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Iron-regulatory protein-1 (IRP-1) is highly conserved in two invertebrate species
Characterization of IRP-1 homologues in *Drosophila melanogaster* and *Caenorhabditis elegans*

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Iron-regulatory protein-1 (IRP-1) plays a dual role as a regulatory RNA-binding protein and as a cytoplasmic aconitase. When bound to iron-responsive elements (IRE), IRP-1 post-transcriptionally regulates the expression of mRNAs involved in iron metabolism. IRP have been cloned from several vertebrate species. Using a degenerate-primer PCR strategy and the screening of data bases, we now identify the homologues of IRP-1 in two invertebrate species, *Drosophila melanogaster* and *Caenorhabditis elegans*. Comparative sequence analysis shows that these invertebrate IRP are closely related to vertebrate IRP, and that the amino acid residues that have been implicated in aconitase function are particularly highly conserved, suggesting that invertebrate IRP may function as cytoplasmic aconitases. Antibodies raised against recombinant human IRP-1 immunoprecipitate the *Drosophila* homologue expressed from the cloned cDNA. In contrast to vertebrates, two IRP-1 homologues (*Drosophila* IRP-1A and *Drosophila* IRP-1B), displaying 86% identity to each other, are expressed in *D. melanogaster*. Both of these homologues are distinct from vertebrate IRP-2. In contrast to the mammalian system where the two IRP (IRP-1 and IRP-2) are differentially expressed, *Drosophila* IRP-1A and *Drosophila* IRP-1B are not preferentially expressed in specific organs. The localization of *Drosophila* IRP-1A to position 94C1-8 and of *Drosophila* IRP-1B to position 86B3-6 on the right arm of chromosome 3 and the availability of an IRP-1 cDNA from *C. elegans* will facilitate a genetic analysis of the IRE/IRP system, thus opening a new avenue to explore this regulatory network.

Keywords: *Drosophila melanogaster*; *Caenorhabditis elegans*; iron-regulatory protein; RNA binding; iron regulation.

The post-transcriptional regulation of mRNAs involved in cellular iron metabolism by iron-regulatory proteins (IRP) and iron-responsive elements (IRE) is widely used in the animal kingdom. In mammals, where this system is best characterized, IRP control the translation of mRNAs for the iron-storage protein ferritin, the erythroid 5-aminolevulinate synthase a rate-limiting enzyme for the main iron-utilization pathway, and the mitochondrial aconitase, by binding to a single IRE located in the 5′ untranslated region (UTR) of the respective messages. IRP bound to multiple IRE in the 3′ UTR of the transferrin receptor mRNA stabilize the transcript, which encodes a critical receptor for cellular iron uptake [1−3].

Two IRP polypeptides (IRP-1 and IRP-2) have been identified in vertebrates and shown to be regulated by iron. IRP bind to IRE in iron-deficient but not in iron-replete cells. The regulation of IRP-1 and IRP-2 occurs post-translationally by distinct mechanisms. Striking similarities were discovered between IRP-1 and mitochondrial [4, 5] and bacterial aconitases [6, 7], which are Fe-S proteins that reversibly convert citrate to isocitrate. This finding led to experiments showing that IRP-1 is converted in iron-replete cultured cells into a cytoplasmic aconitase by insertion of a [4Fe-4S] cluster liganded to three highly conserved cysteine residues [8−10]. The [4Fe-4S] IRP-1 is inactive in IRE binding. Removal or loss of the Fe-S cluster converts the aconitase form into an RNA-binding protein. In contrast to IRP-1, IRP-2 does not exhibit aconitase activity in iron-loaded cells, but is specifically degraded. In iron-deficient cells, IRP-2 is stable and contributes to the total IRE-binding activity [7, 11]. In addition to iron, IRP-1 and IRP-2 are regulated by nitric oxide, and IRP-1 is also controlled by H_2O_2 [1].

In addition to vertebrates, IRE-binding activities have been detected in flies. Using electromobility-shift assays, specific complexes were identified in extracts prepared from a *Drosophila melanogaster* cell line [12] and *Drosophila* embryos [14]. Moreover, functional IRE have been identified in the mRNAs encoding a citric-acid-cycle enzyme, the Fe-S protein subunit of the *D. melanogaster* succinate dehydrogenase [13, 14] and in the *Drosophila* ferritin mRNA [15], suggesting that the IRE/IRP-mediated translational-control mechanism also operates in flies. Here, we report the cloning and characterization of invertebrate IRP, two *D. melanogaster* IRP-1 homologues with 87% sequence identity, and an IRP-1 homologue that was identified in the *Caenorhabditis elegans* genomic sequencing data base.

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Abbreviations. IRE, iron-responsive mRNA element; IRP, iron-regulatory protein; UTR, untranslated region.

