ELSEVIER

Gene 256 (2000) 35–42

GENE
AN INTERNATIONAL JOURNAL ON GENES, GENOMES AND EVOLUTION

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dfh is a *Drosophila* homolog of the Friedreich's ataxia disease gene

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Received 18 January 2000; received in revised form 3 July 2000; accepted 25 July 2000
Received by M. D'Urso

Abstract

A putative *Drosophila* homolog of the Friedreich's ataxia disease gene (*FRDA*) has been cloned and characterized; it has been named *Drosophila* frataxin homolog (*dfh*). It is located at 8C/D position on X chromosome and is spread over 1 kb, a much smaller genomic region than the human gene. Its genomic organization is simple, with a single intron dividing the coding region into two exons. The predicted encoded product has 190 amino acids, being considered a frataxin-like protein on the basis of the sequence and secondary structure conservation when compared with human frataxin and related proteins from other eukaryotes. The closest match between the *Drosophila* and the human proteins involved a stretch of 38 amino acids at C-terminus, encoded by *dfh* exon 2, and exons 4 and 5a of the *FRDA* gene, respectively. This highly conserved region is very likely to form a functional domain with a β sheet structure flanked by α-helices where the sequence is less conserved. A signal peptide for mitochondrial import has also been predicted in the *Drosophila* frataxin-like protein, suggesting its mitochondrial localization, as occurs for human frataxin and other frataxin-like proteins described in eukaryotes. The *Drosophila* gene is expressed throughout the development of this organism, with a peak of expression in 6–12 h embryos, and showing a spatial ubiquitous pattern from 4 h embryos to the last embryonic stage examined. The isolation of *dfh* will soon make available specific *dfh* mutants that help in understanding the pathogenesis of FRDA.

Keywords: *Drosophila*; Frataxin-like proteins; Friedreich's ataxia; Model organism

1. Introduction

Friedreich's ataxia (FRDA), an autosomal recessive disease, is the most frequent form of inherited ataxias in the Caucasian population. The estimated prevalence of this condition is approximately 1 in 50 000, with a carrier frequency of 1 in 100 in the European populations (Winter et al., 1981; Romeo et al., 1983; López-Arlandis et al., 1995). FRDA is characterized by ataxia of all four limbs, dysarthria, muscle weakness and sensory loss as a consequence of degenerative atrophy of the posterior columns of the spinal cord (Harding, 1981). The cervical region is most severely damaged with loss of large cells in the dorsal root ganglia, and loss of large myelinated axons in the peripheral nerve (Ouvrier et al., 1982). Hypertrophic cardiomyopathy is also found in most patients (Harding and Hewer, 1983). Onset of the disease is typically in late childhood, almost always before age 25 (Harding, 1981).

The genetic defect in FRDA is an abnormal expansion of a polymorphic GAA repeat located in the first intron of the X25 gene (*FRDA* by the Human Genome Organization nomenclature). It is composed of seven exons spread over 85 kb of genomic DNA on 9q13 (Campuzano et al., 1996). Most FRDA patients are homozygous for the expanded alleles, and the rest of them (4%) are compound heterozygous carrying the GAA expansion and rare point mutation alleles (Monrós et al., 1997). The expansion mutation causes

Abbreviations: bp, base pair (s); CNS, central nervous system; *D.*, *Drosophila*; *dfh*, *Drosophila* frataxin homolog; EST, expressed sequence tag; FRDA, Friedreich's ataxia; GCG, genetics computer group; kb, kilobase(s); kDa, kiloDaltons; ORF, open reading frame; RACE, rapid amplification of cDNA ends; SDS, sodium dodecyl sulfate; STS, sequence tagged site; *YFH1*, yeast frataxin homolog.

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0378-1119/00/$ - see front matter
PII: S0378-1119(00)00343-7

a severe reduction of the product encoded by the *FRDA* gene (Cossée et al., 1997; Bidichandani et al., 1997; Campuzano et al., 1997), a mitochondrial protein named frataxin (Campuzano et al., 1996, 1997). The exact biological function of frataxin is not well known, but frataxin-like proteins have been described in other species, such as mouse (Koutnikova et al., 1997) and yeast (Babcock et al., 1997; Wilson and Roof, 1997), and suggested for *C. elegans* (Campuzano et al., 1996). It has been possible to detect a highly conserved segment of the frataxin molecule, defining what may be an important functional domain of the protein. The conserved region corresponds to that encoded by exons 3, 4 and 5a of the human frataxin (Pandolfo and Koenig, 1998). Frataxin homologs will be identified in other lower eukaryotes, since a distant homolog has been described in purple bacteria (Gibson et al., 1996).

