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Cloning and molecular characterization of a voltage-dependent anion-selective channel (VDAC) from *Drosophila melanogaster* [1]

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Abstract

A full length voltage-dependent anion-selective channel (VDAC) cDNA was cloned from *Drosophila melanogaster* by expression library screening using an antibody against an insect VDAC protein. The cDNA clone (denoted DmVDAC) is 1082 base pairs (bp) in length and contains an open reading frame (bp 62–907) encoding a 282 amino acid protein which has a predicted molecular mass of 30 550 Da, a predicted p*I* of 6.98 and no cysteines. Hydrophobicity analysis suggests 15 or 16 membrane-spanning domains. The DmVDAC amino acid sequence has variable homology with VDACs from other species ranging from 62% identity with a human VDAC to 23% identity with a *Dictyostelium discoideum* VDAC. DmVDAC has 92% identity with the 38 conserved residues in a VDAC consensus sequence. DmVDAC was expressed in VDAC-null yeast but failed to rescue viability. DmVDAC has 88% identity at the amino acid level and 99% identity at the nucleic acid level with a recently reported *D. melanogaster* VDAC sequence (A. Messina et al., FEBS Lett. 384 (1996) 9–13). Homology analyses with the Messina and other VDAC sequences indicate that the amino acid differences are due to minor errors in the Messina sequence. Southern blots and chromosomal in situ hybridizations suggest a single VDAC gene occurs in the fly with a locus at 32B on the left arm of the second chromosome. © 1997 Elsevier Science B.V.

Keywords: Voltage-dependent anion-selective channel; Cloning; cDNA; Gene mapping; (*Drosophila*)

1. Introduction

Voltage-dependent anion-selective channels (VDACs) are large-pore channels through which adenine nucleotides, metabolites and ions traverse the outer mitochondrial membrane [1–3]. The biophysical properties of VDACs from a wide variety of species have been characterized in planar phospholipid membranes (reviewed in [4,5]) and VDACs have been cloned and sequenced from several species including the human [6–9], rat [10], cow [11,12], *Neurospora crassa* [13], *Saccharomyces cerevisiae* [14,15], potato [16], wheat [17], pea [18] and maize [18]. Isoforms have been identified in humans [19], fish [20], wheat [17] and potato [16].

The idea has been held by some that VDACs are passive diffusion pores, however, recent evidence

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[1] The nucleotide sequence data of the DmVDAC clone reported in this paper have been submitted to the GenBank under accession number U70314.

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suggests that VDACs are regulated by a variety of factors including cytoplasmic enzymes [21–26], a modulator protein [27–29] and metabolites [30–32], and that VDAC dysfunction may be involved in a variety of human diseases [21,25,33–39]. Elucidation of VDAC function has come primarily from in vitro studies and from studies with yeast and an important goal is to identify models in which the role of VDACs in multicellular eukaryotes can be studied in vivo.

The fruitfly, *Drosophila melanogaster*, would be a useful model for such studies, however, there have been few studies of VDACs in insects [20,40]. We have recently isolated and characterized a 31 kDa VDAC protein from the moth *Heliothis virescens* [41] and we describe here the cloning and molecular characterization of a VDAC cDNA from *D. melanogaster* (denoted DmVDAC). The cloning of another VDAC cDNA from the fly was recently reported by Messina et al. [42]. The two fly sequences differ by 12% at the amino acid level, however, they are more than 99% identical at the nucleic acid level. Our analyses show that the two cDNAs represent the same gene and that the differences in amino acid sequence derive from minor errors in the Messina sequence. Our Southern blot and in situ hybridization data indicate a single gene with a locus at 32B on chromosome 2L.

2. Materials and methods

2.1. Library screening

Approximately 150 000 plaques from a *D. melanogaster* cDNA library (Stratagene UniZap XR Canton S embryonic library) were screened with an affinity-purified antibody (R19AP; raised against a 31 kDa VDAC protein from the tobacco budworm *H. virescens*; Ref. [41]) using standard procedures and alkaline phosphatase-labelled secondary antibodies to visualize positive plaques. Eight potential positive clones were identified, two of which had sequence identity with VDAC genes. The clone described here was manually sequenced on both strands using the Sanger procedure and the sequence was then independently confirmed on both strands by automated fluorescent DNA sequencing in the DNA sequencing facility at Monsanto Company, St. Louis, MO.

2.2. Computer data-base searches and analyses

Homology searches and alignments, and hydrophobicity analyses were carried out using the Wisconsin Genetics Computer Group (GCG) software package, version 8.1.

