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Expressed Sequence Tag Analysis of Blood Cells in the Vanadium-Rich Ascidian, *Ascidia sydneiensis samea* –A Survey of Genes for Metal Accumulation

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ABSTRACT—Some species in the family Ascidiidae accumulate vanadium at concentrations in excess of 350 mM, which corresponds to about 10^7 times that found in seawater. The vanadium ions are stored in vacuoles located within vanadium-containing blood cells, vanadocytes. To investigate the phenomenon, an expressed sequence tag analysis (EST) of a cDNA library of *Ascidia sydneiensis samea* blood cells was carried out. Three hundred clones were obtained and sequenced by EST analysis. A similarity search revealed that 158 of the clones (52.7%) were known genes, and 142 of the clones (47.3%) did not have any similarity to genes registered in the SwissProt database. According to the functions of their genes the identified EST clones were categorized into eight types of clones; these consisted of genes; metal-related proteins (29 clones), signal transduction (22 clones), protein synthesis (17 clones), nuclear proteins (17 clones), cytoskeleton and motility (14 clones), energy conversion (3 clones), hypothetical proteins (11 clones), and others (45 clones). The ferritin homologue has a high degree of similarity to that of mammals; the iron-binding sites of ferritin are well conserved including His-118 which is important for capturing Fe^{2+} , also works as a ligand for VO^{2+} .

Key words: EST, vanadium, accumulation, ascidian, ferritin

INTRODUCTION

Ascidians, also known as tunicates or sea squirts (Chordata, Urochordata, Ascidiacea), accumulate extremely high concentrations of vanadium (Henze, 1911). In particular, species belonging to the family Ascidiidae are known to accumulate vanadium in excess of 350 mM, which corresponds to about 10^7 times the concentration of vanadium ion found in seawater (Michibata *et al.*, 1991). Vanadium is accumulated in vacuoles within vanadocytes, which are a type of blood (coelomic) cell (Michibata *et al.*, 1987). The vanadium is reduced to the +3 oxidation state via the +4 oxidation state for storage in the vacuoles (Kanamori and Michibata, 1994), which also contain high concentrations of protons and sulfate ions (Frank *et al.*, 1986; Hirata and Michibata, 1991). To investigate this unusual phenomenon

of vanadium accumulation, we isolated several proteins and genes that are expressed in vanadocytes.

To date, three types of vanadium-associated protein have been isolated, with molecular masses of 12.5, 15, and 16 kDa (Kanda *et al.*, 1997), along with the cDNAs encoding these proteins (unpublished data). In addition, four types of enzyme related to the pentose phosphate pathway that produces NADPH are located in vanadocytes (Uyama *et al.*, 1998a, b, c; Ueki *et al.*, 2000). The pentose phosphate pathway participates in the reduction of vanadium(V) to vanadium(IV) (Kanamori *et al.*, 1999). Furthermore, the cDNA for each of the vacuolar-type H^+ -ATPase (V-ATPase) A, B, and C subunits, which are located on the vacuolar membranes of vanadocytes, has been isolated and analyzed (Uyama *et al.*, 1994; Ueki *et al.*, 1998, 2001). V-ATPase generates a proton-motive force, and is thought to provide the energy for vanadium accumulation (Forgac, 1989; Nelson, 1992)

Our ultimate goal is to clarify the entire mechanism involved in the accumulation and reduction of vanadium in

