The Murine Voltage-dependent Anion Channel Gene Family

CONSERVED STRUCTURE AND FUNCTION

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Voltage-dependent anion channels (VDACs) are pore-forming proteins found in the outer mitochondrial membrane of all eukaryotes. VDACs are the binding sites for several cytosolic enzymes, including the isoforms of hexokinase and glycerol kinase. VDACs have recently been shown to conduct ATP when in the open state, allowing bound kinases preferential access to mitochondrial ATP and providing a possible mechanism for the regulation of adenine nucleotide flux. Two human VDAC cDNAs have been described previously, and we recently reported the isolation of mouse VDAC1 and VDAC2 cDNAs, as well as a third novel VDAC cDNA, designated VDAC3. In this report we describe the structural organization of each mouse VDAC gene and demonstrate that, based on conserved exon/intron boundaries, the three VDAC isoforms belong to a single gene family. The 5′-flanking region of each VDAC gene was shown to have transcription promoter activity by transient expression in cultured cells. The promoter region of each VDAC isoform lacks a canonical TATA box, but all are G+C-rich, a characteristic of housekeeping gene promoters. To examine the conservation of VDAC function, each mouse VDAC was expressed in yeast lacking the endogenous VDAC gene. Both VDAC1 and VDAC2 are able to complement the phenotypic defect associated with the mutant yeast strain. VDAC3, however, is only able to partially complement the mutant phenotype, suggesting an alternative physiologic function for the VDAC3 protein.

Voltage-dependent anion channels (VDACs, also known as mitochondrial porins) are 30–35-kilodalton (kDa) pore-forming proteins found in the outer mitochondrial membrane of eukaryotes (reviewed in Ref. 1). VDACs play a role in the regulated flux of metabolites across the outer mitochondrial membrane, but their exact cellular role is not well understood. VDACs from a variety of organisms have remarkably similar electrophysiological properties (1). Gating of the channel depends upon the transmembrane potential, while its voltage sensitivity is modulated by an intermembrane protein (2). VDACs are “open” at low transmembrane potentials, with a preference for anions such as phosphate, chloride, and adenine nucleotides. At higher transmembrane potentials, VDACs are in a “closed” configuration and more selective for cations (2, 3). VDACs have been shown to reversibly bind several cytosolic kinases, including glycerol kinase and the hexokinase isofoms I–IV (reviewed in Ref. 4). This interaction is believed to allow bound kinases preferential access to mitochondrial ATP derived from oxidative phosphorylation (5, 6). VDACs have been associated with the adenine nucleotide translocator of the inner mitochondrial membrane and octomeric creatine kinase of the intermembrane space (6–8). It has been suggested that this complex has properties resembling the permeability transition pore (9).

A direct demonstration of voltage-gated ATP flux through VDAC was recently reported (10). Physiological concentrations of NADH also affect VDAC permeability, suggesting one possible mechanism for the observed ability of glycolysis to suppress oxidative phosphorylation (the Crabtree effect; Refs. 11 and 12). VDACs have been identified as a component of the peripheral benzodiazepine receptor complex (13), which is linked to steroid biosynthesis (14). Finally, VDACs have been shown to co-purify with the brain γ-aminobutyric acid subunit A receptor complex (15).

cDNAs encoding two human VDAC isoforms were reported by Blachly-Dyson et al. (16). We have previously described the isolation of mouse orthologues for VDAC1 and VDAC2, as well as a novel mouse VDAC termed VDAC3 (17, 18). Each isoform is 65–70% identical to the other isoforms. Phylogenetic analysis indicates that VDAC3 is the more primordial of the vertebrate VDAC genes, suggesting that if the multiple isoforms arose from a gene duplication and divergence event VDAC3 diverged from the primordial VDAC first, with VDAC1 and VDAC2 arising more recently (18). The three mouse genes have been mapped to separate autosomal loci (17, 18).

To determine whether the genomic organization can be correlated with existing structural information about the VDAC protein, and whether the VDAC isoforms arose by gene duplication and divergence or evolutionary convergence, we have characterized the gene structure for each VDAC locus. In addition, we have examined the promoter regions of each VDAC gene by DNA sequence analysis and expression studies to discern any regulatory similarities. Furthermore, to begin to examine the functional properties of VDAC3, the cDNA was expressed in a VDAC-deficient yeast strain and tested for its ability to complement the temperature-sensitive growth phenotype of a VDAC-deficient yeast.

