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Two isoforms of the $Na^+/K^+/2Cl^-$ cotransporter are expressed in the European eel (*Anguilla anguilla*)

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Abstract

Two cDNA isoforms of the NKCC1 secretory cotransporter have been isolated from the European eel. The NKCC1a isoform exhibited mRNA expression in a wide range of tissues in a similar fashion to mammals, whereas NKCC1b was expressed primarily in the brain. The effect of freshwater (FW) to seawater (SW) transfer on NKCC1a expression was dependent on the developmental stage. In non-migratory yellow eels, NKCC1a mRNA expression in the gill was transiently up-regulated 4.3-fold after 2 days but also subsequently by 2.5–6-fold 3 weeks after SW transfer. Gill NKCC1a expression was localised mainly in branchial chloride cells of SW acclimated yellow eels. In contrast to yellow eels, NKCC1a mRNA abundance was not significantly different following SW acclimation in silver eel gill. NKCC1a mRNA abundance decreased in the kidney following SW acclimation and this may correlate with lower tubular ion/fluid secretion and urine flow rates in SW teleosts. Kidney NKCC1a mRNA expression in silver eels was also significantly lower than in yellow eels, suggesting some pre-acclimation of mRNA levels. NKCC1a mRNA was expressed at similar low levels in the middle intestine of FW- and SW-acclimated yellow or silver eels, suggesting the presence of an ion secretory mechanism in this gut segment.

Keywords: Osmoregulation; Teleost fish; CCC; NKCC2; NCC; KCC; BSC1; BSC2

1. Introduction

The mammalian chloride-cation-cotransporter (CCC) gene family currently comprises nine related membrane proteins with differing or unknown ion transporting specificities [1]. Within this family, the $Na^+/K^+/2Cl^-$ cotransporters are found in a wide variety of tissues where they are engaged either in the control of cell volume or trans-epithelial ion transport [2,3]. The $Na^+/K^+/2Cl^-$ cotransporter subfamily comprises two members, the secretory isoform known as NKCC1 and the absorptive isoform known as NKCC2 (also known as BSC2 and BSC1, respectively) which exist as several splice variants, and these isoforms are known to be primarily located in the respective basolateral or apical membrane compartments of transporting epithelial cells [1].

In teleost fish, $Na^+/K^+/2Cl^-$ cotransporter activity has been associated with a number of ion secretory mechanisms. The cotransporter was initially identified as the likely entry pathway for Na^+ and Cl^- ions across the basolateral membrane of 'chloride' cells; the main cell type engaged in ion secretion in the operculum and gill of marine teleosts [4]. This assertion was based on earlier studies which used the loop-diuretic furosemide to inhibit cotransporter-like activity in the operculum of the killifish (*Fundulus heteroclitus* [5,6]). The ion secretory system of chloride cells has been shown to be regulated by changes in plasma osmolality following acute transfer of killifish from freshwater (FW) to seawater (SW) [7].

In mammals, the activity of members of the CCC family is known to be regulated by various protein kinases [2]. Using an antibody which recognises a phosphorylated form of NKCC, Flemmer and Forbush [8] recently demonstrated the presence of a phosphorylated form of NKCC in branchial chloride cells of FW-acclimated killifish and showed a 3–4-fold increase in phosphorylation following transfer of fish to SW. The NKCC cotransporter has also been immunolocalised to branchial chloride cells in the mudskipper (*Periophthalmodon schlosseri*) [9], and in the Atlantic salmon (*Salmo salar*), where the abundance of cotransporter protein was shown to increase by 2.5-fold in the gill of parr following SW transfer [10]. However, in all of these studies

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the isoform specificity of the antibodies for the NKCC cotransporter isoform binding the antibodies was unknown.

The NKCC cotransporter has also been suggested to be involved in ion secretion in the teleost intestine [11–13]. The intestine of marine teleosts is primarily involved in ion absorption associated with the uptake of water from imbibed SW. However, in fed SW killifish, the posterior region of the intestine has been shown to be capable of secreting ions, by a mechanism thought to involve a NKCC cotransporter [13]. Further components of the intestinal secretory mechanism (as found in other vertebrates) such as the CFTR chloride ion channel, are also known to be expressed in this segment of the (killifish) intestine [14], as is the eKir potassium channel, which is associated with ion secretory processes in the eel [15]. Hormonal systems known to control intestinal ion secretion in other vertebrates, such as the hormone guanylin and isoforms of its receptor, GC-C, are also known to be expressed in teleost intestine [16–18]. Together, this data suggests that the teleost intestine should possess an ion secretory mechanism similar to that found in mammals which would include a NKCC1 secretory cotransporter [19].

