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Review

Integrated responses of Na^+/HCO_3^- cotransporters and V-type H^+-ATPases in the fish gill and kidney during respiratory acidosis

S.F. Perry*, M. Furimsky, M. Bayaa, T. Georgalis, A. Shahsavarani, J.G. Nickerson, T.W. Moon

Department of Biology, University of Ottawa, 30 Marie Curie, Ottawa, Ontario, Canada K1N 6N5

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Abstract

Using degenerate primers, followed by 3′ and 5′ RACE and "long" PCR, a continuous 4050-bp cDNA was obtained and sequenced from rainbow trout (*Oncorhynchus mykiss*) gill. The cDNA included an open reading frame encoding a deduced protein of 1088 amino acids. A BLAST search of the GenBank protein database demonstrated that the trout gene shared high sequence similarity with several vertebrate Na^+/HCO_3^- cotransporters (NBCs) and in particular, NBC1. Protein alignment revealed that the trout NBC is >80% identical to vertebrate NBC1s and phylogenetic analysis provided additional evidence that the trout NBC is indeed a homolog of NBC1. Using the same degenerate primers, a partial cDNA (404 bp) for NBC was obtained from eel (*Anguilla rostrata*) kidney. Analysis of the tissue distribution of trout NBC, as determined by Northern blot analysis and real-time PCR, indicated high transcript levels in several absorptive/secretory epithelia including gill, kidney and intestine and significant levels in liver. NBC mRNA was undetectable in eel gill by real-time PCR. In trout, the levels of gill NBC1 mRNA were increased markedly during respiratory acidosis induced by exposure to hypercarbia; this response was accompanied by a transient increase in branchial V-type H^+-ATPase mRNA levels. Assuming that the branchial NBC1 is localised to basolateral membranes of gill cells and operates in the influx mode (HCO_3^- and Na^+ entry into the cell), it would appear that in trout, the expression of branchial NBC1 is transcriptionally regulated to match the requirements of gill pHi regulation rather than to match trans-epithelial HCO_3^- efflux requirements for systemic acid–base balance. By analogy with mammalian systems, NBC1 in the kidney probably plays a role in the tubular reabsorption of both Na^+ and HCO_3^-. During periods of respiratory acidosis, levels of renal NBC1 mRNA increased (after a transient reduction) in both trout and eel, presumably to increase HCO_3^- reabsorption. This strategy, when coupled with increased urinary acidification associated with increased vacuolar H^+-ATPase activity, ensures that HCO_3^- levels accumulate in the body fluids to restore pH.

Keywords: Gill; Kidney; Na^+-HCO_3^- cotransporter; Proton pump; Acid–base balance; HCO_3^- reabsorption

1. Introduction

In teleost fish, acid–base regulation is accomplished by the dynamic modulation of ion transport proteins at the gill and kidney [1–4]. At the gill, the two principal mechanisms of acid–base regulation involve adjustments of the trans-epithelial net fluxes of Na^+ and Cl^- [5]. Owing to obligate relationships between Na^+ uptake and H^+ excretion and Cl^- uptake and HCO_3^- excretion, such adjustments lead to appropriate changes in HCO_3^- levels within the body fluids and corresponding changes in pH. In rainbow trout, it is the Cl^-/HCO_3^- exchanger, thought to be located on the apical membrane of mitochondria-rich (MR) cells, which is predominantly regulated [6]. Although this branchial anion exchanger has yet to be identified, there is recent evidence obtained from Atlantic stingray (*Dasyatis sabina*) [7] implicating a pendrin-like molecule [8–10] or another member of the SLC26 sub-family of HCO_3^- transporters. The mechanism of Na^+ uptake across the freshwater fish gill also is unknown although most current models incorporate conductance through an apical membrane Na^+ channel that is energised by a V-type H^+-ATPase [11,12]. Regulation of Na^+ uptake, at least in trout, appears to be a less important route of maintaining or adjusting acid–base balance. With the discovery of another group of HCO_3^- transporters, the Na^+/HCO_3^- cotransporter (NBC) family [13–15], there has been increasing interest in their potential involvement in branchial ionic and acid–base regulation in fishes. Recently, NBC1 was cloned from Japanese dace (*Tribolodon hako-*

* Corresponding author. Tel.: +1-613-562-5800x6005; fax: +1-613-562-4286.
E-mail address: sfperry@science.uottawa.ca (S.F. Perry).

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nensis) gill where it was implicated in trans-epithelial HCO_3^- transport and acid–base regulation in fish living in acidic water [16]. In the present paper, we report the cloning and characterization of NBC1 in a second fish species, the rainbow trout, and document the temporal changes in NBC1 mRNA expression levels during periods of respiratory acidosis induced by exposure to hypercarbia.

Although the gill is the major site of acid–base equivalent transfer between the body and the environment, the kidney also plays an essential role in systemic acid–base balance owing to its capacity to flexibly modulate HCO_3^- reabsorption [17–19]. During respiratory acidosis, the compensatory increase in HCO_3^- levels within the blood initially arises from increases in net branchial acid excretion with a smaller contribution from net renal acid excretion. However, for this strategy of acid–base balance to be effective, the additional quantities of HCO_3^- being filtered must be reabsorbed, a process requiring increased tubular acid secretion. Thus, elevated rates of acid excretion at the gill must be roughly matched by similar rates of renal acid secretion, to preserve plasma HCO_3^- levels. For this reason, the amount of additional net acid excreted into the urine during acidosis underestimates overall acid secretion by the amount used to reabsorb the additional filtered HCO_3^-. In mammals, the reabsorption of tubular HCO_3^- [20] involves acidification of filtrate at the proximal tubule by H^+ pumping (via the V-type H^+-ATPase [21]) and Na^+/H^+ exchange (via Na^+/H^+ exchanger isoform 3; NHE3). The CO_2 formed by the titration of filtrate HCO_3^- (catalysed by carbonic anhydrase isoform IV; CA IV [22,23]) enters the tubule cells where it is hydrated to HCO_3^- and H^+ (catalysed by CA isoform II; CA II). Finally, the intracellular HCO_3^- is transported into the extracellular fluid by NBC1 on the basolateral membrane [14,24–26]. In the present study, the potential involvement of V-type H^+-ATPase and NBC1 in the renal reabsorption of HCO_3^- during respiratory acidosis in trout and eel was examined using real-time PCR measurements of NBC1 and ATPase mRNA levels.