2.3. Yeast plasmid construction, transformation and functional assay

The DmVDAC coding region was amplified in a thermal cycler using a 5′ primer (5′GACTCCATGGCTCCTCCATCATACA3′) containing an *Nco*I site and a 3′ primer (5′CTGAATGCATTTAGGCCTCCAGCTCCAGAC3′) containing an *Nsi*I site. The product was digested with *Nco*I and *Nsi*I, and inserted into the yeast POR1 gene cut with *Nco*I (as the initiation codon) and *Nsi*I (near the 3′ end of the POR1 coding region). The resulting construct was inserted into the yeast centromere-based plasmid pSEYC58 and into the 2-micron based plasmid pSEY8 [43]. The insert sequence was confirmed by DNA sequencing in the sequencing facility at the Vollum Insititute, Portland, OR. Yeast strains M-3 and M22-2 [44] were transformed [45] with plasmids and grown on SMM plates [46] supplemented with adenine, histidine, tryptophan, lysine and leucine. A minimum of six transformants per transformation were streaked on glycerol-based YPG plates [46] and duplicate plates were grown at 30°C and 37°C with M-3 and M22-2 on the same plate for controls. Transformants were scored as VDAC negative if they grew on YPG at 30°C and grew no better than untransformed M22-2 on YPG at 37°C. Cultures for yeast cell lysates were grown on liquid SMM supplemented as above and extracts were prepared for immunoblots as described [47].

2.4. Southern blots

Genomic DNA was isolated from adult flies [48] and 15 μg aliquots were digested with 10 units each of *Bam*HI, *Eco*RI, *Hin*dIII, *Sac*I or *Xho*I for 2.5 h

at 37°C, electrophoresed on 0.7% agarose gels, blotted to nylon membranes, incubated in 6 × SSC for 2 h, baked at 80°C for 2 h, incubated in prehybridization buffer [48] for 2 h at 55°C and hybridized at 55°C for 16 h with [α-^{32}P]dCTP-labelled DmVDAC (specific activity, 2.4×10^8 cpm/μg) in hybridization buffer [48]. The blots were washed for 15 min in 2 × SSC [48], 0.5% SDS at 25°C, for 15 min in 2 × SSC, 0.1% SDS at 25°C, and for 2 × 60 min in 0.1 × SSC, 0.5% SDS at 68°C. They were then air-dried and subjected to autoradiography for 5 d.

2.5. In situ hybridization

An 18 kb genomic VDAC clone was isolated by screening a *D. melanogaster* genomic library (0–12 h Canton S embryonic, Stratagene) with ^{32}P-labelled DmVDAC cDNA as a probe using standard procedures [48]. The genomic clone was confirmed to contain a VDAC gene by the specific amplification of a VDAC product by polymerase chain reaction using DmVDAC specific primers. The genomic clone was labelled with biotin-14-dATP (BioNick labelling system, Life Technologies), hybridized to fly polytene chromosome squashes [49] and hybridizing bands visualized by means of streptavidin-alkaline phosphatase staining (Life Technologies kit).

3. Results and discussion

3.1. Nucleotide sequence

The nucleotide sequence and the predicted amino acid sequence of the DmVDAC cDNA clone are shown in Fig. 1. The nucleotide sequence has been deposited in the GenBank (accession number U70314). The clone is 1082 base pairs (bp) in length and includes 61 bps of non-coding 5′ sequence, an open reading frame of 845 bp (bp 62–907) and 176 bp of non-coding 3′ sequence. The 3′ sequence contains the termination consensus sequence *aataaa* (bp 1036–1041) and a polyA tail (bp 1075–1082). The *a* and *g* nucleotides in the −3 and +4 positions of the *atg* at bp 61–63 match Kozak's initiation consensus sequence [50] and together with the 5′ in-frame stop codons (asterisks) suggest this is the translation start site. Thus there is no additional 5′ leader sequence in the DmVDAC cDNA as in some VDAC genes [9,10]. The 3′ untranslated sequence lacks an *attta* motif,

```
                                                        *       *
   1      gtggtgtctctgttcctctgcgtttgcacttacactctaaccaaactaaaactcatcaaaa
   1   M  A  P  P  S  Y  S  D  L  G  K  Q  A  R  D  I  F  S  K  G  Y  N
  62 atggctcctccatcatacagcgatttgggcaaacaggctcgcgacatcttcagcaaaggctacaac
  23   F  G  L  W  K  L  D  L  K  T  K  T  S  S  G  I  E  F  N  T  A  G
 128 tttggcctgtggaagctcgatctgaagactaagacctcgtcgggcattgagttcaacacagccgga
  45   H  S  N  Q  E  S  G  K  V  F  G  S  L  E  T  K  Y  K  V  K  D  Y
 194 cactccaaccaggagtctggaaaggtcttcggctccctggagaccaagtacaaggtcaaggactac
  67   G  L  T  L  T  E  K  W  N  T  D  N  T  L  F  T  E  V  A  V  Q  D
 260 ggcctgaccctcaccgagaagtggaacacagacaacacgctgttcaccgaggttgctgtccaggat
  89   Q  L  L  E  G  L  K  L  S  L  E  G  N  F  A  P  Q  S  G  N  K  N
 326 cagctcctcgagggtctgaagctgtccctggagggcaactttgctcctcagtctggaaacaagaac
 111   G  K  F  K  V  A  Y  G  H  E  N  V  K  A  D  S  D  V  N  I  D  L
 392 ggcaagttcaaggttgcctacggccatgagaacgtcaaggccgattcggatgtgaacattgatctg
 133   K  G  P  L  I  N  A  S  A  V  L  G  Y  Q  G  W  L  A  G  Y  Q  T
 458 aagggccccttgatcaatgcctctgccgtgcttggctaccagggatggttggccggctaccagacc
 155   A  F  D  T  Q  Q  S  K  L  T  T  N  N  F  A  L  G  Y  T  T  K  D
 524 gcatttgacacacaacagtccaagctgaccaccaacaactttgcccttggctacaccaccaaggac
 177   F  V  L  H  T  A  V  N  D  G  Q  E  F  S  G  S  I  F  Q  R  T  S
 590 tttgttctgcacacagctgtcaacgatggccaggagttcagcggctcgatcttccaacgcacttcg
 199   D  K  L  D  V  G  V  Q  L  S  W  A  S  G  T  S  N  T  K  F  A  I
 656 gacaagctggatgtgggtgtacagctgtcgtgggccagcggcaccagcaacaccaagttcgccatc
 221   G  A  K  Y  Q  L  D  D  D  A  S  V  R  A  K  V  N  N  A  S  Q  V
 722 ggcgccaagtatcagctggatgatgatgccagcgtgcgcgctaaggtgaacaacgccagccaggtg
 243   G  L  G  Y  Q  Q  K  L  R  D  G  V  T  L  T  L  S  T  L  V  D  G
 788 ggtctgggctaccagcagaagttgcgcgacggagtcaccctgaccctgtccacgctggtcgatggc
 265   K  N  F  N  A  G  G  H  K  I  G  V  G  L  E  L  E  A  *
 854 aagaacttcaatgccggcggccacaagatcggtgtgggtctggagctggaggcctaagtccgtggt
 920 tttcctctagtcgtgaaatccttgaacgttgtctaacatccccacacgcccaacaacaacaacaac
 986 aacaataacagcagccagacaagtgcagaaacaacaacaagatcaaacaaaataaaagatacgata
1052 ttctttaaaaaccaaaaaaaaaaaaaaaaaa
```

