Cloning of rainbow trout SLC26A1: involvement in renal sulfate secretion

Fumi Katoh¹*, Martin Tresguerres¹, Kyung Mi Lee², Toyoji Kaneko², Katsumi Aida², Greg G. Goss¹

¹Dept of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada
²Dept of Aquatic Biosciences, Graduate School of Agricultural and Life Sciences, University of Tokyo, Bunkyo, Tokyo, Japan

*Author for correspondence: Fumi Katoh
Address: Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9
e-mail: fkatoh@ualberta.ca
Tel: 780-492-1288
Fax: 780-492-9234

Running head: SLC26A1 anion exchanger in rainbow trout kidney
ABSTRACT

The kidney plays an important role in ion regulation in both freshwater and seawater fish. However, ion transport mechanisms in the teleost kidney are poorly understood, especially at the molecular level. We have cloned a kidney specific SLC26 sulfate/anion exchanger from rainbow trout (*Oncorhynchus mykiss*) that is homologous to the mammalian SLC26A1 (Sat-1). Excretion of excess plasma sulfate concentration after Na$_2$SO$_4$ injection corresponded to significantly higher expression of the cloned SLC26A1 mRNA. Detailed morphological observation of rainbow trout renal tubules was also performed by light microscopy and transmission electron microscopy. According to the structure of brush border and tubular system in the cytoplasm, renal tubules of rainbow trout were classified into proximal tubule first and second (PI and PII) segments, and distal tubules. In situ hybridization revealed that SLC26A1 anion exchanger mRNA is specifically localized in the PI segment of kidneys from both seawater and freshwater adapted rainbow trout. Using immunocytochemistry, Na$^+$, K$^+$-ATPase and vacuolar type H$^+$-ATPase (V-ATPase) were co-localized to the same cells and distributed in the basolateral and the apical membranes, respectively, of the cells where the SLC26A1 mRNA expressed. These findings suggest that the cloned kidney-specific SLC26A1 is located in kidney proximal tubules and is involved in excretion of excess plasma sulfate in rainbow trout.

Keywords

ion regulation, rainbow trout, kidney, SLC26A1, sulfate/anion exchanger
INTRODUCTION

Plasma ion regulation in teleosts fish is achieved by integrated ion and water transport of the gills, kidney, and intestine (8, 9). The kidney plays an important role in ion regulation in both freshwater and seawater teleosts, although its function is entirely different under the two diametrical environments. Freshwater teleosts face water loading and ion loss through their permeable body surface, and accordingly, the primary kidney function is excretion of excess water and reabsorption of the filtered solutes. Seawater fish, by contrast, gain excess ions, lose water to their surrounding environment, and consequently ingest seawater to prevent dehydration. The sulfate (SO$_4^{2-}$) concentration of normal seawater is around 25 mM, and as a result of water absorption, the intestine generates a high sulfate concentration in the intestinal lumen (50-100 mM). It has been reported that kidneys of marine teleosts primarily excrete the excess plasma sulfate (2, 3, 6, 7, 32, 36, 38, 42, 43, 44, 45) which is actively and passively absorbed across intestinal mucosa (36, 37). Net renal sulfate secretion in marine teleosts was first shown by Renfro and Pritchard (44). Ion transport function in kidney, analyzed using isolated renal tubules, has demonstrated sulfate excretion in proximal tubules of marine teleosts such as the southern flounder (44, 45), winter flounder (3), and both freshwater- and seawater- adapted killifish (6). However, the molecular identity and specific mechanisms of sulfate transport in teleost kidney have not yet been elucidated.

The solute carrier (SLC) 26A anion exchangers are a relatively newly discovered gene family of high versatile anion exchangers, with intriguing roles in normal physiology and human pathophysiology (34). In mammals, eleven members of SLC26A anion exchangers (A1-A11) have been cloned. The proteins of this family member possess low conservation between orthologs even in mouse and human (28,
Three members of the gene family known as genes associated with human genetic diseases, A2 (DTDST); chondrodysplasia, A3 (DRA); congenital chloride-losing diarrhea, A4 (Pendrin); pendred syndrome and hereditary deafness have been in the spotlight (10, 48). The functional analysis of most mammalian homologs of SLC26A anion exchanger have been performed by heterologous expression systems, and have demonstrated that each member of this family of exchangers can transport a wide variety of monovalent and divalent anions. These include sulfate, chloride, iodide, formate, oxalate, hydroxyl ion and bicarbonate (4, 18, 24, 33, 34, 46, 47, 50, 53). The mammalian SLC26A anion exchangers, A1 (Sat-1), A4, A6 (Pat-1) and A7, are localized in kidney (19, 22, 39, 40). Immunoreactivity of A1 and A6 has been reported specifically in the rat basolateral membrane and the rat and mouse brush border of nephron proximal tubule, respectively, where they play a role in the solute reabsorption from the glomerular ultrafilterate (19, 23, 40).

Rainbow trout are anadromous and a model species for ion and acid-base transport. Specifically, the contribution of gills and branchial mitochondria-rich cells to the trout ion regulation has been well studied (11). However, the kidney’s role in ion regulation is less well known and trout have the potential to be used as a model for freshwater and seawater kidney function in teleosts.