2. Materials and methods

2.1. Experimental animals

Rainbow trout (*Oncorhynchus mykiss* Walbaum; 200–300 g, <2 years old) of both sexes were obtained from Linwood Acres Trout Farm. American eel (*Anguilla rostrata*; ~300 g) were obtained from a commercial fishery on the St. Lawrence River (Cornwall, Ontario). All fish were transported in oxygenated water to the University of Ottawa where they were kept in large fibreglass tanks supplied with flowing, aerated and dechloraminated, city of Ottawa tap water ($[Na^+]=0.12$ mmol l^{-1}, $[Cl^-]=0.15$ mmol l^{-1}, $[Ca^{2+}]=0.35–0.40$ mmol l^{-1}, $[K^+]=0.03$ mmol l^{-1}, pH 7.7–8.0). Fish were maintained at 13 °C on a 12-h light:12-h dark photo-period and were fed ad libitum on alternate days using a commercial pelleted fish diet except for the 48 h prior to experimentation.

2.2. Exposure of fish to hypercarbia

Fish were placed into opaque acrylic boxes, allowed to acclimate for 16–24 h and then were exposed to external hypercarbia (7.5 mm Hg) for 1–24 h. Hypercarbia was achieved by gassing a water equilibration column with 1.3–2.5% CO_2 in air (provided by a GF-3/MP gas mixing flowmeter; Cameron Instruments Inc.) to reach a final water PCO_2 (P_WCO_2) of ~7.5 mm Hg. The P_WCO_2 was monitored continuously using a PCO_2 electrode and associated meter (Cameron Instruments) linked to a data acquisition system (Biopac). Deviations in P_WCO_2 from the target of 7.5 mm Hg were corrected by adjustments of gas and/or water flows through the equilibration column. At 1, 2, 3, 6, 12 and 24 h, fish ($N=6$ at each time) were euthanised by a blow to the head. Gill and kidney (posterior 15 mm) tissues were removed, frozen in liquid N_2 and stored at −80 °C until total RNA was prepared. Control fish were also placed into opaque boxes but were maintained under normocarbic conditions for 24 h; tissues were removed at 3, 6, 12 and 24 h. Data from the 3-h period were used as the control points for the 1–3-h hypercarbic fish.

2.3. Molecular cloning of NBC

Primers were designed for nested PCR based on conserved nucleotide sequences determined from a multiple alignment of the NBC gene using vertebrate species obtained from GenBank. Primer sites were selected to give a maximum PCR fragment size of 1 kb. The outer primers were the forward primer 5′ -CATGCAGGGCGTGTTGGAGAG-3′ and the reverse primer 5′ -GTCTCTGTCTCCATCTTCAAR-3′. These primers corresponded to the nucleotides at positions (1492–1512) and (2566–2586), respectively, of the *Ambystoma* kidney NBC [13]. The inner primers were the forward primer 5′ -TAYGATGCTTTCAAGAAGATG-3′ and the reverse primer 5′ -GATAATGGCAAARTCACTGAT-3′ . These primers corresponded to the nucleotides at positions (1793–1813) and (2183–2203) of the *Ambystoma* kidney NBC, respectively. PCR was performed in 25-μl reaction mixtures containing 2.5 mmol l^{-1} $MgCl_2$, 200 μmol l^{-1} of each dNTP, 200 nmol l^{-1} of each primer (in different combinations) and 1 unit of Taq polymerase (Life Technologies) in PCR buffer supplied with the enzyme. The templates for the reactions were 1 μl of either a mixed gill/kidney cDNA library from the rainbow trout [27] or a rainbow trout liver cDNA library (generously provided by J.F. Leatherland, University of Guelph). For PCR, the annealing and extension temperatures were 56 and 72 °C, respectively. PCR products of the expected size (731 and 420 bp) were obtained from the gill/kidney cDNA library and a 420-bp product was obtained from the liver cDNA library. PCR products were cloned using the pGEM®-T Easy

cloning vector system (Promega). Plasmid DNA was obtained for sequencings using the Wizard Plasmid prep kit (Promega). A BLASTp search using the translated 731-bp nucleotide sequence yielded 25 matches with *E* values less than 10^{-50}. The highest identity was with a fragment of the *Ambystoma* NBC1 protein. The 420-bp trout liver sequence was ~100% identical to the trout gill/kidney sequence. Additionally, a 420-bp product with high homology to NBC1 was obtained from eel kidney cDNA.

For 3′ RACE, total gill RNA was isolated using Trizol reagent (Invitrogen) and reverse-transcribed to cDNA using a 3′ RACE adapter primer (Gibco) and Supercript II reverse transcriptase (Gibco). Semi-nested PCR was performed on the cDNA using abridged universal amplification primers (Gibco) with the following forward primers; first round 5′ -TGTCATTCATCCTTTTCCTGG-3′ and second round 5′ -AGCTGATCAGTGATTTTGCC-3′ . For 5′ RACE, the following primers were used with a commercial kit (Generacer; Invitrogen); for cDNA synthesis 5′ -TGGCATCTGTGGCAACCAG-3′ , for first round PCR 5′ -CAACACACCCTGCATGTTTTCTGTA-3′ , for second round (nested) PCR 5′ -GGATGAGGCCACCACAAAACTTT-3′ . PCR products were cloned into pCR2.1 vectors using TOPO TA cloning kits (Invitrogen). At least three unique clones corresponding to each overlapping PCR product were sequenced to produce an assembled consensus sequence.

To obtain a full-length continuous cDNA, gill RNA was reverse-transcribed using Expand Reverse Transcriptase (Boehringer Mannheim) with the primer 5′ -TTCAATAGATTCAGACTTGCTAC-3′ . PCR was performed with Platinum Pfx DNA polymerase using the forward primer 5′ -GGACAAAACTCCGGCAGA-3′ and the reverse primer 5′ -TTGCTACAGTAGTGGGCTCAAAGTAGTC-3′. The resulting PCR product was cloned into TOPO pCR 2.1 and fully sequenced by the primer walking technique.

