ile at the position of human IRP1 L729, and a Lys and Met at the position of human IRP1 R732-F733. These substitutions still permit IRE binding activity. The Lys substitution at position 732 is common among other mammalian IRP1s (Gegout et al., 1999). The substitution of Met at position 733 concurs with Kaldy et al., 1999, who showed that a mutation of F733 to M733 reduced, but did not eliminate, IRE binding activity. We did not study the affinity of the *M. sexta* protein for the human ferritin IRE as part of this work. However, our data suggest that comparison of the insect sequences with mammalian sequences might be helpful in determining the residues and tolerated substitutions of the IRP1 RNA binding site.

The loss of IRP1/IRE binding activity of fat body cytoplasmic extracts in the presence of reducing agents was surprising to us. It is improbable that *M. sexta* IRP1 requires either a disulfide bridge or a partial iron sulfur cluster to maintain conformational structure for IRE interaction because the binding activity of the recombinant *Manduca* IRP1 was not decreased by thiol reducing agent. Thus, we suggest there is another constituent of cytoplasmic extract, activated by this thiol reducing agent that interferes with IRP1 interaction with the IRE . Alternatively, reducing agent could render the IRP1 more susceptible to degradation. This seems less likely since our fat body extracts were

prepared in the presence of protease inhibitors and protein degradation was not observed for cytoplasmic extracts in subsequent experiments.

Fat body IRP1/IRE binding activity is reduced in insects injected with at low concentrations, and binding activity correlates with a significant increase in hemolymph ferritin. This occurs without a change in IRP1 expression. These data agree with findings in mammals that IRP1/IRE interaction is reduced by iron administration and ferritin synthesis is increased without a change in IRP1 expression (Tang et al., 1992, Chen et al., 1997, Hentze and Kuhn, 1996). An increase in ferritin levels in response to iron was also observed for mosquitoes (Pham et al., 1999, Dunkov et al., 1995). We have found that an iron sulfur cluster will form *in vitro* in the *Manduca* IRP1 (Gailer and Winzerling, unpublished). We have also found that the recombinant *Manduca* IRP1 suppresses *in vitro* synthesis of *M. sexta* ferritin subunits by specific interaction with the IRE (Zhang et al., 2000). This information, taken together, supports the hypothesis that *M. sexta* ferritin synthesis *in vivo* is controlled, in part, at the translational level by IRP1.

An important question that needs further evaluation concerns the mechanisms responsible for the absence of down-regulation of IRP1 binding activity and the stabilization of hemolymph ferritin at lowered levels in response to iron at high dosages. We do not know why this occurred. Since there was no significant change in IRP1 mRNA or protein levels, we can speculate that an oxidative response could be responsible for these observations. If we exceeded the capacity of these animals to rapidly bind and store

injected iron, ferric bound to low molecular mass compounds, such as citrate, would be available to generate an oxidative reaction. In mammals, reactive oxygen species increase IRP/IRE binding activity by mechanisms that are not well understood (Hentze and Kuhn, 1996, Hanson and Leibold, 1999, Pantopoulos and Hentze, 1998). In fact, a recent report shows that oxidative stress up-regulates transcription of ferritin message, while IRP1/IRE interaction down-regulates ferritin synthesis (Tsuji et al., 2000). If an oxidative response occurred here, simultaneous iron down-regulation and oxidative up-regulation of IRP1/IRE binding activity could account for the slight increase and plateau that we observed. Ferritin levels significantly declined and then stabilized at higher iron concentrations. We think it probable that ferritin synthesis and secretion continued to respond to iron, but that this response was attenuated by the plateau in IRP1/IRE binding activity. Alternatively, iron-loaded ferritin from hemolymph could have been moved to other tissues. The kinetic studies to evaluate this possibility are beyond the scope of the present work. However, if iron-loaded ferritin was removed from hemolymph in response to iron, we might have expected a dose-responsive fall in hemolymph ferritin rather than a plateau at high iron concentrations.

Late fifth instar larvae are in rapid growth. In order not to influence protein synthesis adversely, we allowed the insects to continue feeding and included a group of non-injected insects to evaluate the effect of injection. Water injection significantly increased IRP1 mRNA and significantly reduced hemolymph ferritin. IRP1 binding activity was increased, but did not reach significance. We think that injection of non-sterile water

probably elicited a mild immune response in these insects. The Dipterans immune response involves nitric oxide up-regulation (Luckhart et al., 1998), and nitric oxide synthase has been sequenced for both Dipterans (Luckhart and Rosenberg, 1999) and Lepidopterans (Nighorn et al., 1998). Work in mammalian cells shows that NO exposure results in an increase in IRP1 binding activity by disassembly of the iron sulfur cluster (Pantopoulos and Hentze, 1995, Wardrop et al., 2000). If an immune response was elicited here, the effects of iron dominated this response.

In summary, we have shown that *M. sexta* IRP1 has high identity and similarity with other proteins of the IRP1 family, and specifically binds to ferritin IRE transcripts. Expression of IRP1 by *M. sexta* fat body is not responsive to iron, whereas IRP1/IRE binding activity of fat body declines in response to iron administered at low iron concentrations, but increases with higher iron dosages. Ferritin levels of hemolymph correspond inversely to the IRP1/IRE binding activity. Our work raises many questions. What are the mechanisms responsible for the loss in IRPI binding activity in the presence of reducing agent? What are the mechanisms involved in the response of IRP1 and hemolymph ferritin to iron at higher dosages? Is IRP1 expressed by other insect tissues besides the fat body? Future studies on the effects of oxidants and infectious agents on the insect IRP1 and hemolymph ferritin will help to answer these questions.

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Figures and Tables

Table 1. ***Manduca sexta*** **IRP1 shows greatest similarity to proteins of the IRP1 family.**

Similarity / Identity	MIRP1	HIRP1	HIRP2	Dirp1a	Dirp1b	CIRP1	Pm-Aco
MIRP1		76.4	69.2	80.4	80.3	76.7	40.0
HIRP1	67.8		72.3	75.6	75.8	78.9	43.9
HIRP2	57.9	61.8		67.8	67.7	71.1	39.7
DIRP1A	73.3	68.0	57.1		89.7	75.6	42.1
DIRP1B	73.0	67.9	57.2	86.0		75.2	41.4
CIRP1	69.9	69.7	61.4	68.8	67.8		41.3
Pm-Aco	32.5	32.0	28.6	32.5	31.7	29.7	

Abbreviations are: *Manduca sexta* IRP1 (MIRP1), human IRP1 (HIRP1), human IRP2 (HIRP2), *Drosophila melanogaster* IRP1A (DIRP1A), *Drosophila melanogaster* IRP1B (DIRP1B), crayfish IRP1 (CIRP1) and porcine heart mitochondrial aconitase (Pm-Aco).

Table 1. Comparison of *Manduca sexta* IRP1 to other IRPs and pig heart mitochondrial aconitase. Upper-right: similarities. Bottom-left: Identities. mIRP1: Manduca IRP1; hIRP1: human IRP1; hIRP2: human IRP2; dIRP1A: Drosophila IRP1A; dIRP1B: Drosophila IRP1B; p-Co: pig heart mitochondrial aconitase.

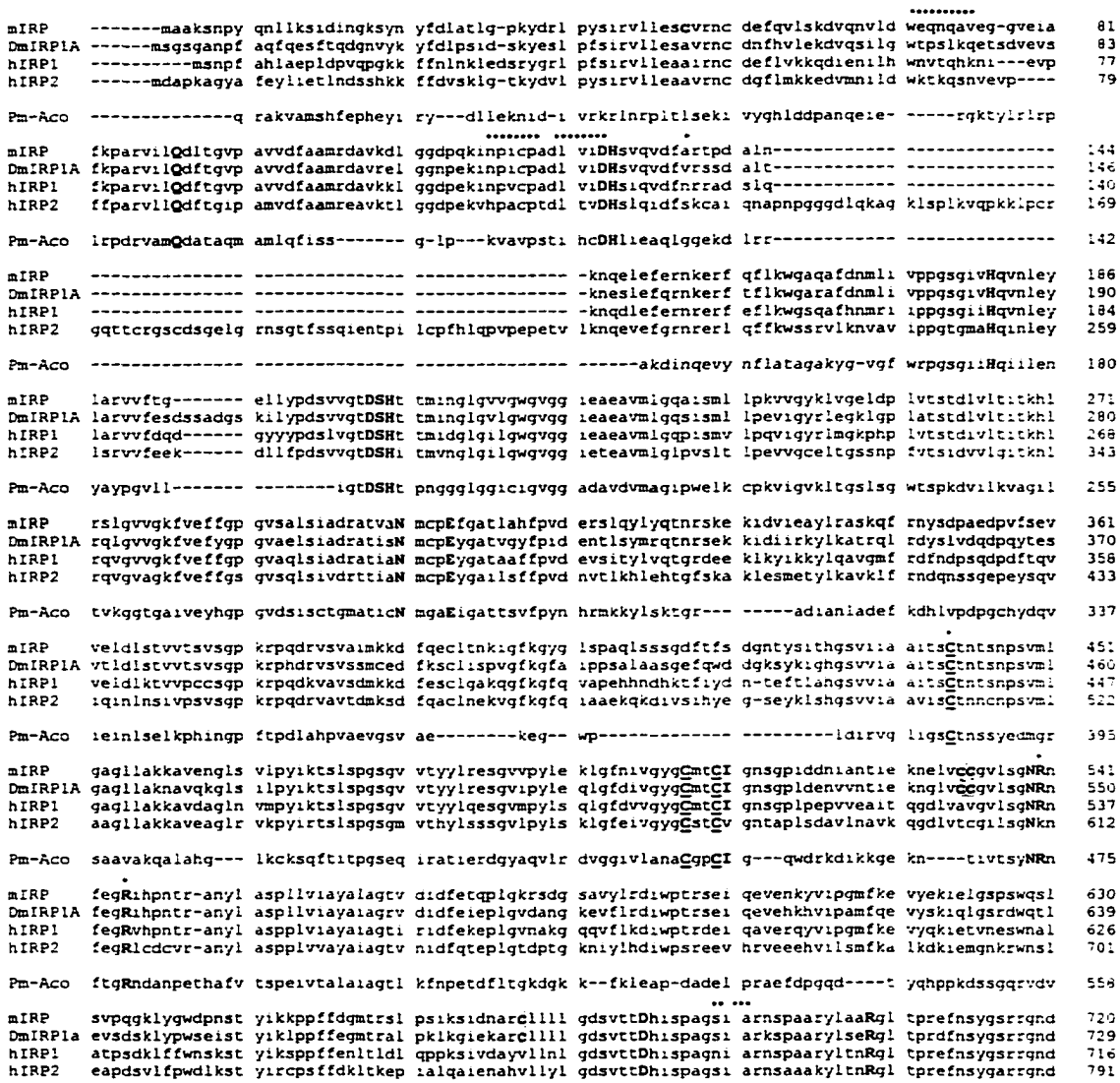


Fig. 1. Residues for iron sulfur cluster formation and aconitase activity and IRE binding are conserved for *Manduca* IRP1. Cys residues for formation of Fe-S cluster are uppercase, bolded and underlined. Amino acid residues necessary for aconitase activity are uppercase and bolded. Residues involved in IRP/IRE interaction are indicated by an asterisk. Abbreviations are *M. sexta* IRP1 (mIRP1), human IRP1 (hIRP1), human IRP2 (hIRP2), *Drosophila melanogaster* IRP1A (DmIRP1A) and porcine heart mitochondrial aconitase (Pm-Aco).

