Mol Genet Genomics (2002) 267: 746–756
DOI 10.1007/s00438-002-0714-1

ORIGINAL PAPER

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# A genetic analysis of the *porin* gene encoding a voltage-dependent anion channel protein in *Drosophila melanogaster*

Received: 28 February 2002 / Accepted: 7 June 2002 / Published online: 23 July 2002
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**Abstract** The voltage-dependent anion channel (VDAC, also known as porin) is an abundant protein in the outer mitochondrial membrane that forms transmembrane channels permeable to solutes. While in mammals at least three different porin genes have been found, only one VDAC-encoding gene, *porin*, has been described so far in *Drosophila melanogaster*. It produces transcripts with alternative 5′ untranslated sequences. Here we report the identification of two *PlacW* insertions in the *porin* gene among a set of *P*-element insertions that have been mapped to the 32B3–4 region on the second chromosome. Homozygotes, as well as *trans*-heterozygotes for these insertions, lack VDAC, and die during the late pupal stage. Function can be restored by precise excision of the *P* transposon, while most deletions in the *porin* locus, produced by imprecise excisions, display the recessive lethal effect of the original mutant alleles. However, one of the deletions was found to be a hypomorphic male-sterile allele producing low levels of the VDAC protein, indicating that the product of the *porin* gene is also essential for male fertility. Analysis of the new mutant alleles also showed that the untranslated exon 1B of the *porin* gene is not required for VDAC synthesis. In the course of our investigation, we found that immediately adjacent to the *porin* gene are three more genes encoding proteins that share homology with the VDAC protein. The possible evolutionary and functional relationships of the *porin*-like genes at 32B3–4 are discussed.

**Keywords** VDAC protein · *Drosophila melanogaster* · Genetic analysis · *PlacW* mobilization · Multigene family

## Introduction

The voltage-dependent anion channel (VDAC, also known as mitochondrial porin) is the most abundant protein found in the outer mitochondrial membrane of all eukaryotes, and constitutes the permeability transition pore together with the adenine nucleotide translocator (ANT) and other molecules which regulate mitochondrial membrane permeability (for reviews, see Bernardi et al. 1994; Colombini et al. 1996; Mannella 1997). VDAC is an integral membrane protein with a molecular mass of 30–32 kDa, whose function as a passive permeability pathway was suggested after purification and incorporation into planar phospholipid bilayers in vitro (De Pinto et al. 1987; Benz 1994). The VDAC pore can be modulated in a number of ways that implicate this molecule as a site for the regulation of mitochondrial function in a cellular context (Liu and Colombini 1992; Holden and Colombini 1993; Lee et al. 1994; Wilson 1997), including a potential role in the mitochondrial release of cytochrome *c* via an interaction with pro-apoptotic molecules (Harris and Thompson 2000; Tsujimoto and Shimizu 2000; Vander Heiden et al. 2000; Bernardi et al. 2001). Although VDACs have been thought to be localized exclusively in the outer mitochondrial membrane, there is some evidence for the presence of VDACs in the cell membrane (Thinnes 1992; Bathori et al. 1999; Buettner et al. 2000).

The indications that VDAC function(s) in vivo are likely to be much more complex than the channel activities observed for purified VDAC reconstituted into planar bilayers suggest that VDAC function(s) might best be elucidated by genetic strategies based on the study of the phenotypic consequences of mutations that suppress or alter the function of the VDAC protein in appropriate model organisms. However, genetic

Communicated by E. K. F. Bautz

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dissection of VDAC function is complicated by the fact that a certain genetic redundancy appears to be associated with VDAC in all systems so far studied. In the yeast *Saccharomyces cerevisiae*, deletion of the *POR1* gene encoding VDAC1, the primary porin expressed in this organism, allowed the identification of a second gene, *VDAC2*, that encodes an additional porin isoform. While VDAC2 is unable to form channels in vitro, its overexpression complements the inability to grow on non-fermentable carbon sources that results from the deletion of *VDAC1* (Blachly-Dyson et al. 1997). In human, rat and mouse, three different VDAC genes encoding distinct, expressed, isoforms have been reported (Blachly-Dyson et al. 1993; Sampson et al. 1997; Rahmani et al. 1998). A further source of complexity may be associated with alternative splicing variants, which have been shown to exist in some cases, but whose function is unknown, although differential expression in different tissues has been reported (Sampson et al. 1998; Buettner et al. 2000). Human and mouse VDAC1 and VDAC2 isoforms show channel-forming activity in vitro and can compensate for VDAC1 deficiency in yeast (Blachly-Dyson et al. 1993), while VDAC3 can only partially complement the yeast mutant (Sampson et al. 1997). Mammalian VDAC isoforms are expressed in a wide variety of tissues; expression of specific VDAC isoforms has been observed in mammalian testes (Sampson et al. 1998; Rahmani et al. 1998; Hinsch et al. 2001).

In *Drosophila melanogaster*, the *porin* gene, which encodes a VDAC that is about 60% identical to the VDAC isoforms in mammals, has been cloned, sequenced and mapped to 32B3–4 on the second chromosome (Messina et al. 1996; Ryerse et al. 1997), and was shown to give rise to two different mRNAs encoding the same protein but carrying alternative 5′UTRs (Oliva et al. 1998). At the level of resolution provided by in situ hybridization and Southern blotting at various stringencies, no evidence could be obtained for the presence of further *porin*-related sequences in the *D. melanogaster* genome. However, we report in this work that three genes that lie adjacent to the *porin* gene at 32B encode proteins with significant homology to VDAC. They probably originated from a single ancestral gene by successive duplication events. Moreover, the availability of the whole *D. melanogaster* genome sequence and the identification of strains containing *P* elements inserted in the *porin* gene allowed us to generate several new mutant alleles of the gene by secondary mobilization of the element, and study the phenotypic consequences of deletions in the 5′UTRs of the *porin* mRNAs. In this report we present a detailed characterization of the phenotypes associated with these new mutant alleles and with the original *porin* insertion alleles, and of their expression patterns as determined by Western blotting and immunofluorescence experiments using antisera raised against the VDAC protein. We show that deletion of different regions of the gene is associated with different phenotypes, ranging from full viability through male sterility to almost complete lethality in the late pupal stage, and demonstrate that such phenotypic variations are correlated with the level of VDAC protein produced.