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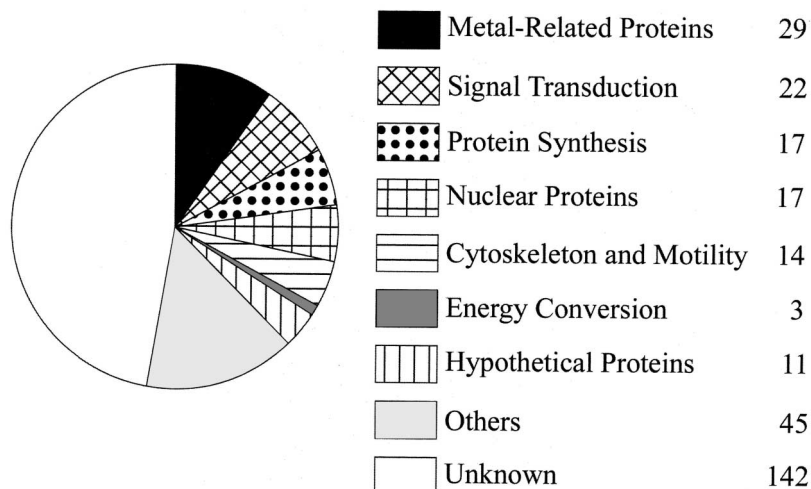


Fig. 1. A summary of the 300 EST clones obtained from the cDNA library of *Ascidia sydneiensis samea* blood cells. The clones obtained were classified into nine categories. One hundred and fifty eight clones showed similarity to the proteins registered in the SwissProt database, and the other 142 clones showed no similarities. In the identified ESTs, 29 clones had similarities with metal-related protein genes.

ascidian vanadocytes. To attain this goal, many more genes and proteins expressed in the blood cells needed to be systematically identified. Therefore, in this study, we first performed an expressed sequence tag (EST) analysis of blood cells from a vanadium-accumulating ascidian species, *A. sydneiensis samea*, although genes expression profile of fertilized eggs, embryos and neural complex based on EST analysis have been reported in some ascidian species (Makabe *et al.*, 2001; Nishikata *et al.*, 2001; Satou *et al.*, 2001; Takamura *et al.*, 2001). Three hundred cDNA clones from a blood cell library were analyzed to determine their 5'-terminal partial nucleotide sequences. The amino acid sequences were then compared with protein sequences registered in the SwissProt database. Twenty-nine of the sequences were found to be similar to gene products that are related to the transport or redox of metals, such as Fe, Ca, Zn, Mg, Co, Cu, and Na. In addition, two sequences were obtained for V-ATPase subunits previously identified in our laboratory (Ueki *et al.*, 1998). These two subunits may participate in maintaining the high acidity within the vanadocyte vacuoles.

MATERIALS AND METHODS

Separation of Blood Cells and RNA extraction to construct the cDNA library

Specimens of the vanadium-rich ascidian, *A. sydneiensis samea*, were collected near the Otsuchi Marine Research Center, which is a branch of the Ocean Research Institute of the University of Tokyo in Otsuchi, Iwate Prefecture, Japan. Blood was drawn from each specimen by making an incision through the lower part of the tunic. To separate blood cells from the serum, the blood was suspended in Ca^{2+} - and Mg^{2+} -free artificial seawater (CMFASW) containing 460 mM NaCl, 9mM KCl, 32mM Na_2SO_4 , 6mM NaHCO_3 , 5mM HEPES and 5mM EDTA at pH 7.0 and centrifuged at $300\times g$ for 10 min at 4°C . The blood cells were resuspended in CMFASW containing 20% sucrose, and were then centrifuged again at $1,500\times g$ for 10 min at 4°C to remove giant cells that have very

acidic contents but no vanadium (Michibata *et al.*, 1990). The remaining blood cells were suspended in a solution containing 4M guanidine thiocyanate (GTC), 0.1% sodium N-lauryl sarcosinate, 5mM EDTA, and 40mM Tris-HCl at pH 7, and the mixture was homogenized by ultrasonication. The homogenate was then added to a solution of 50% cesium trifluoroacetate and 100mM EDTA, and centrifuged at $100,000\times g$ for 16 hr at 15°C using an ultracentrifuge (Model 70P72, Hitachi). The RNA that precipitated was recovered and dissolved in sterilized water. A cDNA library was constructed from the total RNA using Uni-Zap XR vector (Stratagene, La Jolla, CA).