The purpose of this study was 1) to molecularly identify the SLC26A anion exchangers involved in sulfate/anion exchange in the kidney of rainbow trout. 2) to determine localization of the cloned SLC26A trout homolog in the trunk kidney with transmission electron microscopy and light microscopy and 3) to demonstrate the involvement of the cloned SLC26A transport in sulfate transport.

MATERIALS AND METHODS
Experimental animal and tissue preparation

Molecular identification, tissue expression and transmission electron microscopy

Adult rainbow trout (*Oncorhynchus mykiss*) of either sex, were obtained from Alberta Trout Growers and held in 450-l indoor tanks with flowing dechlorinated Edmonton tap water (1.00 mM Na$^+$, 1.05 mM Ca$^{2+}$, 0.54 mM SO$_4^{2-}$), in the Aquatic facility of Department of Biology, University of Alberta, Alberta, Canada. The water temperature was maintained at 11-14°C. The fish were fed daily with commercial fish pellets. Photoperiod was maintained similar to the natural photoperiod in Edmonton, Alberta, Canada.

Some of the rainbow trout were acclimated gradually (~30%, 50%, 70%, and 90% seawater, for one week each) to full-strength seawater (510 mM Na$^+$, 9.38 mM Ca$^{2+}$, 24.6 mM SO$_4^{2-}$), made by Instant Ocean brand salts (Aquarium Systems Inc., OH, USA) in dechlorinated Edmonton tap water, and maintained at least for 1 wk in 190-l circulating indoor holding tanks. The experimental animals in seawater and fresh water were terminally anesthetized with ethyl-$m$-aminobenzoate (1.0 g/l, MS-222), and tissues were immediately dissected out, rapidly frozen in liquid N$_2$, and stored at -80°C for later analysis. For transmission electron microscopy (TEM), small pieces of trunk kidney of both freshwater and seawater fish (N=3 each) were sampled from the middle part of trunk kidney and fixed in 2% paraformaldehyde (PFA)-2% glutaraldehyde in 0.1 M phosphate buffer (PB, pH7.4) for 24 hours, postfixed in 1% osmium tetroxide in 0.1 M PB for 1 h.

In situ hybridization and immunocytochemistry

Adult rainbow trout (*Oncorhynchus mykiss*) from Sagami suisan (Kanagawa, Japan) were held in 1000-l tank with circulating dechlorinated Tokyo tap water
(0.96 mM Na\(^+\), 0.63 mM Ca\(^{2+}\)) in the aquatic facility of Department of Agriculture, University of Tokyo, Japan. The water temperature was maintained at 11°C, and the fish were fed daily with commercial fish pellets. Some of them were acclimated to full-strength seawater (478 mM Na\(^+\), 12.57 mM Ca\(^{2+}\)) as mentioned above, and maintained at least for 1 wk. The trunk kidneys from both freshwater and seawater fish (N= 3 each) were fixed in 4% PFA in 0.1 M PB for 24 h. The trunk kidneys were then cut into small pieces (approximately 5 mm) across the axis, dehydrated in ethanol, and embedded in Paraplast. Serial sections cut at 4 µm thickness were divided into two groups and mounted on MAS coated slides (Matsunami Glass Ind., Ltd., Japan) for \textit{in situ} hybridization and immunocytochemistry (see below).

**cDNA cloning**

Total RNA was extracted using TRIZOL Reagent (Invitrogen) from frozen trunk kidney and gill filaments of seawater rainbow trout. Poly(A\(^+\)) RNA purified with Oligotex mRNA MiniKit (Qiagen) was treated with cMaster RT plus PCR system (Eppendorf) to obtain cDNA. Polymerase chain reactions (PCRs) were performed using Taq DNA polymerase (Eppendorf). The resulting products were ligated into a pCR2.1 vector (Invitrogen), and the nucleotide sequences were determined in an automated DNA sequencer (3100 Genetic Analyzer, Perkin-Elmer/Applied Biosystems) in the Molecular Biology Service Unit in Department of Biology, University of Alberta. The sequences were analysed using GeneTool 2.0 software (BioTools incorporated, www.biotools.com).

Degenerate PCR primers, F1 and R1 (table 1, Fig. 1) were designed on the basis of homology to sequences from previously determined human and mouse SLC26A members in GenBank, and BLAST search on the web site of puffer fish
(http://fugu.hgmp.mrc.ac.uk) and salmon (GRASP, http://web.uvic.ca/cbr/rgasp) to obtain a partial cDNA fragment of a rainbow trout SLC26A anion exchanger. After an initial denaturation at 96°C for 2 min, 35 cycles of PCR were performed, each consisting of 55 sec denaturation at 94°C, 30 sec annealing at 55°C and 90 sec extension at 72°C. Gene-specific primers F2 and R2 (Table 1, Fig. 1) were designed for 3'- and 5’-RACE methods, respectively. The 3’ RACE System for Rapid Amplification of cDNA Ends (Invitrogen) and 5’ RACE System for Rapid Amplification of cDNA Ends Ver.2.0 (Invitrogen) were used according to the supplier’s instructions for 3’ RACE and 5’ RACE with specifically designed primers mentioned above, respectively.

