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Authors: Ueki, Tatsuya, Uyama, Taro, Kanamori, Kan, and Michibata, Hitoshi

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Isolation of cDNAs Encoding Subunits *A* and *B* of the Vacuolar-Type ATPase from the Vanadium-Rich Ascidian, *Ascidia sydneiensis samea*

Tatsuya Ueki[1]*, Taro Uyama[1], Kan Kanamori[2] and Hitoshi Michibata[1]

[1]*Mukaishima Marine Biological Laboratory, Faculty of Science and Laboratory of Marine Molecular Biology, Graduate School of Science, Hiroshima University, Mukaishima-cho 2445, Hiroshima 722-0073, Japan*
[2]*Department of Chemistry, Faculty of Science, Toyama University, Gofuku 3190, Toyama 930-8555, Japan*

ABSTRACT—Vacuolar-type H^+-ATPases (V-ATPases), which are composed of at least ten different subunits, can generate a proton-motive force by hydrolyzing ATP and acidify the contents of various intracellular organelles. Subunits *A* and *B* of V-ATPase have been detected immunologically in ascidian blood cells, predominantly in signet ring cells (vanadocytes), which accumulate vanadium in their vacuoles. The action of V-ATPase in ascidian blood cells has been demonstrated by the fact that bafilomycin A_1, a specific inhibitor of V-ATPases, inhibits the acidification of the vacuoles of vanadocytes. As the next step in studying the function of V-ATPase in vanadocytes, we isolated cDNAs encoding subunits *A* and *B* of V-ATPase from the blood cells of an ascidian, *Ascidia sydneiensis samea*. The nucleotide sequences of the cDNAs for subunits *A* and *B* encoded proteins of 619 and 509 amino acids, respectively, both of which were highly conserved among organisms.

INTRODUCTION

The blood cells of ascidians, especially those in the family Ascidiidae in the suborder Phlebobranchia, accumulate extremely high levels of vanadium from seawater (Henze, 1911). At the same time, the homogenate of the blood cells has been revealed to be extremely acidic (Henze, 1911, 1912, 1913, 1932). Most of the vanadium ions in the vanadocytes are in the +3 oxidation state (Hirata and Michibata, 1991). These unusual phenomena have attracted the interest of investigators because of the possible role of the highly acidic environment in changing or maintaining the redox potential.

Previously, we found that there is a correlation between the concentration of vanadium(III) ions and the pH within the vacuole (Michibata *et al*., 1991). In *Ascidia gemmata*, which is known to contain the highest concentration of vanadium at 350 mM, the vacuoles have the lowest pH of 1.86. Vacuoles of *A. ahodori* containing 60 mM vanadium have a pH of 2.67, and those of *A. sydneiensis samea* containing 13 mM vanadium have a pH of 4.20 (Michibata *et al*., 1991).

Immunocytological studies, using antibodies against subunits *A* and *B* of the vacuolar-type H^+-ATPases (V-ATPases) originated from bovine chromaffin granules, have shown that V-ATPases are localized in the vacuolar membranes of vanadocytes (Uyama *et al*., 1994). A specific inhibitor of V-ATPases inhibits the proton pump in the vacuoles of the vanadocytes, neutralizing the vacuoles' contents (Uyama *et al*., 1994). Thus, one definite function of V-ATPases is to accumulate protons in the vanadocytes. However, it is difficult to explain the extremely low pH observed in ascidian vacuoles by only the action of V-ATPases, since the maximum ΔpH that a V-ATPase can generate under typical physiological conditions is around 4 pH units, based on measured H^+/ATP stoichiometry (Rea and Sanders, 1987).

We have proposed that two mechanisms may be responsible for the proton accumulation in vanadocytes. One is the hydrolysis of the water molecules coordinating with the vanadium(III) ions. In our recent study, we showed that an extremely low pH could be achieved by hydrolysis of the water molecules coordinating with vanadium(III) ions (Kanamori *et al*., unpublished data). The other mechanism involves the extremely tight coupling of ATP hydrolysis and proton pumping by V-ATPase in the vanadocytes. As a first step to assess the latter possibility, we isolated and analyzed cDNA of subunits *A* and *B* of V-ATPase from the blood cells of the vanadium-rich ascidian, *Ascidia sydneiensis samea*.

* Corresponding author: Tel. +81-848-44-1143;
FAX. +81-848-44-5914.