## Materials and methods

### *Drosophila* genetics

Fly stocks were maintained on standard Drosophila media and all crosses were done at 24°C. Genetic markers used are described in Lindsley and Zimm (1992). Stocks carrying a single *PlacW* element insertion in the 32B3–4 region of the second chromosome [*l(2)k05123* and *l(2)k08405*] were kindly provided by the Bloomington Drosophila Stock Center and are described in FlyBase (1999; http://flybase.bio.indiana.edu/). In this work *l(2)k05123* and *l(2)k08405* are renamed $porin^1$ and $porin^2$, respectively. Revertants were obtained from the $porin^1$ and $porin^2$ insertion lines by secondary mobilization of the *P*-element (Robertson et al. 1988). To mobilize the *P* element in the $porin^1$ allele, *y w; porin*$^1$*/CyO* females were crossed to *CyO/Sp; Sb*Δ*2–3(99B)/TM6* males, which carry a stable source of Δ2–3 transposase integrated on the third chromosome, and *y w; porin*$^1$*/CyO; +/Sb*Δ*2–3* male progeny with variegated eye color were collected. Fifty groups (five males each) of such males were crossed to *y w, porin*$^1$*/CyO* females, and *Curly* non-*Stubble* white-eyed progeny were individually crossed to *y w; porin*$^2$*/CyO*. The white-eyed *Cy* lines were propagated and tested for lethality and fertility. Revertants were obtained from the $porin^2$ insertion lines by crossing *y w; porin*$^2$*/CyO* females to *CyO/Sp; Sb*Δ*2–3(99B)/TM6* males, and collecting *y w; porin*$^2$*/CyO; +/Sb*Δ*2–3* male progeny with variegated eye color. Again 50 groups of such males (250 in all) were crossed to *y w, porin*$^2$*/CyO* females, and *Curly* non-*Stubble* white-eyed progeny were individually crossed to *y w, porin*$^1$ */CyO*. The white-eyed *Cy* lines were propagated and tested for lethality and fertility.

To precisely define the lethal stage in individuals homozygous for the $porin^1$ and $porin^2$ alleles, as well as in homozygotes for lethal *porin* alleles obtained by secondary excision of the inserted *P* element, the *CyO* balancer chromosome present in the original strains was replaced by *T(2;3)SM6a-TM6B, Cy Tb*, and the development of fertilized eggs laid by females of the stocks so established was followed up to eclosion. In such stocks, homozygotes for the mutant *porin* alleles can be unambigously identified, starting from the second larval instar, by their wild-type body length, since heterozygotes have the shorter body typical of the *Tb* phenotype. Fertility tests on homozygous vital $porin^2$ revertants showed that an independent female-sterile mutation is present on the second chromosome that carries the original $porin^2$ insertion.

### RNA and DNA techniques

Total RNA for analysis of *porin* expression was obtained from 0.5- to 1-g samples of Oregon-R individuals at various developmental stages (0–3 h embryos, third-instar larvae, and adult flies). Poly(A)$^+$ RNA was prepared by standard methods and reverse transcribed with AMV RT (Roche). The first-strand cDNA was used as the template for PCR amplification with a primer derived from the putative exon 1B of *porin* (Por1R: 5′-TTCCGTTCGT TATCGTCA-3′) and two primers derived from the 3′UTR of the *porin* gene (Por3F: 5′-TTGTCTGGCTGCTGTTAT-3′ and Por2F: 5′-TGGGCGGATGTGGTGATG-3′). The Por3F primer anneals 28 bp upstream of the first polyadenylation site identified in the *porin* gene; the Por2F primer is located between the first and the second polyadenylation site, 174 bp upstream of the second one. To confirm that the fragments obtained were true amplification products, a nested PCR was performed using two new primers derived from the coding sequence of the gene (Por4R: 5′-ATGGCTCCTCCATCATACAG-3′ and Por5F: 5′-TTAGGCCTCCA GCTCCAG-3′). Amplification products were cloned in the pGEM-T vector (Promega) and sequenced by Thermo-sequenase

(Amersham) cycle sequencing using universal primers and the dideoxy chain-termination method.

All standard DNA manipulations were performed as described in Sambrook et al. (1989). Genomic DNAs from heterozygous mutant flies carrying a single *P* element were analyzed using oligonucleotide primers derived from the 31-bp terminal inverted repeat sequence of the *P* element (Pterm: 5′-CGGGACCACCTTA-TGTTATTT-3′) and a primer (Por6F: 5′-TGCCCAAATCGCTGT-ATGA-3′) corresponding to positions 2498–2516 of the genomic *porin* clone EMT1. The primers used for PCR analysis of the excision events were Pterm, Por6F (see above) and two genomic primers corresponding to positions 536–553 (Por7R: 5′-TGTC GTGGTGTCTCTGTT-3′) and 1504–1521 (Por8F: 5′-GCAAT-CAAGGCAGAAAAT-3′), respectively. Another primer (Por9R: 5′-ACGCCATCATCGCTCACA-3′) was based on a stretch of the BACR03D06 genomic sequence (see below) not encompassed by the EMT1 clone. This primer anneals 284 bp upstream of the first base of the EMT1 clone. PCR was performed using the Expand High Fidelity PCR System (Roche). Amplification products were either directly sequenced or cloned into pGEM-T vector (Promega) and then sequenced.

### Sequence data and analysis

Primer positions refer to the EMT1 *porin* genomic sequence of 4494 bp deposited in the EMBL Nucleic Acid Sequence Data Library under Accession No. AJ000880 (Oliva et al. 1998). We used three existing genomic sequences in the database of the Berkeley Drosophila Genome Project (BDGP) (BACR03D06, Accession No. AC007186; BACR19N18, Accession No. AC007147; genomic scaffold, Accession No. AE003630) to correct for obvious errors in the annotation of the EMT1 sequence and to deduce the genomic organization of the *porin* gene and three adjacent genes, *CG17137*, *CG17139* and *CG17140*. The *porin* cDNA sequences as well as expressed sequence tag (EST) sequences obtained by the BDGP were instrumental in defining the possible *porin* transcripts. Homology searches were performed on the BDGP BLAST Server (http://www.fruitfly.org/blast/). Sequence alignments were constructed using CLUSTAL W (version 1.81).

### Immunoblots and immunolocalization

Polyclonal antibodies were raised in mice against *D. melanogaster* VDAC protein purified as described in De Pinto et al. (1989). Purified mitochondrial proteins were electrophoresed on SDS-polyacrylamide gels, and gel slices corresponding to the VDAC protein were excised, electroeluted and dialyzed to remove SDS. Aliquots (50 μg) of purified protein were emulsified with complete Freund's adjuvant and injected into mice. Subsequent boosts were done at 2-week intervals with 20 μg of purified protein emulsified with incomplete Freund's adjuvant. The antigen was injected subcutaneously in a volume of 500 μl.

SDS-PAGE and immunoblotting were carried out as described by Towbin et al. (1979). Protein samples were prepared from four late pupae or adult flies by squashing them on a piece of blotting paper and boiling the paper for 4 min in 50 μl of sample buffer. Samples were electrophoresed on slab gels (1 mm thick), electroblotted onto nitrocellulose membrane (Amersham) and probed with an anti-VDAC antibody diluted 1:2000 and an alkaline phosphatase-conjugated secondary antibody (Sigma). Antibody reactivity was visualized with NBT plus BCIP (Bio-Rad), in alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM TRIS-HCl, pH 9.5).

