Sequence and expression pattern of the *Drosophila melanogaster* mitochondrial porin gene: evidence of a conserved protein domain between fly and mouse

Marta Oliva[a], Angela Messina[a], Gianluca Ragone[b], Corrado Caggese[b], Vito De Pinto[a,*]

[a]*Istituto di Scienze Biochimiche e Farmacologiche, Facoltà di Scienze M.F.N., Università di Catania, viale A. Doria, 6, 95125 Catania, Italy*
[b]*Istituto di Genetica, Facoltà di Scienze M.F.N., Università di Bari, via Amendola 165/A, 70126 Bari, Italy*

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Abstract We have recently cloned a cDNA encoding mitochondrial porin in *Drosophila melanogaster* and shown its chromosomal localization (Messina et al., FEBS Lett. (1996) 384, 9–13). Such cDNA was used as a probe for screening a genomic library. We thus cloned and sequenced a 4494-bp genomic region which contained the whole gene for the mitochondrial porin or VDAC. It was found that this *D. melanogaster* porin gene contains five exons, numbered IA (115 bp), IB (123 bp), II (320 bp), III (228 bp) and IV (752 bp). The exons II, III and IV contain the protein coding sequence and the 3′ untranslated sequence (3′-UTR). The first base in exon II precisely corresponds to the first base of the starting ATG codon. Exon IA corresponds to the 5′-UTR sequence reported in the published cDNA sequence. Exon IB corresponds to an alternative 5′-UTR sequence, demonstrated to be transcribed by 5′-RACE experiments. The exon-intron splicing borders and the length of the exon III perfectly match a homologous internal exon detected in the mouse genes. Such exon encodes a protein domain predicted by sequence transmembrane arrangement models to contain major hydrophilic loops and it is thus suspected to have a conserved distinct function. In situ hybridization experiments confirmed the localization of the genomic clone on the chromosome 2L at region 32B3-4. Together with genomic Southern blotting at various stringencies, the same experiment did not confirm the presence of a second genetic locus on *D. melanogaster* chromosomes. Northern blots demonstrated that the porin gene is a housekeeping one: three messages of approx. 1.2–1.6 kbp are transcribed in every fly developmental stage that was studied. They were shown to derive by an alternative usage of different promoters and polyadenylation sites.

Key words: Porin-pore; Gene; 5′-untranslated region; Protein domain; Alternative transcript; Hydrophilic loop; *Drosophila melanogaster*

1. Introduction

Voltage-dependent anion channels (VDACs) or mitochondrial porins are integral membrane proteins first discovered in the outer mitochondrial membrane where they act as pore-formers [1–5]. The application of DNA molecular techniques to this bioenergetic topic as well unravelled the existence in one and the same species of more cDNAs, and thus transcripts, slightly differing in their amino acid sequences [6–11]. The recent report of the structure and the expression patterns of three different porin genes in mouse confirmed at a molecular level the existence of isoforms [12]. On the other hand, in the many species where porin was isolated [4–9], usually one main protein form (in human: porin31HL [6], also called HVDAC1 [10,11]) was purified and functionally characterized. Immunohistochemical techniques raised the surprising question if this same polypeptide was also localized in the plasma membrane. In human B-lymphocytes, porin was indeed shown in the plasma membrane by indirect immunofluorescence staining [13,14]. Other evidence about the presence of porin in the plasmalemma were independently obtained in various laboratories from different biological sources and with different experimental approaches [15–17].

A differentiated utilization of the various gene products is the obvious hypothesis for the 'porin-redundancy' in the cell. In analogy with bacteria, more porins may be necessary to maintain the outer mitochondrial membrane always permeable. This was shown in yeast porin-null mutants [18,19]. Furthermore, the first discovered porin-deficient human patient showed lack of porin in muscle, where mitochondria were still, but slowly, working, postulating some 'rescue' channel in the outer membrane [20,21]. Another possibility is a different subcellular localization of porin isoforms. Such a hypothesis appeared unlikely based on experimental evidence obtained in mammals. Yu et al. [22] introduced in the isoform cDNA sequences directing the insertion of known epitopes in each protein. Transfection of cultured cells by the modified HVDACs cDNAs provided a convenient way to individually tag each isoform. Experiments with antibodies against the tagged proteins showed that all three isoforms were exclusively localized in mitochondria [22].