As demonstrated for an important number of human genes, analysis of their homologs in model organisms has provided a powerful tool to investigate their functions (Mounkes et al., 1992; Basset et al., 1996; Hahn et al., 1996). In the case of *FRDA*, the first insights about its possible function came from yeast. Knockout of the yeast frataxin homolog (*YFH1*), which also encoded a mitochondrial protein, revealed that it is involved in iron homeostasis and respiratory function (Babcock et al., 1997; Wilson and Roof, 1997). Among model organisms with powerful genetics such as yeast is the fruit fly *Drosophila*. In this species a high number of different genetic tools have been developed and they can be applied for the full characterization of any gene. The isolation of the frataxin homolog in *Drosophila* will allow the use of such strategies in order to investigate the biological function of frataxin in a pluricellular organism with the advantage of easy manipulation and culture. Here we describe the cloning and characterization of the *Drosophila* homolog of the *FRDA* gene.

2. Materials and methods

2.1. Probes

All DNA and RNA probes used in this work were labeled with digoxigenin-11-dUTP (Boehringer Mannheim). Detection of hybrids was performed by colorimetric or chemiluminescent methods according to the manufacturer's protocols.

2.2. Cloning of dfh

A *Drosophila subobscura* genomic library in λEMBL4 was used. 9×10^4 clones were plated, hybridized with the human X25 probes, EST126314 and a 5′ RACE product (Campuzano et al., 1996) and washed at a final stringency of $0.5 \times$ SSC/0.1% SDS at 50°C. Positive clones were analyzed by Southern blot. *Eco*RI, *Hin*dIII and *Pst*I fragments hybridizing to the X25 probes were subcloned in pUC19 and sequenced.

In order to identify *dfh* full-length transcripts, an oligo(dT)-primed *Drosophila melanogaster* cDNA library in λZAPII, made from adult individuals (Stratagene), was screened. Half a million clones were analyzed and hybridized with the *D. melanogaster* X25 open reading frame (ORF) isolated from the cosmid 125a12. This cosmid was provided by the European *Drosophila* Genome Project.

DNA sequencing was carried out automatically by Medigene (Munich, Germany) and by Servei de Seqüenciació de la Universitat de València (Valencia, Spain). Computer-assisted sequence analyses and database searches were conducted with the appropriate program of the GCG software package.

2.3. Southern and Northern blots

2.3.1. Southern blots

Genomic DNAs from *D. algonquin*, *D. melanogaster* and *D. subobscura* were isolated as described previously (Junakovic et al., 1984). Samples of DNA (2 μg) were digested with *Eco*RI, electrophoresed on 0.8% agarose gels and blotted onto nylon membranes. Hybridization was performed using the human X25 probes under the same conditions as described in Section 2.2.

2.3.2. Northern blots

Polyadenylated RNA was isolated from embryos (0–6 h, 6–12 h and 12–24 h), third larval instar, pupae and adults of *D. melanogaster*, by using a quickprep protocol (Pharmacia) and following the manufacturer's specifications. 6 μg of polyA$^+$ RNA from each stage was loaded onto a 33% formaldehyde agarose gel and blotted according to Rueger et al. (1996). Filters were hybridized with *D. melanogaster* X25 in 50% formamide at 68°C. Washes were carried out at 65°C in $0.1 \times$ SSC. Filters were exposed to X-ray film for 4 h. An actin probe was also hybridized as a control for RNA loading. In order to measure the intensity of the hybridization signals, densitometric analysis was performed using the Intelligent Quantifier 2.1 program (Bioimage).

2.4. In situ hybridization

In situ hybridization on polytene chromosomes of *D. melanogaster* was carried out following the method described in de Frutos et al. (1990).

Whole mount in situ hybridization to embryos from 2 to 16 h was performed according to O'Neil and Bier (1994). Digoxigenin labeled riboprobes were made from DAM1 cDNA clone. Sense probe was used as a control of this experiment.