Fig. 1. Nucleotide and predicted amino acid sequence of the DmVDAC cDNA. The clone is 1082 bp in length and includes an open reading frame (bp 62–907) which predicts a 282 amino acid peptide with a molecular mass of 30 635 Da, a p*I* of 7.82 and no cysteines. Asterisks indicate two 5′ in-frame stop codons (bp 38–40 and 47–49) and the termination codon (bp 908–910). Potential glycosylation sites are indicated by underlining. The 3′ sequence contains an *aataaa* termination consensus sequence (bp 1036–1041) and a polyA tail (bp 1065–1082).

thought to signal the rapid degradation of a human VDAC [8] and other [51] mRNAs.

3.2. Amino acid sequence

The DmVDAC cDNA predicts a protein of 282 amino acids with a molecular mass of 30 550 Da. As is the case for most VDACs, the initial methionine is cleaved in both *H. virescens* [41] and *D. melanogaster* (J. Ryerse, unpublished data) VDAC proteins. DmVDAC contains no cysteines, thus disulfide bonds are not required for secondary or tertiary structure. *N. crassa* [15] and *Dictyostelium discoideum* [13] VDACs also lack cysteines. The DmVDAC protein contains two potential N-linked glycosylation sites (Fig. 1, underlined) which may be involved in plasma membrane targeting [11,51]. The predicted p*I* of 6.98 suggests the protein has a net positive charge at neutral pH, consistent with the weak anion-selectivity of most VDACs including those from *D. melanogaster* [40] and *H. virescens* [41].

3.3. Homologies with VDACs from other species

The DmVDAC amino acid sequence is compared with the *D. melanogaster* VDAC sequence of Messina et al. [42] and with VDACs from other species in Fig. 2. The DmVDAC protein has the highest identity (87.9%) with the Messina sequence followed by mammals, *N. crassa*, plants, *S. cerevisiae* and *D. discoideum*. The view has been expressed that VDAC sequence is very poorly conserved among organisms [16], but our data suggest that there is a graded continuum with the highest degree of homology among mammals, a moderate degree between mammals and arthropods and relatively low homology between vertebrates and plants.