For immunofluorescence microscopy, testes from late pupal or adult males were dissected and fixed by the methanol-acetone method (Cenci et al. 1994). The fixed material was then incubated for 90 min with a primary antiserum diluted 1:100, and binding to Nebenkern bodies was detected with a secondary antibody [fluorescein-isothiocyanate (FITC)-labeled goat anti-mouse IgG, from Sigma] diluted 1:150. Nuclear DNA was stained with DAPI. Digital images were obtained using an Olympus AX70 epifluorescence microscope equipped with a cooled CCD camera (Princeton Instruments). Gray-scale images separately obtained for FITC and DAPI fluorescence using specific filters were computer colored and merged to form the final image using Adobe Photoshop software.

## Results

### Identification of alternative 5′UTRs in *porin* transcripts

*porin* cDNA clones were recovered by screening cDNA libraries with polyclonal antisera raised against the purified *D. melanogaster* VDAC protein (Messina et al. 1996). A cDNA fragment containing the *porin* coding sequence recognized three transcripts on Northern blots at all developmental stages assayed: the different transcripts derive in part from the use of alternative polyadenylation sites at the 3′ ends; however, primer extension experiments on poly(A) RNA isolated from Drosophila larvae indicated that two alternative transcription starts might also be used. Further analysis revealed that, although they clearly varied in relative abundance, the transcripts detected differed at the 5′ end only in having alternative 5′UTRs. Comparison of cDNA and genomic sequences including the complete *porin* gene identified two putative alternative exons, which were termed exon 1A (115 bp) and exon 1B (124 bp) respectively. Both exons are followed by GT consensus intron splice sequences, situated 1890 and 1353 bp, respectively, from the ATG codon, which corresponds to the first three bases of exon 2 (Oliva et al. 1998).

To verify the in vivo production of the *porin* transcript containing exon 1B and to study its possible developmental specificity, RT-PCR experiments were performed on poly$(A)^+$ RNAs from *D. melanogaster* embryos, larvae, and adults of both sexes. The primers utilized were Por1R, a primer derived from exon 1B of the *porin* gene, and two primers (Por3F and Por2F) corresponding to sequences upstream of the first and the second putative polyadenylation sites, respectively (Fig. 1A). A single amplification product of the expected length (1065 bp) was observed at all developmental stages using the Por3F primer, but no product was obtained using the Por2F primer (not shown). A nested PCR experiment using two primers derived from sequences of the coding region (Por4R and Por5F) verified that the amplification products originated from the *porin* gene, since a single amplified fragment of the expected length (849 bp) was obtained (Fig. 1B). Sequence analysis of the 1065-bp amplification products confirmed that a transcript which lacks exon 1A and uses the first polyadenylation site in the transcribed sequence is present at all developmental stages tested.

### Identification and phenotypic characterization of mutant alleles of the *porin* gene

To identify mutant alleles of the *porin* gene among *P*-element insertion mutants mapping at the 32B3–4

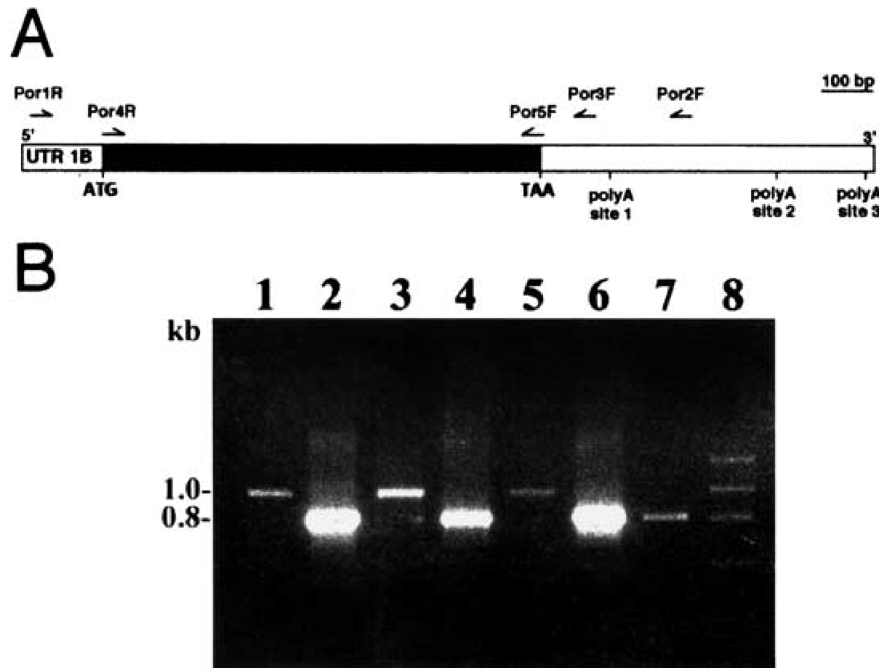


**Fig. 1A, B.** A porin mRNA with an alternative 5′UTR is constitutively expressed in *Drosophila melanogaster*. RT-PCR analysis was performed on poly$(A)^+$ RNA from Oregon-R. **A** Schematic representation of the transcript encompassing sequences complementary to exon 1B. Possible polyadenylation sites are marked. The translated sequence is indicated by the *filled box*. **B** Agarose gel showing amplified RT-PCR products visualized by staining with ethidium bromide. In the first amplification (lanes 1, 3 and 5) the Por1R primer, which is specific for untranslated sequence 1B, was used together with the Por3F primer, derived from the 3′UTR. The resulting products were re-amplified using the Por4R and Por5F primers, derived from the translated sequence (lanes 2, 4 and 6). Lane 1, product of first amplification derived from poly$(A)^+$ RNA isolated from 0–3 h embryos; 2, internal amplification using the amplified fragment shown in lane 1 as template; 3, first amplification using poly$(A)^+$ RNA from third-instar larvae; 4, internal amplification using the amplified fragment shown in lane 3 as template; 5, first amplification with poly$(A)^+$ RNA isolated from adult flies; 6, internal amplification using the fragment shown in lane 5 as template; 7, control obtained by amplification on the *porin* cDNA with the internal primers; 8, $\phi$ X174 DNA digested with *Hae*III. RT-PCR experiments using Por1R and Por2F primers did not produce amplification fragments (data not shown)

region on the second chromosome, we performed PCR with primers derived from the *porin* genomic sequence (Por6F) and a primer derived from the 31-bp terminal inverted repeat sequence of the *P* element (Pterm). Using this strategy, a single specific amplification product was detected in the DNA of flies heterozygous for the recessive lethal *P*-element insertions *l(2)k05123* and *l(2)k08405*. These mutant alleles are renamed $porin^1$ and $porin^2$, respectively, in this work. Sequence analysis showed that in both of these alleles a *P* element is inserted in the first intron of the *porin* transcription unit at position 1009 of the EMT1 genomic clone (Fig. 2A). In both stocks rare adult escapers of both sexes are observed; as the lethal-bearing chromosome is balanced over *CyO*, flies homozygous for the *P*-element insertion are unambiguously identifiable by their wild-type wing phenotype. Such escapers, as well as *trans*-heterozygotes for the two mutant alleles, do not exhibit any evident morphological defects; however, they show locomotory abnormalities and have a short life span.

