Short Communication

Subunit *C* of the Vacuolar-Type ATPase from the Vanadium-Rich Ascidian, *Ascidia sydneiensis samea*, Rescued the pH Sensitivity of Yeast *vma5* Mutants

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Running title: Rescue of *vma5* Mutants by Ascidian cDNA

Key Words: ascidian, metal accumulation, vacuolar H^+-ATPase, vacuole, gene expression.

Abstract: A vanadium-accumulating ascidian, *Ascidia sydneiensis samea*, expresses vacuolar-type H^+-ATPases (V-ATPases) on the vacuole membrane of the vanadium-containing blood cells known as vanadocytes. Previously, we showed that the contents of their vacuoles are extremely acidic and that a V-ATPase-specific inhibitor, bafilomycin A_1, neutralized the contents of the vacuoles. To understand the function of V-ATPase in vanadocytes, we isolated cDNA encoding subunit *C* of V-ATPase from vanadocytes since this subunit has been known to be responsible for the assembly of V-ATPases and to regulate the ATPase activity of V-ATPases. The cloned cDNA was 1,443 nucleotides in length, and encoded a putative 384 amino-acid protein. By expressing the ascidian cDNA for subunit *C* under the control of a galactose-inducible promoter, the pH-sensitive phenotype of the corresponding *vma5* mutant of a budding yeast was rescued. This result showed that the ascidian cDNA for subunit *C* functioned in yeast cells.

INTRODUCTION

Henze (1911) revealed that the blood cells of ascidians, which belong to the family Ascidiidae in the suborder Phlebobranchia, contain extremely high levels of vanadium ions. The homogenate of these blood cells is extremely acidic (Henze, 1911, 1912, 1913, 1932). We have reported a close correlation between the concentration of vanadium(III) ions and the pH within the vacuole (Michibata et al., 1991). Vacuoles of *Ascidia gemmata*, which contain the highest concentration of vanadium at 350 mM, corresponding to 10^7 times the vanadium level in sea water (Michibata and Kanamori, 1998), 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).

Previously, we identified subunits *A* and *B* of vacuolar-type ATPase (V-ATPase) expressed in the vanadocytes by immunological and molecular biological methods (Uyama et

al., 1994, Ueki et al., 1998). In addition, a specific inhibitor of V-ATPases, bafilomycin A_1, inhibits the proton pump in the vacuoles of the vanadocytes, resulting in neutralization of the contents of vacuoles (Uyama et al., 1994). Our preliminary studies showed that inhibition of V-ATPase causes 30 to 40% of the vanadium to leak from the vacuoles. Thus, one definite function of V-ATPases is to accumulate protons in the vanadocytes. The steep proton gradient is thought to be the energy source for accumulating vanadium ions against the steep gradient between the cytoplasm and inside the vacuole of the vanadocytes.

V-ATPases belong to a highly conserved family of proton pumps, which contribute to the acidity of various cellular organelles in eukaryotic cells (Nelson and Klionsky, 1996, Finbow and Harrison, 1997, Forgac, 1999a, b, Wieczorek et al., 1999). The overall structure of V-ATPases is highly related to that of F_0F_1-ATPases/synthases (F-ATPases). Like F-ATPases, V-ATPases are composed of catalytic and membrane sectors. The catalytic sector is composed of at least eight major subunits, *A* to *H* (Forgac, 1999a, b).

In this article, we report that a cDNA fragment encoding one of the peripheral subunits, subunit *C*, of V-ATPase was isolated from a vanadium-accumulating ascidian, *A. sydneiensis samea*, and transformation of an yeast *vma5* mutants with this cDNA rescued the pH-sensitive phenotype of the mutants.