2.4. Phylogenetic analysis

The amino acid sequences of selected vertebrate NBCs were aligned using default settings in Clustal W version 1.8

A

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MSTDKNKVEDEAVLDRGACFVKHVCDEEEVEGHHTVYIGVHVPKSYQRRRRHRRKSSHKERKGRPADNVEGDKSDGENAD

EAAPNILKPLISAAAERIRFILGEEDDGPPPPQLLTELDELLAVDGQEMEWKETARWIKFEEKEEKGGERWSKPHVATLS

LHSLFELKTCIEKGTILLDLEASTLPQVVELITDSQIEIGQLKPELKDQVMYTLLRKHRHQTKKSNLRSLADIGKTVSSA

SRLFSNQDQNGSPATAHRNLTSNSLNDFSDQPDRDQLKNKLMKKLPRDAEASNVLVGGVDFLDTPFVAFVRLQQAAMLGG

LTEVPVPTRFLFVLLGPKSKAKSYHEIGRAIATLMSDEVFHDIAYKAKDRQDLLAGIEEFLDEVIVLPPGEWDPDIRIEP

PKSLPSSDKRKNQYNAGGGGDGPQMNGDTPGHDGGQGGGGGHGHVGEELMNTGKFCGGLILDIKRKLPFFASDFYDAIHI
                TM-1                                    TM-2
QSLSAILFIYLGTVTNAITFGGLLGDATENMQGVLESFLGTALTGAIFCLLGGQPLTILSSTGPVLVFERLLFNFSKDHD
                    TM-3                    TM-4          ****
FDYLEFRLWIGLWSAFFCLVLVATDASSLVKYFTRFTEEGFSSLISFIFIYDAFKKMLKLAHYNPINADYDPNYVTMYDC
                                                                TM-5
RCMPPPDDGNMTGLYIDASAWINNADLSVNATWASLDKKQCVVYGGELIGPACGFVPDVTLMSFILFLGTYTCSMSLKKF
                        TM-6                                                TM-7
KFSRFFPTTVRKLISDFAIILTIVIFCGVDAFVGVDTPKLIVPTEFKPTSPNRGWFVPPFGGNPWWVYLAAALPALLVTI
                ****                    TM-8
LVFMDQQITAVIVNRKEHKLKKGAGYHLDLFWVAILLIVCSFMGLPWYVAATVISIAHIDSLKMETQCSAPGEQPKFLGV
                        TM-9
REQRVTGVFVFVLTGLSVFMAPILKFIPMPVLYGVFLYMGVASLNGVQFLDRLQLLLMPAKHQPDLIYLRHVPQRRIHLF
            TM-10
TFIQALCLALLWVLKSTVAAIIFPVMILALVAVRKAMDYIFSQQDLSYLDDVIPEKDKKKKEDEKRKKKQKKGSIDSEID

FPPEYPYNDKIPSIKISMDIMETEPMLGNKDKASDRPPSSFRDQHSPC
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B

Topology location	Consensus sequence	Trout sequence	Trout AA position
N-terminal cytoplasmic tail	ETARWIKFEE	ETARWIKFEE	133-142
N-terminal cytoplasmic tail	EVPVPTRFLFiLLGP	EVPVPTRFLFVLLGP	323-337
TM-1	AITFGGLLG	AITFGGLLG	497-505
After TM-2	TGPVLVFE	TGPVLVFE	542-549
TM-4	LISFIFIY	LISFIFIY	604-612
DIDS binding site 1	KMiK	KMLK	616-619
TM-7	PALLVTILiF	PALLVTILVF	794-855
DIDS binding site 2	KLKK	KLKK	819-822
Before TM-8	KGAGYHLDL	KGAGYHLDL	822-890
Before TM-9	VREQRVTG	VREQRVTG	880-887
TM-9	IPMPVLYGVFLYMGV	IPMPVLYGVFLYMGV	907-921
TM-10	RvHLFTflQvlCLAlLW	RIHLFTFIQALCLALLW	956-972

Fig. 1. (A) Deduced amino acid sequence of rainbow trout NBC1 showing the positions of the predicted trans-membrane domains (horizontal lines), DIDS binding motifs (denoted by asterisks) and regions (shaded) that are known to be conserved in the HCO_3^- transporter superfamily. (B) A comparison of conserved consensus sequences and DIDS binding motifs in vertebrate NBC1s with rainbow trout NBC1.

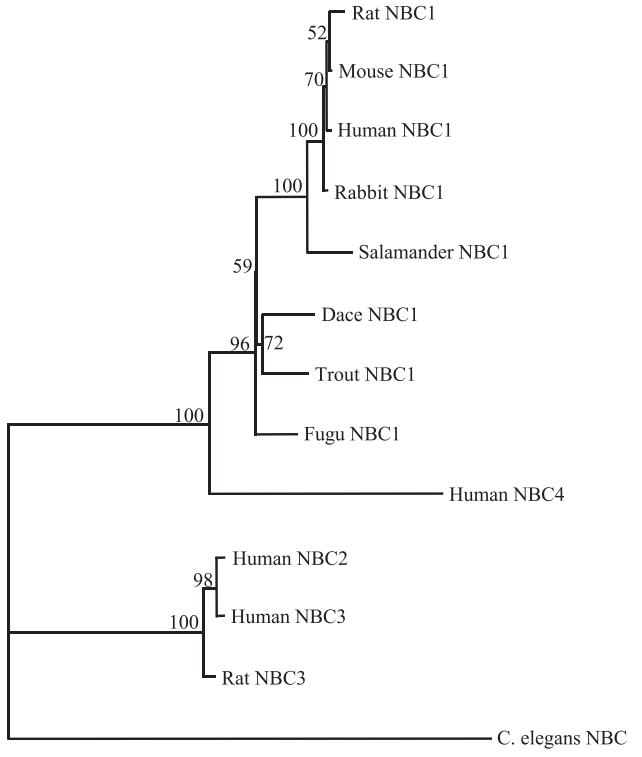

Fig. 2. Phylogenetic relationship of selected vertebrate NBC isoforms inferred from maximum likelihood analysis of NBC amino acid alignment. The position of the trout, dace and fugu NBCs relative to the mammalian NBC1 group indicates that these teleost NBCs are homologous to mammalian NBC1. Horizontal branch lengths are scaled to represent the relative number of amino acid substitutions occurring along a branch and support values at the nodes are indicated as a percentage from 1000 puzzling steps. The *C. elegans* NBC sequence was used as the "outgroup".

