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Na^+/K^+-ATPase α-isoform switching in gills of rainbow trout (*Oncorhynchus mykiss*) during salinity transfer

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Summary

We identified five Na^+/K^+-ATPase α-isoforms in rainbow trout and characterized their expression pattern in gills following seawater transfer. Three of these isoforms were closely related to other vertebrate α1 isoforms (designated α1a, α1b and α1c), one isoform was closely related to α2 isoforms (designated α2) and the fifth was closely related to α3 isoforms (designated α3). Na^+/K^+-ATPase α1c- and α3-isoforms were present in all tissues examined, while all others had tissue specific distributions. Four Na^+/K^+-ATPase α-isoforms were expressed in trout gills (α1a, α1b, α1c and α3). Na^+/K^+-ATPase α1c- and α3-isoforms were expressed at low levels in freshwater trout gills and their expression pattern did not change following transfer to 40% or 80% seawater. Na^+/K^+-ATPase α1a and α1b were differentially expressed following seawater transfer. Transfer from freshwater to 40% and 80% seawater decreased gill Na^+/K^+-ATPase α1a mRNA, while transfer from freshwater to 80% seawater caused a transient increase in Na^+/K^+-ATPase α1b mRNA. These changes in isoform distribution were accompanied by an increase in gill Na^+/K^+-ATPase enzyme activity by 10 days after transfer to 80% seawater, though no significant change occurred following transfer to 40% seawater. Isoform switching in trout gills following salinity transfer suggests that the Na^+/K^+-ATPase α1a- and α1b-isoforms play different roles in freshwater and seawater acclimation, and that assays of Na^+/K^+-ATPase enzyme activity may not provide a complete picture of the role of this protein in seawater transfer.

Key words: gene expression, seawater, freshwater, ion regulation, salmonid, rainbow trout, *Oncorhynchus mykiss*, Na^+/K^+-ATPase.

Introduction

The gill plays an important role in the maintenance of blood ion and acid–base balance in both freshwater- and seawater-acclimated fish (Claiborne et al., 2002; Marshall, 2002). In freshwater-acclimated fish, the gill epithelium actively transports Na^+ and Cl^- from the dilute freshwater into the blood to match passive loss of these ions in the urine and across the body surface (Marshall, 2002). In contrast, seawater-acclimated fish drink seawater to counter osmotic loss of water, and secrete excess Na^+ and Cl^- across the gills. During movement from freshwater to seawater, euryhaline and anadromous fishes must transform their gills from an ion-absorbing epithelium to an ion-secreting epithelium (Marshall, 2002; McCormick, 2001). Associated with this transformation is an increase in gill Na^+/K^+-ATPase activity (for a review, see McCormick, 1995) and an increase in Na^+/K^+-ATPase gene expression (Seidelin et al., 2000; Shikano and Fujio, 1998a,b; Uchida et al., 1996).

Na^+/K^+-ATPase is a membrane protein that couples the exchange of two extracellular K^+ ions for three intracellular Na^+ ions to the hydrolysis of one molecule of ATP (Mobasheri et al., 2000). The functional Na^+/K^+-ATPase is composed of two essential subunits (α and β) that are noncovalently paired to form an αβ-heterodimer. The α-subunit contains the binding sites for ATP, Na^+, K^+ and the cardiac glycoside, ouabain, and is considered the catalytic subunit. The β-subunit is a type II glycosylated polypeptide that is thought to assist in the folding and placement of the α-subunit into the cell membrane (Blanco and Mercer, 1998). A third, nonessential γ-subunit has been identified in mammals (Reeves et al., 1980), and is thought to play a role in modulating Na^+-, K^+- and ATP-binding affinities to the Na^+/K^+-ATPase αβ complex (Béguin et al., 1997; Therien et al., 1999). Thus far, four α-, three β- and one γ-subunit isoforms have been identified in mammals (Mobasheri et al., 2000) and several α- and β-subunits have been identified in fish. Full or partial cDNA sequences for Na^+/K^+-ATPase α1-, α2- and α3-isoforms have been identified in numerous fish species including salmonids (Cutler et al., 1995a,b; Guynn et al., 2002; Schonrock et al., 1991; Semple et al., 2002);

however, little is known about the physiological role of these isoforms.

Among the four Na^+/K^+-ATPase α-isoforms identified in mammals, differences in Na^+, K^+ and ATP binding affinity and sensitivity to ouabain have been described. Characterization of rat Na^+/K^+-ATPase α-and β-isoforms in insect cell lines demonstrated that Na^+ and K^+ affinity varied among Na^+/K^+-ATPase isoform combinations with a rank order of α2β1>α1β1>α3β1 and α1β1>α2β1>α3β1, respectively (see Blanco and Mercer, 1998). Thus, differences in the α-isoforms strongly influence the kinetic properties of Na^+/K^+-ATPase and the tissue distribution of these isoforms may be related to matching isoform-specific kinetic properties with tissue-specific physiological functions. Indeed, the physiological role of these isoforms have recently been examined in several mammalian tissues (e.g. He et al., 2001; James et al., 1999; Woo et al., 2000). However, little is known about the importance of differential Na^+/K^+-ATPase α-isoform expression in organs such as fish gills that must dynamically regulate ion balance.

There is accumulating evidence to suggest that fish gills express multiple Na^+/K^+-ATPase α-isoforms and that the isoform complement may change to meet the ion-regulatory challenges imposed during salinity transfer. Pagliarani et al. (1991) demonstrated that Na^+/K^+-ATPase proteins isolated from freshwater- and seawater-acclimated fish gills had different biochemical properties, suggesting that different Na^+/K^+-ATPase isoforms may be involved in freshwater *versus* seawater ion regulation. Furthermore, Lee et al. (1998) showed increases in Na^+/K^+-ATPase α1- and α3-protein abundance in the gills of tilapia *Oreochromis mossambicus* following transfer from freshwater to seawater. Analysis of Arrhenius plots also provides evidence for the presence of multiple Na^+/K^+-ATPase isoforms in carp *Cyprinus carpio* gills and implicates these isoforms in thermal acclimation (Metz et al., 2003). Overall, changes in Na^+/K^+-ATPase α-isoform expression may be a crucial mechanism to match the kinetic properties of Na^+/K^+-ATPase to changing environmental conditions.

Thus, the objectives of the present study were to determine if multiple Na^+/K^+-ATPase α-isoforms exist in rainbow trout and to examine the expression pattern of these isoforms in trout gills following abrupt transfer from freshwater to 40% and 80% seawater.

Materials and methods

Animals

Rainbow trout *Oncorhynchus mykiss* Walbaum were obtained from Rainbow Springs Trout Farm, Thamesford, Ontario, Canada and held in flow-through well water (11.5°C) at the University of Waterloo for at least 2 weeks before experimentation. These fish were used in the identification and cloning of Na^+/K^+-ATPase α-subunit genes. A second group of trout were obtained from Spring Valley Trout Farm, Langley, British Columbia, Canada and held in Cypress creek water (8°C) at the Department of Fisheries and Oceans, West Vancouver Laboratories, West Vancouver, British Columbia for 1 month before experimentation. These fish were used to investigate Na^+/K^+-ATPase α-isoform expression in gills of trout following abrupt salinity transfer. All trout were fed daily with commercial trout feed. All experimental procedures fully comply with Canadian Council of Animal Care guidelines.

Identification and sequencing of Na^+/K^+-ATPase isoforms

Tissue sampling

Trout were sampled directly from the holding tank and killed by decapitation. Samples of brain, gill, eye, heart, liver, spleen, intestine, kidney, testis, white muscle and red muscle were dissected from the trout and immediately frozen in liquid N_2. Tissue samples were stored at –70°C until analysis.

