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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*

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**ABSTRACT**—Vacuolar-type H<sup>+</sup>-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<sub>1</sub>, 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<sup>+</sup>-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  $\Delta$ pH that a V-ATPase can generate under typical physiological conditions is around 4 pH units, based on measured H<sup>+</sup>/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*.

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## MATERIALS AND METHODS

### PCR amplification

A cDNA library of the blood cells of *A. sydneiensiensis 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<sub>2</sub>. 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. sydneiensiensis 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  $6 \times 10^4$  phages from the cDNA library of *Ascidia sydneiensiensis 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  $6 \times 10^4$  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	GAATAAGGCCAACATTTGTATAATAGTCATAGAGGGAATATTAAATCTGCTAAAAATGAAGCAAGAGGCTTAT	72
1		6
		M K Q E A Y
73	CAAATTCACAAAATCCAGGATATGGAAGGAAAGCATGCTGGGGACTGTGTTTGGGTATCTGGTCCAGTC	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	GTGACTGCCGAGCACATGTCTGGATCTCCATGTACGAGCTGGTTCGTGTAGGCCATTCCGAACCTGGTGGGA	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	GAGATCATTCGTCTTGATGGTGTGCTGCTACAATTCAGTATACGAAGAAACATCTGGCGTAACCTGTGGC	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	GATCCTGTGCTGAGAACTGGAAGCCTTTGTCCGTAGAATTGGGGCCTGGAATTTGAACAACATCTATGAT	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	GGTATTCAGCGTCCCTTGAAGTTATCCAACAGCAGACCAAGTCAATTTACATTCCTCGTGGTGTAAACACG	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	TTAGCTTTGAACCGCAGTGTCTAGTGGGATTTCGCTCCAACCTAAAGCATTCAGGTGGGAAGTCACATCACT	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	GGTGGGACATCTACGGTCTGATTACGAAACCTTTGATTCAACACAGGGTCATGCTCCCTCCCTAAAGCA	576
150	G G D I Y G L I Q E N S L I Q H R V M L P K A	174
577	CGAGGACGAGTTACTTACGTGCTGAAGCTGGCCACTACAATGTTGATGACACCTATTGGAGGTGGAGTTC	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	GATGGCGTGTGCAAGAAGTACAGCATGCTGCAAGTCTGGCTGTCCGTCAACCCAGACCTGTGTGGAGAAG	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	CTGCCAGCTAATTAACCTCTTCTCACTGGGCAGAGAGTCTTGGATGCTCTGTTCCCATCCGTCCAGGGAGGC	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	ACCACCGCATCCAGGCGCCTTCGGCTGTGGCAAGACCTCATCTCCAGTCTGCTCTCGAAATTTCTCCAAC	864