In preliminary investigations, we have reported the presence of NKCC isoforms in teleost fish including the European eel (*Anguilla anguilla*), where the expression of two homologous partial cDNA fragments of NKCC1 isoforms was described in a number of tissues including the gill and intestine (previously called cot1 and cot3 [11,12]).

The object of this study was (1) to complete the cloning and sequencing of both secretory NKCC1 cotransporter isoforms expressed in the European eel, (2) determine their distribution in eel tissues, (3) investigate changes in gene expression associated with the process of acclimation from FW to SW in both non-migratory ‘yellow’, and migratory ‘silver’ eels and, (4) to localise cotransporter protein expression within the branchial epithelium using immunohistochemistry.

2. Materials and methods

2.1. Fish

Adult freshwater sexually immature “yellow” and sexually maturing migratory “silver” eels (*A. anguilla*: 125–1250 g weight) were obtained from local suppliers in Inverness, Blairgowrie and Kelso and transferred to laboratory aquaria at the Gatty Marine Laboratory where they were maintained on a 12 h light/dark cycle. Yellow and silver eels used in experiments were transferred to tanks containing either FW or SW for a period of up to 21 days. Fish were decapitated and pithed before removal of tissues.

2.2. Total RNA extraction

Total RNA was isolated from scrapes of gill arches prepared with a single-sided razor blade. Total RNA extracted from the intestine in the tissue distribution (Fig. 2) and gut segments (Fig. 5) experiments was prepared from scrapings of the intestinal epithelium using a glass slide. Gill and intestinal scrapes were homogenised using a syringe and 16 gauge needle. Total RNA was otherwise prepared from whole tissues homogenised using either a syringe and 16 gauge needle or using a Polytron blender (Kinematica). In the gut segments experiment (Fig. 5), the intestine/rectum was sectioned into three equal length parts, subsequently referred to as the anterior and middle intestine and the posterior intestine/rectum. Total RNA was extracted as previously described [24].