[28]. Maximum likelihood phylogenetic analysis was performed using PUZZLE version 4.0.2 [29]. The following program settings were used: quartet puzzling tree search, compute exact quartet likelihood, 1000 puzzling steps (the *Caenorhabditis elegans* NBC sequence was used as an "outgroup"), branch lengths are not clocklike, and JTT model of substitution. In addition, amino acid frequencies were estimated from the data set and the model of rate heterogeneity was 1 invariable + 8 gamma rates.

2.5. RNA isolation, gel electrophoresis and Northern blotting

Total RNA was isolated using Trizol reagent (Invitrogen). Northern blot analysis was performed using a cDNA insert from the plasmid containing the initially cloned 731-bp fragment as the probe. RNA samples (15 μg) were incubated in loading buffer at 65 °C, electrophoresed through 1.5% (w/v) agarose gels in MOPS (morpholinopropanesulfonic acid) buffer containing 0.6 mol l^{-1} formaldehyde, and then transferred to GeneScreen^{+} membranes (NEN Life Sciences) by capillary action for 24 h. Ambion Millennium RNA markers were used to estimate transcript size. Membranes were pre-hybridised at 65 °C for 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 dodecyl sulfate (SDS) and 10% dextran sulfate (Amersham Pharmacia Biotech). The probe was prepared by PCR amplification of the 731-bp insert followed by incorporation of ^{32}P labelled dCTP (using DNA polymerase fragment I; Gibco), purification (SigmaSpin Post Reaction Purification Column; Sigma) and thermal denaturation. After the addition of probe, hybridisation proceeded for 20 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 a phosphorimager screen (Biorad) for 2 days. In order to confirm equal loading between samples, membranes were re-probed with a homologous β-actin probe (514-bp PCR product) under similar conditions but with an exposure time of only a few hours.

2.6. Quantification of mRNA levels using real-time PCR

Frozen tissue samples were ground to a fine powder under liquid N_2 using a mortar and pestle. Total RNA was

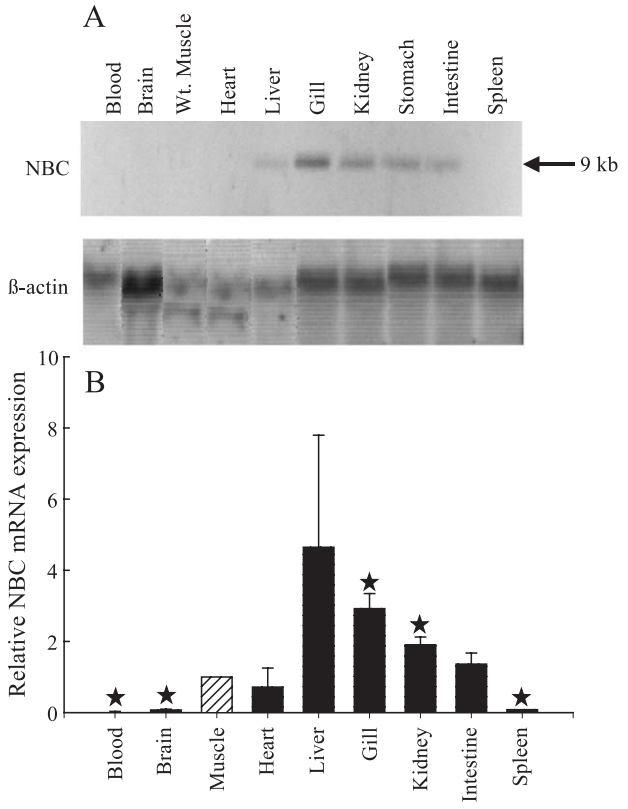

Fig. 3. Tissue distribution of NBC1 in rainbow trout (*O. mykiss*) as determined by (A) Northern blot analysis or (B) real-time PCR. For statistical analysis (two-tailed one sample Student's *t* test; $P < 0.05$) of the PCR results ($N = 3$ for each tissue), data were compared to white muscle, which was given a relative value of 1. Stars denote tissues with relative mRNA levels different than 1 (white muscle).

extracted from 30-mg aliquots of powdered tissue using RNeasy Mini Kits (Qiagen). To remove any remaining genomic DNA, the RNA was treated on-column using RNase-free DNase (30 Ul Qiagen) for 20 min at room temperature (~ 22 °C). The RNA was eluted in 80 μl of nuclease-free H_2O and its quality was assessed by gel electrophoresis and spectrophotometry (Eppendorf Biophotometer). cDNA was synthesised from 2-μg RNA using random hexamer primers (Boehringer Mannheim) and Stratascript reverse transcriptase (Stratagene). NBC1 or V-type H^+-ATPase mRNA levels were assessed by real-time PCR on duplicate samples of cDNA (0.5 μl) using a Hot StarTaq Master Mix kit (Qiagen) and a Stratagene MX-4000 multiplex quantitative PCR system. SYBR Green (Molecular Probes Inc.) and ROX (Stratagene) were used as DNA and reference dyes, respectively. The PCR conditions (final reaction volume = 25 μl) were as follows: cDNA template = 0.5 μl; forward and reverse [primer] = 300 nmol l^{-1}; [Mg^{2+}] = 2.0 mmol l^{-1}; CYBR green = 1:50 000 final dilution; ROX = 1:30 000 final dilution; dNTPs = 200 μmol l^{-1}. The annealing and extension temperatures over 40 cycles were 58 °C (30 s) and 72 °C (30 s), respectively. The following primer pairs were designed using Primer3 softwarehttp://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi):

Trout β-actin forward	5′ -CCAACAGATGTGGATCAGCAA-3′
Trout β-actin reverse	5′ -GGTGGCAGAGCTGAAGTGGTA-3′
Eel β-actin forward	5′ -CACCATGAAGATCAAGATCATYGC-3′
Eel β-actin reverse	5′ -ATTTRCGGTGGACGATGGAG-3′
Trout NBC1 forward	5′ -TGGACCTGTTCTGGGTAGCAA-3′
Trout NBC1 reverse	5′ -AGCACTGGGTCTCCATCTTCAG-3′
Eel NBC1 forward	5′ -ACGGTACCACCACCTGGATA-3′
Eel NBC1 reverse	5′ -TTACAGGCTTCTCCCACCAG-3′
Trout H^+-ATPase forward	5′ -ATGTGGACAGACAGCTGCAC-3′
Trout H^+-ATPase reverse	5′ -CATCAGCATGGTCTTTACGG-3′
Eel H^+-ATPase forward	5′ -AAAGACGTTCAGGCCATG-3′
Eel H^+-ATPase reverse	5′ -GTTTCATAGACTGTCCTGTTGTCA-3′