In Western blotting experiments, antisera raised against the Drosophila VDAC protein failed to recognize any cross-reacting material in extracts from pupae or adult escapers homozygous for each of the mutant alleles. The Western blot obtained using an extract from homozygous $porin^1$ late pupae is shown in Fig. 2B. In the wild-type, antisera recognized a band corresponding to a molecular weight of about 31 kDa in extracts from all tested developmental stages, or from separated body parts or tissues (not shown), pointing to constitutive expression of the *porin* gene.

## Production and phenotypic characterization of reversion events

To confirm that the lethal phenotypes observed in $porin^1$ and $porin^2$ homozygotes are due to the *P*-element insertion at 32B3–4 and to check that the insertions prevent synthesis of VDAC, dysgenic crosses were performed to remobilize the *P* element in the $porin^1$ and $porin^2$ lines. Among 75 white-eyed revertant lines, representing at least 31 independent excision events, we found 63 excision products that were viable in *trans* to the chromosome carrying the other *P*-element insertion (i.e., not the one from which the revertant arose). On the other hand, 12 white-eyed revertant lines displayed the same lethal phenotype as the original mutant alleles. $porin^{2rev9}$, a viable revertant allele, produces male sterility both in homozygotes and when heterozygous with either of the two original *porin* insertion alleles. No

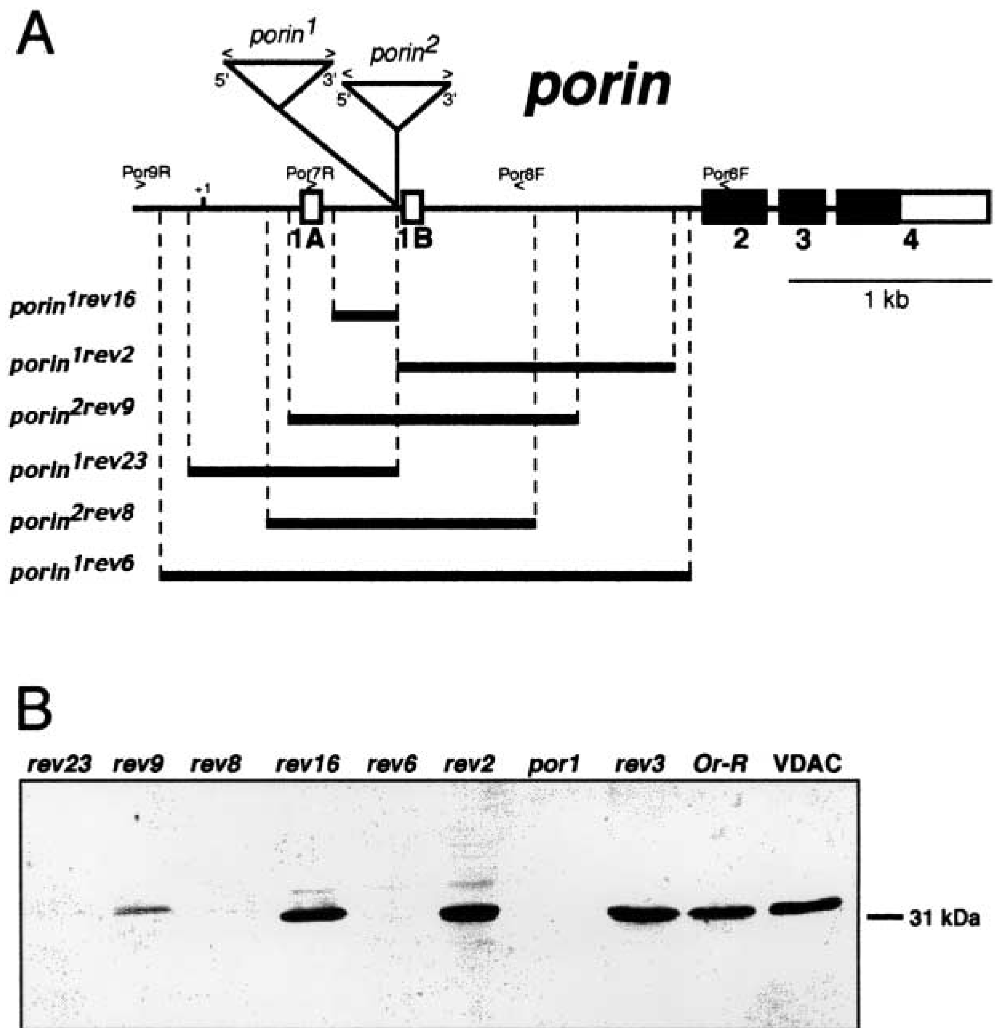


**Fig. 2A, B.** Molecular characterization of *porin* alleles. **A** Schematic representation of the genomic organization of the *porin* gene. The *triangles* indicate *P*-element insertion sites in the *porin*$^1$ [*l(2)k05123*] and *porin*$^2$ [*l(2)k08405*] lines. +1 indicates the first base of the EMT1 genomic clone and the *arrowheads* indicate the positions of the primers used for PCR analysis of revertants (see Materials and methods). Translated exons are indicated by the *filled boxes*. The *horizontal bars* represent regions deleted by imprecise excision of the *PlacW* insertions. **B** Western analysis of VDAC protein. Protein extracts from Oregon-R late pupae (*Or-R*), from late pupae homozygous for the *porin*$^1$ insertion (*por1*) and from late pupae homozygous for the deletion alleles shown in **A** (*rev*) were used. Four pupae were homogenized to obtain each sample. *rev3* is a homozygous viable revertant allele (Table 1). VDAC indicates the purified 31-kDa Drosophila VDAC protein

obvious cytological defects in spermatogenesis are observed in males of the *porin*$^{2rev9}$ line; however, such males produce non-motile sperms only.

For all the revertant lines, viability of homozygotes correlates with the production of VDAC as determined by Western analysis. A pattern indistinguishable from wild-type was obtained from homozygous viable revertant lines, as shown in Fig. 2B for extracts of adult flies of the *porin*$^{2rev3}$ line. The protein is present in lower amounts in extracts from the male-sterile *porin*$^{2rev9}$ line. In contrast, the antiserum failed to recognize any cross-reacting material in extracts from homozygous adult escapers or pupae of lines carrying new lethal alleles induced by remobilization of the *PlacW* element in the *porin* gene.