Enzyme. Aconitate hydratase (EC 4.2.1.3).

Note. M. M. and N. G. contributed equally to this work.

Note. The nucleotide sequence data presented here have been submitted to the EBI database and are available under the accession numbers AJ223247 (*Drosophila* IRP-1A), AJ223248 (*Drosophila* IRP-1B) and Z66567 (*C. elegans* IRP).

MATERIALS AND METHODS

Degenerate primers, PCR and cDNA library screening. Degenerate primers were designed in conserved regions of IRP. The upstream primer (5′-GAGATCTGC$^{C}_{T}$GG$^{CC}_{TT}$T$^{CC}_{TT}$T$^{C}_{T}$GC$^{C}_{T}$A-AIAA$^{G}_{A}$GC$^{C}_{T}$GT-3′) introduces a synthetic *Bgl*II site at the 5′ end and corresponds to amino acid residues AGLLAKKAV in human IRP-1; the downstream primer (5′-CGAATTCGGICC$^{C}_{T}$TT-$^{C}_{T}$GC$^{C}_{T}$GCCCA$^{A}_{G}$TC-3′) corresponds to amino acid residues DWAAKGP in human IRP-1 and bears a synthetic *Eco*R1 site at the 5′ end. PCR amplification (94 °C for 2 min, then 35 cycles of 94 °C for 1 min, 55 °C for 90 s and 72 °C for 2 min, followed by 72 °C for 5 min) yielded a PCR product of the expected length of 1035 bp. The PCR product was excised from an agarose gel, subcloned in the presence of *Srf*I into a modified pBS+ vector where the polylinker had been replaced by an *Srf*I site. Multiple clones were sequenced.

Random-primed radioactive probes were prepared from the subcloned PCR fragments and used to screen a *D. melanogaster* embryonic λ-ZAP library (a kind gift from Steve Cohen, EMBL) and an adult *D. melanogaster* gt10 cDNA library (Clontech).

RNA analysis. Total RNA was prepared with the RNA-Clean system (Angewandte Gentechnologie Systeme GmbH) from adult flies. Poly(A)-enriched mRNA was prepared from total RNA with the Dynabeads mRNA Direct kit (Dynal) as described in the manual. 30 μg of total RNA were subjected to primer-extention analysis [16] using a synthetic oligonucleotide complementary to nucleotides +94 to +114 (relative to the ATG) of *Drosophila* IRP-1A that was end labelled ([γ-^{32}P] ATP, 5000Ci/mmol, Amersham). The primer-extention product was analysed on a 6% polyacrylamide gel in comparison with a sequencing reaction of the pQE vector (Qiagen) using the sequencing primer (5′-ATCACGAGGCCCTTTCG-3′) at position −82.

Approximately 2 μg poly(A)-enriched RNA were resolved electrophoretically in denaturing agarose gels and electrotransferred onto nylon membranes. The RNA was cross-linked to the membrane by means of ultraviolet light and hybridized [17] with a radiolabeled *Drosophila* IRP-1A probe that corresponds to the *Eco*RI/*Xho*I insert of the *Drosophila* IRP-1 A clone.

Cell-free translation and immunoprecipitation. *Drosophila* IRP-1A and IRP-1B cDNAs were transcribed *in vitro*. 100 ng of each capped mRNA was used to programme a standard 50-μl cell-free translation reaction containing 35 μl reticulocyte lysate (Promega), 1 U RNAsin (Promega), 20 μM amino acids (minus methionine) and 30 μCi [^{35}S]methionine (37 TBq/mmol, Amersham). The reactions were incubated at 30 °C for 60 min. 15 μl of each translation reaction were adjusted to 500 μl with buffer A (50 mM Tris/HCl, pH 7.4, 300 mM NaCl and 1% Triton X-100) and 20 μl anti-(human IRP-1) serum [18] or 20 μl preimmune serum were added; the reaction was incubated at 4 °C for 16 h. 50 μl protein-A-Sepharose (Pharmacia) were added and incubated for 2 h at 4 °C. The beads were washed twice with buffer A and once with 10 mM Tris/HCl pH 7.4, and the bound proteins were analyzed by SDS/PAGE and autoradiography.

Chromosome localization. Polytene chromosome spreads, *in situ* hybridizations using biotinylated probes synthesized from *Drosophila* IRP-1A and *Drosophila* IRP-1B, and determination of the map localization of the hybridization signal were carried out as described [19].