MATERIALS AND METHODS

Yeast strains and media

Saccharomyces cerevisiae strain W303-1B (MATα, leu2, his3, ade2, trp1, ura3) and a mutant strain, W303-1B-Δ5 (MATα, *LEU::vatC*, his3, ade2, trp1, ura3), were kindly provided by Dr. N. Nelson (Beltran et al., 1992). Wild-type and mutant strains were grown in YPD medium containing 1% yeast extract, 2% polypeptone, and 2% D(+)-glucose at the appropriate pH. To induce expression of the introduced ascidian V-ATPase C-subunit gene, 2%

D(+)-galactose was used instead of D(+)-glucose. The medium was buffered by 50mM KH_2PO_4 and adjusted to pH 5.0 with KOH, or buffered with 50mM Na_2HPO_4 and adjusted to pH 7.5 with NaOH. Agar plates were prepared by the addition of 2% agar to medium at a given pH. After transformation, the yeast was grown on minimal plates containing 0.67% yeast nitrogen base, 2% glucose, 2% agar, and the appropriate amino acids.

PCR amplification of cDNA fragments of subunit *C* from an ascidian

Total RNA was isolated from the blood cells of *A. sydneiensis samea* as described previously (Uyama et al., 1998b). 10 μg of total RNAs is mixed with 1.5 μg of dT15 primer, denatured at 70°C for 5 min, and chilled quickly on ice. The reaction mixture is set up at 50 μl scale as follows: RNAs and primers shown above, 50mM Tris-HCl(pH8.3), 37.5mM KCl, 1.5mM $MgCl_2$, 10mM DTT, 50 pmole dNTPs, and 200 units M-MLV reverse transcriptase (Gibco BRL). The mixture is incubated at 37°C for 1 hour. 150 μl TE buffer (10mM Tris-HCl, 1mM EDTA, pH8.0) was added to the reaction mixture, and the resulting solution was used as a cDNA pool for the following PCR. Degenerate primers were designed from conserved regions of subunit *C* of V-ATPases. The forward primer was 5'- TGG (C/T)TN AT(A/C/T) (A/T)(C/G)N GCN CC -3' corresponding to the amino acid sequence WLISAP, and the reverse one was 5'- GC CAT TTC CCA (C/T)TG (A/G)AA -3' corresponding to FQWDMA. PCR-reaction medium contained: 5 μl of the cDNA pool, 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 94ºC for 30 sec, 37ºC for 60 sec, and 72ºC for 60 sec. This was followed by a final extension at 72ºC for 5 min. Amplified DNA fragments of the expected length (321 bp) were purified and cloned into pBluescript SK(–) vector (Stratagene).

Screening an ascidian cDNA library for subunit *C* cDNA

A cDNA library of *A. sydneiensis samea* blood cells has been constructed in Uni-Zap XR vector (Stratagene) (Uyama et al., 1998a). The cDNA library was screened with digoxigenin-labeled random-primed DNA probes derived from the cloned PCR fragments of subunit *C*, essentially as described previously (Ueki et al., 1998). Pre-hybridization, hybridization and stringent washes were done at 55ºC. 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 ALFexpress automated sequencer (Amersham Pharmacia Biotech).

Transformation of a yeast mutant strain with a plasmid expressing ascidian subunit *C*

The shuttle vector p416-GALL was purchased from American Type Culture Collection (ATCC). The cDNA fragment containing the entire coding region of ascidian subunit *C* was excised from cDNA clone #903 using *Bam*HI and *Xho*I. The cDNA fragment was ligated into the corresponding site of p416-GALL vector. The resulting plasmid was introduced into *E. coli* JM109 strain. The transformants were cloned and grown for small-scale isolation of the plasmid. Two micrograms of the plasmid were introduced into W303-1B-Δ5 mutant yeast strain by a lithium acetate method (Ito et al., 1983) and the cells were grown at pH 5.0 on a minimal plate containing alanine, histidine, and tryptophane. The transformed yeast clones, which were able to grow on the minimal medium, were used for the following experiments.