MATERIALS AND METHODS

PCR amplification

A cDNA library of the blood cells of *A. sydneiensis samea* has been constructed in Uni-Zap XR vector (Stratagene, USA) (Uyama *et al.*, 1998). Lambda phage DNA was extracted from the amplified cDNA library. Degenerate primers were designed from conserved regions of subunits *A* and *B* of V-ATPase. For subunit *A*, the forward primer was 5'- AT(A/C/T) GT(A/C/G/T) TT(C/T) GC(A/C/G/T) GC(A/C/G/T) ATG -3' and the reverse primer was 5'- (A/G)TC (A/C/G/T)GT (A/G) TA CAT (A/G)TA (A/C/G/T)CC -3'. For subunit *B*, the forward primer was 5'- AT(A/C/T) GT(A/C/G/T) TT(C/T) GC(A/C/G/T) GC(A/C/G/T) ATG -3' and the reverse primer was 5'- (A/G)TC (A/C/G/T)GT (A/G) TA CAT (A/G)TA (A/C/G/T)CC -3'. PCR conditions were as follows: 0.2 μg lambda phage DNA, 200 pmole each primer, 0.2 mM dNTP, 25 mM Tris-HCl (pH 8.3), 50 mM KCl and 1.5 mM $MgCl_2$. The reaction volume was 50 μl. 30 cycles of PCR were run; each cycle consisted of 94°C for 45 sec, 50°C for 45 sec and 72°C for 45 sec. This was followed by a final extension at 72°C for 5 min. Amplified DNA fragments of the expected length (126 bp for subunit *A* and 306 bp for subunit *B*) were cloned into pBluescript SK(–) vector (Stratagene) and sequenced.

Screening of a cDNA library

The same cDNA library was screened with digoxigenin-labeled random-primed DNA probes derived from the cloned PCR fragments. Hybridization was done at 50°C (subunit *A*) or 65°C (subunit *B*) for at least 16 hr in 0.25 M disodium hydrogenphosphate buffer (Na-Pi buffer, pH 7.2), 7% SDS, 1 mM EDTA and 10 ng/ml probes. The membranes were washed three times in 20 mM Na-Pi buffer (pH 7.2), 1% SDS at 40°C (subunit *A*) or 65°C (subunit *B*). They were treated with anti-digoxigenin-AP conjugate (Boehringer Mannheim, Germany). The signals were detected by chemiluminescence using CSPD substrates (Boehringer Mannheim) and Hyper-ECL film (Amersham Pharmacia Biotech, Sweden). Positive plaques were screened again before they were subcloned. The cDNAs were excised as plasmids *in vivo* according to the manufacturer's protocol. Nucleotide sequences were determined using an ABI 377 automated sequencer (Perkin-Elmer Applied Biosystems, USA) or an ALFexpress automated sequencer (Amersham Pharmacia Biotech).

5'-RACE of subunit *A* cDNA

Oligonucleotides, 5'- TAG TTG GAG CGA AAT CCC AC -3' (R1), complementary to the 5' region of cDNA clone #104 of subunit *A* of V-ATPase (*VATA*) were synthesized. The lambda phage DNA from the cDNA library was again used for amplifying a longer 5'-stretch of *VATA* cDNA. cDNA fragments corresponding to the 5' region of *VATA* cDNA were amplified by PCR with the specific primer R1 and a common primer T3, but they did not reach the expected 5'-end of the cDNA. Therefore, another oligonucleotide, 5'- CAG TTC GGA ATG GCC TAC AC -3' (R2), was designed complementary to the cDNA fragment amplified from the cDNA library. The 5'-RACE was performed with a 5'/3' RACE Kit (Boehringer Mannheim) according to the manufacturer's protocol. Briefly, total RNA was extracted from blood cells except for giant cells as described (Uyama *et al.*, 1998). Two micrograms of total RNAs were reverse-transcribed with R1 primer. The cDNA products were used as templates for PCR with R2 and anchored dT primers supplied by the manufacturer. The PCR products were cloned into pBluescript SK(–) vector and sequenced.

RESULTS

Cloning cDNA for V-ATPase subunit *A*

We amplified 126-bp cDNA fragments corresponding to V-ATPase subunit *A* from the cDNA library constructed from whole blood cells of the ascidian *A. sydneiensis samea*. Three clones of subunit *A* were isolated, and were revealed to contain almost identical fragments. Within the amplified region, the ascidian and bovine amino acid sequences were identical at 23 residues out of 30. Using one of the PCR fragments as a probe, we isolated two cDNA clones for subunit *A* out of 6×10^4 phages from the cDNA library of *Ascidia sydneiensis samea* blood cells. We designated the corresponding gene as the ascidian *VATA* gene. The two cDNA clones had almost identical nucleotide sequences. Comparisons of the open reading frame (ORF) of one of the cDNA clones (clone number #104) with those of *VATA* genes from other organisms showed that cDNA clone #104 seemed to lack a 5'-region corresponding to roughly one-hundred amino acids.

In order to isolate the missing region, we designed two specific primers complementary to the cDNA, and amplified this region directly from the cDNA library and a blood cell cDNA pool (see Materials and Methods). The 3'-part of the amplified fragments was identical to the 5'-part of clone #104. We combined the amplified sequences with that of cDNA clone #104 to generate a cDNA sequence for the *VATA* gene (Fig. 1).

The assembled *VATA* cDNA was composed of 2,253 nucleotides including a 21-bp poly(A) tail. The cDNA contained a single, long ORF of 1,860 nucleotides including the termination codon. It encoded a protein of 619 amino acids. The calculated molecular mass of the predicted protein was 69.0 kDa, which showed a good correlation to the expected molecular weight of 70 kDa determined by western blot analyses using antibodies against the 72K subunit (subunit *A*) derived from bovine chromaffin granules (Uyama *et al.*, 1994). The deduced amino acid sequence showed a striking similarity to the known 72K subunit of V-type ATPases from various organisms (Fig. 2). There was 81.7% amino acid identity between the ascidian and the bovine sequence and 68.1% identity between the ascidian and yeast sequence, excluding the intein that is spliced into a DNA endonuclease (Gimble and Thorner, 1992). The nucleotide-binding P-loop motif, GXXXXGKT (Saraste *et al.*, 1990), was also found in the ascidian *VATA* cDNA (Figs. 1, 2).

