Chloride Channel in Vanadocytes of a Vanadium-Rich Ascidian *Ascidia sydneiensis samea*

Tatsuya Ueki, Nobuo Yamaguchi, and Hitoshi Michibata*

Marine Biological Laboratory, Graduate School of Science, Hiroshima University, Mukaishima-cho 2445, Hiroshima 722-0073, Japan

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* Corresponding author.
Tel: +81-848-44-1143, Fax: +81-848-44-5914, e-mail: hmichi@sci.hiroshima-u.ac.jp

Abstract

Ascidians, so-called sea squirts, can accumulate high levels of vanadium in the vacuoles of signet ring cells, which are one type of ascidian blood cell and are also called vanadocytes. In addition to containing high concentrations of vanadium in the +3 oxidation state, the proton concentrations in vanadocyte vacuoles are extremely high. In order to elucidate the entire mechanism of the accumulation and reduction of vanadium by ascidian vanadocytes, it is necessary to clarify the participation of anions, which might be involved as counter ions in the active accumulation of both vanadium and protons. We examined the chloride channel, since chloride ions are necessary for the acidification of intracellular vesicles and coexist with H^+-ATPase. We cloned cDNA encoding a chloride channel from blood cells of a vanadium-rich ascidian, *Ascidia sydneiensis samea*. It encoded a 787-amino-acid protein, which showed striking similarity to mammalian ClC3/4/5-type chloride channels. Using a whole-mount in situ hybridization method that we developed for ascidian blood cells, the chloride channel was revealed to be transcribed in vanadocytes, suggesting its participation in the process of vanadium accumulation.

Keywords: Ascidian, Blood Cells, Chloride Channel, CLC-type, Gene Expression, H^+-ATPase, Metal Accumulation, Vanadium.

1. Introduction

Henze's discovery of high levels of vanadium in the blood (coelomic) cells of an ascidian (so-called seasquirt) collected from the Bay of Naples (Henze, 1911) has attracted the interdisciplinary attention of chemists, physiologists, and biochemists (Michibata et al., 2003). The highest concentration of vanadium, 350 mM, found in the blood cells of *Ascidia gemmata*, is 10^7 times higher than that in seawater; such unusual accumulations have never been reported in organisms other than ascidians (Michibata et al., 1991). The vanadium accumulated in ascidian blood cells is reduced to V(III) via V(IV) and is stored in the vacuoles of vanadocytes (vanadium-containing blood cells), which are called signet ring cells morphologically and are one of the 10 types of ascidian blood cell (Michibata et al., 1987, Hirata and Michibata, 1991). Recently, vanadium localized in living vanadocytes was visualized without fixation, freezing, or staining, using a scanning x-ray microscope installed at the ID21 beamline of the European Synchrotron Radiation Facility (Ueki et al., 2002). The contents of the vanadocyte vacuole are maintained at an extremely low pH of 1.9 by vacuolar-type H^+-ATPase (V-ATPase) (Uyama et al., 1994). Therefore, vanadocytes must hold the key to resolving the unusual mechanism.

From vanadocytes, we have already isolated vanadium-binding proteins (Kanda et al., 1997, Ueki et al., 2003), designated vanabins, and cloned several enzymes related to the pentose phosphate pathway, which produces NADPH, a reducing agent involved in one step of the reduction of V(V) to V(IV) (Uyama et al., 1998a, 1998b, 1998c, Ueki et al., 2000, Kanamori et al., 1999). Furthermore, we have cloned and analyzed cDNAs for the *A*, *B*, and *C* subunits of V-ATPase, which are located on the vacuolar membranes of vanadocytes (Ueki et al., 1998, Ueki et al., 2001). V-ATPase generates a proton-motive force, and is thought to provide the energy for vanadium accumulation.

To elucidate the entire mechanism of accumulation and reduction of vanadium by ascidian vanadocytes, however, it is necessary to clarify the roles of anions in the mechanism, since anions might be involved as counter ions in the active accumulation of both vanadium and protons. As the first step, we cloned a cDNA encoding a chloride channel localized in vanadocytes. We examined this chloride channel because chloride

ions are reported to be necessary for the acidification of intracellular vesicles, and they coexist with V-ATPase (Chuang et al., 1999, al-Awqati, 1995, Günther et al., 1998, Luyckx et al., 1998, Sakamoto et al., 1999).