Analysis for determination of the DNA sequences

The recombinant λ -ZAPII vector was inserted into pBluescript SK (-) plasmids by *in vivo* excision, according to the manufacturer's instructions (Stratagene). We performed cDNA fragment insertion checks by direct PCR to select clones longer than 500bp. The PCR mixture was denatured at 95°C for 2 min, and this was followed by 30 cycles at 95°C for 30 sec, 50°C for 30 sec, and 72°C for 60 sec, with a final 10 min at 72°C using primers T3 and T7 (model PTC-200, MJ Research). The recombinant plasmid DNA isolated by the alkaline lysis method was used as a template for DNA sequencing. The cDNA clones were sequenced using ThermoSequenase

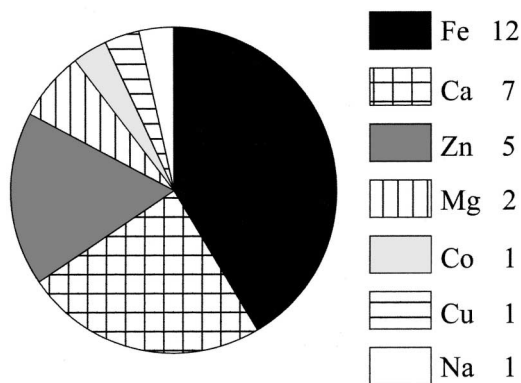


Fig. 2. The classified EST clones that are dependent on metals. Twenty-nine metal-related ESTs were classified into seven groups based on metals.

Table 1. A list of ESTs identified from the blood cells of *Ascidia sydneiensis samea*, excluding giant cells. Using the program BLASTX, EST sequences were compared with the SwissProt database to identify related proteins. The EST number, GenBank registered number, and results of the homology search (Closet Protein name, species, SwissProt accession number, probability and frequency) are shown. (1/3)