RNA extraction and reverse-transcriptase PCR amplification

Total RNA was extracted from tissues using the guanidine thiocyanate method outlined by Chomczynski and Sacchi (1987) using TriPure Isolation Reagent (Roche Diagnostics, Montreal, QC, Canada). Following isolation, RNA was quantified spectrophotometrically and electrophoresed on an agarose–formaldehyde gel (1.2% w/v agarose, 16% formaldehyde) to verify RNA integrity. RNA was stored at –80°C.

First strand cDNA was synthesized from 4 or 5 μg of total RNA isolated from the above tissues using oligo(dT_{15}) primer and RevertAid™ H Minus M-MuLV reverse transcriptase, following the manufacturer's instructions (MBI Fermentas Inc., Burlington, ON, Canada). Partial Na^+/K^+-ATPase α-subunit sequences were obtained using primers determined from conserved regions of *Torpedo californica* (Accession No. X02810), *Catostomus commersoni* (Accession No. X58629), *Anguilla anguilla* (Accession No. X76108), and *Xenopus laevis* (Accession No. U10108). The forward primer (NaKF) was 5′-AAC CCC AGA GAT GCC AA-3′ and the reverse primer (NaKR) was 5′-AAG GCA CAG AAC CAC CA-3′ (see also Semple et al., 2002). Primers were designed with the assistance of GeneTool Lite software (www.biotool.com). Polymerase chain reactions (PCRs) were carried out in a PTC-200 MJ Research themocycler using *Taq* DNA polymerase (MBI Fermentas) and cDNA isolated from the above tissues. Each PCR consisted of 35 cycles: 1 min at 94°C, 1 min at 42°C and 2 min at 72°C. PCR products were electrophoresed on 1% agarose gels containing ethidium bromide and bands of appropriate size were extracted from the gels using QIAEX II gel extraction kit (Qiagen Inc., Mississauga, ON, Canada). The extracted PCR product from each tissue was cloned into a T-vector (pGEM-T easy; Promega; Fisher Scientific, Nepean, ON, Canada), transformed into heat shock competent *Escherichia coli* (strain JM109; Promega, Fisher Scientific, Nepean, ON, Canada) and colonies grown on ampicillin LB-agar plates. Several colonies containing the ligated PCR product were selected and grown overnight in liquid culture. Plasmids were harvested from liquid culture using GenElute

Plasmid Miniprep kit (Sigma-Aldrich, Oakville, ON, Canada) and sequenced using an ABI 377 automated fluorescent sequencer at York University Molecular Biology core facility (Toronto, ON, Canada).

Isoform-specific primers were designed to obtain the complete cDNA sequence using 5′ and 3′ RACE (Smart RACE cDNA amplification kit; BD Bioscience Clontech, Mississauga, ON, Canada). Multiple clones of each fragment were sequenced in both directions at least twice, and a majority-rule consensus for the full-length cDNA transcript was developed for each isoform. Sequence assembly and analysis were performed using GeneTool Lite software. Comparison with published sequences in GenBank was made with the BLAST algorithm (Altschul et al., 1997) and multiple alignments were produced with ClustalW (Thompson et al., 1994). Putative transmembrane regions were identified by hydrophobicity analysis using AnTheProt (http:\antheprot-pbil.ibcp.fr/ie_sommaire.html). cDNA sequences have been deposited into GenBank with accession numbers given in Table 1.

Tissue distribution

The distribution pattern of Na^+/K^+-ATPase α-subunit mRNA was determined using isoform-specific PCR and ethidium bromide stained gels. Alignment of the Na^+/K^+-ATPase α-isoform sequences were used to determine regions that were unique to each isoform and PCR primers were designed in these areas. PCR reactions were performed with the isoform-specific primers and cDNA obtained from the dissected tissues.

Phylogenetic analysis

Amino acid (aa) sequences were deduced from the nucleotide sequence of each isoform using GeneTool Lite software. Protein sequences or deduced aa sequence of other Na^+/K^+-ATPase α-subunits were obtained from GenBank: human α1 (X04297); human α2 (XP010502); human α3 (NM000703); human α4 (Q13733); rat α1 (D00189), rat α2 (NM012505), rat α3 (NP036638); rat α4 (NP074039); chicken α1 (J03230); chicken α2 (17041294); chicken α3 (P24798); mouse α4 (Q9WV27); horse α1 (P18907); sheep α1 (P04074); dog α1 (P50997); pig α1 (P05024); killifish α1 (AY057072); killifish α2 (AY057073); tilapia α1 (U82549); tilapia α3 (AF109409); electric eel (AF356351); frog (P30714); goldfish α3 (BAB60722); Torpedo ray (P05025); whitesucker (X58629), eel (Q92030); Xenopus α1 (Q92123); zebrafish α1 (AF286372); zebrafish α2 (AF286373); zebrafish α3 (AF286374); zebrafish α4 (AF308598); zebrafish α5 (AF308599); zebrafish α6 (AY008374); zebrafish α7 (AY008375); zebrafish α8 (AY008376); fruitfly (AAF55826). Sequences were aligned using ClustalW and phylogenetic analysis was performed using the neighbour-joining method with complete deletion of gaps using MEGA2 software (Kumar et al., 2001). The support for each node was assessed using 500 bootstrap replicates. Isoforms were named according to their position in the phylogenetic tree.

Table 1. *Accession number and size of coding, 5′- and 3′-untranslated region (UTR) of each Na^+/K^+-ATPase α-isoform*

Isoform	Accession number*	Length		
		5′-UTR	Coding region	3′-UTR
α1a	AY319391	30	3087	135
α1b	AY319390	153	3087	213
α1c	AY319389	159	3075	202
α2	AY319387	21	3036	825
α3	AY319388	117	3033	1440

*Sequences have been deposited into GenBank.

Sliding window analysis

To determine the percentage variation between the cDNA and aa sequences of the five Na^+/K^+-ATPase α-isoforms, we performed sliding window analysis. Sliding window analysis quantifies the variation between aligned sequences by counting the average number of differences between isoforms within overlapping windows. For the present analysis, we used an overlapping window of 50. Sliding window analysis was performed using MEGA software (version 1.01).

Salinity transfer experiment

Experimental protocol

1 week before experimentation, 46 fish were acclimated in each of 6 × 60 liter buckets supplied with 1 l min^{-1} Cypress creek water. Each bucket was held in a large tank supplied with well water to maintain water temperature at 8°C. Fish were not fed during the acclimation period or during the salinity transfer experiment. 1 h before the salinity transfer (Pre on graphs), four fish were removed from each tank and their tissues sampled (see below). At time zero (vertical broken line on graphs), the water level in each bucket was lowered to 12 liters and then immediately replaced with either freshwater and/or seawater to yield salinity equivalent to 0%, 40% or 80% seawater. Fish were held under static/renewal conditions where 75% of the water was replaced daily. Salinity was monitored daily using a handheld refractometer. Each transfer condition was duplicated and four fish were sampled from each tank at 2, 4, 8, 24, 48, 72, 120, 240 and 360 h after transfer, yielding a total of eight fish per treatment per time point. There were no statistically significant differences for any measurements between duplicate tanks, so all data were pooled and are presented as a single point.

Tissue sampling

Individual fish were netted from each tank with minimal disturbance and rapidly killed by concussion. Blood samples were taken by caudal puncture using heparinized needles and stored briefly on ice. Plasma was separated from blood cells by centrifugation and plasma was frozen in liquid N_2. Plasma samples were used for the measurement of osmolality, $[Na^+]$, $[Cl^-]$ and [cortisol]. Immediately following blood sampling, second and third gill arches were quickly dissected from each fish and frozen in liquid N_2.