The DmVDAC sequence is compared with a VDAC consensus sequence [8] in Fig. 3. Thirty-five of the 38 residues (92.1%) which are conserved in all four VDACs evaluated (Fig. 3, underlined) are also conserved in the DmVDAC sequence. The consensus sequence contains 58 additional residues which are conserved in three of the four VDACs evaluated, of which 39 (67.2%) are conserved in the fly. Many of the conserved residues are charged (e.g., lysines and aspartic acids) and these are thought to be essential

```
      1                                                  50
DmVDAC  ..MAPPSYSD LGKQARDIFS KG.YNFGLWK LDLKTKTSSG IEFNTAGHSN
VDACME  ..******** ********* **.******* ********** **********
VDACH2  ..CI****A* ***V*****N **.FG***V* **V***SC** V**S*S*S**
VDACRB  ..CI**P*A* ***A*****N **.FG***V* **V***SC** V**S*S*S**
VDACH1  ..AV**T*A* ***S***V*T **.*G***I* ******SEN* L**TSS*SA*
VDACNC  ..**V*AF** IA*S*N*LLN *DF*HLAAGT IEV*SN*PNN VA*KVT*K.S
VDACMZ  MVV*VGL*T* I**KT**LLY *DYN..THQ* FC*T*SSPN* VAITA**TRK
VDACPO  MGKG*GL*TE I**K***LLY *DYQ..SDH* FSTI*YSPT* VVITSS*SKK
VDACSC  ..*S**V*** ISRNIN*LLN *DF*HATPAA FDVQ*T*AN* *K*SLKAKQP
VDACDD  ..*N*GL*A* *T*PTA*FIK *DFAETFKLD TTF*G*YG*I VAVT.....D

      51                                                 100
DmVDAC  QESGKVFGSL ETKYKVKDYG LTLTEKWNTD NTLFTEVAVQ DQLLEGLKLS
VDACME  ********** ********* ********* ********** **********
VDACH2  TDT***T*T* *****WCE** **F******* ***G**I*IE **ICQ****T
VDACRB  TDT***S*T* *****WCE** **F******* ***G**I*IE **ICQ****T
VDACH1  T*TT**T*** ****RWTE** **F******* ***G**IT*E ***AR****T
VDACNC  THDKVTS*A* *G*FTD*PN* **V*QT***A *A*E*K*EMA *N*AK***AE
VDACMZ  N**..I**E* H*QI*N*K.. **VDV*A*SE SD*LTTIT*D EFGTP***SI
VDACPO  GDL..FLADV N*QL*N*N.. V*TDI*VD*N SN***TIT*D E.AAP***TI
VDACSC  VKD*PLSTNV *A*LND*QT* *G**QG*SNT *N*Q*KLEFA .N*TP***NE
VDACDD  IKDSG*VA*I QP*ADFTK*. ..*GKVS*GN F*VD*NGVKK GEFTIENIIP

      101                                                150
DmVDAC  LEGNFAPQSG NKNGKFKVAY GHENVKADSD VNIDLKGPLI NASAVLGYQG
VDACME  **ATLLLSLE TRTASSR.S* ********** ********** **********
VDACH2  FDTT*S*NT* K*S**I*SS* KR*CINLGC* *DF*FA**A* HG***F**E*
VDACRB  FDTT*S*NT* K*S**I*S** KR*CINLGC* *DF*FA**A* HG***F**E*
VDACH1  FDSS*S*NT* K**A*I*TG* KR*HINLGC* MDF*IA**S* RGAL****E*
VDACNC  GIFS*L*ATN ARGA**NLHF KQS*FHGRAF FDL.****TA *ID*IV*HE*
VDACMZ  INLVV...PD QRS**LEFQ* L**YAGVNAS *GLN.SN*MV *L*GAF*SKA
VDACPO  *SFRV...PD QRS**LE*Q* L*DYAGICTS *GLT.AN*IV *F*G*V*TNI
VDACSC  *ITSLT*GV. A*SAVLNTTF TEPFFT*RGA FDLC**S*TF VGDLTMAHE*
VDACDD  GLKAV*NGDS KQ*FSTEFQ* KKDKIAFTL. ..FGHNNKSF *T*LAFLINP

      151                                                200
DmVDAC  WLAGYQTAFD TQQSKLTTNN FALGYTT.KD FVLH.TAVND GQEFSGSIFQ
VDACME  ********** ********** ***A.*P.QG LCSA.HSCQR C*********
VDACH2  ******MT** SAK****R** **V**R*.G* *Q**.*N*** *T**G***Y*
VDACRB  ******MT** SAK****RS* **V**R*.G* *Q**.*N**N *T**G***Y*
VDACH1  ******MN*E *AK*RV*QS* **V**K*.DE *Q**.*N*** *T**G***Y*
VDACNC  F***ASAGY* V*KAAI*GYS A*V**HA.PT YSAAI**TDN LSV**A*YYH
VDACMZ  LSV*VDVS** *AT*DF*KY* A**SL*S.P* LIASLHLN*H *DTLVA*YYH
VDACPO  IAL*TDVS** *KTGDF*KC* AG*SF*N.A* L*ASLNLN*K *DNLTA*YYH
VDACSC  IVG*AEFGY* ISAGSISRYA M**S*FA.** YS*GA*.L*N E*ITTVDF**
VDACDD  TFSVGVQ*EG NAKNT*KNV* ATITIRPRP* VFVSIVDRFM DKQILL*TLY

      201                                                250
DmVDAC  RTSDKLDVGV QLSWASGTSN TK..FAIGAK YQLDDDASVR AKVNNASQVG
VDACME  ********** ********** **..SV**** *******R** **********
VDACH2  KVCED**TS* N*A*T***NC *R..*G*A** ****PT**IS *****S*LI*
VDACRB  KVCEDF*TS* N*A*T***NC *R..*G*A** ****PT**IS *****S*LI*
VDACH1  KVNK**ETA* N*A*TA*N** *R..*G*A** **I*P**CFS *****S*LI*
VDACNC  KVNSQVEA*S KAT*N*K*G* *.VGLEVAT* *RI*PVSF*K G*I*DRGVAA
VDACMZ  LVKNHSGTA* GAELSHSM*R NESTLIF*SQ HS**PHTTIK IRF**YGMAS
VDACPO  TV*PLTSTA* GAEVNHSF*T NENIITVGTQ HR**PLT**K *RI**FGKAS
VDACSC  NVNAFLQ**A KATMNCKLP* SNVNIEFATR *LP*ASSQ*K ***SDSGI*T
VDACDD  TATS**SFAG DVTVDLKA*E KAPS*NV*TQ *KI*SASLLK *****NRK*N

      251                                          289    %ID  Ref
DmVDAC  LGYQQKLRDG VTLTLSTLVD GKNFNAGGHK IGVGLELEA      -    -
VDACME  ********T* ********** ********** *********    87.9 [42]
VDACH2  V**T*T**P* *K****A*** **SI****** V*LA*****    62.1  [9]
VDACRB  V**T*T**P* *K****A*** **S******* L*LA*****    61.4 [10]
VDACH1  ***T*T*KP* IK****A*L* ***V****** L*L***FQ*    58.0  [9]
VDACNC  IA*NVL**E* ***GVGASF* TQKLDQAT** V*TSFTF*S    31.1 [15]
VDACMZ  ALV*HEW*PK SFV*I*GD** T*AIEKST.* V*LS*VLKH    29.2 [18]
VDACPO  ALL*HEW*PK SLF*V*GE** T*SVDK*A.* F*LA*A*KP    28.0 [16]
VDACSC  *A*K*L**P* ***GVGSSF* ALKLSEPV** L*WS*SFD*    27.6 [14]
VDACDD  IS*IYNTSNN TKFV*GWN*N T***KQ*.NT F*ATVN*TL    22.8 [13]
```