3. Results

3.1. Cloning and sequence analysis of Drosophila frataxin homolog

Southern blot analysis performed in several *Drosophila* species using human X25 probes (Campuzano et al., 1996) showed that the *FRDA* gene is conserved in *Drosophila* (data not shown). In order to isolate a X25 homolog in this organism a genomic library from *D. subobscura* was screened. Three of the clones identified were partially sequenced and an ORF of 558 bp was predicted. The putative protein encoded by this ORF showed a high similarity to the human frataxin. We also found, by database searches, a sequence homologous to the *D. melanogaster* STS 125a12 (accession number E32434, Fig. 1). It has been mapped at 8C/D region on the X chromosome of this species and cloned in the cosmid with the same name as the STS. *Eco*RI and *Hin*dIII cosmid fragments hybridizing to the *D. subobscura* X25 ORF were subcloned and sequenced. A 573 bp ORF with strong similarity to that found in *D. subobscura* was identified in *D. melanogaster*, predicting a protein product with high sequence similarity to frataxin protein, as expected. On the basis of these homologies, we therefore named the *Drosophila* gene *Drosophila* frataxin homolog (*dfh*), following the same nomenclature applied for the yeast counterpart (Babcock et al., 1997; Wilson and Roof, 1997). The *Drosophila* X25 ORF and its genomic flanking sequences are deposited in the GenBank with accession numbers AF209098 for *D. subobscura* and AF208492 for *D. melanogaster*. In *D. melanogaster* this sequence coincides with the predicted gene CG8971, recently annotated by the *Drosophila* Genome Project.

D. subobscura X25 ORF has the putative initiation methionine at position 631 and the first in-frame stop codon (TAG) at nucleotide 1254 (Fig. 1), yielding a protein of 185 amino acids. For *D. melanogaster*, two in-frame ATG codons were identified, but the first one, at position 1222, corresponds to the postulated start codon of the *D. subobscura* X25 ORF. The first in-frame stop codon (TAA), in *D. melanogaster*, was found at nucleotide 1863 (Fig. 1), yielding a putative product of 190 amino acids. In both cases a single intron was predicted. It extended from position 933 to 997 and from position 1539 to 1608 in *D. subobscura* and *D. melanogaster*, respectively. We identified, in both *Drosophila* sequences, several transcription signals (Fig. 1) such as putative CAAT boxes (position 291 and 379 in *D. subobscura*; 819 and 862 in *D. melanogaster*) and a putative TATA box (position 411 in *D. subobscura*; 1058 in *D. melanogaster*) and a binding site for the zinc finger transcriptional activator YY1/UCRB (Natesan and Gilman, 1993) at position 205 in *D. subobscura* and 875 in *D. melanogaster*.

We looked for a full-length *dfh* cDNA by screening an adult cDNA library from *D. melanogaster* using genomic probes encompassing the X25 ORF of this species. Two independent but overlapping clones were isolated, DAM1 and DAM7, with 0.9 and 0.5 kb, respectively (Fig. 1). The first one contained the entire *D. melanogaster* X25 ORF identified in the 125a12 cosmid, meanwhile DAM7 was truncated at the 5′ end. DAM1 (GenBank Accession No. AJ002208) and DAM7 (GenBank Accession No. AF208491) represent two alternative transcripts of *dfh* depending on the use of two different polyadenilation signals, located 27 bp (DAM7) and 259 bp (DAM1) downstream of the stop codon, not affecting in any case the encoded product.

When comparing *dfh* cDNA with the corresponding genomic sequence, we confirmed the boundaries and size of the *dfh* intron. It is 69 bp long, dividing the coding sequence of *D. melanogaster* in exon 1 with 340 bp, and exon 2 with 282 or 551 bp depending on the alternative transcript. Since we did not look for *dfh* cDNA in *D. subobscura*, we inferred the gene organization in this species by comparison with *D. melanogaster*.