Collection and *in situ* hybridizaton of *Drosophila* embryos. After several rounds of precollection, embryos were collected for 2 h at 25 °C on apple juice plates, developed for up to 22 h and harvested. *In situ* labeling of RNA was performed essentially as described [20]. Ppu101-digested *Drosophila* IRP-1A and *Drosophila* IRP-1B plasmids were used as templates to synthesize digoxigenin-labeled antisense RNA probes, using the digoxigenin RNA-labeling-kit (Boehringer Mannheim). These probes only contain the 3′-UTR of *Drosophila* IRP. A *nubbin* probe was prepared as described in [21] and a *fuzhi-taratzu* probe as described in [22].

RESULTS

Cloning of two IRP from *D. melanogaster*. Degenerate primers were designed that correspond to conserved regions of IRP that are divergent from mitochondrial aconitases (see Materials and Methods; Fig. 1). The amplification of a cDNA library from *Drosophila* embryos yielded a PCR product of the expected size of 1035 bp, which was subcloned and sequenced. Ten clones were analyzed and two highly related IRP-like sequences (*Drosophila* IRP-1A and *Drosophila* IRP-1B) were detected (Fig. 1). Eight clones contained *Drosophila* IRP-1A, one clone *Drosophila* IRP-1B, and one clone a contaminating *Escherichia coli* aconitase (*acnA*) sequence, an enzyme displaying substantial similarity with eucaryotic IRP [6]. The PCR primers described here were used to amplify IRP-like cDNAs from *Lampetra fluviatilis* [22a], *Manduca sexta* (Winzerling, J., personal communication) and *Anopheles gambiae* (Smith, A. and Kafatos, F., personal communication).

Radiolabeled probes were prepared from the subcloned PCR fragments and used to screen a *Drosophila* embryonic λ-ZAP library and an adult *Drosophila* λgt10 cDNA library (Clontech). The complete cDNA sequence of *Drosophila* IRP-1A was obtained. Sequence analysis of 12 partially overlapping clones containing the *Drosophila* IRP-1A sequence revealed an ORF of 2718 nucleotides (Fig. 1) that was similar to known IRP coding regions. Primer-extention analysis of *Drosophila* IRP-1A mRNA suggests the presence of a short 5′ UTR of only 15 nucleotides (Fig. 2 A). Ten nucleotides of the 5′ UTR were found in the *Drosophila* IRP-1A cDNA (Fig. 1). The 3′ UTR is 113 bp long and contains a consensus polyadenylation hexanucleotide signal followed by a poly(A) tail (Fig. 1). The size of the hybridization signal obtained by probing a Northern blot with a radiolabeled *Drosophila* IRP-1A cDNA corresponds well to the expected length of 2830 bp for the entire cDNA of *Drosophila* IRP-1A (Fig. 2 B). Two additional clones of *Drosophila* IRP-1A, which are identical (with the exception of the 5′ UTR sequence, Fig. 1) to the *Drosophila* IRP-1A cloned by us, were subsequently identified in the *Drosophila* sequencing project data base [accession numbers LD13363 (AA438959) and LD13354 (AA438954)].

Drosophila IRP-1B, which we detected among the subcloned PCR fragments, was not found by screening the *Drosophila* embryo and adult cDNA libraries. However, a 5′ expressed sequence tag containing an IRP-like sequence [accession number LD06822 (AA263732)] is listed in the *Drosophila* genome project data base. LD06822 is different from our full-length *Drosophila* IRP-1A clone, and sequence analysis of the total LD06822 cDNA revealed that it is identical to the PCR fragment termed *Drosophila* IRP-1B. This *Drosophila* IRP-1B cDNA is approximately 2.9 kb in length (Fig. 1; including UTR) and the ORF *Drosophila* IRP-1A and 1B display 76% identity at the nucleic acid level. In contrast to the coding regions, the 3′ UTR of *Drosophila* IRP-1A and 1B differ in length and display little similarity with the exception of the sequences boxed in Fig. 1.

The finding that *Drosophila* IRP-1B is underrepresented among the IRP clones obtained by PCR (10%) in two cDNA libraries and in the *D. melanogaster* expressed-sequence-tag library (only one of four clones) suggests that *Drosophila* IRP-1B is less prevalent in the fly than *Drosophila* IRP-1A.

The two IRP homologues from *D. melanogaster* are highly related to vertebrate IRP-1. The two related IRP (IRP-1 and

Fig. 1. Sequence alignment of *D. melanogaster* IRP-1A and 1B. The cDNA sequence of *Drosophila* IRP-1A cloned from an adult *D. melanogaster* gt10 cDNA library (Clontech) is shown. Base substitutions in the coding region of *Drosophila* IRP-1B (LD 06822) are indicated; the 5′ and 3′ UTR of *Drosophila* IRP-1B are shown. Translation start and stop codons and the consensus polyadenylation hexanucleotide signal are underlined. A conserved region in the 3′ UTR is boxed. The locations of the degenerate PCR primers are indicated by arrows above the *Drosophila* IRP-1A sequence. The *Ppu*101 restriction sites used to linearize the templates to synthesize the 3′ UTR RNA probes for *in situ* hybridizations, and the sequences of the 5′ cDNA adapter downstream of *Eco*RI are indicated in bold letters. The *Drosophila* IRP-1A cDNA shown here differs from the *Drosophila* IRP-1A cDNAs (LD 13363 and LD 13354) listed in the *Drosophila* sequencing project data-base in the 5′ UTR sequence (5′-GCCACA-3′).