MATERIALS AND METHODS

Isolation of VDAC Genomic Clones—To generate isoform-specific probes, the polymerase chain reaction (PCR) was used to amplify the

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3’-untranslated region of each mouse VDAC isoform (from nucleotides 959–1277 for VDAC1, 942–1276 for VDAC2, and 1022–1428 for VDAC3 (17, 18). Each PCR product was used to generate [α-32P]dCTP (NEN Life Science Products) random primer-labeled probes. The three probes were spotted onto a 120 base pair denaturing gel (Stratagene, La Jolla, CA). Hybridizations were carried out in Blotto (1.5 M NaCl, 50 mM Tris, 1% SDS, 0.5% nonfat dried milk) at 65°C. Purified phage were isolated by three rounds of sequential purification, and restriction mapping was performed using standard methods.

**VDAC Gene Structures**—Genomic fragments that hybridized to the full-length cDNAs were subcloned into pBluescript KS vectors using established protocols. The fragments were sequenced using Sanger and Reverse primers on an Applied Biosystems model 373A automated fluorescent DNA sequencer. When necessary, complementary oligonucleotides were used to complete the sequencing of all VDAC exons. When the genomic clone did not contain the entire genomic structure of the VDAC isoform, a 5’ intronic fragment from the genomic clone was used to screen the genomic library as before. The new genomic clone isolated was then hybridized with both cDNA and 5’-RACE probes until the entire genomic structure of each isoform was characterized. Nucleotide sequence analysis was performed using the GCG software package (51).

3’-RACE Amplification—Total RNA was extracted using guanidinium isothiocyanate from embryonic stem (ES) cells as described above. The RNA (~20 µg/lane) was fractionated on a 1% agarose/formaldehyde gel and transferred to Hybond N+ membranes (Amersham Life Science). To prevent probe cross-hybridization the membranes were probed with the 3’-untranslated regions of each VDAC isoform labeled as before. Hybridizations were carried out overnight at 65°C in Blotto. The membranes were washed twice in 2x standard saline citrate, 0.1% SDS for 10 min and exposed for 16–24 h at ~80°C.

**VDAC Zoo Blot**—Ten micrograms of genomic DNA from the species listed in Fig. 2 were digested with EcoRI, separated by electrophoresis on a 0.7% agarose gel, and Southern-blotted onto a Hybond N+ membrane (Amer sham Life Science). Each full-length VDAC cDNA was labeled as before and used to probe the blot. Hybridization was carried out in Blotto at 55°C overnight. After hybridization the filter was washed several times in 3x SSC, 0.1% SDS at 37°C and set to expose for several days. The blot was stripped after each hybridization by washing with 0.1x SSC, 0.1% SDS at 85°C.

**CAT Reporter Gene Constructs**—CAT (chloramphenicol acetyltransferase) reporter plasmid constructs of the 5’-flanking region of each mouse VDAC isoform were prepared by standard cloning techniques. The VDAC1 5’-flanking 918-base pair (bp) fragment was generated by PCR using a sense oligonucleotide upstream of the first exon and an antisense oligonucleotide from the 5’-untranslated region (5’-GCTT-GATATCGAATTCCTCCGTC3’- and 5’-GGAGACGCAGCCGACTAC3’, respectively). The predicted start codon is located 4 bp into the second exon. The PCR product was subcloned into a plasmid T-vector (20) and sequenced. The restriction enzymes XbaI and AatII were used to subclone the DNA in both orientations in the pCAT Basic and Enhancer vectors using adapter oligonucleotides. The pCAT Basic vector, which lacks a promoter and enhancer, and the pCAT Enhancer vector, which lacks a promoter, were used as negative controls, while the pCAT Control vector containing both the promoter and enhancer of simian virus 40 (SV40) was used as a positive control. DNA was prepared using a Qiagen plasmid miniprep kit (Qiagen, Santa Clarita, CA).

