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

Authors: Yamaguchi, Nobuo, Togi, Akiko, Ueki, Tatsuya, Uyama, Taro, and Michibata, Hitoshi

Source: Zoological Science, 19(9) : 1001-1008

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.19.1001

ZOOLOGICAL SCIENCE **19**: 1001–1008 (2002)

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

Nobuo Yamaguchi[1], Akiko Togi[2], Tatsuya Ueki[1], Taro Uyama[1] and Hitoshi Michibata[1]*

[1]*Marine Biological Laboratory, Graduate School of Science, Hiroshima University, Mukaishima-cho 2445, Hiroshima 722-0073, Japan*
[2]*Minase Research Institute, Sekisui Chemical Co., Ltd., 2-1 Hyakuyama Shimamoto-cho, Mishima-gun, Osaka 618-8589, Japan*

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

* Corresponding author: Tel. +81-848-44-1143;
FAX. +81-848-44-5914.
E-mail: hmichi@sci.hiroshima-u.ac.jp

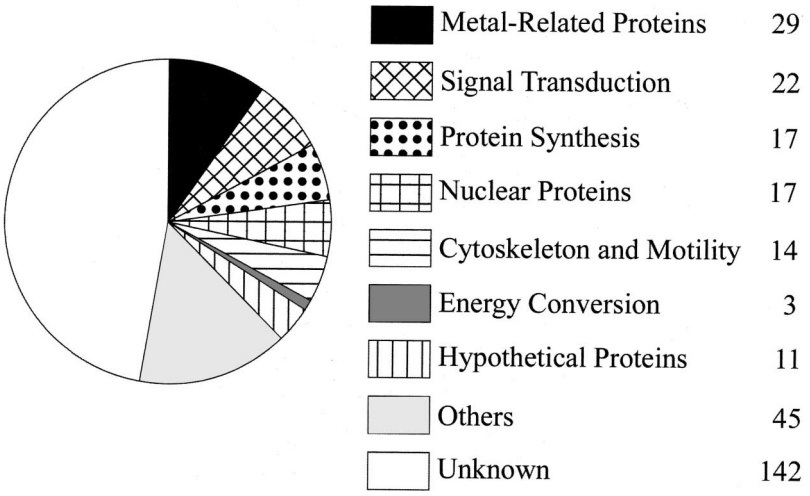

Fig. 1. A summary of the 300 EST clones obtained from the cDNA library of *Ascidia sydneiensis samea* blood cells. The clones obtained were classified into nine categories. One hundred and fifty eight clones showed similarity to the proteins registered in the SwissProt database, and the other 142 clones 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×*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×*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×*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

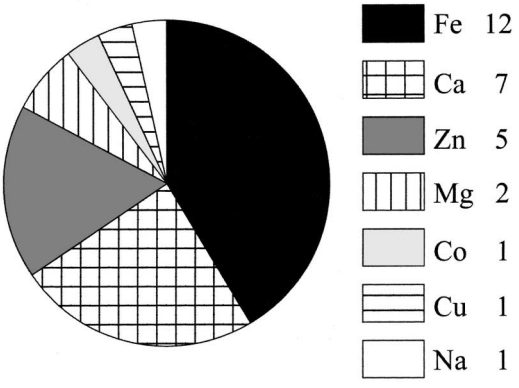

Fig. 2. The classified EST clones that are dependent on metals. Twenty-nine metal-related ESTs were classified into seven groups based on metals.

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.	Closet 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	Apolipophorins 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.

ACKNOWLEDGEMENTS

We would like to express our hearty thanks to Mr. K. Morita of the Otsuchi Marine Research Center for his kind cooperation in collecting the materials. This work was supported in part by Grant-in-Aids from the Ministry of Education, Culture, Sports, Science and Technology of Japan (#11440244 and #11559014 to H. M.).

