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CLONING AND MOLECULAR CHARACTERISATION OF THE TROUT (*ONCORHYNCHUS MYKISS*) VACUOLAR H^+-ATPase B SUBUNIT

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Summary

The current model of transepithelial ion movements in the gill of freshwater fish incorporates an apically oriented vacuolar H^+-ATPase (H^+V-ATPase; proton pump) that is believed to facilitate both acid excretion and Na^+ uptake. To substantiate this model, we have cloned and sequenced a cDNA encoding the B subunit of the rainbow trout (*Oncorhynchus mykiss*) H^+V-ATPase. The cloning of the B subunit enabled an examination by northern analysis of its tissue distribution and expression during external hypercapnia.

Degenerate oligonucleotide primers to the B subunit of the H^+V-ATPase were designed and used in a semi-nested polymerase chain reaction (PCR) to amplify an 810 base pair (bp) product from a trout gill/kidney cDNA library. This PCR product was cloned and sequenced and then used to screen the same cDNA library. The assembled 2262 bp cDNA included an open reading frame coding for a deduced protein of 502 amino acid residues. A BLAST search of the GenBank nucleotide database revealed numerous matches to other vertebrate and invertebrate H^+V-ATPase B subunits. Protein alignment demonstrated that the trout H^+V-ATPase B subunit is more than 85 % identical and more than 90 % similar to those in other vertebrate species.

An initial analysis of H^+V-ATPase mRNA tissue distribution revealed significant expression in blood. Although a comparison of perfused tissues (blood removed) with non-perfused tissues demonstrated no obvious contribution of the blood to total tissue H^+-ATPase mRNA levels, all subsequent experiments were performed using perfused tissues. Levels of H^+V-ATPase mRNA expression were high in the gill, kidney (anterior or posterior), intestine, heart and spleen, but lower in liver and white muscle. Exposure of the fish to 12 h of external hypercapnia (water P_{CO_2}=7.5 mmHg; 1 kPa) was associated with a transient increase (at 2 h) in the levels of H^+V-ATPase B subunit mRNA in gill and kidney; liver mRNA levels were unaffected.

These results are consistent with the hypothesis of an apically localised plasma membrane H^+V-ATPase in the freshwater trout gill and that the expression of this proton pump is increased during periods of acidosis, at least in part because of an increased steady-state level of H^+V-ATPase mRNA.

Key words: proton pump, H^+-ATPase, gill, kidney, hypercapnia, acidosis, cDNA, mRNA, northern analysis, rainbow trout, *Oncorhynchus mykiss*.

Introduction

The vacuolar H^+-ATPase (H^+V-ATPase) is expressed ubiquitously in eukaryotic cells as a housekeeping enzyme that acidifies intracellular organelles (Nelson, 1992). Within the animal kingdom, the H^+V-ATPase is also expressed specifically on the plasma membrane of various ion-transporting epithelia or acid-secreting cells (for reviews, see Stevens and Forgac, 1997; Forgac, 1998; Nelson and Harvey, 1999; Wieczorek et al., 1999a). These tissues include the insect midgut and Malphigian tubules (Klein et al., 1991; Novak et al., 1992; Bertram and Wessing, 1994; Wieczorek et al., 1999b), amphibian (Brown et al., 1987) and reptile (Steinmetz, 1988) bladder, amphibian skin (Harvey, 1992; Jensen et al., 1997; Ehrenfeld and Klein, 1997; Ehrenfeld, 1998), mammalian kidney (Brown et al., 1988; Gluck, 1992; Gluck and Nelson, 1992), rat epididymis (Brown et al., 1997), bovine corneal epithelium (Torres-Zamorano et al., 1992) and mammalian osteoclasts (Laitala-Lenonen et al., 1996). In more recent years, the gills of freshwater teleosts (Lin and Randall, 1993; Laurent et al., 1994; Sullivan et al., 1995) and crustaceans (Zare and Greenaway, 1998) have also been identified as sites of plasma membrane H^+V-ATPase activity.

The gill is an important site of ionic and acid–base regulation. In freshwater fish, the uptake of Cl^- and Na^+ from the environment is linked to equimolar excretion of basic (e.g. HCO_3^-) and acidic (e.g. H^+) equivalents, respectively (Heisler, 1984, 1986, 1989, 1993; Goss et al., 1992b; Marshall, 1995; Claiborne, 1998). Thus, acid–base balance and ionic regulation are intricately linked in freshwater fish (McDonald et al., 1989,

1991; Cameron and Iwama, 1989; Wood and Goss, 1990; Wood, 1991). The exact mechanisms underlying this linkage, however, have not yet been established, although several working models have been proposed. For Na^+ uptake, the traditional view (Krogh, 1938) of an apically located electroneutral Na^+/H^+ antiporter has been revised to a model (Avella and Bornancin, 1989) favouring apical membrane Na^+ channels that are energised by an H^+V-ATPase. According to this scheme, the outward pumping of H^+ across the apical membrane of gill epithelial cells creates a favourable electrochemical gradient for the inward diffusion of Na^+ through Na^+ channels (Lin and Randall, 1995; Perry and Fryer, 1997). Evidence for this model has arisen from theoretical arguments (Avella and Bornancin, 1989; Potts, 1994), pharmacological studies (Lin and Randall, 1991) and the detection of H^+-ATPase activities in the gill using biochemical (Lin and Randall, 1993; Kultz and Somero, 1995) and histological (Laurent et al., 1994; Lin et al., 1994; Sullivan et al., 1995, 1996; Wilson et al., 1997) methods.

The activity of the rainbow trout branchial H^+V-ATPase is increased during respiratory acidosis (Lin and Randall, 1993; Sullivan et al., 1995), presumably as a mechanism to modulate internal pH. The increased H^+V-ATPase activity could reflect transcriptional or post-transcriptional regulation of mRNA levels and/or translational or post-translational regulation of H^+V-ATPase protein activity/localisation. Evidence in support of transcriptional/post-transcriptional regulation was provided by Sullivan et al. (1996), who demonstrated, using *in situ* hybridisation, that the levels of H^+V-ATPase mRNA were increased during exposure of rainbow trout to external hypercapnia. That study, however, was limited by the use of a heterologous oligonucleotide probe derived from the sequence of bovine renal H^+V-ATPase E subunit. Similarly, previous immunocytochemical studies of trout gill H^+V-ATPase have been constrained by the reliance on heterologous antibodies (Lin et al., 1994; Sullivan et al., 1995). The production of homologous probes and antibodies for the trout gill H^+V-ATPase has been restricted by a lack of information on the molecular structure of the trout gene. Thus, the goals of the present study were to clone and characterise the B subunit of the trout H^+V-ATPase and to use a homologous probe to examine by northern analysis the expression and tissue distribution of the B subunit mRNA during external hypercapnia.