Cloning the cDNA of subunit *B*

We used a similar procedure to clone subunit *B* cDNA. We cloned and determined the sequences of eight PCR fragments amplified from the same blood cell cDNA library. The identity of the nucleotide sequences between the fragments was at least 93% (data not shown). Within the amplified region, the ascidian and bovine amino acid sequences were at least 95% identical. Using one of the PCR fragments as a probe, two positive clones were isolated out of 6×10^4 phages. Both were almost identical and contained a nearly full-length cDNA. We designated the corresponding gene as *VATB* and determined the complete nucleotide sequence of one of the cDNA clones (#105, Fig. 3).

The ascidian *VATB* cDNA clone had an insert of 2,017 nucleotides. The insert contained a single, long open reading frame of 1,530 nucleotides including the termination codon,

```
   1 GAATAAGGCCAACATTTGTATAATAGTCATAGAGGGAATATTAATTCTGCTAAAATGAAGCAAGAGGCTTAT    72
   1                                                       M  K  Q  E  A  Y     6
  73 CAAATTCCCAAAATCCAGGATATGGAAAAGGAAAGCATGCTGGGGACTGTGTTTGGTGTATCTGGTCCAGTC   144
   6  Q  I  P  K  I  Q  D  M  E  K  E  S  M  L  G  T  V  F  G  V  S  G  P  V    30
 145 GTGACTGCCGAGCACATGTCTGGATCCTCCATGTACGAGCTGGTTCGTGTAGGCCATTCCGAACTGGTGGGA   216
  30  V  T  A  E  H  M  S  G  S  S  M  Y  E  L  V  R  V  G  H  S  E  L  V  G    54
 217 GAGATCATTCGTCTTGATGGTGATCGTGCTACAATTCAAGTATACGAAGAAACATCTGGCGTAACTGTTGGC   288
  54  E  I  I  R  L  D  G  D  R  A  T  I  Q  V  Y  E  E  T  S  G  V  T  V  G    78
 289 GATCCTGTGCTGAGAACTGGAAAGCCTTTGTCCGTAGAATTGGGGCCTGGAATTTTGAACAACATCTATGAT   360
  78  D  P  V  L  R  T  G  K  P  L  S  V  E  L  G  P  G  I  L  N  N  I  Y  D   102
 361 GGTATTCAGCGTCCCCTGGAAGTTATCCAACAGCAGACCAAGTCAATTTACATTCCTCGTGGTGTAAACACG   432
 102  G  I  Q  R  P  L  E  V  I  Q  Q  Q  T  K  S  I  Y  I  P  R  G  V  N  T   126
 433 TTAGCTTTGAACCGCAGTGCTCAGTGGGATTTCGCTCCAACTAAAGCATTCCAGGTGGGAAGTCACATCACT   504
 126  L  A  L  N  R  S  A  Q  W  D  F  A  P  T  K  A  F  Q  V  G  S  H  I  T   150
 505 GGTGGGGACATCTACGGTCTGATTCAGGAAAACTCTTTGATTCAACACAGGGTCATGCTCCCTCCTAAAGCA   576
 150  G  G  D  I  Y  G  L  I  Q  E  N  S  L  I  Q  H  R  V  M  L  P  P  K  A   174
 577 CGAGGACGAGTTACTTACGTCGCTGAAGCTGGCCACTACAATGTTGATGACACCCTATTGGAGGTGGAGTTC   648
 174  R  G  R  V  T  Y  V  A  E  A  G  H  Y  N  V  D  D  T  L  L  E  V  E  F   198
 649 GATGGCGTTGTCAAGAAGTACAGCATGCTGCAAGTCTGGCCTGTCCGTCAACCCAGACCCTGTGTGGAGAAG   720
 198  D  G  V  V  K  K  Y  S  M  L  Q  V  W  P  V  R  Q  P  R  P  C  V  E  K   222
 721 CTGCCAGCTAATTACCCTCTTCTCACTGGGCAGAGAGTCTTGGATGCTCTGTTCCCATCCGTCCAGGGAGGC   792
 222  L  P  A  N  Y  P  L  L  T  G  Q  R  V  L  D  A  L  F  P  S  V  Q  G  G   246
 793 ACCACCGGCATCCCAGGCGCCTTCGGCTGTGGCAAGACCGTCATCTCCCAGTCGCTCTCGAAATTCTCCAAC   864
 246  T  T  G  I  P  G  A  F  G  C  G  K  T  V  I  S  Q  S  L  S  K  F  S  N   270
 865 AGCGACATCATCGTCTACGTGGGCTGCGGTGAGCGTGGCAACGAGATGTCCGAAGTGCTGAGAGATTTCCCC   936
 270  S  D  I  I  V  Y  V  G  C  G  E  R  G  N  E  M  S  E  V  L  R  D  F  P   294
 937 GAGCTGACCGTCTACATGTACGGGAGGGAAGAATCCATCATGAAACGTACCACGCTGGTGGCCAACACATCG  1008
 294  E  L  T  V  Y  M  Y  G  R  E  E  S  I  M  K  R  T  T  L  V  A  N  T  S   318
