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Molecular Cloning of cDNAs Encoding the Proteolipid Subunit of the Vacuolar H^+-ATPase of *Acetabularia acetabulum*

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Abstract: Three cDNAs (named pVC1, pVC2 and pVC3) have been isolated from a giant alga, *Acetabularia acetabulum* that encoded the N, N′-dicyclohexylcarbodimide-binding 16 kDa proteolipid subunit of V-ATPase. The open reading frames of pVC1, pVC2 and pVC3 predicted the polypeptides of 164, 167 and 168 amino acids with the molecular masses of 16.5, 16.7 and 16.8 kDa, respectively. Seventy nine percent identity between pVC1 and pVC2 or pVC3 and 95% identity between pVC2 and pVC3 was observed. PVC1 and pVC2/pVC3 showed extensive divergences in their 3′ -untranslated region, while pVC2 and pVC3 possessed the same 3′ -untranslated region. The deduced amino acid sequences of the three cDNA clones showed extensive similarities with that of proteolipids of oat (75 to 80%), bovine (55%) and yeast (55%) V-ATPase. Based on hydropathy plot, four membrane-spanning domains were predicted, in which domain IV was especially conserved among different species. This domain showed 96-100% identity in amino acid sequences between the *A. acetabulum* and the oat proteolipid in which a glutamate residue is included, the putative N, N′-dicyclohexylcarbodimide-binding residue.

Key words: *Acetabularia acetabulum*, molecular cloning, V type ATPase, proteolipid subunit

Introduction

The tonoplast H^+-pumping ATPase in higher plants utilizes the energy of ATP hydrolysis to pump protons into the vacuolar lumens (Sze, 1985; Rea and Sanders, 1987). The proton motive force drives secondary active transport of various ions and metabolites such as Ca^{2+}, anions, amino acids, and sugar into vacuoles (Rea and Sanders, 1987). In plant cells vacuoles not only serve as storage compartments but are also important for regulating cytoplasmic pH and cytoplasmic ion levels (e.g. Ca^{2+}) and thus maintaining cytoplasmic homeostasis and cell turgor (Boller and Weimken, 1986). The tonoplast ATPase belongs to a class of vacuolar-type ATPase (V-ATPases) found on membranes of acidic compartments in many eukaryotes such as fungal vacuoles, bovine chromaffin granules and brain clathrin-coated vesicles (Forgac, 1989) and on the plasma membrane of archaebacteria (Konishi *et al.*, 1987).

The eukaryotic V-ATPase is a multimeric enzyme consisting of 3-10 different subunits with a mass of about 450-759 kDa (Forgac, 1989). Three subunits common to the eukaryotic V-ATPases are the major polypeptides of 67-73 (70) and 57-60 (60) kDa and a 16-17 kDa proteolipid (Randall and Sze, 1986; Parry *et al.*, 1989; Arai *et al.*, 1988; Kane *et al.*, 1989; Moriyama and Nelson, 1989; Gluck and Caldwell, 1987). The primary amino acid sequences of the 70 kDa and the 60 kDa polypeptides have been deduced recently from cDNA or genomic DNA sequences (Bowman *et al.*, 1988; Manolson *et al.*, 1988; Zimniac *et al.*, 1988). The 70 and 60-kDa polypeptides are thought to be catalytic and regulatory subunits, respectively, which participate in catalysis analogous to the interaction between the α and β subunits of the F_1F_0 –ATPase. These two large polypeptides from part of a peripheral sector that can be removed from vacuolar membranes by chaotropic anions (Moriyama and Nelson, 1989; Lai *et al.*, 1988; Bowman *et al.*, 1989), an observation confirmed by the hydrophobic nature of the deduced amino acid sequences of cloned 70- and 60-kDa subunit genes (Bowman *et al.*, 1988; Manolson *et al.*, 1988; Zimniac *et al.*, 1988). The 16-kDa polypeptide is a major subunit of the membranous sector that binds N,N′-dicyclohexylcarbodimide (DCCD), an inhibitor of the V-ATPase activity and potential H^+ pore blocker (Kaestner *et al.*, 1988; Arai *et al.*, 1987; Rea *et al.*, 1987). The DCCD-binding subunit can be extracted with chloroform / methanol like the 8-kDa proteolipid of the F_0F_1-ATPase. Sun *et al.* (1987) presented evidence for H^+ translocation by the purified and reconstituted 16 KDa proteolipid from coated vesicle V-ATPase; however, it is unclear whether an H^+ pore is formed by 16-kDa alone or in combination with other integral subunits. To understand further the structure, function and biosynthesis of the 16-kDa proteolipid we have obtained three cDNAs encoding the proteolipid subunit of V-ATPase from *Acetabularia acetabulum*, a unicellular marine alga and is one of the most ancient eukaryotes.