Materials and methods

Experimental animals

Rainbow trout [*Oncorhynchus mykiss* (Walbaum); 200–300 g, less than 2 years old] of both sexes were obtained from Linwood Acres Trout Farm. All fish were kept in large fibreglass tanks supplied with flowing, aerated and dechlorinated city of Ottawa tapwater (14 °C). Fish were maintained on a 12 h:12 h light:dark photoperiod and were fed *ad libitum* on alternate days using a commercial pelleted fish diet but were not fed for 48 h prior to experimentation.

Surgical procedures

Fish were anaesthetised in a 1:12 000 (mass:volume) solution of benzocaine (ethyl-*p*-aminobenzoate) cooled to 10 °C. After cessation of breathing movements, the fish were transferred to an operating table and the gills were irrigated with the same anaesthetic solution throughout surgery. An indwelling polyethylene cannula (Clay-Adams PE 50; i.d. 0.58 mm, o.d. 0.97 mm) was inserted into the dorsal aorta according to the basic method of Soivio et al. (1975). After surgery, the fish were revived and allowed to recover for 24 h while constrained in opaque acrylic boxes supplied with flowing water (>2 l min^{-1}).

cDNA library construction and screening

To increase the expression of mRNA for the H^+V-ATPase and for other genes involved in ionic and acid–base regulation (for future studies), different fish were subjected to a variety of treatments prior to the harvesting of tissue. Treatments included 24 h of external hypercapnia (N=2), intra-arterial infusion of $NaHCO_3$ for 24 h (N=2), 1 week of intramuscular cortisol (4 mg kg^{-1}; hemisuccinate salt) injections (N=4) or exposure to ion-poor water for 1 week (N=4). Hypercapnia was achieved by gassing a water equilibration column with 1.3 % CO_2 in air (Cameron flowmeter) to reach a final water P_{CO_2} (Pw_{CO_2}) of approximately 7.5 mmHg (1 kPa). Pw_{CO_2} was monitored continuously using a CO_2 electrode and associated meter (Cameron Instruments Inc.). Deviations in Pw_{CO_2} from the target of 7.5 mmHg were corrected by adjustments of gas and/or water flows through the equilibration column. To induce metabolic alkalosis, iso-osmotic $NaHCO_3$ (140 mmol l^{-1}) was infused *via* the dorsal aorta cannula at a rate of 800 μmol kg^{-1} h^{-1} (Goss and Wood, 1990) using an infusion pump. Ion-poor water was prepared by mixing tapwater with deionised water derived from a reverse osmosis unit. Normal tapwater and the ion-poor water were analysed daily for Na^+, Ca^{2+} and K^+ levels using flame emission spectroscopy (Varian model 250 atomic absorption spectrophotometer). For normal tapwater, the ion concentrations (in mmol l^{-1}) were Na^+, 0.135; Ca^{2+}, 0.391; K^+, 0.025. For ion-poor water, the ion concentrations (in mmol l^{-1}) were Na^+, 0.026–0.054; Ca^{2+}, 0.014–0.033; K^+, 0.005. Fish were not fed during the duration of the ion-poor water treatment.

Fish were killed by a blow to the head, and the gill and kidney tissues were harvested and immediately frozen in liquid nitrogen prior to storage at –80 °C. Total RNA was isolated as described below from a mixture of gill and kidney tissues obtained from the four treatment groups. A random-primed cDNA library was prepared from poly(A^+) RNA in the cDNA vector λ-Zap Express by Strategene Inc. The amplified library represented 10^6 recombinants with an average insert size of 1.0 kb (information supplied by Stratagene).

Protocols for media preparation, plaque growth, plaque lifts and hybridization and the *in vitro* excision of the pBK-CMV phagemid were taken from the instruction manual that accompanied the λ-Zap Express *Eco*RI library.

Polymerase chain reaction (PCR) using library cDNA as template

Degenerate primers were designed for semi-nested PCR on the basis of sequence comparisons of selected vertebrate (Bernasconi et al., 1990; Nelson et al., 1992; Bartkiewicz et al., 1995; Laitala-Lenonen et al., 1996) and invertebrate (Novak et al., 1992; Davies et al., 1996) H^+V-ATPase B subunit protein sequences available in GenBank. For the first round of PCR amplification, the forward 5′ primer (VA1) was the 20mer 5′-TGYGARTTYACNGGNGAYAT-3′, and the reverse 3′ primer (VA7) was the 20mer 5′-TTCATNGCY-TGNACRTCRTT-3′. These primers correspond to amino acid residues CEFTGDI (positions 106–112) and KDVQAMK (positions 424–430) in the bovine kidney H^+V-ATPase B subunit sequence (Nelson et al., 1992; GenBank accession no. AAA30394). For the second PCR reaction (see below), the forward 5′ primer (VA3) was the 20mer 5′-GARGARATGATHCARACNGG-3′, and the reverse 3′ primer remained VA7. Primer VA3 corresponds to amino acid residues EEMIQTG (positions 161–167 in the bovine kidney H^+V-ATPase B subunit). The positions of these three primers on the trout sequence are depicted in Fig. 1.

PCR was performed in 50 µl reaction mixtures containing 3.5 mmol l^{-1} $MgCl_2$, 200 µmol l^{-1} of each dNTP, 250 nmol l^{-1} of each primer and 1 unit of Taq polymerase (Life Technologies) in PCR buffer supplied with the enzyme. Following a denaturation step of 94 °C for 5 min, the reaction was subjected to 30 cycles of 94 °C for 0.5 min, 52 °C for 1 min and 72 °C for 1.5 min, with a final extension for 5 min at 72 °C in a PTC-100 (M. J. Research) thermal cycler. For the first round of amplification, 10 µl of the amplified cDNA library phage lysate was used as template with the primers VA1 and VA7. For the second round, 2 µl of the first PCR reaction was used as template with primers VA3 and VA7. This yielded a PCR product of the expected size (810 bp).

The PCR product was cloned into pCR II-TOPO vector (TOPO II TA cloning kit; Invitrogen) and sequenced. A search of GenBank revealed that the cloned cDNA (termed SFP1) exhibited approximately 85 % nucleotide identity with other known vacuolar H^+V-ATPase B subunits. Furthermore, the pSFP1 insert hybridised with cloned B subunit cDNA of *Drosophila melanogaster* (clone C10 of VHA55 courtesy of Dr Julian Dow).

RNA isolation, gel electrophoresis and northern analysis

Total RNA was isolated by homogenisation in guanidinium thiocyanate (Chomcyzynski and Sacchi, 1987) as modified by Chomcyzynski and Mackey (1995).