1009 AACATGCCCGTCGCCGCTAGAGAGGCTTCCATTTACACTGGAATCACCTTGTCCGAATACTTCCGTGATATG  1080
 318  N  M  P  V  A  A  R  E  A  S  I  Y  T  G  I  T  L  S  E  Y  F  R  D  M   342
1081 GGATACAACGTGTCCATGATGGCCGACTCGACGTCTCGATGGGCCGAGGCTCTTCGTGAGATCTCCGGTCGT  1152
 342  G  Y  N  V  S  M  M  A  D  S  T  S  R  W  A  E  A  L  R  E  I  S  G  R   366
1153 CTTGCCGAGATGCCCGCCGACTCCGGCTACCCCGCCTACTTGGGTGCCAGGCTGGCTTCCTTCTACGAGCGA  1224
 366  L  A  E  M  P  A  D  S  G  Y  P  A  Y  L  G  A  R  L  A  S  F  Y  E  R   390
1225 GCCGGCCGCGTCACGTGCCAGGGAAGTCCCAGCCGCGAAGGCAGCGTCTCCCTCGTCGGAGCTGTGTCGCCC  1296
 390  A  G  R  V  T  C  Q  G  S  P  S  R  E  G  S  V  S  L  V  G  A  V  S  P   414
1297 CCCGGTGGTGACTTTTCGGATCCTGTCACGTCGGCGACGCTCGGTATCGTCCAGGTCTTCTGGGGTCTGGAC  1368
 414  P  G  G  D  F  S  D  P  V  T  S  A  T  L  G  I  V  Q  V  F  W  G  L  D   438
1369 AAGAAGCTCGCCCAGCGAAAGCACTTTCCTTCGGTCAACTGGCTGATCAGTTACTCCAACTACATGAGATCG  1440
 438  K  K  L  A  Q  R  K  H  F  P  S  V  N  W  L  I  S  Y  S  N  Y  M  R  S   462
1441 CTGGACGACTTCTACGACAAGAACTACCCCGACTTCGTTCCACTCCGTGTCAAGGTCAAGGAGATCCTCCAG  1512
 462  L  D  D  F  Y  D  K  N  Y  P  D  F  V  P  L  R  V  K  V  K  E  I  L  Q   486
1513 GAGGAAGAGGAGCTCTCCGAAATCGTCCAGCTCGTCGGAAAAAGCTCCCTCGCCGAGACCGACAAAATCACG  1584
 486  E  E  E  E  L  S  E  I  V  Q  L  V  G  K  S  S  L  A  E  T  D  K  I  T   510
1585 CTGGAGGTCGCCAAGATCATCAAGGACGATTTCCTGCAGCAAAACGGCTTTTCGGCTTACGACAGGTACTGC  1656
 510  L  E  V  A  K  I  I  K  D  D  F  L  Q  Q  N  G  F  S  A  Y  D  R  Y  C   534
1657 CCCTTCTACAAAACCGTCGGCATGATCCGCAACATGATCGCTTTCTACGACATGGCGCGCGCCAGCGTGGAG  1728
 534  P  F  Y  K  T  V  G  M  I  R  N  M  I  A  F  Y  D  M  A  R  A  S  V  E   558
1729 TCGACCGCCCAGTCGGACAACAAGATCACCTGGGCGATCATACGCGAAAACCTGAGCGACATCCTGTACCGG  1800
 558  S  T  A  Q  S  D  N  K  I  T  W  A  I  I  R  E  N  L  S  D  I  L  Y  R   582
1801 CTCTCGTCGATGAAGTTCAAGGACCCGATCAAGGACGGGGAGGCGAAGATCAAGCGCGAAATCGAGCAGCTC  1872
 582  L  S  S  M  K  F  K  D  P  I  K  D  G  E  A  K  I  K  R  E  I  E  Q  L   606
1873 TACGACGACATGCAAAACGGCTTCAGGAATTTGGAAGAATAGGCGATTTTTTTTCTCGTTTCTCCGATTGTT  1944
 606  Y  D  D  M  Q  N  G  F  R  N  L  E  E  *                               619
1945 TTTTGTGTTTGTTCGAGGACCGCTTTCGACGACCGACCGACCGCCCCCCGTGCAATATTTCAGGCTTCGTCT  2016
2017 CTTTTTTTGACCTTCGAGCACCGAGGTCCACGACCTTGGGCTCCCGCGTAAAAGTCCCTCTCGTCCCCGTTC  2088
2089 GTGATTCCCGGTGATCCTGCCGCCCCCGGATCCCAGTGATCGTGTATTTCTCGTTTGTGGCACTCCCGTTCG  2160
2161 GAAAATAAAACAACTTATTGTGCGTAACAGAAAAAAATTTACGTCTTGGTTATACACAGGGCTGTGTCGTTT  2232
2233 AAAAAAAAAAAAAAAAAAAAA                                                     2253
```

Fig. 1. Nucleotide and amino acid sequences of *VATA* cDNA assembled from cDNA clone #104 and two 5'-RACE products from *Ascidia sydneiensis samea*. The regions corresponding to the forward and reverse primers are shown by dotted lines. The P-loop motif is underlined. Clone #104, isolated by the cDNA library screening, starts at the 376th nucleotide of this sequence, the first round 5'-RACE product at the 124th nucleotide, and the second round product at the 1st nucleotide. This sequence is stored in the DDBJ, GenBank, and EMBL nucleotide databases under accession number AB016483.