**Transfection and CAT Assays**—NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, and cells were plated at a density of 1.5 × 10⁴ cells/60 mm plate. The following day, transfections were carried out using 1 µg of CAT construct DNA, 8 µg of LipofectAMINE (Life Technologies, Inc.), and 0.2 µg of the β-galactosidase expression vector pCMVβ (Promega, Madison, WI) in serum-free medium (Opti-MEM, Life Technologies, Inc.) following the manufacturer’s recommended protocol. The cells were harvested 48 h after transfection, and lysed by three freeze-thaw cycles. Cell extracts (20 µl) were heated for 10 min at 65°C and assayed for CAT activity, as described by Sambrook et al. (49). CAT assay results were quantified using a Betascope Betascanner (InnteliGenetics, Cambridge, MA). CAT activities are expressed as a percentage of the pCAT control.

**Expression of Mouse VDACs in Yeast**—For each of the three VDAC isoforms oligonucleotide-directed mutagenesis was used to create NcoI sites at the start codon for both VDAC1 and VDAC2, and an AflIII site for VDAC3. The same strategy was used to generate NsiI sites in the 3’-untranslated region of each gene. This allowed for the complete open reading frame of each VDAC DNA to precisely replace the yeast VDAC gene previously cloned in a yeast single-copy shuttle vector (kindly provided by M. Forte, Oregon Health Sciences University, Portland, OR; Ref. 22). The oligonucleotides for each gene were used to amplify the cDNA inserts, the products were digested with the appropriate restriction enzymes, and the fragments were subcloned to replace the yeast VDAC gene between the NcoI and NsiI sites. From the starting ATG codon, the length of each cDNA insert was 1111 bp for VDAC1, 1079 bp for VDAC2, and 1014 bp for VDAC3. The three constructs were then introduced into the VDAC-deficient yeast strain M22-2 by lithium acetate transformation (23). The yeast were streaked onto media containing 2% glycerol as the sole carbon source and incubated at 30°C or 37°C for 6 days.

**RESULTS AND DISCUSSION**

The importance of VDAC proteins for normal metabolic homeostasis was recently emphasized by the report of a child with a mitochondrial myopathy who was shown by Western analysis to have a partial deficiency of VDAC1 protein (24). The disorder appeared to be somewhat tissue-specific, with a greater deficiency of VDAC1 in skeletal muscle than fibroblasts. The patient’s skeletal muscle mitochondria also exhibited reduced rates of pyruvate oxidation and ATP production. Since VDACs are expressed in nearly all tissues and have been shown to bind various kinases in liver, fat, and brain tissues and in several tumor cell lines, a global role for VDACs in metabolic homeostasis has been suggested (4). However, despite the extensive electrophysiologica characterization of the VDAC channels from numerous organisms, the functional roles of the VDAC isoforms in metabolic homeostasis are not well understood. To begin to understand the genetic basis for VDAC function we have determined the structural and regulatory regions of the murine genes encoding the three VDAC isoforms.

**Structure of the Mouse VDAC Genes**—The VDAC1 gene spans approximately 28 kb, and is made up of 9 exons (Fig. 2). The predicted start codon is located 4 bp from the second exon. Sequencing of the first 5’-untranslated exon of VDAC1 identified 41 bp of cDNA sequence previously obtained by 5’-RACE (17). Since high promoter activity is detected in the region immediately 5’ to this sequence, transcription/translation is predicted to occur at or close to this sequence. In addition to the splice sites, functional genes contain five distinct VDAC3 processed pseudogenes, each without an open reading frame, were isolated and sequenced from the genomic library (data not shown).

The VDAC2 gene, unlike VDAC1 or VDAC3, is encoded by 10 exons, with the additional exon constituting part of the 5’-untranslated region. VDAC2 spans approximately 12 kb, with.
the 132-bp 5'-untranslated region (17) encoded in the first two exons. The predicted start codon of VDAC2 is located 26 bp into the second exon. A single VDAC2 processed pseudogene lacking an open reading frame was also isolated (data not shown).