Northern analysis was performed using the cDNA insert from SFP1 plasmid (pSFP1) as the probe. RNA samples (20 µg) were incubated in loading buffer at 65 °C and electrophoresed through 1.5 % (w/v) agarose gels in Mops buffer containing 0.6 mol l^{-1} formaldehyde, as described by Sambrook et al. (1989), and then transferred to GeneScreen+ membranes (NEN Life Sciences) by capillary action (Sambrook et al., 1989). Membranes were prehybridised at 65 °C for 2–4 h in a buffer containing 6× SSC (0.9 mol l^{-1} NaCl, 0.09 mol l^{-1} sodium citrate, pH 7.0), 5× Denhardt's (1× Denhardt's is 0.1 % Ficoll 400 000, 0.1 % polyvinylpyrrolidone, 0.1 % bovine serum albumin), 100 µg ml^{-1} single-stranded herring sperm DNA, 1 % sodium dodecylsulphate (SDS) and 10 % dextran sulphate (Amersham Pharmacia Biotech). After the addition of probe to a concentration of 10^6 to 2×10^6 cts min^{-1} ml^{-1}, hybridisation proceeded for 16 h at 65 °C in the same solution. Following hybridisation, the membranes were washed several times at 65 °C with 0.1× SSC, 0.1 % SDS and exposed to BioMax film plus intensifying screen (Kodak) at −80 °C for up to 4 days.

To confirm equal loading between samples, membranes were re-probed with a salmon (*Oncorhynchus kisutch*) 18S rRNA probe (Hervio et al., 1997; courtesy of Dr Robert H. Devlin) under similar conditions but with an exposure time of hours.

DNA protocols

All DNA restriction, agarose gel electrophoresis, ligations and sub-cloning steps were performed using standard procedures (Sambrook et al., 1989). DNA transformation into competent DH5αF′ (Life Sciences) was according to Hanahan (1985).

DNA fragments were isolated from agarose gels by centrifugation through a 0.45 µm filter followed by purification using a QIAquik purification kit (Qiagen).

Plasmid DNA was isolated using the Wizard kit (Promega). Sequencing of plasmid DNA employed the Thermosequenase kit (Amersham Pharmacia Biotech) and labelled primers (LI-COR).

DNA for probes was labelled with [α-^{32}P]dCTP (Amersham, 370 MBq ml^{-1}) by the random-primer method (Feinberg and Vogelstein, 1983). Unincorporated nucleotides were removed by spin columns (Sambrook et al., 1989). Prior to use, the labelled DNA was denatured by boiling for 5 min and then rapid chilling in ice.

Southern transfer and hybridisation using aqueous conditions at 65 °C were performed as described in Sambrook et al. (1989). A final wash at 65 °C with 0.1× SSC, 0.1 % SDS was performed to ensure high stringency.

Sequence analysis

GenBank searches were performed with the standard BLAST algorithms at the National Center for Biotechnology Information (NCBI) using the default settings (Altschul et al., 1990). Sequence alignments were carried out on derived protein sequences using CLUSTAL W (Thompson et al., 1994). PHYLIP (Phylogeny Inference Package) version 3.5c (Felsenstein, 1993) was used to construct phylogenetic trees. The aligned sequences were bootstrapped with PHYLIP 3.57c (Felsenstein, 1993) using 'Seqboot', which created 100 multiple data sets. Distances were calculated with 'Protdist' using the PAM-Dayhoff matrix, and trees were created with 'Fitch' using 10 jumbles per data set for a total of 1000 trees. A consensus tree was chosen by 'Consense' and

drawn with Treeview version 1.4 (D. M. Roderic; http://taxonomy.zoology.gla.ac.uk/rod/rod.html) using *Cyanidium caldarium* (red algae) as an outgroup. For routine DNA manipulations such as contig assembly, sequence editing, sequence translation, etc., DNAMAN (Lynnon Biosoft) was used.

Expression of H^+V-ATPase B subunit mRNA as estimated by northern hybridisation

Tissue distribution

Fish were anaesthetised, and a blood sample (3–5 ml) was withdrawn by caudal puncture and was frozen in liquid N_2. After a lethal blow to the head, the following tissues were removed, frozen in liquid N_2 and stored at –80 °C; gill, kidney (anterior and posterior halves), intestine, spleen, liver, heart and white muscle. Total RNA was prepared for gel electrophoresis, northern transfer and northern hybridization as described above.

Initial results demonstrated specific hybridisation in the blood, and therefore the possible contribution of blood H^+V-ATPase mRNA to tissue levels was assessed by comparing perfused and non-perfused tissues. For perfusion, the fish was first injected (*via* the caudal vessels) with a solution of heparin (1000 i.u. of ammonium heparin), anaesthetised and then killed by a blow to head. The fish was placed on ice, the heart was exposed and a polyethylene cannula (Clay Adams PE 160) was inserted through a cut in the ventricle into the bulbus arteriosus and secured with ligatures. The gills and systemic circulation were perfused with 240 ml of ice-cold Cortland saline (Wolf, 1963) over a 5 min period. At this point, the fluid returning to the heart appeared to be bloodless, and inspection of the gills revealed that more than 90 % of the tissue was devoid of blood. Those areas that still retained blood (usually areas of blood clotting) were dissected away and discarded.

Effects of external hypercapnia

Fish were placed into opaque acrylic boxes, allowed to acclimate for 16–24 h and then exposed to external hypercapnia (P_{wCO_2}=7.5 mmHg; 1 kPa) for 2–12 h as described above. At 0, 2, 4, 6 and 12 h, fish (N=2 at each time) were killed and perfused with saline (see above). Gill, total kidney and liver tissues were removed, frozen in liquid N_2 and stored at –80 °C until total RNA was prepared.

The levels of H^+V-ATPase B subunit mRNA in tissues from hypercapnic fish were estimated from northern blots after sequential hybridisation with the insert from pSFP1 (cDNA probe for H^+-ATPase) and 18S rRNA probe (to correct for unequal loading of total RNA). This was achieved by superimposition of the developed X-ray films (one for H^+-ATPase mRNA and one for 18S rRNA) to yield an image suitable for scanning. The films were scanned, saved as Tiff files and imported into a commercial software package (PaintShop Pro 4.0). Using the software, square areas representing the middle of each band were selected at high magnification, and the luminance was determined automatically (a luminance of 0 represents pure black and a luminance of 255 represents pure white). Although luminance under grey-scale conditions varies in a linear fashion from black (0) to white (255), the contrast of the scanned images was adjusted to yield luminance values roughly between 40 and 150. Below and above these values, it was noted that luminance differences, as detected from the software, were not easily discerned by eye. For each total RNA sample, the specific H^+V-ATPase and 18S bands were analysed for luminance in triplicate; these values were averaged and presented graphically as the ratio of H^+V-ATPase/18S luminance. To confirm the validity of this technique, a series of filled boxes of increasing and known luminance were drawn using Powerpoint software. These boxes were printed onto transparent acetates and scanned as described above. The luminance values as measured using PaintShop Pro were then compared with the actual luminance values, yielding a significant linear correlation (r^2=0.99).