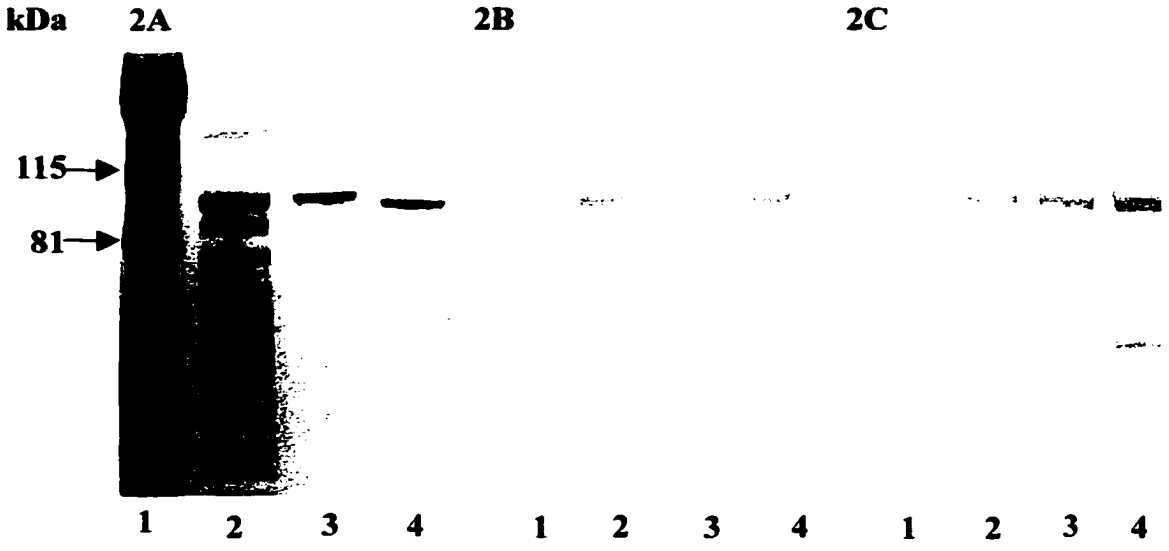


Fig. 2. Recombinant *Manduca sexta* IRP1 reacts with rabbit anti-rat IRP1-specific serum. **2A.** SDS-PAGE of lysate and recombinant IRP1. Recombinant *M. sexta* IRP1 was purified from bacterial lysate as described in the methods. Bacterial lysate (8.4 μg), recombinant MIRP (0.5 μg) or recombinant HIRP (0.5 μg) were separated by SDS-PAGE on a 10-20% gradient gel conducted as described and stained with Coomassie Blue. Molecular weight standards were used for relative size determination. Abbreviations are recombinant *Manduca* IRP1 (MIRP1) and human IRP1 (HIRP1). **2B.** Recombinant *M. sexta* IRP1 is identified by Penta-HIS antibody. SDS-PAGE and immunoblots were conducted as describe in the methods. **2C.** Recombinant *M. sexta* IRP1 is identified with rabbit anti-rat IRP1-specific serum. SDS-PAGE and immunoblots were conducted as describe in the methods.

3A

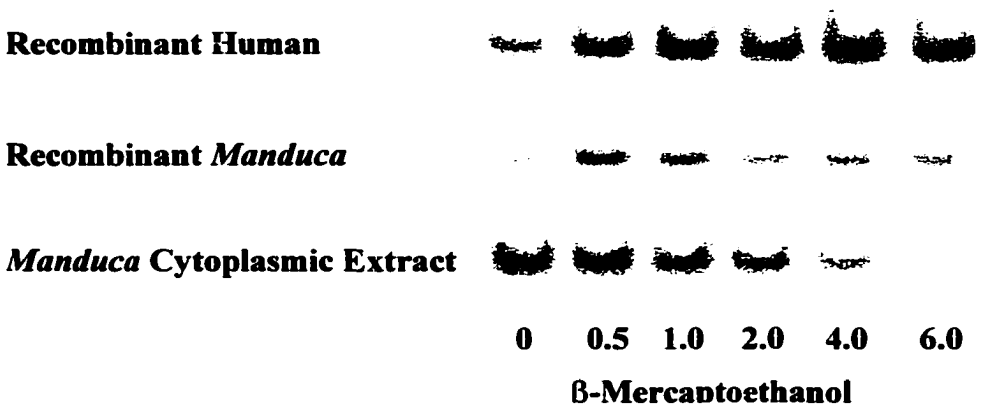
	1	2	3	4	5	6
mIRP1	-	+	+	+	+	+
lysate	+	-	-	-	-	-
mIRE*(50 fM)	+	+	+	+	+	+
mIRE (2500 fM)	-	-	+	-	-	-
mIRE (5000 fM)	-	-	-	+	-	-
mIRE (10000 fM)	-	-	-	-	+	-
nonspecific RNA	-	-	-	-	-	+

3B

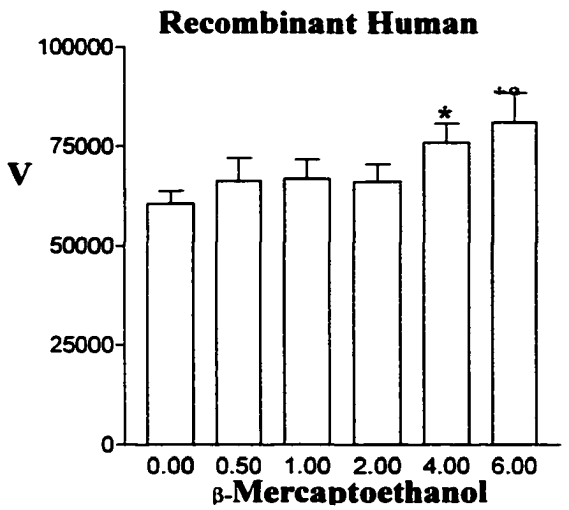
	1	2	3	4	5	6	7	8	9
hIRP1	+	+	+	+	+	+	-	-	-
mIRP1 -	-	-	-	-	-	+	+	+	
hIRE* (50 fM)	+	+	+	-	-	-	+	+	+
mIRE* (50 fM)	-	-	-	+	+	+	-	-	-
hIRE (5000 fM)	-	+	-	-	-	-	-	+	-
mIRE (5000 fM)	-	-	-	-	+	-	-	-	-
nonspecific RNA	-	-	+	-	-	+	-	-	+

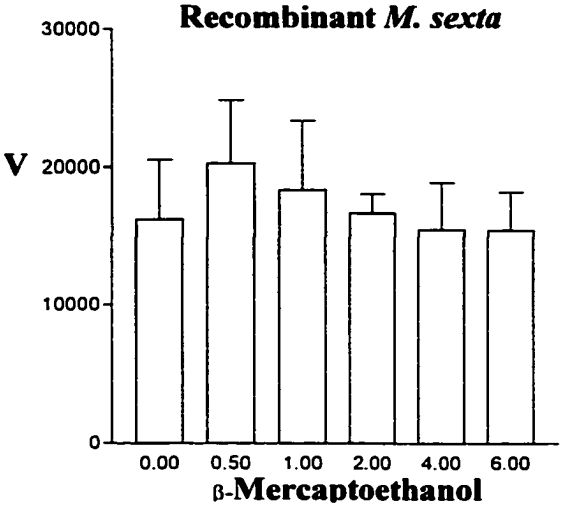
Fig. 3. Recombinant *Manduca* IRP1 binds specifically with ferritin IRE transcripts. **3A.** Recombinant *Manduca* IRP1 binds specifically *M. sexta* ferritin IRE transcripts. Reactions were conducted using recombinant *Manduca* IRP1(50 ng), [P^{32}]-labeled *Manduca* IRE transcript, in the presence of increasing concentrations of trace-labeled *Manduca* IRE transcript or nonspecific RNA (RNA ladder, Stratagene) and evaluated by electrophoretic mobility shift assay (EMSA) as described in the methods. Lane 1, bacterial lysate without *Manduca* IRP1 plasmid (negative control), and lanes 2-5, competition assays with increasing concentrations 0, 50, 100 and 200-fold excess of *Manduca* IRE transcript, respectively, and lane 6, nonspecific RNA. **3B.** Recombinant *Manduca* IRP1 binds human ferritin IRE transcripts. Reactions were conducted and EMSA was done as described in the methods. Lanes 1, human IRP1/human IRE, lane 2, human IRP1/human IRE and 100-fold trace-labeled human IRE, lane 3, human IRP1/human IRE and nonspecific RNA, lanes 4, human IRP1/*Manduca* IRE, lane 5, human IRP1/*Manduca* IRE and 100-fold trace-labeled *Manduca* IRE, lane 6, human IRP1/*Manduca* IRE and nonspecific RNA, lane 7, *Manduca* IRP1/Human IRE, lane 8, *Manduca* IRP1/Human IRE and 100-fold excess human IRE, lane 9 *Manduca* IRP1/Human IRE and nonspecific RNA. *50 fM of [^{32}P]-labeled transcript.

4A


Recombinant Human
Recombinant *Manduca*
Manduca Cytoplasmic Extract
0
0.5
1.0
2.0
4.0
6.0
β-Mercaptoethanol

4B


Recombinant Human
V
100000
75000
50000
25000
0
0.00
0.50
1.00
2.00
4.00
6.00
β-Mercaptoethanol


Recombinant *M. sexta*
V
30000
20000
10000
0
0.00
0.50
1.00
2.00
4.00
6.00
β-Mercaptoethanol

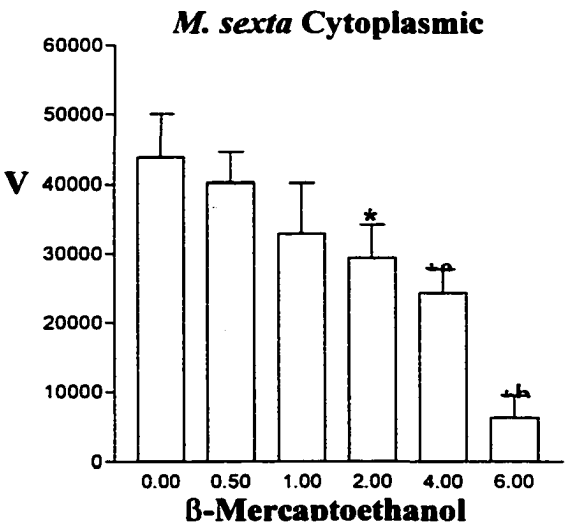

M. sexta Cytoplasmic
V
60000
50000
40000
30000
20000
10000
0
0.00
0.50
1.00
2.00
4.00
6.00
β-Mercaptoethanol

Fig. 4. β-Mercaptoethanol reduces *M. sexta* fat body IRP/IRE interaction.

Reactions of recombinant human IRP1 (50 ng), recombinant *Manduca* IRP1 (50 ng) or 5th instar *M. sexta* fat body cytoplasmic extract (15 ug of total protein) with radio-labeled ferritin IRE transcripts of the same species in the presence of increasing concentrations of ∃-mercaptoethanol (0.065, 0.13, 0.26, 0.52 and 0.78 M) were evaluated by EMSA as described in the methods. Representative panels of each assay are shown at the top of each graph. Quantitative results represent triplicate experiments conducted at the same time under the same conditions and shifts from each experiments were scanned by a laser densitometer as described in the methods. Data are shown as means ± S.D. * = significantly different from the control (0 M β-mercaptoethanol) $p<0.05$. **a** = significantly different from control $p<0.01$. **b** = significantly different from control $p<0.001$.