Materials and Methods

The research work has been carried out (1993-1996) in the Department of Biopharmaceutical Science, Faculty of Pharmaceutical Sciences, The Graduate School of Natural Science and Technology, Okayama University, Okayama, Japan.

Restriction enzymes, DNA polymerase klenow fragment, Taq polymerase, ExTaq polymerase and NuSeive GTG agarose were purchased from Takara (Kyoto, Japan). T_4 DNA ligase was from Gibco BRL (Gaithersburg, USA), and Bacto-tryptone and yeast-extract were from DIFCO laboratories (Detroit, USA). Agarose was from Nippon Gene Co. Ltd. (Toyama, Japan). Other reagents of analytical grade were from Wako Pure Chemicals Ind. Ltd. (Osaka, Japan).

Strain and culture conditions: *A. acetabulum* was grown axenically in artificial sea water as described by Schimid and Giesecke (1984) with 10 hours illumination and 14 hours darkness at 22°C.

Bacterial strain, plasmid and growth media: *Escherichia coli* strain XL-1 Blue (STRATAGENE, La Jolla, CA) was used as a host for preparation of plasmid DNAs and pBluescript II SK (+) (STRATAGENE, La Jolla, CA) as a vector plasmid. For cultivation of *E. coli*, LB medium (Luria Bertani medium, 1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl) was used with 100 μg/ml sodium salt of ampicillin.

Primer synthesis: Oligonucleotides were synthesized on the basis of conserved regions of eukaryotic V-ATPase proteolipid subunit and specific DNA sequences of *A. acetabulum* were from respective cDNA fragments (Table 1).

DNA source: The cDNA library (about 2.3×10^5 clones and 2×10^{10} pfu/ml) was constructed into the bacteriophase λgt11 at *EcoRI* site (Yang and Groot, 1992) using poly $(A)^+$ RNA prepared from total RNA of *A. acetabulum* isolated according to the method of Li-Weber and Schweiger (1985).

Total RNA was isolated from *A. acetabulum* using Plant-A-Extraction Kit of CLONTECH (California, USA) following the manufacturer's instruction.

Table 1: Oligonucleotides used for PCR amplification of V-ATPase, Proteolipid subunit of *A. acetabulum*

VC1	5′ GGBGCBGCYTACGGKACDGC 3′
VC3	5′ CCRACRATRCCRATDGC 3′
VC25	5′ TGAACTTCCTCCAGATGCCTTG 3′
VC58	5′ AGAAGCAGAAGCCTTGGAAGCC 3′
DT-adapter1	5′ AACTGGAAGAATTCGCGGCCGCAGGAAT$_{18}$ 3′

B: G/C/T, Y: C/T, K: G/T, D: A/G/T, R: A/G

Table 2: Characteristics of V-ATPase, proteolipid subunit of *A. acetabulum*

Gene product:	pVC1 (combined product of pVC25 and pVC10) PVC2 (combined product of pVC58 and pVC51) PVC3 (combined product of pVC58 and pVC74)
Structural features:	pVC1 ; 707 bp in length (from 67 to 561) pVC2 ; 782 bp in length (from 51 to 554) pVC3 ; 790 bp in length (from 56 to 562) ORF ; 80% (pVC1/pVC2), 78% (pVC1/pVC3), 95% (pVC2/pVC3) matching 5′-UTR; 58% (½), 53% (1/3), 50% (2/3) 3′-UTR; 54% (½), 54% (1/3), 99% (2/3)
Codon Usage:	All codon used. TAA & TAG; Q
(G + C) content:	pVC1 ; 45% in ORF pVC2 ; 49.1% in ORF pVC3 ; 46.2% in ORF
Structural Features of Proteins:	
	pVC1 ; 164 amino acids with predicted Mr. of 16,466. Isoelectric point 9.08, DCCD binding site at Glu-142. Alignments (% identity); 75% (oat), 54% (bovine), 51% (yeast).
	pVC2; 167 amino acids with predicted Mr. of 16,698. Isoelectric point 8.72, DCCD binding site at Glu-147. Alignments (% identity); 80% (oat), 56% (bovine), 56% (yeast).
	pVC3; 168 amino acids with predicted Mr. of 16,843. Isoelectric point 8.72, DCCD binding site at Glu-148. Alignments (% identity); 81% (oat), 56% (bovine), 56% (yeast).