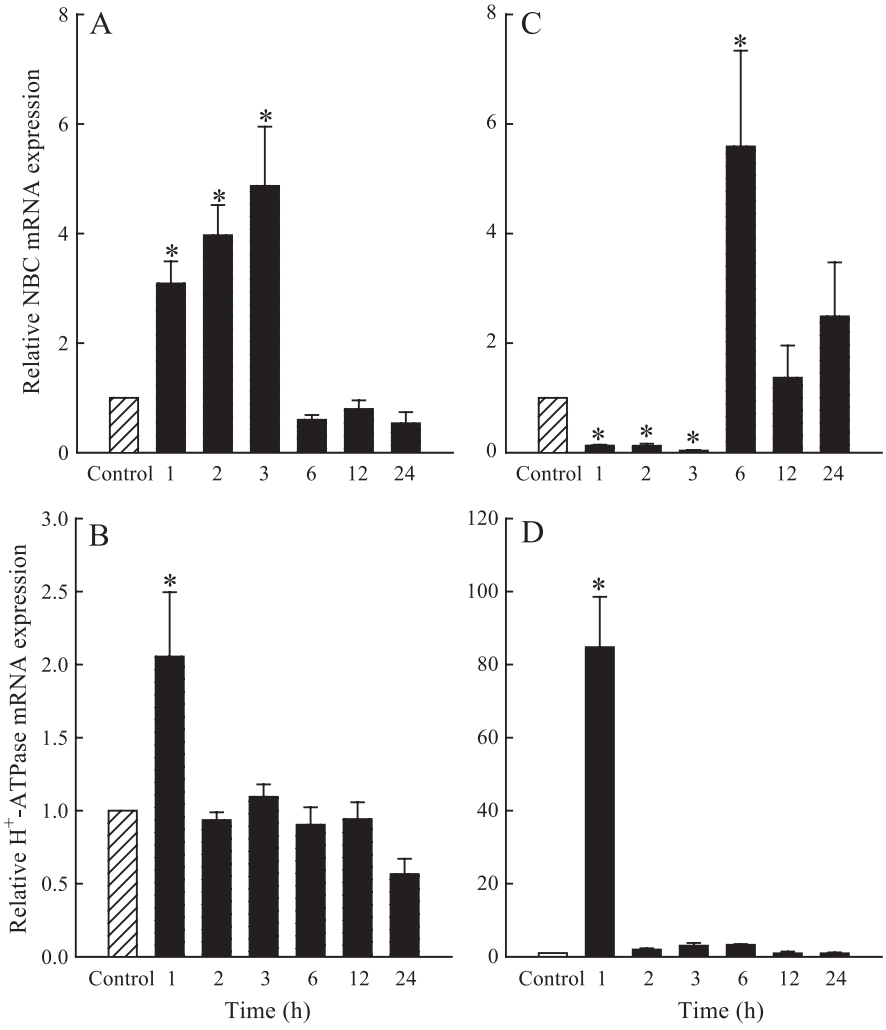

Fig. 4. The effects of exposure to hypercarbia (7.5 mm Hg) on relative levels of (A, C) NBC1 or (B, D) V-type H^+-ATPase mRNA levels in rainbow trout (*O. mykiss*) gill (A, B) or kidney (C, D). Control (cross-hatched) data (taken at 3, 6, 12 and 24 h) were assigned a value of 1 and statistically compared to the corresponding hypercarbia periods (the 3-h control data were used for the 1-, 2- and 3-h hypercarbia groups); statistical differences (two-tailed one sample Student's *t* test; $P < 0.05$) from the corresponding control group are denoted by asterisks. Note the different *Y*-axis scales in panels B and D.

The specificity of the primers was verified by cloning (TOPO TA cloning kit; Invitrogen) and sequencing of the amplified products. To ensure that SYBR green was not being incorporated into primer dimers or nonspecific amplicons during the real-time PCR runs, PCR products were analysed by gel electrophoresis in initial experiments; single bands of the expected size were obtained in all instances. Furthermore, the construction of SYBR green dissociation curves after completion of 40 PCR cycles revealed the presence of single amplicons for each primer pair. Relative expression of mRNA levels was determined (using actin as an endogenous standard) by a modification of the delta-delta Ct method [30]. Amplification efficiencies were determined from standard curves generated by serial dilution of plasmid DNA.

3. Results

The deduced amino acid sequence of trout NBC1 (GenBank accession numberAAN52239) is presented in Fig. 1A. A BLAST search of the GenBank protein database demonstrated that the trout gene shared high homology with several vertebrate NBCs and in particular, NBC1. Regions of the protein known to be highly conserved within the vertebrate NBCs bear striking resemblance to the trout sequence (Fig. 1B). A prediction of the secondary structure using TMAP [31] revealed a protein with a long NH_2-terminal cytoplasmic tail and 10 membrane-spanning domains (Fig. 1A). Two DIDS binding motifs were identified at positions 616–619 (KMLK) and 819–822 (KLKK).

A phylogenetic relationship of selected vertebrate NBC isoforms inferred from maximum likelihood analysis of NBC amino acid alignment was generated (Fig. 2). The position of the trout NBC relative to the mammalian NBC1 group and its close grouping with Japanese dace and pufferfish (*Fugu rubripes*) NBC1 indicate that the trout NBC is homologous to NBC1.

The tissue distribution of NB1C1 in trout was investigated by Northern blot analysis (Fig. 3A) or real-time PCR (Fig. 3B). Relatively high levels of a single 9-kb transcript were detected in liver, gill, kidney, stomach and intestine; NBC1 mRNA was barely detectable in blood, spleen and brain. Although a detailed tissue distribution of NBC1 in eel was not performed, mRNA was undetectable in gill using real-time PCR.

In trout, exposure to hypercarbia resulted in a transient (first 3 h) three- to fivefold increase in gill NBC1 mRNA levels (Fig. 4A) and a brief (at 1 h only) increase in V-type H^+-ATPase mRNA levels (Fig. 4B). Renal NBC1 mRNA exhibited a biphasic response during hypercarbia; levels were markedly diminished during the first 3 h of hypercarbia and then tended to be elevated for the remaining 24 h (only the 6-h time point, however, was statistically significant; Fig. 4C). Renal V-type H^+-ATPase mRNA levels were markedly (~ 80-fold) but transiently (1 h only) increased during hypercarbia (Fig. 4D).