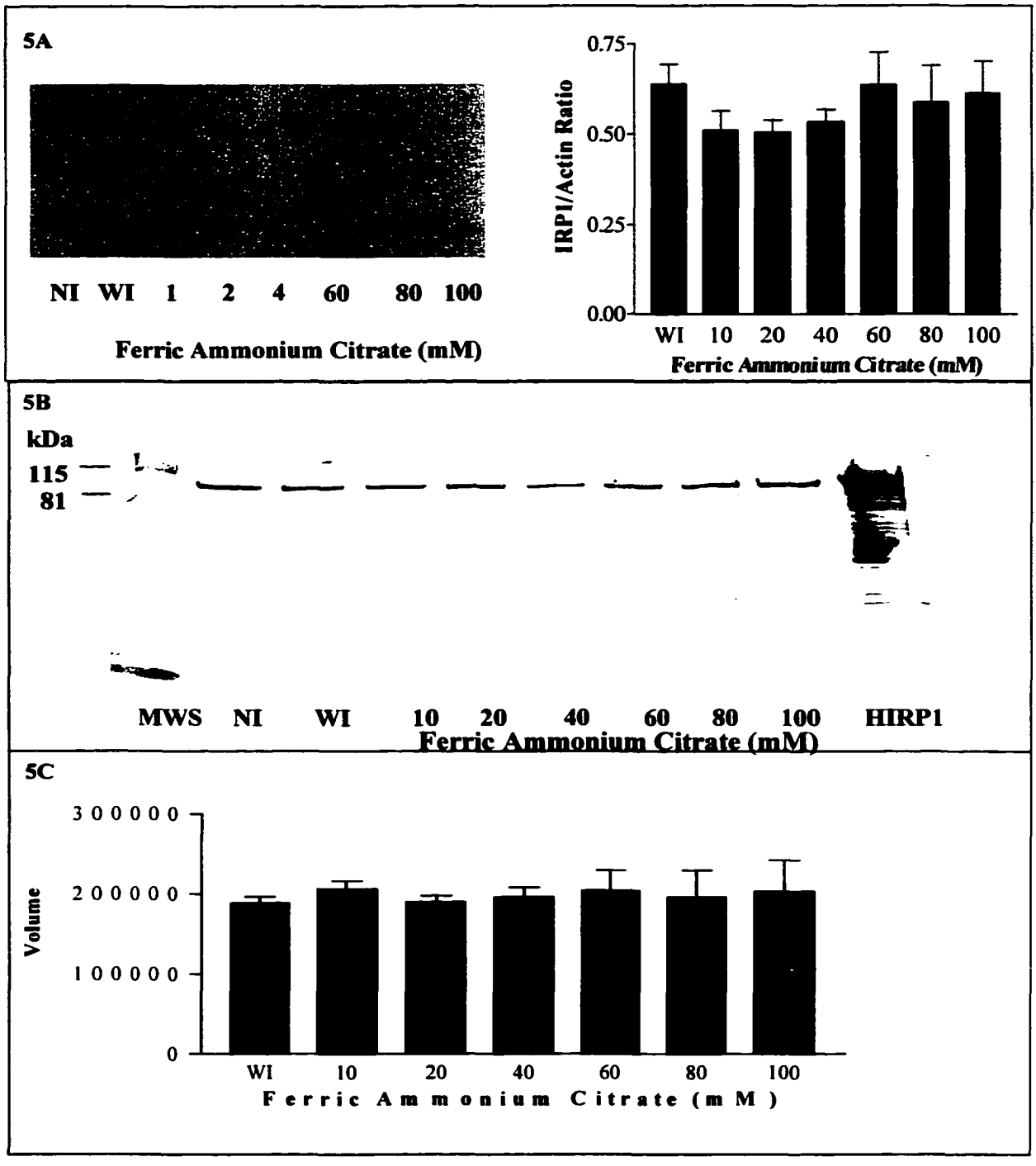

5A
NI WI 1 2 4 60 80 100
Ferric Ammonium Citrate (mM)
IRP1/Actin Ratio
0.75
0.50
0.25
0.00
WI 10 20 40 60 80 100
Ferric Ammonium Citrate (mM)
5B
kDa
115
81
MWS NI WI 10 20 40 60 80 100 HIRP1
Ferric Ammonium Citrate (mM)
5C
Volume
300000
200000
100000
0
WI 10 20 40 60 80 100
Ferric Ammonium Citrate (mM)

Fig. 5. *M. sexta* fat body IRP expression is not responsive to iron administration. Either 10 µl of water or 10 µl of FAC solution of different concentrations as designated (10-100 mM) was injected into fourth-day, 5th instar *M. sexta* larvae; the insects were allowed to continue feeding for 16 hrs. Fat body was dissected and prepared as described in the methods. Each group represents tissues of 7 insects. **5A.** *Manduca sexta* IRP1 mRNA remains constant after injection of iron. Total RNA was purified and quantitative RT-PCR was conducted as described in the Methods. The reaction aliquots were run on an agarose gel as described and quantified by laser densitometry. **5B.** *Manduca sexta* fat body IRP1 remains constant with iron administration. Immunoblot was done as described in the methods. Panels above the graphs show a representative assay; graphs represent analysis of triplicates. Data are shown as means $\pm$ S.D. Abbreviations are: molecular weight standards (MWS), not injected (NI), water injected (WI), and mM FAC concentration (10-100). * = significantly different from not injected at $p < 0.05$.

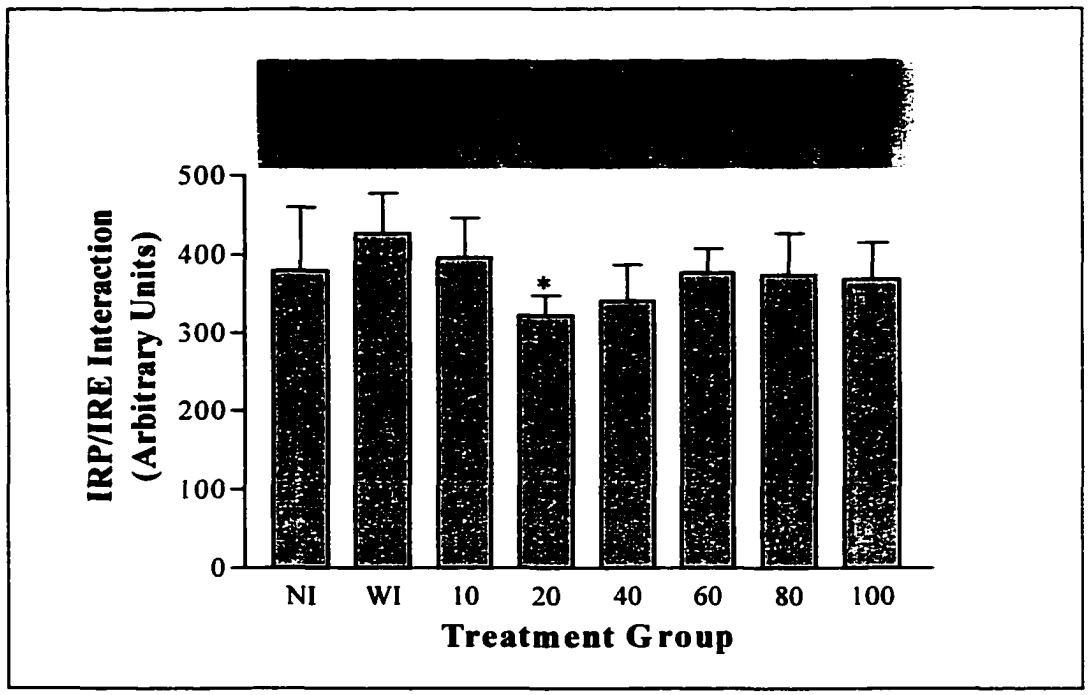


Fig. 6. *M. sexta* fat body IRP1/IRE binding activity is reduced by iron administration. Either 10 μl of water or 10 μl of FAC solution of different concentrations as designated was injected into fourth-day, 5th instar *M. sexta* larvae and the insects were allowed to continue feeding for 16 hrs. Fat body was dissected and prepared as described in the methods. Cytoplasmic extract from fat body was prepared and EMSA was conducted as described in the methods. Each treatment group represents 7 insects. Upper panel is a representative shift assay; graph represents analysis of triplicates. Data are shown as means ± S.D. Abbreviations are: not injected (NI), water injected (WI), and mM FAC concentration (10-100). * = significantly different from water injected $p<0.05$.

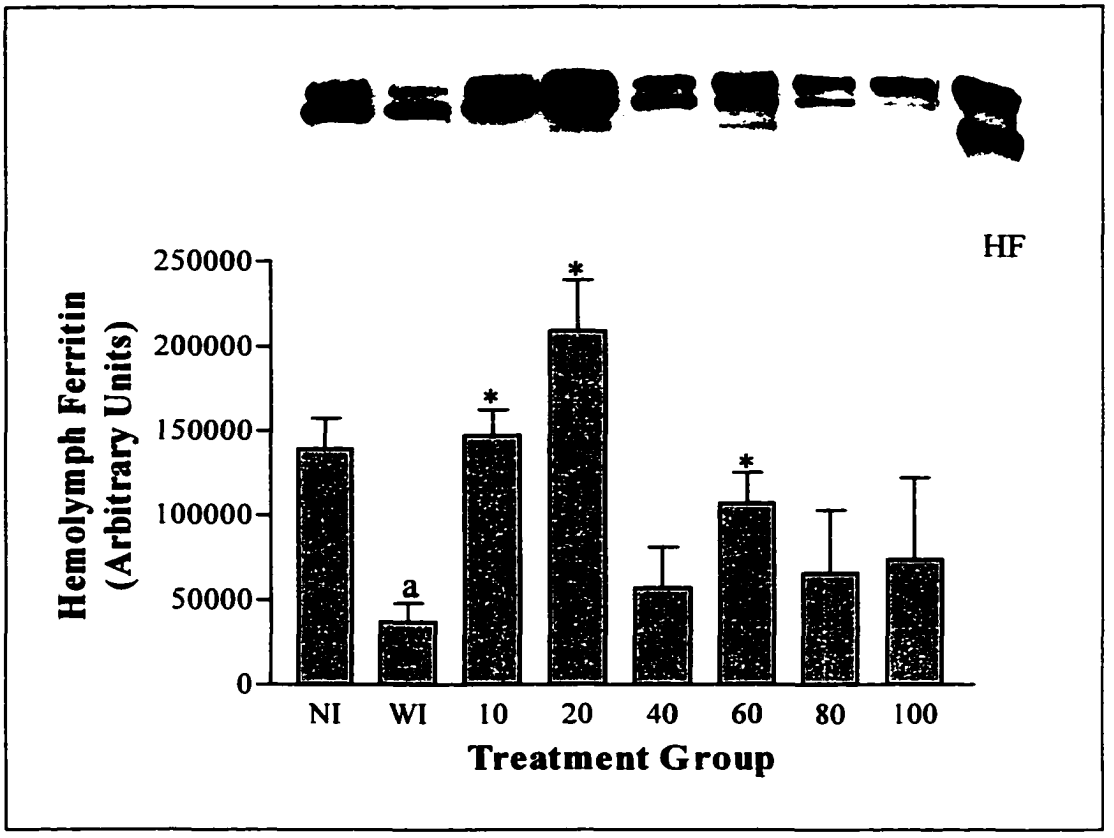


Fig. 7. *M. sexta* hemolymph ferritin is responsive to iron administration. Either 10 µl of water or 10 µl of FAC solution of different concentrations as designated was injected into fourth-day, 5th instar *M. sexta* larvae and the insects were allowed to continue feeding for 16 hrs. Hemolymph was collected as described in the methods. Hemolymph proteins were separated on a 10-20% gradient Mini gel (BioRad) and immunoblot was conducted as described in the methods . Each treatment group represents 7 insects. Upper panel is a representative immunoblot; graph represents analysis of triplicates. Data are shown as means ± S.D. Abbreviations are: not injected (NI), water injected (WI), mM FAC concentration (10-100) and purified hemolymph ferritin (HF, positive control). * = significantly different from water injected $p<0.05$. **a** = significantly different from not injected $p<0.05$.

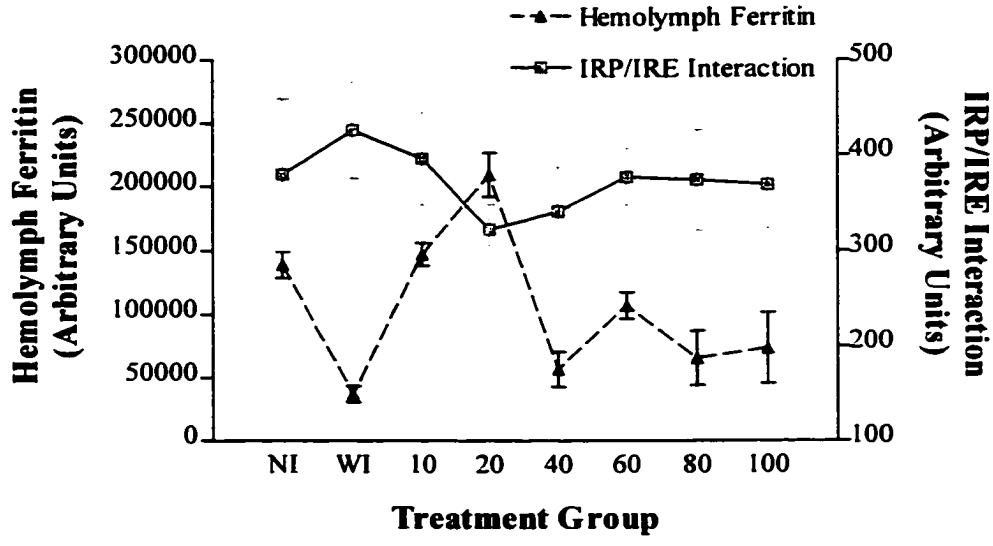


Fig. 8. Hemolymph ferritin is inversely related to fat body IRP1/IRE binding activity in response to iron administration. Hemolymph ferritin was assessed by immunoblot (see Legend Fig.7) and IRP1/IRE interaction was evaluated by EMSA (see Legend Fig. 6). Abbreviations are: not injected (NI), water injected (WI), and mM FAC concentration (10-100).

