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Drosophila ferritin mRNA: alternative RNA splicing regulates the presence of the iron-responsive element

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Abstract Several mRNAs encoding the same ferritin subunit of *Drosophila melanogaster* were identified. Alternative RNA splicing and utilisation of different polyadenylation sites were found to generate the transcripts. The alternative RNA splicing results in ferritin transcripts with four unique 5′ untranslated regions. Only one of them contains an iron-responsive element. The iron-responsive element was found to bind in vitro specifically to human recombinant iron regulatory protein 1. Furthermore, the ferritin subunit mRNAs are differentially expressed during development. Our data provides the first molecular evidence that the presence of iron-responsive element in a ferritin mRNA is regulated by alternative RNA splicing.

Key words: Alternative RNA splicing; Ferritin; Iron; Iron-responsive element; *Drosophila melanogaster*

1. Introduction

The iron-storing protein, ferritin, has been found and characterised in animals, plants, fungi, and bacteria [1–3]. This protein plays a key role in iron homeostasis. Most ferritins have a similar structure and consist of 24 polypeptides, forming a hollow shell. The cavity can store up to 4500 iron atoms as an Fe(III) inorganic complex (ferrihydrite and phosphate). In mammals, the apoprotein consists of two different subunits, the heavy chain (H) and the light chain (L), with a molecular weight (M_r) of 440 000 [2]. The mammalian ferritin is intracellular and is located in the cytoplasm, but small traces of ferritin also exist in the serum [2]. The ferritin of insects is larger (M_r about 660 000) and contains more than two different subunits [1,3]. They are usually secreted and glycosylated, which explains their localisation in the endoplasmic reticulum, the secretory pathway, as well as in the haemolymph [1].

The expression of ferritin subunits have been shown to be regulated both at the transcriptional and at the posttranscriptional level. The regulation at the posttranscriptional level is controlled by the labile iron pool of the cell, and is depending on a *cis*-acting RNA stem-loop structure, the iron-responsive element (IRE), which is located in the 5′ untranslated region (5′ UTR) of the ferritin subunit mRNA, and *trans*-acting proteins, the iron regulatory protein (IRP) [4–9]. The IRE consists of a conserved loop, 5′-CAGUGN-3′, and a bulged C, which is located six nucleotides upstream of the loop and interrupts the stem [4–9]. An IRE-like structure has been shown to exist in all characterised ferritin subunit mRNAs of animals, except for the yolk ferritin of the snail *Lymnaea stagnalis* L. [10], and the soma and yolk ferritins of *Schistosoma mansoni* [11]. IREs have also been found in other transcripts encoding proteins involved in iron homeostasis, such as the transferrin receptor, the erythroid 5-aminolevulinate synthase (eALAS), and the proton-coupled metal ion transporter [4–9,12]. In addition, mRNAs encoding citric acid cycle enzymes are regulated by IREs. In mammals this regulation has been demonstrated for mitochondrial aconitase [4–9], whereas the iron sulphur subunit of succinate dehydrogenase (SDH-Ip) has been found to be regulated via the IRE/IRP system in *Drosophila* [13–15]. The IRPs can recognise and bind to the IRE, and this normally occurs in iron-depleted cells. The IRE binding activity can be modulated directly or indirectly by many other stimuli, e.g. phosphorylation, nitric oxide, hydrogen peroxide, ascorbate, oxygen, and interferon-γ/lipopolysaccharide [4–9,16–19]. Binding of IRP to IRE in the 5′ UTR inhibits the translation of the mRNA, while binding of IRPs to several IREs in the 3′ UTR protects the mRNA against rapid degradation [4–9]. The IRE/IRP system is important for regulation of the uptake, storage, and utilisation of iron and to keep the iron level at a non-toxic level.

In this study we identified several mRNAs encoding the same ferritin subunit in *D. melanogaster*. These transcripts are differentially expressed during development and generated by alternative RNA splicing and differential polyadenylation. An IRE was found in the 5′ UTR of some ferritin cDNAs, and we showed that this IRE binds in vitro specifically to human recombinant iron regulatory protein 1. Interestingly, alternative RNA splicing was found to regulate the presence of the IRE in the 5′ UTR of the ferritin transcripts. These results suggest that translation of the ferritin subunit in *D. melanogaster* can be independent of the IRE/IRP system, which will result in ferritin subunit expression even when the cellular iron concentration is low.

2. Materials and methods

2.1. Cloning of ferritin cDNA

The *D. melanogaster* cDNA was synthesised using 1 μg of poly(A)-rich RNA from 4-day-old larvae of the Canton S strain (Clontech) and the Marathon kit (Clontech). Two degenerated primers and 25 ng of the cDNA were used in a polymerase chain reaction (PCR) as described by Huang et al. [20]. These primers were designed according to two conserved regions of ferritins, EEREH(A/G)EKL and LGE(F/Y)LFDK, respectively. The PCR product was analysed by agarose gel electrophoresis and a single band with the expected size of about 370 bp was revealed. The product was directly sequenced by the Sanger method following the dye terminator cycle sequencing ready reaction kit (Perkin Elmer), and the deduced amino acid sequence shows high similarity to other known sequences of ferritin.