RT-PCR analysis of ascidian and yeast *vatc* gene expression

Yeast cells were grown in YPD medium (pH 7.5) containing 2% D(+)-galactose at 30°C until the stationary phase. The cells were collected by centrifugation, suspended in RNA extraction buffer (0.5 M NaCl, 0.2 M Tris-HCl, 10 mM EDTA, 1% SDS, pH 7.5), and RNA

was extracted using glass beads according to Elder et al. (1983). After DNaseI digestion, 5 μg of total RNA was mixed with 1.5 μg of oligo-dT15 primer, and reverse-transcribed by Superscript MMLV RTase (Gibco BRL). Specific primer sets for detecting ascidian and yeast *subunit C* gene transcripts were as follows: For yeast *vatC* transcripts, 5'-TGA TTG CAG AGG ATG CTG AG-3' and 5'-GGC CAA TCT TAC CAA CTG GA-3'; For ascidian *VATC* transcripts, 5'-TTG GAA CCC TGG ATT CGT TA-3' and 5'-CGA CCT TGG AGA GGA TTT CA-3'. For PCR, cDNA products corresponding to 100 ng of total RNA, 50 pmole each primer, 0.2 mM dNTP, 25 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$ were mixed in a reaction volume of 50 μl. Thirty cycles of PCR were run; each cycle consisted of 94°C for 30 sec, 50°C for 30 sec and 72°C for 30 sec. One fifth of the reaction products were separated on 3% agarose gels and stained with ethidium bromide.

RESULTS AND DISCUSSION

V-ATPase consists of two large complexes of subunits: the catalytic V_1 sector and the transmembrane V_0 sector. The catalytic V_1 sector is composed of at least eight different subunits, designed as subunits *A* to *H* (Forgac, 1999a, b). Of these, both genetic analysis (Beltran et al., 1992) and biochemical studies (Peng et al., 1993, Xie, 1996, Zhou et al., 1998) have shown that subunit *C* plays an important role in the regulation of V-ATPase activity. Subunit *C* appears to be located on the outside of the catalytic V_1 sector. Subunits *C*, *D*, and *E* can all be crosslinked to the *c* subunits of the membrane V_0 sector (Puopolo et al., 1992), suggesting that they play a role in the attachment and coupling of the V_1 and V_0 sectors.

Therefore, in this study, we first amplified 321-bp partial cDNA fragments of V-ATPase subunit *C* from a cDNA pool constructed from whole blood cells of the ascidian *A. sydneiensis samea*. Two positive clones were isolated, and revealed to contain almost identical DNA sequences. Within the amplified region, the ascidian and bovine amino acid

sequences were identical at 53 residues out of 91 (data not shown). Using one of the PCR fragments as a probe, we isolated two cDNA clones for subunit *C* out of 6×10^4 phages from the *A. sydneiensis samea* blood cell cDNA library. We designated the corresponding gene as the ascidian *VATC* gene. The two cDNA clones had almost identical nucleotide sequences (data not shown).

The ascidian *VATC* cDNA was composed of 1,443 nucleotides, including a 24-bp poly (A) tail. The cDNA contained a single, long ORF of 1,155 nucleotides including the termination codon. It encoded a protein of 384 amino acids. The calculated molecular mass of the predicted protein was 44.1 kDa. The deduced amino acid sequence showed a striking similarity to the known subunit *C* of V-ATPases from various organisms, including yeast (Beltran et al., 1992), mammals (Nelson et al., 1990, van Hille et al., 1993), plants (Viereck et al., 1996), insects (McSwain et al., 1997), and slime molds (SWISS-PROT, P54648). The ascidian subunit *C* cDNA encoded a protein closely related to mammalian subunit *C*, and less closely related to its counterparts in the other organisms. There was 69.8% amino acid identity between the ascidian and bovine sequences and 46.8% identity between the ascidian and yeast sequences.

Yeast provides the best-known system for functional studies of V-ATPase subunits. One can readily interrupt a gene encoding an individual subunit in yeast and try to complement the phenotype by expressing cDNA encoding a specific V-ATPase subunit. A chimeric yeast/bovine gene encoding Vma5p/vma5 complemented the phenotype of an interrupted mutation for the subunit *C* gene (Beltran et al., 1992).

To determine whether the ascidian *VATC* transcripts are functional, we constructed a plasmid containing the entire cDNA sequence of the ascidian *VATC* gene under the control of a galactose-inducible promoter on the URA3-bearing shuttle vector. The plasmid was introduced into a yeast W303-1B-Δ5 mutant strain that completely lacks an endogenous *vatC* gene (Beltran et al., 1992) and was selected on minimal plates lacking uracil.