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APPENDIX B. REPRESSION OF *MANDUCA SEXTA* FERRITIN

SYNTHESIS BY IRP1/IRE INTERACTION

(Accepted by ***Insect Molecular Biology*** for publication)

Repression of Manduca sexta ferritin synthesis by IRP1/IRE Interaction

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Running Title: ***IRP1 represses M. sexta ferritin translation***

Keywords: *Manduca sexta*, ferritin synthesis, IRE, IRP1, translational control, iron

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Abstract

Mammalian ferritin subunit synthesis is controlled at the translational level by iron regulatory protein 1 (IRP1)/iron responsive element (IRE) interaction. Insect hemolymph ferritin subunit messages have an IRE in the 5'-untranslated region (UTR). We show that recombinant *M. sexta* IRP1 represses the *in vitro* translation of both the heavy and light chain ferritin subunits from this species without altering transcription. Deletion of either the 5'-UTR or the IRE from the mRNA abolishes IRP1 repression. Our studies indicate that translational control of ferritin synthesis by IRP/IRE interaction could occur in insects in a manner similar to that of mammals. To our knowledge this is the first report of the control of insect ferritin synthesis by IRP1/IRE interaction. Further, this is the first indication that synthesis of a secreted ferritin subunit also can be controlled in this manner.

1. Introduction

All living organisms require iron. In vertebrates, intracellular iron is stored in ferritin (reviewed by Rouault & Klausner, 1997; Eisenstein & Blemings, 1998; Aisen *et al.*, 1999). Ferritin protects cells from the potential toxic effects of free iron and makes iron accessible when required. Vertebrates synthesize ferritin in response to increased intracellular iron, as well as during inflammation and infection (reviewed by Weiss *et al.*, 1995; Hanson & Leibold, 1999). Because ferritin plays a vital role in iron metabolism and responds to inflammation and infection, we are studying translational control of ferritin synthesis in insects.

Mammalian ferritin is composed of 24 heavy (~21 kDa) and light chain (~19 kDa) subunits configured as a hollow sphere (Harrison & Arosio, 1996; Chasteen & Harrison, 1999). Subunit synthesis is controlled at the translational level by the binding of the iron regulatory proteins, IRP1 and IRP2, to an iron responsive element (IRE) found in the 5'-untranslated region (UTR) of the subunit mRNA (Hentze & Kuhn, 1996; Eisenstein & Blemings, 1998). When intracellular iron is low, IRP binding to the IRE represses ferritin translation (Gray & Hentze, 1994; Muckenthaler *et al.*, 1998a). When intracellular iron concentration is increased, IRP2 is rapidly degraded. In contrast, IRP1 is converted to cytoplasmic aconitase by the formation of an iron sulfur cluster in the protein core and can no longer bind to the IRE. Thus, ferritin synthesis proceeds in response to increased iron availability.

Several insect ferritin subunits have been cloned and sequenced (Dunkov *et al.*, 1995; Charlesworth *et al.*, 1997; Lind *et al.*, 1998; Georgieva *et al.*, 1999) including two from *Calpodes ethlius* (Nichol & Locke, 1999) and one from *Manduca sexta* (Pham *et al.*, 1996). Many of these messages have an IRE in the 5'-UTR. In addition, IRP1 has been sequenced from *Drosophila melanogaster* (Muckenthaler *et al.*, 1998b), and we recently sequenced the IRP1 from *M. sexta* (Zhang *et al.*, 2001). The insect IRP1s are similar to and share epitopes with the mammalian IRP1s. Recombinant *M. sexta* IRP1 binds specifically to transcripts of either the human or *M. sexta* ferritin mRNA IRE. When *M. sexta* late 5^{th} instar larvae are exposed to iron, hemolymph ferritin increases and fat body IRP1 binding activity declines (Zhang *et al.*, 2001). An IRE also is present in the mRNA of *D. melanogaster* succinate dehydrogenase subunit b (SDhb, Kohler *et al.*, 1995; Melefors, 1996). This IRE is functional *in vivo* as the synthesis of SDhb is responsive to iron administration. These data, taken together, suggested to us that the IRE of *M. sexta* ferritin mRNA could be an active translational control site.

We wanted to know whether the *M. sexta* IRP1 would repress synthesis of heavy or light chain hemolymph ferritin subunits from this insect. The ferritin subunit we previously cloned from *M. sexta* is similar to the vertebrate ferritin light chain (LCH, Pham *et al.*, 1996). We now report the cloning and sequencing of a second ferritin subunit for *M. sexta* that is a homologue of the vertebrate ferritin heavy chain. We show that recombinant *M. sexta* IRP1 represses the *in vitro* translation of either subunit. Repression occurs only when the IRE is present. Deletion of either the 5'-UTR or the IRE from the

mRNA abolishes IRP1 repression. Our studies indicate that translational control of ferritin synthesis via IRP/IRE interaction could occur in insects in a manner similar to that of mammals.

2. Results

2.1. Cloning of the M. sexta ferritin heavy chain homologue (HCH)

The cDNA and deduced amino acid sequence for a second *M. sexta* ferritin subunit is shown in Fig. 1. The deduced amino acid sequence has a 20-residue signal sequence (underlined) followed by a mature protein of 22,003 daltons. The N-terminal of the predicted protein (bold-faced print) matches the amino terminal sequence of the 24-kDa hemolymph ferritin subunit (TQCHVPVNIQREWI, Winzerling *et al.*, 1995). Although work by others indicates that some lepidopteran hemolymph ferritin subunits are glycosylated (Nichol & Locke, 1989; Nichol & Locke, 1989; Ketola-Pirie, 1990; Winzerling *et al.*, 1995), computer analysis of our deduced amino acid sequence reveals no glycosylation sites (H. Nichol, personal communication).

Ferritin subunits have five α-helices and an internal loop, and the heavy chain subunits, when folded appropriately, form a ferroxidase site that allows ferrous to ferric conversion for iron uptake (Harrison & Arosio, 1996). The ratio of the heavy and light chains in ferritin depends on iron status; heavy chains predominate when rapid uptake of iron is desirable (Harrison *et al.*, 1998). Our *M. sexta* ferritin deduced amino acid sequence shows that the principle subunit structure is preserved (Fig. 2A) and the amino acid residues that constitute the ferroxidase center are conserved (Fig. 2A, bold-faced print, Harrison & Arosio, 1996). The higher mass of the insect subunit relative to mammalian ferritin heavy chain

subunits reflects additional residues found in the N-terminus and the internal loop. Comparative sequence analysis (Fig. 2B) shows that our subunit has greater identity to the human heavy chain (33%) that to the human light chain (25%), as well as substantial identity (48%) to the *Aedes aegypti* heavy chain homologue (HCH, Dunkov *et al.*, 1995). From these data, we conclude that we have sequenced the *M. sexta* homologue of the mammalian ferritin heavy chain. Although this is the smaller hemolymph ferritin subunit (24 kDa, Winzerling *et al.*, 1995), it has greatest similarity to the heavier subunits from mammals, as well as the conserved ferroxidase sites. For these reasons, we refer to this as a homologue of the vertebrate ferritin heavy chain. During the writing of this publication, we discovered that an EST (Assession number BE015464) was cloned from the antennae of female *M. sexta* moth that matches our cDNA sequence corresponding to nt 375-820.

Vertebrate IRE consensus structure is defined as a well conserved six-base loop, a five-base pair upper stem followed by a C or G bulge and a lower stem of at least five base pairs (Hentze & Kuhn, 1996). The CAGUGN bases of the loop and the bulge are required for IRP1 binding (Theil, 1994, Huang *et al.*, 1996). In addition, the total length of the stem, as well as the flanking regions, can influence IRP1/IRE interaction. The *M. sexta* HCH message has an IRE in the 5'-UTR (Fig. 1, double underlined). A comparison of the *M. sexta* HCH IRE with IREs from other ferritin messages shows that the consensus structure is preserved and the bases of the loop and the C bulge are conserved (Fig. 3, upper panel). The consensus IREs of the *M. sexta* HCH and LCH differ at the second

base pair of the upper stem (Fig. 3, lower panel) and the HCH IRE has a longer predicted stem based on nucleotide pairing than the LCH IRE. While the insect ferritin IREs are very well conserved, the *Drosophila* SDhb IRE differs suggesting that the affinity of the IRP1 for this IRE might vary from that of the ferritin IREs (Henderson *et al.*, 1996). In *Drosophila melanogaster*, the ferritin IRE can be removed from the HCH message by alternative splicing (Lind *et al.*, 1998; Georgieva *et al.*, 1999). Computer analysis of the *M. sexta* ferritin HCH 5'-UTR sequence reveals no apparent splicing sites in this region (H. Nichol, personal communication). The HCH mRNA of *C. ethlius* (Nichol & Locke, 1999) and *A. aegypti* (Dunkov *et al.*, 1995) also have no apparent splice sites in the 5'-UTR. These data suggest that control of ferritin synthesis differs among insects.

2.2. Translational repression of ferritin HCH and LCH by recombinant IRP1

We wanted to know whether the *in vitro* translation of *M. sexta* HCH ferritin subunits could be repressed by recombinant *M. sexta* IRP1. In order to answer this question, we evaluated the *in vitro* transcription/translation of the ferritin subunit cDNA in the presence of recombinant *M. sexta* IRP1. Recombinant *M. sexta* IRP1 was purified from *Escherichia coli* lysate (Zhang *et al.*, 2001) to >90% homogeneity (Fig. 4A) and identified by Penta-HIS monoclonal antibody (Fig. 4B). *In vitro* ferritin synthesis efficiency can vary with the translation system used (Theil, 1994; Paraskeva *et al.*, 1999). We used the wheat germ lysate system because it contains no endogenous IRP1 (Oliveira *et al.*, 1993) that reacts with ferritin IRE transcript and mRNA translates with equal efficiency

without regard to position of the IRE in the 5'-UTR (Paraskeva *et al.*, 1999). *In vitro* transcription/translation experiments were conducted using linear DNA constructs with the ferritin HCH or LCH clone containing the 5'-UTR and the full open reading frame (ORF) inserted downstream of a T7 promotor. These constructs were transcribed and translated in the presence or absence of the recombinant *M. sexta* IRP1. We differentiated transcription and translation products by labeling the transcribed message with [^{35}S]-CTP and the translated protein with [^{35}S]-methionine.

The results show that when the IRP1 is present, ferritin synthesis is repressed (Fig. 5A, lanes 1-6) without affecting transcription as indicated by the mRNA levels (Fig. 5A, lanes 7-12). Repression occurs for both subunits assayed separately (Fig. 5A, lanes 1-2, LCH; lanes 3-4, HCH) or together (Fig. 5A, lanes 5-6). When we removed the 5'-UTR from ferritin mRNA, the IRP1 failed to repress either LCH or HCH ferritin synthesis (Fig. 5B). We also found that translation of insect ferritin is repressed by recombinant human IRP1 (RNA Gel Shift Kit, MBI Fermentas, Amherst, NY). It appears that human IRP1 is more efficient at repressing translation of the insect ferritin mRNA than the *M. sexta* IRP1. This could reflect the fact that the *M. sexta* protein was not as pure as the commercial human recombinant IRP1 that was used as a positive control. Alternatively, the specific bases of the *M. sexta* IRE or the residues of the human IRP1 that differ from the *M. sexta* IRP1 could enhance human IRP1 binding affinity to the *M. sexta* IRE. Others have shown that alterations in the IRP1 residues or in the bases of the IRE can enhance the affinity of IRP1 for an IRE (Theil, 1994; Theil *et al.*, 1994; Kaldy *et al.*,

1999). In nature, it would seem important to the organism that the affinity of the IRP1 for the IRE be sufficient to permit translational control, yet allow release of the protein in the presence of iron. From these studies, we conclude that *in vitro* synthesis of both ferritin subunits is repressed by the *M. sexta* IRP1 by a translational mechanism that involves a site in the 5'-UTR of the mRNA. We hypothesize that the site of control is the IRE because the human IRP1 also repressed translation of the *M. sexta* HCH and LCH.

2.3. The IRE of the ferritin message is the site of IRP1 interaction and translational control

To determine whether the IRE was the site of repression by the *M. sexta* IRP1, we deleted the IRE from the 5'-UTR of the HCH clone and evaluated whether the IRP1 could repress translation of this message. The deleted sequence corresponded to the HCH IRE as shown in Fig. 3 (lower panel). *In vitro* transcription/translation experiments were conducted using the deletion clone in the presence or absence of the recombinant IRP1. Figure 6A shows that when the IRE is present in the 5'-UTR, translation repression occurs in response to the IRP1 concentration. In contrast, when the IRE has been removed, translation is not repressed by the recombinant IRP1. These data show that the IRE is the site responsible for *in vitro* translational repression by the IRP1, and that IRP1/IRE interaction is necessary for the repression of synthesis that we observed here. Most mRNA transcribed *in vivo* is capped. To evaluate whether capped message would

be repressed by IRP1/IRE interaction, we translated capped ferritin HCH mRNA in the presence of various concentrations of *Manduca* IRP1. As shown in Fig. 6B, when the IRE is present, IRP1 represses translation of capped ferritin HCH mRNA. In contrast, when the IRE is not present, repression does not occur.