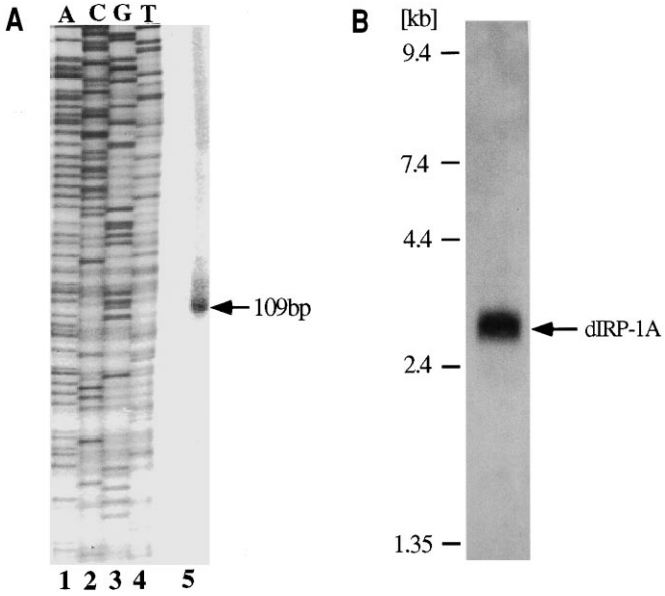

Fig. 2. mRNA analysis of *Drosophila* IRP-1A. (A) Primer extension analysis of *Drosophila* IRP-1A using a synthetic oligonucleotide complementary to nucleotides +94 to +114 (relative to the ATG) of *Drosophila* IRP-1A cDNA. The primer-extension product (109 bp; lane 5) was analyzed on a 6% polyacrylamide gel in comparison to a sequencing reaction (lanes 1−4). (B) Northern blot analysis of poly(A)-enriched RNA using a random-prime-labeled DNA probe specific for *Drosophila* IRP-1A. The size of *Drosophila* IRP-1A transcript (approximately 2.8 kb) was determined in comparison to a RNA ladder (GIBCO BRL); the fragment sizes are indicated on the left. Similar experiments yielded no specific signals with *Drosophila* IRP-1B clones (data not shown).

IRP-2) that have been identified in vertebrates are 57% identical at the amino acid level. The most distinctive differences between the two proteins are a 73-aa insertion in IRP-2, which is encoded by a separate exon and mediates protein degradation in iron-replete cells, and the substitution of several aconitase active-site residues in IRP-2 [23, 24]. To further consider whether the two IRP homologues found in *D. melanogaster* and tentatively designated *Drosophila* IRP-1A and 1B correspond to the two types of IRP found in vertebrates, we generated a multiple-sequence alignment of a representative set of IRP and pig mitochondrial aconitase by using the program Clustal W [25]. The deduced amino acid sequences of *Drosophila* IRP-1A and 1B show 86% sequence identity. All amino acids that have been implicated as aconitase active-site residues in crystallographic studies [26−29] and by site-directed mutagenesis [30] are conserved in both *D. melanogaster* homologues (Fig. 3). This includes those amino acids that play a role in aconitase substrate recognition (Q72, S166, R447, R452, R580, S643 and R644), in catalysis (D100, H101, H147, D165, H167, M170, E262 and S642) and in the interaction with the Fe-S cluster (N258, C358, C421, C424 and N446). Additional residues implicated [24] to be relevant for the structure and/or function of aconitases because of their conservation in the whole family of Fe-S isomerases (aconitases, isopropylmalate isomerases and IRP) are also conserved in *Drosophila* IRP-1A and 1B (Fig. 3): G144, I145, E152, T168, G175, G180, G182, G209, D219, E239 (previously mislabeled in [24] as E237), G242, M259, G264, P328, I355, T359, N360, G413, N448, S465, P466, G476, G639, G656 and S663. The high level of conservation of these features suggests that *Drosophila* IRP-1A and 1B belong to the IRP-1 family and may function as cytoplasmic aconitases.