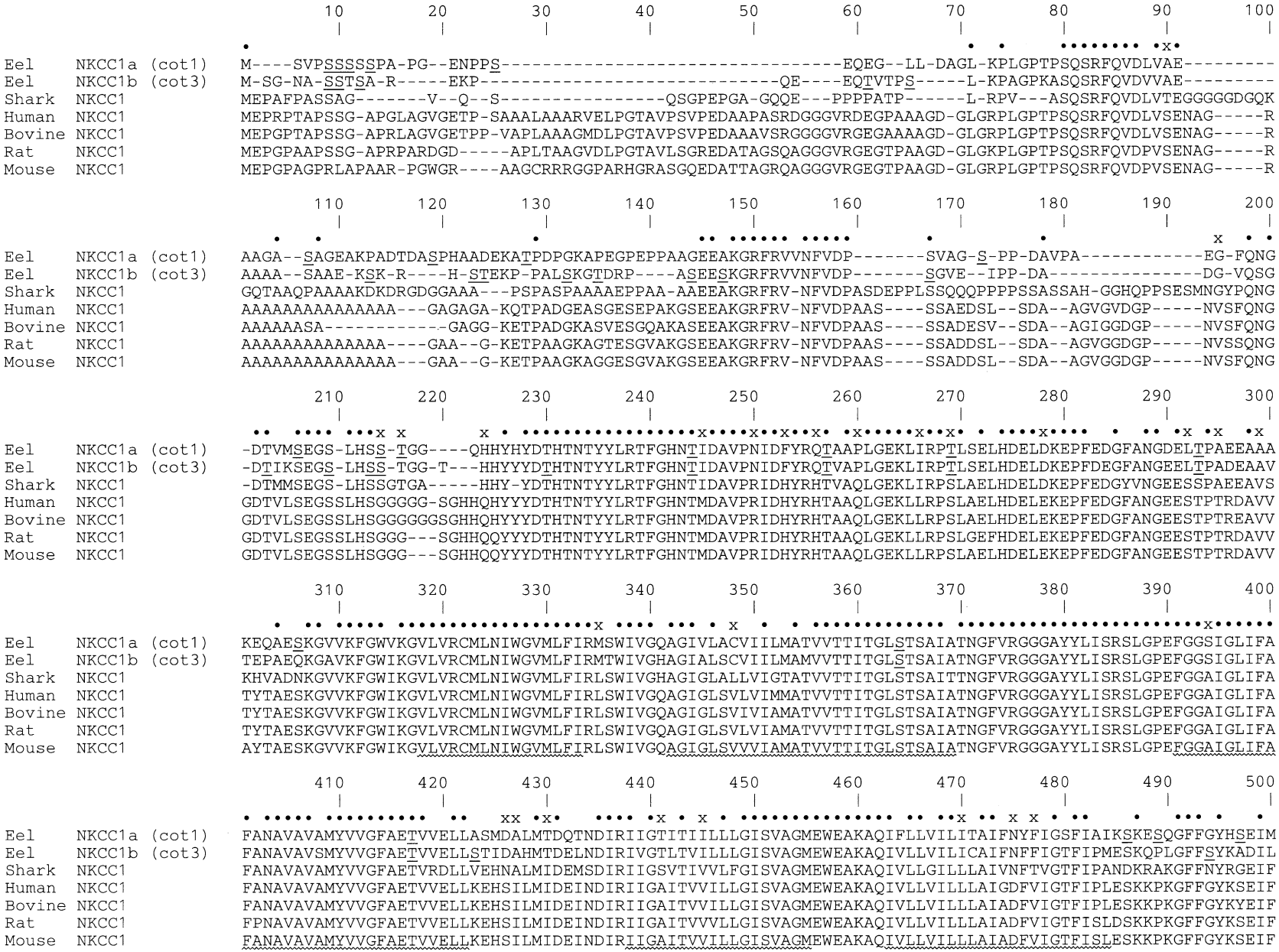
2.3. cDNA cloning and sequencing

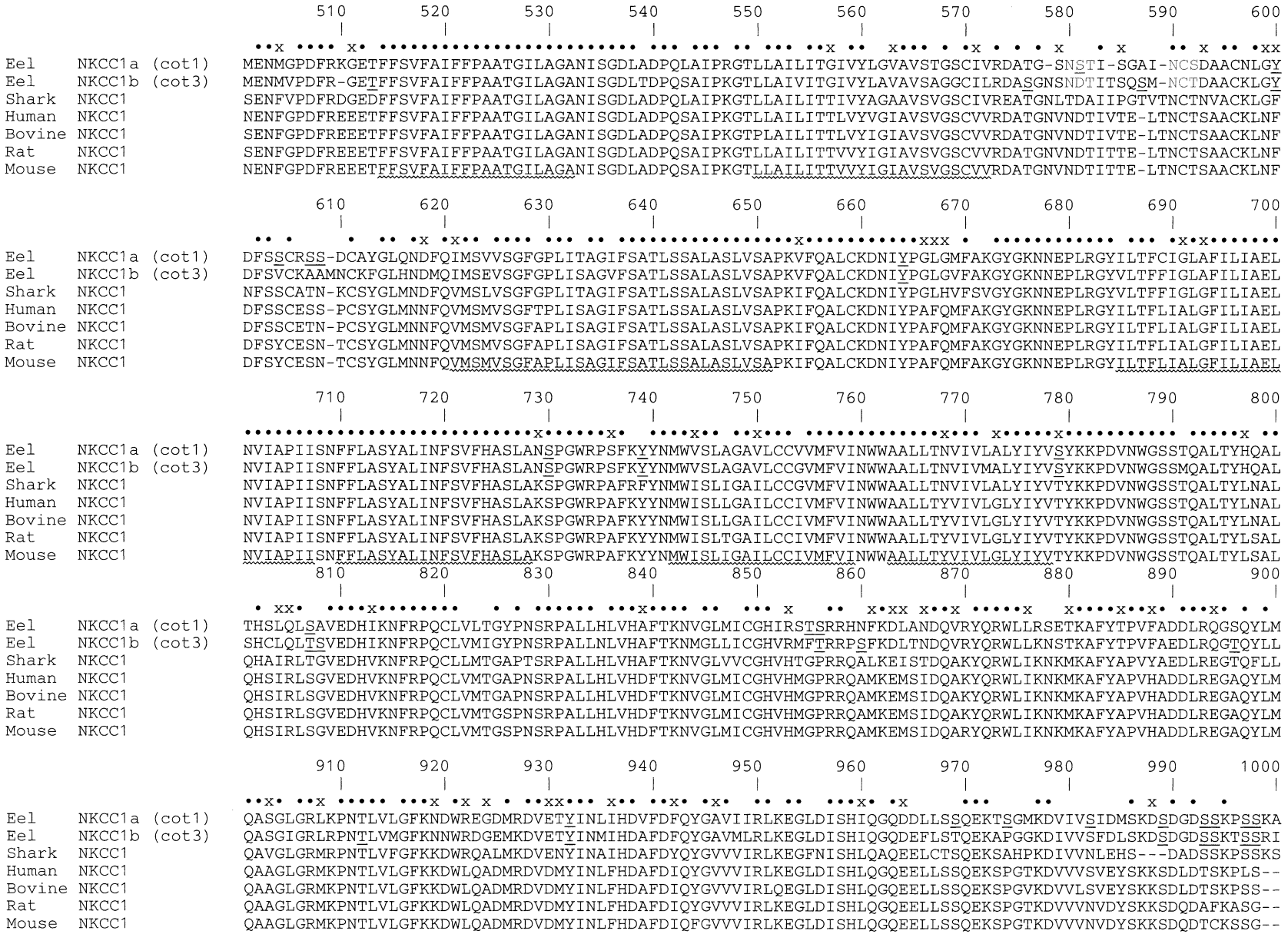
The original cDNA fragments amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) used degenerate primers whose design was based on an amino acid alignment of the NKCC1 cotransporter from shark rectal gland [20] and the sodium chloride cotransporter (NCC) from winter flounder bladder [21]. This was because these were the only CCC sequences available at the initiation of this project (see Table 1, [11]). These original primers, amplified a 755 nucleotide (nt) fragment (originally designated cot1) from 7-day SW-acclimated eel gill mRNA (using RNA samples pooled from three fish) and a 707 nt (originally designated cot3) fragment from 3-week SW-acclimated eel brain total RNA (using tissue samples pooled from 10 fish). For amplification and cloning of the initial fragments, the RNA extraction, RT-PCR, cloning and sequencing protocols were performed