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

Martina MUCKENTHALER, Niki GUNKEL, Dmitrij FRISHMAN, Anna CYRKLAFF, Pavel TOMANCAK and Matthias W. HENTZE

1 European Molecular Biology Laboratory, Heidelberg, Germany
2 Martinsried Institute for Protein Sequences/GSF, Martinsried, Germany

(Received 23 December 1997) – EJB 97 1815/2

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. Species 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 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.
MATERIALS AND METHODS

Degenerate primers, PCR, and cDNA library screening. Degenerate primers were designed in conserved regions of IRP. The upstream primer (5′-GAGATTTGCAGGTTTCTCCCATGGTC-3′) introduces a BglII site at the 5′ end and corresponds to amino acid residues 44-54 of cytoplasmic aconitase (AcnB) of human IRP-1: the downstream primer (5′-GGTGATCCGTCAGGTTCGCCTGCAGTA-3′) introduces a BglII site at the 5′ end and corresponds to amino acid residues D85-A86-G87-C88 in human IRP-1: the downstream primer (5′-GGAATTCGGTCCATTCGCTGCAGTG-3′) introduces a BglII site at the 5′ end and corresponds to amino acid residues D241-K242-Q243 of Srf, which is a transcription factor in human IRP-1. A TG) of Srf was subcloned in the presence of 1 length of 72° primer-extention analysis [1].

In situ hybridization. In situ hybridization was essentially as described [20]. Ppu101-digested Drosophila IRP-1A and Drosophila IRP-1B cDNAs were used as probes 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 fuchi-tarazumi 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 910 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 Lepidoptera (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 D. melanogaster embryonic λ-ZAP library (a kind gift from Steve Cohen, EMBL) and an adult D. melanogaster gt10 cDNA library (Clontech).

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 μl U RNASin (Promega), 20 μl amino acids (minus methionine) and 30 μl [35S]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 (30 mM Tris/HisCL 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/HisCl 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 hybridization 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 fuchi-tarazumi probe as described in [22]. 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 IRP-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’ UTRs 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 Ppu101 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 EcoRI 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’).
at the amino acid level. The most distinctive differences between chromosomes 1N448, S465, P466, G476, G639, G656 and S663. The high level early blastoderm (stage 5) through gastrulation (stage 7) and homologues (Fig. 3). This includes those amino alternative method to map genes on polytene chromosomes [3].

aconitase active-site residues in crystallographic studies [26]. Using a probe against Drosophila IRP-1A, 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 isomerasers 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 (H145, 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. The 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 anti-serum 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 [35S]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 germ band extension (stage 9) up to stage 13, when the neural anlagen and the gut are established. To distinguish between Drosophila IRP-1A 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. 2. mRNA analysis of Drosophila IRP-1A.](image-url)
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 aconitate. Aconitate active-site residues defined in crystallographic studies are indicated in red, and additional residues [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. *, aconitate 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 aconitate sequence as in the Protein Data Bank [36] entry 7acm.brk. The first 28 pig aconitate residues, missing in the crystal structure, are shown in italics.
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. 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-
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 vertebral 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 staining 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 Fc-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.

We thank Dr Steve Cohen for the *D. melanogaster* embryonic λ-ZAP library. During this work, M. M. was a recipient of a European Molecular Biology Organisation postdoctoral fellowship and N. G. a recipient of a Deutshe Forschungsgemeinschaft postdoctoral fellowship.

**REFERENCES**