First-strand cDNA was synthesized from total RNA (ca. 5 μg) of *A. acetabulum* in a 33 μl reaction volume using Ready-to-Go T-primed First-strand Kit (RTG Kit from Pharmacia Biotech., Uppsala, Sweden).

Polymerase chain reaction: The PCR technique was performed according to the manufacturer's thermal cycler instructions (Perkin – Elmer, Cetus, Emerville, CH). 5′- and 3′-Rapid Amplification of cDNA End (RACE) were carried out by the use of Marathon™ cDNA Amplification Kit of CLONETECH. Briefly, first-strand cDNA was synthesized from total RNA (ca. 1 μg) with a primer at the base of poly (A)$^+$ tail in a 10 μl reaction volume, then second-strand cDNA with Marathon cDNA adapter according to the manufacturer's protocol. Double-stranded cDNA was used for further amplification with gene-specific oligonucleotides and the adapters (AP-1 or NAP-1 supplied by the manufacturer).
5′-RACE of the proteolipid subunit of the V-ATPase was performed using double stranded cDNA with Marathon Kit. Oligonucleotides (pVC25, pVC58, Table 1) specific for *A. acetabulum* proteolipid subunits were used for amplification with 5′-AP-1. 3′-RACE of the proteolipid subunit of the V-ATPase was carried out using first-strand cDNA with RTG Kit. Oligonucleotides VC-1 and adapter1 nested to poly d(T) were used for amplification. The temperature program was 1 min at 94°C, 2 min at 55°C, 3 min at 72°C for 40 cycles.

Cloning and sequencing: The PCR reaction mixtures were treated with Klenow fragment (2 U/50 μl) at 37°C for 30 min. After gel electrophoresis, the product of interest was extracted from the gel using Qiaex DNA extraction kit (Diagen, Duesseldorf, FRG) and ligated into the multiple cloning site (*Eco*RV site) of pBluescript II SK(+). Nucleotide sequencing of double stranded templates was performed with SequiTherm Long-Read cycle sequencing kit (Epicentre Tech., Chicago, USA) using a Li-Cor DNA sequencer, model 4000L (Lincoln, USA).

Results

Isolation of cDNAs encoding the proteolipid subunit of *Acetabularia acetabulum:* Our initial strategy for cloning the cDNA encoding the proteolipid of *A. acetabulum* was to screen the cDNA library with the oligonucleotides VC1 and VC3 which were synthesized from highly conserved regions of V-ATPase proteolipid subunit of higher plants (Konishi, 1994) (Table 1). As a result, a cDNA fragment (260 bp) possibly encoding the proteolipid subunit of *A. acetabulum* was obtained. Further attempts to obtain a full length of the cDNA were not successful using a cDNA library because of unspecific amplification of λgt11 forward and reverse primers. The first-strand cDNA generated by RTG kit was thus used to obtain the 3′ -end carrying cDNA clone with oligonucleotides VC1 and poly d(T)-adapter1(Table 1). An aliquot (5 μl / 50 μl) of the first polymerase chain reaction mixture was amplified with the same primers and the procedure was repeated. The temperature program was 1 min at 94°C, 2 min at 55°C and 3 min at 72°C for 40 cycles. After the third amplification, an approximately 650 bp cDNA was specifically amplified, subjected to ligation, transformation and selection. After minipreparation of plasmids, recombinants with the predicted size were amplified by PCR using oligonucleotides VC1 and VC3. Six positive clones designated as pVC17, 25, 35, 39, 58 and 69 were selected and sequenced. Among six cDNA clones, pVC58 showed extensive divergences in its codon usage and 3′-untranslated region, while the other five cDNA clones appeared to be the same accounting for misreading of Taq polymerase (data not shown). In order to obtain cDNA clones containing a complete open reading frame and 5′-end of the proteolipid subunit of *A. acetabulum*, two antisense oligonucleotides, VC25 and VC58 (Table 1), were synthesized from the clones pVC25 and pVC58, respectively and used for the amplification of cDNAs synthesized from total RNA with Marathon Kit.