Results

A two-step strategy was used to isolate a cDNA encoding the B subunit of the rainbow trout (*Oncorhynchus mykiss*) H^+V-ATPase. In the first step, the available sequence information from cloned B subunits was used to design degenerate primers for semi-nested PCR of a trout gill/kidney cDNA library. An 810 base pair (bp) cDNA fragment was amplified and cloned to produce the plasmid pSFP1. Sequence analysis confirmed the identify of this DNA insert, and it was subsequently used as a probe for all further hybridization experiments. In a second step, the cDNA library was screened, and several hybridizing clones were isolated, analysed and sequenced, thus allowing the assembly of the cDNA contig of 2262 bp (GenBank accession no. AF140022) depicted in Fig. 1. This cDNA included an open reading frame of 1506 bp, encoding a protein of 502 amino acid residues (nucleotide positions 21–1527; Fig. 1). The full-length sequence of the 3′ untranslated region was not obtained, as indicated by the absence of a poly-A tail and polyadenylation signal.

A BLASTn search of the GenBank database produced 19 matches with E values of less than 10^{-50}. Each of these matches corresponded to a vertebrate or invertebrate H^+V-ATPase B subunit and showed 82–83 % nucleotide identity with the trout sequence. The best match was to the H^+V-ATPase B subunit of European eel (*Anguilla anguilla*), which has recently been cloned from swimbladder gas gland tissue (H. Niederstaetter and B. Pelster; GenBank accession no. AF099743). The reported coding sequence of the eel H^+V-ATPase B subunit (1533 bp) is slightly larger than the trout coding sequence (1506 bp; Fig. 1). At the protein level, the trout sequence displays 95 % amino acid identity and 97 % similarity with the eel H^+V-ATPase B subunit. An alignment of the amino acid sequence of the trout H^+V-ATPase B subunit with other known vertebrate and invertebrate sequences (Fig. 2) revealed striking homology, with amino acid identities ranging between 85 % (sea squirt; *Ascidia sydneiensis* Samea) and 95 % (eel; *Anguilla anguilla*).

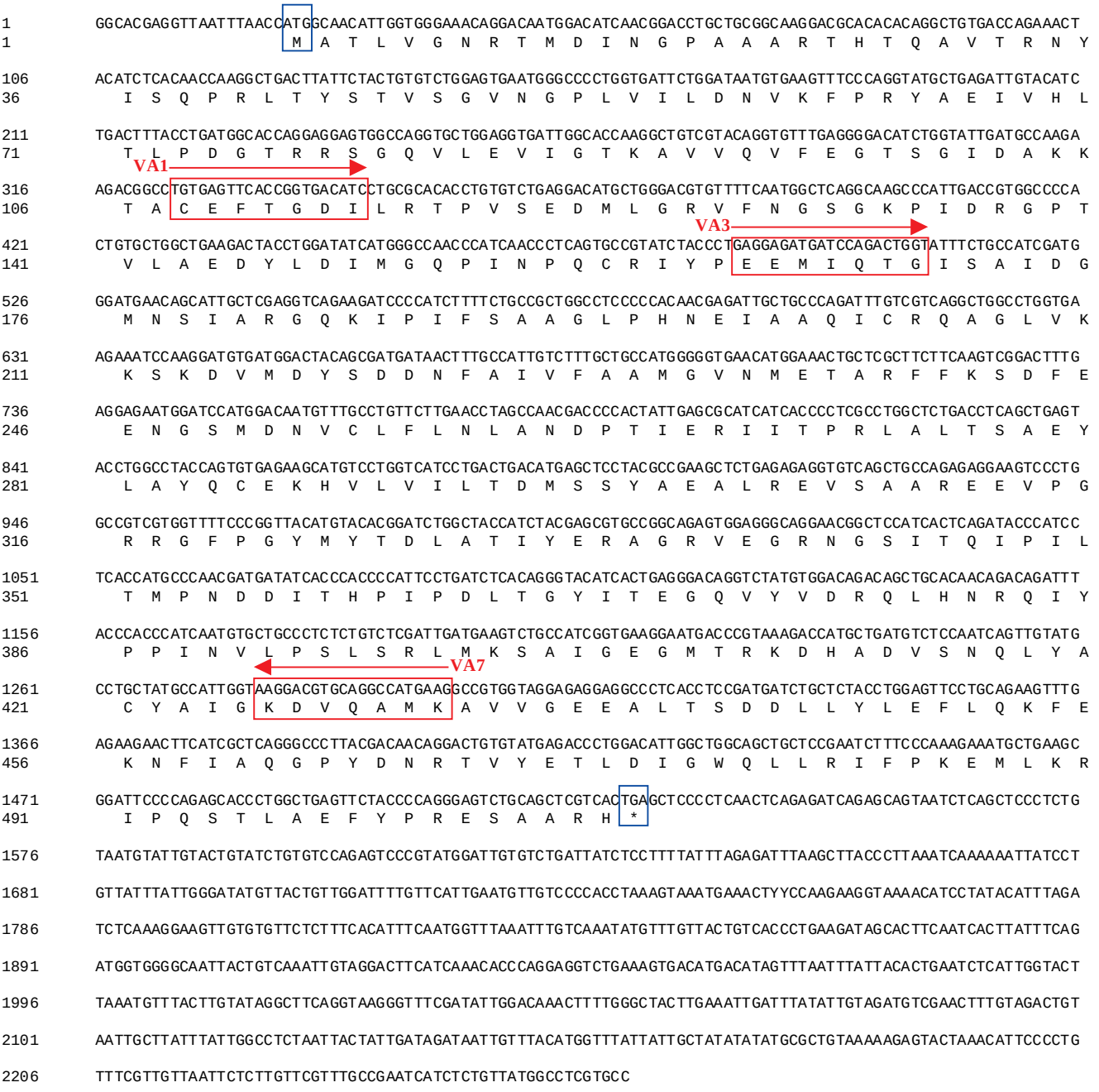


Fig. 1. The nucleotide and translated amino acid sequences of the trout (*Oncorhynchus mykiss*) vacuolar H^+V-ATPase B subunit (GenBank accession no. AF140022). The start and stop codons are indicated by blue boxes. The red boxes indicate the positions of the primers used for semi-nested polymerase chain reaction (PCR; round 1 amplification used primers VA1 and VA7; round 2 amplification used primers VA3 and VA7; see Materials and methods for details). The asterisk indicates the stop codon.