The IRP-2-type 73-amino-acid insertion corresponding to the 'proteolysis' domain in IRP-2 (Fig. 3) is missing from *Drosophila* IRP-1A and 1B. Furthermore, the five aconitase active-site residues that are conserved in IRP-1 but typically substituted in IRP-2 (I145, T168, I355, R447 and S562 in IRP-1 are substituted with M, I, V, K and N, respectively, in IRP-2, Fig. 3) are also conserved in *Drosophila* IRP-1A and 1B. It was speculated previously that these five residues may be especially important for aconitase function and/or unique roles of IRP-2. These results assign both *D. melanogaster* IRPs as homologues of vertebrate IRP-1. IRP-2-type proteins, as known from mammalian cells, have not been identified in *D. melanogaster.*

Immunoprecipitation of *Drosophila* IRP-1A and 1B by anti-(human IRP-1) Ig. The similarity of *Drosophila* IRP-1A and 1B to vertebrate IRP suggests that they are genuine IRP. To evaluate this conclusion experimentally, we expressed both *Drosophila* IRP in a cell-free translation system and subjected the translation products to immunoprecipitation using a rabbit antiserum raised against recombinant human IRP-1 [18]. The cDNAs for both *Drosophila* IRP were transcribed *in vitro* and translated in rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Translation products of the expected size of 97 kDa, corresponding to full-length *Drosophila* IRP-1A and 1B, were obtained (Fig. 4). No translation products were generated in control reactions lacking *Drosophila* IRP mRNAs, suggesting that the smaller products seen in lanes 2 and 3 were derived either from premature translation termination and/or IRP degradation. Only anti-(human IRP-1) serum (Fig. 4) but not the preimmune serum precipitated the full-length *Drosophila* IRP-1A and 1B and some of the smaller fragments. This experiment shows that *Drosophila* IRP-1A and 1B are related immunologically to human IRP-1.

Chromosomal localization of *Drosophila* IRP-1A and 1B. We determined the localization of the *Drosophila* IRP on polytene chromosomes [19]. Using a probe against *Drosophila* IRP-1A, a strong signal was obtained at chromosome position 94C1-8 on the right arm of the third chromosome (3R), marking the chromosomal location of *Drosophila* IRP-1A (data not shown). Probing with IRP-1B resulted in a strong signal at chromosome position 86B3-6 on 3R, marking the chromosomal location of *Drosophila* IRP-1B (data not shown). In addition, weak signals were seen with the *Drosophila* IRP-1A probe at position 86B3-6 and with the *Drosophila* IRP-1B probe at position 94C1-8, suggesting cross-hybridization between the IRP-1A and *Drosophila* IRP-1 B probes due to the high similarity (76%) of the cDNAs. We were not able to detect any significant hybridization signal at position 3R83, a location that was suggested previously as a possible localization of a *Drosophila* IRP homologue using an alternative method to map genes on polytene chromosomes [31].

***Drosophila* IRP-1A and 1B mRNA are uniformly expressed in the *Drosophila* embryo.** Mammalian IRP-1 and IRP-2 are preferentially expressed in different tissues. IRP-1 mRNA and protein is enriched in liver, intestine and kidney, while IRP-2 mRNA and protein are expressed to higher levels in intestine and brain [11, 32]. To test whether *Drosophila* IRP-1A and 1B mRNAs are differentially expressed, we used an *in situ* RNA-staining assay to determine mRNA distribution and relative RNA levels of *Drosophila* IRP-1A and 1B during embryonic development. The developmental stages investigated span from early blastoderm (stage 5) through gastrulation (stage 7) and germband extention (stage 9) up to stage 13, when the neural anlagen and the gut are established. To distinguish between *Drosophila* IRP-1 A and 1B we used probes corresponding to the 3′ UTR of the transcripts, which show little similarity to each other (Fig. 1). The results suggest that *Drosophila* IRP-1A and 1B