In this work we show the structure of the *Drosophila melanogaster* porin gene, pointing out the surprising similarities to the more evolved mouse genes, and we present a novel characteristic of such a polymorphic molecular system consisting of the alternative transcription of the 5′-UTR mRNA region. This finding and its relationship to 'porin redundancy' will be discussed.

2. Materials and methods

2.1. Isolation of porin genomic clones and DNA sequencing

A published *D. melanogaster* cDNA clone encoding porin (7T21; acc. no. X92408; [23]) was used as a probe to screen a genomic library in EMBL3 derived from Canton-S strain. DNA sequencing was accomplished using the dideoxy chain termination method with Sequenase version 2.0.

*Corresponding author. Fax: (39) (95) 337195.
E-mail: e113vd01@area.ba.cnr.it

Abbreviations: RACE, rapid amplification of cDNA ends; UTR, untranslated region; VDAC, voltage dependent anion channel; HVDAC, human VDAC; MVDAC, mouse VDAC

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2.2. 5′- and 3′-RACE-PCR experiments

D. melanogaster Oregon-R third instar larvae poly(A)$^+$ RNA were used for 5′-RACE-PCR experiments. The RACE system by Boehringer was used, following the manufacturer's protocol. 5′-end PCR amplification of the cDNA was performed using two nested primers (5′-ACGAGGTCTTGGTCTTCA-3′ and 5′-TGCCCAAATCGCTGTATGA-3′). The amplified PCR products were subcloned into a T-vector plasmid (Promega) and sequenced. 3′-end PCR amplification was performed using a single primer (5′-GTGAACAACGCCAGCCAGGTG-3′).

2.3. Southern, Northern blotting and in situ hybridization to polytene chromosomes

Southern experiments on fly genomic DNA and hybridization with an amplified probe corresponding to the porin coding sequence were performed by standard procedures. The amplified probe corresponding to the protein coding sequence of porin was obtained by PCR experiments with the primers termed DmPorATG (5′-CGCGAATTCCATATGGCTCCTCCATCATACAG-3′) and DmPorTAA (5′-CGCAAGCTTTTATTAGGCCTCCAGCTCCAG-3′) containing the starting and the stop codon in the cDNA sequence. Poly(A)$^+$ RNA was selected using oligo-(dT) cellulose chromatography. RNA concentration was estimated by A_{260nm} and by an internal standard, a DNA coding rp49 [24]. In situ hybridization to polytene chromosomes was performed essentially as described in [25], modified for non-radioactive biotinylated probes.

3. Results

3.1. The D. melanogaster porin gene structure

We used the cDNA termed 7T21, containing the whole transcript sequence for *D. melanogaster* porin (1370 bp), to screen an EMBL3 genomic library of the same organism. The clone EM1T was chosen for further analysis. Subcloned fragments of this genomic clone were sequenced, until the sequence corresponding to the full length cDNA [23] was identified. In Fig. 1 the position of the sequenced genomic subclones is shown. Furthermore Fig. 1 shows the structure of the genomic sequence. The whole sequence of the 4494-bp *D. melanogaster* genomic fragment containing the mitochondrial porin gene was stored in the EMBL database (acc. no. AJ000880).

The exons of porin were identified by comparison of the genomic sequences with the cDNA. The consensus rules for splice sites that predict conservation of the dinucleotides GT and AG, respectively, next to the 5′ and 3′ boundaries of the introns were fulfilled. In this way it was found that the *D. melanogaster* porin gene contained four exons separated by three introns. The beginning of the first exon was deduced by 5′-RACE-PCR experiments which elongated the cDNA 7T21 by 41 bp. The 5′-UTR homologous regions present slight dissimilarities between the cDNA and that present in the genome. Four nucleotides, three corresponding to positions 566–568 in the genomic sequence and an A located just prior to the starting ATG, were missing in our cDNA.