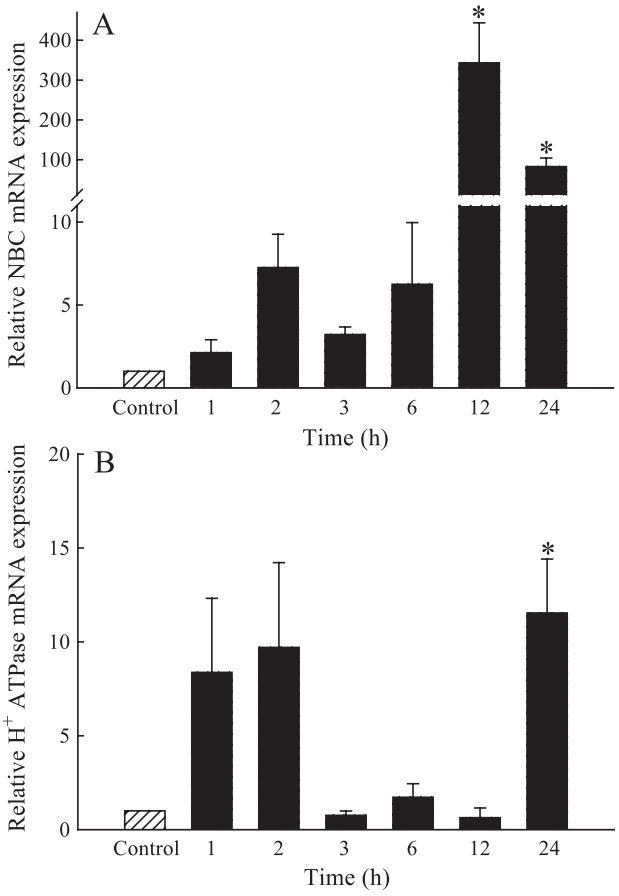

Fig. 5. The effects of exposure to hypercarbia (7.5 mm Hg) on relative levels of (A) NBC1 or (B) V-type H^+-ATPase mRNA levels in American eel (*A. rostrata*) kidney. Control (cross-hatched) data (taken at 3, 6, 12 and 24 h) were assigned a value of 1 and statistically compared to the corresponding hypercarbia periods (the 3-h control data were used for the 1-, 2- and 3-h hypercarbia groups); statistical differences (two-tailed one sample Student's *t* test; $P < 0.05$) from the corresponding control group are denoted by asterisks.

In eel gill, NBC1 mRNA was undetectable under all conditions; V-type H^+-ATPase mRNA levels were unchanged throughout hypercarbia (data not shown). In eel kidney, NBC1 mRNA was elevated by 100–300-fold during the final 12 h of hypercarbia (Fig. 5A). Renal V-type H^+-ATPase RNA levels also were increased at 24 h (Fig. 5B).

4. Discussion

Two distinct families of HCO_3^- transporters are now recognized [9]. One family, termed solute carrier 4 (SLC4), contains the Na^+-independent Cl^-/HCO_3^- exchangers [32,33], the Na^+-dependent anion exchangers [10] and the NBCs [10,14,15,34,35]). Another family, termed solute carrier 26 (SLC26), was originally thought to be comprised of specific sulfate transporters but is now known to be

composed of several genes coding for proteins that are able to transport a wide variety of anions, including HCO_3^- [8,36–38]. Unlike the situation for mammals, little is known about the physiological roles of the various HCO_3^- transporters in fishes, and indeed, most members of these gene families have yet to be identified in any fish species. In the present study, we report the cloning of the rainbow trout NBC, only the second full-length member of the NBC family to be reported in fish.

4.1. Rainbow trout NBC1

Based on a phylogenetic analysis in which the trout NBC groups closely with vertebrate NBC1s including the teleost fish Japanese dace *T. hakonensis* [16], we have tentatively identified the trout NBC as NBC1. A defining feature of NBC1 is its electrogenicity whereby it transports >2 HCO_3^- for each Na^+ [15]. We have not yet attempted functional studies of the trout NBC to confirm its electrogenic nature. However, a previous study [39] provided strong evidence for an electrogenic NBC in trout liver that operates in the influx mode (HCO_3^- entry into the cell) to play a key role in hepatocyte pHi regulation. As part of the initial strategy to clone trout NBC, a liver cDNA library was used as a template for PCR. The 420-bp PCR product obtained using NBC degenerate primers was identical to the PCR products obtained from the gill/kidney cDNA library and therefore the gill/kidney NBC would appear to be identical to the liver NBC, an isoform that is known to be electrogenic [39]. Thus, on the basis of phylogenetic analysis and our previous knowledge of the trout liver NBC, it is highly probable that the clone reported in this study is a homolog of NBC1.

At the protein level, the trout NBC is 80% identical to Japanese dace NBC1 [16] and 78% identical to a pufferfish sequence that presumably is also NBC. Interestingly, the first DIDS binding motif (KMLK) in the trout sequence (Fig. 1B) is different from all previously reported vertebrate NBC1 isoforms (KMIK). The second DIDS binding motif (KLKK), however, is identical to all other NBC1 sequences except mouse (KLQK).

4.2. Tissue distribution of NBC1

Unlike in the Japanese dace, which appeared to express high levels of NBC1 mRNA only in the gill [16], the trout exhibited high levels in several tissues including gill, kidney, intestine and liver. In the liver, NBC1 serves to regulate pHi during acid–base challenges and in the kidney NBC1 plays a key role in HCO_3^- reabsorption. The role of NBC1 in the intestine is unknown although it is conceivable that it contributes to HCO_3^- excretion into the intestinal lumen [40]. Because base excretion into the intestine is enhanced during seawater acclimation, it would be interesting to determine whether intestinal NBC1 mRNA levels change accordingly as a function of external salinity. Although it is likely that the branchial NBC1 is involved in acid–base regulation, it is less obvious as to whether it is strictly regulating pHi or whether it might also be contributing to systemic pH balance by regulating HCO_3^- movement across the gill epithelium (see below). To discern between these possibilities requires precise knowledge of the stoichiometry of NBC and the membrane potential at its site of operation.