First, we isolated a cDNA containing the conserved region of the chloride channel of the ClC3/4/5 family from an *Ascidia sydneiensis samea* blood cell cDNA library, and cloned a nearly full-length cDNA from blood cell cDNA library. The deduced amino acid sequence showed striking similarity to ClC3/4/5 chloride channel genes. The chloride channel gene was transcribed in vanadocytes (signet ring cells), suggesting the participation of the chloride channel in the process of vanadium accumulation.

2. Materials and Methods

2.1. Cloning chloride channel cDNA from Ascidia sydneiensis samea

We designed the following three degenerate primers from the conserved regions of known ClC-type chloride channel genes: ACLCF: 5'-ATA GGN AAR GAR GGN CC-3', BCLCF: 5'-GGT ACW GGN ATH CCN GA-3', and ACLCR: 5'-TCC GAT NGG NGC NCC RAA-3'. Primers ACLCF and BCLCF corresponded to the amino acid sequences Gly-Lys-Glu-Gly-Pro and Gly-Ser-Gly-Ile-Pro-Glu, respectively, which are common to all types of human ClC channel. The amino acid sequence Phe-Gly-Ala-Pro-Ile-Gly of primer ACLCR is common to human ClC3-, ClC4-, and ClC5-type chloride channels.

Phage DNA was isolated from a cDNA library of *A. sydneiensis samea* blood cells (Uyama et al., 1998a). The first-round PCR reaction was set up as follows: 500 ng of λphage DNA, 200 pmols each of primers BCLCF and ACLCR, 0.2 mM of each dNTP, 1× reaction buffer, and 2.5 units of *Taq* DNA polymerase (TaKaRa, Inc.). The reaction volume was 50 μl. After denaturation at 94°C for 1.5 min, 30 cycles of PCR were done (94°C for 20 sec, 50°C for 40 sec, and 72°C for 40 sec) followed by a final extension at 72°C for 4.5 min. After the reaction, 1 μl of the first-round reaction mixture was taken as the template for the second-round reaction. The second-round reaction used the same amounts of primers ACLCF and ACLCR as the first reaction, but each PCR cycle was 94°C for 15 sec, 50°C for 15 sec, and 72°C for 15 sec. The PCR products were separated by gel electrophoresis using 3% LMP agarose in TAE buffer. The band of the expected

size was excised and cloned into pBluescript vector and its nucleotide sequence was determined by the dideoxy method using an ALFexpress DNA sequencer and ThermoSequenase kit (Amersham Pharmacia Biotech, Ltd.).

The cDNA fragment was excised from the vector and labeled with alkaline phosphatase using an AlkPhos Direct Labeling kit (Amersham Pharmacia). The cDNA library of *A. sydneiensis samea* blood cells mentioned above was screened with the probe. Hybridization essentially followed the manufacturer's protocol. Positive phages were re-screened until they were cloned. The cDNAs were excised in vivo, subcloned into pBluescript vector, and sequenced. Putative transmenbrane domains were predicted by HMMTOP2.0 method (Tusnády, 1998) using the deduced amino acid sequence and by comparing it with CLC-3/4/5 chloride channels.

2.2. In situ hybridization on Ascidia sydneiensis samea blood cells

Blood cells were extracted from adult *A. sydneiensis samea* and suspended in artificial seawater (460 mM NaCl, 9 mM KCl, 32 mM Na_2SO_4, 5 mM HEPES, 6 mM $NaHCO_3$, 1 mM EDTA). After centrifugation at 300 × g for 10 min at 4°C, giant cells were removed and the resulting blood cells were re-suspended in an appropriate volume of artificial seawater. One drop of blood cell suspension was put on a coverslip coated with 3-aminopropyltriethoxysilane (Sigma Chemical Co.). After incubation at RT for 15 min, the blood cells became attached to the coverslip. Then, the coverslip was dipped in a fixative (4% paraformaldehyde in high-salt PBS; hsPBS: 450 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4) on ice for 4 hr, and washed twice in hsPBT (0.05% Tween20 in hsPBS) for 10 min and twice in hsPBS for 10 min.