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A *D. melanogaster* 5′ stretch λgt11 cDNA library, made from poly(A)-rich mRNA of 4-day-old larvae of the Canton S strain (Clontech), was screened at 65°C using a probe. The probe was [α-^{32}P]dCTP-labelled by random priming of the above-mentioned PCR product, using a Megaprime Labeling Kit (Amersham Life Science). Out of 300 000 screened, 14 positive clones were detected. These clones were amplified by PCR, using a pair of λgt11-specific primers, according to the protocol for the cDNA Insert Screening Amplimers kit (Clontech). The PCR fragments were purified using QIAquick PCR Purification Kit (Qiagen), and then subcloned into T-vectors, which were prepared from pBluescript SK$^+$ (Stratagene) according to Marchuk et al. [21]. The clones were subsequently partially or fully sequenced on both strands by using either vector-specific primers or clone-specific primers.

The 5′ part of the cDNA (bp 1–43) was obtained by using the 5′ RACE System for Rapid Amplification of cDNA Ends, version 2.0 (Gibco BRL). Two ferritin-specific primers were designed after the sequence of the phage clones obtained in the library screening. The rapid amplification of cDNA end (RACE) method was done according to the manufacturer's instruction except that the first strand cDNA reaction was performed at 50°C instead of 42°C to prevent formation of secondary structures of the RNA. A total of 0.5 μg of poly(A)-rich RNA and 10 pmol of the ferritin-specific primer, 5′-GAACAGCGAGGGAGCACTTGAAATC-3′, were used in the first reaction and 10 pmol of the nested ferritin-specific primer, 5′-CTTTAGTTTGATTTGCTTCTGCTG-3′, was used in the PCR amplification step. The PCR was performed on a Gene Amp PCR System 2400 (Perkin Elmer) at 96°C for 5 min followed by 30 cycles at 95°C for 45 s, 55°C for 45 s, and 72°C for 90 s and ended by an extension step at 72°C for 7 min. The PCR products were cloned into T-vectors and sequenced as described before.

2.2. Plasmid construction

A plasmid (pDfer) containing the ferritin IRE of *D. melanogaster* was constructed. Two ferritin-specific primers, CGGTGGAGCTCAAGCCCATCAAAT and CGGGGGATCCAAATAGGCGTTGAAA, were synthesised. The sense primer corresponds to bp 108–122 of the ferritin cDNA sequence and the 5′ end of the oligonucleotide contains an extra region with a *Sac*I restriction site (underlined). The antisense primer corresponds to bp 190–175 and has a *Bam*HI site (underlined) in the 5′ part. The IRE region was amplified by PCR in a total volume of 100 μl containing 50 ng of a T-vector with an insert corresponding to bp 20–410 of the cDNA, 200 pmol of each primer, 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 200 μM of each dNTP. The PCR product was purified and subcloned as described before into a T-vector. The resulting construct, which has a T7 promoter (derived from pBluescript KS$^+$, Stratagene) upstream of the IRE region, was cut with *Sac*I restriction enzyme, purified and religated to get rid of the region between the *Sac*I site in the vector and the *Sac*I site in the insert. The final plasmid construct (pDfer) can be linearised by *Bam*HI to generate a template for T7 polymerase transcription. The RNA sequence will be as shown in Fig. 1A. The sequence of the plasmid construct was confirmed by DNA sequencing.

2.3. In vitro transcription

The RNA transcripts were produced by in vitro transcription. pDfer and pSPT-fer [22] were linearised by *Bam*HI and used as templates. [α-^{32}P]UTP-labelled RNA transcripts were synthesised using the RNA transcription kit (Stratagene) and the T7 polymerase in vitro transcription was performed according to the manufacturer's instruction. Large scale transcription of unlabelled transcripts were performed as previously described by Neupert et al. [22], with 5 μg of the plasmids pDfer and pSPT-fer, linearised by *Bam*HI, and I-12.CAT and I-19.CAT linearised by *Dde*I [23]. All RNA transcripts were purified on Croma spin+DEPC H_2O-10 columns (Clontech).

2.4. Electrophoretic mobility shift assay (EMSA)

For EMSA reactions, a 12 μl sample volume was used containing 25 mM Tris, pH 7.4, 40 mM KCl, 1% Triton X-100, and 5 ng human recombinant IRP1, which was expressed and purified according to Gray et al. [23]. In some samples the recombinant IRP1 was reduced with 2% 2-mercaptoethanol before addition of radiolabelled IRE (1.5×10^4 cpm). For the competition experiment, 100-fold molar excess of cold RNA was mixed with the labelled RNA before adding them to the protein sample. The RNA/protein binding reaction was allowed to proceed at room temperature for 20 min. 1 μl of 50 mg/ml heparin was added and incubated for 5 min to reduce non-specific binding. RNA/protein complexes were run on a 4% non-denaturing polyacrylamide gel (59 acrylamide:1 bisacrylamide) in 1×TBE buffer (90 mM Tris-borate, pH 8.3, 2 mM EDTA) for 2–3 h at 7.5 V/cm. The gel was dried and subjected to autoradiography.