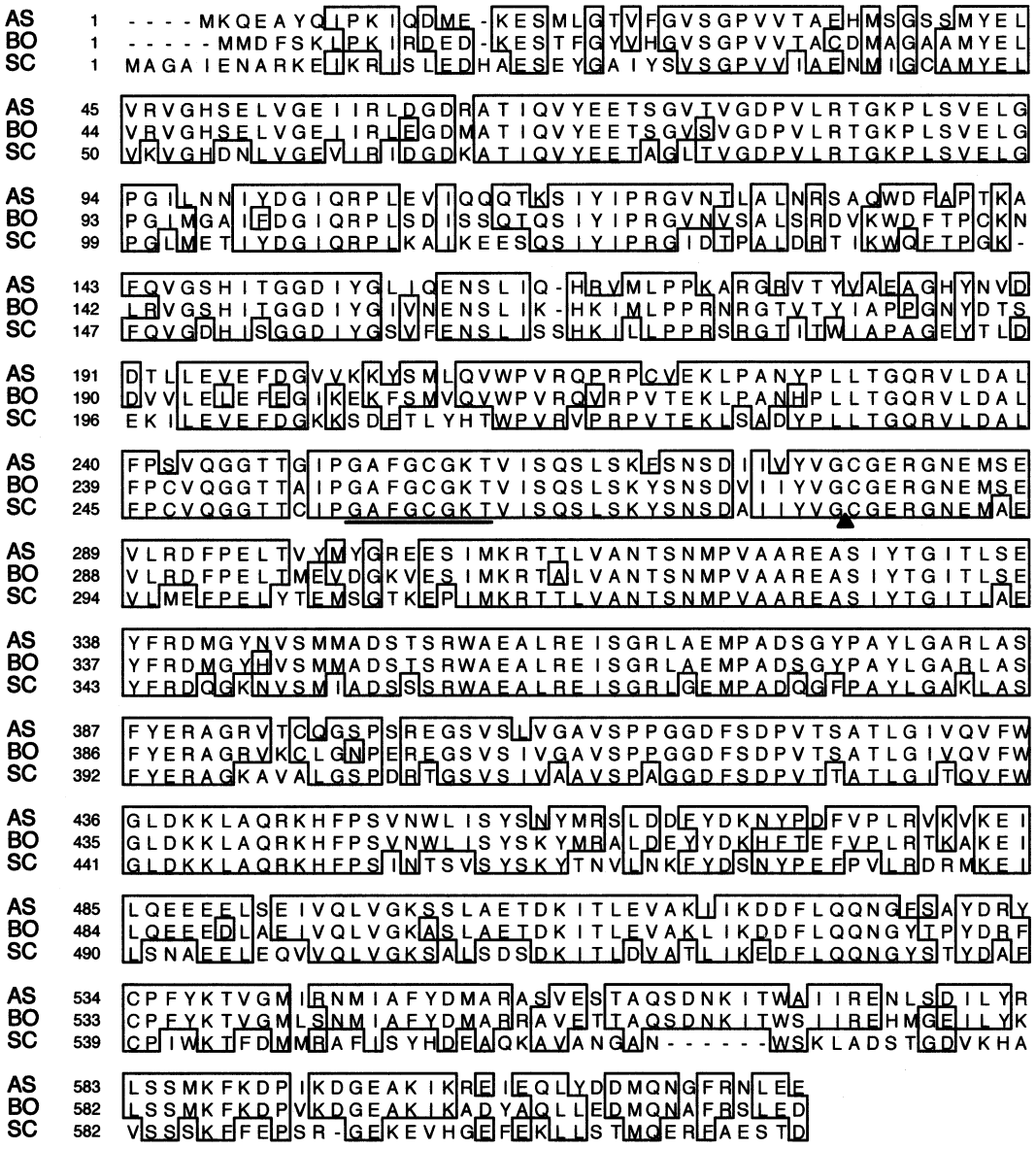

Fig. 2. Alignment of the amino acid sequences of subunit *A* of V-ATPases from various organisms. Identical residues are boxed. The P-loop motif is underlined. The solid triangle shows the position of protein splicing, which generates a DNA endonuclease (Gimble and Thorner, 1992) in yeast. Abbreviations: AS, *Ascidia sydneiensis samea* (this study); BO, bovine (Puopolo *et al*., 1991); SC, budding yeast (Hirata *et al*., 1990).

encoding a protein of 509 amino acids. Its calculated molecular mass was 56.7 kDa, which also showed a good correlation to the molecular weight of the antigen detected by anti bovine subunit *B* antibody (Uyama *et al*., 1994). The deduced amino acid sequence of the *VATB* cDNA was very similar to known sequences of V-ATPase subunit *B* from various organisms (Fig. 4).