essentially as described in Ref. [22]. Comparison of these eel cDNA sequences with those from mammalian species revealed that these two eel CCC isoforms represented apparent duplicate copies of the NKCC1 isoform from mammals [12].

Table 1
Degenerate CCC oligonucleotide primer sequences and their position within the derived encoded amino acid (aa) alignment in Fig. 1

Name and position in alignment	Degenerate primer nucleotide sequences
Original sense at aa 810–821	GARGAYCAYGTXAARAAYTWYMGXCCXCARTGYYT
Original antisense at aa 1053–1064	AYRTCIATXGTXYYYTTXCCYTGYTTYTTYTGRAA
Modified sense at aa 810–820	GARGAYCAYRTXAARAAYTWYMGXCCXCARTG
Modified antisense at aa 1057–1067	ARCCAXYAXAYRTCXATXGTXYYYTTXCCYTG
3′ End antisense at aa 1241–1250	ARTARAAXGTXARXACRYTYTBXTGRTT

The sequences use standard IUPAC codes except for I = inosine and X = inosine/cytosine wobbles (see Ref. [22]).


Eel NKCC1a (cot1)
Eel NKCC1b (cot3)
Shark NKCC1
Human NKCC1
Bovine NKCC1
Rat NKCC1
Mouse NKCC1