The VDAC3 gene is encoded by 9 exons and spans approximately 16 kb. The predicted start codon is found 3 bp into the second exon, and the first and second exons contain the entire 78 bp of 5'-untranslated sequence predicted by 5'-RACE (18). A single VDAC3 processed pseudogene lacking an open reading frame was isolated, as well as an intronless VDAC3-like sequence with a complete open reading frame (data not shown; GenBank™ U89990). This sequence differs from the VDAC3 cDNA sequence at only eight nucleotide positions, five of which are in the predicted coding region, leading to three silent codon changes and two amino acid substitutions; a glycine to glutamic acid substitution and tryptophan to glycine substitution. Despite the presence of an open reading frame, the lack of conserved amino acid substitutions suggests it is a processed pseudogene under no evolutionary selection.

**VDAC Exon/Intron Boundaries**—The exon/intron junctions were sequenced for each gene, and the size of all introns was determined by DNA sequencing, restriction site mapping, and/or PCR amplification (Table I). All exon/intron splice junctions follow the GT-AG rule and conform to the established consensus exon boundary sequences (48). The VDAC exon/intron boundaries correspond to the transmembrane regions proposed for the human VDAC protein structure (Fig. 1; Ref. 25). It may be argued that the introduction of introns into the VDAC gene defines a modular organization to the different transmembrane regions of the VDAC protein, however it may also simply be a chance occurrence. The genomic conservation of all coding exon/intron boundaries is indicative of a gene family, and strongly suggests gene duplications and divergence as the origin of the three VDAC isoforms. The 5'-untranslated region exon/intron boundaries and the intron sizes are not conserved, suggesting the gene duplications were ancient evolutionary events that followed the introduction of introns, and that these sequences may have evolved a new function after the duplication of the genes, or alternatively are under no functional constraints. The splice acceptor boundaries of the third exon of VDAC2 and the second exon of VDAC3 are conserved. In VDAC3 the site is located within the 5'-untranslated region, while in VDAC2 it is located in the coding region. This demonstrates a single instance of coding and non-coding splice site conservation between the VDAC isoforms. The same boundary site in the 5'-untranslated region of the VDAC1 gene differs by a single nucleotide (Table I).

**VDAC Polyadenylation Signals**—The human VDAC1 gene was reported to contain 854 bp of 3'-untranslated sequence with a consensus polyadenylation site (16). Since the original mouse VDAC1 cDNA did not contain a consensus polyadenylation site (17), 3'-RACE was used to identify the polyadenylation site(s). Using this technique, a 873-bp 3'-untranslated region was sequenced, and, in contrast to the human VDAC1 gene, two consensus polyadenylation signals (AATAAA) at nucleotides 1737 and 1748 were identified. The latter signal is most likely the primary polyadenylation signal used, both because of its proximity to the poly(A) tract and because the signal at nucleotide 1737 is not conserved in the human 3'-untranslated sequence. The mouse VDAC1 3'-untranslated region has approximately 70% DNA sequence conservation with the human VDAC1 cDNA. This region is 20 bp longer than the
human VDAC1 3'-untranslated sequence, but the high degree of sequence similarity between the two regions further supports that these genes are orthologues.

Blachly-Dyson et al. (16) suggested the existence of alternative polyadenylation sites in the human VDAC2 gene based upon finding two sets of human cDNA clones with differing 3'-untranslated sequence lengths. Four of the human cDNA clones terminated at a position equivalent to nucleotide 1301 in the mouse VDAC2 gene. The mouse VDAC2 gene was previously shown to be encoded by multiple transcripts, based on Northern blot analysis (Ref. 17; Fig. 2). 3'-RACE was used to determine if the multiple VDAC2 transcripts are due to alternative polyadenylation signals. Three distinct PCR products were generated by 3'-RACE (data not shown), each of which was subcloned and sequenced. The 3'-untranslated region of VDAC2 contains one aberrant and two canonical polyadenylation signals. The two longer products can be accounted for by consensus polyadenylation signals found at nucleotides 1462 and 1637, respectively. The shortest product, corresponding to the strongest signal on a Northern blot of ES cell RNA (Fig. 2), terminates in a 4-bp region from nucleotides 1300 to 1303, with no consensus polyadenylation signal present. This 4-bp region is completely conserved in the human VDAC2 3'-untranslated region. Less than 5% of mRNAs are generated by non-consensus polyadenylation signals (26), although the most proximal alternative polyadenylation signal used by the mouse and human VDAC2 genes is not apparent. The lack of a consensus polyadenylation signal in VDAC2 may cause the termination site of the short transcript to be less precise, thus accounting for the 4-bp region of termination identified by 3'-RACE. 3'-Untranslated regions have been shown to control mRNA turnover, translation efficiency, and the subcellular location of transcripts (27–30); it is possible that the shorter 3'-untranslated region plays a specific role in regulating VDAC2 gene expres-
During development, Ha et al. (31) has suggested the existence of two alternative VDAC2 amino termini in humans based upon the isolation of variant cDNAs. The possibility that the mouse VDAC2 gene also generates alternative amino termini has not been exhaustively addressed, although no alternative VDAC2 cDNAs were identified in the course of these studies.