246	T T G I P <u>G A F G C G K T</u> V I S Q S L S K F S N	270
865	AGCGACATCATGCTCTACGTGGGCTGCGGTGAGCGTGGCAACAGATGTCGGAAGTCTGAGAGATTTC	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	GAGTGACCGTCTCATGTACGGGAGGAAGAATCCATCATGAAACGTACCACGCTGGTGGCAACACATCG	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	AACATGCCCGTCGCCGTAGAGAGCTTCCATTACACTGGAATCACCTTGTCCGAATACTTCCGTGATATG	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	GGATACAACGTGTCCATGATGGCCGACTCGACGTCTCGATGGGCGGAGGCTCTTCTGTGAGATCTCCGGTCT	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	CTTGCCGAGATGCCCGGACTCCGGCTACCCGCTTACTTGGGTGCCAGGCTGGCTTCTTCTACGAGCGA	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	GCCGGCCGCTCACGTGCCAGGAAGTCCAGCCGGAAGGCAGCGTCTCCCTCGTCCGAGCTGTGTGCCCC	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	CCCGGTGTGACTTTTCGGATCCTGTACGTGCGCGACGCTCGGTATCGTCCAGGTCTTCTGGGCTCTGGAC	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	AAGAAGCTCGCCAGCGAAAGCACTTTCCTTCGGTCAACTGGCTGATCAGTTACTCCAACATACATGAGATCG	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	CTGGACGACTTCTACGACAAGAACTACCCGACTTCGTTCCACTCCGTGTCAAGGTCAAGGAGATCCTCCAG	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	GAGGAAGGAGCTCTCCGAAATCGTCCAGCTCGTCCGAAAGCTCCCTCGCCGAGACCGACAAATCACG	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	CTGGAGGTGCCAAGATCATCAAGGACGATTTCTTCGAGCAAAACGGCTTTTCGGCTTACGACAGGTACTGC	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	CCCTTCTACAAAACCGTCGGCATGATCCGCAACATGATCGCTTTCTACGACATGGCGCGCCAGCGTGGAG	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	TCGACCGCCAGTCGGACAACAAGATCACCTGGGCGATCATACGCGAAACCTGAGCGACATCCTGTACCGG	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	CTCTCGTGTGAGTTCAAGGACCCGATCAAGGACGGGAGGCGAAGATCAAGCGGAAATCGAGCAGCTC	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	TACGACGACATGCAAAACGGCTTCAGGAATTTGGAAGAATAGGCGATTTTTCCTCGTTCTCCGATTGTT	1944
606	Y D D M Q N G F R N L E E *	619
1945	TTTTGTGTTTGTTCGAGGACCGCTTTTCGACGACCGACCGACCGCCCCCGTGCAATATTTACGGCTTCGTCT	2016
2017	CTTTTTTGGACCTTCGAGCACCAGGTCACGACCTTGGGCTCCCGCTAAAAGTCCCTCTCGTCCCGGTT	2088
2089	GTGATTCGGGTGATCCTGCCGCCCGGATCCCAAGTATCGGTGATTCTCGTTTGTGGCACTCCCGTTTCG	2160
2161	GAATAAAACAACTTATGTGCGTAACAGAAAAAATTTACGCTTTGGTTATACACAGGGCTGTGTGCTTT	2232
2233	AAAAAAAAAAAAAAAAAAAA	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.