Due to the lack of a functional subunit *C* of V-ATPase, the W303-1B-Δ5 mutants could not grow at pH 7.5, but could grow at pH 5.0 (Fig. 1). The wild-type strain could grow at either pH 7.5 or pH 5.0. The transformants bearing the ascidian *VATC* gene were able to grow at pH 7.5 when gene expression was induced by the addition of D(+)-galactose (Fig. 1) . Without induction with D(+)-galactose, the transformants could not grow at pH 7.5 (data not shown).

To confirm the expression of the ascidian and yeast *VATC/vatC* genes, we performed an RT-PCR analysis with specific primer sets for *VATC/vatC* transcripts of each organism. As shown in Fig. 2, the wild-type yeast strain expressed yeast *vatC* gene transcripts, while the W303-1B-Δ5 mutants did not express any *vatC* transcripts. In the transformants bearing a plasmid expressing the ascidian *VATC* gene, only ascidian *VATC* gene transcripts were detected. Thus, the transformants recovered the ability to grow at pH 7.5 by expressing the ascidian *VATC* gene.

To examine the pH within the cell, we allowed the transformed yeast cells to uptake a pH indicating fluorescent reagent, acridine orange. The color of fluorescence excited by blue-violet light was green within the vacuole of transformed yeast cell bearing a plasmid expressing the ascidian *VATC* gene, suggesting that the pH within the vacuole of transformed cells is neutral. In addition, the transformed cells could grow on medium at pH 7.5, but their growth rate was slightly lower than that of wild-type yeast cells (data not shown). Therefore, we concluded that the proton pumping activity of the chimeric V-ATPase complex is lower than that of the wild type.

We cannot exclude the possibility that the introduced ascidian subunit *C* was not fully functional because its transcription, translation, or incorporation into the yeast V-ATPase complex was not efficient. By using a specific antibody against V-ATPase C-subunit, we hope to examine the expression and incorporation of ascidian subunit *C* proteins. Analysis of proton pumping activity is also necessary. We are now extending the analysis to other

subunits of ascidian V-ATPase to identify the subunit(s) responsible for the unusual phenomenon.

ACKNOWLEDGMENTS

We would like to thank Mr. T. Morita and all the staff at the Otsuchi Marine Center, Otsuchi, Iwate, Japan, for their help in collecting adult ascidians. Thanks are also due to Mr. N. Abo in our laboratory, who collected some of the animals and kept them healthy in an aquarium. This work was supported in part by a Sasakawa Scientific Research Grant (12-364M) and Grants-in-Aid from the Ministry of Education, Science, and Culture of Japan (#11440244 and #11559014). The nucleotide and amino acid sequence reported in this paper has been registered in GenBank/DDBJ/EMBL with the accession number AB035809.

REFERENCES

Beltran, C., Kopecky, J., Pan, Y.-C. E., Nelson, H., and Nelson, N. (1992). Cloning and mutational analysis of the gene encoding subunit C of yeast vacuolar H^+-ATPase. *J. Biol. Chem.,* **267,** 774-779.

Elder, R. T., Loh, E. Y., and Davis, R. W. (1983). RNA from the yeast transposable element Ty1 has both ends in the direct repeats, a structure similar to retrovirus RNA. *Proc. Natl. Acad. Sci. U S A,* **80,** 2432-2436.

Finbow, M. E., and Harrison, M. A. (1997). The vacuolar H^+-ATPase: a universal proton

pump of eukaryotes. *Biochem. J.*, **324,** 697-712.

Forgac, M. (1999a). Structure and properties of the vacuolar H^+-ATPases. *J. Biol. Chem.*, **274,** 12951-12954.

Forgac, M. (1999b). Structure, function and regulation of the vacuolar H^+-ATPases. *FEBS Letters*, **440,** 258-263.

Henze, M. (1911). Untersuchungen über das Blut der Ascidien. I. Mitteilung. Die Vanadiumverbindung der Blutkörperchen. *Hoppe-Seyler's Z. Physiol. Chem.*, **72,** 494-501.