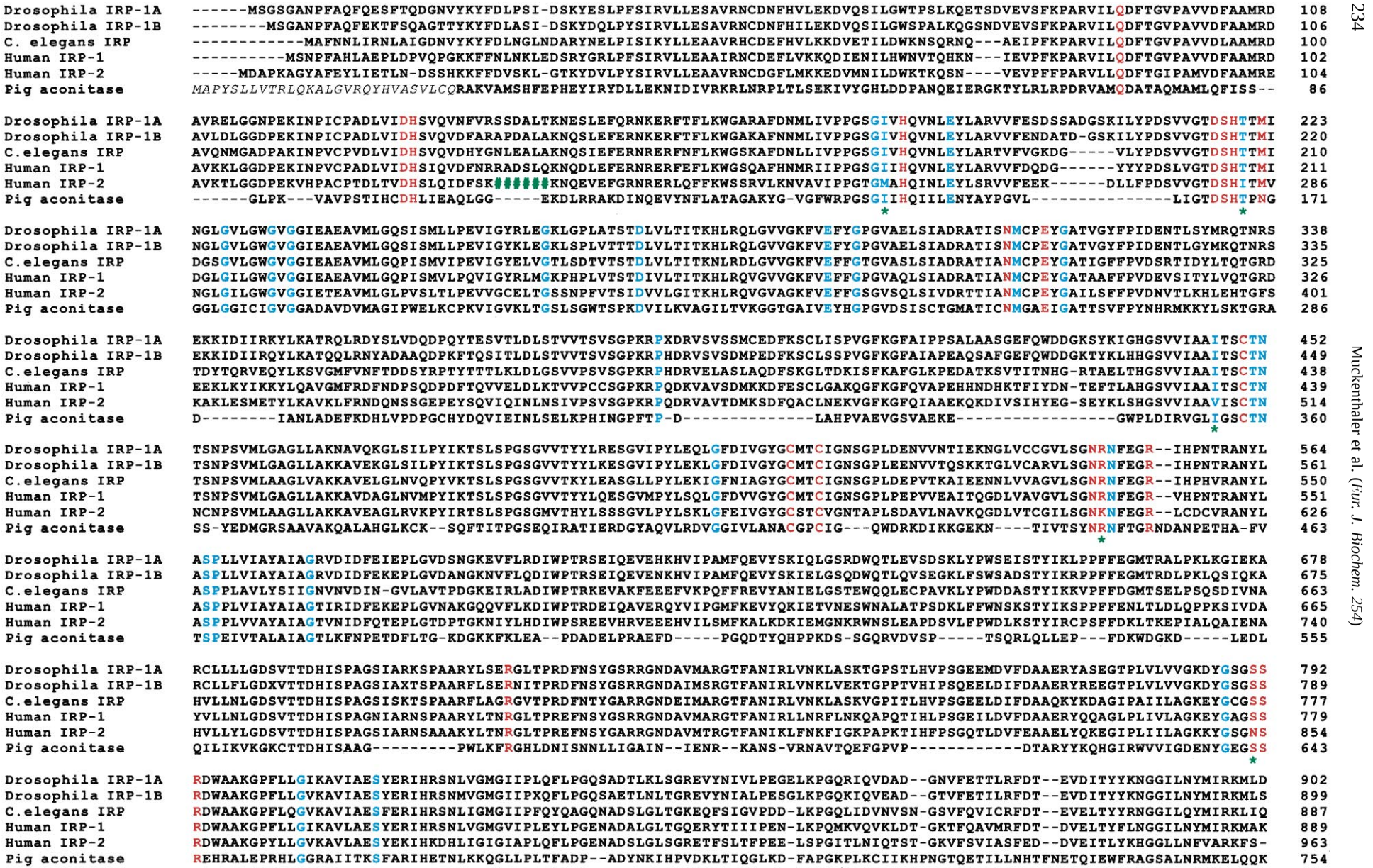

Fig. 3. IRP-1 like proteins identified in *D. melanogaster* and *C. elegans*. A multiple-sequence alignment was performed of the IRP family (as representatives, human IRP-1 and human IRP-2 are shown) and the pig mitochondrial aconitase. Aconitase active-site residues defined in crystallographic studies are indicated in red, and additional residues shown earlier [24] to be absolutely conserved in all members of the Fe-S isomerase family are indicated in blue. #, the position of the 73-residue insertion in the human IRP-2. *, aconitase active-site residues that are not conserved in human IRP-2. For consistency with earlier work [24], residue numbers are given according to the pig aconitase sequence as in the Protein Data Bank [36] entry 7acn.brk. The first 28 pig aconitase residues, missing in the crystal structure, are shown in italics.

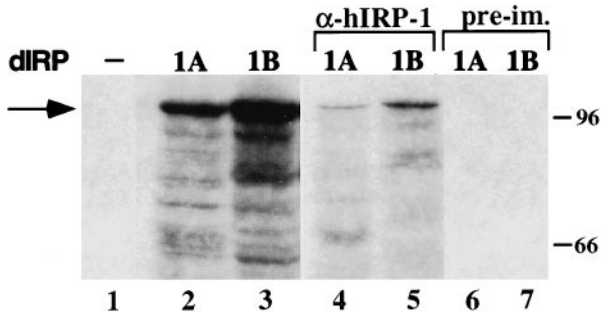

Fig. 4. *Drosophila* IRP-1A and 1B are immunologically related to human IRP-1. Capped *Drosophila* IRP-1A and 1B mRNAs were transcribed *in vitro* and translated in a reticulocyte cell-free translation system (lanes 2 and 3). No *Drosophila* IRP mRNA was added to the sample shown in lane 1. The translation products were either precipitated with anti-(human IRP-1) serum (α-hIRP-1, lanes 4 and 5) or preimmune serum (pre-im.; lanes 6 and 7). The positions of the full-length *Drosophila* IRP (dIRP; left) and the molecular-mass standards (right) are indicated.