510 520 530 540 550 560 570 580 590 600
Eel NKCC1a (cot1) MENMGPDFRKGETFFSVFAIFFPAATGILAGANISGDLADPQLAIPRGTLLAILITGIVYLGVAVSTGSCIVRDATG-SNSTI-SGAI-NCSDAACNLGY
Eel NKCC1b (cot3) MENMVPDFR-GETFFSVFAIFFPAATGILAGANISGDLTDPQSAIPKGTLLAIVITGIVYLAVAVSAGGCILRDASGNSNDTITSQSM-NCTDAACKLGY
Shark NKCC1 SENFVPDFRDGEDFFSVFAIFFPAATGILAGANISGDLADPQLAIPKGTLLAILITTIVYAGAAVSVGSCIVREATGNLTDAIIPGTVTNCTNVACKLGF
Human NKCC1 NENFGPDFREEETFFSVFAIFFPAATGILAGANISGDLADPQSAIPKGTLLAILITTLVYVGIAVSVGSCVVRDATGNVNDTIVTE-LTNCTSAACKLNF
Bovine NKCC1 SENFGPDFREEETFFSVFAIFFPAATGILAGANISGDLADPQSAIPKGTPLAILITTLVYIGIAVSVGSCVVRDATGNVNDTIVTE-LTNCTSAACKLNF
Rat NKCC1 SENFGPDFREEETFFSVFAIFFPAATGILAGANISGDLADPQSAIPKGTLLAILITTVVYIGIAVSVGSCVVRDATGNVNDTITTE-LTNCTSAACKLNF
Mouse NKCC1 NENFGPDFREEETFFSVFAIFFPAATGILAGANISGDLADPQSAIPKGTLLAILITTVVYIGIAVSVGSCVVRDATGNVNDTITTE-LTNCTSAACKLNF
610 620 630 640 650 660 670 680 690 700
Eel NKCC1a (cot1) DFSSCRSS-DCAYGLQNDFQIMSVVSGFGPLITAGIFSATLSSALASLVSAPKVFQALCKDNIYPGLGMFAKGYGKNNEPLRGYILTFCIGLAFILIAEL
Eel NKCC1b (cot3) DFSVCKAAMNCKFGLHNDMQIMSEVSGFGPLISAGVFSATLSSALASLVSAPKVFQALCKDNIYPGLGVFAKGYGKNNEPLRGYVLTFFIGLAFILIAEL
Shark NKCC1 NFSSCATN-KCSYGLMNDFQVMSLVSGFGPLITAGIFSATLSSALASLVSAPKIFQALCKDNIYPGLHVFSVGYGKNNEPLRGYVLTFFIGLGFILIAEL
Human NKCC1 DFSSCESS-PCSYGLMNNFQVMSMVSGFTPLISAGIFSATLSSALASLVSAPKIFQALCKDNIYPAFQMFAKGYGKNNEPLRGYILTFLIALGFILIAEL
Bovine NKCC1 DFSSCETN-PCSYGLMNNFQVMSMVSGFAPLISAGIFSATLSSALASLVSAPKIFQALCKDNIYPAFQMFAKGYGKNNEPLRGYILTFLIALGFILIAEL
Rat NKCC1 DFSYCESN-TCSYGLMNNFQVMSMVSGFAPLISAGIFSATLSSALASLVSAPKIFQALCKDNIYPAFQMFAKGYGKNNEPLRGYILTFLIALGFILIAEL
Mouse NKCC1 DFSYCESN-TCSYGLMNNFQVMSMVSGFAPLISAGIFSATLSSALASLVSAPKIFQALCKDNIYPAFQMFAKGYGKNNEPLRGYILTFLIALGFILIAEL
710 720 730 740 750 760 770 780 790 800
Eel NKCC1a (cot1) NVIAPIISNFFLASYALINFSVFHASLANSPGWRPSFKYYNMWVSLAGAVLCCVVMFVINWWAALLTNVIVLALYIYVSYKKPDVNWGSSTQALTYHQAL
Eel NKCC1b (cot3) NVIAPIISNFFLASYALINFSVFHASLANSPGWRPSFKYYNMWVSLAGAVLCCGVMFVINWWAALLTNVIVMALYIYVSYKKPDVNWGSSMQALTYHQAL
Shark NKCC1 NVIAPIISNFFLASYALINFSVFHASLAKSPGWRPAFRFYNMWISLIGAILCCGVMFVINWWAALLTNVIVLALYIYVTYKKPDVNWGSSTQALTYLNAL