Fig. 2. Comparison of the DmVDAC amino acid sequence with the Messina et al. [42] fly sequence (VDACME) and with VDACs from human (VDACH1 and VDACH2), rat (VDACRB), *N. crassa* (VDACNC), *S. cerevisiae* (VDACSC), *D. discoideum* (VDACDD), maize (VDACMZ) and potato34 (VDACPO). %ID, percent identity with the DmVDAC sequence. Asterisks indicate the same residue as in the DmVDAC sequence and dots are spacings introduced by computer alignment. Ref, reference.

for ion selectivity [44]. A GLK domain (residues 97–98) is conserved among the 10 VDAC sequences shown in Fig. 2, although in *D. discoideum* it is offset by computer alignment. This domain must be

```
          1                                                  50
DmVDAC    MAPPSYSDLGKQARDIFSKGYNFGLWKL..DLKTKTSSGIEFNTAGHSNQ
VDACME    **************************..******************** 
Ha con    ..PP.Y.D..K.A.D..NK.F.........DVKT...NG..F...G....

          51                                                 100
DmVDAC    ESGKVF..GSLETKYKVKDYGLTLTEKWNTDNTLFTEVAVQDQLLEGLKL
VDACME    ******..**************************************** 
Ha con    ........G.LE.K......GLT.T..WNT.N.L.T.....D.L..GLK.

          101                                                150
DmVDAC    SLEGNFAPQSGNKNGKFKVAYGHENVKADSDVNIDLK.GPLINASAVLGY
VDACME    ***ATLLLSLETRTASSR.S***************.************ 
Ha con    ....SF.P.T.K..AK.....K..........D.....GP...G....G.

          151                                                200
DmVDAC    QGWLAGYQTAFDTQQSKLTTNNFALGYTTKDFVLHTAVNDGQE.FSGSIF
VDACME    ************************A.*PQGLCSAHSCQRC**.****** 
Ha con    EG.LAG.....D..K...T....AVGV......L....ND....F..S.Y

          201                                                250
DmVDAC    QRTSDKLDVGVQLS..WASGTSNTKFAIGA.KYQLDDDASVRAKVNNASQ
VDACME    **************..*********SV***********R*********** 
Ha con    QKVN............W............A.KY..DP.....AKVN.S..

          251                                  291
DmVDAC    VGLGYQQKLRDGVTLTLSTLVDGKNFNAGGHKIGVGLELEA
VDACME    **********T**************************** 
Ha con    ....Y.Q.LRPGV.L...A..D........HK.G.......
```

Fig. 3. Comparison of the DmVDAC and Messina (VDACME) *D. melanogaster* VDAC [42] sequences with the consensus sequence of Ha et al. [8]. The underlined residues in the consensus sequence are conserved in all four sequences evaluated. The remaining residues are conserved in three of the four sequences evaluated. 92% of the absolutely conserved residues and 67% of the 3/4 conserved residues are conserved in the DmVDAC sequence. Dots in the *D. melanogaster* sequences represent alignment adjustments.

important for VDAC structure or function given it's conservation among the widely divergent species. The GLK triad is not absolutely conserved, however, since L is replaced by V in the pea [18] and by W in a recently cloned yeast VDAC (E. Blachly-Dyson and M. Forte, unpublished data). D17, K21 and T85 are also highly conserved among VDACs and likely play critical roles in channel function. In general, however, relatively few amino acids are absolutely conserved among VDACs, consistent with their tolerance for considerable divergence in primary structure without significant effect on secondary structure and channel function [16].