Gill Na^+/K^+-ATPase enzyme activity

Gill Na^+/K^+-ATPase activity was measured on crude gill homogenates using the methods outlined by McCormick (1993). This assay couples ouabain-sensitive ATP hydrolysis to the oxidation of NADH *via* pyruvate kinase and lactate dehydrogenase. Briefly, gill filaments (~50 mg) were cut from each arch on ice and immediately homogenized in SEI buffer (250 mmol l^{-1} sucrose, 10 mmol l^{-1} EDTA·Na_2, 50 mmol l^{-1} imidazole, pH 7.3) with 0.1% sodium deoxycholate. Gill homogenates were centrifuged at 5000 ***g*** for 30 s and the supernatant immediately frozen in liquid N_2. To determine Na^+/K^+-ATPase activity, homogenates were thawed and 10 μl was assayed for ATPase activity in the absence or presence of 0.5 mmol l^{-1} ouabain. Each assay was run in triplicate and the coefficient of variation was always <10%. Homogenate [protein] was measured using the bicinchoninic acid method (Sigma-Aldrich) with bovine serum albumin standards. Na^+/K^+-ATPase activities were calculated by subtracting the ouabain sensitive ATPase activity from total ATPase activity and are expressed in μmol mg^{-1} protein h^{-1}.

Plasma analysis

Plasma osmolality was measured using a Wescor 5500 vapour pressure osmometer (Wescor Inc., UT, USA). Plasma [Na^+] was measured using a FLM2 flame photometer (Radiometer, Copenhagen, Denmark). Plasma [Cl^-] was measured using a CMT 10 chloride titrator (Radiometer). When possible, all assays were done in duplicate. Plasma [cortisol] was determined in duplicate using a [^{125}I] radioimmunoassay (Medicorp Inc., Montreal, QC, Canada). The coefficient of variation between duplicates was <15%.

Gill Na^+/K^+-ATPase α-isoform expression

Na^+/K^+-ATPase α-subunit isoform expression was estimated using quantitative real-time PCR (qRT-PCR). Isoform-specific primers were designed using Primer Express software (version 2.0.0; Applied Biosystems Inc., Foster City, CA, USA). Primer sequences were as follows: Na^+/K^+-ATPase α1a forward 5′ GGC CGG CGA GTC CAA T 3′, Na^+/K^+-ATPase α1a reverse 5′ GAG CAG CTG TCC AGG ATC CT 3′ (product size 66), Na^+/K^+-ATPase α1b forward 5′ CTG CTA CAT CTC AAC CAA CAA CAT T 3′, Na^+/K^+-ATPase α1b reverse 5′ CAC CAT CAC AGT GTT CAT TGG AT 3′ (product size 81), Na^+/K^+-ATPase α1c forward 5′ GAG AGG GAG ACG TAC TAC TAG AAA GCA 3′, Na^+/K^+-ATPase α1c reverse 5′ CAG CAA GAC AAC CAT GCA AGA 3′ (product size 69); Na^+/K^+-ATPase α3 forward 5′ CCA GGT ATT GAG TTC CGT GTG A 3′, Na^+/K^+-ATPase α3 reverse 5′ CAG CCT GAA ATG GGT GTT CCT 3′ (product size 66), and elongation factor-1α forward 5′ GAG ACC CAT TGA AAA GTT CGA GAA G 3′, elongation factor-1α reverse 5′ GCA CCC AGG CAT ACT TGA AAG 3′ (product size 71).

Total RNA was extracted from ~20 mg of frozen gill tissue using TriPure reagent and quantified spectrophotometrically. First-strand cDNA was synthesized from 5 μg of total RNA using the protocols outlined above. Quantitative RT-PCR was performed on an ABI Prism 7000 sequence analysis system (Applied Biosystems Inc., Foster City, CA, USA). PCR reactions contained 2 μl of cDNA, 4 pmoles of each primer and Universal SYBR green master mix (Applied Biosystems Inc.) in a total volume of 20 μl. All qRT-PCR reactions were performed as follows: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min Melt curve analysis was performed following each reaction to confirm the presence of only a single product of the reaction. In addition, representative PCR products were electrophoresed on a 2.0% agarose gel to verify that only a single band was present. Negative control reactions were performed for all samples using RNA that had not been reverse transcribed to control for the possible presence of genomic DNA contamination. Genomic DNA contamination was present in all samples, but never constituted more than 1:4096 starting copies for Na^+/K^+-ATPase α1a, 1:16 384 starting copies for Na^+/K^+-ATPase α1b, 1:2048 starting copies for Na^+/K^+-ATPase α1c, 1:1024 starting copies for Na^+/K^+-ATPase α3, or 1:16 384 starting copies for the control gene elongation factor-1α (EF-1α; Accession No. AF498320). As a result, only a negligible fraction of the qRT-PCR signal was attributable to genomic DNA. One randomly selected control sample was used to develop a standard curve relating threshold cycle to cDNA amount for each primer set. All results were expressed relative to these standard curves and mRNA amounts are normalized relative to EF-1α. Post-transfer samples are expressed relative to the pre-transfer freshwater gill samples. All samples were run in duplicate and the coefficient of variation between duplicate samples was always <10%. To reduce cost, pre-transfer controls and 8 h, 5 day and 15 day post-transfer samples were analyzed first. If there were differences in Na^+/K^+-ATPase α-isoform expression at one of these time points, then the complete time course was analyzed; however, if no differences in expression were observed then no further analysis was performed.

The relative quantity of Na^+/K^+-ATPase-α1a, -α1b, -α1c and -α3, mRNA in trout gills was estimated for each gene in the pre-transfer freshwater trout using the formula Efficiency^{-Ct}, where efficiency is the slope of the standard curve and Ct is the threshold cycle number. These quantities were also expressed relative to EF-1α.

Statistical analysis

All data are presented as means ± S.E.M. (*N*, number of fish). At each time point, values for trout held in 40% and 80% seawater were compared to freshwater controls using a non-parametric Kruskal–Wallis test. Significance was set at α=0.05, and when obtained, Dunn's *post-hoc* test was used to identify where significant differences occurred.

Results

Identification and characterization of Na^+/K^+-ATPase α-subunit isoforms in trout

In the present study we identified five, full-length cDNA sequences coding for Na^+/K^+-ATPase α-isoforms in rainbow

trout. Alignment of the trout with other vertebrate Na^+/K^+-ATPase α-isoforms indicated that our trout isoforms were 69–92% similar to other Na^+/K^+-ATPase α-subunit isoforms known in GenBank. Phylogenetic analysis of the five trout isoforms indicated that three Na^+/K^+-ATPase α-isoforms group closely with other vertebrate Na^+/K^+-ATPase α1 isoforms (designated α1a, α1b and α1c; Fig. 1), one trout isoform grouped closely with α2 isoforms (designated α2), and the fifth trout isoform grouped closely with α3 isoforms (designated α3). Na^+/K^+-ATPase α1a and α1b were very closely related (Fig. 1).

These five cDNA sequences have coding regions of 3033–3087 nucleotides, yielding an open reading frame of 1011–1029 aa (Table 1). The five Na^+/K^+-ATPase α-isoforms were 72.6–89.0% and 74.6–89.2% similar at the cDNA (coding region) and aa levels, respectively (Table 2). The probable start codon (ATG) was between 21 and 159 nucleotides from the 5′ end of the cDNA and the probable stop codon (TAT/TAC) was between 135 and 1440 nucleotides from the 3′ poly(A)$^+$ tail (Table 1). Among the five Na^+/K^+-ATPase α-isoforms, the 5′- and 3′-UTRs were only 10–35% and 3–28% similar, respectively, except for Na^+/K^+-ATPase α1a and α1b, which shared 90% and 38% similarity in the 5′ and 3′ UTRs, respectively.