3. Discussion

Insect iron metabolism has been reviewed (Locke & Nichol, 1992; Winzerling & Law, 1997). In Lepidopterans, intracellular ferritin is found primarily in the vacuolar system and is secreted into hemolymph (Nichol & Locke, 1989). Lepidopteran hemolymph ferritin occurs in relatively high concentrations and is increased (Zhang *et al.*, 2001) and loaded with iron in response to iron administration (Nichol & Locke, 1989; Nichol & Locke, 1990; Winzerling *et al.*, 1995). These findings are in contrast to those for vertebrates, where intracellular ferritin is mostly cytosolic, and secreted ferritin is found at very low concentrations in the blood and does not store iron (Linder *et al.*, 1996; M. Linder, personal communication).

Lepidopteran hemolymph ferritin is composed of several subunits (Nichol & Locke, 1989; Winzerling *et al.*, 1995). We have sequenced the cDNA for a second *M. sexta* hemolymph ferritin subunit. The deduced amino acid sequence contains a signal for secretion and the predicted matured protein has an N-terminal that matches that of the 24-kDa hemolymph subunit. The residues that constitute the active ferroxidase center of ferritin heavy chain subunits are conserved (Harrison & Arosio, 1996) and the matured protein shows greater similarity to mammalian heavy chains than to light chains. These data show that we have sequenced a homologue of the vertebrate ferritin heavy chain.

Our work shows that recombinant *M. sexta* IRP1 represses *in vitro* synthesis of either the

M. sexta HCH or LCH hemolymph ferritin subunit. *In vitro* repression occurs at translation, not transcription, and requires IRP1 interaction with an IRE that is found in the 5'-UTR of the subunit message. These findings agree with those for vertebrates where translation of ferritin is repressed *in vitro* and *in vivo* by IRP1/IRE interaction (Hentze & Kuhn, 1996; Eisenstein & Blemings, 1998). To our knowledge this is the first report of the control of ferritin subunit synthesis in an insect by IRP1/IRE interaction. It is also the first indication that synthesis of a secreted ferritin subunit is controlled by IRP1/IRE interaction in any species.

Although our studies clearly show that either human or insect IRP1 can repress translation of both subunits, repression is not complete. Others have noted that both the lysate system used and the concentration of translation initiation factors (Theil, 1994; Paraskeva *et al.*, 1999) influence *in vitro* repression efficiency. On the other hand, repression could be partial in insects allowing a constitutive synthesis of ferritin that is attenuated as needed by iron availability. Translational repression by IRP1/IRE interaction occurs by different mechanisms depending on the position of the IRE relative to the mRNA cap (Muckenthaler *et al.*, 1998a; Paraskeva *et al.*, 1999). When the IRE is within 60 nt of the mRNA cap, IRP1 binding to the IRE prevents small ribosomal subunit recruitment to the eIF4F complex, and thereby fully represses translation (Muckenthaler *et al.*, 1998a). In contrast, when the IRE is located at a greater distance from the cap, the ribosomal complex can attach to the mRNA and scan for a translational start codon; in this case, IRP1/IRE interaction results in partial repression of translation (Paraskeva *et al.*,

1999). The ferritin IRE of either LCH or HCH mRNA is >60 nt from the cap. Perhaps, a scanning or other mechanism exists in insects that permits partial translational repression of IRE-containing mRNA.

Is the IRE an active site of translational control of ferritin synthesis by the IRP1 *in vivo* in insects? Available evidence to date indicates this is the case. Both *M. sexta* ferritin messages have IREs in the 5'-UTR, and recombinant *M. sexta* IRP1 binds to transcripts of these IREs (Zhang *et al.*, 2001). IRP1/IRE interaction represses translation of both subunits *in vitro*. In addition, *M. sexta* hemolymph ferritin is increased in response to iron administration and correlates negatively with fat body IRP1/IRE binding activity (Zhang *et al.*, 2001). In Dipterans, ferritin synthesis also is responsive to iron administration and is subject to translational control (Dunkov *et al.*, 1995; Pham *et al.*, 1999) and the IRP1 is well conserved (Rothenberger *et al.*, 1990; Muckenthaler *et al.*, 1998b).

In summary, we have demonstrated translational repression of *in vitro* synthesis of *M. sexta* ferritin HCH or LCH, secreted ferritin subunits, by recombinant *M. sexta* IRP1. The IRE in the 5'UTR of the ferritin message is required for IRP1 mediated repression. Our work indicates that ferritin synthesis in insects is controlled at the translational level by IRP/IRE interaction in a manner similar to that seen in vertebrates, and that IRP1/IRE interaction could control the synthesis of secreted ferritin subunits.

4. Experimental Procedures

4.1. Cloning and sequencing of the M. sexta ferritin HCH

Two degenerate primers, 5'-GCIGCIG(A)C(A)IGAA(G)GAA(G)A(C)GIGAA(G)CA-3'(corresponding to nt 458-480) and 5'-T(C)TTA(G)TCA(G)AAIAA(G)A(G)AAT(C) TCICCIA-3' (corresponding to nt 801-823) were designed from the conserved amino acid sequences of ferritin heavy chain subunits. Reverse transcriptase-polymerase chain reaction (RT-PCR) was conducted using these primers with total RNA purified from *M. sexta* fat body as template. A ~360 bp fragment was purified (DNA and Gel Band Purification Kit, Amersham Pharmacia Biotech Inc), cloned (T/A Cloning Kit, Invitrogen, La Jolla, CA) and sequenced (Sequinase Kit, USB, Cleveland, OH). The fragment was labeled with digoxigenin (DIG, DNA Labeling Mix, Roche Laboratories) by PCR and used to screen an *M. sexta* 5^{th} instar lambda-ZAPII midgut cDNA library (a gracious gift from Dr. Michael A. Wells, University of Arizona). Positive clones were obtained and excised *in vivo* (Lambda-ZAPII cDNA Library, Stratagene). The clone with the longest insert was sequenced in both directions by automation (Macromolecular Facility, University of Arizona, Tucson, AZ), as well as sequenced manually (Sequinase, USB) and the results were compared. To determine whether there was more upstream sequence, 5'-RACE (Gibco BRL) was conducted using the *M. sexta* midgut cDNA library as template. This procedure yielded a product that extended the sequence by 10nt. The cDNA and deduced amino acid sequences were analyzed using Genetics Computer

Group (Devereux *et al.*, 1984) and database searches were done using BLAST program (Altschul *et al.*, 1990). Potential splicing sites were analyzed using Analysis Tools, (Berkeley *Drosophila* Genome Project, personal communication, www.fruitfly.org)

4.2. DNA constructs for in vitro transcription/translation

DNA templates for in vitro transcription/translation of the M. sexta ferritin subunits were generated using the following primers: ferritin HCH subunit sense primer: 5'-TAGGGCCCGTCAACACGAGATCCTTTTGTTATC-3' (MS5-total, ApaI site is underlined) and antisense primer: 5'-GCGATGGGCTACAATGCGCGG-3'(MS3-total) and ferritin LCH subunit sense primer: 5'-TTGGGCCCGGCTGTTTTTATCTCCGC-3' (163-4A) and antisense primer: 5'-CTGGTACCCGGCCTTTAGATTTTACAGCC-3'(163-4B, KpnI site is underlined). The G to C substitution (HCH sense primer, underlined and bold-faced print) was used to eliminate the out-of-frame start codon. PCR was conducted using these primers with the ferritin cDNA clones (Pham et al., 1996, LCH) with high fidelity polymerase, Pfu (Strategene). The product was purified (Amersham Pharmacia Biotech, Inc.) and cloned (T/A Cloning Kit). In order to remove a fragment of the vector that contained a start codon, the clones were digested with ApaI and the larger fragment containing the ferritin cDNA was circularized (Clone A and Clone B were named for the HCH and LCH template, respectively).

4.3. Deletion clones

To make DNA constructs without the 5'-UTR, specific primers corresponding to the open reading frames (Hch: 5'-AGGGGCCCATGAAGGCTATCCTGTTA-3') and (Lch: 5'-TCGGGCCCATGAACCCAATCACTTTC-3') were synthesized with restriction sites ApaI (underlined) added to their 5' ends. For the HCH subunit without 5'-UTR, PCR was conducted with primers Hch and MS3-total using Clone A as template, and for LCH subunit without 5'-UTR, primers Lch and 163-4B were used with Clone B as template. PCR products were cloned (T/A Cloning Kit) and digested with ApaI. Clones with the correct orientation were ligated and named as Clone C (HCH) and Clone D (LCH). The sequences were verified. To make the HCH subunit DNA construct with the IRE deleted (IRE(-)), two primers corresponding to the flanking regions of the IRE, 5'-CCAACA AATATGAAATAAAACG-3' (163-89A) and 5'-AGTACTGTTGTATAAATAACCC-3' (163-89B) were synthesized and PCR was done with primer pairs M13F/163-89A and M13R/163-89B using Clone A as template. The products were ligated and amplified using primers MS5-total and MS3-total. Appropriate PCR products were cloned. Clones with the correct orientation were digested with ApaI to remove the fragment between the T7 transcription promoter and insert. The correct sequence of the final product was verified and named HCH-IRE(-).

4.4. In vitro transcription/translation assays

In vitro transcription/translation assays of uncapped RNA were conducted using the

TNT-coupled Wheat Germ Extract System (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, equal amounts of the appropriate DNA template made linear by EcoRI digests were added to the transcription/translation reaction buffer. The RNA transcripts generated from the reaction were labeled with [^{35}S]-CTP (10 mCi/ml, 3000 Ci/mmol), the protein synthesized from the message(s) was labeled with [^{35}S]-methionine (10 mCi/ml, 1000Ci/mmol; Amersham Life Science Products, Arlington Heights, IL). Two μl of reaction from each assay were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 15% homogeneous gel); the gels were dried and exposed overnight to X-ray film (Custom X-ray Imaging Service, Inc., Phoenix, AZ).

4.5. Capped RNA for in vitro translation

Capped RNAs were transcribed from Clone A and the HCH-IRE(-) DNA templates using the RiboMax Large Scale RNA Production System (Promega) with the addition of Ribo m^{7}G Cap Analogue (Promega). Capped RNA was purified from the cap analogue according to the manufacturer's instructions and RNA concentration was determined by absorbance at λ_{260} . *In vitro* translations were conducted using Wheat Germ Extract (Promega) according to the manufacturer's instructions. Translation products were analyzed as described for the coupled transcription/translation reaction.

4.6. Expression and purification of the M. sexta IRP1 and human IRP1

The IRP1/pTrcHis2B clone was expressed in *E. coli* strain BL21-CodonPlus(DE3)-RIL cells as described elsewhere (Zhang *et al.*, 2001). The expression clone for the recombinant human IRP1 was a kind gift (Dr. M. Hentze, EMBL, Heidelberg, Germany). The purification of the recombinant human IRP1 or the recombinant *M. sexta* IRP1 was done using Ni^{2+}-NTA agarose (Qiagen, Germany) and Q Sepharose Fast Flow (Pharmacia Biotech, Piscataway, NJ) as described previously (Zhang *et al.*, 2001). Protein was evaluated using bovine serum albumin as a reference standard (Bradford, 1976).

4.7. SDS-PAGE and immunoblot assay for recombinant IRP1

Protein (0.5 μg/μl) was loaded onto 4-15% SDS-gradient Phast gels (Phamacia). Electrophoresis was conducted and the gels were silver-stained according to manufacturer's instructions (Pharmacia Biotech). For immunoblots, the proteins were transferred to nitrocellulose membrane (BioRad, Hercules, CA) using the Phast Immunoblot System according to the manufacturer's instructions (Pharmacia Biotech). Blots were washed, blocked and incubated with antibodies according to the manufacturer's instructions (QIAexpress Detection and Assay Handbook, 1999, Valencia, CA). Penta-HIS antibody was used at 1:500 dilution and the anti-mouse alkaline phosphatase conjugate antibody (Jackson ImmunoResearch Laboratories, West

Grove, PA) was used at a dilution of 1:2000. In addition, two 1 min washes in 100 mM NaCl, 5.0 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5 were done immediately before development. Blots were developed in alkaline phosphatase substrate (BioRad) until the bands were visualized. Low molecular weight standards (BioRad) were used for the SDS-PAGE, and 6xHIS Protein Ladder (Qiagen) was used as standards for the immunoblots.