A representative northern blot depicting the tissue distribution of the H^+V-ATPase B subunit mRNA is illustrated in Fig. 3A. Detectable levels of H^+-ATPase B subunit mRNA were observed in all tissues examined. Levels of H^+V-ATPase mRNA expression were high in the gill, kidney (anterior or posterior), intestine, heart and spleen, but lower in liver and white muscle. Qualitatively similar tissue distribution patterns were observed in replicate blots of identical tissues and in tissue derived from four different animals. Although blood exhibited significant hybridisation against the H^+V-ATPase probe, a comparison of non-perfused (containing blood) and perfused (to remove blood) tissues (Fig. 3B) demonstrated no obvious contribution of blood mRNA to total tissue H^+-ATPase mRNA levels. Nevertheless, to ensure that northern blots represented specific tissue mRNA, all subsequent experiments were performed using tissues obtained from perfused fish.

The size of the hybridizing mRNA was found to be 3.2 kb

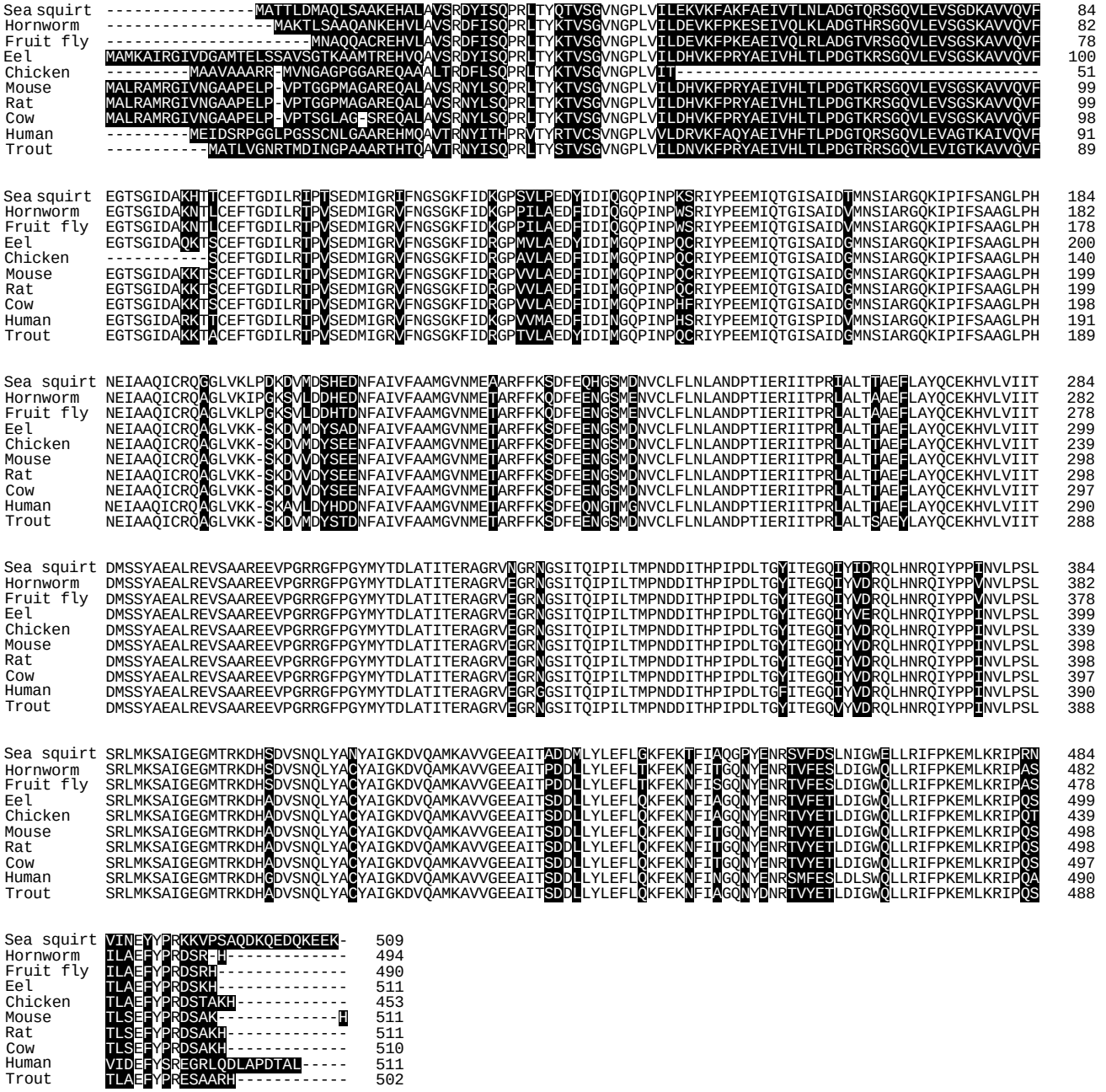
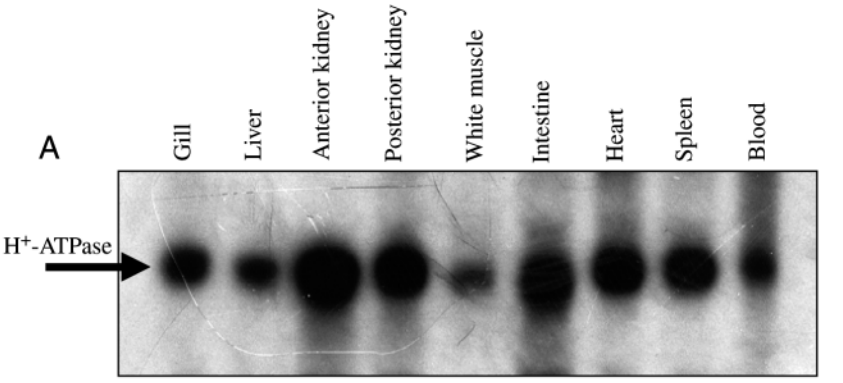


Fig. 2. Protein alignment of the trout (*Oncorhynchus mykiss*; GenBank accession no. AF140022) H^+V-ATPase B subunit with selected vertebrate and invertebrate B subunit sequences, including sea squirt (*Ascidia sydneiensis* Samea, GenBank accession no. AB016484), tobacco hornworm (*Manduca sexta*, GenBank accession no. X64354), fruit fly (*Drosophila melanogaster*, GenBank accession no. X67839), European eel (*Anguilla anguilla*, GenBank accession no. AF099743), chicken (*Gallus gallus*, GenBank accession no. U20766), mouse (*Mus musculus*, GenBank accession no. Y12634), rat (*Rattus norvegicus*, GenBank accession no. Y12635), cow (*Bos taurus*, GenBank accession no. M88690) and human (*Homo sapiens*, GenBank accession no. M25809). Conserved amino acids (100 % identity) among taxa are not highlighted. Sequences were aligned using CLUSTAL W (Thompson et al., 1994), and the alignment was prepared from DNAMAN (Lynnon BioSoft).

in all tissues examined to date. Thus, the trout B subunit mRNA is similar to the size (3.0–3.5 kb) typically found for most vertebrate B subunit mRNAs (Bernasconi et al., 1990; Nelson et al., 1992; Puopola et al., 1992; Bartkiewicz et al., 1995).