DISCUSSION

V-ATPases that can generate a proton-motive force by hydrolyzing ATP are known to acidify the contents of various intracellular organelles including clathrin-coated vesicles, endosomes, lysosomes, Golgi-derived vesicles, multivesicular bodies and chromaffin granules that belong to the central vacuolar system, and to make an electrochemical gradient of H^+ linked to the uptake of certain small molecules (Forgac, 1989, 1992; Nelson, 1992). We are focusing our attention on the close relationship between the high concentration of protons and the high level of vanadium in vanadocytes (Michibata *et al*., 1991), which may provide a key to clarify the energetics of the accumulation of vanadium in ascidians.

In this experiment, we successfully isolated full-length cDNAs of subunits *A* and *B* of the V-ATPase from the vanadium-rich ascidian *Ascidia sydneiensis samea*. Since antibodies against subunits *A* and *B* from bovine chromaffin granules detected antigens specific to vanadocytes (Uyama *et al*., 1994), we suppose that these cDNAs were derived from the

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   1 TCTACATCATTGCATCTTGTCAAAATAGATCAAAATGGCCACCACGTTAGACATGGCGCAACTTTCTGCAGC    72
   1                                   M  A  T  T  L  D  M  A  Q  L  S  A  A    13
  73 TAAAGAGCACGCTTTGGCTGTGAGCAGAGATTACATTTCCCAGCCACGTTTAACATACCAAACCGTATCCGG   144
  13  K  E  H  A  L  A  V  S  R  D  Y  I  S  Q  P  R  L  T  Y  Q  T  V  S  G    37
 145 TGTCAACGGGCCGCTGGTCATTTTGGAAAAAGTGAAGTTTGCTAAGTTCGCCGAAATTGTAACCTTGAACTT   216
  37  V  N  G  P  L  V  I  L  E  K  V  K  F  A  K  F  A  E  I  V  T  L  N  L    61
 217 GGCTGACGGTACACAGCGCAGTGGACAGGTGCTGGAAGTCAGTGGAGACAAAGCTGTCGTCCAGGTGTTCGA   288
  61  A  D  G  T  Q  R  S  G  Q  V  L  E  V  S  G  D  K  A  V  V  Q  V  F  E    85
 289 AGGCACGTCAGGTATTGATGCCAAGCACACAACATGCGAGTTCACGGGCGACATTTTAAGGATTCCAACCTC   360
  85  G  T  S  G  I  D  A  K  H  T  T  C  E  F  T  G  D  I  L  R  I  P  T  S   109
 361 GGAAGACATGCTCGGTCGTATCTTCAACGGATCTGGAAAACCGATCGACAAGGGGCCATCTGTTTTGCCTGA   432
 109  E  D  M  L  G  R  I  F  N  G  S  G  K  P  I  D  K  G  P  S  V  L  P  E   133
 433 AGACTACTTGGATATTCAGGGTCAGCCCATTAATCCCAAGTCTCGTATTTACCCAGAGGAGATGATTCAAAC   504
 133  D  Y  L  D  I  Q  G  Q  P  I  N  P  K  S  R  I  Y  P  E  E  M  I  Q  T   157
 505 TGGCATCTCTGCCATTGACACCATGAACAGCATCGCTCGTGGACAGAAAATTCCCATCTTCTCCGCTAATGG   576
 157  G  I  S  A  I  D  T  M  N  S  I  A  R  G  Q  K  I  P  I  F  S  A  N  G   181
 577 TTTGCCGCACAACGAAATCGCCGCCCAGATTTGTCGACAGGGCGGTTTGGTCAAGCTCCCTGACAAGGACGT   648
 181  L  P  H  N  E  I  A  A  Q  I  C  R  Q  G  G  L  V  K  L  P  D  K  D  V   205
 649 CATGGACAGTCACGAGGACAACTTTGCTATTGTGTTCGCTGCTATGGGTGTGAACATGGAAGCAGCTAGATT   720
 205  M  D  S  H  E  D  N  F  A  I  V  F  A  A  M  G  V  N  M  E  A  A  R  F   229
 721 TTTCAAGTCCGACTTTGAGCAGCACGGCTCCATGGACAACGTGTGCCTCTTCCTTAACCTTGCAAACGATCC   792
 229  F  K  S  D  F  E  Q  H  G  S  M  D  N  V  C  L  F  L  N  L  A  N  D  P   253
 793 GACCATTGAGAGGATCATTACACCTCGTATTGCACTTACGACTGCTGAGTTCCTGGCCTACCAGTGCGAGAA   864
 253  T  I  E  R  I  I  T  P  R  I  A  L  T  T  A  E  F  L  A  Y  Q  C  E  K   277
 865 GCACGTGCTGGTCATCCTCACCGACATGAGTTCCTATGCTGAAGCTCTGAGAGAGGTGTCAGCCGCCAGAGA   936
 277  H  V  L  V  I  L  T  D  M  S  S  Y  A  E  A  L  R  E  V  S  A  A  R  E   301