## PCR analysis of revertants

We performed a detailed PCR analysis of the recovered *PlacW* mobilization events using a primer derived from the 31-bp terminal inverted repeat sequence of the *P* element (Pterm) and four primers (Por7R-Por8F and Por9R-Por6F) derived from genomic sequences flanking the *porin*$^1$ and *porin*$^2$ insertions. When necessary, the amplified fragments were sequenced to obtain further information on the mobilization event. The results are summarized in Table 1. From 43 out of 63 homozygous viable revertant lines, amplified fragments of approximately the length expected for the wild-type allele were obtained. As shown by sequencing of several amplification products, the events that gave rise to such reversions were either precise excisions of the inserted *P* element (e.g., *porin*$^{2rev3}$) or excisions that left a few nucleotides derived from the *P*-element terminal inverted repeats at the insertion site (Table 1). Therefore, the lethal phenotype can be reverted by precise or almost precise excision of the *PlacW* transposon in the *porin* gene, strongly supporting the notion that the phenotype is due to the insertion. In 17 homozygous viable lines an amplified fragment of up to 5 kb – significantly larger than expected from wild-type DNA – was observed. This group may include instances of imprecise excision of the remobilized transposon that leave a substantial fraction of the *P*-element at the insertion site, and more complex rearrangements. In contrast, the homozygous viable *porin*$^{1rev16}$, *porin*$^{1rev2}$ and *porin*$^{2rev9}$ lines produced an

**Table 1.** Summary of *porin* alleles and phenotypes

| Allele[a] | Phenotype of allele/*porin*[1b] | Phenotype of allele/*porin*[2b] | Mutational event | VDAC protein present |
|---|---|---|---|---|
| *porin*[1] | Lethal | Lethal | *PlacW* insertion | – |
| *porin*[2] | Lethal | Lethal | *PlacW* insertion | – |
| *porin*[2rev3] | MF | MF | Perfect excision | + |
| *porin*[2rev14] | MF | MF | 25-bp P insertion | + |
| *porin*[2rev7] | MF | MF | 54-bp P insertion | + |
| *porin*[2rev2] | MF | MF | ~300-bp P insertion | + |
| *porin*[1rev18] | MF | MF | ~2.5-kb P insertion | + |
| *porin*[1rev21] | MF | MF | ~5.0-kb P insertion | + |
| *porin*[1rev17] | Lethal | Lethal | ~9.0-kb P insertion | – |
| *porin*[1rev16] | MF | MF | 365-bp deletion | + |
| *porin*[1rev23] | Lethal | Lethal | 1052-bp deletion | – |
| *porin*[1rev2] | MF | MF | 1346-bp deletion | + |
| *porin*[2rev8] | Lethal | Lethal | 1344-bp deletion | – |
| *porin*[2rev9] | MS | MS | 1438-bp deletion | ± |
| *porin*[1rev6] | Lethal | Lethal | 2642-bp deletion | – |

[a] The suffixes *1rev* and *2rev* refer to revertant alleles derived by mobilization of the *P* element in the lines *l(2)k05123* (*porin*[1]) and *l(2)k08405* (*porin*[2]), respectively
[b] MF, male fertile; MS, male sterile

amplified band with a lower molecular weight than expected. As shown by sequencing, the mobilization events that gave rise to these lines deleted some genomic sequences flanking the *P*-element insertion site (Fig. 2A). Thus, a segment of 365 bp was removed from the region between exons 1A and 1B in *porin*[1rev16], while *porin*[1rev2] is characterized by a deletion of 1346 bp encompassing exon 1B as well as most of the intronic sequences between exon 1B and exon 2. *porin*[2rev9], identified by phenotypic and Western analysis as a male-sterile hypomorphic allele of the *porin* gene (see above), has a 1438-bp deletion with a breakpoint 40 bp downstream of the putative transcription start site, and lacks both exon 1A and exon 1B plus some intronic sequences (Fig. 2A).

Of the 12 white-eyed lines derived from *PlacW* mobilization events that gave rise to new *porin* alleles still associated with a lethal phenotype, nine produced amplified fragments that included *P*-element-derived sequences larger than 5 kb. In contrast, *porin*[1rev6], *porin*[2rev8] and *porin*[1rev23] produced amplified fragments smaller than expected, and, as shown by sequencing, represent deletions that remove genomic sequences (but no coding sequences) flanking the *P*-element insertion site in the *porin* gene (Fig. 2 and Table 1).

Taken together, the results of the molecular analysis reported above show that exon 1B is not necessary for VDAC synthesis or for viability, while exon 1A and/or sequences of the 5′ untranscribed region of the *porin* gene are required for male fertility.

In accordance with the results of Western analysis, indirect immunofluorescence experiments, using antisera raised against VDAC, on testes of males from lines carrying wild-type *porin* alleles or revertant alleles that are homozygous viable revealed a strong cytoplasmic signal. In spermatids, the signal appears to be exclusively concentrated in Nebenkern bodies, which are mitochondrially derived. On the other hand, no signal was observed in lines carrying *porin* alleles associated with a lethal phenotype. Interestingly, a weaker and often irregularly distributed signal was observed in the Nebenkern in testes from the male-sterile *porin*[2rev9] line (Fig. 3).

## Three *porin*-like genes are located adjacent to the *porin* gene at 32B3–4

We previously suggested, on the basis of Southern and in situ hybridization experiments, that a single gene encodes the VDAC protein in *D. melanogaster* (Oliva et al. 1998). This contrasts with the fact that, as far as we know, in all other organisms studied, a small gene family is present that encodes different VDAC isoforms. However, a search for homologies in the BDGP database (release 2) revealed that the 31-kDa VDAC protein encoded by the *porin* gene shares significant homology with the predicted proteins encoded by three genes that flank the *porin* gene at 32B3–4 (*CG17137*, BLASTP *P* value $4.3e^{-64}$; *CG17139*, BLASTP *P* value $5.1e^{-22}$; and *CG17140*, BLASTP *P* value $2.3e^{-26}$). Figure 4A shows the genomic organization of the four genes as deduced in silico by alignment of the genomic sequences with the EST sequences in the BDGP collection. The four transcription units are all on the same strand, and in all of them the coding sequence is interrupted by two introns of similar length. The first of the two introns that interrupt the coding sequence in the *porin* gene occurs at exactly the same position in each of the flanking genes, and the second at almost the same position. Furthermore, the phase in which the introns occur appears to be conserved. On the other hand, the intron in the 5′UTR is present only in the *porin* gene. The distances separating the four *porin*-like genes are very short: there are only 180 bp between the *porin* EST that extends farthest in the 3′ direction (RH09077.3′, Accession No. BI573443) and the closest *CG17137* EST (GH26967.5′, Accession No. AI513856). Similarly, alignment with the genomic sequences of the *CG17137*