2.5. Analysis of the 5′ UTR of the ferritin transcript

Comparison of the clones, obtained from the cDNA library, resulted in an unexpected diversity of the 5′ UTR of the ferritin cDNA. To investigate this in further detail, two oligonucleotides, corresponding to bp 47–62 (5′-GCTCCACTGAAAAATC-3′) and 409–395 (5′-AACAGCGAGGGAGCA-3′), respectively, were synthesised. 10 pmol of each primer and 50 ng of cDNA were used for PCR in 100 μl final volume containing 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTPs, and 1.5 units of Taq DNA polymerase (Amersham Life Science), which had been inactivated with 1 μg TaqStart antibody (Clontech). The PCR was performed as follows: a hot start at 96°C for 5 min; denaturing at 94°C for 45 s, annealing at 45°C for 45 s, and extension at 72°C for 1 min, for 30 cycles; and finally an extension step at 72°C for 7 min. PCR-amplified products were analysed by agarose gel electrophoresis, and cloned into a T-vector as previously described. The insert size of the clones was determined by running PCR with the same primers and conditions as above, and subsequently analysing the products on an agarose gel. Two independent clones of each size were sequenced.

2.6. Genomic library screening and cloning of 5′ flanking sequence of the ferritin gene

An EMBL3 genomic library, which has been constructed from *D. melanogaster* DNA partially digested with *Mbo*I restriction enzyme, was screened with [α-^{32}P]dCTP-labelled fragments covering the 5′ UTR (bp 20–410) of the cDNA. From 100 000 screened, nine positive phage clones were detected. DNA of three clones were purified using the Wizard lambda preps DNA purification system (Promega). The genomic region of interest was amplified by PCR and the same primers and conditions were used as described in Section 2.5, except that 50 ng DNA of the genomic phage clones were used as template. The PCR products were purified, cloned and sequenced as previously described.

2.7. Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was prepared from normally reared *D. melanogaster* Canton S strain by Trizol extraction according to protocol provided by the manufacturer (Gibco BRL). RNA was extracted from 0–4 h embryos, first, second and third instar larvae, pupae 3 days after pupation, 3–4-day-old adult males and females as well as from separate collections of heads, thoraces and abdomens from adult flies. Total RNA from cultured mbn-2 cells (a *Drosophila* haemocyte cell line) was also prepared [24]. 4 μg of each total RNA was treated by 7.5 units of DNase I (Pharmacia Biotech) in a final volume of 10 μl for 20 min at room temperature. The reaction was terminated by addition of 1 μl of 25 μM EDTA and the DNase I was heat-inactivated at 65°C for 15 min. The RNA was precipitated and resolved in 10 μl of water. 1.5 μg of DNase I-treated RNA was used for the first stand cDNA synthesis. The RNA was mixed with 10 pmol of a primer corresponding to bp 438–421. The mixture was denatured at 70°C for 10 min before adding of 2 μl first strand buffer (0.25 M Tris, pH 8.3, 0.375 M KCl, and 15 mM $MgCl_2$), 1 μl of dNTP (10 mM of each dNTP), 2.5 μl of 0.1 M dithiothreitol, and 200 units of M-MLV reverse transcriptase (Gibco BRL) to a final volume of 25 μl. The synthesis was performed at 42°C for 50 min. The synthesis was stopped by heating at 70°C for 10 min and the RNA was degraded by incubation with RNase H for 10 min at 37°C. The PCR amplification step was performed as described in Section 2.5, using 1 μl of the reverse transcription mixture as template. The PCR-amplified products were resolved by agarose gel electrophoresis. The RT-PCR was done three times, and the same results were obtained. Furthermore, PCR was also performed on total RNA, which had been treated in the same way as the samples except that no reverse transcriptase was added in the first stand synthesis, and that did not result in any PCR products.