To confirm the nucleotide sequence obtained by the 5’- and 3’- RACE methods, 3 PCR reactions were performed with gene-specific primers, F4 and R5 (Table 1, Fig. 1). One of the sequencing reactions was obtained by placing a transposon containing universal priming sites into DNA at random locations using GPS-1 Genome Priming System (New England Biolabs, Inc., MA, USA). According to the first obtained sequence obtained by the GPS-1 Genome Priming System, sequence primers, F3, F6, F7, R3, R6, R7, were designed for further sequencing reactions.

**Sequence analysis of cloned SLC26A anion exchanger**

The hydropathy profile of the deduced amino acid sequence was analyzed using PepTool version 2.0 software (BioTools incorporated) according to the algorithm of Kyte and Doolittle. Predicted topology of the membrane domain of the anion exchanger was derived using the web servers: PredictProtein (www.predictprotein.org/), SOSUI (http://sosui.proteome.bio.tuat.ac.jp/~sosui/proteome/sosuiframe0.html) and HMMTop
(www.enzim.hu/hmmtop/). The phylogenic relationship between amino acid sequences of the cloned SLC26A and previously found human homologs was analyzed using ClustalW program on DDBJ web site (www.ddbj.nig.ac.jp/). We used a neighbor-joining bootstrap method (1000 times repeated) with gaps ignored and the tree was figured by TreeView Win 3.2 software (http://taxonomy.zoology.gla.uk/rod/rod.html).

**Northern blotting**

The brain, white muscle, intestine, gill, eye, head kidney, trunk kidney, heart, liver, and blood were removed from seawater-adapted rainbow trout (N=3) and total RNA was isolated from these tissues, as described above. 20 μg of total RNA was electrophoresed on a 1.2% formamide-agarose gel and transferred to nylon membrane (Hybond-N, Amersham Biosciences). A cDNA corresponding to nucleotides (1473-2121) in SLC26A was labeled with [α-32P] dCTP (Amersham Biosciences) using Random Primers DNA Labeling System (Invitrogen), and then purified with QIAquick nucleotide removal kit (Qiagen). The membrane was hybridized with the radioisotope-labeled probe (24 X 10^5 cpm/ml) in 1% BSA, 0.5M NaH_2PO_4, 1mM EDTA, 7% SDS at 65°C for 24 h, the membrane was exposed to a screen for 2 wks. Specific bands were visualized using a PhosphoImager (Molecular Dynamics).

**Sulfate injections**

Adult rainbow trout (~250 g) of either sex, taken from holding facilities at the University of Alberta, were injected with 150 μl of 1M Na_2SO_4 under anesthesia with MS-222 and placed in 190-l tanks after their recovery. At 0 h (N=8, initial controls), 3 h (N=7), 12 h (N=8), or 24 h (N=8) after injection, fish were overdosed by MS-222
and the trunk kidneys and blood were sampled. The experiments were performed with less than 10 fish in a tank, and feeding was stopped 1 day before the injections and during the experiments. Total RNA were isolated from the tissues and Northern blot analysis was performed using 10 µg of total RNA, as described above. The hybridized signals and ethidium bromide stained 18S ribosomal RNA bands were quantified using ImageQuant (Molecular Dynamics) and Scion image beta-4.0.2 (http://www.scioncorp.com/), respectively. To correct for differences in RNA sample loading, the quantitation of SLC26A1 mRNA in each sample was given by the ratio of the hybridized signal/18S ribosomal RNA. Significant differences were examined using one-way ANOVA, followed by Dunnett’s test which compares the value to the initial control group (0 h).

Transmission electron microscopy

After dehydration in ethanol, the samples were transferred to propylene oxide and embedded in Spurr’s resin. Ultrathin sections were cut with a diamond knife, mounted on grids, stained with uranyl acetate and lead citrate, and observed with TEM (JEM1010, JEOL, Tokyo, Japan).

In situ hybridization

We utilized in situ hybridization to determine the cellular localization of the cloned SLC26A anion exchanger in the trunk kidney. These specifically designed oligonucleotide three antisense probes (44 bases each, corresponding to nucleotide sites; 734-778, 1395-1439, 1785-1829) were labeled with digoxigenin (DIG) using DIG oligonucleotide tailing kit, 2nd generation (Roche). To hybridize the probes to specific mRNA fragments, the deparaffined sections were incubated sequentially with:
1) 0.01M phosphate buffered saline (PBS, pH 7.4), 2) 0.2 N HCl for 15 min, 3) 2 µg/ml protease K in PBS for 30 min at 37°C, 4) 4% PFA in 0.1M PB for 10 min for post-fixation, 5) PBS for 1 min at room temperature, 6) 0.2% glycine in PBS for 15 min (twice), 7) distilled water (DW) for 1 min for washing, 8) The sections were pre-hybridized in 40% formamide in 2X SSC for 2 hours for pre-hybridization, 9) DIG-labeled probes (1µg/ml each) diluted in hybridization buffer (40% formamide, 2X SSC, 20 mM Tris-HCl (pH 7.6), 1X Denhardt’s, 0.1% tween-20, 25 µg/ml calf thymus DNA and 10% dextran sulfate) overnight at 37°C, 10) 2X SSC for 15 min, 11) 1X SSC for 15 min at 40°C (twice), 12) 2X SSC for 15 min, 13) PBS for a few min. The sections which possess the hybridized mRNA were sequentially incubated to detect the signals with: 1) 2% normal goat serum (NGS) in PBS for 30 min, 2) anti-DIG-gold (Roche) diluted 1:30 with PBS containing 1% bovine serum albumin (BSA) for 30 min at room temperature, 3) PBS for 5 min, 4) DW for 3 min (five times). The gold particles which exist at the localization of hybridized probes were emphasized using silver enhancing kit (British BioCell international) after incubation for 15 min at 20°C. The sections were dehydrated in series of ethanol and xylene, mounted with Permount, and observed under a light microscope (Nikon) equipped with a differential interference contrast optics. In addition, serial sections were stained with Periodic acid/Shiff (PAS, WAKO chemical, Osaka, Japan) to examine the general morphology of the trunk kidney and counter-stained with Hematoxylin.