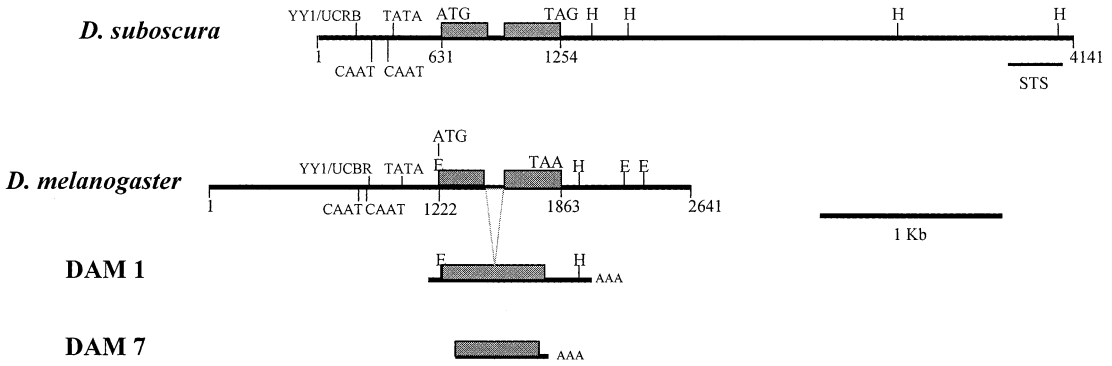

Fig. 1. Genomic region of *dfh* locus in *D. subobscura* and *D. melanogaster*. DAM1 and DAM7 are alternative *dfh* transcripts of the latter species. The corresponding sequences are deposited in the GenBank with the accession number indicated in Section 3.1. Coding regions are shown by filled boxes and the intron by a thick line. STS indicates the position of the *D. subobscura* sequence homologous to *D. melanogaster* STS125a12. E, *Eco*RI and H, *Hin*dIII restriction sites.

Two exons of 303 and 570 bp, respectively, are separated by a 66 bp intron in *D. subobscura*; this confirms the prediction made from the splicing signals found in genomic DNA. No alternative forms for the latter exon were predicted in this species, since only one polyadenilation signal was found.

All these data show that *dfh* is spread over a 1 kb region approximately, much smaller in size than in the case of the human gene. In situ hybridization on polytene chromosomes of *D. melanogaster*, using the *dfh* cDNA as probe, confirmed that this gene is located on the *Drosophila* X chromosome at 8C/D section (Fig. 2).

3.2. The predicted dfh protein and alignment with other frataxins

The *D. melanogaster dfh* cDNAs are predicted to encode a protein of 190 amino acids with a molecular weight of 21 kDa. The *D. subobscura* X25 ORF encodes a potential product with similar molecular weight but different in length (185 amino acids). This is due to the differences observed in the *dfh* coding region of both species. Codons 10, 11 and 39–41 of *D. melanogaster dfh* protein were deleted in *D. subobscura* (Fig. 3). When comparing the amino acid sequence of the potential *dfh* protein (DFH) of both species, an interesting feature was also observed. The degree of sequence identity was different along the protein, it being possible to identify two different regions. The C-terminal two-thirds of the protein showed the strongest homology, sharing 77% identity (87.5% similarity) between the two *Drosophila* species. Meanwhile the major differences were found at the N-terminus with only 15% identity (41% similarity), where the deletions/insertions cited above were also identified. A mitochondrial cleavage signal peptide was predicted in this region, using the program PSORT (Gavel and von Heijne, 1990; Nakai and Kanehisa, 1992). The putative cleavage site was located at amino acid position 58 in *D. melanogaster* and at position 53 in *D. subobscura*, giving a potential processed product of 133 amino acids in both cases.

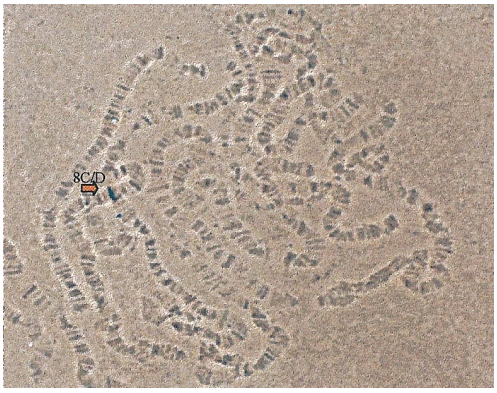

Fig. 2. In situ hybridization on *D. melanogaster* polytene chromosomes using *dfh* cDNA as probe. A single hybridization signal is observed at 8C/D section on X chromosome.

Secondary structure predictions using the NNSSP program (Yi and Lander, 1993) suggested, in both *Drosophila* species, the presence of putative β sheet regions interspersed between residues 105 and 140, which were flanked by an α-helical structure around amino acids 40 to 95 and 155 to 170. A helical structure for the N-terminal 20 residues in *D. melanogaster* and N-terminal 12 amino acids in *D. subobscura* was also predicted. No transmembrane domain was determined in any case. This protein profile is very similar to that described for the human frataxin (Campuzano et al., 1996).