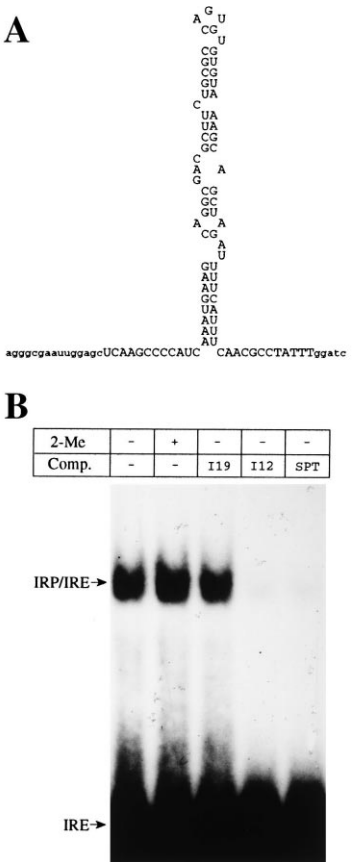

Fig. 1. A: The RNA sequence produced by T7 polymerase in vitro transcription of *Bam*HI-linearized pDfer plasmid. Most of the transcript (capital letters) correspond to a part of the 5′ UTR including the IRE of the mRNA encoding a ferritin subunit of *D. melanogaster*. The nts C_1 and G_5 in the loop are basepaired [35,36]. B: Electrophoretic mobility shift assay for IRE binding of recombinant human IRP1. Excess of radiolabelled *Drosophila* ferritin IRE was incubated with recombinant human IRP1. Then the mixture was resolved on a nondenaturing polyacrylamide gel. Then indicated 2% 2-mercaptoethanol (2-Me) was present in the binding reaction (lane 2). For competition experiments (Comp., lanes 3–5) a 100-fold molar excess of non-radiolabelled IRE was premixed with the probe before adding of the protein. The competitor RNAs (I19, I12 and SPT) have been transcribed from the vector I-19.CAT, I-12.CAT and pSPT-fer, respectively. I12 and SPT are both derived from human ferritin H IRE while I19 is identical to I12 except a C deletion in the loop, which will prevent binding of IRP.

2.8. Northern blot analysis

The same total RNA samples that were used for the RT-PCR were also analysed by Northern blot. 15 μg of total RNA from each sample was loaded on a 1% denaturing agarose gel (2% formaldehyde) and separated electrophoretically in 1×MOPS buffer (20 mM 3[*N*-morpholino]propanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA, pH 7.0). A size standard (G3191, Promega) was run parallel with samples. The gel was treated with 700×100 μJ in a Stratalinker (Stratagene) and soaked twice in 10×SSC (1.5 M NaCl and 1.5 mM sodium citrate, pH 7.0) for 30 min. RNA was transferred with 20×SSC to Amersham nitrocellulose membrane or to nylon membrane (Hybond N^+) by overnight capillary transfer. RNA was crosslinked to the membrane with 1200×100 μJ in the Stratalinker. Prehybridisation and hybridisation were carried out at 65°C in 6×SSC, 5×Denhardt's solution (0.1% of bovine serum albumin, Ficoll (Pharmacia Biotech), and polyvinylpyrollidone), 0.5% SDS, and 20 μg/ml denatured salmon sperm DNA. The ferritin transcripts were detected by the same probe as used in the cDNA library screening. This corresponds to bp 584–936 of the cDNA. All stringency washes were performed at 65°C for 15 min with two initial washes in 2×SSC/0.1% SDS, two subsequent washes in 1×SSC/0.1% SSC and a final wash in 0.2×SSC/0.1% SDS. Northern blot analysis was also performed at a temperature of 60°C instead of 65°C and with probes corresponding to bp 108–223 and bp 1260–1357. These probes are specific for transcripts containing a cDNA1-like 5′ UTR, and a poly(A) tail after the second poly(A) signal, respectively. The membranes were then subjected to autoradiography. Furthermore, the same membranes were also hybridised with a probe corresponding to the gene encoding the *Drosophila* ribosomal protein 49 [25].

2.9. Sequence analysis

The cDNA sequences and the deduced amino acid sequences were analysed with the MacVector 4.1.4 software (Kodak, New Haven, CT), and compared to data bases, using the BLAST network (National Center for Biotechnology Information, Bethesda, MD). The 5′ UTR of the cDNA was examined for the presence of secondary structures by the program Mulfold version 2.0 [26]. The nucleotide sequences reported in this paper have been submitted to the EMBL Nucleotide Sequence Databases with accession numbers: ferritin cDNA: Y15629; cDNA1: Y15630; cDNA2: Y15631; cDNA3: Y15632; and cDNA4: Y15633.

3. Results

3.1. Cloning of the cDNA encoding a ferritin subunit of D. melanogaster

The phage clones, obtained from a larval cDNA library, and the 5′ RACE clones revealed a ferritin cDNA sequence of 1357 bp. The 5′ UTR varied from 104 to 328 bp, and the 3′ UTR was 414 bp in length. In the 5′ UTR a stable stem-loop structure was found between bp 134 and 194. The structure contains a loop (5′-CAGTGT-3′), and a bulged C, located six nucleotides upstream of the loop, interrupting the stem. This agrees well with the consensus sequence of a special RNA stem-loop, the iron-responsive element (IRE) [4–9]. The start codon (bp 329–331) represents a good consensus sequence for initiation of translation with an A at position 326, and a G at position 332 [27]. Two putative poly(A) sites starting at positions 1087 and 1329, respectively, were found downstream of the stop codon (TAA). The cDNA has an open reading frame of 615 bp corresponding to a 23 kDa ferritin subunit with 205 amino acid residues. The deduced amino acid sequence starts with a secretory signal peptide and has a putative cleavage site between residues 19 and 20 [27,28], and the cleaved polypeptide will have a mass of 21 kDa. A putative glycosylation site (N-A-S) starting at amino acid residue 54 is present.