Antibodies

The antiserum (NAK121) was raised in a rabbit against a synthetic peptide corresponding to a part of the highly conserved region of the chum salmon Na⁺, K⁺-ATPase α-subunit (20). The antibody used to detect the V-ATPase was the
affinity-purified antibody from a polyclonal antiserum raised in a rabbit against a synthetic peptide. The antigen region was designed based on the highly conserved region in the A-subunit of killifish V-ATPase (21).

**Western blot analysis for antibody to Na\(^+\), K\(^+\)-ATPase and V-ATPase**

Western blot analysis was performed according to the method described by Tresguerres et al. (51) with some minor modifications. Frozen trunk kidney samples were weighed, pulverized in 1: 10 w/v of ice-cold homogenization buffer (250 mM sucrose, 1 mM EDTA, 30 mM Tris, 100 mg/ml PMSF and 2 mg/ml pepstatin, pH 7.4) in a porcelain grinder on ice, and sonicated for 20 seconds. Debris was removed by centrifugation (4200 x g for 15 min at 4\(^{\circ}\)C), and membranes were pelleted by a centrifugation at 20800 x g for 60 min at 4\(^{\circ}\)C. The resulting pellets were resuspended in homogenization buffer and an aliquot was saved for BCA protein determination analysis (Pierce, IL, USA). The remaining sample was combined with 2X Laemmli buffer (25) for Western analysis. 20 µg (for Na\(^+\), K\(^+\)-ATPase) or 50 µg (for V-ATPase) of total protein was separated in a 7.5 % polyacrylamide gel (45 min at 180 V) and transferred to a nitrocellulose membrane. Following blocking (5% chicken ovalbumin in 0.5 M Tris-buffered saline (TBS) with 0.1 % Triton X, pH 8.0, overnight at 4\(^{\circ}\)C), the membranes were incubated with primary antibodies to either Na\(^+\), K\(^+\)-ATPase or V-ATPase (1:2500 in blocking buffer) at 4\(^{\circ}\)C overnight. After washing in TBS- 0.2% Triton X, the membrane was blocked briefly for 15 min, and incubated with the fluorescent secondary antibody (1:2500 in blocking buffer), IR800 labeled donkey anti-rabbit (Li-Cor Inc., NE, USA) at 4\(^{\circ}\)C overnight. The blots were visualized using the Odyssey infrared imaging system (Li-Cor Inc.).
**Immunocytochemistry**

The deparaffined sections were incubated sequentially with: 1) 0.6% H$_2$O$_2$ for 30 min, 2) 2% NGS for 30 min, 3) anti-Na$^+$, K$^+$-ATPase serum or anti-V-ATPase overnight at 4°C, 4) biotinylated goat anti-rabbit IgG for 30 min, 5) ABC for 1 h, 6) 0.02% 3, 3’-diaminobenzidine tetrahydrochloride containing 0.005% H$_2$O$_2$ for 4 min. Anti-Na$^+$, K$^+$-ATPase serum and V-ATPase antibody was diluted 1:4000 and 1:100, respectively, with 0.01M PBS containing 2% NGS, 0.1% BSA, 0.02% keyhole limpet hemocyanin and 0.01% sodium azide.

**Ion concentration measurements**

Na$^+$ and Ca$^{2+}$ concentrations were measured using an atomic absorption spectrophotometer (Perkin Elmer 3300), and SO$_4^{2-}$ concentrations were examined by ion chromatography (Dionex DX 500 system, Dionex Corp., CA, USA).

**RESULTS**

**cDNA cloning and sequence analysis of SLC26A anion exchanger**

RT-PCR was performed with trunk kidney and gill filaments RNA using degenerate primers, F1 and R1 (Table 1, Fig. 1). A single PCR product with the expected length (~1000 bp) was obtained only from the trunk kidney, not from the gill filaments. Following 3’ and 5’ RACE, a full-length cDNA encoding the SLC26A exchanger (3258 bases) was cloned from trunk kidney and we obtained the deduced amino acid sequence (693 amino acids) of the putative SLC26A transporter (Fig. 1). The cDNA sequence has been deposited in the database (Gen Bank, accession number AY512964) and the nucleotide sequence and amino acid sequence showed 52% of identity from human SLC26A1 (Sat-1). The rainbow trout homolog of SLC26A1 has
potential N-glycosylation sites as shown in Fig. 1. Bioinformatics analysis for conserved protein domains suggests that the cloned trout SLC26A1 has conserved domains typical for previous discovered SLC26A anion exchangers. These include SLC26A transporters signature (PS 01130, amino acid residue 71-92), the sulfate transporter family conserved amino acid sequence (pfam00916, amino acid residue 176-486), the sulfate transporter and anti-sigma factor antagonist (STAS) domain (pfam01740, amino acid residue 516-644), and anti-sigma regulatory factor, SpoIIAA (COG1366, amino acid residue 586-640) (Fig. 1).