A BLASTP 2 search (Altschul et al., 1997) with DFH of *D. melanogaster* disclosed the highest homology to the mouse and human frataxins (BLAST probability $= 2 \times 10^{-32}$ and 2×10^{-31}, respectively), following the corresponding proteins of *C. elegans* (BLAST probability $= 1 \times 10^{-17}$) and of two species of *Saccharomyces* (*S. pombe*, BLAST probability $= 3 \times 10^{-17}$; *S. cerevisiae*, BLAST probability $= 5 \times 10^{-15}$). Multiple sequence alignments of these proteins using the CLUSTAL program (Thompson et al., 1994) showed that the *Drosophila* sequences align much better at the central and the C-terminal regions with the human frataxin and other eukaryotic homologs, meanwhile no alignment was possible in the N-terminal region (Fig. 3). The closest match involved a stretch of 38 amino acids encoded by *dfh* exon 2, and corresponding to exons 4 and 5a of the *FRDA* gene. A hydrophobic moment profile was obtained for each frataxin-like protein using the algorithm of Eisenberg et al. (1984). As we can see in Fig. 3, all proteins analyzed have the same hydrophobic pattern in the central and the C-terminal regions, where two hydrophilic peaks at position 100 and between 180 and 190 were obtained. However, this pattern was very different in the N-terminus even between the two *Drosophila* species analyzed. From these results, it could be suggested that the frataxin conserved region shows a hydrophobic nature, being flanked by more hydrophilic amino acids sequences.

3.3. Expression studies of dfh

The expression pattern of *dfh*, in *D. melanogaster*, was determined by both Northern analysis at different developmental stages, and RNA in situ hybridization on whole mount embryos. When analyzing mRNAs from different embryonic stages, third larval instar, pupae and adults, a $\sim$1 kb major transcript was detected (Fig. 4), in agreement with the predicted size of the DAM1 mRNA. This transcript was identified in all stages examined but with different intensity, detecting a peak of expression in 6–12 h embryos, stages characterized by the differentiation of most tissues. A fainter