3.2. Drosophila ferritin IRE binds in vitro to human recombinant IRP1

Electrophoretic mobility shift assay (EMSA) was performed to verify that the putative IRE was able to bind IRP. The EMSA (Fig. 1B) showed that the *Drosophila* ferritin IRE (Fig. 1A) binds to human IRP1 with an affinity similar to that of other IREs. Competition experiments were performed to verify the specificity of the RNA/protein binding. A 100-fold molar excess of both cold human ferritin H like IRE (I-12.CAT, lane 4 in Fig. 1B) and cold human ferritin H IRE (pSPT-fer, lane 5 in Fig. 1B) significantly reduced the binding of the radiolabelled *Drosophila* IRE to the human IRP1, whereas a 100-fold molar excess of the human mutated IRE was without any affect (lane 3 in Fig. 1B). The reducing agent, 2-mercaptoethanol, slightly stimulated the IRE binding activity of the IRP1 (compare lanes 1 and 2 in Fig. 1B). If human ferritin H IRE was used as radiolabelled RNA, a 100-fold molar excess of cold *Drosophila* ferritin IRE was able to abol-

A

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Genomic GCTCCACTGAAAAATCAGATTAAAGCTGTCAGCAGAAGGTACTGGAAAGCATTCGAGAAGTCGAAAATAATCCCAATCAAGCCCATCAAATTGAAGCAGG 100
cDNA1   GCTCCACTGAAAAATCAGATTAAAGCTGTCAGCAGAAGGTACTGGAAAGCATTCGAGAAGTCGAAAATAATCCCAATCAAGCCCATCAAATTGAAGCAGG 100
cDNA2   GCTCCACTGAAAAATCAGATTAAAGCTGTCAGCAGAAG---------------------------------------------------------- 38
cDNA3   GCTCCACTGAAAAATCAGATTAAAGCTGTCAGCAGAAG---------------------------------------------------------- 38
cDNA4   GCTCCACTGAAAAATCAGATTAAAGCTGTCAGCAGAAG---------------------------------------------------------- 38

Genomic CGACGCCTTCTGCGCCAGTGTGTGTAAAGGCAGCTAGATTTTCAATTTCAACGCCTATTTAACATTgtagctCAACTCGAGTCGCCTCATTCTCATTTGT 200
cDNA1   CGACGCCTTCTGCGCCAGTGTGTGTAAAGGCAGCTAGATTTTCAATTTCAACGCCTATTTAACATTGTAGCTCAACTCGAGTCGCCTCATTCTCATTTGT 200
cDNA2   ----------------------------------------------------------CTCAACTCGAGTCGCCTCATTCTCATTTGT  68
cDNA3   ---------------------------------------------------------------------------------------------- 38
cDNA4   ---------------------------------------------------------------------------------------------- 38

Genomic GCATTTcaagcAAATCAAACTAAAGTGAAAAACAAATTTTGGTTCTGTGATTTTGTGTgaagaCTACGTTCGACGATCAAAGatgGTGAAACTAATTGCT 300
cDNA1   GCATTTCAAGCAAATCAAACTAAAGTGAAAAACAAATTTTGGTTCTGTGATTTTGTGTGAAGACTACGTTCGACGATCAAAGatgGTGAAACTAATTGCT 300
cDNA2   GCATTTCAAGCAAATCAAACTAAAGTGAAAAACAAATTTTGGTTCTGTGATTTTGTGTGAAGACTACGTTCGACGATCAAAGatgGTGAAACTAATTGCT 168
cDNA3   ----------CAAATCAAACTAAAGTGAAAAACAAATTTTGGTTCTGTGATTTTGTGTGAAGACTACGTTCGACGATCAAAGatgGTGAAACTAATTGCT 128
cDNA4   ----------------------------------------------------------ACTACGTTCGACGATCAAAGatgGTGAAACTAATTGCT  76

Genomic AGCCTGCTCCTGTTGGCCGTGGTGGCCCAGGCCTATGGAGATTTCAAGTgtaagtactaatgt                                        363
cDNA1   AGCCTGCTCCTGTTGGCCGTGGTGGCCCAGGCCTATGGAGATTTCAAGTGCTCCCTCGCTGTT                                        363
cDNA2   AGCCTGCTCCTGTTGGCCGTGGTGGCCCAGGCCTATGGAGATTTCAAGTGCTCCCTCGCTGTT                                        231
cDNA3   AGCCTGCTCCTGTTGGCCGTGGTGGCCCAGGCCTATGGAGATTTCAAGTGCTCCCTCGCTGTT                                        191
cDNA4   AGCCTGCTCCTGTTGGCCGTGGTGGCCCAGGCCTATGGAGATTTCAAGTGCTCCCTCGCTGTT                                        139
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B