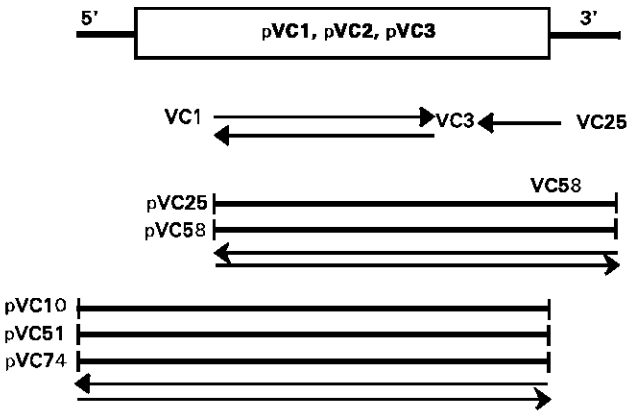

Fig.1: The relationship of cDNA clones encoding the proteolipid subunit of *A. acetabulum* V-ATPase. Open box shows open reading frame and the bars untranslated regions. Arrows indicate direction and extent of sequence read with the sequencing primers.

As a result three cDNA clones, pVC10 with VC25 and AP-1, and pVC51/pVC74 with VC58 and AP-1 were obtained and sequenced. They were combined with the respective 3′-end carrying cDNA clone and designated as pVC1 (pVC10/pVC25), pVC2 (pVC51/pVC58) and pVC3 (pVC74/pVC58). The map of these cDNA clones and sequencing strategy are shown in Fig. 1. Complete nucleotide and deduced amino acid sequences of pVC1, pVC2 and pVC3 are given in Fig. 2-A, B and C. The characteristics of these three cDNA clones encoding the proteolipid subunit of the V-ATPase are summarized in Table 2. The amino acid sequences of pVC1 and pVC2/3 were about 80% identical, while those of pVC2

```
A.   ACTTGCTAACATATACTACAACTTTATACTGTGCCGTTTGATACACGTCTCTCTGATACAACA   63
     AAT ATG GCA GAC GAA ACT AAA GGC CCG GTT CAA TTG ACA GCT TCT TTC   111
        M  A  D  E  T  K  G  P  V  Q  L  T  A  S  F                   15
     TAC GGC TTC TTA GGT GCT GCG TTT GCC CTA ATA TTT TCT TGT ATG GGT   159
  I  Y  G  F  L  G  A  A  F  A  L  I  F  S  C  M  G                   31
     GCC GCC TAC GGT ACA GCT AAA TCC GGT ATC GGT ATT GCC CAA ATG GGC   207
     A  A  Y  G  T  A  K  S  G  I  G  I  A  Q  M  G                   47
     GTG ATG AAA CCC GAG CTG GTG ATG AAA TCA ATT GTG CCT GTT GTG ATG   255
     V  M  K  P  E  L  V  M  K  S  I  V  P  V  V  M                   63
     GCT GGT GTT TTG GGA ATT TAT GGA TTG ATT ATT GCT GTC ATT ATC TCC   303
 II  A  G  V  L  G  I  Y  G  L  I  I  A  V  I  I  S                   79
     ACA AAT GTT AAG AAA ACA GGG TAT ACT TTA TAC GAT GGA TAT GCA CAC   351
     T  N  V  K  K  T  G  Y  T  L  Y  D  G  Y  A  H                   95
     ATG GGC GCC GGT ATT GCG TGC GGT ATG GCT GGT ATG CCG GCA GGA ATG   399
III  M  G  A  G  I  A  C  G  M  A  G  M  P  A  G  M                   111
     GCA ATT GGT ATA GTG GGA GAC GCC GGT GTT CGT GCC AAT GCT TAA CAA   447
     A  I  G  I  V  G  D  A  G  V  R  A  N  A  Q  Q                   127
     CCC AAA TTG TTT GTG GGG GTG ATT TTA ATT CTT ATT TTC GCC GAG GCT   495
     P  K  L  F  V  G  V  I  L  I  L  I  F  A  E  A                   143
     TTG GCT TTG TAT GGT TTG ATT GTT GGA ATC ATT CTT GCT TCA AAG GCA   543
 IV  L  A  L  Y  G  L  I  V  G  I  I  L  A  S  K  A                   159
     TCT GGA GGA AGT TCA TGA TTGATGGCATATGTTTTTTTATATTATTTTTGAATCCGA   600
     S  G  G  S  S  end                                               164
     ATTTGATGAAAAAATGGAGAGATAAAAGGTAAGGGGGGAGCTGTAATTATGATGATTTGGTAA  663
     GAAATAATTTGCTAATGATTGATTGAACTGATCTTACCGGGGATAAAAAAAAAAAAAAAAAA   725
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Fig. 2A: Nucleotide and deduced amino acid sequences of pVC1 (A).
Underlined sequences of pVC1 represent hyperphobic domains I, II, III and IV in descending order. The number of nucleotides or amino acids are shown at right.

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B.   CTTTTGTCTATTATATTTCTTCTCTCGTTAAATAATTTGTACCCTGAAAA ATG GCA ACT   59
                                    M  A  T                          3
     GCT GAG ACT CCT TCT GCC ACC CCT GCT GCT GGA AAT GAT ACC GCT CCA   107
      A  E  T  P  S  A  T  P  A  A  G  N  D  P  A  P                  19
     TTT TTT GGC TTT ATG GGT GCA GCT TCT GCT TTG GTT TTT GCT TGT ATG   155