Sliding window analysis of the trout Na^+/K^+-ATPase α-isoforms identified three areas of low aa variability (<10% heterogeneity) common to the five isoforms (Fig. 2D). These areas of low variability were located 320–360, 580–630 and 690–755 aas from the N terminus. Located within these areas of low variability were the predicted ATP binding site (g in Fig. 2C), which was fully conserved in all trout isoforms, and a highly conserved 'hinge' sequence found in all P-type ATPases (VAVTGDGVNDSPALRKADIGVAM; h in Fig. 2C) (Mobasheri et al., 2000). The only variation among isoforms in the 'hinge' sequence was a conservative aa substitution in the Na^+/K^+-ATPase α1a isoform. Two areas of high aa variability (>50% heterogeneity) were found among the isoforms. The first area of high variability included the 40 N-terminal aas (ns; Fig. 2A,D) and the second was a 12-aa segment located approximately 490 aa from the N terminus (cs; Fig. 2B,D). Sliding window analysis of the Na^+/K^+-ATPase α-isoform cDNA sequences identified two areas of >75% variability (Fig. 2E), which correspond to the two areas of high variability observed in the aa sequence (ns and cs; Fig. 2C).

Hydrophobicity analysis of each Na^+/K^+-ATPase α-isoform revealed nine putative transmembrane domains (f in Fig. 2C), which is in good agreement with the 8–10 transmembrane domains predicted to be present in mammalian sequences (Mobasheri et al., 2000). One predicted transmembrane domain was located in an area of low variability, while the other eight transmembrane domains were located in areas of high variability (cf. Fig. 2C,D).

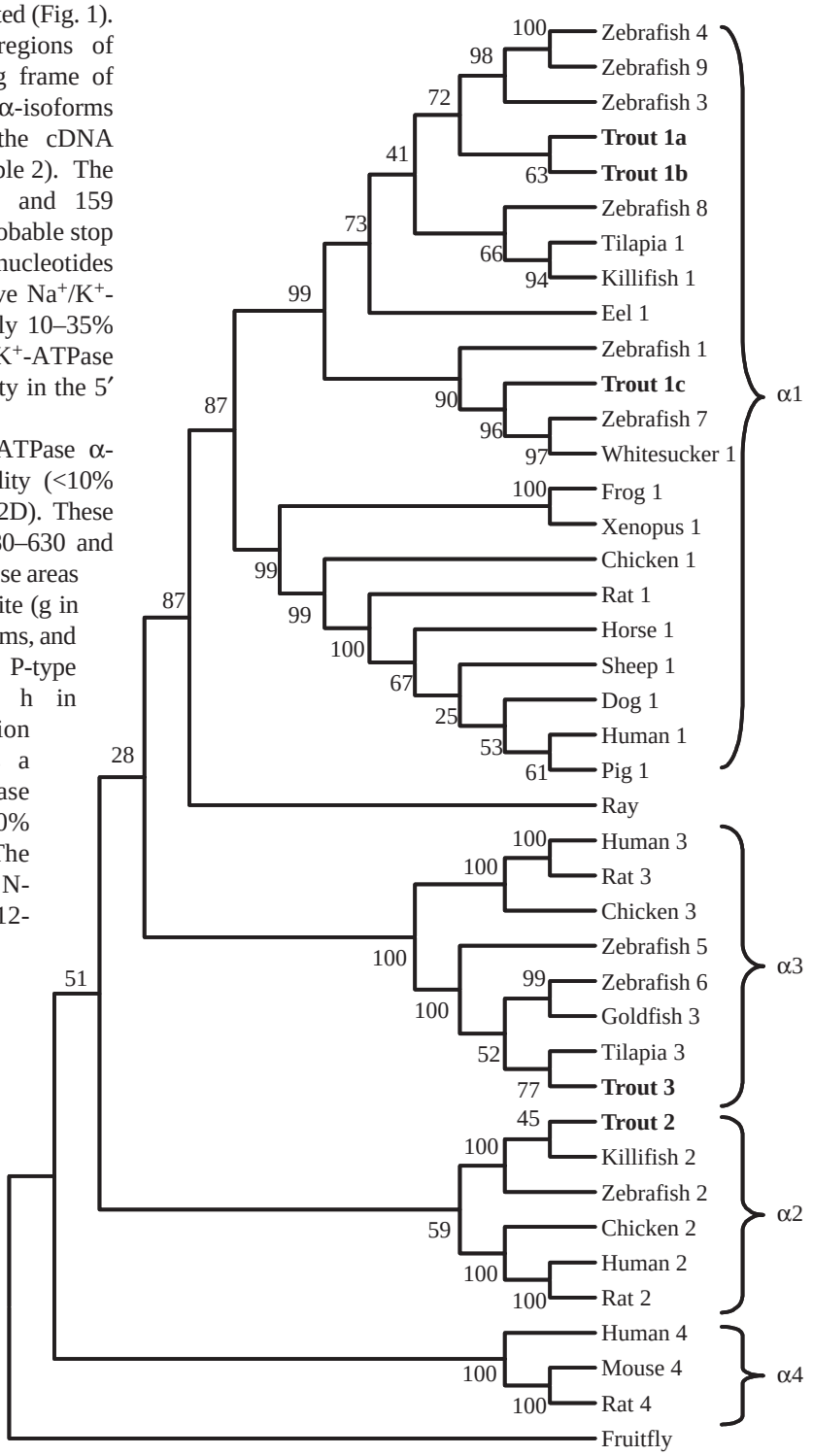

Fig. 1. Phylogenetic analysis of Na^+/K^+-ATPase α-isoform amino acid sequences. Numbers presented at each branch point represent bootstrap values from 500 replicates. Bold-face type indicates Na^+/K^+-ATPase α-isoforms identified in the present study and each is named according to its position in the phylogenetic tree. Fruitfly Na^+/K^+-ATPase was used as an outgroup.

Table 2. *cDNA and amino acid similarity matrix for Na^+/K^+-ATPase α-isoforms from trout*

	cDNA/amino acid identity (%)				
Isoform	α1a	α1b	α1c	α2	α3
α1a	100/100	89.0/86.3	83.8/82.5	72.6/75.8	74.3/74.6
α1b		100/100	87.7/89.2	74.1/81.5	76.5/80.8
α1c			100/100	74.5/81.9	78.1/82.5
α2				100/100	78.8/82.8
α3					100/100

The full coding regions of each Na^+/K^+-ATPase α-isoform genes were used to develop this matrix.

Similarity indices were obtained using BioEdit software.

Each trout Na^+/K^+-ATPase α-isoform had a tissue-specific distribution. Na^+/K^+-ATPase α1c and α3 isoforms were found in all tissues examined (Fig. 3). Na^+/K^+-ATPase α1c was expressed in brain, eye, gill, heart, kidney, spleen, intestine, liver, white muscle, red muscle and testis. Na^+/K^+-ATPase α3 was ubiquitously expressed. The Na^+/K^+-ATPase α1b was expressed in brain, eye, gill, kidney, liver, spleen, intestine, testis, white muscle and red muscle. The most restricted tissue distributions were observed for Na^+/K^+-ATPase α1a and α2 isoforms (Fig. 3). Na^+/K^+-ATPase α1a isoform was found only in the gills and heart of trout, while the α2 isoform was expressed in white muscle, red muscle, brain and eye.

Salinity transfer

Cortisol

Trout held in freshwater during the pre-transfer period (Pre) had plasma [cortisol] of 15–20 ng ml^{-1} (Fig. 4). Transfer of trout from freshwater to freshwater (control; 0‰ salinity), 40% seawater (14.4±0.11‰; N=18), or 80% seawater (27.8±0.09‰; N=18) and subsequent holding for 15 days did not significantly affect plasma [cortisol] at any time (P>0.05).