| METAL-RELATED PROTEINS | | | | | | |
|------------------------|-----------------------|-----------------------------------------------|----------------|---------------|-------|-------|
| EST No. | GenBank Accession No. | Closet Protein | Closet species | Accession No. | Prob. | Freq. |
| 009 | AU249187 | Fibrillin 1 precursor (MP340) | Human | P35555 | 5e-19 | |
| 010 | AU249187 | Goliath protein (G1 protein) | Fruit fly | Q06003 | 8e-14 | |
| 017 | AU249189 | Ferritin heavy chain | Sheep | P18685 | 3e-22 | 6 |
| 032 | AB084793 | Annexin A7 | Frog | Q92125 | 2e-10 | |
| 051 | AB084797 | Regulator of nonsense transcripts 1 | Human | Q92900 | 4e-62 | |
| 058 | AU249200 | Calcium-transporting ATPase | Shrimp | P35316 | 4e-96 | |
| 074 | AU249206 | Na/K-transporting ATPase alpha-1 chain | Chicken | P09572 | 4e-59 | |
| 106 | AU249219 | Cytochrome P450 3A29 | Pig | P79401 | 4e-13 | |
| 108 | AU249220 | Succinate dehydrogenase | Fruit fly | Q94523 | 1e-69 | |
| 124 | AU249225 | Calmodulin, flagellar | Bacteria | P53440 | 2e-11 | |
| 128 | AU249227 | Ovotransferrin | Bird | P56410 | 4e-17 | |
| 129 | AB084804 | Nuclear accomodation of mitochondria 7 | Yeast | P30771 | 2e-08 | |
| 132 | AU249230 | Matrix metalloproteinase-14 precursor | Rabit | Q95220 | 1e-14 | 2 |
| 136 | AU249232 | NRAMP 2 (Metal ion transporter DCT1) | Rat | O54902 | 1e-05 | |
| 142 | AU249187 | Fibrillin 2 precursor | Mouse | Q61555 | 6e-17 | 3 |
| 147 | AU249236 | Cytochrome P450 3A2 | Rat | P05183 | 3e-40 | |
| 164 | AU249241 | NADH-ubiquinone oxidoreductase | Bovine | P17694 | 1e-45 | |
| 185 | AU249247 | DnaJ homolog subfamily A member 1 | Mouse | P54102 | 5e-18 | |
| 202 | AU249256 | Thyroid receptor interacting protein 6 | Human | Q15654 | 1e-29 | |
| 254 | AU249271 | Ceruloplasmin precursor | Mouse | Q61147 | 6e-26 | |
| 300 | AU249287 | Methionine aminopeptidase 2 | Rat | P38062 | 4e-51 | |
| SIGNAL TRANSDUCTION | | | | | | |
| EST No. | GenBank Accession No. | Closet Protein | Closet species | Accession No. | Prob. | Freq. |
| 003 | AU249183 | Protein kinase C, gamma type | Mouse | P05697 | 9e-13 | |
| 005 | AU249185 | Signal recognition particle 72 kDa protein | Human | O76094 | 1e-27 | |
| 019 | AB084792 | Tumor necrosis factor receptor 1 precursor | Pig | P50555 | 6e-05 | |
| 033 | AU249194 | Proto-oncogene tyrosine-protein kinase YRK | Chicken | Q02977 | 1e-41 | |
| 067 | AU249204 | Ras GTPase-activating-like protein IQGAP1 | Mouse | Q9JKF1 | 5e-28 | 2 |
| 075 | AU249207 | Ras-related protein Rab-14 | Human | P35287 | 7e-73 | 2 |
| 080 | AB084800 | Swiss cheese protein | Fruit Fly | Q9U969 | 3e-30 | |
| 085 | AU249209 | GTP-binding protein G(i) | Star Fish | P30676 | 3e-70 | 2 |
| 123 | AU249224 | GTP-binding protein beta subunit-like protein | Zebrafish | O42248 | 7e-39 | |
| 133 | AU249231 | Protein kinase C, brain isozyme | Fruit Fly | P05130 | 7e-24 | |
| 139 | AU249233 | Ras GTPase-activating-like protein IQGAP1 | Human | P46940 | 2e-25 | |
| 175 | AU249244 | cAMP-dependent protein kinase type I | Human | P31321 | 4e-45 | |
| 207 | AU249257 | MAP kinase-activated protein kinase 2 | Human | P49137 | 5e-50 | |
| 214 | AU249260 | Transforming protein RhoA | Mouse | Q9QUI0 | 3e-37 | |
| 231 | AU249264 | Tyrosine-protein kinase STK | Hydra | P17713 | 3e-16 | |
| 233 | AB084806 | TAK1-binding protein 1 | Human | Q15750 | 1e-24 | |
| 252 | AB084808 | Vegetatible incompatibility protein HET-E-1 | Funji | Q00808 | 3e-21 | |
| 272 | AU249277 | Channel associated protein of synapse-110 | Human | Q15700 | 5e-30 | |
| 276 | AU249280 | GTP-binding protein G(I)/G(S)/G(T) | Rat | P54311 | 2e-55 | |

Table 1. continued (2/3)