The VDAC3 transcript has previously been shown to terminate at a single polyadenylation site (18).

Cross-species Conservation—The evolutionary conservation of the VDAC genes, suggested by previous electrophysiologic studies of VDACs isolated from different species (1, 16) is further supported at a DNA sequence level by the “zoo blot” results (Fig. 3). Each VDAC isoform gives a unique hybridization pattern (data not shown). These results suggest that each of the mouse VDAC genes is evolutionarily conserved in a number of distantly related species, including cow, chicken, sea urchin, *Caenorhabditis elegans*, and *Drosophila melanogaster*. Some of the hybridizing bands do overlap from the different isoforms, reflecting sequence identity with more than one mouse VDAC isoform or, alternatively, a genomic repeat sequence. VDAC1 gives the most complicated hybridization pattern, perhaps reflecting the abundance of pseudogenes for this isoform encountered in the mouse. This high level of sequence conservation and the functional conservation suggested by the ability of VDACs from other species to complement the yeast VDAC-deficient phenotype implies that the VDAC isoforms function in conserved pathways across phyla.

**DNA Sequence Analysis of the Transcription Control Regions**—Sequence analysis of the 5′-flanking region of each VDAC isoform reveals several characteristic features. The promoter regions of all three VDAC isoforms lack canonical TATA boxes and are G+C-rich, a characteristic of housekeeping gene promoters (32). In these regions, VDAC1 (493 bp; GenBank™ U89987), VDAC2 (952 bp; GenBank™ U89988), and VDAC3 (529 bp; GenBank™ U89988), have an average G+C content of 69.8%, 60.6%, and 68.7%, respectively (Fig. 4). The transcription initiation sites for each VDAC gene were predicted using the TSSG and TSSW computer programs. For VDAC1 the transcription initiation site is predicted to be nucleotide 453, 41 bp upstream of the first confirmed 5′-untranslated exon nucleotide (Fig. 4). VDAC2 transcription initiation sites are predicted to be nucleotides 656 and 956. The latter site is the fourth nucleotide in the first 5′-untranslated exon. The VDAC3 transcription initiation site is predicted to be nucleotide 474, 56 bp upstream of the first 5′-untranslated exon.

A data base search for transcription factor binding motifs revealed multiple sites in the 5′-flanking sequences of each VDAC gene, including a number of Sp1 binding sites. It is common for at least one Sp1 binding site to be located within the promoter of a housekeeping gene (32); VDAC1 contains two Sp1 sites, VDAC2 contains 10 Sp1 sites, while VDAC3 contains five Sp1 sites. VDAC1 and VDAC2 also have a sterol repressor element 1 (SRE-1) binding site within the predicted promoter regions. The SRE-1 octanucleotide sequence appears to enhance transcription in the absence of sterols but is transcriptionally inactive in the presence of sterols. Therefore this sequence may contribute to the maintenance of cholesterol homeostasis in cells (33). VDAC proteins have been identified as a component of the peripheral benzodiazepine receptor complex.

2 These programs can be found on the Baylor College of Medicine Gene Finder home page (http://dot.imgen.bcm.tmc.edu:9331/gene-finder/gf.html).
plex, which has been implicated in the metabolism of cholesterol for steroidogenesis (14). Since it has been shown previously that in vitro VDAC channel activity requires the presence of sterols (34), it is noteworthy that a SRE-1 site is located in the promoter region of these two genes, and suggests a role for sterols in the regulation of VDAC1 and VDAC2 expression, or perhaps indirectly in the regulation of cholesterol import into mitochondria.