Plasma ions

Transfer of trout from freshwater to 40% and 80% seawater resulted in a rapid (2 h) and salinity-dependent increase in plasma osmolality, which remained elevated compared to freshwater controls for the 15 day post-transfer period (Fig. 5A). Similarly, plasma [Na^+] and [Cl^-] increased in a salinity-dependent manner following transfer from freshwater to 40% and 80% seawater (Fig. 5B,C) and, for the most part,

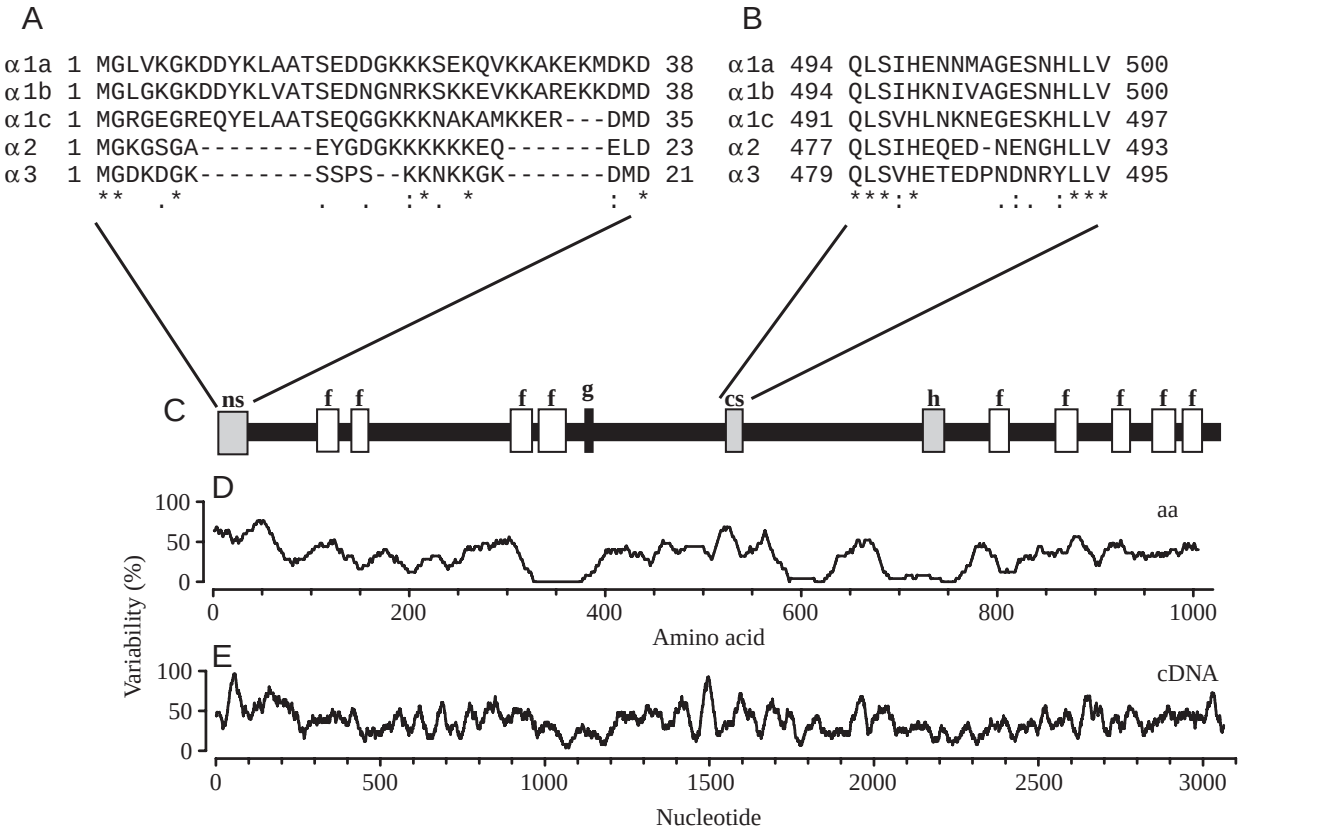

Fig. 2. Sequence analysis of trout Na^+/K^+-ATPase α-isoforms. (A) N-terminal isoform-specific identity sequence (ns), (B) central isoform-specific identity sequence (cs), (C) predicted structure of the Na^+/K^+-ATPase α-subunit protein showing (f) transmembrane domains, (g) ATP binding site, and (h) a highly conserved area found in all P-type ATPases. Asterisks indicate conserved aas among all isoforms; colons indicate the presence of a conservative aa substitution; stops indicate a semi-conservative aa substitution. (D,E) Percentage variability among isoforms was determined by sliding window analysis on the predicted aa sequence (D) and cDNA sequence (E). Sliding window analysis used overlapping windows of 50 to assess variability.

remained elevated compared to the freshwater controls throughout the 15 day post-transfer period.

Gill Na⁺/K⁺-ATPase enzyme activity

Gill Na^+/K^+-ATPase activity did not change following transfer from freshwater to either freshwater or 40% seawater (Fig. 6). Furthermore, gill Na^+/K^+-ATPase activity was unaffected during the first 5 days following transfer from freshwater to 80% seawater; however, compared to the freshwater controls, gill Na^+/K^+-ATPase activity increased 1.9- and 2.4-fold at 10 and 15 days post-transfer, respectively (Fig. 6).

Na⁺/K⁺-ATPase α-isoform expression

Of the four Na^+/K^+-ATPase α-isoforms found in freshwater trout gills, α1a was expressed at the highest level (28.4±5.1-fold greater than EF-1α; *N*=8), followed by α1b, α1c and α3, which were expressed at 6.3±1.2, 0.13±0.03 and 0.000023±0.000009, respectively (expressed relative to EF-1α). Following seawater transfer, two Na^+/K^+-ATPase α-isoforms (α1a and α1b) were differentially expressed in trout gills (Fig. 7A,B), while the other two isoforms (α1c and α3) were found not to respond to salinity transfer (Tables 3 and 4). Furthermore, using qRT-PCR, we verified that Na^+/K^+-ATPase α2 expression did not change following seawater transfer (data not shown). Na^+/K^+-ATPase α1a mRNA was expressed at high levels in trout held in freshwater and dramatically decreased within 1 day following transfer from freshwater to 40% and 80% seawater (Fig. 7A). In contrast, trout transferred from freshwater to 80% seawater experienced a transient increase in Na^+/K^+-ATPase α1b mRNA compared to the freshwater controls (Fig. 7B). Transfer of trout from freshwater to 40% seawater did not affect gill Na^+/K^+-ATPase α1b mRNA for the first 5 days post-transfer, but significant decreases in gill Na^+/K^+-ATPase α1b mRNA were observed at 10 and 15 days post-transfer (Fig. 7B).