      F  E  G  F  M  G  A  A  S  A  M  V  F  A  C  M                  35
     GGA GCT GCC TAT GGT ACT GCT AAA TCT GGA GTG GGT ATT GCT TCC ATG   203
      G  A  A  Y  G  T  A  K  S  G  V  G  I  A  S  M                  51
     GGG GTT ATG AGA CCG GAA TTG GTT ATG AAG TCT ATT GTC CCT GTT GTC   251
      G  V  M  R  P  E  L  V  M  K  S  I  V  P  V  V                  67
     ATG GCG GGT GTG TTG GGT ATT TAC GGT TTA ATC ATT GCC GTG ATC ATC   299
      M  A  G  V  L  G  I  Y  G  L  I  I  A  V  I  I                  83
     AGT ACA AAC GTG AAG CGT GAC GTG TAC AAG TTG TAC GAC GGG TAT GCT   347
      S  T  N  V  K  R  D  V  Y  K  L  Y  D  G  Y  A                  99
     CAC TTG TCT GCA GGT TTG GCA TGC GGC CTT GCC GGT TTG CCC GCC GGA   395
      H  L  S  A  G  L  A  C  G  L  A  G  L  P  A  G                  115
     ATG GCC ATC GGA ATC GTA GGT GAT GCC GGA GTT CGC GCA AAC GCA CAA   443
      M  A  I  G  I  V  G  D  A  G  V  R  A  N  A  Q                  131
     CAA CCC AAA TTG TTG GTC GGT ATG ATC CTT ATT CTA ATT TTT GCC GAG   491
      Q  P  K  L  F  V  G  M  I  L  I  L  I  F  A  E                  147
     GCA CTG GCA CTG TAC GGA TTG ATT GTG GGA ATT ATC TTG GCT TCC AAG   539
      A  L  A  L  Y  G  L  I  V  G  I  I  L  A  S  K                  163
     GCT TCT GCT TCT TGA GGCATGTATGCGGTTATTATATTTTTCATAGGTGGATATGGAT   597
      A  S  A  S  end                                                  167
     AAATTTTTTTTGTTGCTTAAAAAGAATAAGAAATATAAATTCAGATGGATGGATATGGTTTTAA  660
     TAATGTCTTTTAGCATAGATTTTTATGAATTGGTTTTATATTGATTGACAAGAATAATAATAG   723
     TAAATTGGATATGTTTTAAAGTTTTAACATTTATATGTAAGCAAAGTTAATTTGGATTTAAAA   786
     AAAAAAAAAAAAAAAAA                                                804
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Fig. 2B: Nucleotide and deduced amino acid sequences of pVC2 (B).
Underlined sequences of pVC1 represent hyperphobic domains I, II, III and IV in descending order. The number of nucleotides or amino acids are shown at right.

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C.  CTTTTTTAAACCGTTGTATAACCAATTCGCCAGTTTCTATCCAAAACACATTAAA ATG TCT      61
                                  M  S                                    2
    GCA ACC CCG TCT GAA ACC CTG TCA ACT GCT AGT GCC GGT AAC GAT ACC      109
     A  T  P  S  E  T  L  S  T  A  S  A  G  N  D  T                     18
    GCA CCT TTC TTT GGG TTC ATG GGA GCA GCT TCT GCT TTG GTC TTC GCT      157
     A  P  F  F  G  F  M  G  A  A  S  A  L  V  F  A                     34
    TGT ATG GGT GCC GCG TAT GGT ACC GCA AAG TCC GGC GTG GGT ATT GCC      205
     C  M  G  A  A  Y  G  T  A  K  S  G  V  G  I  A                     50
    CCT ATG GGT GTA ATG AGA CCC GAG TTG GTT ATG AAG AGT ATT GTT CCC      253
     P  M  G  V  M  R  P  E  L  V  M  K  S  I  V  P                     66
    GTT GTT ATG GCG GGT GTG TTG GGT ATC TAC GGA CTT ATT ATT GCT GTC      301
     V  V  M  A  G  V  L  G  I  Y  G  L  I  I  A  V                     82
    ATT ATT AGT ACC AAT GTT AAA AGG GAC GTT TAT AAA TTA TAT GAT GGA      349
     I  I  S  T  N  V  K  R  D  V  Y  K  L  Y  D  G                     98
    TAT GCC CAC TTG TCT GCT GGA CTT GCT TGT GGA CTT GCA GGT TTA CCT      397
     Y  A  H  L  S  A  G  L  A  C  G  L  A  G  L  P                    114
    GCT GGT ATG GCT ATT GGA ATT GTG GGT GAT GCT GGT GTG CGT GCT AAT      445
     A  G  M  A  I  G  I  V  G  D  A  G  V  R  A  N                    130
    GCC TAA CAA CCT AAG CTG TTT GTC GGT ATG ATT TTG ATT TTG ATT TTC      493