4.3. Cellular localisation and proposed orientations of NBC1

4.3.1. The gill—a suggestion for revised nomenclature

To unambiguously discuss the cellular localisation of NBC in the fish gill, it is first important to note and clarify the existing confusion surrounding cell nomenclature in freshwater-adapted species. The fish gill contains several cell types, of which the pavement cells and the MR cells (also frequently referred to as chloride cells) traditionally have been viewed as forming two distinct categories [41]. This nomenclature, however, is confusing and inappropriate because it is now recognised that a sub-population of pavement cells is enriched with mitochondria [3,42,43]. On the basis of mitochondria densities and membrane affinity for peanut lectin agglutinin (PNA), three cell types have been identified; mitochondria-poor PNA^- cells, MR PNA^- cells and MR PNA^+ cells [44,45]. Thus, the situation in the fish (trout) gill appears to be analogous to the mammalian collecting duct in which there are PNA^- and PNA^+ MR cells (termed α and β intercalated cells, respectively) as well as mitochondria-poor principal cells. A similar nomenclature has been applied to frog skin, an epithelium containing MR and principal cells. It is proposed, therefore, that the term "pavement cell" be replaced by "principal cell" to describe the mitochondria-poor epithelial cells of the gill. In freshwater fish, the MR cells should be exclusively referred to as such and use of the term "chloride cell" should be restricted to the gills of seawater fishes where this MR cell is known to play a role in Cl^- excretion [46]. Although there is indirect evidence to suggest that the PNA^- and PNA^+ MR cells of the trout gill are analogous to the renal α (acid-secreting) and β (base-secreting) intercalated cells, respectively [47], it is probably premature to assign similar nomenclature to the MR gill cells. A further complicating issue is that Pisam et al. [48], while recognising two types of MR cells in the fish gill, arbitrarily named these α and β MR cells, a nomenclature that may be opposite to that implied by analogy to the collecting duct. Until function can be specified with certainty, it is probably best to refer to the two MR sub-types (whenever possible) as PNA^- and PNA^+. This terminology will be employed in the remainder of this discussion.

In a recent study, Hirata et al. [16] demonstrated specific localization of NBC1 to the basolateral membrane of MR cells in Japanese dace gill. Interestingly, and in accordance with the view that there are two types of MR cells, NBC1

was located in only a subset of MR cells. On the basis of increased NBC1 expression on exposure of fish to acidic water, a model was proposed (partly replicated in Fig. 6) in which NBC operates in the efflux mode to move HCO_3^- from the cytosol into the interstitial fluids. For NBC to operate in such an efflux mode at physiological membrane potential, a stoichiometry of 3 HCO_3^- to 1 Na^+ would be required [15–26]. In mammals, the proximal tubule appears to be the exclusive site where NBC operates in an efflux mode [15]. In other tissues, NBC operates in an influx mode to bring HCO_3^- into the cell. This may reflect the fact that the stoichiometry of NBC1 in non-renal tissues is 2 HCO_3^- to 1 Na^+, and thus it is incapable of overcoming unfavourable chemical gradients [15]. While it is conceivable that the orientation of gill NBC1 may be analogous to the proximal tubule, it is also possible that it operates in the influx mode; both possibilities are illustrated in Fig. 6. In support of the latter, Wood and Part [49] demonstrated that pHi regulation in cultured gill principal cells was, in part, dependent on inwardly directed Na^+/HCO_3^- cotransport. Assuming that gill NBC1 is operating in the influx mode, its increased expression during hypercarbia (this study) or exposure to acidic water [16] would be consistent with regulation of intracellular acidosis. On the other hand, if operating in the efflux mode, increased expression of NBC1 at such times would be consistent with a role in systemic (whole body) acid–base regulation. Clearly, further studies are required to discern between these two possibilities.

Unlike in trout and dace, NBC1 mRNA was undetectable in the gill of American eel. The eel is unusual (although not unique) amongst the teleosts owing to its paucity of branchial MR cells and extremely low rates of Cl^- uptake from the water [54]. Given that in dace, the NBC1 is restricted to a sub-type of MR cell [16], its absence in the eel may simply reflect the absence of this particular cell type. By analogy with the β intercalated cell of the collecting duct, which is known to contain an apical anion exchanger, it is tempting to speculate that it is the PNA^+ MR cell that is absent (or scarce) in eel because it is this cell type that is believed to be the site of Cl^- uptake via an apical anion exchanger [50].

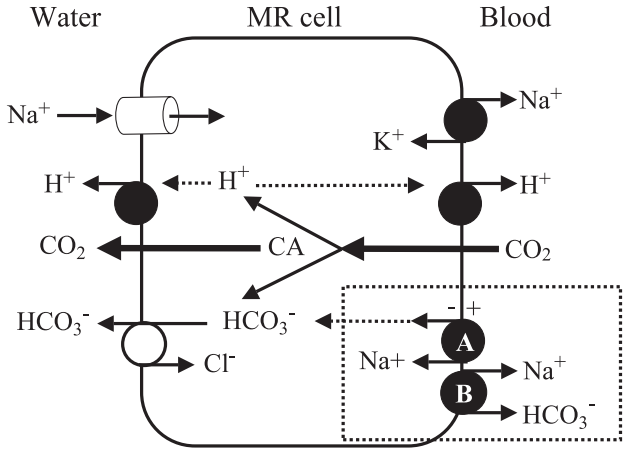

Fig. 6. A model showing two possible modes of NBC-mediated HCO_3^- transport across the basolateral membrane of a rainbow trout gill MR cell. One possibility (A) is that NBC operates in the influx mode to bring HCO_3^- into the cell for intracellular pH (pHi) regulation as well as to provide a counter-ion for an apical membrane Cl^-/HCO_3^- exchanger. A second possibility (B) is that NBC operates in the efflux mode to move HCO_3^- into the blood at variable rates depending on systemic acid–base status. Because two types of MR cells exist and it is unknown in which type the NBC is expressed, the cell in this schematic represents a hybrid MR cell exhibiting features of both MR cell types. Thus, the V-type H^+-ATPase is shown on both apical (PNA^- MR cell?) and basolateral (PNA^+ MR cell?) membranes. CA refers to carbonic anhydrase; see text for further details.