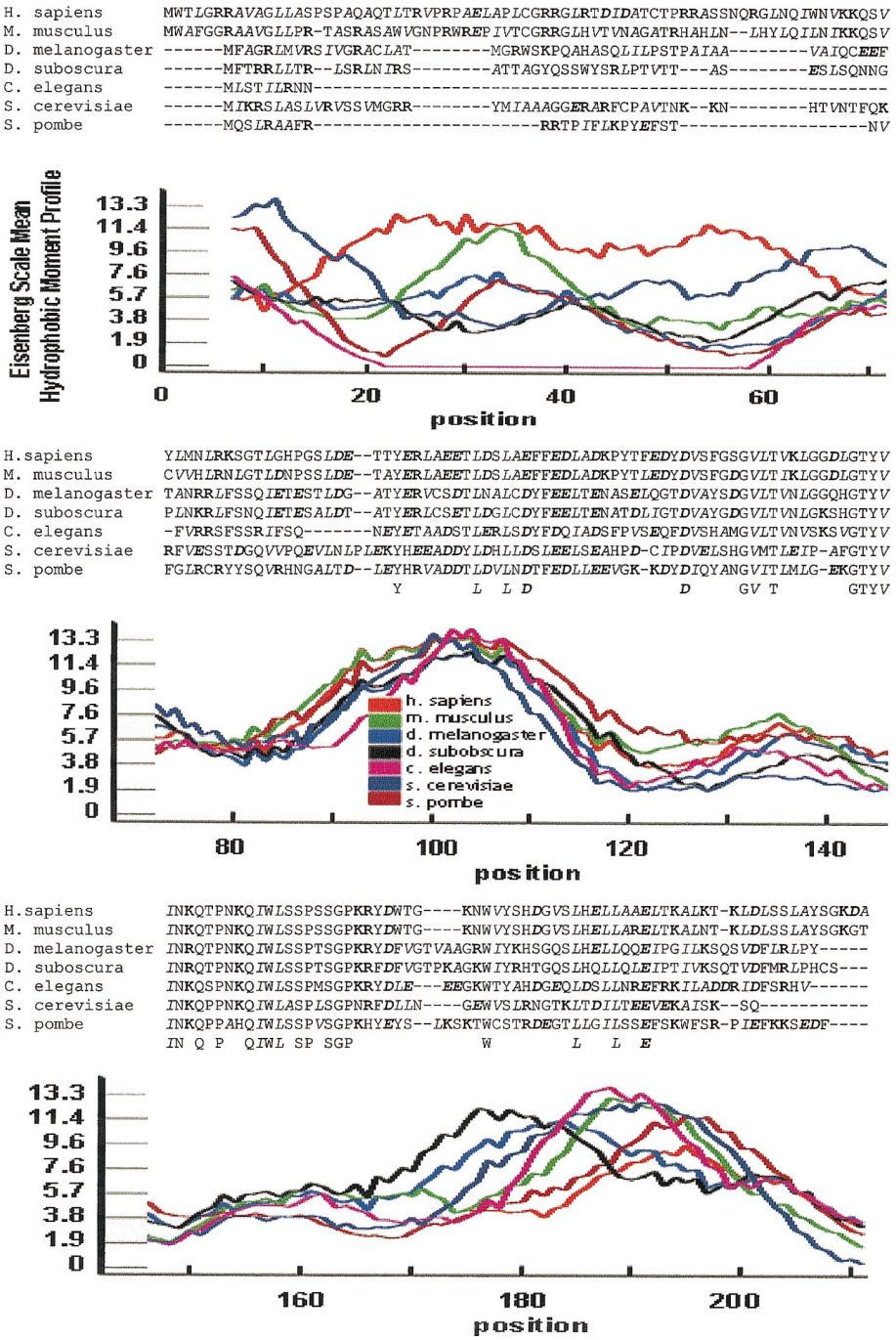

Fig. 3. Multiple alignments of frataxin-like proteins and Eisenberg hydrophobic moment profile. Basic, acidic and aliphatic amino acids are indicated in bold, in bold and italics, and in italics, respectively. A conserved region is observed from residues 132 to 169 where the invariant amino acids are indicated on the bottom. Note also the similar hydrophobic pattern in the central and C-terminus in all proteins analyzed.

band corresponding in size to DAM7 transcript was also identified in 0–6 h embryos mainly. It indicated a very low level of expression of the DAM7 mRNA throughout the development of *D. melanogaster*. In fact it was less frequent than DAM1 in the cDNA library screened. On the other hand, the existence of the DAM7 transcript was not predicted in *D. subobscura*, possibly indicating a lower biological significance than DAM1 in *Drosophila*.

RNA in situ hybridization was performed in *Drosophila* embryos from 2 to 16 h, using sense and antisense *dfh* riboprobes. Four independent in situ hybridization experiments were performed, and 200 embryos were analyzed in each case. A control experi-

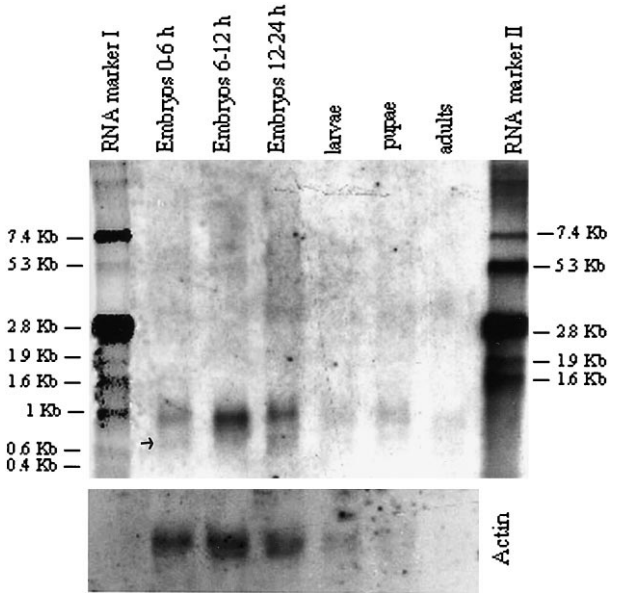

Fig. 4. Northern blot analysis of the *dfh* gene. PolyA$^+$ RNA from different developmental stages hybridized to genomic probe encompassing *dfh* coding sequences. A 1 kb major transcript (DAM1 mRNA) is detected in all developmental stages analyzed. The fainter band corresponding to DAM7 transcript is indicated by an arrow.

ment was conducted in parallel using a *Drosophila* gene which maps on X chromosome, and shows a specific expression pattern in CNS and epidermis (our unpublished results). The hybridization with the antisense *dfh* probe revealed a ubiquitous expression of *dfh* in the *Drosophila* embryos of the different stages examined (Fig. 5A–C). The intensity of hybridization signals was significantly higher than the background level detected by the sense probe (Fig. 5E). The *dfh* pattern contrasted with that obtained in the control experiment (Fig. 5D) and was first detected in 4 h embryos and persisted for the following embryonic stages analyzed.