     A  Q  Q  P  K  L  F  V  G  N  I  L  I  L  I  F                    146
    GCT GAG GCA TTG GCT CTG TAC GGA TTG ATT GTT GGA ATC ATT TTG GCT      541
     A  E  A  L  A  L  Y  G  L  I  V  G  I  I  L  A                    162
    TCC ATG GCT TCT GCT TCT TGA GGCATGTATACGGTTATTATATTTTTCATAGGTGG      597
     S  K  A  S  A  S END                                               168
    ATATGGATAAATTTTTTTGTTGCTTAAAAAGAATAAGAAATATAAATTCAGATGGATGGATAT     660
    GGTTTTAATAATGTCTTTTAGCATAGATTTTTATGAATTGGTTTTATATTGATTGACAAGAAT     723
    AATAATAGTAAATTGGATATGTTTTAAAGTTTTAACATTTATATGTAAGCAAAGTTAATTAGG     786
    ATTTAAAAAAAAAAAAAAAAAAAAA                                           812
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Fig. 2C: Nucleotide and deduced amino acid sequences of pVC3 (C).
Underlined sequences of pVC1 represent hyperphobic domains I, II, III and IV in descending order. The number of nucleotides or amino acids are shown at right.

and pVC3 were 95% identical. Small differences were observed in their 5′-untranslated regions and N-terminal 12 amino acid sequences between pVC2 and pVC3. DNA and amino acid sequences of the rests were completely identical. The clones, pVC2 and pVC3 are, therefore, judged to be the subtypes with splicing differences of the same genomic DNA. In contrast, pVC1 was clearly different from pVC2/pVC3 in its codon usage, 5′- and 3′-untranslated regions.

Homology to proteolipid subunits of V-ATPase from other organisms: The proteolipid of *A. acetabulum* V-ATPase shared obvious homology with the proteolipid of oat, bovine and yeast (Table 2). The *A. acetabulum* contained 75 to 80% identical amino acid residues in comparison with the oat proteolipid and about 55% when compared with either bovine or yeast proteolipid.

The hydropathy profiles of the *A. acetabulum* proteolipid revealed four (I-IV) hydrophobic domains (Fig. 2-A). The four domains corresponding to amino acid residues 19-42, 63-84, 105-126, 139-163 in pVC1 were remarkably similar to those in oat (Lai *et al.*, 1991), bovine (Mandel *et al.*, 1988) and yeast (Nelson and Nelson, 1989) proteolipids. These four stretches of 20-25 amino acids were highly conserved and were thought to span the membrane (Mandel *et al.*, 1988). Domain IV was especially conserved in which *A. acetabulum* amino acid sequences were 96-100% identical with that of the oat sequence.

Domain IV might be a functionally conserved region since it included the putative DCCD-binding site. By analogy with the F_0 proteolipid, DCCD is thought to inhibit H^+-pumping V-ATPase by reacting with carboxyl groups of glutamate or aspartate (Solioz, 1984) found in the hydrophobic environment of the membrane (Bowman *et al.*, 1989). Glu-142, Glu-147 and Glu-148 in *A. acetabulum* proteolipid (Fig. 2-A, B and C), like Glu-142 in oat (Lai *et al.*, 1991), Glu-139 in bovine (Mandel *et al.*, 1988) or Glu-137 in yeast (Nelson and Nelson, 1989) proteolipid, was a potent candidate for DCCD modification since glutamate residue was in a hydrophobic domain.

Discussion

cDNA encoding the proteolipid of the vacuolar H^+-ATPase was cloned and sequenced from various sources (Nelson *et al.*, 1989). The genes encode a highly hydrophobic protein with a molecular mass of about 16 kDa, which is double the size of the proteolipids of F type ATPases. Moreover, hydropathy plots revealed four transmembrane segments of the proteolipids from mitochondria, chloroplasts and bacteria (Dow *et al.*, 1992; Hoppe and Sebald, 1984; Schneider and Altendorf, 1987). Recently Lai *et al.* (1991) demonstrated the presence of a small multigene family in oat genome. This group also suggested that different gene copies coding for isoforms of the 16-kDa proteolipid in oat. The data presented here gives credence to these suggestions.