**Transient Expression of VDAC-CAT Plasmids**—To determine if the predicted promoter region of each VDAC gene is capable of directing transcription, plasmids were constructed containing the different 5' flanking regions of each VDAC gene placed upstream of a promoter-less CAT gene. A VDAC1 5'-flanking 918-bp fragment containing 19 bp of the first 5'-untranslated exon was found to direct expression of the CAT gene in both the sense and antisense orientations. A pCAT Basic vector containing a shorter 513-bp VDAC1 5'-flanking fragment with 19 bp of the first 5'-untranslated exon was subsequently generated and found to have greater promoter activity in the sense orientation than the 918-bp fragment, while the antisense orientation lacked any significant promoter activity (Fig. 5), suggesting there may be silencer sequences in the first 406 bp of the larger VDAC1 fragment. These results indicate that a minimal VDAC1 promoter fragment is transcriptionally active in only the sense orientation.

It has been reported previously that the promoters of several housekeeping genes demonstrate transcriptional activity in both orientations when linked to a reporter gene in transfection assays (35–38). It is possible that elements of the VDAC1 promoter may also direct the transcription of a second gene aligned in the opposite orientation, as has been demonstrated with other bidirectional promoters (39). Alternatively, the promoter of a second gene may be in a head-to-head configuration with the VDAC1 promoter (40).

A VDAC2 5'-flanking 1014-bp fragment containing 62 bp of the first 5'-untranslated exon, as well as a longer 5'-flanking fragment of approximately 5 kb in length, were subcloned into the pCAT Basic vector in both orientations. The 1014-bp and 5-kb fragments were essentially equivalent in activity to the pCAT Control vector that contains the SV40 early promoter and enhancer. In contrast, when the fragments were placed in the antisense orientation, no transcriptional activity was detected.

The genomic clone containing the first 5'-untranslated exon of VDAC3 has only an additional 529 bp of upstream genomic sequence, thus limiting the size of the putative promoter region that was tested. A 549-bp fragment, including 20 bp of the first 5'-untranslated exon, was subcloned into both the pCAT Basic and Enhancer vectors, and was found to have promoter activity in the sense orientation. In the pCAT Basic vector the fragment had approximately 16% of the activity obtained with the control SV40 early promoter, while the pCAT Enhancer vector was able to induce this activity by 3-fold (Fig. 4). Again, the opposite orientation was found to lack significant promoter activity.

The promoter region of VDAC1, although imparting significant transcriptional activity, has the least amount of activity of the three isoforms examined. Addition of an enhancer sequence to the VDAC1 and VDAC3 promoter regions (VDAC1-9E and VDAC3-5E, respectively in Fig. 5) led to higher transcriptional activity, suggesting that additional regulatory elements for these VDAC genes are not present in the DNA fragments.

**FIG. 5. Transient transfection analysis of the VDAC promoter regions.** The 5'-flanking fragments of each VDAC gene were subcloned into pCAT Basic and Enhancer vectors in both orientations. These constructs were transfected into NIH3T3 cells and assayed for CAT activity (see “Materials and Methods”). The data presented are from at least three independent sets of experiments, with the standard error of the mean indicated by bars.