| PROTEIN SYNTHESIS | | | | | | |
|---------------------------|----------------------------------------------------------|-----------------------------------------------|----------------|---------------|-------|-------|
| EST No. | GenBank Accession No. | Closet Protein | Closet species | Accession No. | Prob. | Freq. |
| 034 | AU249195 | 60S ribosomal protein L4A (L1A) | Frog | P08429 | 1e-63 | |
| 084 | AU249208 | Elongation factor 1-beta | Frog | P30151 | 2e-36 | |
| 103 | AU249217 | Elongation factor 1-alpha | Insect | P19039 | 1e-76 | 3 |
| 146 | AU249235 | 60S ribosomal protein L32 (RP49) | Fruit Fly | P46615 | 3e-38 | |
| 150 | AU249238 | Eukaryotic initiation factor 4A-like NUK-34 | Human | P38919 | 7e-57 | |
| 195 | AU249252 | Eukaryotic initiation factor 4F subunit P82-3 | Plant | Q03387 | 4e-16 | |
| 239 | AU249266 | 60S ribosomal protein L3 | Bovine | P39872 | e-106 | |
| 241 | AU249267 | 60S ribosomal protein L44 (L41) | Yeast | P52809 | 3e-48 | 2 |
| 244 | AU249268 | 60S ribosomal protein L19 | Mouse | P22908 | 2e-35 | |
| 263 | AU249275 | Transcription initiation factor IIB | Rat | P29053 | 2e-70 | |
| 265 | AU249276 | Eukaryotic initiation factor 4A-II | Mouse | P10630 | 6e-71 | 2 |
| 275 | AU249279 | Elongation factor Tu mitochondrial precursor | Bovine | P49410 | 2e-69 | |
| 296 | AU249286 | Elongation factor 1-gamma | Shrimp | P12261 | 4e-17 | |
| NUCLEAR PROTEINS | | | | | | |
| EST No. | GenBank Accession No. | Closet Protein | Closet species | Accession No. | Prob. | Freq. |
| 039 | AU249196 | NDRG3 protein (Fragment) | Human | Q92597 | 3e-27 | |
| 062 | AU249201 | Exportin 1 | Yeast | P14068 | 7e-33 | |
| 070 | AU249205 | Histone H3 | Plant | P08437 | 8e-14 | |
| 090 | AU249212 | Transcription regulator protein BACH2 | Mouse | P97303 | 4e-11 | |
| 096 | AU249215 | 80 kDa nuclear cap binding protein | Human | Q09161 | 1e-27 | |
| 113 | AB084802 | Developmental protein cactus | Fruit Fly | Q03017 | 1e-11 | |
| 115 | AU249222 | C-Rel proto-oncogene protein | Chicken | P16236 | 9e-48 | |
| 116 | AU249223 | Probable ATP-dependent RNA helicase p47 | Pig | Q29024 | 2e-63 | 2 |
| 120 | AB084803 | CCAAT/enhancer binding protein epsilon | Human | Q15744 | 2e-07 | |
| 127 | AU249226 | Pre-mRNA splicing factor | Human | Q92620 | 1e-61 | |
| 130 | AU249228 | DNA polymerase zeta catalytic subunit | Human | O60673 | 7e-57 | |
| 188 | AU249248 | csgAB operon regulatory protein | Bacteria | O54294 | 8e-35 | |
| 226 | AU249262 | Putative pre-mRNA splicing factor | Plant | O22899 | 4e-37 | |
| 257 | AU249273 | Heat shock 70 kDa protein cognate 4 | Insect | Q9U639 | e-103 | |
| 288 | AB084811 | X box binding protein-1 | Human | P17861 | 3e-18 | |
| CYTOSKELETON AND MOTILITY | | | | | | |
| EST No. | GenBank Accession No. | Closet Protein | Closet species | Accession No. | Prob. | Freq. |
| 105 | AU249218 | Vacuolar ATP synthase subunit B | Insect | P31401 | 1e-83 | |
| 213 | AU249259 | Mitochondrial uncoupling protein 2 | Rat | P56500 | 3e-33 | |
| 250 | AU249270 | Vacuolar ATP synthase catalytic subunit A | Human | P38607 | e-107 | |
| HYPOTHETICAL PROTEINS | | | | | | |
| EST No. | Closet Protein | Closet species | Accession No. | Prob. | Freq. | |
| 018 | WD-repeat protein 7 (Fragment) | Human | Q9Y4E6 | 1e-27 | | |
| 100 | Hypothetical 51.2 kDa protein in PET54-DIE2 | Yeast | P50079 | 1e-15 | | |
| 107 | Hypothetical protein ybhE | E. coli | P52697 | 2e-58 | | |
| 122 | Hypothetical 84.5 kDa protein in CPS1-FPP1 | Yeast | P46995 | 2e-30 | | |
| 152 | Hypothetical 39.4 kDa protein in MET1-SIS2 | Yeast | P36151 | 8e-10 | | |
| 154 | Hypothetical protein ygcP | E. coli | Q46906 | 2e-14 | | |
| 197 | Hypothetical 94.3 kDa Trp-Asp repeats containing protein | Yeast | Q03177 | 2e-22 | | |
| 206 | Hypothetical 83.6 kDa protein R05D3.2 in chromosome III | Nematoda | P34535 | 7e-32 | | |
| 237 | Hypothetical 46.7 kDa protein C3H1.10 in chromosome I | Yeast | Q10075 | 4e-46 | | |
| 258 | Hypothetical 52.8 kDa protein T05E11.5 in chromosome IV | Nematoda | P49049 | 5e-14 | | |
| 293 | Hypothetical 54.9 kDa protein C02F5.7 in chromosome III | Nematoda | P34284 | 1e-12 | | |