REFERENCES

Aisen P, Wessling-Resnick M, Leibold EA (1999) Iron metabolism. Bioinorg Chem 3: 200–206

Blackwell JM, Searle S, Goswami T, Miller EN (2000) Understanding the multiple functions of Nramp1. Microbes Infect 2: 317–321

Forgac M (1989) Structure and function of vacuolar class of ATP-driven proton pumps. Physiol Rev 69: 765–796

Frank P, Carlson RMK, Hodgson KO (1986) Vanadyl ion EPR as a non-invasive probe of pH in intact vanadocytes from *Ascidia ceratodes*. Inorg Chem 25: 470–478

Grady JK, Shao J, Arosio P, Santambrogio P, Chasteen ND (2000) Vanadyl(IV) binding to mammalian ferritins. An EPR study aided by site-directed mutagenesis. J Inorg Biochem 80: 107–113

Harrison PM, Arosio P (1996) The ferritins: molecular properties, iron storage function and cellular regulation. Biochim Biophys Acta 1275: 161–203

Henze M (1911) Untersuchungen über das Blut der Ascidien I. Mitteilung. Die Vanadium-verbindungen der Blutkörperchen. Hoppe-Seyler's Z Physiol Chem 72: 494–501

Hirata J, Michibata H (1991) Valency of vanadium in the vanadocytes of *Ascidia gemmata* separated by density-gradient centrifugation. J Exp Zool 257: 160–165

Kanamori K, Michibata H (1994) Raman spectroscopic study of the vanadium sulfate in blood cell homogenate of the ascidian, *Ascidia gemmata.* J Mar Biol Assoc UK 74: 279–286

Kanamori K, Sakurai K, Kinoshita M, Uyama T, Ueki T, Michibata H (1999) Direct reduction from vanadium(V) to vanadium(IV) by NADPH in the presence of EDTA. A consideration of the reduction and accumulation of vanadium in the ascidian blood cells. J Inorg Biochem 77: 157–161

Kanda T, Nose Y, Wuchiyama J, Uyama T, Moriyama Y, Michibata H (1997) Identification of a vanadium-associated protein from the vanadium-rich ascidian, *Ascidia sydneiensis samea*. Zool Sci 14: 37–42

Kusakabe T, Araki I, Satoh N, Jeffery WR (1997) Evolution of chordate actin genes: evidence from genomic organization and amino acid sequences. J Mol Evol 44: 289–298

Makabe KW, Kawashima T, Kawashima S, Minokawa T, Adachi A, Kawamura H, Ishikawa H, Yasuda R, Yamamoto H, Kondoh K, Arioka S, Sasakura Y, Kobayashi A, Yagi K, Shojima K, Kondoh Y, Kido S, Tsujinami M, Nishimura N, Takahashi M, Nakamura T, Kanehisa M, Ogasawara M, Nishikata T, Nishida H (2001) Large-scale cDNA analysis of the maternal genetic information in the egg of *Halocynthia roretzi* for a gene expression catalog for ascidian development. Development 128: 2555–2567

Michibata H (1996) The mechanism of accumulation of vanadium by ascidians: Some progress towards an understanding of this unusual phenomenon. Zool Sci 13: 489–502

Michibata H, Hirata J, Uesaka M, Numakunai T, Sakurai H (1987) Separation of vanadocytes: Determination and characterization of vanadium ion in the separated blood cells of ascidian, *Ascidia ahodori*. J Exp Zool 244: 33–38

Michibata H, Iwata Y, Hirata J (1991) Isolation of highly acidic and vanadium-containing blood cells from among several types of blood cell from Ascidiidae species by density-gradient centrifugation. J Exp Zool 257: 306–313

Michibata H, Kanamori K (1998) Vanadium in the Environment. Part 1: Chemistry and Biochemistry, Edt. by Nriagu JO, John Wiley & Sons Inc, pp 217–249

Michibata H, Uyama T, Hirata J (1990) Vanadium containing cells (vanadocytes) show no fluorescence due to the tunichrome in the ascidian *Ascidia sydneiensis samea*. Zool Sci 7: 55–61

Michibata H, Uyama T, Kanamori K (1998) The accumulation mechanism of vanadium by ascidians- An interdisciplinary study between biology and chemistry on extraordinary high levels and reduced form of vanadium in vanadocytes. Am Chem Soc