Exposure of trout to external hypercapnia for 2 h caused an apparent, transient 2.5-fold increase in H^+V-ATPase B subunit mRNA levels (Fig. 4) in the gill tissue (the low sample size, N=2, precluded statistical confirmation). Within 4 h, H^+V-ATPase mRNA levels had returned to pre-hypercapnia levels and remained stable for the duration of the 12 h experiment. A similar pattern was observed in the kidney tissue (Fig. 5), with an apparent transient 1.5-fold increase in mRNA levels after 2 h of hypercapnia. In contrast, the levels of H^+V-ATPase B



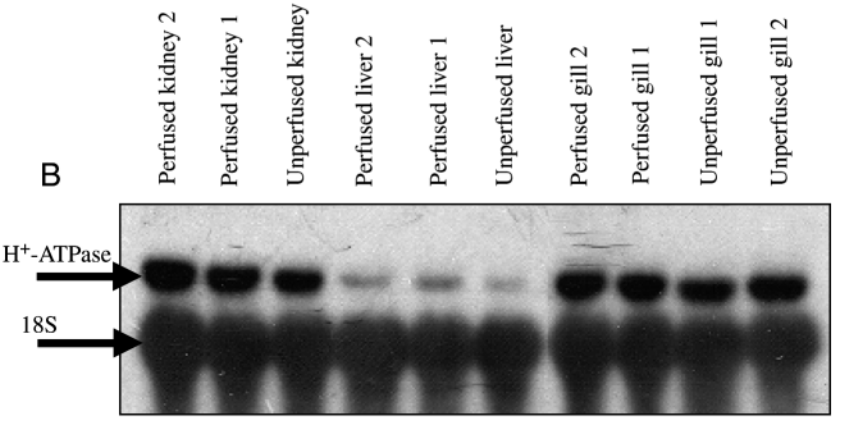
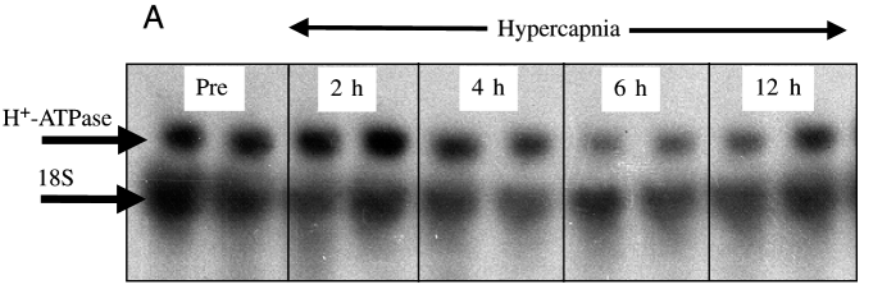


Fig. 3. Northern transfers of RNA isolated from selected rainbow trout (*Oncorhynchus mykiss*) tissues hybridized with a homologous H^+V-ATPase B subunit cDNA probe derived from SFP1 (810 base pairs) and/or a salmon (*Oncorhynchus kisutch*) 18S rRNA clone. Gels were loaded with 20 μg per lane of total RNA obtained from (A) non-perfused fish (i.e. the tissues still contained blood) or (B) both non-perfused and perfused fish (i.e. the blood had been removed from tissue *via* saline perfusion).



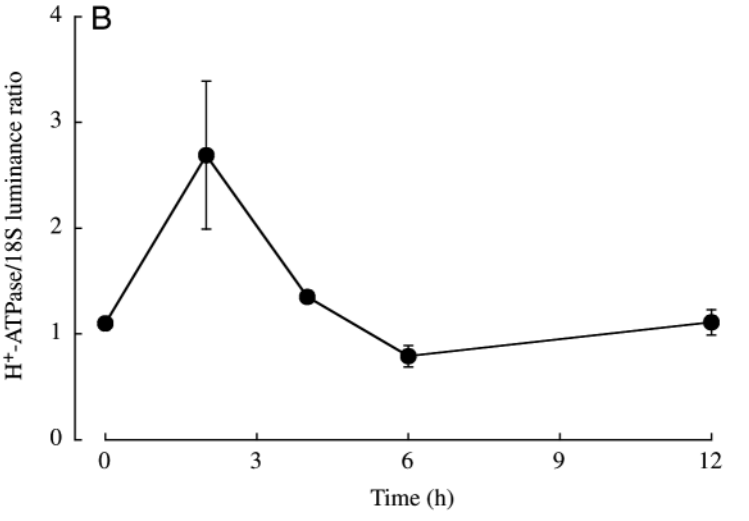


Fig. 4. (A) Northern transfer of trout (*Oncorhynchus mykiss*) gill tissue showing the temporal effects of external hypercapnia (N=2 at each sampling time) on the levels of vacuolar H^+V-ATPase B subunit mRNA. Transfers were hybridized with a homologous H^+V-ATPase B subunit cDNA probe derived from SFP1 (810 base pairs) and subsequently with a salmon (*Oncorhynchus kisutch*) 18S rRNA clone. Gels were loaded with 20 μg per lane of total RNA obtained from perfused fish. Pre, pre-treatment. (B) The results were quantified by calculating (in triplicate) the luminance ratios of H^+-ATPase to 18S rRNA (see Materials and methods). Values are means ± S.E.M.