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Figures

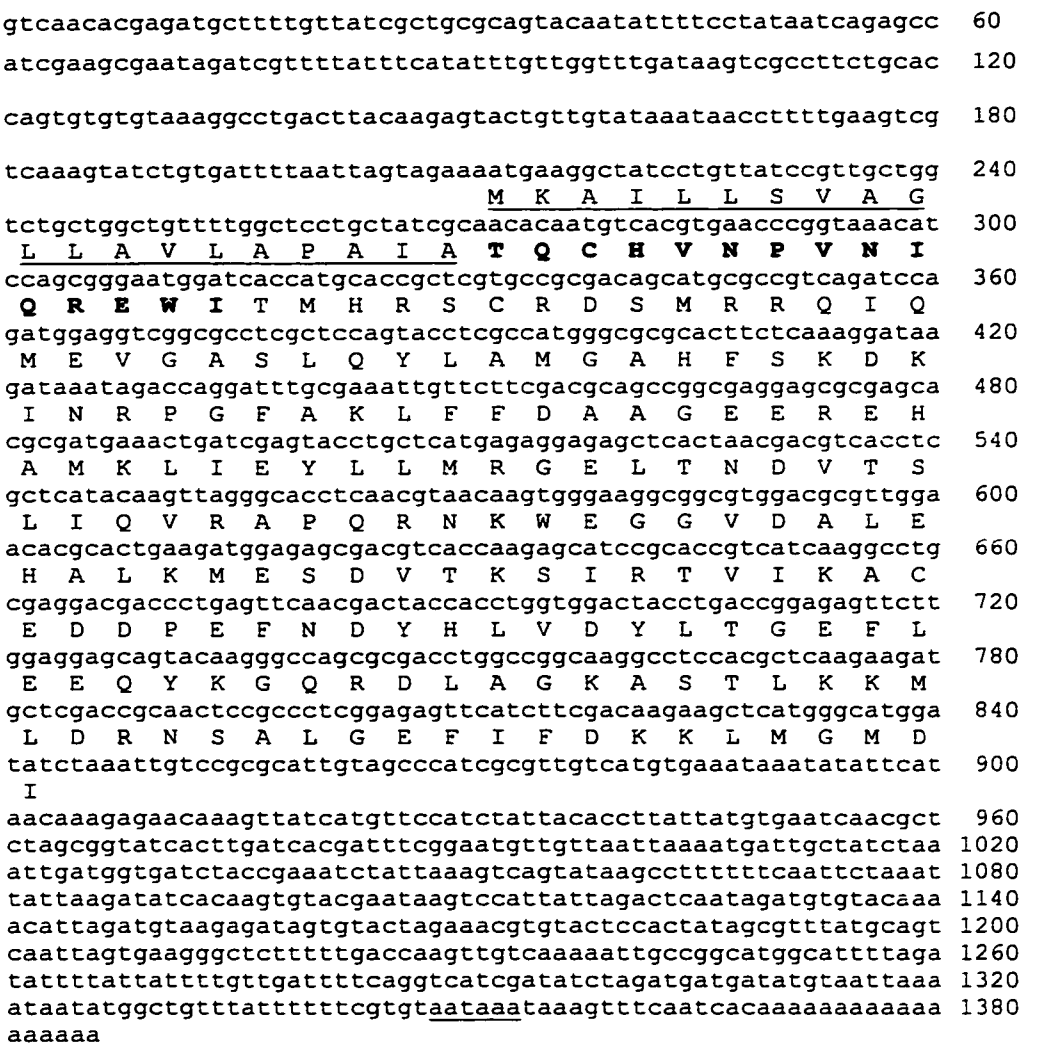

Figure 1. *Manduca sexta* ferritin heavy chain homologue cDNA and deduced amino acid sequence (Assession number AY032659). *M. sexta* total RNA was purified from 5th instar larvae and random primers were used for first strand cDNA synthesis, PCR was done using degenerate primers designed from the conserved residues of ferritin heavy chain sequences, and the PCR product was used to screen an *M. sexta* cDNA library as described in the methods. Positive clones were sequenced from two directions, and the 5'-UTR sequence also was evaluated by 5'-RACE as described in the methods. The IRE sequence is double underlined, the polyadenylation signal and the N-terminal leader sequence are underlined, and the N-terminal sequence of the matured HCH is bold-faced print.

2A

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                       A-helix                     B-helix
HumanL   ------------------------------------------MSSQIRQNYSTDVEAAV
HumanH   ---------------------------------------MTTASTSQVRQNYHQDSEAAI
MsHCH    ---------------MKAILLSVAGLLAVLAPAIATQCHVNPVNIQREWITMHRSCRDSM
AaHCH    ----------MMKSVFFGVVAITVAILSIYQETAQAQEQTVGATDNYQWDSVDDQCLAAL
MsLCH    MNPITFFVACLLALCGAVAADTCYQDVSLDCSQVSNSLTLPNCNAVYAEYGHHGNVAKEM

               A-helix                     B-helix
HumanL   NSLVNLYLQASYTYLSLGFYFDRDDVALEGVSHFFFELAEEKREGYERLLKMQNQRGGR-
HumanH   NRQINLELYASYVYLSMSYYFDRDDVALKNFAKYFLHQSHEEREHAEKLMKLQNQRGGR-
MsHCH    RRQIQMEVGASLQYLAMGAHFSKDKINRPGFAKLFFDAAGEEREHAMKLIEYLLMRGELT
AaHCH    HRQINKEFDASIIYLKYAAYFAQEKINLPGFEKFFFHAAAEEREHGIKLIEYALMRGKAP
MsLCH    QAYAALHLERSYEYLLSSSYFNNYQTNRAGFSKLFRKLSDDAWEKTIDLIKHITMRGDEM

             L-Loop               C-helix
HumanL   -----ALFQDIKKPAEDEWGKTPDAMKAAMALEKKLNQALLDLHALGSART----DPHLC
HumanH   -----IFLQDIKKPDCDDWESGLNAMECALHLEKNVNQSLLELHKLATDKN----DPHLC
MsHCH    --NDVTSLIQVRAPQRNKWEGGVDALEHALKMESDVTKSIRTVIKACEDDPE-FNDYHLV
AaHCH    --VDKHFKLNYDHEVPTVTTG-ESALETALQKEVEVTRSIRGVIKACEDGS---NDFHLA
MsLCH    NFAQRSTQKSVDRKNYTVELHELESLAKALDTQKELAERAFFIHREATRNSQHLHDPEVA

                D-helix                    E-helix
HumanL   DFLETHFLDEEVKLIKKMGDHLTNLHRLGGPEAG----LGEYLFERLTLKHD----  175
HumanH   DFIETHYLNEQVKAIKELGDHVTNLRKMGAPESG----LAEYLFDKHTLGDSDNES  183
MsHCH    DYLTGEFLEEQYKGQRDLAGKASTLKKMLDRNSA----LGEFIFDKKLMGMDI---  211
AaHCH    DYLTGEYLDEQHKGQRELAEKIATLKKMKKSAPK----LGEFLFDKNHM-------  209
MsLCH    QYLEEEFIEDHAKTIRNLAGHTTDLKRFVSGDNGQDLSLALYVFDEYLGKTV----  232
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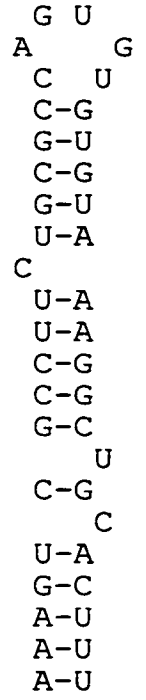
2B

% Similarity / % Identity	Human H	Human L	*Aedes* HCH	*Manduca* HCH	*Manduca* LCH
Human H	-	67.4	45.4	43.3	38.0
Human L	55.3	-	40.1	42.4	36.0
***Aedes* HCH**	36.3	26.2	-	56.9	34.0
***Manduca* HCH**	33.3	25.0	48.0	-	35.4
***Manduca* LCH**	28.0	25.0	25.7	25.4	-

Figure 2. Comparative analysis of ferritin subunits. **(2A)** Alignment of the *M. sexta* HCH ferritin subunit with subunits from other species. The N-terminal secretion signal sequence is bold-faced and underlined, and conserved residues for ferroxidase are bold-faced print. The five α-helixes are marked by lines above the sequence (Grossman *et al.*, 1992; Huang *et al.*, 1996). Abbreviations and references are: human ferritin light (L) and heavy (H) chain subunits (Boyd *et al.*, 1984; Boyd *et al.*, 1985), *Aedes aegypti* heavy chain homologue (*Aa*HCH, Dunkov *et al.*, 1995) and *Manduca sexta* light chain homologue (*Ms*LCH, Pham *et al.*, 1996). **(2B)** Pairwise identities and similarities of the ferritin subunits of different species.

***M. sexta* HCH**	GCCUU **C** UGCAC **CAGUGU** GUGUA AAGGC
***M. sexta* LCH**	GCCUU **C** UGCGC **CAGUGU** GUGUA AAGGC
***C. ethlius* HCH**	GCCUU **C** UGCGC **CAGUGU** GUGUA AAGGC
***D. melanogaster* HCH**	GCCUU **C** UGCGC **CAGUGU** GUGUA AAGGC
***A. aegypti* HCH**	ACCUU **C** UGUGC **CAGUGU** GUAUA AAGGU
***N. lugens* HCH**	GCCUU **C** UAUAC **CAGUGU** GUGUA AAGGC
***D. melanogaster* SDhb**	AAUUG **C** AAACG **CAGUGC** CGUUU CAAUU
Snail-S	UCUUG **C** UGCGU **CAGUGA** ACGUA CAGAC
Crayfish HCH	CUCCG **G** GUCGC **CAGUGU** GUGAA CGAGC
Human-H	UCCUG **C** UUCAA **CAGUGC** UUGGA CGGAA
Rana-H	UCUUG **C** UUCAA **CAGUGU** UUGAA CGGAA
Rat-H	UCCUG **C** UUCAA **CAGUGC** UUGAA CGGAA
Human-L	UCUUG **C** UUCAA **CAGUGU** UUGAC GAACA

***Ms*LCH**



***Ms*HCH**

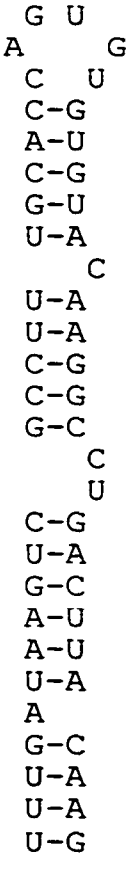


Figure 3. Alignment and comparison of the IREs from different species. Abbreviations and references are *Manduca sexta* heavy and light chain homologues (*Ms*HCH, *Ms*LCH respectively), *Calpodes ethlius* (*C. ethlius* HCH, Nichol & Locke, 1999), *Drosophila melanogaster* (*D. melanogaster* HCH, Charlesworth *et al.*, 1997), *Aedes aegypti* (*A. aegypti HCH,* Dunkov *et al*, 1995) succinate dehydrogenase subunit b (SDhb, Au & Scheffler, 1994), *Nilaparvata lugens* (*N. lugens* HCH, Du *et al.*, 2000), *Lymnaea stagnalis* soma ferritin (Snail S, Von Darl *et al.*, 1994), *Pacifastacus leniusculus* (crayfish HCH, Huang *et al.*, 1996), *Rana catesbeiana* ferritin heavy chain (Rana H, Didsbury *et al.*, 1986) and Rat H (Murray *et al.*, 1987).