The coverslip was incubated in a pre-hybridization buffer (12 × SSC, 200 μg/ml yeast RNA, 5 × Denhardt's solution, 0.05% Tween20) at 37°C for 1 hr. Digoxigenin-labeled RNA probes were prepared using T3 or T7 RNA polymerase and digoxigenin-11-dUTP (Roche Diagnostics) from a plasmid containing a cDNA fragment (nucleotide 1 to 683) of the chloride channel. Probes were denatured at 70°C for 10 min and added to the pre-hybridization buffer at a concentration of 1 μg/ml. After incubation for 15 hr at 37°C, the coverslip was washed serially in 4 × SSC with 0.05% Tween20 at 37°C for 20 min, 2 × SSC with 0.05% Tween20 at 37°C for 20 min, 1 × SSC with 0.05% Tween20 at 37°C for 20 min, PBT at RT for 10 min, and PBS at RT for 10 min. The

coverslip was incubated in 10% blocking reagent (Roche) in PBS at RT for 2 hr, and then in 1:1500 diluted rhodamine-conjugated anti-digoxigenin antibody (Roche) at RT for 1 hr. The coverslip was washed three times in PBT at RT for 10 min and twice in PBS at RT for 10 min, and observed by fluorescent microscopy.

3. Results

3.1. Cloning an ascidian chloride channel homologue

In order to identify the chloride channel gene expressed in the vanadocytes of a vanadium-rich ascidian, *A. sydneiensis samea*, we first isolated a cDNA fragment containing a conserved region of the ClC3/4/5 family chloride channel from an *A. sydneiensis samea* blood cell cDNA library. The amplified fragment was 156 bp, as expected. The deduced amino acid sequence, G-K-E-G-P-L-V-H-I-S-A-C-C-G-N-V-F-S-A-L-F-P-K-Y-Y-S-N-E-A-K-K-R-E-M-L-S-A-A-A-A-A-G-V-S-V-A-F-G-A-P-I-G, showed striking similarity to that of ClC3/4/5 chloride channel genes. The amino acid sequence of the ascidian cDNA fragment was 82.7, 78.8, and 75.0% identical to the corresponding regions of the human ClC3, ClC4, and ClC5 chloride channel proteins, respectively, but the difference of identity was not significant to clarify which type the ascidian cDNA belongs to.

Fig. 1

Using this fragment as a probe, we screened 60,000 phages of the cDNA library and obtained two positive clones. The two phage clones contained >99% identical DNA sequences for their over wrapping region, and the difference was thought to be due to polymorphism. The longer cDNA clone was chosen as a representative one (Fig. 1). This cDNA encoded a protein of 787 amino acids, which showed 53, 54, and 53% identity to the human ClC3, 4, and 5 chloride channel proteins, respectively. The four separate regions that compose the chloride ion-binding site of the *E. coli* ClC chloride channel (Dutzler et al., 2002), which are conserved in ClC-type chloride channels, were also found in the predicted sequence (Fig. 1). The deduced amino acid sequence of this chloride channel has 31, 53, 54, 53, and 28% identity with human ClC2, ClC3, ClC4, ClC5, and ClC7, respectively. Furthermore, the sequence has 28, 22, and 23% identity with *Saccharomyces cerevisiae* GEF1, *Escherichia coli* ClC, and *Methanococcus* ClC, respectively. Since the deduced amino acid sequence clearly clustered with

ClC3/4/5-type chloride channels in a molecular phylogenetic analysis, we determined that this cDNA was an ascidian ClC3/4/5 homologue (Fig. 2).

Fig. 2

3.2. Expression of the chloride channel in signet ring cells

To ascertain whether signet ring cells express this gene, we developed a method of whole mount in situ hybridization using *A. sydneiensis samea* blood cells. Blood cells were fixed in paraformaldehyde and hybridized with digoxigenin-labeled RNA probes derived from the cDNA for the chloride channel gene. The signals were detected using anti-digoxigenin antibodies conjugated with rhodamine. As seen in Fig. 3, the signet ring cells were stained with antisense probes, but not with sense probes, indicating that the chloride channel gene was transcribed in signet ring cells. The other major types of cells, such as morula, compartment, and pigment cells, fluoresced similarly with antisense and sense probes.

4. Discussion

In this study, we first cloned a cDNA encoding the CLC3/4/5 type chloride channel gene in blood cells of the vanadium-rich ascidian, *A. sydneiensis samea*, and demonstrated that the chloride channel is present in vanadocytes. We initially performed PCR reactions using several combinations of forward and reverse primers for ClC-type chloride channels. We only succeeded in amplifying a ClC3/4/5-type chloride channel homologue from the blood cell cDNA library using the primer sets described in the Materials and Methods.