**FIG. 6. Complementation of the yeast VDAC-deficient strain using the mouse VDAC genes.** A yeast strain in which the chromosomal VDAC gene is deleted (Δ) (2) was transformed with a single copy yeast shuttle vector containing the wild-type VDAC gene (WT) or the mouse VDAC1, VDAC2, or VDAC3 cDNA constructs as described under “Materials and Methods.” The resulting strains were then streaked on media containing 2% glycerol as the sole carbon source and incubated at 30 °C or 37 °C. Note that while VDAC1 and VDAC2 fully complement the temperature-sensitive phenotype, VDAC3 can only partially complement the defect. A small number of more rapidly growing colonies can be seen in the VDAC3 sector.
analyzed. Although VDAC2 consistently directed the highest level of activity in the transient transfection assays, by North-ern analysis the in vivo expression of the three isoforms is fairly comparable, with the exception of a lack of VDAC1 ex-pression in testes (17). The distribution of these regulatory elements must also vary between the genes, since comparably sized fragments of each VDAC gene were examined, and each gave different levels of promoter activity. Alternatively, in vitro transcription assays may not reflect in vivo regulation, due to altered mRNA stability or the rate of protein turnover. It is also possible that other VDAC promoters exist within each locus, particularly for the VDAC2 gene, which is expressed as multiple sized transcripts. The VDAC2 promoter, because it directs a high level of expression and appears to be expressed ubiqui-tously in mouse tissues, may be useful as a heterologous pro-moter in transgenic mouse experiments. The minimal active region and in vivo regulation of this promoter can be analyzed to investigate this possibility.

Expression of Mouse VDACs in Yeast—To examine the abil-ity of the mouse VDAC isoforms to form a functional VDAC protein, each mouse VDAC cDNA was expressed in yeast lack-ing the endogenous yeast VDAC gene (22). Such yeast are viable but have a temperature-sensitive growth phenotype on media containing glycerol as the sole carbon source (41–43). The biochemical basis for this observed phenotype is unknown. Mouse VDAC cDNA constructs were generated which contain the complete mouse coding region flanked by the yeast VDAC promoter and 5′-untranslated region and the yeast 3′-untrans-lated region. Thus, each mouse VDAC plasmid differs only within the coding region, with all relevant control elements in common. The yeast shuttle vector used is a single copy plasmid, and in combination with the yeast VDAC regulatory elements should approximate the endogenous levels of VDAC expression in yeast. Like their human orthologues (16), introduction of either mouse VDAC1 or VDAC2 into the mutant yeast elimi-nated the temperature-sensitive growth defect (Fig. 6). Thus, VDAC1 and VDAC2 cDNAs appear to encode VDAC proteins that are able to substitute for the endogenous activity of the yeast VDAC gene.

Phylogenetic analysis indicates that VDAC3 is the more ancient of the three VDAC isoforms. The placement of VDAC3 on a separate branch of a phylogenetic tree also suggests that this protein may have a physiological function distinct from that of VDAC1 and VDAC2 (18). This prediction is indirectly supported by the observation that VDAC3 does not rescue the temperature-sensitive phenotype completely, but generates a lower level of growth under the restrictive conditions. A small number of rapidly growing colonies are seen when yeast expressingeVAD33 are grown at the restrictive temperature. The basis for the vigorous growth found in this subpopulation of VDAC3-expressing yeast is currently under investigation.

Because the molecular basis of the VDAC-deficient yeast phenotype is unknown, the inability of VDAC3 to completely rescue this phenotype is also not understood. It is unlikely that the VDAC3 protein is simply not expressed efficiently in the yeast because the transcriptional elements used for the three isoform constructs are identical and the VDAC3 translation initiation site in the yeast construct matches the yeast consensus translation initiation sequence more closely than the mammalian “Kozak” consensus sequence (44, 47). Since mammalian VDACs are known to interact with various cytosolic kinases, the VDAC3 protein may be unable to interact with certain kinases necessary for full complementation, and therefore only partial complementation is observed. It is worth noting that of the three mouse VDAC isoforms only VDAC3 contains a leucine zipper motif (amino acids 150–171; LAGYQMSLDTAKSKL-

SQNNFAL), as determined by a protein motif search, whereas all other recognized motifs (e.g. PKC or CR2 phosphorylation sites, myristoylation sites) are conserved between the isoforms. This motif is found in a region of the protein predicted to form an exposed cytoplasmic loop (25).

Information gained from defining the gene structure of each VDAC isoform provides insights into the history of VDAC evo-lution and the reagents necessary to examine whether transcrip-tional control of VDAC expression occurs. Although the existence of other as yet undiscovered mouse VDAC isoforms has not been completely excluded, screenings of several cDNA and genomic libraries, and data base searches for related ESTs have failed to identify any additional VDAC-like sequences. Data from the experimental work described in this paper can be used to design targeting vectors for generating mutations at each locus to genetically define VDAC function.

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