mRNAs are uniformly expressed throughout the embryo (Fig. 5). Neither transcript is particularly enriched in any embryonic structure or specific cell type, in contrast to the control stainings with *fuzhi-taratzu* (segmental staining) and *nubbin* (segregated neuroblasts after stage 11). Comparison of *in situ* hybridizations stained with *Drosophila* IRP-1A or *Drosophila* IRP-1B probes of similar specific activity for an identical period of time showed that the signal for *Drosophila* IRP-1B was consistently weaker, in accord with the idea that *Drosophila* IRP-1B expression is lower in the fly.

Comparative sequence analysis of a *C. elegans* IRP. A further invertebrate IRP was identified by screening the data base of the *C. elegans* genome project (cosmid ZK455). The similarity of this sequence to the human iron-responsive-element-binding protein (the old term for IRP-1) had been noticed before. A predicted peptide sequence of 887 amino acids was aligned to all known IRP and the pig mitochondrial aconitase. All residues implicated in aconitase function are conserved in *C. elegans* IRP, including those that are substituted in IRP-2-type proteins (Fig. 3). Furthermore, the insertion of amino acids that is typically found in IRP-2 is not present, suggesting that the *C. elegans* IRP is of the IRP-1 type. The *C. elegans* IRP-1 shows 63% identity to human IRP-1 and 63% identity to *Drosophila* IRP-1A and 1B at the amino acid level. The identification of a *C. elegans* IRP indicates that the post-transcriptional IRE/IRP-mediated control mechanism is conserved in *C. elegans*.

DISCUSSION

The mechanisms by which IRP-1 and IRP-2 regulate the expression of mRNAs that encode proteins involved in cellular iron metabolism have been studied in great detail in mammalian cells. In this paper, we strengthen the evidence that the IRE/IRP regulatory system is not confined to vertebrates, but also operates in invertebrate species. We report the characterization of two IRP-1 homologues from *D. melanogaster* (*Drosophila* IRP-1A and 1B), with 86% identity to each other, and the identification of an IRP-1 clone from *C. elegans.* Two related IRP (IRP-1 and IRP-2) have been identified in vertebrates. They are most notably distinguished by a 73-amino-acid segment unique to IRP-2, which mediates protein degradation in iron-replete cells and by several aconitase active-site residues that are substituted in IRP-2 in comparison with IRP-1, consistent with the data that IRP-2 does not display aconitase activity [23, 24]. Multiple se-