AS	1	- - - - MKQEAYQLPKIQDME - KESMLGTVFGVSGPVVTAEHMSGSSMYEL
BO	1	- - - - MMDFSKLPKIRDED - KESTFGYVHGVSQPVVTAACDMAGASMYEL
SC	1	MAGA IENARKEIKRLISLEDHAESIEYGA IYSVSGPVVTAENMIGCIAMYEL
AS	45	VRVGHSELVGEIIRLDGDRATIQVYEETSGVTVGDPVLRGTGKPLSVELG
BO	44	VRVGHSELVGEIIRLEGDMA TIQVYEETSGVSVGDPVLRGTGKPLSVELG
SC	50	VKLVGHDLNLVGEVIRIDGDKATIQVYEETAGLITVGDPVLRGTGKPLSVELG
AS	94	PGILNNIYDGIQRPLEVIQQQTKSIYIPRGVNTLALNRSQAQWDFAPTKA
BO	93	PGIMGAIIFDGIQRPLSDISSQTQSIYIPRGYNVSAISRQDVKWDFTPCKN
SC	99	PGLMETIYDGIQRPLKAIKEESQSIYIPRGIDTPALDRITIKWQFTPGK -
AS	143	EQVGSHITGGDIYGLIQENS LIQ - HRVMLPPKARGBVTYVAEAGHYINVD
BO	142	LRVGSHTGGDIYGLIVNENSLIK - HKIMLPPRNRGTVTYIAPPGNYDTS
SC	147	EQVGDIHISGGDIYGSVFENSLISSHKILLPPRSIRGTITWAPAGEYITLD
AS	191	DTLLEVEFDGVVKKYSMLQVWPVVRQPRPCVEKLPANYPLLTGQRVLDAL
BO	190	DVVLELEFEIGIKKFSMVQVWPVVRQVRPVTEKLPANHPLLTGQRVLDAL
SC	196	EKILLEVEFDGKKSDFTLYHTWPVVRVPRPVTEKISADYPLLTGQRVLDAL
AS	240	FPSVQGGTTGIPGAFGCGKTVISQSLSKFSNSDIIVYVGCGERGNEMSE
BO	239	FPCVQGGTTAIPGAFGCGKTVISQSLSKYNSDVIIVYVGCGERGNEMSE
SC	245	FPCVQGGTTCIPGAFGCGKTVISQSLSKYNSDAIIIVYVGCGERGNEMAE
AS	289	VL R D F P E L T V Y M Y G R E S I M K R T T L V A N T S N M P V A A R E A S I Y T G I T L S E
BO	288	V L R D F P E L T M E I V D G K V E S I M K R T A L V A N T S N M P V A A R E A S I Y T G I T L S E
SC	294	V L M E F E L I Y T E M S G T K E P I M K R T T L V A N T S N M P V A A R E A S I Y T G I T L A E
AS	338	YFRDMGYNVSMMA DSTSRWAEALREISGR LAEMPADSGYPAYLGARLAS
BO	337	YFRDMGYHVSMMADSTSRWAEALREISGR LAEMPADSGYPAYLGARLAS
SC	343	YFRDQGNVSMIADSSSRWAEALREISGR LGEMPADCGFPAYLGAKLAS
AS	387	FYERAGRVTCQGSPSREGSVSLVGAVSPPGGDFSDPVT SATLGIVQVFW
BO	386	FYERAGRVKCLGNPEREGSVSIVGAVSPPGGDFSDPVT SATLGIVQVFW
SC	392	FYERAGKAVALLGSPDRITGSVSVIAAVSPAAGGDFSDPVTATLGITQVFW
AS	436	GLDKKLAQRKHFP SVNWLISYSN YMRSLDD EYDKNYPDFVPLRVKVKKEI
BO	435	GLDKKLAQRKHFP SVNWLISYSKYMBALDEYDKHFEFVPLRTKAKEI
SC	441	GLDKKLAQRKHFP SINTSVISYSKYTNV LNKFYDSNYPEFPVLRDRMKEI
AS	485	LQEEEEELSEIVQLVGKSSLAETDKITLEVAKLIKDDFLQONGESAYDRY
BO	484	LQEEEDLAEIVQLVGKASLAETDKITLEVAKLIKDDFLQONGYTPYDRF
SC	490	LISNAIEELEQVVQLVGKSSALSDSDKITLIDVATLIKEDFLQONGYSTYDAE
AS	534	CPFYKTVGMIRNMIAFYDMARASVESTAQSDNKITWAIIRENLSIDILYR
BO	533	CPFYKTVGMLSNMIAFYDMABRAVETTAQSDNKITWSIIRFHMGEILYK
SC	539	CP IWKTFDMMBIAFISYHDEAQA VANGAN - - - - WSKLADS TGDV KHA
AS	583	LSSMKFKDP IKDGEAKIKREIEQLYDDMQNGFRNLEE
BO	582	LSSMKFKDPVKDGEAKIKADYAIQLLEDMQNAIFRISLED
SC	582	VSSSKKFFELPSR - GEKEVHGEFEKLLISTMQERFAESTD