4. Discussion

In this work a potential *Drosophila* homolog of the Friedreich's ataxia disease gene has been isolated and characterized. For this, we have cloned the orthologous genes of two *Drosophila* species, *D. melanogaster* and *D. subobscura*. Our group has been working on the latter species for several years, but the genetic knowledge of this species is much less developed than for *D. melanogaster*. To most scientists *Drosophila* means *D. melanogaster* because this species has been more extensively and intensively studied than any other, displaying a high number of well-characterized mutants with diverse phenotypes. These reasons led us to use *D. melanogaster* in this study in spite of starting with *D. subobscura*. Nevertheless, working in two related species strengthened our findings about the existence of a *Drosophila* frataxin homolog.

On the basis of the sequence and secondary structure conservation found between the predicted protein of *dfh* and the human frataxin, we concluded that the *Drosophila* gene is the homolog of the *FRDA*. Multiple alignments of DFH with frataxin and with the related protein sequences from several eukaryotes confirmed that most of the frataxin sequence has been conserved throughout evolution. Several invariant amino acids have been identified in the central and C-terminal regions of these proteins, and the highest conserved region of the frataxin sequence (Campuzano et al., 1996; Pandolfo

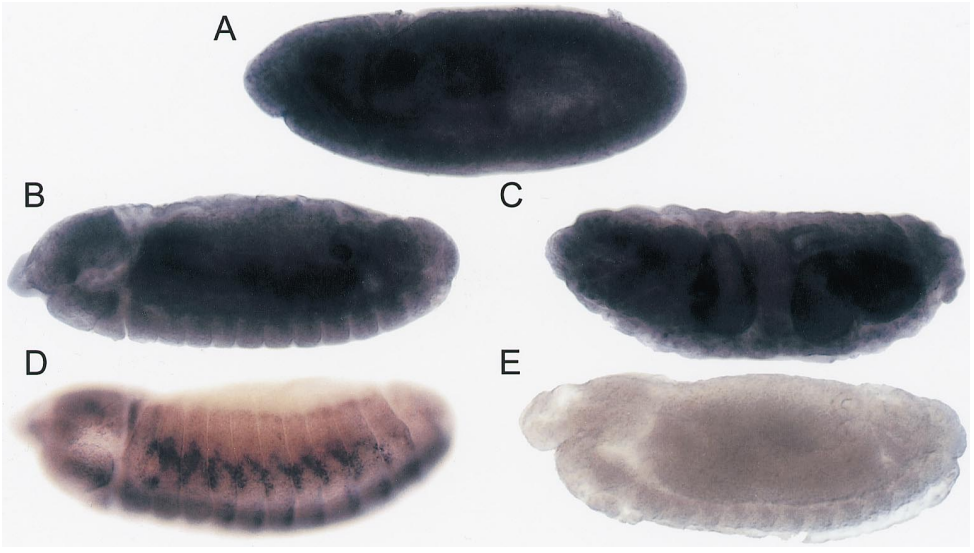

Fig. 5. RNA in situ hybridization using antisense (**A** to **C**) and sense (**E**) *dfh* riboprobes. The preparation in (**D**) was hybridized with a probe labeling principally the CNS and epidermis. (**A**) Embryos at stage 9 (3:40–4:20 h) of Campos-Ortega and Hartenstein (1985); (**B**, **D** and **E**) embryos at stage 13 (9:20–10:20 h); (**C**) embryos at stage 16 (13–14 h).

and Koenig, 1998) becomes more reliable with the identification of its *Drosophila* homolog. In a stretch of 38 amino acids encoded by exons 4 and 5a of *FRDA* gene, 33 are identical in DFH encoded by *dfh* exon 2. Secondary structure predictions for all these proteins using the NNSSP program indicated that the conserved sequence is most likely to form a β sheet flanked by α-helices, features reported previously for the human, yeast and nematode proteins (Campuzano et al., 1996; Gibson et al., 1996). This region of high sequence conservation defines what may be an important functional domain of these proteins. In fact, several FRDA patients from three Southern Italian families showed a missense mutation in this domain (Campuzano et al., 1996). Moreover, experimental data have demonstrated that this region can functionally substitute the corresponding domain of *YFH1* in yeast (Wilson and Roof, 1997).