Henze, M. (1912). Untersuchungen über das Blut der Ascidien. II. Mitteilung. *Hoppe-Seyler's Z. Physiol. Chem.*, **79,** 215-228.

Henze, M. (1913). Über das Vorkommen freier Schwefelsäure im Mantel von *Ascidia mentula*. *Hoppe-Seyler's Z. Physiol. Chem.*, **88,** 345-346.

Henze, M. (1932). Über das Vanadiumchromogen des Ascidienblutes. *Hoppe-Seyler's Z. Physiol. Chem.*, **213,** 125-135.

Ito, H., Fukada, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.*, **153,** 163-168.

McSwain, J. L., Luo, C., deSilva, G. A., Palmer, M. J., Tucker, J. S., Sauer, J. R., and Essenberg, R. C. (1997). Cloning and sequence of a gene for a homologue of the C subunit of the V-ATPase from the salivary gland of the tick *Amblyomma americanum* (L). *Insect Mol.*

Biol., **6,** 67-76.

Michibata, H., Iwata, Y., and 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., and Kanamori, K. (1998) Selective accumulation of vanadium by ascidians from sea water. In *Vnadiums in the Environment. Part 1: Chemistry and Biochemistry* (Ed., Nriagu, J. O.), John Wiley & Sons, Inc., 217-249.

Nelson, H., Mandiyan, S., Noumi, T., Moriyama, Y., Miedel, M. C., and Nelson, N. (1990). Molecular cloning of cDNA encoding the C subunit of H^+-ATPase from bovine chromaffin granules. *J. Biol. Chem.,* **265,** 20390-20393.

Nelson, N., and Klionsky, D. J. (1996). Vacuolar H^+-ATPase: from mammals to yeast and back. *Experientia,* **52,** 1101-1110.

Peng, S. B., Stone, D. K., and Xie, X. S. (1993). Reconstitution of recombinant 40-kDa subunit of the clathrin-coated vesicle H^+-ATPase. *J. Biol. Chem.*, **268,** 23519-23523.

Puopolo, K., Sczekan, M., Magner, R., and Forgac, M. (1992). The 40-kDa subunit enhances but is not required for activity of the coated vesicle proton pump. *J. Biol. Chem.*, **267,** 5171-5176.

Ueki, T., Uyama, T., Kanamori, K., and 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.

Uyama, T., Moriyama, Y., Futai, M., and 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., Suhama, Y., Kanamori, K., and Michibata, H. (1998a). 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., Kinoshita, T., Takahashi, H., Satoh, N. Kanamori, K., and Michibata, H. (1998b). 6-Phosphogluconate dehydrogenase is a 45-kDa antigen recognized by S4D5, a monoclonal antibody specific to vanadocytes in the vanadium-rich ascidian, *Ascidia sydneiensis samea*. *J. Biochem.*, **124**, 377-382.

van Hille, B., Vanek, M., Richener, H., Green, J. R., and Bilbe, G. (1993). Cloning and tissue distribution of subunits C, D, and E of the human vacuolar H^+-ATPase. *Biochem. Biophys. Res. Commun.*, **197,** 15-21.

Viereck, R., Kirsch, M., Loew, R., and Rausch, T. (1996). Down-regulation of plant V-type H^+-ATPase genes after light-induced inhibition of growth. *FEBS Lett.*, **384,** 285-288.

Wieczorek, H., Grüber, G., Harvey, W. R., Huss, M., and Merzendorfer, H. (1999). The plasma membrane H^+-ATPase from tobacco hornworm midgut. *J. Bioenerg. Biomem.*, **31,** 67-74.

Xie, X. S. (1996). Reconstitution of ATPase activity from individual subunits of the clathrin-coated vesicle proton pump. The requirement and effect of three small subunits. *J. Biol. Chem.,* **271,** 30980-30985.

Zhou, Z., Peng, S. B., Crider, B. P., Slaughter, C., Xie, X. S., and Stone, D. K. (1998). Molecular characterization of the 50- and 57-kDa subunits of the bovine vacuolar proton pump. *J. Biol. Chem.,* **273,** 5878-5884.