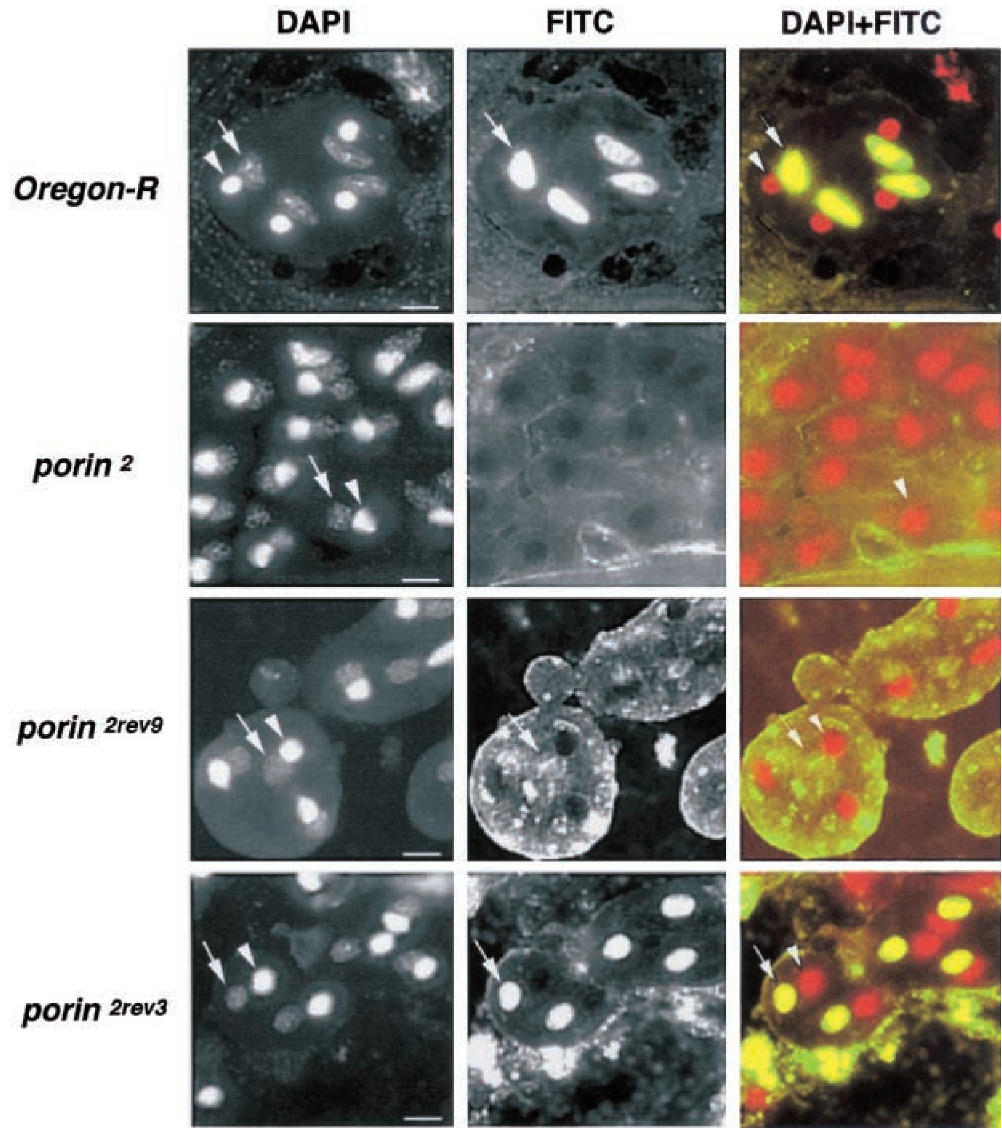


**Fig. 3.** Immunolocalization of VDAC protein in the mitochondrially derived Nebenkern bodies of *D. melanogaster* secondary spermatids. Squashes of testes from a wild-type (Oregon-R) male, and from adult males homozygous for the $porin^2$ insertion, the $porin^{2rev9}$ male-sterile allele or the viable $porin^{2rev3}$ revertant allele were incubated with an anti-VDAC antibody, and binding to Nebenkern bodies was detected with a FITC-labeled secondary antibody. DNA was stained with DAPI. Portions of a cyst with 64 secondary spermatids at the onion stage before differentiation (Fuller 1993) are shown. Gray scale images separately obtained recording DAPI and FITC fluorescence by specific filters were computer colored (DAPI red and FITC yellow) and merged to give the final image (DAPI + FITC). The *arrows* indicate Nebenkern bodies; *arrowheads* indicate nuclei. *Bars* = 10 μm

EST furthest 3′ (LP07660.3′, Accession No. AI294320) and the closest *CG17139* EST (AT29460.5′, Accession No. BG633740) shows that at most 120 bp separate the two genes. Finally, the putative stop codon of the *CG17139* coding sequence (no 3′ EST is available for this gene) is only 50 bp from the genomic position corresponding to the end of the closest *CG17140* EST (AT02648.5′, Accession No. BF494210). Of particular interest is EST AT03918.5′ (Accession No. BF495093), which apparently results from the splicing of the second *CG17139* exon to the first *CG17140* exon. In the BDGP EST collection there are about 250 sequences derived from the *porin* gene vs. only 14 derived from *CG17137,* four from *CG17139* and 12 from *CG17140*. This suggests that the *porin* transcript may be much more abundant in vivo.

In Fig. 4B the VDAC protein is aligned with the proteins putatively encoded by the three flanking genes. VDAC and CG17137 sequences differ from the CG17139 and CG17140 sequences because the latter pair possess an additional sequence preceding the standard N-terminus. The CG17137 protein is also characterized by the insertion of a 10-amino acid stretch at position 290. This insertion codes for an acidic cluster and is only found in this protein. By pairwise analysis, the highest sequence conservation (42% identity, 65% similarity) is observed between VDAC and CG17137, and between CG17139 and CG17140 (47% identity, 67% similarity), while CG17139 and CG17140 show significantly lower homology with the VDAC protein (23% identity, 42% similarity and 26% identity, 44% similarity, respectively).

A pattern of putative amphipathic beta-strands (the main repeated structural motif of pore-forming proteins; Schulz 2000) is detected in all four proteins by secondary-structure predictive tools (Jacoboni et al. 2001). However, the presence of additional amino acids at the N-terminal ends of CG17139 and CG17140 does not allow a meaningful comparison with the other sequences. In the VDAC and CG17137 sequences both