3.4. Secondary structure

VDACs are thought to consist of a single peptide which forms a thin-walled β-barrel [52–54] with a cytoplasmic, α-helical N-terminus [55–57] and with either 12 [44] or 16 [16,18,55] anti-parallel amphipathic membrane-spanning domains. Kyte and Doolittle [58] hydropathy profiles for *D. melanogaster*, human, potato and yeast VDACs are compared in Fig. 4. All are characterized by a hydrophilic N-terminus and a series of alternating hydrophobic and hydrophilic domains along the proteins. The fly, human and potato profiles are generally similar and are most consistent with 15 or 16 membrane-spanning domains. DmVDAC, like other VDACs, has a high glycine content permitting extensive peptide folding for β-barrel formation.

3.5. Complementation analysis

To determine whether DmVDAC could substitute for the major VDAC gene in yeast, the DmVDAC coding region was placed between the promoter and termination sequences of the yeast VDAC1 gene, POR1. This construct was introduced into yeast lacking the POR1 gene on a low copy number centromere-based plasmid [59] and on a multicopy plasmid [60]. Yeast cells lacking the POR1 gene can grow on glycerol-based media (YPG) at 30°C, but not at 37°C [44], while yeast with a wild-type POR1 gene grow well at both temperatures. The yeast cells failed to grow on YPG at 37°C following transformation with either construct, indicating the fly gene could not functionally substitute for the yeast gene. Inability to complement is somewhat surprising as two human VDAC genes restored growth [9], and DmVDAC has 58–62% identity with human VDACs (Fig. 2). Two potato VDACs, however, also failed to rescue VDAC-deficient yeast [16]. We confirmed by nucleotide sequencing that the DmVDAC constructs contained the appropriate initiation and termination codons and had no missing or altered bases (not shown). In addition, we confirmed that DmVDAC protein was synthesized in transformed null yeast and targeted to the mitochondria by staining extracts of

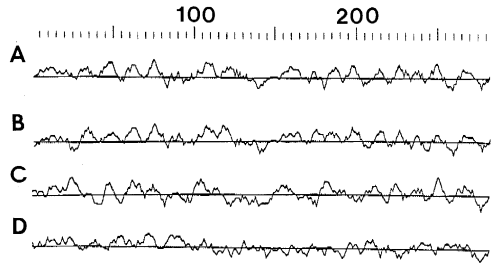

Fig. 4. Hydrophilicity profiles of (A) DmVDAC, (B) human, (C) potato and (D) yeast VDACs. Amino termini are to the left, residue numbers are at the top. Hydrophilic residues are above and hydrophobic residues are below the horizontal lines.

yeast and mitochondrial fractions on blots with a DmVDAC protein antibody (data not shown; Ab described in Ref. [41]). The reason for the lack of complementation therefore remains to be determined.

3.6. Southern blots

In order to evaluate the complexity of VDAC genes in *D. melanogaster*, we carried out a Southern blot analysis of genomic DNA cut with five restricton enzymes (*Bam*HI, *Eco*RI, *Hin*dIII, *Sac*I, *Xho*I) and probed at 55°C with a ^{32}P-labelled DmVDAC polymerase chain reaction product (Fig. 5). Lanes 1, 2, 3 and 5 contain single hybridizing bands indicating that the DmVDAC gene is a single copy gene. Lane 4 contains several bands due to the presence of internal *Xho*I sites in the DmVDAC clone. DmVDAC cDNA was used as a positive control and λDNA size markers as a negative control (not shown). Similar results were obtained in a duplicate experiment in which the hybridization was carried out at 50°C (not shown).

3.7. Are there VDAC isoforms in flies?

VDAC isoforms have been identified in the human [9], in plants [16,17], and may also occur in fish [20]. The differences in the DmVDAC and Messina sequences (Fig. 2) raised the possibility that these might also be isoforms, however, the following sequence analysis suggests that these differences result from sequencing errors rather than from the cDNAs being isoforms.

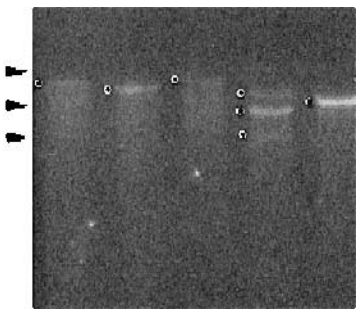

Fig. 5. Southern blot analyses of *D. melanogaster* genomic DNA cut with (1) *Bam*HI, (2) *Hin*dIII, (3) *Sac*I, (4) *Xho*I, and (5) *Eco*RI and probed at 55°C with ^{32}P-labelled DmVDAC cDNA. Single hybridizing bands (dots) in lanes 1, 2, 3 and 5 suggest a single VDAC gene occurs in *D. melanogaster*. Lane 4 has several bands because the VDAC gene contains internal *Xho*I sites. Size standards of 7, 4 and 3 kb are indicated with arrowheads.