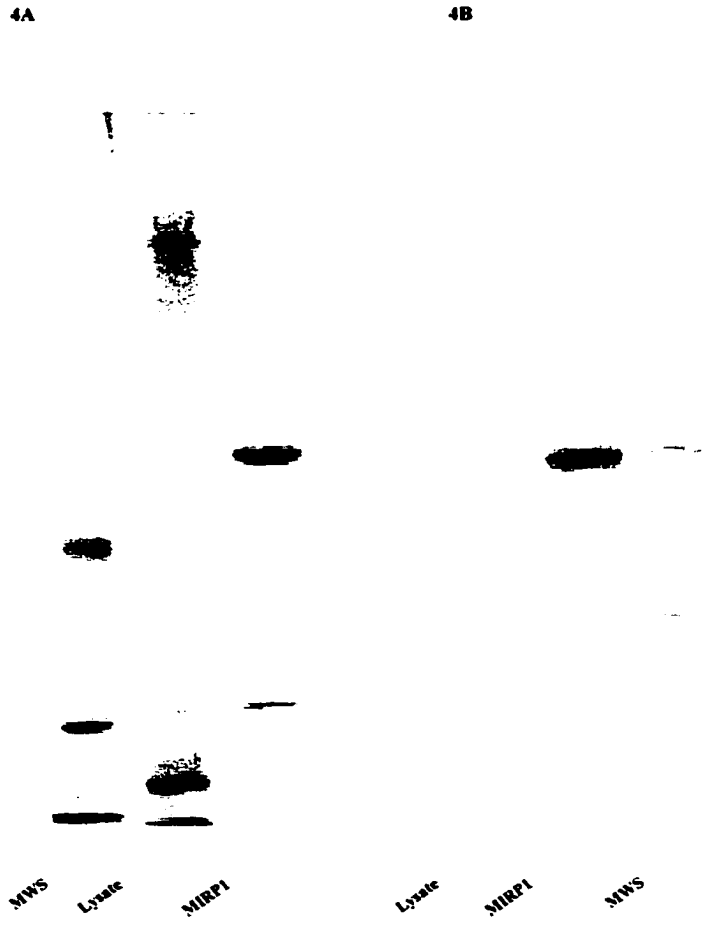


Figure 4. Purification and identification of recombinant *M. sexta* IRP1. **(4A)** Relative purity of recombinant *M. sexta* IRP1. Lysate and pooled fractions of partially purified M. sexta IRP1 were compared by SDS-PAGE on a 4-15% gradient Phast gel. Gel and silver stain were done according to the manufacturer's instructions (Pharmacia Biotech). **(4B)** Identification of the recombinant *M. sexta* IRP1 by monoclonal Penta-HIS antibody. Samples were separated on a 4-15% gradient Phast gel, transferred to nitocellulose membrane, incubated with monoclonal Penta-HIS antibody and developed using alkaline phosphatase as described in the methods. Abbreviations are molecular weight standards (MWS) and *Manduca* IRP1 (MIRP1).

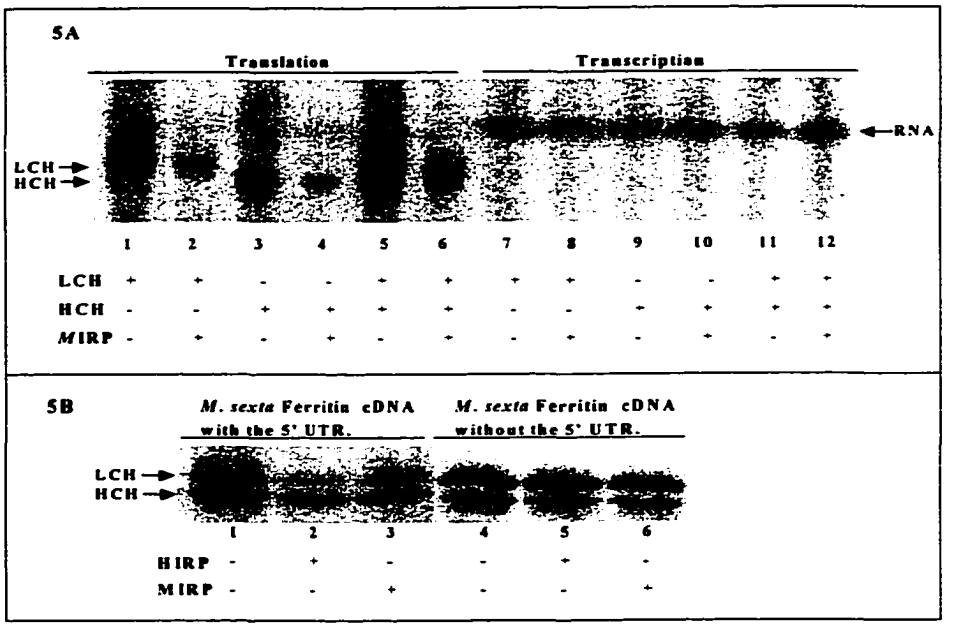


Figure 5. IRP1 represses translation of the M. sexta LCH and HCH. (5A) M. sexta ferritin HCH and LCH in vitro translation, but not transcription, is repressed by the IRP1. Constructs were prepared, the sequences were verified, and transcription/translation assays were conducted as described in the methods. Translated protein is labeled with [^{35}S]-methionine (10 mCi/ml, 1000Ci/mmol) and RNA is labeled with [^{35}S]-CTP (10 mCi/ml, 3000 Ci/mmol). Each lane represents 2 :l of reaction, separated by SDS-PAGE. Dried gels were developed by autoradiography. Template and the presence of IRP in the reaction are designated below the lane. (5B). In vitro translation of M. sexta ferritin in the absence of the 5'-UTR is not repressed by the IRP1.

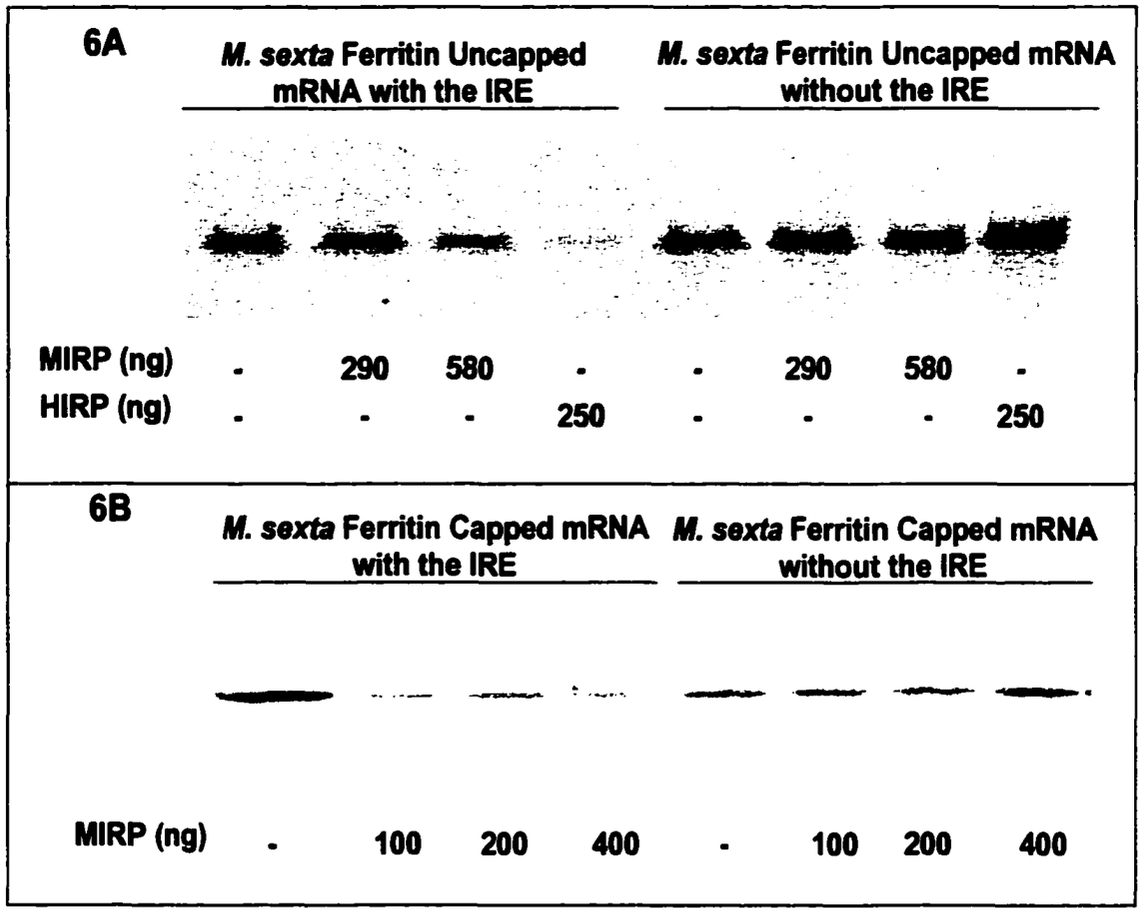


Figure 6. The IRE is the active site for IRP1 translational repression. (**6A**) IRP1 represses translation of uncapped RNA only when the IRE is present. An IRE (-) clone of the HCH subunit was constructed as described in the methods, the sequence was verified, and the reactions and gels were conducted as above. The amount of the IRP in the reaction is shown below the lanes. (**6B**) IRP1/IRE interaction represses translation of capped ferritin HCH RNA. Abbreviations are: heavy or light chain homologue (HCH or LCH), *M. sexta* IRP1 (MIRP)) and human IRP1 (HIRP).

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APPENDIX C. CLONING AND MOLECULAR CHARACTERIZATION OF TWO MOSQUITO IRON REGULATORY PROTEINS

(Accepted by ***Insect Biochemistry and Molecular Biology*** for publication)

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.

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, Dimopoulos et al., 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 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) (Weiss et al., 1995, Pantopoulos and Hentze, 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 *D. 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, Nichol and Locke, 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 also is 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'-GAGATCTGG(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 deduced 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 λ-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 λ-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

Anophles gambiae (Suakoko, 4a r/r) were raised at 28°C (75% humidity, 12-hr 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 Plasmodium 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 hr 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 hr (75% humidity, with a 12-hr light/dark cycle) before dissection and RNA extraction. The *A. gambiae* cell line Sua1B was challenged with various elicitors for 8 hr 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-[^{32}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 primers used were as follows: S7A, 5'-GGCGATCATCATCTACGT-3',

S7B 5'-GTAGCTGCTGCAAACTTCGG-3'; GNBPA, 5'-GCAACGAGAATCTGTACC -3'; GNBPB, 5'-TAACCACCAGCAACGAGG-3'; *A. gambiae* IRPA, 5'- GAAAGC TTGGGACTGACG-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 His6-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^{2+}-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 hr, and exposed to 1.0 mM IPTG for 5 hr. The cells were

collected by centrifuge (1800*g*), washed and stored at -20°C until the protein purification was conducted. Following lysis (Gray et al., 1993), the sample was loaded onto Ni^{2+}-NTA agarose (Qiagen) and eluted with 100 mM imidazole in 24 mM HEPES, pH 7.6, 150 mM KOAc, 1.5 mM $MgCl_2$, 0.5% glycerol. The fraction containing the recombinant IRP1 was adjusted to 10 mM imidazole with the same buffer and loaded onto Ni^{2+}-NTA agarose (1 ml bed volume). Recombinant IRP1 was eluted in a 10-100 mM imidazole gradient (40 bed volumes) in the same buffer. Fractions from 20-35 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 (Anoph.* IRE, unpublished). Double stranded DNA templates with the following sequences were synthesized (Gibco BRL, Grand Island, NY):

Aedes IRE template:5'-GAAAGCTTCGAGTCACCTTCTGTGCCAGTGTGTATAA AGGTTGACAACGGATCCCC-3', 3'-CTTTCGAAGCTCAGTGGAAGACACGGTCA

CACATATTTCCAACTGTTGCCTAGGGG-5'; *Anoph.* IRE template: 5'-GAAAGC TTAAGCTGTCGACCTTCTGTGCCAGTGCGTATAAAGGCCGGACAACTTGATCC CC-3', 3'-CTTTCGAATTCGACAGCTGGAAGACACGGTCACGCATATTTCCGGCCTG TTGAACTAGGGG-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 Fermantas, 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 α- [^{32}P]-CTP (56 μCi, > 3x10^{6} mCi/mmol; Amersham). Following transcription, transcripts were ethanol precipitated, suspended in DEPC-treated water, and quantified from α[^{32}P]-CTP incorporation. In order to quantify transcripts for competition assays, the transcripts were labeled with trace levels of α-[^{35}S]-CTP (0.01 μCi, 10 mCi/ml; Amersham). α-[^{35}S]-CTP incorporated at this concentration was not detectable by autoradiography for film exposed at -80°C for several days. The sequences of the four RNA transcripts were: human IRE: 5'-GGGAUCCUGCUUCAACAGUGCUUGGACG GAACC-3', *Aedes* IRE: 5'-CGAGUCACCUUCUGUGCCAGUGUGUAUAAAGGUGA CAACG-3', *Anoph.* IRE: 5'GAAAGCUUAAGCUGUCGACCUUCUGUGCCAGUGCGU

AUAAAGGCCGGACAACUUGAUC-3', and NSL: 5'-

GAAAGUCGCCUUCUGUGCCA

GUGUGUAUAAAGGCCACUUUC-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 hr 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 10.0-50.0x10^6 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 18,500*g* for 30 min at 4°C. The cytoplasmic extract (supernatant) was stored separately at –80°C until analysis by western blotting and EMSA were conducted. EMSA (Leibold and Munro, 1988) was done as a 20 µl reaction. Recombinant *A. aegypti* IRP1 (50 ng), recombinant human IRP1 (50 ng), or *A. gambiae* cytoplasmic extract (26 µg) was added to 16.9 mM HEPES, 0.84 mM $MgCl_2$, 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 volts for 2 hr 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 volts for 1 hr. 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 2 hr. using BLOTTO and probed for 1.5 hr. 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 hr. 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 Fermantas) 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 predicted 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 (Frishman and Hentze, 1996, Beinert et al., 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. 2A). 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 (Figs. 2B and 2C). Thus, IRP1/IRE recognition occurred between insect and mammalian RNAs and proteins. The specificity of binding was demonstrated by competition assays (Fig. 2C). Binding of the recombinant IRP1 to IRE-containing transcripts was reduced effectively by 25 to 100-fold excess of unlabelled 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.