Fig. 3

Four major structurally unrelated types of chloride channel have been identified in other organisms: the cystic fibrosis transmembrane conductance regulator, ligand-gated receptor channels, the ClC (chloride channel) family (ClC-1 to -7, ClCK, and ClCL) and the CLIC (chloride intracellular channel) family (CLIC-1 to -5). The number of ClC orthologues varies for each organism. The *E. coli* and *S. cerevisiae* genome projects have shown that each species possesses a single ClC-type chloride channel gene. In contrast, mammals possess many orthologues, as well as other types of chloride channel. For example, *Homo sapiens* has at least nine members of the ClC family and five members of the CLIC family chloride channels. BLAST search against EST database of another ascidian *Ciona intestinalis* (http://ghost.zool.kyoto-u.ac.jp/indexr1.html) using amino

acid sequences for *A. sydneiensis samea* and human CLC-type chloride channels revealed that at least four EST clusters encode CLC-type chloride channels (CLC-2, -3/4/5, -6 and -7) (data not shown).

Chloride channels play important roles in maintaining cell volume, transepithelial transport, setting the membrane potential, bone resorption, and the response to certain neurotransmitters (Jentsch and Günther, 1997, Dutzler et al., 2002). It is well known that the disease cystic fibrosis results from mutations in the cystic fibrosis transmembrane conductance regulator, which forms a chloride channel expressed in the epithelial tissues of many organs (Akabas, 2000), and that Dent's disease, an inherited disorder characterized by hypercalciuria, nephrolithiasis, nephrocalcinosis, rickets, low-molecular-weight proteinuria, Fanconi's syndrome, and renal failure, is caused by mutations in the renal ClC5 chloride channel (Wrong et al., 1994). These chloride channels localize to either plasma membranes or intracellular membranes. Chloride channels present in the latter membranes play important roles in the acidification of intracellular compartments and in exocytosis. Al-Awqati (al-Awqati, 1995) first reported that open Cl^- channels favor the accumulation of H^+ in the Golgi apparatus and some endosomes. Günther et al. demonstrated that ClC5 colocalizes with V-ATPase in endocytic vesicles (Günther et al., 1998). Luyckx reported that the localization of ClC5 in the endosomal compartment of the renal tubule segment is consistent with a role in providing counter-ion conductance for electrogenic V-ATPase (Luyckx et al., 1998). Sakamoto et al. reported that ClC-5 likely works cooperatively with a H^+ pump as an essential element in the acidification of endosomes and proton secretion into the lumen (Sakamoto et al., 1999).

In ascidian vanadocytes, the contents of vacuoles are extremely acidic and there is a strong inverse correlation between pH and vanadium level; for example, *A. gemmata* vacuoles contain the highest concentration of vanadium (350 mM) and have the lowest pH (1.86); *A. ahodori* vacuoles contain 60 mM vanadium and have a pH of 2.67, and those of *A. sydneiensis samea* contain 13 mM vanadium and have a pH of 4.20 (Michibata et al., 1991). Furthermore, we have shown that V-ATPase is localized in the vacuolar membrane of vanadocytes, and a specific inhibitor of V-ATPase, bafilomycin A_1, neutralized the contents of the vacuoles, suggesting that the extreme acidity of the contents is maintained by the V-ATPase (Uyama et al., 1994). Since such extremely low

pH values have never been reported in organisms other than ascidian species, it is a matter of primary concern whether the chloride channel coexists in ascidian vanadocytes. As shown in Fig. 3, our experiment revealed that the chloride channel gene was transcribed in vanadocytes, suggesting that the chloride channel plays a role in providing counter-ion conductance for the electrogenic V-ATPase. We have been developing a yeast cell system that expresses ascidian V-ATPase (Ueki et al., 2001), and the development of an expression system for both the chloride channel and V-ATPase is expected to clarify the participation of the chloride channel in the unusual acidification of the vacuoles of vanadocytes.