Fig. 2 shows the hydropathy analysis of the predicted amino acid sequences of the cloned SLC26A1 rainbow trout homolog. The hydropathy profile shows the possible transmembrane domains of this homolog. Programs from three different servers, PredictProtein, SOSUI, and TMMTop yield highly divergent models of the rainbow trout homolog of the SLC26A, these included 10-, 11- and 10-transmembrane domains, respectively. PredictProtein program derived the most reasonable membrane topology suggesting 10 transmembrane domains with intracellular amino- and carboxyl- terminal ends. This is in congruence with current predicted structures of mammalian SLC26A family members. These characteristics, in addition to its position in the phylogenic tree (Fig. 3), confirms that cloned SLC26A1 is the rainbow trout homolog of mammalian SLC26A1 (Sat-1).

**Tissue distribution of SLC26A1 mRNA**

Tissue distribution of the rainbow trout homolog of SLC26A1 anion exchanger was examined by Northern blot analysis with total RNA from seawater adapted rainbow trout tissues. As shown in Fig. 4A, hybridized signals were detected as major band of approximately 3.3 kb and found in the trunk kidney, but not in other tissues.
examined. It was confirmed that the size of the mRNA was in agreement with that of the cDNA for SLC26A1 rainbow trout homolog.

**SLC26A1 mRNA expression after excess sulfate injection**

Plasma SO$_4^{2-}$ concentration also showed a transient increase (p<0.01) at 3 h after the injection, but was rapidly restored by 24 h to the level that was not significantly different from the level of 0 h (initial control) (Fig. 5A). Correspondingly, SLC26A1 mRNA expression level in trunk kidney became significantly (p<0.01, about 2.5 times) elevated at 3 h after Na$_2$SO$_4$ injection followed by a decrease to the initial expression level by 12 h (Fig. 5B).

**Ultrastructure of kidney tubules**

In rainbow trout trunk kidney, tubules were classified into three clearly differentiated fine structures, the first segment (PI) and second segment (PII) of proximal tubules and distal (DS) tubules (Fig. 6 A-F). Although every cell in these three tubules has a rich population of mitochondria and basolateral tubular system as common fine structures, these three segments are characterized by the shape of their mitochondria. Cells in PI and DS segments have longer mitochondria than those in PII cells (Fig. 6B, D, F). In addition, the apical membranes of the cells in PI segments are equipped with long and numerous brush borders pointing into the tubular lumen, while cells in PII segments have less extended brush borders. Cells in DS have distinctly less developed brush borders compared to PI and PII segments. The PI segment is signalized by pinocytotic vesicles and lysosomes in the sub-apical region of the cells. These PI tubule cells also have small electrodense dots just below the brush borders. DS segments were distinguished from PI and PII segments by the extended tubular
system in the cytoplasm which seemed lattice like (Fig. 6E, F). The tubular system in the cytoplasm was confined to the basolateral region of the cells in PI and PII segments (Fig. 6B, D). There were no remarkable differences in the fine structure between tissues from seawater (pictures not shown) and freshwater (Fig. 6) trout.

**Western blot analysis**

Since the antibodies which were used for immunocytochemistry are heterologous, it is important to demonstrate specificity in rainbow trout. The specificities of the antibodies were tested in kidney by Western blot analysis. The antibody to V-ATPase recognized the two major bands both with ~70 kDa (Fig. 7A). The antiserum to Na\(^{+}\), K\(^{+}\)-ATPase a major protein band with a size of ~100 kDa (Fig. 7B).

**Specific localization of SLC26A anion exchanger in the first segment of proximal tubules**

The three probe regions were chosen because they have sufficient GC content to serve as a probe, and were unlikely to cross-hybridize to any other mRNA. In both seawater- and freshwater-adapted rainbow trout trunk kidney, hybridization of SLC26A1 anion exchanger probes to a section of the trunk kidney showed that the mRNA is expressed most intensely in a region corresponding to the cytoplasm of the PI (Fig. 8A). The PAS staining of serial sections of the trunk kidney (Fig. 8B) demonstrated the prominent brush border projecting into the lumen of the first segment of proximal tubules and identified the tubules as belonging to the PI. The proximal tubules are PAS-positive since the surfaces of the microvilli are coated with a particularly dense glycocalyx which thought to afford physical and chemical protection to the microvilli. However, some of the PAS-positive PI lack signal for the
cloned SLC26A1 suggesting heterogeneity even with the PI segments (Fig. 8A, B). SLC26A1 mRNA localization and expression were unchanged between seawater (pictures not shown) and freshwater (Fig. 8A, B) fish.

**Co-localization of Na\(^+\), K\(^+\)-ATPase and V-ATPase with SLC26A1 mRNA**

Na\(^+\), K\(^+\)-ATPase immunoreactivity was detected in the basolateral membrane of every tubule in the trunk kidney with more intensive immunoreactivity with Na\(^+\), K\(^+\)-ATPase found in DS compared to the PT (Fig. 8C). The ultrastructure of DS shows a more extended basolateral membrane, reaching close to the subapical region of cells. Na\(^+\), K\(^+\)-ATPase staining in the proximal tubules was confined to the basolateral region of the cells (Fig. 6F, E).