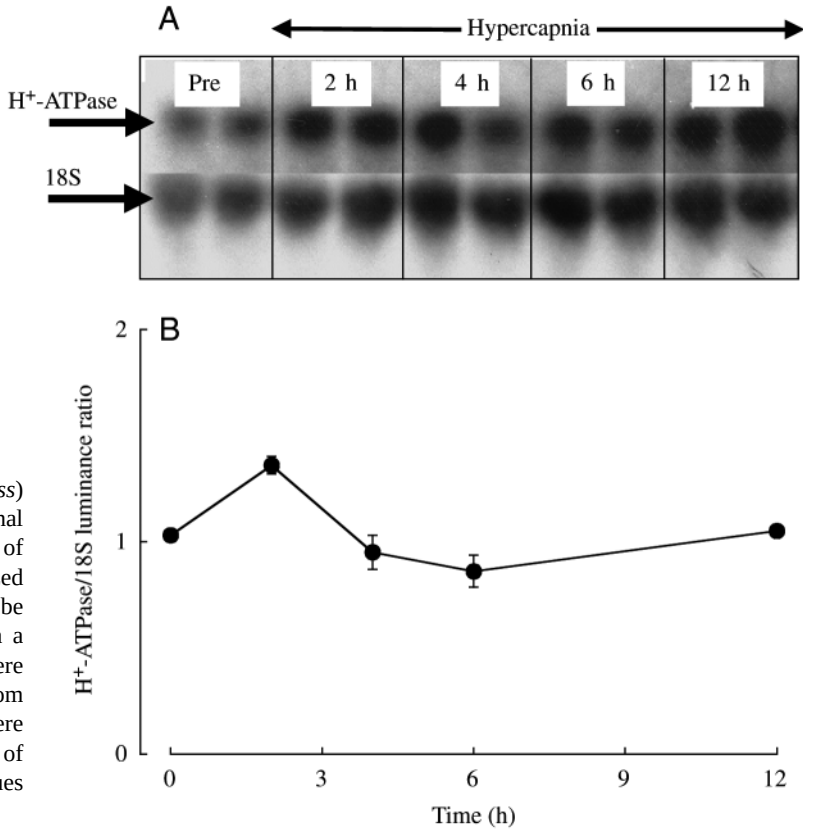


Fig. 5. (A) Northern transfer of trout (*Oncorhynchus mykiss*) kidney tissue showing the temporal effects of external hypercapnia (*N*=2 at each sampling time) on the levels of H^+V-ATPase B subunit mRNA. Transfers were hybridized with a homologous H^+V-ATPase B subunit cDNA probe derived from SFP1 (810 base pairs) and subsequently with a salmon (*Oncorhynchus kisutch*) 18S rRNA clone. Gels were loaded with 20 µg per lane of total RNA obtained from perfused fish. Pre, pre-treatment. (B) The results were quantified by calculating (in triplicate) the luminance ratios of H^+-ATPase to 18S rRNA (see Materials and methods). Values are means ± S.E.M.

subunit mRNA in liver were low and constant throughout the hypercapnia period (H^+V-ATPase/18S luminance ratios ranged between 0.79 and 0.71 during 12 h of hypercapnia).

Discussion

Previous studies on the molecular biology of the H^+V-ATPase in animals have focused almost exclusively on mammalian, avian and invertebrate species. Surprisingly, there is little molecular information available for the lower vertebrates despite the fact that the H^+V-ATPase has been implicated in acid–base regulation and ion transport in amphibian skin, reptile bladder (Brown and Breton, 1996) and fish gill (Lin and Randall, 1995). To our knowledge, no subunit of the H^+V-ATPase has yet been cloned from any amphibian or reptile and, within the fishes, sequence data have been obtained from only a single species (*Anguilla anguilla*; H. Niederstaetter and B. Pelster; GenBank accession no. AF099743). Thus, the present paper is only the second report on the molecular biology of a lower vertebrate H^+V-ATPase and is the first to establish, using a homologous probe, the tissue distribution and effects of respiratory acidosis on the levels of H^+V-ATPase mRNA.

The H^+V-ATPase is thought to be expressed ubiquitously in all eukaryote cells, where it is associated with endomembranes and intracellular organelles (Nelson and Harvey, 1999). In addition to its presumed role as a universal housekeeping enzyme, the H^+V-ATPase is targeted specifically to the apical plasma membrane of numerous animal epithelia (for reviews, see Stevens and Forgac, 1997; Forgac, 1998; Nelson and Harvey, 1999; Wieczorek et al., 1999a). The resultant electrogenic flux of protons establishes voltage gradients across the plasma membrane that are coupled to numerous physiological processes including ionic and acid–base balance. Using heterologous antibodies, previous studies (Sullivan et al., 1995; Perry, 1997; Perry and Fryer, 1997; Wilson et al., 1997; Goss et al., 1998) have demonstrated that the H^+V-ATPase in the fish gill, as in the mammalian kidney, is targeted specifically at the apical plasma membrane. Thus, although the present study did not attempt to immunolocalise the H^+V-ATPase using homologous antibodies, it is assumed that the cloned B subunit is indeed targeted at the apical plasma membrane.

The H^+V-ATPase consists of an extracellular catalytic sector (termed V_1) and a transmembrane H^+-conducting sector (termed V_o). The catalytic sector contains eight different subunits (A–H) of which the A (approximately 70 kDa) and B (approximately 56 kDa) subunits have been studied most extensively. Because of the large existing nucleotide database for the B subunit and its postulated role in regulating the activity of the H^+-ATPase (Nelson and Klionsky, 1996), we opted to focus on this subunit in the present study. In mammals, there are two isoforms of the B subunit that are encoded by different genes. Isoform I (also termed the kidney isoform) is localised preferentially within renal tissue (Sudhof et al., 1989; Nelson et al., 1992), whereas isoform II (also termed the brain

isoform) is expressed ubiquitously in all tissues that have been examined (Puopolo et al., 1992; Nelson et al., 1992). The two isoforms are largely homologous at the nucleotide level except for small regions at the carboxy and amino terminals, where marked differences are observed (Nelson et al., 1992). It is interesting that the rainbow trout H^+-ATPase B subunit, although cloned from a gill/kidney cDNA library, appears to be of the isoform II subtype on the basis of sequence alignment and phylogenetic analysis (Fig. 6). Similarly, the eel B subunit also is grouped with the isoform II genes (Fig. 6). It is unclear whether a second isoform (i.e. isoform I) exists in the rainbow trout genome, although the results of a genomic Southern blot are inconsistent with a multi-gene family in this species (D. A. Johnson, unpublished data).