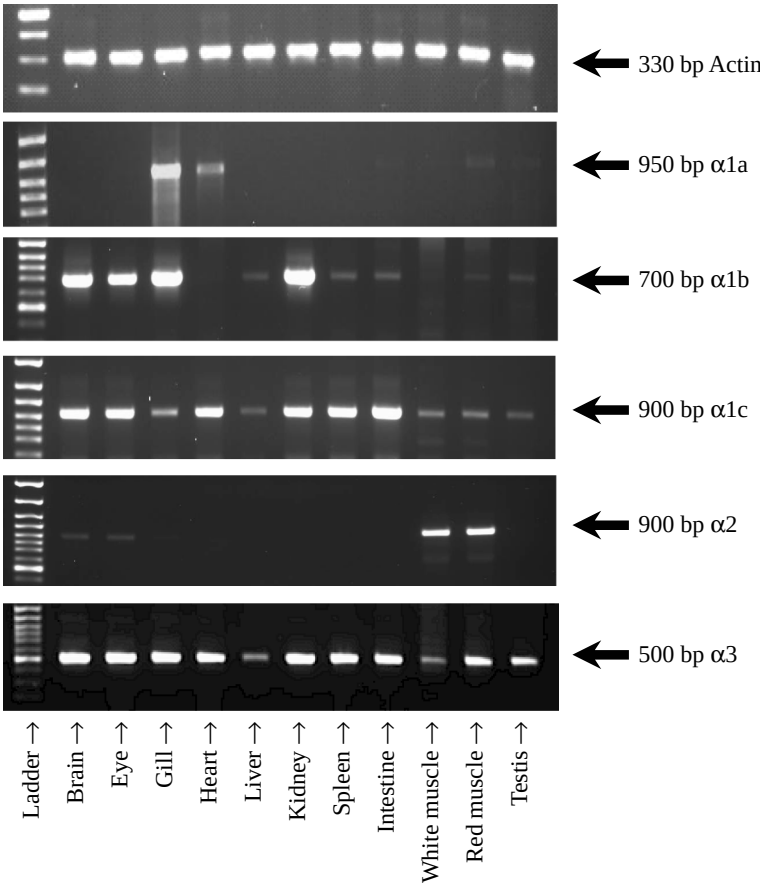

Fig. 3. Tissue distribution of Na^+/K^+-ATPase α1a, α1b, α1c, α2 and α3 isoforms in brain, eye, gill, heart, liver, kidney, spleen, intestine, white muscle, red muscle and testis. Tissue distribution was determined using isoform-specific PCR (see text for more details) and ethidium bromide stained gels. Ethidium bromide stained gels give qualitative estimates of expression level, not quantitative. Actin is included as an internal control.

Table 3. *Na^+/K^+-ATPase α1c-mRNA expression in gills of rainbow trout before (Pre) and 8 h, 5 days and 15 days following abrupt transfer to freshwater, 40% seawater and 80% seawater*

	Expression of Na^+/K^+-ATPase α1c (relative to EF-1α)			
Treatment	Pre	8 h	5 days	15 days
Freshwater	1.00±0.11	1.38±0.24	1.57±0.05	1.05±0.08
40% seawater	1.00±0.05	1.15±0.10	1.69±0.05	1.18±0.13
80% seawater	1.00±0.13	1.30±0.30	1.89±0.11	1.19±0.11

Na^+/K^+-ATPase α1c mRNA expression is relative to EF-1α expression, and all data following the transfer are relative to the pre-transfer freshwater gill samples.

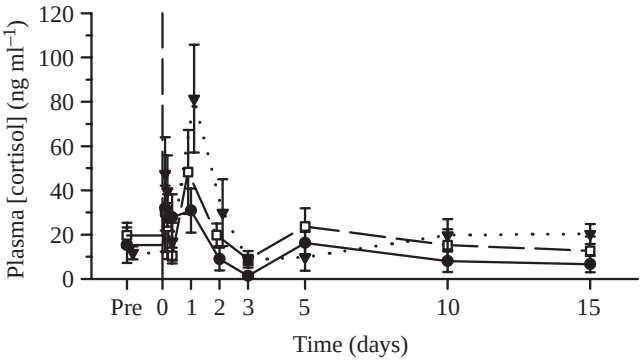

Fig. 4. Plasma cortisol concentrations in trout held in freshwater (Pre) and following transfer to freshwater (filled circles), 40% seawater (open squares) and 80% seawater (filled triangles). Vertical broken line represents the abrupt salinity transfer. Symbols are offset for clarity where necessary. Data are means ± S.E.M. (*N*=5–8).

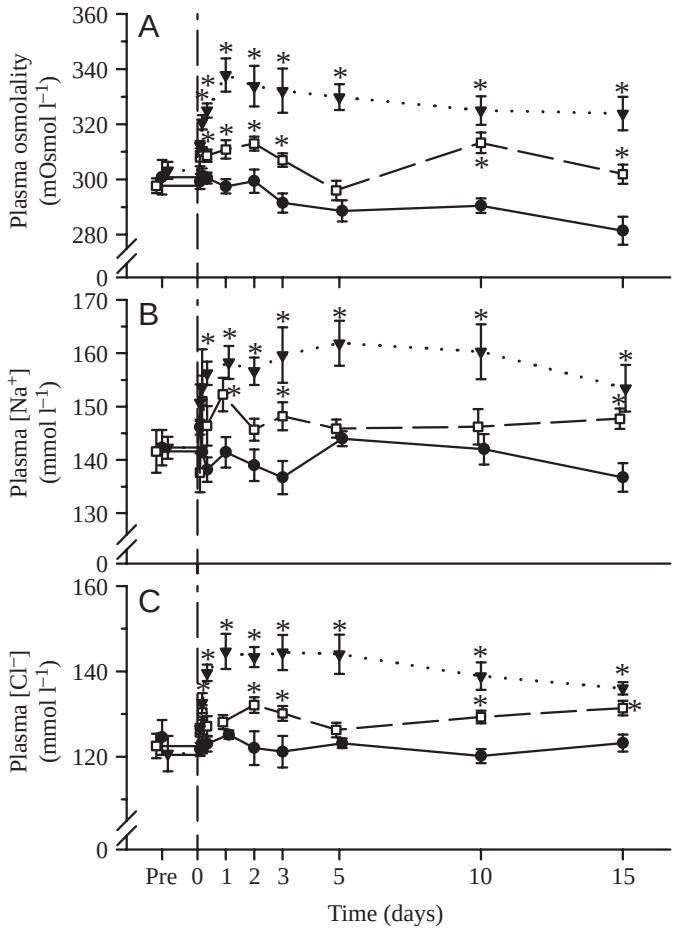

Fig. 5. (A) Plasma osmolality, (B) [Na^+] and (C) [Cl^-] in trout held in freshwater (Pre) and following transfer to freshwater (filled circles), 40% seawater (open squares) and 80% seawater (filled triangles). Vertical broken line represents the abrupt transfer. Symbols are offset for clarity where necessary. Data are means ± S.E.M. (*N*=5–8). Asterisks indicate significant difference from the control (freshwater) value.

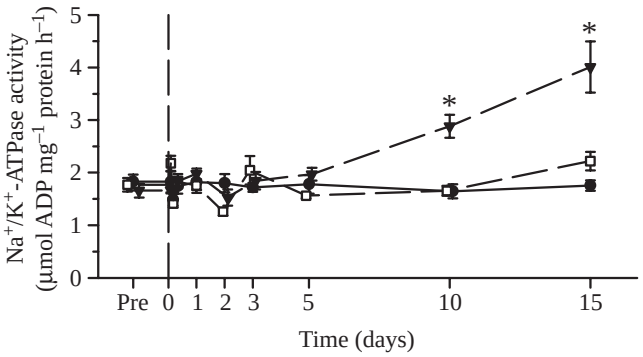

Fig. 6. Gill Na^+/K^+-ATPase activity in trout held in freshwater (Pre) and following transfer to freshwater (filled circles), 40% seawater (open squares) and 80% seawater (filled triangles). Vertical broken line represents the abrupt transfer. Symbols are offset for clarity where necessary. Data are means ± S.E.M. (*N*=8). Asterisks indicate significant difference from the control (freshwater) value.