Table 1. continued (3/3)

| OTHERS | | | t | | | |
|---------|-----------------------|----------------------------------------------|---------------|---------------|-------|-------|
| EST No. | GenBank Accession No. | Closest Protein | Close species | Accession No. | Prob. | Freq. |
| 004 | AU249184 | Onconeural ventral antigen-1 | Human | P51513 | 6e-23 | |
| 012 | AU249188 | Somatostatin receptor type 5 | Mouse | O08858 | 1e-16 | |
| 015 | AB084790 | Auxilin | Bovine | Q27974 | 4e-13 | |
| 016 | AB084791 | Immediate-early protein. | virus | Q01042 | 3e-11 | |
| 020 | AU249190 | Adenylyl cyclase-associated protein | Hydra | P40122 | 6e-07 | |
| 021 | AU249191 | Beta-hexosaminidase beta chain precursor | Human | P07686 | 8e-45 | |
| 025 | AU249192 | Thioredoxin-dependent peroxide reductase | Mouse | P20108 | 2e-70 | |
| 031 | AU249193 | Phosphatidylserine synthase I | Hamster | Q00576 | 8e-28 | |
| 035 | AB084794 | Very-long-chain acyl-CoA synthetase | Rat | P97524 | 7e-09 | |
| 043 | AB084795 | Probable G protein-coupled receptor GPR7 | Human | P48145 | 3e-17 | |
| 048 | AU249198 | Glycerol-3-phosphate acyltransferase | Rat | P97564 | 3e-11 | |
| 050 | AB084796 | Neuromodulin | Bird | Q98987 | 2e-10 | |
| 052 | AU249199 | Ubiquitin-conjugating enzyme E2 G1 | Human | Q99462 | 2e-69 | |
| 060 | AB084798 | Ubiquitin-protein ligase pub1 | Yeast | Q92462 | 2e-11 | |
| 061 | AB084799 | Sporulation protein SPS19 | Yeast | P32573 | 3e-08 | |
| 064 | AU249202 | ATP-binding cassette, sub-family A | Human | O95477 | 3e-31 | |
| 066 | AU249203 | ADP-ribosylation factor 1 | Potozoan | Q25761 | 1e-38 | |
| 086 | AU249210 | Heat shock cognate 71 kDa protein | Bovine | P08109 | 2e-65 | |
| 087 | AU249211 | Proteasome subunit beta type 5 precursor | Rat | P28075 | 7e-65 | |
| 095 | AU249214 | 2-3'-dephospho-CoA synthase | E.coli | P77231 | 8e-43 | |
| 097 | AB084801 | Sorting nexin 9 | Human | Q9Y5X1 | 1e-07 | |
| 131 | AU249229 | Septin 7 | Mouse | O55131 | 4e-54 | |
| 144 | AU249234 | Neutrophil cytosol factor 2 | Bovine | O77775 | 2e-23 | |
| 155 | AU249239 | CD63 antigen | Rat | P28648 | 1e-09 | |
| 189 | AU249249 | Syntaxin 1A | Bovine | P32850 | 7e-26 | |
| 196 | AU249253 | Ubiquitin | Sponge | P14792 | 9e-36 | 4 |
| 198 | AU249254 | T-complex protein 1, theta subunit | Human | P50990 | 4e-67 | |
| 201 | AU249255 | Histidine ammonia-lyase | Rat | P21213 | 2e-64 | |
| 212 | AU249258 | Adapter-related protein complex 1 gamma 1 | Mouse | P22892 | 7e-47 | |
| 224 | AU249261 | Serine hydroxymethyltransferase | Human | P34897 | 5e-13 | |
| 230 | AU249263 | Protein disulfide isomerase precursor | Bovine | P05307 | 3e-37 | |
| 232 | AB084805 | Sorting nexin 6 | Human | Q9UNH7 | 1e-08 | |
| 235 | AU249265 | Nck-associated protein 1 | Rat | P55161 | 9e-36 | |
| 247 | AU249269 | Phosphomannomutase 2 | Human | O15305 | 5e-54 | |
| 249 | AB084807 | Retinal-binding protein (RALBP) | Squid | P49193 | 3e-10 | |
| 255 | AU249272 | Metastasis-associated protein MTA1 | Human | Q13330 | 1e-76 | |
| 259 | AB084809 | Apolipoporphins precursor | Insect | Q25490 | 1e-05 | 2 |
| 269 | AB084810 | PAK-interacting exchange factor beta | Rat | O55043 | 3e-45 | |
| 277 | AU249281 | 4-aminobutyrate aminotransferase | Rat | P50554 | 9e-25 | |
| 280 | AU249282 | TolB protein precursor | Bacteria | P19935 | 1e-63 | |
| 292 | AU249284 | Inter-alpha-trypsin inhibitor heavy chain H4 | Human | Q14624 | 2e-27 | |