 937 GGAGGTGCCCGGTCGTCGTGGTTTCCCCGGTTACATGTACACGGATCTTGCCACCATCTACGAGCGAGCCGG  1008
 301  E  V  P  G  R  R  G  F  P  G  Y  M  Y  T  D  L  A  T  I  Y  E  R  A  G   325
1009 TCGTGTCAACGGCAGAAACGGATCCATCACCCAGATTCCCATTCTCACTATGCCCAACGACGATATTACCCA  1080
 325  R  V  N  G  R  N  G  S  I  T  Q  I  P  I  L  T  M  P  N  D  D  I  T  H   349
1081 TCCCATTCCCGATTTGACCGGTTACATTACCGAGGGACAGATCTACATCGACAGGCAGCTGCACAACAGGCA  1152
 349  P  I  P  D  L  T  G  Y  I  T  E  G  Q  I  Y  I  D  R  Q  L  H  N  R  Q   373
1153 GATCTACCCGCCCATCAACGTCCTGCCTTCGCTTTCCCGTCTCATGAAGTCGGCCATCGGCGAGGGAATGAC  1224
 373  I  Y  P  P  I  N  V  L  P  S  L  S  R  L  M  K  S  A  I  G  E  G  M  T   397
1225 CAGAAAGGACCACTCCGACGTTTCCAACCAGCTGTACGCTAACTACGCCATCGGAAAGGACGTGCAGGCTAT  1296
 397  R  K  D  H  S  D  V  S  N  Q  L  Y  A  N  Y  A  I  G  K  D  V  Q  A  M   421
1297 GAAGGCCGTGGTCGGCGAGGAGGCTCTAACCGCAGACGACATGCTTTACTTGGAGTTCTTGGGCAAATTCGA  1368
 421  K  A  V  V  G  E  E  A  L  T  A  D  D  M  L  Y  L  E  F  L  G  K  F  E   445
1369 GAAGACCTTTATCGCGCAAGGTCCGTACGAGAACCGCAGCGTCTTCGACTCGCTCAACATCGGCTGGGAGCT  1440
 445  K  T  F  I  A  Q  G  P  Y  E  N  R  S  V  F  D  S  L  N  I  G  W  E  L   469
1441 CCTGCGAATCTTCCCCAAGGAGATGCTGAAGCGAATCCCGCGGAACGTCATCAACGAGTACTACCCGAGAAA  1512
 469  L  R  I  F  P  K  E  M  L  K  R  I  P  R  N  V  I  N  E  Y  Y  P  R  K   493
1513 GAAGGTGCCCTCCGCCCAGGACAAGCAGGAGGACCAGAAGGAAGAGAAGTGAGCGAGAGAGAGAGGCCGACC  1584
 493  K  V  P  S  A  Q  D  K  Q  E  D  Q  K  E  E  K  *                        509
1585 GTAGAAGAAGAAAAAGAGAGAGAGAATTATCTCGCTTTTTCGCCCCTTTTTTCTTTTTTCGATCGTCGTTTC  1656
1657 TCGTGTTTTCCTCTTTTCCGCTTCTGTTTCGTATCAGTTTTTTTTTTCAACCAGATTCACTTAGAGCACCTT  1728
1729 CAAAGGTCGCGACGAACCCGAAAAGCACGAAATGTGAGCGCGCACGTAGCTTTTCTCGCTTAGACGCTTCCG  1800
1801 TATTCGCTCGCCCTCATCCAGACTCGACTCTCCCCCCCCTAACGCAGCAAAACGCCTCCTTTCCAAACTACG  1872
1873 GTGTTGTCCTATTGATTGCTCGTCGTGTTCGTTTGCCCCCGCCCCTGAGGTCCCCGTCGTCCGTGTGGAAAC  1944
1945 ATGTCGCGAAACCGATTCGTTTTGTGTGCAATAAAAGCTATCCGACACAGAAAAAAAAAAAAAAAAAAAAA  2016
2017 A                                                                        2017
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Fig. 3. Nucleotide and amino acid sequence of *VATB* cDNA clone #105 isolated from *Ascidia sydneiensis samea*. The regions corresponding to the PCR primers are shown by dotted lines. This cDNA sequence is stored in the DDBJ, GenBank, and EMBL nucleotide databases under accession number AB016484.

transcripts in the vanadocytes. The subunit composition and cDNA sequences of V-ATPases in various living organisms have been reviewed (Finbow and Harrison, 1997) and some differences in these V-ATPases related to intracellular components or the species of living organisms have been reported. In general, V-ATPase is composed of at least five different subunits, denoted as subunits *A* to *E*. Of these, direct chemical labeling and sequence homology studies show that subunits *A* and *B* play an important role in binding and catalyzing ATP. The highly conserved amino acid sequences of subunits *A* and *B* in living organisms imply that they have important roles. In some organisms, more than one isoform has been reported for each subunit. There are two isoforms of subunit *A* in the human and chick, and two of subunit *B* in the