Discussion

We identified five complete Na^+/K^+-ATPase α-isoform cDNA sequences in rainbow trout. Complete cDNA sequences of Na^+/K^+-ATPase α-isoforms have previously been identified in several teleost fish including white sucker *Catostomus commersoni* (α1; Schonrock et al., 1991), European eel *Anguilla anguilla* (α1; Cutler et al., 1995a), the notothenid *Trematomus bernacchii* (α1, α2, α3; Guynn et al., 2002) and *Fundulus heteroclitus* (α1, α2; Semple et al., 2002). Nine Na^+/K^+-ATPase α-isoforms have been identified in zebrafish *Danio rerio* (Rajarao et al., 2001), of which six are closely related to vertebrate α1-isoforms, two are related to α3-isoforms, and one is related to α2-isoforms. Partial Na^+/K^+-ATPase α-subunit clones have also been identified in rainbow trout (Kisen et al., 1994) and the spiny dogfish *Squalus acanthias* (α3; Hansen, 1999). However, the physiological roles of these Na^+/K^+-ATPase α-isoforms in fish have not yet been assessed.

Since the discovery of Na^+/K^+-ATPase α-isoforms in vertebrate tissues (Sweadner, 1979), considerable effort has focused on elucidating the biochemical differences among isoforms and the underlying molecular basis for these differences (see Blanco and Mercer, 2001). Two areas of high variability were present among the trout Na^+/K^+-ATPase α-isoforms. One area of high variability was located within the first 40 aa from the N terminus (Fig. 2A), and a second area of high variability was within a central 12 aa region starting approximately 490 aa from the N terminus (Fig. 2B). The functional significance of variation in the N-terminal and central isoform-specific regions has not been fully characterized; however, there is consensus that these regions may be involved in the regulation of Na^+/K^+-ATPase activity by protein kinases C (PKC; Efendiev et al., 2000; Pierre et al., 2002). Within the N-terminal region of rat Na^+/K^+-ATPase α1-isoform, the specific targets of PKC phosphorylation are Ser 16 and Ser 23 (Efendiev et al., 2000). Trout Na^+/K^+-ATPase α1a-, α1b-, α1c- and α3-isoforms all possess Ser 16, but only α1a and α1b possess a serine close to position 23 (Ser 24). Furthermore, there are several amino acids that differ among trout isoforms in the central isoform-specific region. These sequence differences may allow for isoform-specific regulation of Na^+/K^+-ATPase activity by PKC.

Further insight into the physiological roles of the trout Na^+/K^+-ATPase α-isoforms can be gained from their tissue distribution (Mobasheri et al., 2000). In rats, the Na^+/K^+-ATPase α1-isoform is ubiquitously expressed, while Na^+/K^+-ATPase α2 and α3 are expressed only in muscle and excitable tissues, respectively (Mobasheri et al., 2000). Na^+/K^+-ATPase α4 is found only in rat testis (Woo et al., 2000). Based upon tissue distribution, the rat Na^+/K^+-ATPase α1-isoform is thought to be a 'housekeeping' enzyme, while Na^+/K^+-ATPase α2-, α3- and α4-isoforms are thought to fulfil tissue-specific physiological functions. The tissue distributions of trout Na^+/K^+-ATPase α1c and α2 are in good agreement with the tissue distribution patterns of rat α1 and α2, respectively.

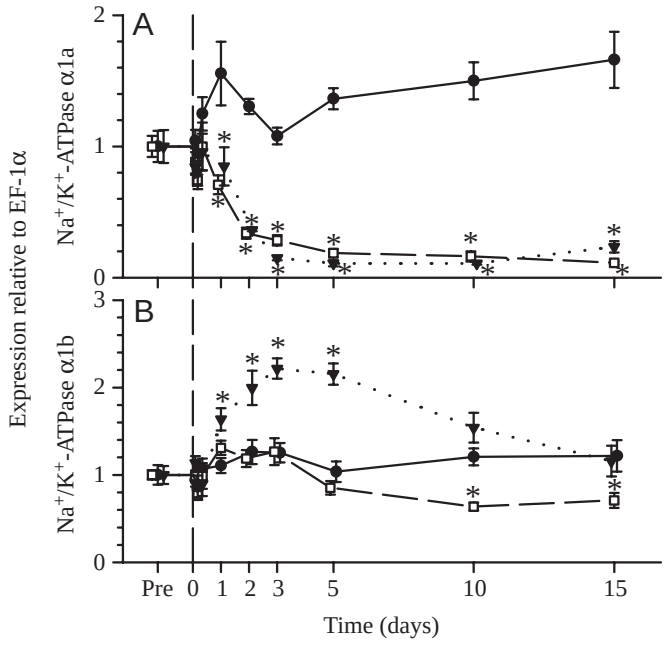

Fig. 7. Gill Na^+/K^+-ATPase (A) α1a and (B) α1b mRNA in trout held in freshwater (Pre) and following transfer to freshwater (filled circles), 40% seawater (open squares) and 80% seawater (filled triangles). mRNA expression is normalized to the control gene, EF-1α, and all data following the transfer are expressed relative to the pre-transfer freshwater gill samples. Vertical broken line represents the abrupt transfer. Symbols are offset for clarity where necessary. Asterisks indicate significant difference from the control (freshwater) value.

However, trout Na^+/K^+-ATPase α1a and α1b show a restricted tissue distribution and unlike the rat, trout Na^+/K^+-ATPase α3 shows a ubiquitous tissue distribution (Fig. 3) and no α4 isoform was identified in trout tissues (Fig. 1). Therefore, by analogy to the rat, trout Na^+/K^+-ATPase α1c and α3 probably function as 'housekeeping' enzymes, while trout Na^+/K^+-ATPase α1a, α1b and α2 are likely to have specific physiological functions. For example, Na^+/K^+-ATPase α2 is found predominately in muscle, suggesting that this isoform may have the kinetic properties appropriate for supporting excitation-contraction coupling. Na^+/K^+-ATPase α1a and α1b are found in a number of common tissues including gill and red muscle, but no precise functional role can be ascribed from the tissue distribution (Fig. 3).

During seawater transfer, many anadromous and euryhaline fish increase their gill Na^+/K^+-ATPase enzyme activity to facilitate ion secretion against a concentration gradient (Marshall, 2002). These increases in gill Na^+/K^+-ATPase activity are, in general, either preceded by or accompanied by increases in Na^+/K^+-ATPase α-subunit mRNA. Specifically, increases in Na^+/K^+-ATPase α-subunit mRNA during salinity transfer have been shown in Atlantic salmon *Salmo salar* (D'Cotta et al., 2000; Singer et al., 2002), brook trout *Salmo trutta* (Madsen et al., 1995; Seidelin et al., 2000), the European eel (Cutler et al., 1995a), and the European sea bass

Table 4. *Na^+/K^+-ATPase α3-mRNA expression in gills of rainbow trout before (Pre) and 8 h, 5 days and 15 days following abrupt transfer to freshwater, 40% seawater and 80% seawater*

	Expression of Na^+/K^+-ATPase α3 (relative to EF-1α)			
Treatment	Pre	8 h	5 days	15 days
Freshwater	1.00±0.15	1.44±0.22	1.67±0.67	0.85±0.11
40% seawater	1.00±0.07	1.10±0.37	1.67±0.77	0.80±0.10
80% seawater	1.00±0.07	1.10±0.28	1.07±0.17	1.24±0.38

Na^+/K^+-ATPase α3 mRNA expression is relative to EF-1α expression, and all data following the transfer are relative to the pre-transfer freshwater gill samples.

Dicentrarchus labrax (Jensen et al., 1998). Increases in Na^+/K^+-ATPase α1 and α3-subunit protein abundance also occur in tilapia gills following seawater transfer (Lee et al., 2003, 1998). However, there is discordance among studies in the degree and timing of these changes in Na^+/K^+-ATPase activity, α-subunit mRNA expression and protein abundance. In addition, few studies have attempted to determine whether Na^+/K^+-ATPase α-isoforms play a role in facilitating freshwater or seawater acclimation.