**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

1	TCTACATCATTGCATCTTGTCAAAATAGATCAAAATGGCCACCACGTTAGACATGGCGCAACTTTCTGCAGC	72
1	M A T T L D M A Q L S A A	13
73	TAAAGAGCACGCTTTGGCTGTGAGCAGAGATTACATTTCCAGCCACGTTTAAACATACCAAACCGTATCCGG	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	TGTCAACGGGCGCTGGTCATTTTGGAAAAAGTGAAGTTTGCTAAGTTCGCCGAAATTGTAACCTTGAACCTT	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	GGCTGACGGTACACAGCGCAGTGGACAGGTGCTGGAAGTCAGTGGAGACAAAGCTGTCTGCCAGGTGTTCTGA	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	AGGCACGTCAGGTATTGATGCCAAGCACACAACATGCCAGTTCACGGGCGACATTTTAAGGATTCCAACCTC	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	GGAAGACATGCTCGTTCGTATCTTCAACGGATCTGGAACACCGATCGACAAGGGGCCATCTGTTTTCCTCGA	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	AGACTACTTGGATATTTCAGGGTCAGCCCATTAATCCCAAGTCTCGTATTTACCCAGAGGAGATGATTCAAAC	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	TGGCATCTCTGCCATTGACACCATGAACAGCATCGCTCGTGGACAGAAATTTCCCATCTTCTCCGCTAATGG	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	TTTGGCCGACAAACGAAATCGCCGCCAGATTGTGTCACAGGGCGGTTTGGTCAAGCTCCCTGACAAGGACGT	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	CATGACAGTTCACGAGGACAACCTTGTCTATTTGCTGCTGCTATGGGTGTGAACATGGAAGCAGCTAGATT	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	TTTCAAGTCCGACTTTGAGCAGCACGGCTCCATGGACAACGTGTGCTCTTCTTAACTTGCAAACGATCC	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	GACCATTTGAGAGGATCATTACACCTCGTATTGCACTTACGACTGCTGAGTTCCTGGCCTACCAGTGCAGAA	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	GCACGTGCTGGTCATCCTCACCGACATGAGTTCCTATGCTGAAGCTCTGAGAGAGGTGTACGCCGCCAGAGA	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	GGAGGTGCCCGGTCGTCGTGGTTTCCCGGTTACATGTACACGGATCTTGCCACCATCTACGAGCGAGCCGG	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	TCGTGTCAACGGCAGAAACGGATCCATCACCCAGATTCCCATTTCTCACTATGCCCAACGAGATATTACCCA	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	TCCCATTTCCCGATTGTACCGGTTACATTACCGAGGGACAGATCTACATCGACAGGCAGCTGCACAACAGGCA	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	GATCTACCCGCCATCAACGTCCTGCCTTTCGGTCTCATGAAGTCGGCCATCGGCGAGGGAATGAC	1224
373	I Y V P P I N V L P S L S R L M K S A I G E G M T	397
1225	CAGAAAGGACCACTCCGACGTTTCCAACACGCTGTACGCTAACTACGCCATCGGAAAGGACGTGCAGGCTAT	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	GAAGGCCGTGGTCGGCGAGGAGGCTCTAACCGCAGACGATGCTTTACTTGGAGTTCCTTGGGCAAATTCGA	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	CCTGCGAATTTCCCAAGGAGATGCTGAAGCGAATCCCGCGGAACGTCATCAACGAGTACTACCCGAGAAA	1512
469	L R I F P K E M L R I P R N V I N E Y Y P R K	493
1513	GAAGGTGCCCTCCGCCAGGACAAGCAGGAGGACCAGAAGGAAGAGAAGTGAGCGAGAGAGAGAGGCCGACC	1584
493	K V P S A Q D K Q E D Q K E E K *	509
1585	GTAGAAGAAGAAAAGAGAGAGAGATTATCTCGCTTTTTCGCCCTTTTTCCTTTTTCGATCGTCGTTTC	1656
1657	TCGTGTTTTCTCTTTTCCGCTTCTGTTTCGTATCAGTTTTTTTTTTTCAACCAGATTCACTTAGAGCACCTT	1728
1729	CAAAGTTCGCGACGAACCCGAAAAGCACGAAATGTGAGCGGCACGTAGCTTTTCTCGCTTAGACGCTTCCG	1800
1801	TATTCGCTCGCCCTCATCCAGACTCGACTCTCCCCCCCCTAACGCAGCAAAACGCCTCCTTTCCAAACTACG	1872
1873	GTGTTGTCTTATTGATGCTCGTCTGTTGCTTTGCCCGCCCGTGAAGTCCCGCTCGTCCGTGTGGAAC	1944
1945	ATGTCGCGAAACCGATTCTGTTTGTGTGCAATAAAAGCTATCCGACACAGAAAAA	2016
2017	A	2017