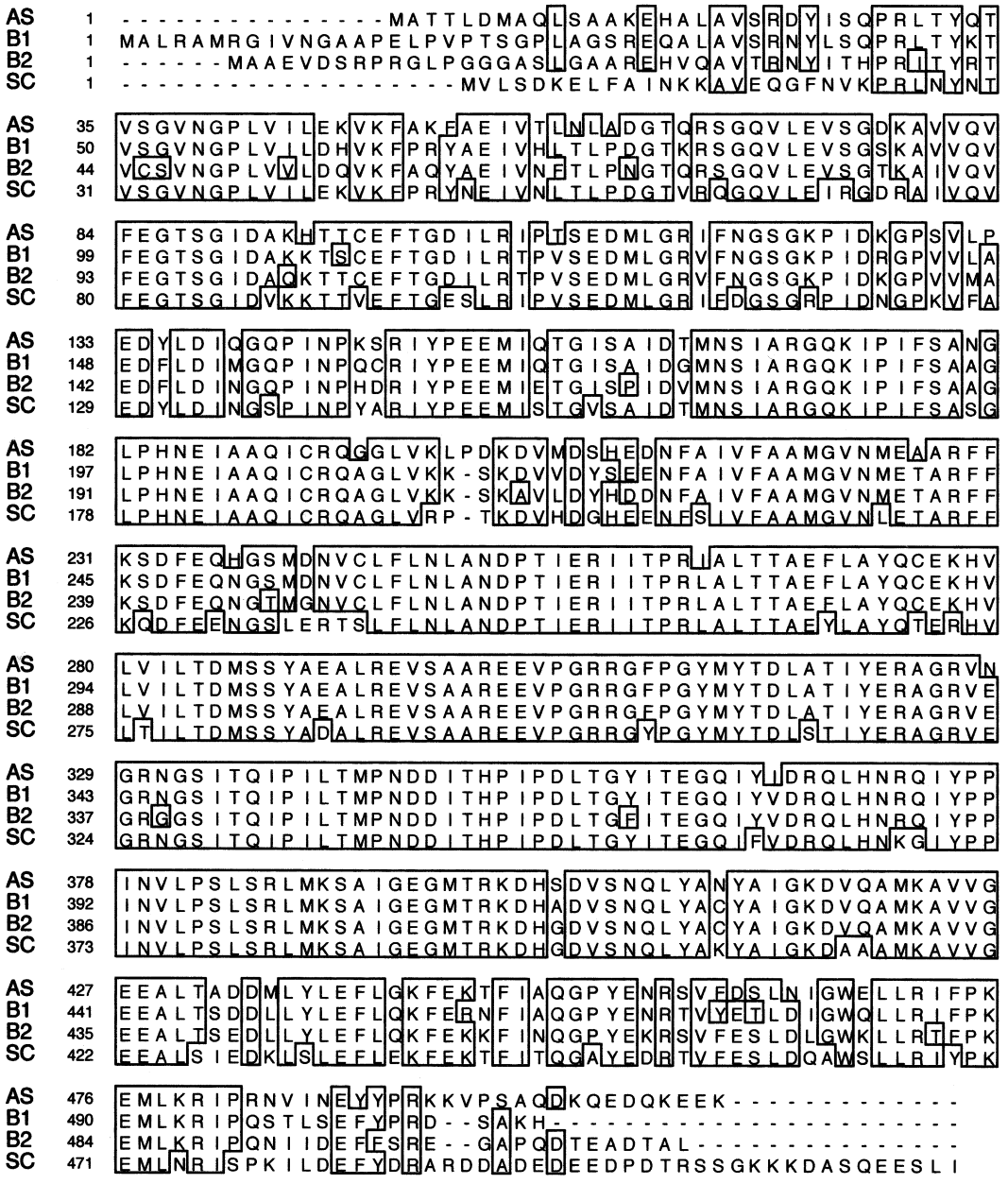

Fig. 4. Alignment of the amino acid sequences of subunit *B* of V-ATPases from various organisms. Identical residues are boxed. Abbreviations: AS, *Ascidia sydneiensis samea* (this study); B1, bovine brain-type isoform (Puopolo *et al.*, 1992); B2, bovine renal-type isoform (Nelson *et al.*, 1992); SC, budding yeast (Nelson *et al.*, 1989).

human and bovine. The ascidian subunit *B* isolated in this study is more closely related to the vertebrate homologs than the diverged isoform from bovine renal cells. So far, we have not found any evidence of other isoforms in this ascidian species.

On the one hand, it is difficult to explain the very low pH values observed in ascidian vacuoles by the action of V-ATPase alone because, in general, the maximum ΔpH that a V-ATPase can generate is around 4 pH units (Rea and Sanders, 1987). We have found that the extremely low pH of the vanadocyte vacuoles is partly explained by the release of protons with the hydrolysis of water molecules coordinated to vanadium(III) ions (Kanamori *et al.*, unpublished data). However, it is still possible that the V-ATPase in the vanadocytes has an unusually tight coupling of ATPase activity and proton pumping and can generate such low pH values in the vacuole. Recently, the subunit composition of V-ATPase in the lemon juice sac, whose vacuole has a pH of 2.5, was reported to differ from that in other organs, and the authors suggested that it may be responsible for the low pH (Müller *et al.*, 1997). To determine whether V-ATPase actually functions in vanadocytes and how much proton-pumping activity the ascidian V-ATPase has, the subunit composition and the role of each subunit in vanadocytes must be clarified and will be our

future subjects of study.

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