Fig. 8E and F show that the V-ATPase antibody labels the inter-microvillar membrane, where TEM showed the presence of electro-dense micro dots (Fig. 6B), in the PI segment. Some of the cells also have diffuse signal throughout the microvilli. Hence, we demonstrate that both Na\(^+\), K\(^+\)-ATPase and V-ATPase are co-localized with SLC26A1 in the cells of PI. There were no remarkable differences in localization of these two transporter proteins between seawater (pictures not shown) and freshwater fish (Fig. 8C-G).

**DISCUSSION**

The kidney, gills and intestine are important organs for ion regulation in teleosts. Recently, the molecular identity and specific localization of various ion transporters have been identified in the teleost gills (9, 31, 49, 52). However, the ion-transport mechanisms in the kidney and intestine of teleosts are less understood. In this study, we have identified a kidney-specific SLC26A1 anion exchanger in renal tubules of
rainbow trout, *Oncorhynchus mykiss*. The specific localization of the trout homolog of the SLC26A1 mRNA was observed in the first segment of the proximal tubule (PI), co-localized with the ion transport proteins V-ATPase and Na\(^{+}\), K\(^{+}\)-ATPase.

Among the human homologs of SLC26 anion exchanger family, rainbow trout SLC26A1 is most closely related to the human SLC26A1 (Sat-1), according to the deduced amino acid sequence. Sat-1 is reported to act primarily as a sulfate/oxalate/bicarbonate exchanger in the proximal tubule of the mammalian kidney (19). The identity compared with recently reported eel (*Anguilla japonica*) homolog of SLC26A1 was 75% (35). The cloned SLC26A1 has all the features of other SLC26A transporters including the transporter signature PS01130, the sulfate transporter family conserved amino acid sequence (pfam00916), the anti-sigma factor antagonist (STAS) domain (pfam01740), and anti-sigma regulatory factor (SpoI1AA) (COG1366). The STAS domain has disease-associated mutants in humans, and the SpoI1AA is a statistically similar site of bacterial antisigma-factor antagonists to the C terminal domain of the SLC26A anion exchangers which normally found in STAS domain (1). These findings strongly suggest that the cloned SLC26A1 represents the rainbow trout homolog of mammalian SLC26A1 (Sat-1).

The topology of SLC26A1 trout homolog was analyzed by the different three programs and resulted in predictions of 10-, 11-, and 10-transmembrane domains, respectively. Previous predicted topologies of SLC26A anion exchangers suggest that the family members have 10 to 14 transmembrane helices (34). Human, mouse, rat and eel SLC26A1 transmembrane domain predictions have been performed based only on computer based algorithms, and they suggest 12 -transmembrane domains with both N- and C- terminal ends located intracellularly (4, 26, 35, 41). However, it is difficult to predict the exact topologies of SLC26A anion exchangers, and different
computer programs produce ambiguous results regarding the numbers of transmembrane segments for members of this family (55), as has been observed in the present study. Zeng J. et al. (55) examined the location of N- and C-terminal domains of Prestin (SLC26A5) using the antibodies to the synthetic epitopes located in N- and C-terminal ends, and showed that both ends are cytoplasmic. In SLC26A6, it has been also suggested the both N- and C-terminal ends are intracellular using tagged isoforms and antibodies to the tags (27). In terms of functional regulation, the STAS domain in C-terminal is likely an intracellular region. Although the trout homolog of the SLC26A1 might have more than 10 transmembrane domains according to hydropathy plot, the most reasonable prediction for it was derived by the PredictProtein program which suggested a 10 transmembrane domain protein with intracellular C- and N-termini.

Fish kidneys lack of a great deal of similarity to mammalian kidneys (12, 54). In regards to basic structure and morphology, the fish kidney consists of only head and trunk kidney. Embryologically, head kidney derived from pronephros, and trunk kidney from mesonephros. The head kidney is the anterior portion of the kidney and consists of primarily of lymphoid tissue while the trunk kidney is composed of many nephrons and interstitial lymphoid tissue. However, nephrons of teleosts are devoid of the loop of Henle found in higher vertebrates. In the present study, light microscopy showed distinct features of each tubule type in rainbow trout trunk kidney, and TEM observations revealed both unity and differences in ultrastructure between segments of nephron. Although every cell in the renal tubules has a rich population of mitochondria, differences in the mitochondrial fine structure and their distribution were observed. This is consistent with observations in English sole where the mitochondria are well-developed and extended throughout the cytoplasm in PII (5).
Our observations showed the equally developed mitochondria both in PI and PII with larger numbers of smaller mitochondria appearing to be present in PII of rainbow trout. The extensive brush border and large lysosomes in PI tubules observed in this study have also been shown in English sole. Moreover, lysosomes were PAS positive (5) as has also been observed in this study. In addition, similar structural feature of proximal tubules, such as brush border and lysosomes were reported in mammalian kidney (29) suggesting functional similarity of this segment to vertebrate kidney tubules. The DS was differentiated from proximal tubules essentially by the prominent basolateral infoldings and less microvilli on the lumen from our observation, in agreement with the previous observation in southern flounder (5), while this segment of the mammalian kidney shows fewer mitochondria (29).