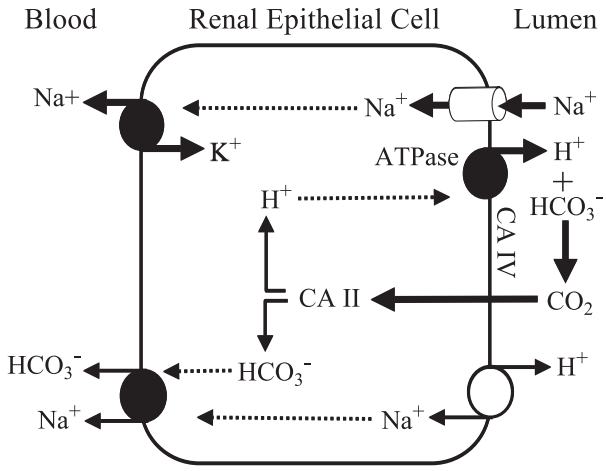

Fig. 7. A model depicting the proposed involvement of NBC1 and V-type H^+-ATPase in renal HCO_3^- reabsorption. Acid added to the filtrate by the H^+-ATPase and a putative Na^+/H^+ exchanger (NHE3?) titrates luminal HCO_3^- to CO_2 in the presence of membrane bound carbonic anhydrase (CA) isoform IV. CO_2 enters the renal epithelial cell by diffusion where it is hydrated to H^+ and HCO_3^- in the presence of CA isoform II. The HCO_3^- thus formed is moved into the blood via NBC1 on the basolateral membrane and the H^+ is pumped into the lumen by the apical membrane H^+-ATPase.

4.3.2. The kidney

In mammals, renal HCO_3^- reabsorption largely occurs in the proximal tubule and is almost entirely dependent on a basolateral NBC1 operating in the efflux mode [34]. Little is known about the mechanism of renal HCO_3^- reabsorption in fishes but we suggest that as in mammals, a basolateral NBC1 may play an important role (Fig. 7). As depicted in Fig. 7, the intracellular HCO_3^- is generated by the hydration of CO_2 that is catalysed by a cytosolic form of CA. On the basis of sequence analysis of a recently cloned trout cytosolic CA (K.M. Gilmour and S.F. Perry; unpublished data), it is likely that it is a CA II-like isoform. We propose that the H^+ required to titrate tubular HCO_3^- is provided by an apical membrane V-type H^+-ATPase and a Na^+/H^+ exchanger that has yet to be identified. As in mammals [23], we speculate that a membrane-bound isoform of CA (CA IV) catalyses the dehydration of HCO_3^- within the tubule. In support of this idea, a type IV-like CA isoform was recently cloned from trout kidney where it appears to be uniquely expressed (K.M. Gilmour and S.F. Perry; unpublished data).

In trout and eel, renal NBC1 mRNA was increased during exposure to hypercarbia, results which are consistent with NBC playing a role in the increased HCO_3^- reabsorption that occurs under these conditions. However, in eel, the response was delayed by ~12 h whereas in trout, the increase was actually preceded by a profound reduction of NBC1 mRNA levels. The basis for the delayed response in eel or the biphasic response in trout is unknown but may reflect an initial reliance on the basolateral NBC for renal pHi regulation whereby the rate of Na^+/HCO_3^- cotransport out of the cell is slowed to correct intracellular acidosis.

4.4. Integrated responses of NBC1 and V-type H^+-ATPase

As reported previously [27,51,52], exposure of trout to hypercarbia resulted in increased levels of branchial V-type H^+-ATPase mRNA. Thus, increased transcription of V-type H^+-ATPase may contribute to the increased H^+-ATPase activity [53] or protein levels [54] that accompany hypercarbic exposure in trout. However, the magnitude of the increase in H^+-ATPase mRNA is small and not universally observed when fish are exposed to acidic conditions. In this study, for example, levels of H^+-ATPase mRNA in the eel gill were unchanged during hypercarbia. Similarly, no increase in H^+-ATPase mRNA levels was noted in the Japanese dace exposed to water of pH 3.5 [16] or in Atlantic salmon (*Salmo salar*) exposed to hypercarbia or hyperoxia [55]. There are three possible explanations for these findings. First, it is conceivable that increased H^+-ATPase activity during acidosis relies more on posttranslational modifications (e.g. phosphorylation, intracellular shuttling) than transcriptional changes. Second, increased H^+-ATPase activity may not be crucial to increasing net acid excretion during acidosis. Certainly, in trout, it is generally accepted that increases in net acid excretion at the gill are largely accomplished by decreased rates of Cl^-/HCO_3^- exchange [56,57] rather than by mechanisms associated with increased Na^+ uptake (e.g. Na^+ uptake linked to apical H^+-ATPase; Fig. 6). In contrast, however, because the American eel displays barely detectable rates of Cl^-/HCO_3^- exchange, it relies exclusively on mechanisms linking Na^+ uptake to H^+ excretion to correct blood acidosis [57]. Thus, assuming that transcriptional regulation is important to regulate H^+-ATPase activity, it was surprising that no changes in mRNA levels were detected in eel gill during hypercarbia. A third possibility to explain the modest effect or absence of any effect of acidosis on H^+-ATPase mRNA levels is that measuring total gill mRNA may obscure changes that are occurring within different MR cell types. For example, if analogous to the mammalian collecting duct, acidosis would be expected to increase apical H^+-ATPase activity in the PNA^- MR cells while decreasing basolateral H^+-ATPase activity [58,59] in the PNA^+ MR cells. Globally, the net result of such opposite changes might be that no detectable changes or only small changes in H^+-ATPase mRNA levels are revealed.

The situation at the kidney appears to be more straightforward. Simultaneous increases in H^+-ATPase activity on the apical membrane and Na^+/HCO_3^- cotransport on the basolateral membrane are appropriate responses to increase the tubular reabsorption of HCO_3^- and thus aid in the correction of blood acidosis.

4.5. Conclusions

NBC1 and V-type H^+-ATPase are expressed in the gill and kidney of trout where they are likely involved in both intracellular and systemic acid–base regulation. Further research is required, however, to elucidate the cellular location of both transporters and the orientation/directionality of the NBC. It is becoming increasingly clear that the fish gill shares remarkable functional homology with the mammalian kidney. Thus, research on the gill should be aimed specifically at confirming that the gill epithelium contains MR cell sub-types that are functionally analogous to the α and β intercalated cells of the mammalian kidney.

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