The changes in plasma osmolality, $[Na^+]$, $[Cl^-]$ (Fig. 5A–C) and gill Na^+/K^+-ATPase activity (Fig. 6) following transfer to 80% seawater are in good agreement with previous studies in rainbow trout and Coho salmon *Oncorhynchus kisutch* during seawater acclimation (Fuentes et al., 1997; Wilson et al., 2002). Transfer of trout from freshwater to 40% seawater had only minor affects on plasma osmolality, $[Na^+]$ and $[Cl^-]$ (Fig. 5A–C) and did not increase gill Na^+/K^+-ATPase activity (Fig. 6). Trout held in 40% seawater probably do not increase Na^+/K^+-ATPase activity because 40% seawater is nearly iso-osmotic with their blood and therefore does not pose severe ionoregulatory stress to fish.

Of the four Na^+/K^+-ATPase α-isoforms expressed in trout gills (α1a, α1b, α1c and α3; Fig. 3), two were differentially regulated in response to seawater transfer. Expression of Na^+/K^+-ATPase α1b mRNA increased in trout gills in response to seawater transfer while the expression of Na^+/K^+-ATPase α1a mRNA decreased in response to seawater transfer. The present study is the first to demonstrate isoform switching during seawater transfer and suggests that differential expression of Na^+/K^+-ATPase α1a- and α1b-isoforms during seawater transfer may be an important feature underlying the transformation of the fish gill from an ion-absorbing epithelium to an ion-secreting epithelium. These results, obtained using isoform-specific qRT-PCR, are in contrast to all previous studies that showed a general increase in Na^+/K^+-ATPase α-subunit mRNA following seawater transfer, similar to the increase observed for Na^+/K^+-ATPase α1b (Fig. 7B). In fact, if we sum our estimates for all Na^+/K^+-ATPase α-isoforms monitored, we predict, based upon our quantitative estimates of Na^+/K^+-ATPase α-isoform expression, that there

would be 3.5- and 2.2-fold decreases in total Na^+/K^+-ATPase α-subunit expression (relative to EF-1α) 3 days following transfer from freshwater to 40 and 80% seawater, respectively. Similarly, at 15 days following transfer of trout from freshwater to 40 and 80% seawater, we predict that total Na^+/K^+-ATPase α-subunit expression would be 34- and 17-fold lower than in the freshwater controls. Clearly, previous studies using northern blot analysis were biased toward monitoring a Na^+/K^+-ATPase α1b-like isoform in fish gills during seawater transfer and thus do not provide a complete picture of the role Na^+/K^+-ATPase in freshwater and seawater acclimation.

Despite decreases in total Na^+/K^+-ATPase α-isoform mRNA in trout gills following transfer from freshwater to 80% seawater, there was a 2.4-fold increase in gill Na^+/K^+-ATPase activity at 15 days (Fig. 6). The increase in gill Na^+/K^+-ATPase activity following transfer to 80% seawater was probably initiated by the transient increase in Na^+/K^+-ATPase α1b mRNA expression (Fig. 7A) and sustained by translational controls. Furthermore, post-translational modification of Na^+/K^+-ATPase (e.g. Na^+/K^+-ATPase γ-subunit binding; Béguin et al., 1997; Therien et al., 1999) may also contribute to the sustained elevation of Na^+/K^+-ATPase activity following seawater transfer.

Na^+/K^+-ATPase α1a is expressed at high levels in the freshwater fish gill and decreases within 1 day following transfer from freshwater to 40% and 80% seawater. This downregulation of Na^+/K^+-ATPase α1a during seawater transfer suggests that this isoform may have kinetic properties favourable for ionregulation in freshwater. Cation binding affinity to Na^+/K^+-ATPase is modulated by differences in the amino acid residues present in the 4th, 5th and 6th transmembrane domains of the α-subunit (Mobasheri et al., 2000). Among the trout Na^+/K^+-ATPase α-isoforms, the amino acid sequences of the 5th and 6th transmembrane domains are highly variable, while the amino acid sequence of the 4th transmembrane is well conserved. For the most part, the differences between trout Na^+/K^+-ATPase α-isoforms tend to separate Na^+/K^+-ATPase α1a from the other four isoforms. In particular, within the 5th predicted transmembrane domain (Fig. 2B), 7 out of 21 amino acids differ in Na^+/K^+-ATPase α1a compared to the other four isoforms. It is tempting to speculate that these differences in amino acid sequence in Na^+/K^+-ATPase α1a may alter cation binding affinity and facilitate Na^+ and Cl^- uptake from freshwater.

Gene expression in fish gills during seawater transfer is thought to be primarily mediated by changes in circulating hormones (McCormick, 1995). Following seawater transfer, many studies have reported increases in plasma [cortisol] (Marshall et al., 1999; McCormick, 2001; Wilson et al., 2002), which has been implicated in initiating changes in gene expression. For example, a large and rapid (1 h) increase in plasma [cortisol] was found to precede an increase in cystic fibrosis transmembranse conductance regulator (CFTR) expression in *Fundulus heteroclitus* gills following seawater transfer (Marshall et al., 1999; Singer et al., 1998). In the present study, seawater transfer did not significantly affect plasma [cortisol]; however, at 1 day post-transfer, there was a highly variable, salinity-dependent increase in plasma [cortisol]. This variable increase in plasma [cortisol] agrees well with a secondary spike in [cortisol] observed by Marshall et al. (1999) and coincides with changes in Na^+/K^+-ATPase α1a and α1b mRNA in trout gills following seawater transfer (c.f. Figs 4 and 7A,B). Therefore, it is possible that changes in plasma cortisol may be involved in the regulation Na^+/K^+-ATPase α1a and α1b transcription in trout. It should be noted, however, that additional hormones are known to change in response to salinity transfer (e.g. insulin-like growth factor, growth hormone; McCormick, 1996), and may influence Na^+/K^+-ATPase gene expression.

Changes in gill morphology during salinity transfer may also influence the pattern of Na^+/K^+-ATPase gene expression. Trout gills are composed of multiple cell types including respiratory pavement cells and ion-regulating mitochondria-rich (MR; chloride) cells. Recently, two distinct populations of MR cells have been isolated from trout gills (Galvez et al., 2002; Goss et al., 2001) and there is accumulating evidence that these two MR cell populations may differ in their complement of ion transporters; thus it seems likely that these two cell populations may be differentially proliferated during freshwater and seawater acclimation. Differential changes in cell proliferation during salinity transfer may explain some of the observed changes in Na^+/K^+-ATPase α-isoform expression (Fig. 7A,B). For example, if Na^+/K^+-ATPase α1a is expressed in only the MR-cell type that proliferates in freshwater and decreases in number or size during seawater transfer, then decreases in whole gill Na^+/K^+-ATPase α1a mRNA may reflect changes in cell proliferation and not changes in global gene expression. Future research should examine the relationship between Na^+/K^+-ATPase α1-isoform expression and MR-cell population during salinity transfer.

In conclusion, in the present study we identified five Na^+/K^+-ATPase α-isoforms in rainbow trout and showed that each isoform had a tissue specific distribution pattern. Two of the Na^+/K^+-ATPase α-isoforms (α1a and α1b) were differentially expressed in gills during transfer from freshwater to 80% seawater. Expression of Na^+/K^+-ATPase α1b was upregulated in response to seawater acclimation suggesting a role in ion secretion, while expression of Na^+/K^+-ATPase α1a was downregulated in response to seawater acclimation, suggesting a role in ion uptake from freshwater. Clearly, Na^+/K^+-ATPase α-isoform switching during seawater transfer provides new insight into the importance of this gene in fish ion regulation.

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