In contrast to the differences between DmVDAC and human amino acid sequences, which are spread out along the length of the proteins, the divergence between the fly proteins is concentrated in two domains (Figs. 2 and 3). The partial homology between the Messina fly sequence and the human VDAC sequence breaks down in these domains, whereas the partial homology between DmVDAC and the human VDAC sequence persists across these regions. Further, of the two absolutely conserved residues in the consensus sequence [8] which are located in the regions of difference between the fly sequences, both are conserved in the DmVDAC sequence but neither is conserved in the Messina sequence (Fig. 3). Similarly, of the 10 residues in the two divergent regions which are conserved in 3/4 of the clones included in the consensus sequence, there are seven matches in the DmVDAC sequence compared with only one match in the Messina sequence.

The foregoing suggested that the differences between the two fly sequences might arise from minor errors in one of the cDNA sequences. Computer analysis of the nucleic acid sequences in the open reading frames of the two cDNAs indicated an identity of greater than 99% (Fig. 6) consistent with the idea that one of the cDNAs contained sequence errors and with the idea that they might represent one and the same gene. With this in mind, we confirmed that the DmVDAC sequence was correct by independent automated fluorescent DNA sequencing at Monsanto Company and at the Vollum Institute. We were then able to identify several missing, mis-assigned and mis-ordered bases in the Messina sequence. These errors accounted for all of the differences in amino acid sequence. For example, the missing G358 (see Fig. 6; base position numbers refer to position in the DmVDAC sequence) initiates a set of 16 divergent amino acids (Figs. 2 and 3) before the coding sequence returns to the correct reading frame due to missing T406 and G407 bases. Similarly, the missing G572 initiates the second domain of divergent amino acids before returning to the correct reading frame due to missing C582 and G617 bases. The mis-assignment of A for C271 in the DmVDAC sequence is without consequence since both codons yield leucine. However, the mis-assignments of C for T714, T for C717, and G for C754 (Fig. 6) result in incorrect amino acid assignments of S, V and R instead of F, A

and A as in the DmVDAC sequence (Figs. 3 and 6). The mis-ordered ACG versus GAC at bps 815–817 in the DmVDAC sequence results in assigning a T instead of a D as in the DmVDAC sequence. Additional differences were also found in the 5′ and 3′ non-coding regions.

We used in situ hybridization of polytene chromosome squashes as another approach to evaluate whether there are VDAC isoforms in the fly. An (18 kb) genomic VDAC clone was obtained by screening a fly genomic library with the DmVDAC cDNA and labelled genomic clone was used as the in situ hybridization probe. A single hybridizing band was consistently observed with a cytogenetic locus at 32B on the left arm of the second chromosome (Fig. 7). The locus can be precisely positioned because it is immediately distal to the 32C band on 2L (inset in Fig. 7). An in situ hybridization analysis by Messina et al. [42] identified two VDAC loci in the fly, at 31E on chromosome 2L and at 79D on chromosome 3L. Our results probably differ from those of Messina et al. because we used a large (18 kb) genomic probe whereas they used a much smaller (1.4 kb) cDNA probe. Larger probes are more likely to yield prominent hybridization signals. It is of interest to note, however, that there is a band at about 32B on chromosome 2L in the situ hybridization figure in the Messina paper.