When facing respiratory acidosis, trout respond by increasing branchial acid excretion (Wood et al., 1984). This excretion serves to increase the levels of HCO_3^- within the body fluids, and blood pH is thus slowly (hours to days) restored to normal levels (Heisler, 1993; Claiborne, 1998). Concomitantly, the kidney also secretes additional acid, which ensures reabsorption of the greater quantities of HCO_3^- present in the initial renal filtrate (Wheatly et al., 1984; Perry et al., 1987b). This additional HCO_3^- reabsorption ensures that the HCO_3^- accumulating in the blood is not excreted in the urine. Previous studies have established that the enhanced branchial acid excretion is linked to the combined effects of decreased Cl^- uptake (Perry et al., 1987a) and increased Na^+ uptake (Wood et al., 1984; for a review, see Goss et al., 1992b). Until recently, the rise in Na^+ uptake during respiratory acidosis was attributed to an increase in the rate of Na^+/H^+ exchange. However, in the light of the revision to the classical model of Na^+ uptake across the freshwater fish gill (see Introduction), an alternative explanation for increased branchial Na^+ uptake and acid excretion during acidosis is an elevation in H^+V-ATPase (proton pumping) activity. By analogy to the mammalian kidney (for reviews, see Gluck and Nelson, 1992; Brown et al., 1992; Brown and Breton, 1996), an increase in H^+ pumping activity could reflect the insertion by endocytosis of H^+V-ATPase-containing vesicles into the apical membrane. Indeed, a recent study by Laurent et al. (1994) provided morphological evidence for H^+V-ATPase-containing vesicles in gill pavement cells of the catfish *Ictalurus nebulosus*. These vesicles displayed an increased rate of fusion with the apical membrane during hypercapnic acidosis. In addition to increased trafficking of H^+V-ATPase-containing vesicles to the apical membrane, the total activity of the gill H^+V-ATPase is enhanced during respiratory acidosis, as demonstrated by assaying H^+V-ATPase activity in crude gill homogenates of rainbow trout (Lin and Randall, 1993). This greater activity reflects, at least in part, increased expression of the H^+V-ATPase protein (Lin et al., 1994; Sullivan et al., 1995). On the basis of the present study employing a homologous probe for northern analysis, it is apparent that a component of the increased H^+V-ATPase expression is related to increased steady-state levels of B subunit mRNA. It is unclear whether the increased mRNA levels reflect enhanced gene transcription and/or a greater stability of the mRNA. It is reasonable to assume, however, that at least a portion of the increase in mRNA levels is related to transcriptional regulation.

This is the first study to assess the effects of acidosis on the levels of renal H^+V-ATPase mRNA of a fish. After 2 h of hypercapnia, there was an apparent 1.5-fold increase in the level of H^+-ATPase B subunit mRNA. Although excess acid excretion by the trout kidney during respiratory acidosis is negligible compared with branchial excretion, an increase in the rate of acid secretion into the urine at such times is essential to promote additional HCO_3^- reabsorption. The present data support a role for the H^+V-ATPase in regulating urine acidification and hence HCO_3^- reabsorption.

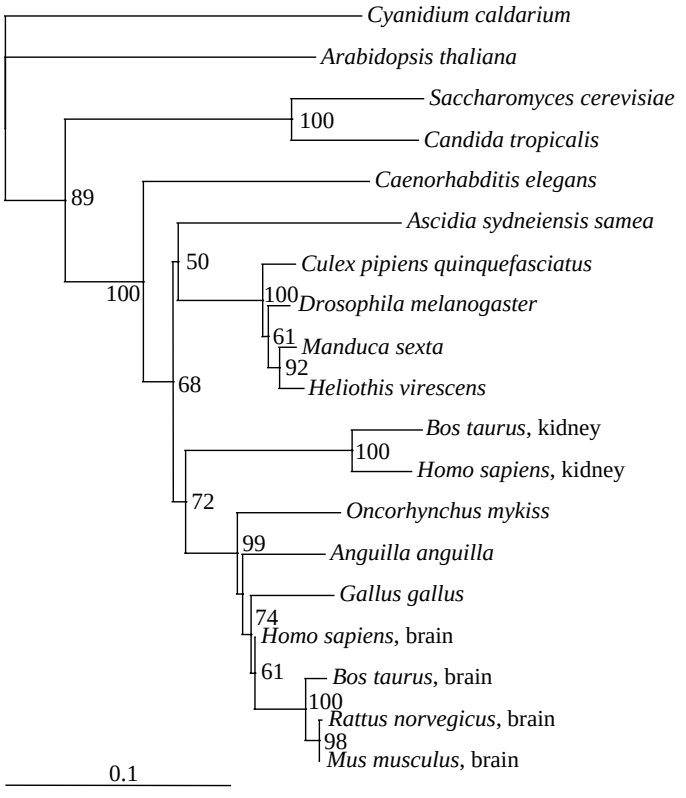


Fig. 6. Phylogenetic tree of H^+V-ATPase B subunits using the following GenBank nucleotide accession numbers: AF140022 (*Oncorhynchus mykiss*), X64354 (*Manduca sexta*), X67839 (*Drosophila melanogaster*), AF099743 (*Anguilla anguilla*), U20766 (*Gallus gallus*), M88691 (*Bos taurus*), M25809 (*Homo sapiens*; kidney), AF140022 (*Oncorhynchus mykiss*), AF037468 (*Culex pipiens quinquefasciatus*), AB016484 (*Ascidia sydneiensis*), S61797 (*Heliothis virescens*), U41015 (*Caenorhabditis elegans*), X54875 (*Candida tropicalis*), J04450 (*Saccharomyces cerevisiae*), J04185 (*Arabidopsis thaliana*), U17101 (*Cyanidium caldarium*), Y12634 (*Mus musculus*), M60346 (*Homo sapiens*; brain), M88690 (*Bos taurus*) and Y12635 (*Rattus norvegicus*). Bootstrap values are indicated at the nodes (values less than 50 are not shown). The scale bar indicates distance in units of fraction of amino acids differing between two sequences. See Materials and methods for further details.

It is important to emphasise that, because of the low sample size, the present data on H^+V-ATPase B subunit mRNA levels, as obtained from northern blots, are qualitative rather than quantitative. Clearly, it will be important to apply quantitative or semi-quantitative techniques in any future studies that assess the effects of physiological challenges on H^+V-ATPase mRNA expression.

The localisation of the trout gill or kidney H^+V-ATPase is either unknown (kidney) or currently under debate (gill). For the gill, arguments have been made for a specific localisation to the chloride cell (Lin and Randall, 1991), the pavement cell (Laurent et al., 1994; Sullivan et al., 1995, 1996; Kultz and Somero, 1995) or both cell types (Lin et al., 1994; Lin and Randall, 1995; Perry, 1997). The chloride and pavement cells both have an abundant supply of carbonic anhydrase (Rahim et al., 1988), an essential component of the proton pump/Na^+ uptake mechanism that ensures a continuous supply of H^+ (*via* CO_2 hydration). Although the chloride cells (also termed mitochondria-rich cells) possess a much higher density of mitochondria, recent morphological examinations of pavement cells have identified a subset of cells that exhibit abundant mitochondria, in particular in fish that are exposed to hypercapnia. (Goss et al., 1992a,b, 1994, 1995; Laurent and Perry, 1995). Thus, these pavement cells are presumably able to generate sufficient ATP to fuel the H^+V-ATPase.

In summary, the trout gill and kidney express an H^+V-ATPase possessing a B subunit that is highly homologous to that of other known vertebrate H^+V-ATPases and, in particular, to isoform II (brain isoform). The levels of B subunit mRNA increase transiently in both tissues during hypercapnia, and this response is likely to contribute to the increased H^+V-ATPase activity observed previously in gill (Lin and Randall, 1993; Sullivan et al., 1995) and kidney (Perry and Fryer, 1997) during respiratory acidosis.

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