Symp Ser 711: 248–258
Michibata H, Uyama T, Ueki T, Kanamori K (2001) The Biology of Ascidians. Edt. by Sawada H, Yokosawa H, Lambert CC, Springer-Verlag, Tokyo, pp 363–373
Nelson N (1992) The vacuolar H^+-ATPaseóone of the most fundamental ion pumps in nature. J Exp Biol 172: 19–27
Nishikata T, Yamada L, Mochizuki Y, Satou Y, Shin-i T, Kohara Y, Satoh N (2001) Profiles of maternal expressed genes in fertilized eggs of *Ciona intestinalis*. Dev Biol 238: 315–331
Sabbioni E, Rade J, Bertolero F (1980) Relationships between iron and vanadium metabolism: the exchange of vanadium between transferrin and ferritin. J Inorg Biochem 12: 307–315
Satou Y, Takatori N, Yamada L, Mochizuki Y, Hamaguchi M, Ishikawa H, Chiba S, Imai K, Kano S, Murakami DS, Nakayama A, Nishino A, Sasakura Y, Satoh G, Shimotori T, Shin-i T, Shoguchi E, Suzuki MM, Takada N, Utsumi N, Yoshida N, Saiga H, Kohara Y, Satoh N (2001) Gene expression profiles in *Ciona intestinalis* tailbud embryos. Dev Biol 238: 315–331
Sawada H, Yokosawa H, Lambert CC (2001) The Biology of Ascidians. Springer-Verlag,Tokyo, pp 470
Takamura K, Oka N, Akagi A, Okamoto K, Okada T, Fukuoka T, Hogaki A, Naito D, Oobayashi Y, Satoh N (2001) EST analysis of genes that are expressed in the neural complex of *Ciona intestinalis* adults. Zool Sci 18: 1231–1236
Ueki T, Uyama T, Kanamori K, Michibata H (1998) Isolation of cDNAs encoding subunits *A* and *B* of the vacuolar-type ATPase from the vanadium-rich ascidian, *Ascidia sydneiensis samea*. Zool Sci 15: 823–829
Ueki T, Uyama T, Yamamoto K, Kanamori K, Michibata H (2000) Exclusive expression of transketolase in the vanadocytes of the vanadium-rich ascidian, *Ascidia sydneiensis samea*. Biochim Biophys Acta 1494: 83–90
Ueki T, Uyama T, Kanamori K, Michibata H (2001) Subunit C of the vacuolar-type ATPase from the vanadium-rich ascidian *Ascidia sydneiensis samea* rescued the pH sensitivity of yeast ma5 mutants. Marine Biotechnol 3: 316–321
Uyama T, Kinoshita T, Takahashi H, Satoh N, Kanamori K, Michibata H (1998a) 6-phosphogluconate dehydrogenase is a 45kDa-antigen recognized by a monoclonal antibody S4D5 specific to vanadocytes in the vanadium-rich ascidian, *Ascidia sydneiensis samea*. J Biochem 124: 377–382
Uyama T, Moriyama Y, Futai M, Michibata H (1994) Immunological detection of a vacuolar-type H^+-ATPase in the vanadocytes of the ascidian *Ascidia sydneiensis samea*. J Exp Zool 270: 148–154
Uyama T, Ueki T, Takahashi H, Suhama Y, Kanamori K, Michibata H (1998b) A 100-kDa antigen recognized by a newly prepared monoclonal antibody specific to the vanadocytes of the vanadium-rich ascidian, *Ascidia sydneiensis samea*, is glycogen phosphorylase. Zool Sci 15: 815–821
Uyama T, Yamamoto K, Kanamori K, Michibata H (1998c) Glucose-6-phosphate dehydrogenase in the pentose phosphate pathway is localized in vanadocytes of vanadium-rich ascidian, *Ascidia sydneiensis samea*. Zool Sci 15: 441–446
Wada H, Satoh N (1994) Details of the evolutionary history from invertebrates to vertebrates, as deduced from the sequences of 18S rDNA. Proc Natl Acad Sci USA 91: 1801–1804

(Received May 20, 2002 / Accepted July 8, 2002)