5′-RACE-PCR experiments were performed on *D. melanogaster* Oregon-R third instar larvae poly(A)$^+$ RNA, first transcribed by reverse transcriptase. Nested primers were designed in the DNA protein-coding region. Several positive clones were detected and sequenced. Sequence analysis showed the expression of two different 5′-untranslated extensions fused to the coding sequence. The ratio between the two 5′-UTRs was 10:1 in favor of the 5′-UTR corresponding to the cDNA 7T21 sequence; surprisingly the second sequence corresponded to another genomic region, enclosed between exon I and II. Furthermore this sequence was followed by the -GT splice-site canonical sequence. Both this observation and the finding of the transcribed message among the 5′-RACE clones suggested to us that such a message, alternative to the most usual one, could be really transcribed in the cell.

The exon corresponding to the cDNA was indicated as exon IA, while the new exon was named exon IB. Exon II (320 bp) and exon III (228 bp) exclusively contain coding sequences. In particular, the first base in exon II precisely corresponds to the first base of the starting ATG codon. Exon IV contains the remaining 298 coding bp, together with the stop triplet TAA and the 3′-UTR sequence.

Two putative polyadenylation sites were detected in the 3′-untranslated region at positions 3607 and 3904. 3′-RACE-PCR experiments and Northern blotting experiments with partial internal probes (Fig. 4B) confirmed the utilization of both these two alternative polyadenylation sites; cDNAs with different lengths in this portion of the message were reported too [23,26].

Promoter analysis was especially intriguing due to the low over-representation of specific sequences in *Drosophila* promoters: i.e. about one-half of *Drosophila* promoters does not contain a recognizable TATA box at the appropriate po-

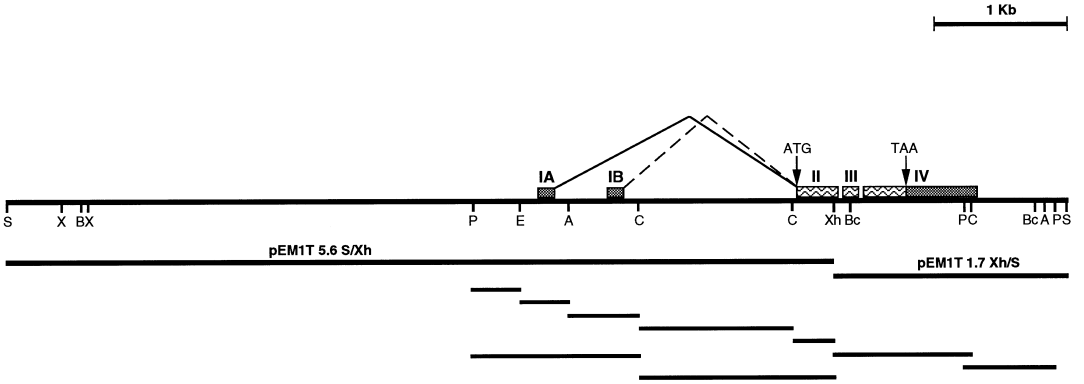

Fig. 1. Structural organization of *D. melanogaster* porin gene (clone EM1T). Exons are shown as boxes above the line and are numbered IA (486–600), IB (1014–1137), II (2485–2805), III (2881–3108), IV (3178 to the end). The positions of the ATG starting codon and of the TAA stop codon are evidenced. Exon II precisely starts with the ATG. Coding regions are clearer, while non-coding regions are shadowed. ER sites are indicated (A: *Acc*I; B: *Bam*HI; Bc: *Bcl*I; C: *Cla*I; E: *Eco*RI; P: *Pst*I; S: *Sal*I; X: *Xba*I; Xh: *Xho*I). IA and IB are two alternative leader exons. Under the gene structure the clones sequenced are reported. A *Sal*I/*Sal*I clone of about 7 kbp from an EMBL3 *D. melanogaster* genomic library was analyzed.