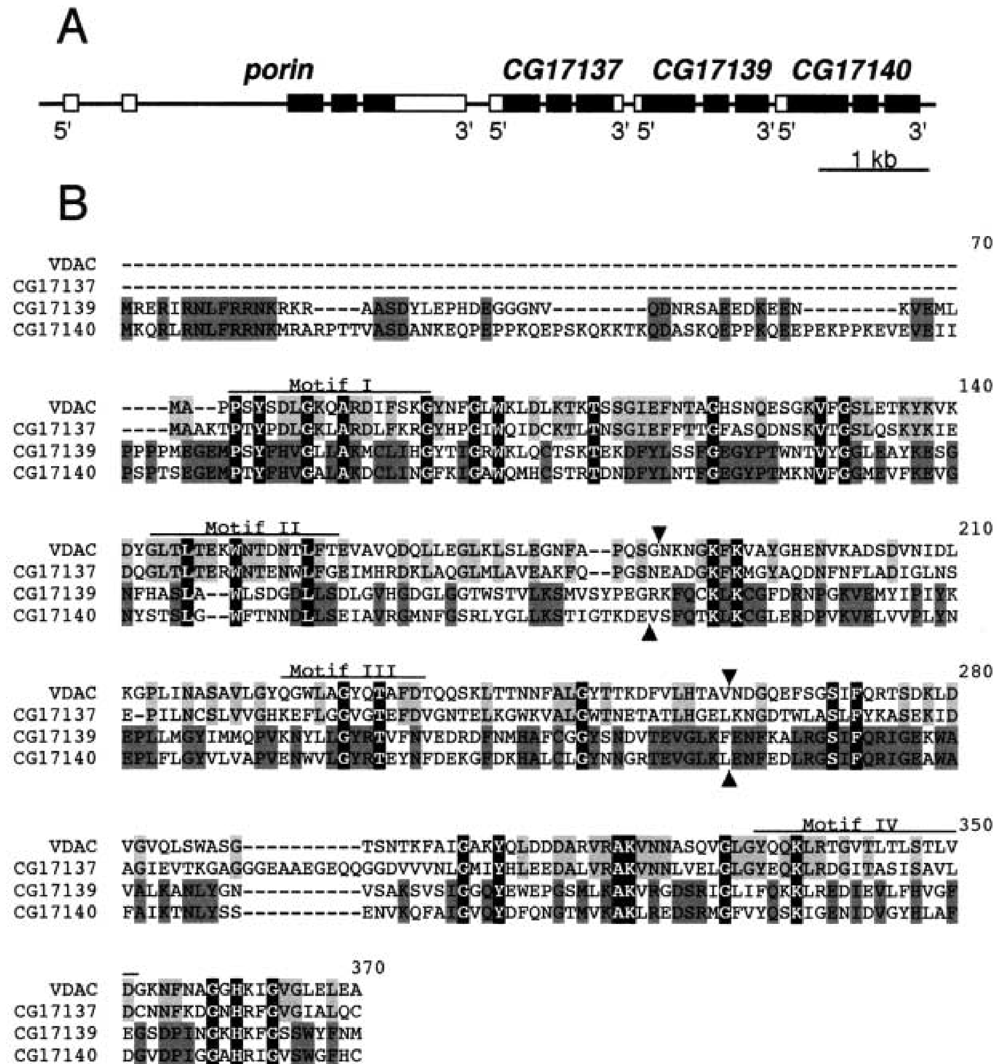


**Fig. 4A, B.** Genomic organization of the *D. melanogaster porin*-like genes at 32B and alignment of their deduced products. **A** Organization and exon/intron structure of the *porin* gene and of three flanking genes. Translated exons are indicated by the *filled boxes*. **B** The sequence of the VDAC protein encoded by the *porin* gene was aligned with the amino acid sequences encoded by the genes *CG17137*, *CG17139*, and *CG17140*. The *arrowheads above* the alignment indicate intron positions in the *porin* and *CG17137* genes; the *arrowheads below* indicate intron positions in *CG17139*, and *CG17140*. Amino acids that are conserved in all four sequences are *highlighted in white*; *light gray boxes* indicate amino acids conserved in VDAC and in the putative CG17137 product, and *dark gray boxes* indicate amino acids conserved in the putative CG17139, and CG17140 products. The four-element EUKARYTPORIN signature motif, present in VDAC and CG17137, is *overlined*. All sequences are available from either the SWISSPROT or SPTREMBL database. The Accession Nos. are as follows: VDAC, Q94920; CG17137, Q9VKP2; CG17139, Q9VKP3 and CG17140, Q9VKP4

the highest identity and the best-supported secondary-structure predictions are concentrated in the outer regions of the protein, not in its central part. This result confirms that the region of the VDAC protein with the least predictable secondary structure is the central one-third of the sequence (Rauch and Moran 1994; Mannella et al. 1996), where regulatory regions may reside (Sampson et al. 1997). A search for functional domains in the PRINTS database revealed that the VDAC and CG17137 proteins contain the EUKARYTPORIN motif (PRINTS: PR00185) that provides a signature for the family, while no statistically significant match above the E-value threshold was found either in the CG17139 or in the CG17140 protein. The four-element porin signature motif is highlighted above the VDAC sequence in Fig. 4B.

## Discussion

Although in vitro experiments using the purified proteins incorporated into planar phospholipid bilayers have been decisive in establishing the role of VDAC as the pore-forming protein of the outer mitochondrial membrane, and have allowed detailed studies on the effect of voltage changes on ion channels, such reconstituted systems are likely to represent an oversimplification of what actually occurs in the cell. In fact, interest in the VDAC protein has expanded over the past few years as a result of studies that suggest that it may be intimately involved in the mechanisms by which proteins of the Bcl-2 family regulate cell death. Since the experimental data indicating a role for VDAC in cell death are based on in vitro studies, generation of mutations that suppress or alter the function of the protein would establish a system in which the many biological functions currently ascribed to VDAC could be tested in vivo.

A model organism of choice for this type of study is *D. melanogaster*. A gene encoding a VDAC protein has been cloned from this species and mapped at 32B3–4 on the second chromosome. We found by 5′RACE-PCR experiments on poly(A)$^{+}$ RNA and by sequence analysis that alternative 5′-untranslated extensions can precede the coding sequence of the *porin* mRNA. The apparently

constitutive expression of at least two alternative transcripts of the *D. melanogaster porin* gene prompted us to start a detailed genetic and functional analysis by searching for *P*-element insertions in the *porin* gene among stocks reported to have single *P*-element insertions in the 32B3–4 region. Two stocks, *l(2)k05123 (porin$^1$)* and *l(2)k08405 (porin$^2$)*, were identified that carry a *PlacW* insertion in the *porin* gene. Western analysis of protein extracts from the rare adult escapers present in both stocks or from late pupae homozygous for either the *porin$^1$* or the *porin$^2$* allele showed that VDAC was absent. The escapers did not show gross morphological abnormalities, but clearly their locomotor system was affected, as shown by problems in locomotion and in flight, and they had a very reduced life span. Impaired energy metabolism is the most likely basis for such defects. In comparison, when mouse embryonic stem (ES) cell lines bearing homozygous deletions in one of the three mouse VDAC genes are produced, they are viable, but have a reduced respiration rate. The viability of these mutant ES cells is assumed to be due to the vicarious activity of the genes encoding the other VDAC isoforms, as it was impossible to obtain double knockout cells (Wu et al. 1999). Mice homozygous for a deletion of the VDAC1 gene have also been generated (Anflous et al. 2001), and again the major problem found appeared to be an impairment of energy metabolism. In the human, a case of VDAC deficiency has been reported: the patient suffered from psychomotor retardation and minor dysmorphism. Skeletal muscle from this patient had a decreased oxidation rate for a variety of substrates and showed a dramatic decrease in the level of VDAC1 protein, but skin fibroblasts from this patient had only moderately reduced VDAC levels, indicating that the defect is tissue-specific (Huizing et al. 1996).