Another frataxin domain is a mitochondrial targeting peptide at the N-terminus of the protein (Gibson et al., 1996). Computer analyses have also predicted such a signal in the yeast and mouse homologs (Koutnikova et al., 1997) and several experimental data have confirmed the organelle localization of the human and yeast frataxins (Babcock et al., 1997; Koutnikova et al., 1997; Wilson and Roof, 1997). A mitochondrial cleavage signal peptide has also been suggested for *Drosophila* in this study, indicating that DFH might be a nuclear-encoded mitochondrial protein as well. All these data support the fact that frataxin and its related proteins, where DFH is included, are members of the same conserved protein family.

It is interesting to note that when comparing DFH amino acid sequences of the two *Drosophila* species analyzed, we observed the same feature as when frataxin-like proteins from more distant species are considered. In all cases, two different regions on the protein could be identified on the basis of the sequence conservation degree along the protein. The central and C-terminal regions showed the strongest homology in all cases, sharing 77% identity between the two *Drosophila* species. The major differences were restricted at the N-terminus with only 15% identity between these two species. Despite the N-terminal sequences being so different in all species investigated, these regions showed the typical features of a mitochondrial signal peptide in all cases: a putative α-helix with abundant positively charged amino acids but rare negatively charged residues. Nevertheless, considerable constraints must also operate on the N-terminus of these proteins to assure their mitochondrial import.

Frataxin shows tissue-specific expression being high in the primary sites of degeneration in FRDA (Campuzano et al., 1996). The developmental expression of the mouse homolog has been analyzed in embryo and adults, indicating its predominance in tissues with a high rate of metabolism, but differences in the level of frataxin expression between human and mouse were also observed (Koutnikova et al., 1997). Differences between human and *Drosophila* are expected too, but surprisingly a uniform expression of *dfh* was observed in *Drosophila* embryos. Moreover, the encoded protein could be needed along all the *Drosophila* life cycle, since it is expressed from embryo to adult, including larval stages and pupation. This difference could be due to evolutionary modification of the control mechanisms. Further study on the molecular basis underlying the expression and regulation of frataxin is needed to answer these questions.

The exact function of frataxin still remains unknown. It could not initially be inferred from its amino acid sequence since no similar proteins of known function have been identified (Campuzano et al., 1996). In this regard, model organisms have been very useful in assigning function to human genes. The isolation of the yeast frataxin homolog has been crucial in the knowledge that frataxin is involved in iron homeostasis. Yeast lacking *YFH1* accumulates iron in mitochondria, resulting in oxidant damage, and loss of mitochondrial DNA and loss of mitochondrial respiratory activity (Babcock et al., 1997; Foury and Cazzalini, 1997; Wilson and Roof, 1997). These findings have suggested that free-radical toxicity is responsible for Friedreich ataxia. In recent years, *D. melanogaster* has gained widest popularity as a model organism in the study of the biological function of human genes (Banfi et al., 1996, 1997; Caggese et al., 1999). The fruit fly is a powerful system for such an analysis since any coding sequence within its genome can be mutated and analyzed, and unlike yeast within the context of a pluricellular organism with developmental patterning and differentiated tissues. Human and *Drosophila* share pathways for these processes (Krumlauf, 1992), as well as for others such as learning and behavior (Kandel and Abel, 1995) and intercellular signaling (Pawson and Bernstein, 1990). The power of genetics in this species makes it possible to identify the components of such pathways more efficiently than in more complex organisms such as mammals. In addition, eight small deficiencies and four P element insertions have been reported in the 8C/D chromosomal region where *dfh* has been mapped. These invaluable tools will help in analyzing the phenotype of *dfh* mutants and investigating the other proteins interacting with frataxin in a common biochemical pathway, which in turn would contribute to establishing the nature of the mitochondrial defect in FRDA.

Acknowledgements

We wish to thank Dr. Rosa de Frutos and Dr. Nuria Paricio for their valuable comments and discussions. We

also thank the Servicio Central de Soporte a la Investigación Experimental de la Universitat de València, for access to DNA analysis resources and databases. This work was supported by grants from the Generalitat Valenciana (GV97-VS-25-88, M.D.M.) and from the Comisión Interministerial de Ciencia y Tecnología (SAF97-0082, F.P.). J.M.B., J.C. and J.A.N. are recipients of fellowships from the Generalitat Valenciana.

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