The primary structure of ClC-type chloride channels is well conserved among eukaryotes and prokaryotes, from humans to bacteria, throughout their length. So far three-dimensional structures of ClC chloride channels have been determined by X-ray crystallography in two prokaryotes: *E. coli* and *S. enterica* (Dutzler et al., 2002). They contain 18 α-helices and exhibit an antiparallel pseudo-two-fold symmetry. The overall hydrophobicity profile and sequences of the putative transmembrane helices of the ascidian chloride channel are similar to those of ClC chloride channels in other organisms, including *E. coli* , and the four separate regions that compose the ion-binding site were completely conserved (Fig. 1), suggesting that the ascidian chloride channel has an analogous structure and functions in a similar manner.

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Figure Legends

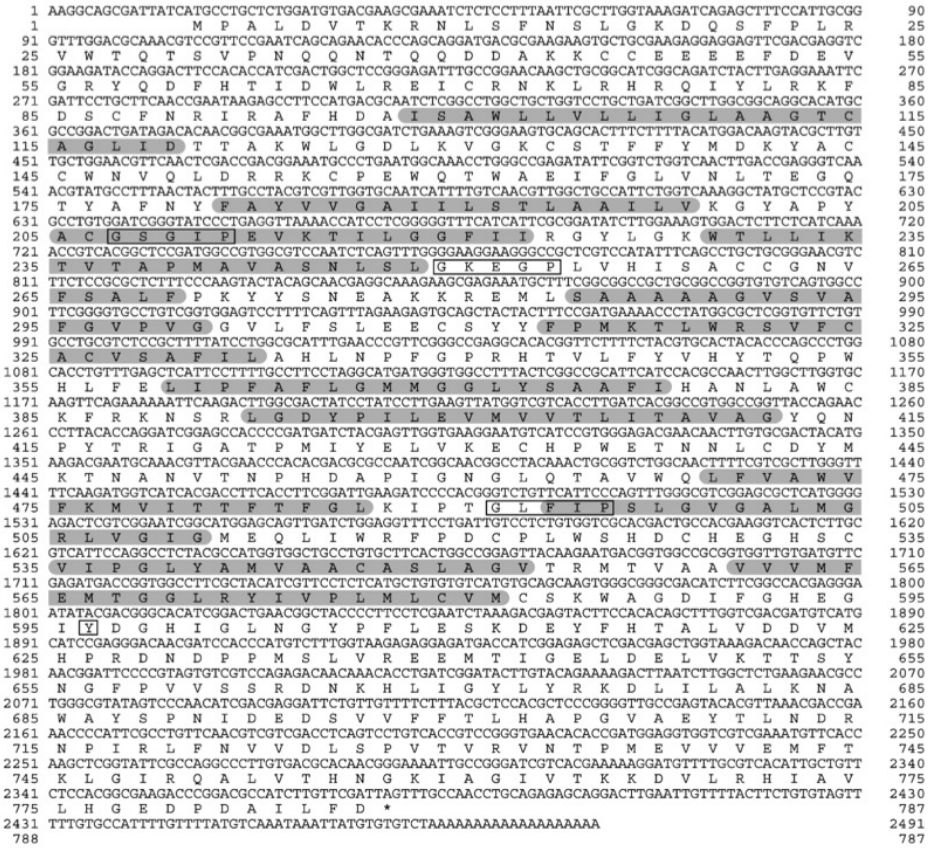

Fig. 1. Nucleotide and amino acid sequences of the cDNA for the ClC3/4/5-type chloride channel gene of *A. sydneiensis samea*. The deduced amino acid sequence is shown using one-letter abbreviations below the corresponding nucleotide sequence. The four conserved regions that make-up the chloride-ion binding site are boxed. Twelve putative transmembrane domains are shaded. The nucleotide and amino acid sequences reported in this paper have been entered in GenBank/DDBJ/EMBL under accession number AB083819.