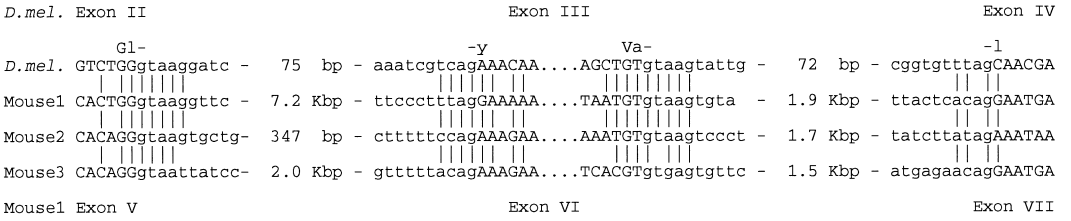

Fig. 2. Comparison of the genomic boundaries of the *D. melanogaster* exon III to corresponding exons in mouse genes. The sequence of exon-intron boundaries determined in the *D. melanogaster* porin gene were compared to those from three mouse genes [12]. Exon and intron sequences are shown in upper- and lowercase letters, respectively. The length of introns and the names of the exons bordered by introns are shown. Amino acids are also shown above the respective interrupted codons. A consensus bar was placed every time an identity among the *D. melanogaster* gene sequence and at least two mouse gene sequences were detected.

sition [27]. Concerning exon IA, a TATA sequence is placed at 441, but its position is −45 with respect to the transcription starting site indicated in this work; also a CAAT sequence was found on the reverse strand at 405 (−80). A CCAAT, also on the reverse strand, was found at 920 (−84 with respect to the transcription starting site of exon IB); between this location and the IB transcription starting site two regions rich in GC (933 and 946), but without canonical sequences, and no putative TATA-box were observed (Fig. 1B).

A neural network promoter prediction (NNPP) on the whole genomic sequence was performed (at the site www.hgc.lbl.gov/projects/promoter.html). NNPP is a method that finds promoters in a DNA sequence; the program is defined as a time-delay neural network consisting of two layers, one recognizing the TATA-box and one recognizing the 'Initiator' spanning the transcription start site. The two-layers analysis is combined in one output unit, which gives scores between 0 and 1 [28]. Sequences between 431 and 481 and between 929 and 979, respectively, gave scores of 0.84 and 1.00. Such sequences are located just before the 5′-RACE predicted transcription starting points. The program predicted a few other putative promoter sequences and especially one placed between 104 and 154; but, interestingly, the highest score (1.00) was found for the promoter located just before the exon IB, which codes the alternative 5′-UTR.

3.2. Porin exon/intron boundaries

According to the exon/intron organization, the protein structure of the *Drosophila melanogaster* porin can be divided into three amino acidic portions of similar size (107, 76 and 99 amino acids). A model of the transmembrane arrangement of the eukaryotic porin has been proposed in previous papers; it is based on the sensitivity of protein epitopes to peptide-specific antibodies and on the cleavability of specific sites, in addition to computer-based secondary-structure predictions [29–31]. Such a model consists of 16 transmembrane amphipathic β-strands forming the pore walls and connected by generally short loops; it stresses the analogy of the mitochondrial porin with the elucidated bacterial porin crystalline structures. Detection of likely transmembrane β-strands using the Gibbs sampler confirmed that the predicted analogy with the bacterial porins is meaningful [32]. The predicted N-terminal amphipathic α-helix [4] is the only structural feature distinct from eukaryotic porins in comparison with bacterial ones. Both biochemical experiments and computer data indicated the presence of long hydrophilic loops in the middle of the sequence [29–32]. The meaning of these loops, if existing, is still unknown. Bacterial porins have a long loop (L3) folded into the barrel, contributing to a constriction of the channel where the charge distribution affects ion selectivity [33]. Also voltage gating in bacterial porin might be caused by a conformational transition involving residues in L3 [33].

The recent discovery of three mouse porin gene structures [12] pointed out that such genes are part of a family, since all the exon-intron boundaries and the sequences of the exons encoding the proteins are highly conserved among the three genes. Therefore, the mouse VDAC exons corresponded to protein transmembrane regions with respect to the above summarized model since the exon-intron splice sites could be localized at the loops connecting the β-strands [12]. The longest mouse exons coding for protein sequence are exons 6 (in MVDAC1 and MVDAC3) and 7 (in MVDAC2) [12]; they contain the two largest hydrophilic loops in the sequence. It is interesting to notice that these exons perfectly match the corresponding *Drosophila melanogaster* exon, exon III (Fig. 2). Not only the size of the coding exons are identical (228 bp), but also the boundaries at the exon-introns splice sites are very well conserved (Fig. 2). The finding of such a sequence homology between fly and human, whose time of divergence was estimated to be at least 800 million years [34], may indicate that this protein domain has an important functional or structural meaning.