FIGURE LEGENDS

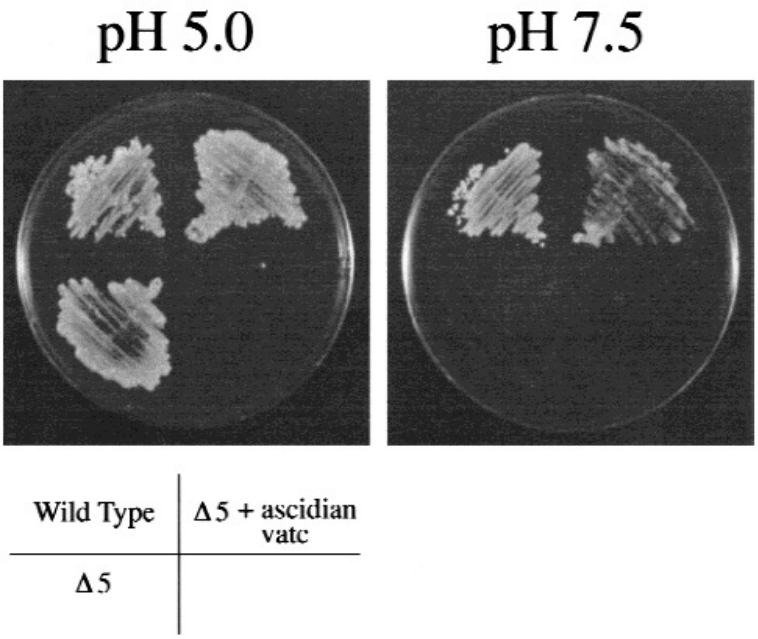

Figure 1. Rescue of the yeast subunit *C* mutants by expression of the ascidian *VATC* gene. The wild-type strain, W303-1B-Δ5 mutant strain (Δ5), and W303-1B-Δ5 strains transformed with a plasmid expressing ascidian subunit *C* were cultured on YPD plates at the indicated pH. The wild-type strain could grow at low and neutral pH, but the Δ5 mutant could only grow at low pH. The Δ5 mutants that expressed the ascidian subunit *C* gene recovered the ability to grow at neutral pH.

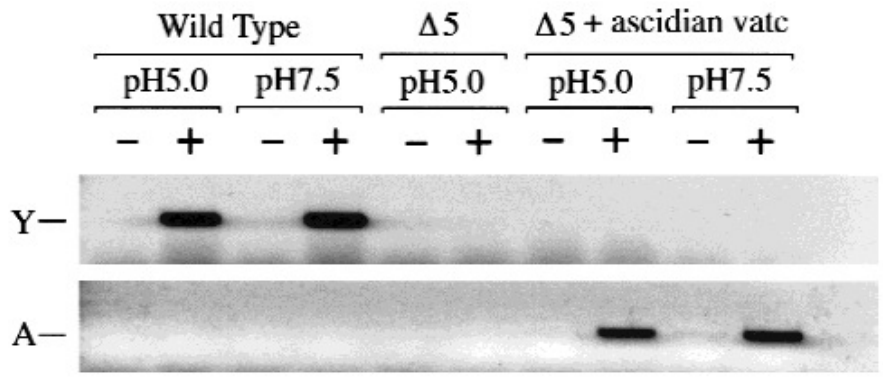

Figure 2. Expression of yeast and ascidian subunit *C* mRNAs detected by RT-PCR using specific primer sets. Total RNA was extracted from the wild-type strain, W303-1B-Δ5 mutant strain (Δ5), and W303-1B-Δ5 strains transformed with a plasmid expressing ascidian subunit *C* cultured in YPD liquid media at the indicated pH. The Δ5 mutants cannot grow at pH 7.5. After reverse-transcription, PCR reactions were done with specific primer sets for yeast or ascidian subunit *C* gene transcripts (+). As a negative control, reverse-transcription was omitted (–). In the wild-type strain, transcripts of yeast subunit *C* gene were detected (Y). In the Δ5 mutant strain, no transcripts were detected. In the transformed strains, ascidian-specific PCR products were detected (A), indicating the expression of the transformed ascidian gene.