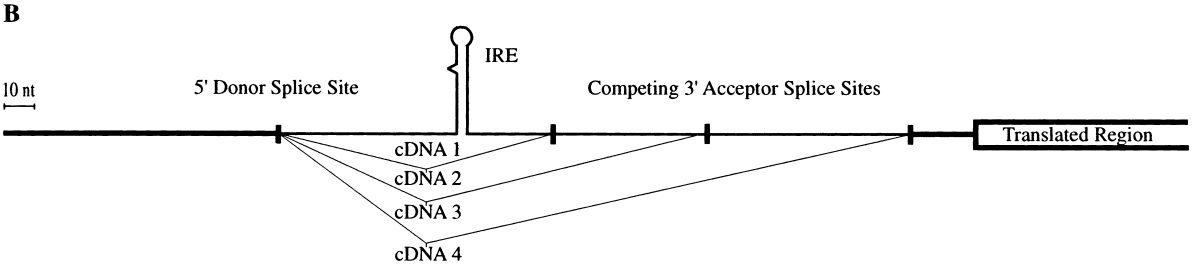

Fig. 2. Alignment of cDNA1–4 with the 5′ flanking region of the ferritin gene of *D. melanogaster* and schematic representation of the alternative splice events in the 5′ UTR. A: PCR was performed on cDNA and genomic phage clones using a sense and an antisense primer (the underlined sequences show the position of the primers). Four different cDNA clones (cDNA1–4) were obtained. Alignment of the cDNA sequences with the genomic sequences (Genomic) shows that cDNA2–4 have a deletion. One 5′ donor splice site and three different competing 3′ acceptor splice site were found (indicated in italics). The intron contains an IRE-like structure (marked in bold). A new intron begin at position 350 in the genomic sequences and are in lowercase letters. The start codon (ATG) is indicated in italics and bold. The nucleotide positions of the sequences are shown to the left. B: Three different splice events seem to occur (indicated by the V-shaped lines). cDNA1 is unspliced and keeps the IRE-containing intron. cDNA2–4 have lost the intron and thereby also the IRE. The sequences which are identical in cDNA1–4 are marked in bolder lines. The positions of the 5′ donor splice site and the three competing 3′ acceptor splice sites are also shown.

ish the binding of the human recombinant IRP1 to the same extent as in the contrary case (data not shown).

3.3. Alternative RNA splicing of the 5′ UTR

The 5′ UTR varied between different cDNA clones. The first part of the 5′ UTR (bp 1–84) was identical, but downstream of this region to nucleotide 309, the sequences differed. To investigate this further, PCR was used to amplify the region of interest. Four different PCR products, cDNA1–4, were isolated (Fig. 2A). The PCR product of cDNA1 was 363 bp, cDNA2 231 bp, cDNA3 191 bp, and cDNA4 139 bp long. Of particular interest was the finding that only cDNA1 contains the IRE.

To clone and study the 5′ flanking region of the ferritin gene, a genomic library screening was first performed. PCR was then used to isolate this region. Comparison of the sequences of the cDNAs and the genomic sequence (Fig. 2A) revealed the existence of alternative RNA splicing, which results in diverse 5′ UTRs of the ferritin transcript (Fig. 2B). The genomic sequence was identical to cDNA1 from bp 1 to 349, and both of these sequences contain a putative 5′ donor splice site, exon-AAG↓GTACTG-intron (indicated in italics in Fig. 2A). The six first nucleotides in the 5′ donor splicing site agree with the reported consensus 5′ donor splice site ((A,C)AG↓GT(A,G)AGT) [29]. At position 350 in the genomic sequence, a new intron begins. Three competing 3′ acceptor splice sites were identified (indicated in italics in Fig. 2A). The consensus sequence for the 3′ acceptor splice site is $(C,T)_nN(C,T)AG\downarrow G$ where n=11 [29]. Generally, the 3′ acceptor splice site is more diverse than the donor site. In alternatively spliced introns it has been demonstrated that the 3′ splice site shows a poor match to the consensus sequence, both in having AAG instead of (C,T)AG at the splice site and in having a poor polypyrimidine tract flanking the splice site [30]. Both the 3′ splice sites used in cDNA3 and cDNA4, respectively, have AAG instead of (C,T)AG. All of the splice acceptor and donor sequences agree with the GT/AG rule [31]. These data suggest that cDNA1 retains the intron, while the cDNA2–4 have lost it and thereby also the IRE.

3.4. Analysis of the ferritin transcripts from different developmental stages

Total RNAs from embryos, first, second, and third instar larvae, pupae and adults (males and females), but also from different parts of the adult flies (heads, thoraces and abdomens) and from mbn-2 cells [24] were prepared. The different RNA samples were analysed by RT-PCR (Fig. 3A) and Northern blot (Fig. 3B). The RT-PCR shows that both unspliced and spliced transcripts exist in all developmental