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. 3A). Either undiluted blood meal or a blood meal diluted 50% with plasma also did not alter the messenger RNA levels by 12 hr or 24 hr post-feeding

(Fig. 3B). 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) GNBP marker (Fig. 3C). 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 4B). In contrast, EMSA results revealed that the binding activity of the *A. gambiae* IRP1 for *A. gambiae* IRE was upregulated following bacterial challenge (Fig. 4A).

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 also is 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 IRP 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, Frishman and Hentze, 1996, Beinert et al., 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 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 are 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 (Yu et al., 1992, Tang 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, Melefors, 1996, Gray et al., 1996). Synthesis 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 hypothesis.

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 up regulates 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, Pantopoulos et al., 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 also is 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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Figures

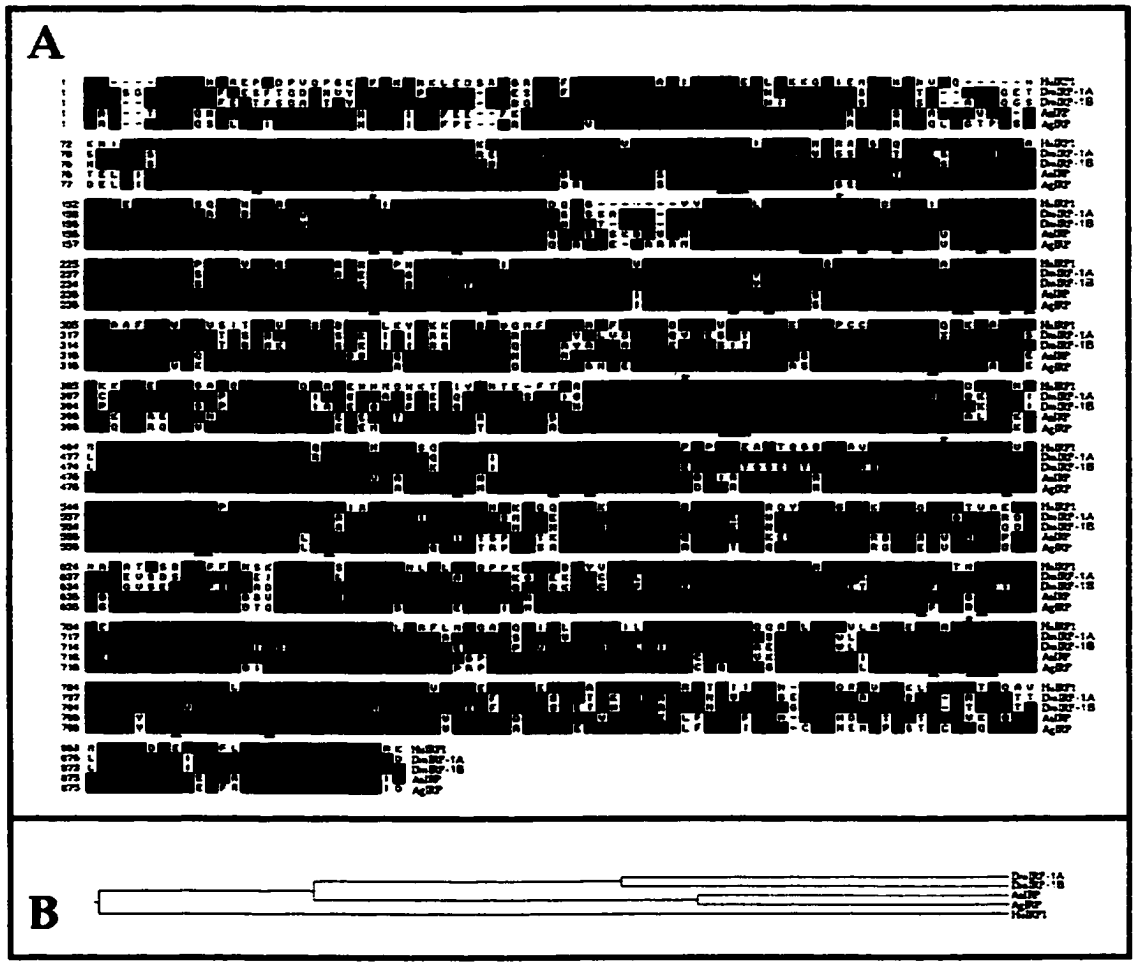

Fig. 1. Alignment and dendogram of deduced amino acid sequences. Protein sequences of the mosquito and other IRP1s were aligned using the BLAST program (GCG). A. Alignment of human (Hu) IRP1, *Drosophila* (Dm) IRP1a, *Drosophila* (Dm) IRP1b, *A. aegypti* (Aa) IRP1, and *A. gambiae* (Ag) IRP1. The amino 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 asteriscs. **B.** Neighbor-Joining tree of five IRP1s drawn by ClustalW.

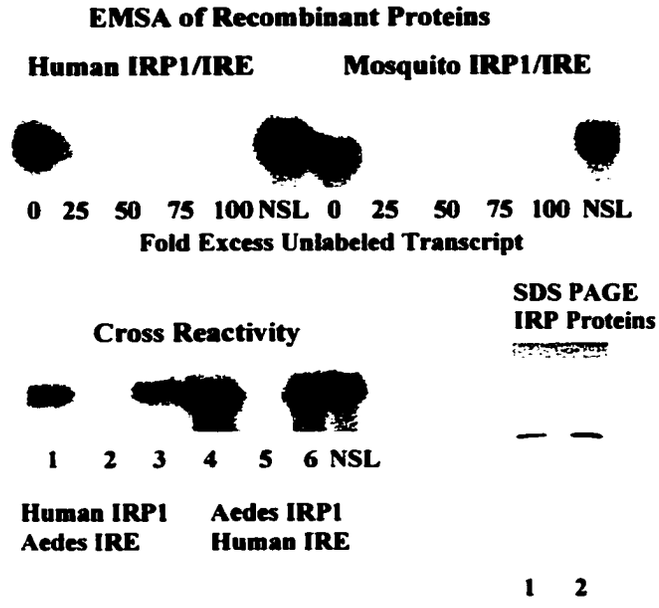

Fig. 2. Electrophoretic mobility shift assay of recombinant *Aedes aegypti* iron regulatory protein 1 (IRP1). Recombinant *A. aegypti* IRP1 or human IRP1 were over-expressed in *E. coli*, purified from cytoplasmic extracts and the purity evaluated by SDS-PAGE as described in the Methods. EMSA was conducted according to Leibold and Munro (1988) as adapted for the insect transcripts and described in the Methods. **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 specie: Lanes 1-3: human IRP1/[^{32}P]-*Aedes* IRE transcript analyzed alone (lane 1), with 100-fold excess trace labeled [^{35}S]-*Aedes* IRE transcript (lane 2), or 100-fold excess trace labeled [^{35}S]-NSL transcript (lane 3). Lanes 4-6: *Aedes* IRP1/[^{32}P]-human IRE transcript analyzed alone (lane 4), with 100-fold excess trace labeled [^{35}S]-human IRE transcript (lane 5), or with 100-fold excess trace labeled [^{35}S]-NSL transcript (lane 6). **C**: Human or *Aedes aegypti* recombinant protein (50 ng) and [^{32}P]-IRE transcripts (50 fmol) together with[^{35}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.

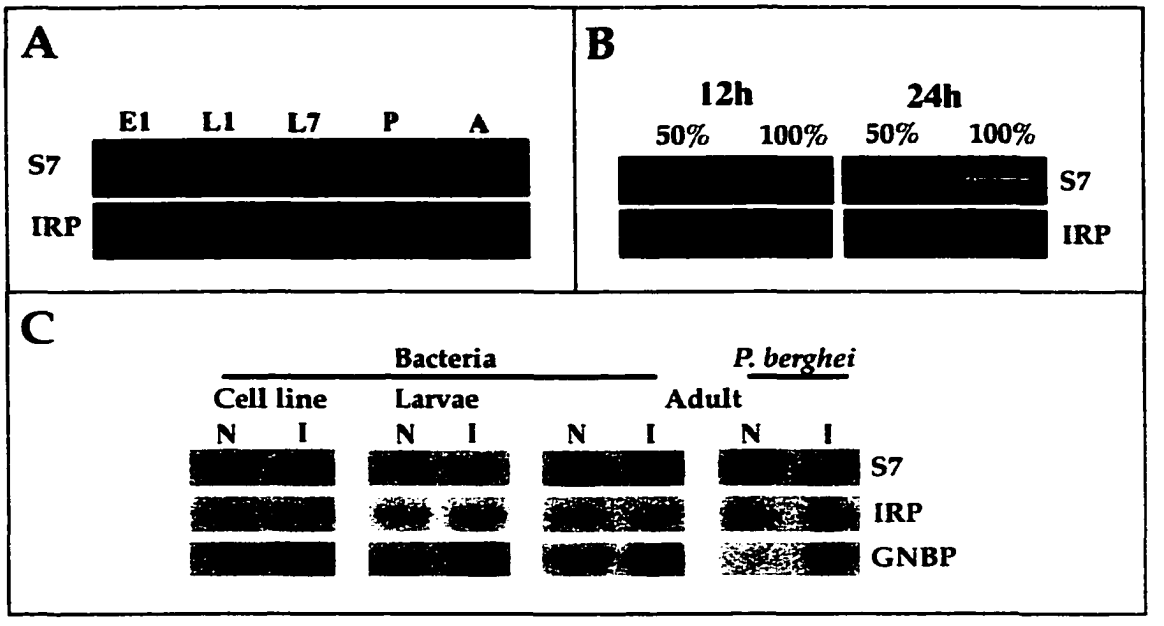

Fig. 3. Expression analysis of *A. gambiae* IRP mRNA levels by RT-PCR. Ribosomal protein S7 gene expression level was used as the normalization standard in the RT-PCR assay and GNBP gene expression level was used as a positive control for immune induction in the assays of both bacterial and parasitic 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 hr and 24 hr after blood feeding on 50% diluted (50%) and undiluted (100%) blood. **C**: the AgIRP message levels in naive (N) and bacterially challenged (I) cells, larvae and adults, and in *Plasmodium berghei* infected (I) adults 24 hr after feeding on an infected mouse.

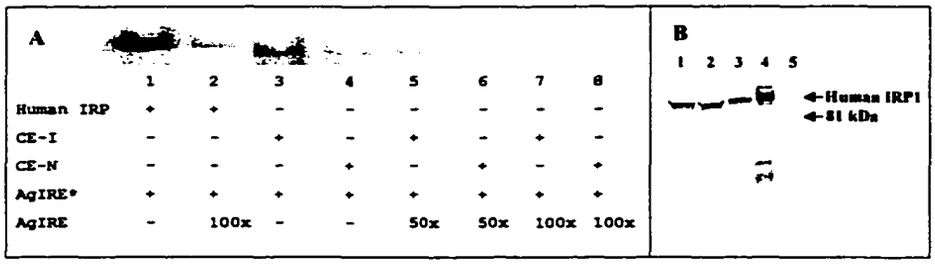

Fig. 4. IRP1 protein and binding activity of cytoplasmic extracts of infected *A. gambiae Sua1B* cells. A: Infection increases IRP1/IRE binding activity of mosquito cells. EMSA was conducted using cytoplasmic extracts prepared from naive (CE-N) or LPS treated (CE-I) *A. gambiae* Sua1B cells. Recombinant human IRP1 was used as a positive control. Samples were prepared with cytopasmic 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.

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