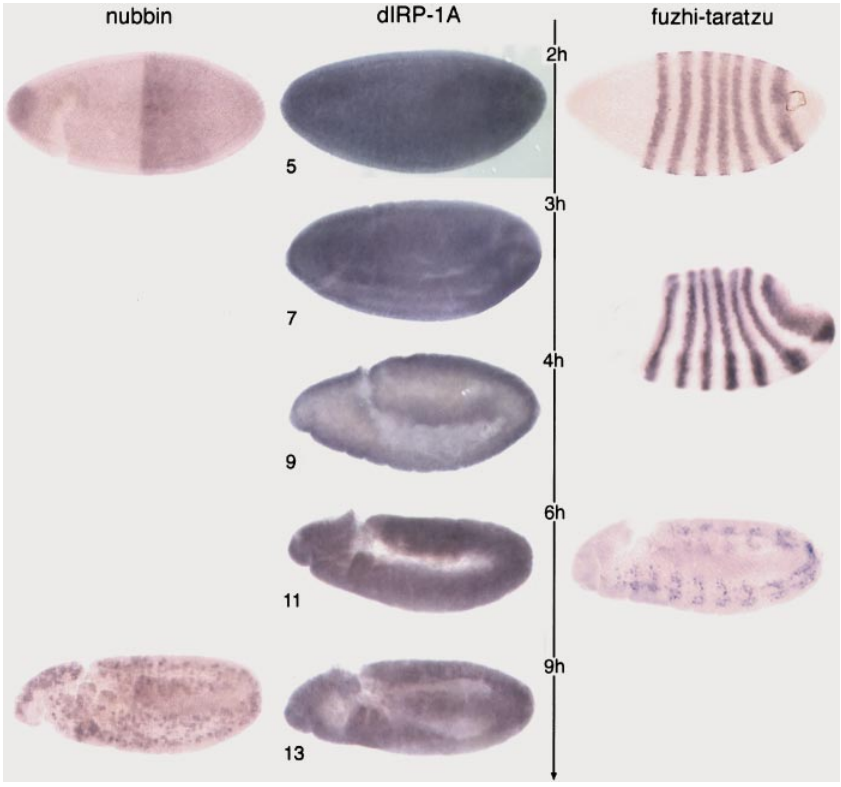

Fig. 5. *Drosophila* IRP-1A mRNA is expressed uniformly in the developing *D. melanogaster* embryo. Staged embryos (times after fertilization are indicated in hours) were subjected to an *in situ* RNA-staining assay using part of the *Drosophila* IRP-1A 3′ UTR as a probe (dIRP-1A). As controls, a *nubbin* probe [21], that visualizes segregated neuroblasts, and a *fuzhi-taratzu* probe [22], that highlights 7 segmental stripes in early embryogenesis and particular cells in 14 stripes at later stages, were used.

quence analysis shows that *Drosophila* IRP-1A and 1B and the IRP from *C. elegans* belong to the vertebrate IRP-1 class. They do not contain an IRP-2−like amino acid insertion and all aconitase active-site residues are of the IRP-1 type (Fig. 3). Direct sequence comparison excludes the possibility that one of the *Drosophila* IRP is the mitochondrial aconitase, as both *Drosophila* IRP show high similarity to vertebrate IRP (67% to human IRP-1), but little similarity to known mitochondrial aconitases (27% to pig mitochondrial aconitase).

Furthermore, we identified two identical *Drosophila* 5′ expressed sequence tags (LD05952 and LD02708) with high similarity to vertebrate mitochondrial aconitases, which are different from both *Drosophila* IRP sequences. The *Drosophila* mitochondrial aconitase clones, in contrast to the mammalian ones, do not contain an IRE in their 5′ UTR [14, 33, 34]. Conversely, the Fe-S protein subunit of succinate dehydrogenase contains a functional IRE in *Drosophila*, but not in the mammalian mRNAs [13, 14]. Therefore, the regulatory influence of the IRE/IRP system on the mitochondrial citric acid cycle is conserved between *Drosophila* and mammals, but it appears that the targets are switched from succinate dehydrogenase in *Drosophila* to mitochondrial aconitase in mammals.

Whether *D. melanogaster* also contains an IRP-2 type protein is not clear. Such a protein has not been detected either by PCR amplification (although the primers in principle should not discriminate against IRP-2−like proteins), or by screening cDNA libraries and expressed-sequence-tag databases. Why does *D. melanogaster* contain two IRP-1−type homologues? One reason could be that the IRP expressed from one gene locus was not sufficient and gene duplication therefore offered an advantage. This could explain the high level of conservation between the two *Drosophila* IRP (86%). Alternatively, as was suggested for mammalian IRP-1 and IRP-2 [35], the *Drosophila* IRP could recognize IRE with different sequence specificity, and thus might control the expression of different subsets of IRE-containing mRNAs. It is also possible that the expression of *Drosophila* IRP-1A and 1B could be regulated by different mechanisms in response to iron and/or by different signals, such as NO or reactive-oxygen intermediates. Furthermore the *Drosophila* IRP could be expressed during different developmental stages or in different cell types. This hypothesis was tested by *in situ* hybridization of different embryonic stages. Both *Drosophila* IRP probes uniformly label all embryonic stages investigated (Fig. 5), although in control reactions specific stainings for neuroblasts and structural components of the embryo were obtained. Although the later embryonic stages investigated contain virtually all inner organs of the fly, it remains to be determined whether adult structures, such as the eyes or wings, exhibit any differential expression of *Drosophila* IRP-1A and 1B.

One might speculate that gene duplication of IRP occurred prior to the diversion of invertebrates and vertebrates. While in vertebrates the IRP developed into IRP-1 and IRP-2 by insertion of a 73-amino-acid exon mediating iron-dependent protein degradation, in invertebrates comparatively fewer changes have occurred. Consistent with this idea, additional IRP-1 homologues were not detected screening databases of the human genome sequencing project.

Drosophila IRP 1A and 1B are IRP-1−like proteins that are immunologically related to human IRP-1 (Fig. 4) and show high sequence similarity to vertebrate IRP in general. Target sites (IRE) for *Drosophila* IRP have been identified in the Fe-S protein subunit of the *D. melanogaster* succinate dehydrogenase [13, 14] and in the ferritin mRNA [15]. Due to the potential to apply genetic and transgenic techniques in *D. melanogaster* and *C. elegans*, the identification of IRP in these two species opens the possibility to explore the IRP/IRE regulatory network in these organisms. Furthermore, the *Drosophila* system will allow us to investigate the role of IRP in early development. It was shown previously that the IRE-binding activity decreases between embryonic stages 5 and 7 [14]. The cloning of *Drosophila* IRP-1A and 1B will help to investigate why the levels of IRP are regulated during embryogenesis.

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