In this context, the phenotype associated with homozygosity for the *porin$^1$* or *porin$^2$* allele, both of which prevent the expression of the gene product, is in accord with what is observed in higher eukaryotes, where the main effect of knocking out the gene encoding the quantitatively dominant VDAC protein seems to be an impairment of energy metabolism; this effect may be lethal when no functional compensation can be invoked. Still, the late pupal lethality and the viability of rare escapers in which the VDAC protein is undetectable by Western blotting is not surprising, as in *Drosophila* several mutants for genes that encode mitochondrial products are known to produce similar pupal-lethal phenotypes. Mitochondria of maternal origin could be sufficient to allow some individuals to survive the crucial stages of development and eclose, or partial rescue by products of other genes could permit the survival of such individuals.

To characterize the effects of discrete deletions in the *porin* transcription unit, we remobilized the *P* elements inserted in the *porin$^1$* and *porin$^2$* alleles by performing dysgenic crosses. The analysis of the revertants supported the notion that the lethal phenotype can be reverted by precise or almost precise excision of the *PlacW* transposon, thus confirming that the inability to synthesize VDAC due to the *P*-element insertion is the cause of the lethality. Deletions of genomic sequences flanking the *P*-element insertion site provided useful information on the genetic role of the sequences involved. The removal of most of the region between exon 1A and exon 1B (*porin$^{1rev16}$*), as well as the removal of exon 1B together with most of the downstream intron (*porin$^{1rev2}$*), produced homozygous vital lines expressing apparently normal amounts of wild-type VDAC, as shown by Western blotting. This shows that the transcription of exon 1B is dispensable for fly development, and poses the question whether this transcript has any specific function. The alternative 5′UTR does not add any pre-sequence to the protein, thus its functional role, if any, must be connected to the polynucleotide sequence itself. One attractive hypothesis is the involvement of this alternative *porin* transcript in a mechanism of differential targeting (Lithgow et al. 1997).

Deletions encompassing exon 1A were also obtained. The removal of this exon together with a few bases upstream of the predicted transcription start (*porin$^{2rev9}$*) results in a lower expression of the VDAC protein. Interestingly, males homozygous for the *porin$^{2rev9}$* allele are sterile. While no obvious cytological defects in spermatogenesis were observed, such males produce non-motile sperms only. The male-sterile phenotype associated with the *porin$^{2rev9}$* allele may correlate with the evidence that specific VDAC isoforms are expressed in mammalian testes. The cause of the defect is likely to be a reduction in the ATP supply to the motor proteins of the flagellum. The *porin$^{2rev8}$* deletion removes 1344 bp starting at a 5′ breakpoint corresponding to position 307 of the genomic clone, while the *porin$^{2rev9}$* deletion removes 1458 bp starting at position 446. Remarkably, the difference between the 5′ breakpoints of *porin$^{2rev8}$* and the *porin$^{2rev9}$*, both of which are located in a putative promoter region, seems to be responsible for the switch from a male-sterile to a lethal phenotype. The fact that the *porin$^{1rev23}$* deletion, which extends further upstream in the putative promoter sequence but does not encompass exon 1B, does not allow production of the VDAC protein and is homozygous lethal further supports the notion that the sequences immediately upstream of exon 1A are required for the normal function of the *porin* gene, probably as parts of a promoter.

## The *porin*-like genes in *D. melanogaster*

We show that three genes located adjacent to the *porin* gene at 32B encode proteins with significant homology to VDAC. These genes are organized in a tandem array, and the lengths of the intergenic regions, as determined by comparing the most extended ESTs available for each of the genes, are remarkably short, ranging from 180 to 50 bp. It is apparent that the four genes share a similar organization of the coding exons (filled boxes in Fig. 4A). Both the homology of their deduced products

and the conservation of their genomic structure strongly suggest that the *porin* gene and the *CG17137*, *CG17139* and *CG17140* genes that flank it at 32B3–4 originated from a common ancestor by consecutive duplication events. Tandem pairs of genes are quite common in *D. melanogaster*. The degree of similarity between members of a pair and the extent to which they are functionally related is variable, and presumably depends both on the time of duplication and, if they remain functional, on functional constraints. In the majority of cases, tandemly duplicated genes are transcribed independently. In some instances, however, they are transcribed dicistronically. This is the case, for example, for the Drosophila genes *sesB* and *Ant2*, which code for adenine nucleotide translocase and produce a transcript that by alternative splicing gives rise to separate mRNAs that share a 5′ untranslated exon (Zhang et al. 1999). Although clear experimental evidence is still lacking, the *porin* gene and the three adjacent *porin*-like genes might also be expressed as polycistronic transcripts. This is suggested by the fact that the sequences separating the genes are extremely short and lack canonical promoters; moreover, an EST (AT03918.5′) exists that contains fused *CG17139* and *CG17140* sequences.

In mammals, there are three different VDAC genes encoding proteins with 68–75% amino-acid sequence identity. The Drosophila VDAC protein encoded by the *porin* gene is equally similar to all three mammalian protein (showing about 60% sequence identity); each of the proteins encoded by the three flanking genes is equally similar to the three mammalian VDAC isoforms but the overall degree of identity is lower, ranging from 34–36% (CG17137) to 20–23% (CG17139 and CG17140). It is worthy noting, however, that VDAC proteins from different species show very little conservation of primary amino acid sequence. For example, human and yeast VDACs are almost indistinguishable on the basis of functional characteristics such as channel gating and selectivity properties, yet they show less than 30% sequence identity. On the other hand, a general pattern of secondary-structure motifs is constantly conserved which suggests that the pore is formed from a series of sided beta sheets in which hydrophobic amino acids face the membrane interior and adjacent hydrophilic amino acids line the aqueous ion conduction pathway (for review, see Gabriel et al. 2001).

Despite their sequence and secondary-structure similarities, and assuming that they are transcribed independently, *porin* and the three flanking genes appear to be functionally distinct. In fact, both the relative abundance of the ESTs in the BDGP collection (in which sequences derived from the *porin* mRNA are much more frequent than those derived from transcription of the three flanking genes) and the isolation of mutant alleles of the *porin* gene with a recessive lethal or male-sterile effect argue that the other *porin*-like genes at 32B are not able to compensate for the loss of the *porin* gene product, and so probably have different functions in the cell. This is consistent with the possible acquisition of new functional roles by members of a small multigene family by modification of a regulatory region while the structural motifs of the protein are conserved overall. The essential role of the VDAC protein in cellular energy metabolism, and the potentially fatal consequences of its loss in *D. melanogaster* (as shown in this work) and in other organisms, may have favored an increase in the numbers of VDAC isoforms able to compensate for each other, as appears to be the case both in mammals and in yeast. The suggested role of porin as part of larger macromolecular complexes (Krimmer et al. 2001) and in important biological processes like apoptosis and spermatid maturation will prompt further investigations on the function of this protein, the role of its isoforms, and its relationships with other molecules, with special attention to the cellular and developmental context.

**Acknowledgements** This work was supported by grants from Progetti di Interesse Nazionale (PRIN), Centro Eccellenza (CE) and the Ministero per Università e la Ricerca Scientifica e Tecnologica (MURST) to C.C.

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