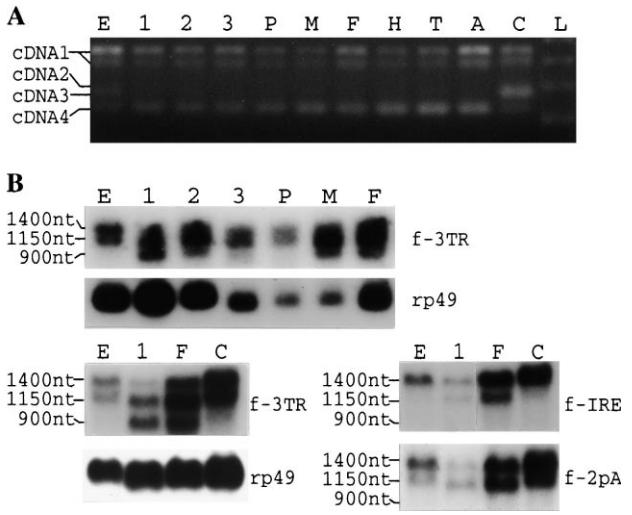

Fig. 3. Analysis of total RNA extracted from different developmental stages, separate parts of adult flies, and a cell line of *D. melanogaster*. Lane E, embryos (0–4 h); lane 1, first instar larvae; lane 2, second instar larvae; lane 3, third instar larvae; lane P, pupae (3 days); lane M, adult males (2–3 days); lane F, adult females (2–3 days); lane H, heads; lane T, thoraces; lane A, abdomens; and lane C, mbn-2 cells. A: RT-PCR was performed to amplify the alternatively spliced region of the ferritin mRNA. The PCR products were analysed by agarose (2%) gel electrophoresis. The size of cDNA1–4 is marked on the left. Lane L represent a 100 bp DNA ladder (Pharmacia Biotech). B: Northern blot analysis using probes (f-3TR, f-IRE and f-2pA) which correspond to different parts of the ferritin subunit cDNA of *D. melanogaster*. A rp49 probe was used as a control. The size of the transcript is indicated on the left and the probes used are indicated on the right.

stages, in head, thorax and abdomen of adult flies and in mbn-2 cells. The RT-PCR product, corresponding to cDNA1, appeared as two bands on a 2% agarose gel (Fig. 3A). This is probably due to formation of secondary structures of the DNA. In larvae, pupae and adults, the transcript corresponding to cDNA4 seems to be more common than transcripts corresponding to cDNA2 or cDNA3. In embryos, transcripts corresponding to the cDNA3 and cDNA4 exist in approximately the same amount, but in mbn-2 cells the cDNA3 transcript seems to be more common than the transcripts corresponding to cDNA2 and cDNA4.

Northern blot analysis with a probe specific for the 3′ part of the translated region of ferritin transcripts (f-3TR) showed the existence of at least three different ferritin mRNAs with sizes of approximately 900, 1150 and 1400 nucleotides (nt), respectively (Fig. 3B). In the second and third instar larva, and in the different adult samples, the 1150 nt transcript was commoner than the other two. The amount of the 1400 nt transcript was approximately the same as the 900 nt transcript in second instar larvae and adults, while it was more abundant in third instar larvae. In first instar larvae the 900 nt transcript was slightly more common than the 1150 nt transcript, and twice as common as the 1400 nt transcript. The mbn-2 cells had the highest concentration of the 1400 nt transcript, followed by the 1150 nt transcript and the concentration of the 900 nt transcript was significantly lower. Only the two largest transcripts were detected in embryos and pupae. The amounts of the two transcripts were approximately the same in pupae, whereas the amount of the 1400 nt transcript was more abundant than the 1150 nt transcript in embryos.

To investigate the result from the first Northern blot analysis further, probes specific for the unspliced mRNAs (cDNA1) and for mRNAs having a poly(A) tail after the second putative polyadenylation signal were used. The cDNA1-specific probe (f-IRE) detected the 1400 nt transcript in all samples, but also the 1150 nt transcript in most of the samples (some samples shown in Fig. 3B). The larger transcript was more abundant in all samples, except in first instar larvae. In this case the amount of the two transcripts were approximately the same. The 1150 nt transcript showed approximately the same distribution among the developmental stages as the 900 nt transcript described above, i.e. was absent (or very uncommon) in embryos and pupae, present at a low amount in third instar larvae and in mbn-2 cells, present in second instar larvae, and abundant in first instar larvae, and in adults. The probe corresponding to the 3′ terminal part of the cDNA (f-2pA) detected both the 1150 and the 1400 nt transcripts in all samples (some samples shown in Fig. 3B). The 1400 nt transcript was twice as common than the 1150 nt in embryos, whereas the amount of the smaller was nearly twice the amount of the larger transcript in first instar larvae. The mbn-2 cells also contained more of the larger transcript. The amounts of the two transcripts within each of the other samples were approximately the same. The 900 nt transcript was not detected by either the cDNA1-specific probe or the probe specific for the last part of the 3′ UTR, showing that none of this regions is present in the smallest ferritin transcript.

4. Discussion

The cDNA sequence of the *Drosophila* ferritin shares 99.4% identity at the DNA level with the recently published sequence reported by Charlesworth et al. [32]. The deduced amino acid sequences show 100% identity and correspond to the 24 and/or the 26 kDa ferritin subunit of *D. melanogaster* [32]. The resemblance between the sequences suggest that these are cDNAs corresponding to the same gene, but different alleles. Many insect ferritins are glycosylated and a putative glycosylation site (N-A-S) starting at residue 54 in the deduced amino acid sequence of the cDNA was found. The cDNA sequence reported here has a 3′ UTR that is 229 nucleotide residues longer than the 3′ UTR reported by Charlesworth et al. [32]. The 3′ UTR has two different putative poly(A) signals. One of the phage cDNA clones contains a poly(A) region 21 nt downstream of the first poly(A) signal. This is in the range of 11–30 nt, which are normally found downstream of the signal [33], and this suggests that both signals may be used. The gene encoding human ferritin H also has two alternative poly(A) signals, which are used in a tissue- and age-specific way [34]. By analysing the 5′ UTR for secondary structures, an IRE-like stem-loop structure was identified. We demonstrated in vitro that this IRE binds to human recombinant IRP1 [23]. This binding is specific because excess of cold transcripts containing human IRE were able to reduce the binding of the radiolabelled transcript, whereas the same amount of cold transcript containing a mutated non-functional human IRE was unable to do this.