D. melanogaster exon II, indeed, encodes for a predicted transmembrane unit of the protein, containing the N-terminal amphipathic α-helix and six β-strands. Quite symmetrically, the exon IV encodes the remaining seven β-strands located at the C-terminal. The overall picture envisages two strictly transmembrane domains bordering the central large-loops-containing domain. Comparing the fly gene with the mouse gene structures it can be hypothesized that the insertion of new introns during evolution did not perturb this kind of gene organization.

3.3. How many genes for porin in Drosophila melanogaster?

Fig. 3 shows a Southern blot comparison of different *D. melanogaster* strains genomic DNA, digested by various restriction enzymes and assayed by an amplified probe containing only the protein coding DNA sequence (see Section 2). We used this probe since the highest level of homology among porin isoforms was detected in such regions [10,11,35]. High stringency conditions (65°C) revealed a hybridization pattern typical for a single-gene condition (Oregon-R, the strain we mostly used for our experiments). Two bands are visible only after digestion by *Bcl*I and *Xho*I, two ERs which were known to be present in the genomic sequence containing the protein

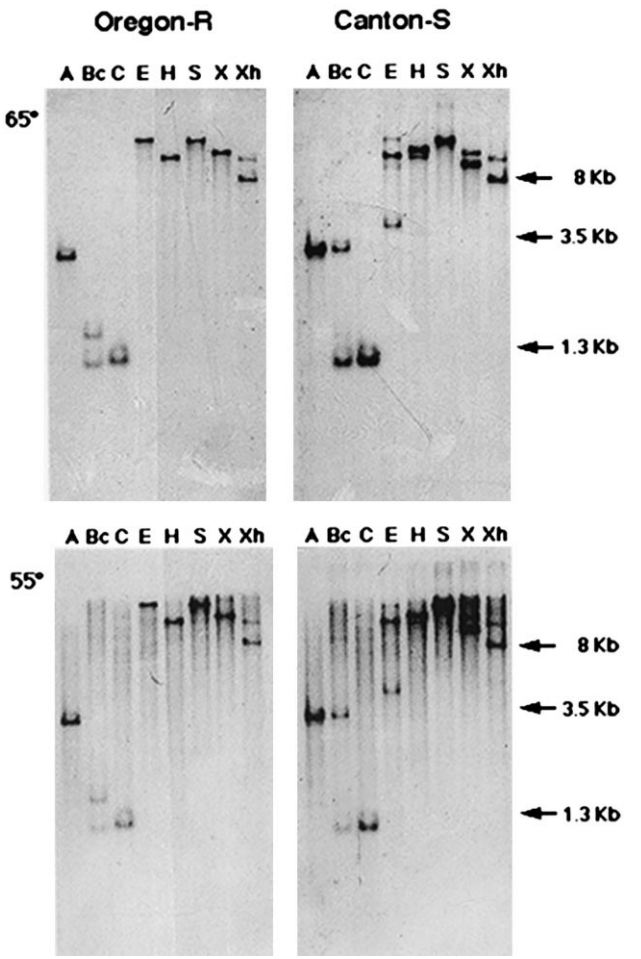

Fig. 3. Southern blot analyses of *D. melanogaster* Oregon-R and Canton-S genomic DNAs. Genomic DNAs were cut with ERs (A: *Acc*I; Bc: *Bcl*I; C: *Cla*I; E: *Eco*RI; H: *Hin*dIII; S: *Sal*I; X: *Xba*I; Xh: *Xho*I), blotted and probed with an amplified DNA corresponding to the fly protein coding sequence. Hybridization and washing were performed at 65°C (upper panels) or at 55°C (lower panels). M.W. markers position is indicated.

coding sequence (see Fig. 1). The comparison with the genomic DNA from the flies Canton-S showed restriction length polymorphism between the two strains. Ryerse et al. [26] previously showed a Southern blot experiment: they defined their analysis as performed at low stringency and with cDNA porin as a probe, but if their hybridization was performed at 55°C nevertheless final washings were "for 2×60 min in 0.1× SSC, 0.1% SDS at 68°C" [26], thus at high stringency conditions. In such conditions their pattern showed more bands after *Xho*I digestion, which is not in agreement with the sequence of the gene, but could be explained by polymorphisms. Unfortunately, Ryerse et al. [26] did not quote the fly strain which was their genomic DNA source.