We conclude that the two fly VDAC cDNAs represent the same gene and that there is a single VDAC gene in *D. melanogaster* with a cytogenetic locus at 32B on 2L.

```
DmVDAC   62 ATGGCTCCTCCATCATACAGCGATTTGGGCAAACAGGCTCGCGACATCTT 111
            ||||||||||||||||||||||||||||||||||||||||||||||||||
VDACME   71 ATGGCTCCTCCATCATACAGCGATTTGGGCAAACAGGCTCGCGACATCTT 120

        112 CAGCAAAGGCTACAACTTTGGCCTGTGGAAGCTCGATCTGAAGACTAAGA 161
            ||||||||||||||||||||||||||||||||||||||||||||||||||
        121 CAGCAAAGGCTACAACTTTGGCCTGTGGAAGCTCGATCTGAAGACTAAGA 170

        162 CCTCGTCGGGCATTGAGTTCAACACAGCCGGACACTCCAACCAGGAGTCT 211
            ||||||||||||||||||||||||||||||||||||||||||||||||||
        171 CCTCGTCGGGCATTGAGTTCAACACAGCCGGACACTCCAACCAGGAGTCT 220

        212 GGAAAGGTCTTCGGCTCCCTGGAGACCAAGTACAAGGTCAAGGACTACGG 261
            ||||||||||||||||||||||||||||||||||||||||||||||||||
        221 GGAAAGGTCTTCGGCTCCCTGGAGACCAAGTACAAGGTCAAGGACTACGG 270

        262 CCTGACCCTCACCGAGAAGTGGAACACAGACAACACGCTGTTCACCGAGG 311
            ||||||||| |||||||||||||||||||||||||||||||||||||||||
        271 CCTGACCCTAACCGAGAAGTGGAACACAGACAACACGCTGTTCACCGAGG 320

        312 TTGCTGTCCAGGATCAGCTCCTCGAGGGTCTGAAGCTGTCCCTGGAGGGC 361
            |||||||||||||||||||||||||||||||||||||||||||| |||
        321 TTGCTGTCCAGGATCAGCTCCTCGAGGGTCTGAAGCTGTCCCTGGA.GGC 369

        362 AACTTTGCTCCTCAGTCTGGAAACAAGAACGGCAAGTTCAAGGTTGCCTA 411
            |||||||||||||||||||||||||||||||||||||||||||  | ||
        370 AACTTTGCTCCTCAGTCTGGAAACAAGAACGGCAAGTTCAAGGT..CGTA 417

        412 CGGCCATGAGAACGTCAAGGCCGATTCGGATGTGAACATTGATCTGAAGG 461
            ||||||||||||||||||||||||||||||||||||||||||||||||||
        418 CGGCCATGAGAACGTCAAGGCCGATTCGGATGTGAACATTGATCTGAAGG 467

        462 GCCCCTTGATCAATGCCTCTGCCGTGCTTGGCTACCAGGGATGGTTGGCC 511
            ||||||||||||||||||||||||||||||||||||||||||||||||||
        468 GCCCCTTGATCAATGCCTCTGCCGTGCTTGGCTACCAGGGATGGTTGGCC 517

        512 GGCTACCAGACCGCATTTGACACACAACAGTCCAAGCTGACCACCAACAA 561
            ||||||||||||||||||||||||||||||||||||||||||||||||||
        518 GGCTACCAGACCGCATTTGACACACAACAGTCCAAGCTGACCACCAACAA 567

        562 CTTTGCCCTTGGCTACACCACCAAGGACTTTGTTCTGCACACAGCTGTCA 611
            |||||||||| ||||||||| ||||||||||||||||||||||||||||||
        568 CTTTGCCCTT.GCTACACCA.CAAGGACTTTGTTCTGCACACAGCTGTCA 615

        612 ACGATGGCCAGGAGTTCAGCGGCTCGATCTTCCAACGCACTTCGGACAAG 661
            ||||| ||||||||||||||||||||||||||||||||||||||||||||
        616 ACGAT.GCCAGGAGTTCAGCGGCTCGATCTTCCAACGCACTTCGGACAAG 664

        662 CTGGATGTGGGTGTACAGCTGTCGTGGGCCAGCGGCACCAGCAACACCAA 711
            ||||||||||||||||||||||||||||||||||||||||||||||||||
        665 CTGGATGTGGGTGTACAGCTGTCGTGGGCCAGCGGCACCAGCAACACCAA 714

        712 GTTCGCCATCGGCGCCAAGTATCAGCTGGATGATGATGCCAGCGTGCGCG 761
            || || |||||||||||||||||||||||||||||||| |||||||||||
        715 GTCCGTCATCGGCGCCAAGTATCAGCTGGATGATGATGCCAGGGTGCGCG 764

        762 CTAAGGTGAACAACGCCAGCCAGGTGGGTCTGGGCTACCAGCAGAAGTTG 811
            ||||||||||||||||||||||||||||||||||||||||||||||||||
        765 CTAAGGTGAACAACGCCAGCCAGGTGGGTCTGGGCTACCAGCAGAAGTTG 814

        812 CGCGACGGAGTCACCCTGACCCTGTCCACGCTGGTCGATGGCAAGAACTT 861
            |||  ||||||||||||||||||||||||||||||||||||||||||||||
        815 CGCACGGGAGTCACCCTGACCCTGTCCACGCTGGTCGATGGCAAGAACTT 864

        862 CAATGCCGGCGGCCACAAGATCGGTGTGGGTCTGGAGCTGGAGGCC 907
            |||||||||||||||||||||||||||||||||||||||||||||
        865 CAATGCCGGCGGCCACAAGATCGGTGTGGGTCTGGAGCTGGAGGCC 910
```

Fig. 6. DmVDAC and the Messina et al. *D. melanogaster* sequences are more than 99% identical at the nucleic acid level suggesting that the two sequences represent the same gene. As discussed in the text, the 12.1% difference between the two sequences at the amino acid level is due to missing, mis-assigned and mis-ordered bases in the Messina nucleotide sequence.

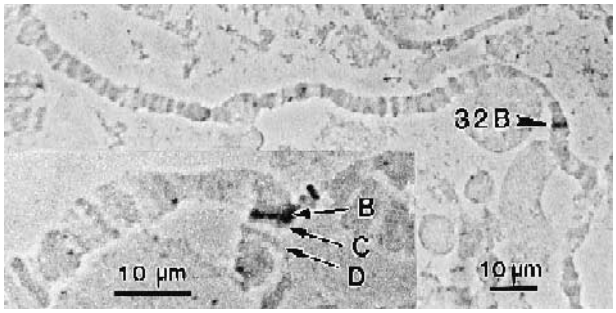

Fig. 7. In situ hybridization to *D. melanogaster* polytene chromosomes using a genomic VDAC clone as a probe indicates there is a single VDAC gene in the fly located at 32B on the left arm of chromosome 2. The inset illustrates the fine structure of the region at higher magnification in which the hybridizing band is localized immediately distal to the 32C band.

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