(Amersham, U.K.) with M13 reverse primer and the DNA sequencer ALF ExpressII (Amersham). For sequencing, the PCR was run using the same protocol as above. Using the program BLASTX,

each approximately 500bp DNA sequence was compared with the SwissProt database to identify related proteins. To determine the full-length DNA sequences of the ferritin H-subunit, we sequenced

the DNA, as already described, using M13 universal and reverse primers.

RESULTS AND DISCUSSION

There are at least 2,300 ascidians in class Ascidiacea of subphylum Urochordata. Recent molecular biological data suggest that ascidians are more closely related to vertebrates than to other invertebrates (Wada and Satoh, 1994; Kusakabe *et al.*, 1997). Therefore, as primitive chordates, ascidians are thought to hold the key to understanding the evolutionary traits and physiological functions of higher chordates. Ascidians and higher chordates have some properties in common, including the mechanism for fertilization and the self- and non-self recognition mechanisms. However, ascidians also have some properties that higher chordates do not have, *e.g.*, vanadium accumulation and asexual reproduction (*cf.* Sawada *et al.*, 2001).

Of the ascidian-specific properties, the unusual ability to accumulate high levels of vanadium in blood cells has attracted the attention of chemists, physiologists, and biochemists (Michibata, 1996; Michibata and Kanamori, 1998; Michibata *et al.*, 1998; Michibata *et al.*, 2001). Blood cells, including the vanadocytes, play a leading role in this phenomenon. This study carried out an EST analysis using whole blood cells, but giant cells were not used because the highly acidic content of the vacuoles of these cells interferes with RNA extraction.