Low stringency hybridization (55°C, with the same washing solutions used at 66°C, a condition that in our hands usually is able to detect up to the 70% sequence homology between the probe and the blotted DNA) did not improve the number of visible bands, but only enhanced the unspecific background.

Our result is thus a clear indication for a single porin gene in the *Drosophila melanogaster* genome. We previously reported the hybridization of two loci in the fly genome by a cDNA for the mitochondrial porin [23]. During our previous screenings for cDNAs we also found copies of another species (termed 1T7, acc. no. X95692). The combined utilization of both cDNAs in in situ hybridization experiments possibly gave us misleading results. A more careful evaluation of this point was performed in this work, with the aim to shed light on the number of homologous genes for porin(s) existing in this organism. The genomic clone EM1T and its subclones

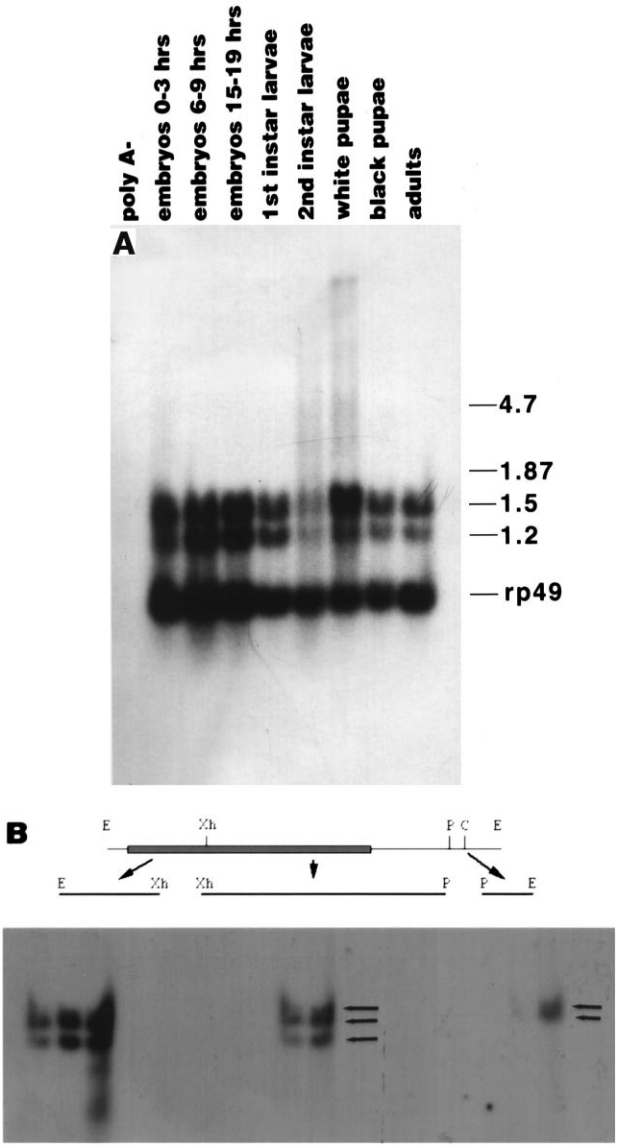

Fig. 4. A: Northern blot analysis of *Drosophila melanogaster* poly-(A)$^+$ mRNA by porin cDNA. *D. melanogaster* poly(A)$^+$ mRNAs were collected at different developmental stages of the fly. The poly(A)$^+$ mRNA content in each lane was normalized by means of rp 49, a message constitutively expressed in the various developmental stages of the fly. The hybridization probe was the porin full length cDNA 7T21. The relative position of RNA size markers is indicated. B: Northern blot analysis of *D. melanogaster* porin transcripts by porin cDNA internal subclones. Three different internal subclones were obtained from the porin full length cDNA 7T21. They represent the 5′ terminal part (*Eco*RI/*Xho*I), the central core of the sequence (*Xho*I/*Pst*I) and the 3′ extension. Such clones were used as probes and hybridized to *D. melanogaster* mRNA panels. While the 5′ end and the central core of the sequence hybridized with the whole group of mRNAs, the 3′ end hybridized only to the two largest mRNAs.