**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 chemi-

cal 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

AS	1	-----MATTLDMAQLSAAKEHALAVSRDYISQPRLT YQT
B1	1	MALRAMRGIVNGAAPLPVPTSGPLAGSRREQALAVSRNLYLSQPRLT YKT
B2	1	-----MAAEVDSPRPLPGGGASLGAAREHVAVTBNYITHPRIT YRT
SC	1	-----MVLSDEKELFAINKKAVEQGFNVKPRINYNNT
AS	35	VSGVNGPLVILEKVKFAKEAEIVTLNLADGTQRSGQVLEVSGDKAVVQV
B1	50	VSGVNGPLVILLDHVKFPRYAEIVHLTLPDGTQRSGQVLEVSGSKAVVQV
B2	44	VCSVNGPLVLVDQVKFAQYAEIVNETLPLNGTQRSGQVLEVSGTKAIVQV
SC	31	VSGVNGPLVILEKVKFPRYNEIVNLTLDPDGTVRGQGVLEIRGDRALVQV
AS	84	FEGTSGIDAKHTTCEFTGDLIRIPTSEDMLGRI FNGSGKPIDKGPSVLP
B1	99	FEGTSGIDAKKTSCEFTGDLIRTPVSEDMLGRVFNNGSGKPIDRGPVVL
B2	93	FEGTSGIDAKTTCEFTGDLIRTPVSEDMLGRVFNNGSGKPIDRGPVVM
SC	80	FEGTSGIDVKKTTVEFTGESLRIPVSEDMLGRIEDGSGRPIDNGPKVFA
AS	133	EDYLDIQGQPINPKSR IYPEEMIQTGISAIDTMNSIARGQKIPIFSANG
B1	148	EDFLDIMGQPINPQOR IYPEEMIQTGISAIDGMNSIARGQKIPIFSAA
B2	142	EDFLDINGQPINPHDR IYPEEMIETGISPIDVMNSIARGQKIPIFSAA
SC	129	EDYLDINGSPINPIYARIYPEEMIETGVSAIDTMNSIARGQKIPIFSAS
AS	182	LPHNEIAAQICRQGLVKLPDKDVMDSHEDNFAIVFAAMGVNMEARFF
B1	197	LPHNEIAAQICRQAGLVKK-SKDVVDYSEIENFAIVFAAMGVNMETARFF
B2	191	LPHNEIAAQICRQAGLVKK-SKAVLDYHDDNFAIVFAAMGVNMETARFF
SC	178	LPHNEIAAQICRQAGLVRP-TKDVHDGHEENFSLVFAAMGVNLETARFF
AS	231	KSDFEQHGSMDNVCLFLNLANDPTIERIITPRIALTTAEFLAYQCEKHV
B1	245	KSDFEQNGSMDNVCLFLNLANDPTIERIITPRIALTTAEFLAYQCEKHV
B2	239	KSDFEQNGTIMGNVCLFLNLANDPTIERIITPRIALTTAEFLAYQCEKHV
SC	226	KQDFEENGSLERTSLFLNLANDPTIERIITPRIALTTAEFLAYQTEFHV
AS	280	LVILTDMSSYAEALREVSAAREEVPGRRGFPGYMYTDLAT IYERAGR V
B1	294	LVILTDMSSYAEALREVSAAREEVPGRRGFPGYMYTDLAT IYERAGR V
B2	288	LVILTDMSSYAEALREVSAAREEVPGRRGFPGYMYTDLAT IYERAGR V
SC	275	LTILTDMSSYADALREVSAAREEVPGRRGYPGYMYTDLIST IYERAGR V
AS	329	GRNGSITQIPILTMPNDDITHPIPDLTGYITEGQIYVDRQLHNRQIYPP
B1	343	GRNGSITQIPILTMPNDDITHPIPDLTGYITEGQIYVDRQLHNRQIYPP
B2	337	GRNGSITQIPILTMPNDDITHPIPDLTGFITEGQIYVDRQLHNRQIYPP
SC	324	GRNGSITQIPILTMPNDDITHPIPDLTGYITEGQIFVDRQLHNKGLIYPP
AS	378	INVLPSSLRLMKSAIGEGMTRKDHSDVSNQLYANYAIGKDVQAMKAVVG
B1	392	INVLPSSLRLMKSAIGEGMTRKDHADVSNQLYACYAIGKDVQAMKAVVG
B2	386	INVLPSSLRLMKSAIGEGMTRKDHGDVSNQLYACYAIGKDVQAMKAVVG
SC	373	INVLPSSLRLMKSAIGEGMTRKDHGDVSNQLYAKYAYIGKDAAMKAVVG
AS	427	EEALTADDMLYLEFLGKFEKTFIAAGPYENRVSFEDSLNIGWELLRIFPK
B1	441	EEALTSDLLLYLEFLQKFEKTFIAAGPYENRRTVYETLDIGWQLLRIFPK
B2	435	EEALTSEDLLLYLEFLQKFEKTFINQGPYENRVSFESLDLGWQLLRIFPK
SC	422	EEAISIEDKLSLEFLGKFEKTFITQGAIEDRTVFESLDQAWSLLRIFPK
AS	476	EMLKRIPRNVINELYPRKKVPSAQDKQEDQKEEK-----
B1	490	EMLKRIPQSTLSEFYPRD--SAKH-----
B2	484	EMLKRIPQNIIDEFESRE--GAPQDTEADTAL-----
SC	471	EMLNRSPKILDEFYDRARDDADEDEEDPDTRSSGKKKDKASQEESLI

**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  $\Delta$ pH 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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