We have isolated three cDNAs (pVC1, pVC2 and pVC3) from *A. acetabulum* that encoded the proteolipid subunit of V-ATPase. Several lines of evidence support this conclusion. The molecular mass deduced the open reading frames of the clones pVC1, pVC2 and pVC3 (16.5,16.7 and 16.8 kDa, respectively, Table 2) agrees remarkably well with that estimated from SDS-PAGE analysis of the purified proteolipid (Bowman *et al.*, 1989). Both cDNA and deduced amino acid sequence of the three clones were nearly identical (Fig. 2-A, B and C). The deduced amino acid sequences of these three clones showed extensive similarities with those of proteolipids of oat (Lai *et al.*, 1991), bovine (Mandel *et al.*, 1988) and yeast V-ATPase (Nelson and Nelson, 1989) (Table 2), particularly in domain IV with the putative DCCD binding site. The hydropathy profiles of the *A. acetabulum* proteolipid revealed four hydrophobic transmembrane segments which are remarkably similar to those in oat (Lai *et al.*, 1991), bovine (Mandel *et al.*, 1988) and yeast (Nelson and Nelson, 1989). The three polypeptide have the basic pI values. Nevertheless, we can not eliminate the possibility that some of the cDNA clones encode a 16-kDa proteolipid-like polypeptide from a membrane protein complex other than V-ATPase. To date there is no evidence for any other polypeptide with structure and function similar to the proteolipid of V-ATPase in higher plants. In contrast, a 16-kDa protein co-

isolating with gap junctions from bovine brain (Darmietzel *et al.*, 1989) and a 15-kDa proteolipid found in mediatophore preparations from *Torpedo* electric organ (Birman *et al.*, 1990) showed extensive similarity to the proteolipid of V-ATPase. In animals it is suggested that the proteolipid of V-ATPase plays more diversified roles in intercellular communication (Darmietzel *et al.*, 1989; Birman *et al.*, 1990). Divergences in the codon usage, 5′- and 3′-untranslated regions of the cDNA clones of *A. acetabulum* proteolipid (Fig. 2-A, B and C) could provide evidence for the existence of a small multigene family in the *A. acetabulum* genome. Sequence analysis of the two most similar cDNA clones, pVC2 and pVC3, revealed that both clones contain distinct N-terminal polypeptide sequence and 5′-untranslated regions (Fig. 2-B and C), indicating that two polypeptides could be the differentially spliced products from one gene. More extensive differences among clones, pVC1 and pVC2/pVC3 further suggest that they perhaps represent different gene copies coding for isoform of the 16-kDa proteolipid in *A. acetabulum*. However, the clone pVC1 and the clones pVC2/pVC3 were most probably derived from two different loci since their codon usages, 5′- and 3′-untranslated regions were completely different (Fig. 2-A, B and C). Genetic mapping and other studies are required to establish the extent and significance of this small multigene family in *A. acetabulum* genome. In contrast, there is only a single copy of the DCCD-binding proteolipid gene in yeast genome (Nelson and Nelson, 1989).

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