were then used as probes for in situ hybridization to polytenic salivary gland chromosomes. In this kind of experiment, hybridization confirmed the localization at one of the loci indicated in [23], which was more precisely fixed on chromosome 2L, at the region 32B3-4 (not shown). In this experiment no evidence for a second locus was obtained.

The overall conclusions from these experiments are in favor of a single-copy gene for porin. The existence of more porin genes in *Drosophila* would thus imply a large sequence or structural difference of the genes.

3.4. D.melanogaster porin transcription analysis

Northern blotting experiments were performed on poly(A)$^+$ RNA fractions purified from whole insects at different developmental stages, using as a probe the porin cDNA (Fig. 4A). The Northern blot indicated a housekeeping pattern since no significant differences in banding pattern was found. Three mRNAs were identified by the clone 7T21 with differences ranging from some tens of basepairs for the two higher M.W. messages to about two hundred basepairs for the lower M.W. transcript. In order to have more insight into the structure of such mRNAs, three cDNA subclones were used as probes. These subclones corresponded to the *Eco*RI/*Xho*I 5′ end of the cDNA, to the *Pst*I/*Eco*RI 3′ end of the cDNA and the inner portion *Xho*I/*Pst*I (Fig. 4B). The analysis of the same poly(A)$^+$ RNA fraction with the three different probes indicated that the smallest mRNA was a form truncated at the 3′ extremity and thus originated from the first polyadenylation site. Both the two major mRNAs were instead recognized by the other two probes (Fig. 4B). This result could be explained by the existence of two different mRNA messages complementary to significant parts of the sequence encoded in the 5′ distal 341 bp of the cDNA (from the beginning to the *Xho*I site): this result is in agreement with the suggestion of the utilization of two different promoters.

4. Conclusions

In this work we showed the structure of the *Drosophila melanogaster* gene encoding the mitochondrial porin or VDAC and localized on the chromosome 2L at 32B3-4. As expected, the gene structure in the smaller fly genome is more compact than in the mouse genes [12]. Anyway, some features concerning the gene regions coding the protein(s) seem conserved among the invertebrate and the mammalian genes. The exon-intron boundaries homology and, moreover, the conservation of a large protein-encoding exon support the existence of a homologous putative protein domain in the porin structural organization.

Furthermore we reported, for the first time, the existence of alternative porin transcripts. Such transcripts differ only in the 5′-UTR region, while the protein encoded by the two messages is identical. We are presently working to get meaningful experimental evidence about the utilization of these two transcripts. On the other hand, many papers in the literature already reported the co-existence of the same porin-protein both in the outer mitochondrial membrane and in the plasma membrane [13–17]. Yu et al. [22] denied such a possibility on the basis of epitope-tagged porin subcellular localization studies; nevertheless, their results do not rule out a differentiated targeting of transcripts containing alternative 5′-untranslated sequences.

Lithgow et al. [36] recently focussed their attention on the mechanisms involved in the transport of mRNAs for proteins from the nucleus to ribosomes placed in the vicinity of the target subcellular compartments. *Cis*-acting localization signals have been identified for a number of mRNAs and all lie within the 3′-untranslated region (3′-UTR) [37]. No data are yet available about the role of 5′-UTR region in this process. Thus our working hypothesis is that transcripts with different 5′-UTR regions could be responsible in the targeting of the same protein to different subcellular compartments.

The very high degree of homology, here reported also at a genomic level, between higher mammals and the fly *D. melanogaster* indicates that our approach could be useful to clarify the genetic system which underlies the 'porin redundancy' and whose defect is responsible for pathological events in human [20,21].

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