The 5'-terminal partial nucleotide sequences of 300 cDNA clones (270 distinct genes) from the *A. sydneiensis*

samea blood cell cDNA library were analyzed. Using the program BLASTX, the amino acid sequences were then compared with known protein sequences registered in the SwissProt database. When a sequence probability was higher than 1e-05, it was classified as "no sequence similarity", while sequences that probability less than 1e-05 were categorized as "sequence similarity". As shown in Fig. 1, cDNAs with sequence similarity were placed into one of eight categories, according to the function of the gene. The categories were genes related to: metal-related proteins (29 clones), signal transduction (22 clones), protein synthesis (17 clones), nuclear proteins (17 clones), cytoskeleton and motility (14 clones), energy conversion (3 clones), hypothetical proteins (11 clones), and others (45 clones). These clones were registered into GenBank database without hypothetical proteins. There were 142 clones that showed no similarity with known proteins, although some of these clones might represent novel genes related to metal accumulation processes. Twenty-nine metal-related clones were further subdivided into seven categories, as shown in Fig. 2. Gene names and similarity scores are described in Table 1. Among them, the 12 clones have been identified as homologues of iron-related genes, such as the ferritin H-subunit, transferrin and Nramp.

The six cDNA clones of the 12 clones were those encoding the ferritin subunit. Ferritin consists of 24 subunits of two types, H- and L-subunits, which form a shell-like structure with a hollow interior (Aisen *et al.*, 1999). Based on the results of matching DNA sequences with the coded the ferritin genes, the various ferritin sequences appear to be



Fig. 3. The alignment of amino acid sequences between the ascidian ferritin subunit and that of other organisms, based on the BLASTX search of the SwissProt database. Boxes indicate the iron-binding residue. The residue of Histidine118 (gray box) is reported to be a vanadium-binding site in the human ferritin H-subunit.

encoded by the same gene. The similarity search using the program BLASTX showed that the ascidian ferritin subunit is similar to some mammal ferritin H-subunits (Fig. 3). In the human ferritin H-subunit, eight residues, Glu-27, -61, -62, -64, -107, Gln-58, -141, and His-65, are considered to be iron-binding sites (Harrison and Arosio, 1996). These residues were conserved in the ascidian ferritin subunit, with the exceptions of Gln-58, Glu-64, and His-65. His-118, a vanadium-binding site in mammalian ferritin H-subunits, was also conserved in the ascidian ferritin subunit (Grady *et al.*, 2000). Site-directed mutagenesis and EPR spectroscopy revealed that mammalian recombinant apoferritins bind to vanadium in the +4 oxidation state (VO^{2+}). These authors also showed that His-118, which is important for capturing Fe^{2+} , also works as a ligand for VO^{2+} . As shown in Fig. 3, the ascidian ferritin subunit has a high degree of similarity with ferritin H-subunit of mammals, and the iron-binding sites, including His-118, are also well conserved. These results suggest that ferritin is involved in the accumulation of vanadium in ascidian blood cells.

This study showed that ascidian transferrin is similar to mammalian transferrin, an iron-transport protein that has also been reported to bind the vanadium ion (Sabbioni *et al.*, 1980). Further evidence is required to determine whether the genes are found exclusively in vanadocytes, and whether the ferritin and transferrin in ascidian blood cells actually bind with vanadium. Nramp, natural resistance-associated macrophage protein, is a chemiosmotic ion/proton exchanger that has an usually broad substrate range, including Fe^{2+} , Zn^{2+} , Mn^{2+} , Co^{2+} , Cd^{2+} , Cu^{2+} , Ni^{2+} , and Pb^{2+} (Blackwell *et al.*, 2000). Therefore, it is also worth examining whether the vanadium ion is a substrate of Nramp in ascidian blood cells.

Genes encoding several metal transporters and metalloproteins, such as Na^+/K^+ -ATPase and ceruloplasmin, were also identified in this study. Furthermore, we found cDNAs encoding subunits A and B of V-ATPase, both of which have been previously identified in our laboratory (Ueki *et al.*, 1998b). These subunits are involved in maintaining the highly acidic conditions within the vanadocyte vacuoles.

The remaining ESTs, accounting for approximately 47% of all the clones obtained, showed no similarity with any genes in the database. This might reflect the unusual physiological phenomenon of vanadium accumulation in vanadium-rich ascidians.

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