Comparison of the 5′ UTR from different clones, derived from the phage library, we found that this region differed between some clones. This diversity was further investigated by performing PCR on cDNA and cloning of the genomic

sequence of the 5′ flanking region of the ferritin gene. The PCR resulted in four subset of cDNAs (corresponding to cDNA1–4 in Fig. 2) and this suggests that ferritin subunit transcripts with at least four different 5′ UTR exist in *D. melanogaster*. Further evidence for the presence of a set of ferritin transcripts in *Drosophila* was obtained from the database of expressed sequence tags (dbEST). By comparing the cDNA1–4 with the dbEST, we found sequences corresponding to cDNA1, cDNA3 and cDNA4. Alignment of the sequences of the PCR products and the genomic sequence reveals the existence of alternative RNA splicing of the 5′ UTR of the ferritin transcript. Alternative RNA splicing is usually used to alter the open reading frame of a transcript, which will result in different translational products. In this case, the alternative splicing only affects the 5′ UTR. Interestingly, only cDNA1 contains an IRE. All of the spliced cDNAs (cDNA2–4) have lost their IRE-containing intron. These transcripts will be insensitive to the iron-controlled regulation by the IRE/IRP system. As a consequence, cells containing ferritin transcripts without the IRE intron should be able to express ferritin even when the cellular iron concentration is low.

Total RNAs from different developmental stages of *D. melanogaster*, but also from separate parts of adult flies and from the *Drosophila* haemocyte cell line, mbn-2, were analysed by RT-PCR and Northern blot. The RT-PCR showed that both unspliced and spliced ferritin transcripts exist in all analysed samples. The Northern blot analysis showed that at least three different ferritin transcripts are present. The size of the largest transcript (1400 nt) is similar to the size of the cDNA reported here. The other two transcripts may be generated by alternative splicing and/or differential polyadenylation. A transcript with a 5′ UTR corresponding to cDNA4 is 224 nt shorter than an unspliced transcript, and a transcript with a poly(A) tail after the first poly(A) site is 241 nt shorter than a transcript with a poly(A) tail after the second site. Therefore, the smallest transcript (900 nt) may be generated by both splicing of the 5′ UTR and using the first poly(A) signal, because these two events will give a transcript with approximately the same size as the transcript detected in the Northern blot, while the middle transcript (1150 nt) may be generated by either splicing of the 5′ UTR or using the first poly(A) signal. Further Northern blot analysis with probes specific for unspliced transcripts or transcript containing the very end of the 3′ UTR supported these events. The Northern blot analysis suggests that the splicing event does not change drastic during development, except between the embryonic and the first instar larval stages. Instead, the splicing of the ferritin 5′ UTR may be tissue-specific in *Drosophila*. The amount of the smallest transcript (900 nt) compared to the other transcripts varied significantly among different developmental stages. The same pattern was seen for the 1150 nt transcript in the Northern blot analysis with a cDNA1-specific probe, and this may be due to the fact that the two poly(A) signals are alternatively used in an age-specific way as in humans [34]. If this is the case, the utilisation of the different polyadenylation sites varies significantly during the development of *D. melanogaster*, e.g. the first poly(A) site is only commonly used in first instar larvae and in adults, and is not or unusually used in embryos and pupae.

Eight different mRNAs encoding the same ferritin subunit seem to exist in *Drosophila*, and this diversity may be a valuable advantage for the organism to be able to regulate the expression of the ferritin subunit. The distribution of apo- and holoferritins has been studied in insects including *D. melanogaster* [1]. The differences in the expression of ferritin in different tissues could be due to regulatory events, both at the transcriptional and at the posttranscriptional level. However, considering the findings reported here, it is likely that cells in ferritin-rich tissues, e.g. midgut, pericardial cells, and malpighian tubules, and/or in the fat body, which synthesised the haemolymph ferritin, contain more of the spliced transcripts without the IRE intron than the unspliced transcript, and that most of the expression of ferritin in these cells is not regulated at the translational level by the IRPs. The importance of this is still unknown, but perhaps certain insects need a continuous high amount of stored apoferritin as a buffer to be able to rapidly take care of excess iron. Another explanation may be that ferritin of insects has more functions than that of vertebrates, such as iron transport and excretion. Furthermore, the secreted ferritins of snail and *Schistosoma mansoni* have been shown to function as yolk storage protein and the transcripts encoding these proteins lack IREs [10,11].

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