In our Western blot analysis, the antibodies recognized one major specific protein band, of approximately 100 kDa for Na\(^+\), K\(^+\)-ATPase, and two major bands for V-ATPase at approximately 70kDa. These are close to the predicted size for these transport proteins and suggest good cross-reactivity for these heterologous antibodies. It is possible that there exist two V-ATPase A-subunits with different but close molecular masses in rainbow trout, as is the case in humans (17) and chickens (13). In the immunocytochemistry using the antibody to Na\(^+\), K\(^+\)-ATPase, a difference in the distribution of immunoreactivity was observed between the proximal and distal tubules. The difference may reflect the intensity and localization of the tubular system (basolateral membrane) of the cells, which can be observed with TEM, and supports that Na\(^+\), K\(^+\)-ATPase is located in the basolateral membrane of every cell in both proximal and distal tubules.

There have been 11 SLC26 genes identified and 6 of them (A1, 2, 4, 6, 7 and 11) have been reported to be expressed in the mammalian kidney (34). Specifically,
immunolocalization of SLC26 A1 and A6 has been observed in the proximal tubules of the rat and mouse nephron (19, 23, 40). The expression of SLC26A1 anion exchanger mRNA was shown by Northern blot analysis to be solely expressed in trunk kidney in seawater-adapted rainbow trout. In situ hybridization showed specific localization of SLC26A1 anion exchanger mRNA in the PI segment of the nephron in both seawater and fresh water fish. This result is consistent with the previous reports in mammals as mentioned above.

The SLC26 anion exchanger family is known to transport a large number of monovalent and divalent anions. The previously cloned rat SLC26A1 expressed in Xenopus oocyte and Sf9 cells demonstrated Na+-independent sulfate and oxalate transport (4, 19). Although the sulfate concentration in seawater is ~25 mM, it has been reported that plasma sulfate concentration is ~0.2 mM, and that in urine is ~70 mM in the marine teleosts, the long-horned sculpin and southern flounder (14, 30). Many previous studies have suggested that proximal tubule excretes sulfate in marine teleosts (2, 3, 6, 7, 32, 36, 38, 42, 43, 44, 45), however, molecular mechanisms of sulfate excretion in teleosts have not been revealed. In the membrane vesicles of kidney tubules of southern flounder, Renfro and Pritchard (44) showed that sulfate transport from interstitium to cell can be trans-stimulated by a basolateral membrane pH gradient and it was an electroneutral, Hg- and DIDS- inhibitable process. In isolated renal tubules, sulfate secretion is Na+ gradient dependent, although there is no effect of a Na+ gradient in the basolateral membrane vesicles. The study in brush border vesicles also suggested the presence of an anion exchanger that secretes sulfate from the cell to the lumen because the transport stimulated by the counter ion gradient (45). Pelis and Renfro reviewed the previous findings on teleosts sulfate transport and proposed a hypothetical model of sulfate excretion in proximal tubules (38). They
suggested that different sulfate/anion exchangers (sulfate and 2HCO₃⁻/2OH⁻/2Cl⁻/oxalate) exist in the apical and basolateral membranes of the proximal tubules, and Na⁺, H⁺-exchanger on either apical or basolateral membranes is required to prevent the tendency for intracellular acidification. In their model, Na⁺, K⁺-ATPase located on the basolateral membrane generates the Na⁺ gradient for Na⁺, H⁺-exchanger by Na⁺ transport into the interstitium. We also identified Na⁺, K⁺-ATPase in the basolateral membrane in the cells of proximal tubules while we also detected the specific localization of V-ATPase in the brush border membrane and subapical region. The Na⁺, K⁺-ATPase localization agrees with the proposed model in the review by Pelis and Renfro (38). Similarly the V-ATPase can transport protons from cytoplasm to lumen and it is consistent with the proposed model in terms of the function. That is, excretion of protons to prevent the intracellular acidification and/or membrane depolarization (Fig. 9).

In this study, an acute increase in cloned SLC26A1 mRNA expression was detected at 3 h after freshwater rainbow trout were injected with excess sulfate. At the same time, it was observed that plasma sulfate concentration of injected rainbow trout reached at least 6 times higher than normal plasma level (0.45 mM) and the excess plasma sulfate rapidly decreased to the initial level. These results strongly suggest the hypothesis that kidney specific rainbow trout SLC26A1 is involved in sulfate excretion. In both seawater and fresh water, in situ hybridization showed the quite similar localization and expression of rainbow trout SLC26A1 in the present study. In the eel, the SLC26A1 has been cloned from kidney, and localized to proximal tubules of the kidney (35). Interestingly in their study, the mRNA expression was greater in freshwater- than seawater- adapted eels, and they suggested that the eel SLC26A1 is involved in sulfate reabsorption across the basolateral membrane possibly contributing
to osmoregulation in fresh water. However, it should be noted that $SO_4^{2-}$ in plasma of freshwater eels is $\sim$37.5 mEq (35) while that of freshwater rainbow trout was 0.36 mM in this study.