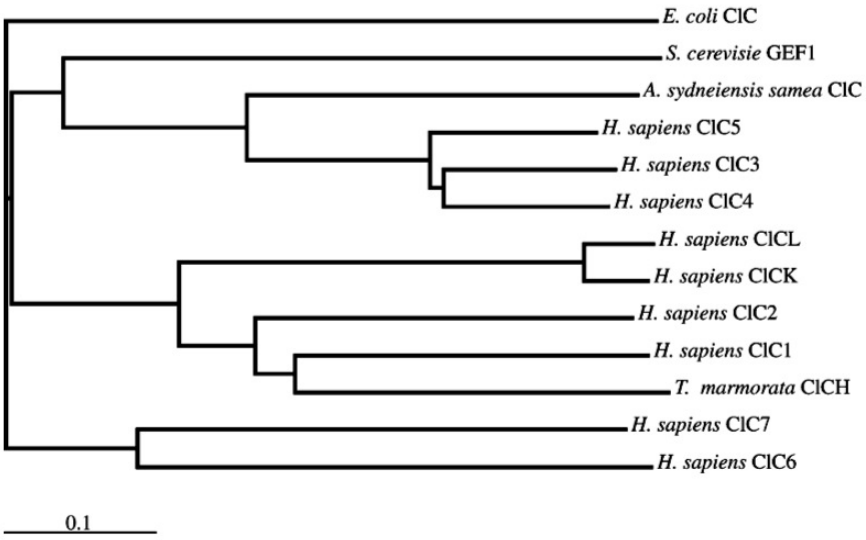

Fig. 2. Molecular phylogenetic analysis of the amino acid sequences of chloride channel genes using the neighbor-joining method (Saitou and Nei, 1987). Sequences were retrieved from the SwissProt Protein Database under the following accession numbers: *Escherichia coli* ClC (P37019), *Saccharomyces cerevisiae* GEF1 (P37020), *Torpedo marmorata* ClCH (P21564), *Homo sapiens* ClC1 (P35523), ClC2 (P51788), ClC3 (P51790), ClC4 (P51793), ClC5 (P51795), ClC6 (P51797), ClC7 (P51798), ClCK (P51800), and ClCL (P51801). The *A. sydneiensis samea* ClC-type chloride channel protein sequence was aligned with them, and the distances among sequences were calculated using ClustalW software (Higgins et al., 1992). Note that the ascidian chloride channel gene clearly clusters with mammalian ClC3/4/5-type chloride channel genes.

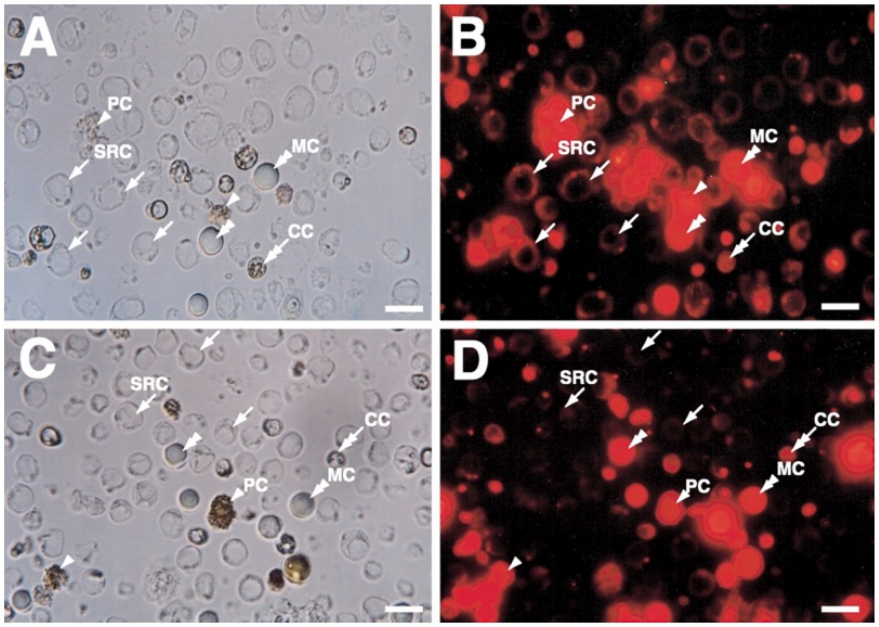

Fig. 3. Whole mount in situ hybridization of the ascidian ClC3/4/5-type chloride channel gene on *A. sydneiensis samea* blood cells. The blood cells were hybridized with antisense (A and B) and sense (C and D) digoxigenin-labeled RNA probes. The probes were detected using anti-digoxigenin antibodies conjugated with rhodamine and observed under a Nomarski optical microscope (A and C) or a fluorescence microscope (B and D). Note that signet ring cells (SRC) are stained with antisense probes (B), but not with sense probes (D), indicating that the chloride channel gene is expressed in signet ring cells. Since morula cells (MC), compartment cells (CC), and pigment cells (PC) are stained with both antisense and sense probes, it is unclear whether these types of cell express this gene. Scale bars: 10 μm.