In the mammalian kidney, reabsorption of filtered sulfate occurs primarily in the proximal tubules and required for sulfate homeostasis in mammals (16). Although the sulfate homeostasis in freshwater fish have been less demonstrated than that in seawater fish, the kidney of freshwater teleosts should be a site of net sulfate reabsorption because of the low levels of sulfate and limitation of drinking in freshwater environments (38). Hence, the SLC26A1 anion exchanger expressed in the PI in freshwater rainbow trout might be involved in sulfate reabsorption. Interestingly, in some tubules identified as PI, no SLC26A1 signal was observed. This raises the interesting possibility that subtypes of cells exist within the segment possibly transporting a variety of ions, such as Na$^+$, Cl$^-$, Mg$^{2+}$, SO$_4^{2-}$, Ca$^{2+}$, HCO$_3^-$ (15). This is significant in that this is the first time that cells in the PI may differentially express certain transporters.

**PERSPECTIVES**

SLC26A1, sulfate/anion exchanger, was cloned from seawater adapted rainbow trout, and excretion of excess plasma sulfate corresponded to the increase in the SLC26A1 expression. The exchanger was distributed specifically in the first segment of proximal tubules and colocalized with both Na$^+$, K$^+$-ATPase in basolateral membrane and V-ATPase in the apical membrane. However, some PI segments without the signals were also observed. This finding suggested the existence of more than one type of mitochondria-rich cell in the first segment of proximal tubules in terms of function. These findings provide an important step to further our
understanding of sulfate transport mechanism in fish renal tubules at the molecular level, the physical roles SLC26 anion exchangers in teleosts, and relatively unexplored kidney physiology in teleosts.

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37. Pelis RM and Renfro JL. Active sulfate secretion by the intestine of winter


FIGURE LEGENDS

Fig. 1
Nicreotude sequence of the cDNA encoding the SLC26A1 from rainbow trout kidney and the deduced amino acid sequence. Designed primer sites are shown in highlighted site, and potential N-linked glycosylation sites are circled. The amino acid positions in boxes are SLC26A transporters signature (71-92) and STAS domain (516-644), respectively.

Fig. 2
Hydropathy profile of cloned SLC26A1 by the Kyte-Doolittle method. Upper lines labeled 1-10 are deduced transmembrane domain predicted by PredictProtein. This putative protein model has N- and C- terminal ends located intracellularly.

Fig. 3
Phylogenetic tree of SLC26 family members of human and cloned rainbow trout homolog. The numbers between branches show bootstrap values (0-1000) which was analyzed using NJ bootstrap method with gaps ignored. The scale bar represents a genetic distance of 0.1 amino acid substitutions per site.

Fig. 4
Tissue distribution and quantification of rainbow trout SLC26A1 mRNA determined by northern hybridization in seawater-adapted fish. Hybridization major signal was detected at approximately 3.3 kb in trunk kidney (A, B).
Plasma sulfate level (A) and SLC26A1 mRNA level (B) in trunk kidney following injection of Na$_2$SO$_4$. Asterisks indicate significant differences (p<0.01) compared with the initial values (0 h) of plasma sulfate level and mRNA expression.

Transmission electron micrographs of rainbow trout renal tubules, the first segment (A, B) and the second segment (C, D) of proximal and distal tubules (E, F) from freshwater rainbow trout. Arrows, arrowheads, and both arrows indicate basolateral membrane, subapical electro-dense dots, and the development of brush borders, respectively. No remarkable differences were found in seawater fish (not shown). t, tubular lumen; m, mitochondria; n, nuclei; asterisks, pinocytotic vesicles and lysosomes. Scale bar, 5 μm.

Western blot analysis for Na$^+$, K$^+$-ATPase (A) and vacuolar-type H$^+$-ATPase (V-ATPase) (B) proteins expressed in rainbow trout trunk kidney. Positions of bands (arrows) of Na$^+$, K$^+$-ATPase and V-ATPase are expected sizes, ~100 kDa and 70 kDa, respectively.

In situ hybridization of SLC26A1 (A), and immunocytochemistry with anti-Na$^+$, K$^+$-ATPase (C) and V-ATPase (E, F). PAS stained serial sections show the structural feature of the trunk kidney (B for A, D for C, G for E). Arrow heads and arrows (A) indicate the first segment of proximal tubules with and without SLC26A1 mRNA.
expression in trunk kidney, respectively. Enlarged view (F) shows the subapical immunolocalization of V-ATPase. PI, the first segment of proximal tubules; PII, the second segment of proximal tubules; D, distal tubules. Scale bars, 50 um (A-E and G); 25 um (F).

Fig. 9
Model of sulfate excretion in the kidney proximal tubules. Sulfate transport through basolateral membrane mediates SO$_4^{2-}$/OH$^-$ exchange, and the presence of SO$_4^{2-}$/anion exchanger in brush border membrane is indicated. The cloned SLC26A1 is placed in the apical membrane as SO$_4^{2-}$/anion exchanger. The apically located V-ATPase prevent intracellular acidifications, and basolateral Na$^+$, K$^+$-ATPase generate the Na$^+$ gradient for Na$^+$ transport through the apical membrane.
### Table 1. Primer sequences

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</table>
Fig. 1
Fig. 5

**A**

Graph showing plasma $\left[\text{SO}_4^{2-}\right]$ (mM) over time (h) from 0 to 24 hours.

**B**

Graph showing mRNA (arbitrary units) over time (h) from 0 to 24 hours.
Fig. 9