Human NKCC1 NVIAPIISNFFLASYALINFSVFHASLAKSPGWRPAFKYYNMWISLLGAILCCIVMFVINWWAALLTYVIVLGLYIYVTYKKPDVNWGSSTQALTYLNAL
Bovine NKCC1 NVIAPIISNFFLASYALINFSVFHASLAKSPGWRPAFKYYNMWISLLGAILCCIVMFVINWWAALLTYVIVLGLYIYVTYKKPDVNWGSSTQALTYLNAL
Rat NKCC1 NVIAPIISNFFLASYALINFSVFHASLAKSPGWRPAFKYYNMWISLTGAILCCIVMFVINWWAALLTYVIVLGLYIYVTYKKPDVNWGSSTQALTYLSAL
Mouse NKCC1 NVIAPIISNFFLASYALINFSVFHASLAKSPGWRPAFKYYNMWISLIGAILCCIVMFVINWWAALLTYVIVLGLYIYVTYKKPDVNWGSSTQALTYLSAL
810 820 830 840 850 860 870 880 890 900
Eel NKCC1a (cot1) THSLQLSAVEDHIKNFRPQCLVLTGYPNSRPALLHLVHAFTKNVGLMICGHIRSTSRRHNFKDLANDQVRYQRWLLRSETKAFYTPVFADDLRQGSQYLM
Eel NKCC1b (cot3) SHCLQLTSVEDHIKNFRPQCLVMIGYPNSRPALLNLVHAFTKNMGLLICGHVRMFTRRPSFKDLTNDQVRYQRWLLKNSTKAFYTPVFAEDLRQGTQYLL
Shark NKCC1 QHAIRLTGVEDHVKNFRPQCLLMTGAPTSRPALLHLVHAFTKNVGLVVCGHVHTGPRRQALKEISTDQAKYQRWLIKNKMKAFYAPVYAEDLREGTQFLL
Human NKCC1 QHSIRLSGVEDHVKNFRPQCLVMTGAPNSRPALLHLVHDFTKNVGLMICGHVHMGPRRQAMKEMSIDQAKYQRWLIKNKMKAFYAPVHADDLREGAQYLM
Bovine NKCC1 QHSIRLSGVEDHVKNFRPQCLVMTGAPNSRPALLHLVHDFTKNVGLMICGHVHMGPRRQAMKEMSIDQAKYQRWLIKNKMKAFYAPVHADDLREGAQYLM
Rat NKCC1 QHSIRLSGVEDHVKNFRPQCLVMTGSPNSRPALLHLVHDFTKNVGLMICGHVHMGPRRQAMKEMSIDQAKYQRWLIKNKMKAFYAPVHADDLREGAQYLM
Mouse NKCC1 QHSIRLSGVEDHVKNFRPQCLVMTGSPNSRPALLHLVHDFTKNVGLMICGHVHMGPRRQAMKEMSIDQARYQRWLIKNKMKAFYAPVHADDLREGAQYLM
910 920 930 940 950 960 970 980 990 1000
Eel NKCC1a (cot1) QASGLGRLKPNTLVLGFKNDWREGDMRDVETYINLIHDVFDFQYGAVIIRLKEGLDISHIQGQDDLLSSQEKTSGMKDVIVSIDMSKDSDGDSSKPSSKA
Eel NKCC1b (cot3) QASGIGRLRPNTLVMGFKNNWRDGEMKDVETYINMIHDAFDFQYGAVMLRLKEGLDISHIQGQDEFLSTQEKAPGGKDIVVSFDLSKDSDGDSSKTSSRI
Shark NKCC1 QAVGLGRMRPNTLVFGFKKDWRQALMKDVENYINAIHDAFDYQYGVVVIRLKEGFNISHLQAQEELCTSQEKSAHPKDIVVNLEHS---DADSSKPSSKS
Human NKCC1 QAAGLGRMKPNTLVLGFKKDWLQADMRDVDMYINLFHDAFDIQYGVVVIRLKEGLDISHLQGQEELLSSQEKSPGTKDVVVSVEYSKKSDLDTSKPLS--
Bovine NKCC1 QAAGLGRMKPNTLVLGFKKDWLQADMRDVDMYINLFHDAFDIQYGVVVIRLQEGLDISHLQGQEELLSSQEKSPGVKDVVLSVEYSKKSDLDTSKPSS--
Rat NKCC1 QAAGLGRMKPNTLVLGFKKDWLQADMRDVDMYINLFHDAFDIQYGVVVIRLKEGLDISHLQGQEELLSSQEKSPGTKDVVVNVDYSKKSDQDAFKASG--
Mouse NKCC1 QAAGLGRMKPNTLVLGFKKDWLQADMRDVDMYINLFHDAFDIQFGVVVIRLKEGLDISHLQGQEELLSSQEKSPGTKDVVVNVDYSKKSDQDTCKSSG--

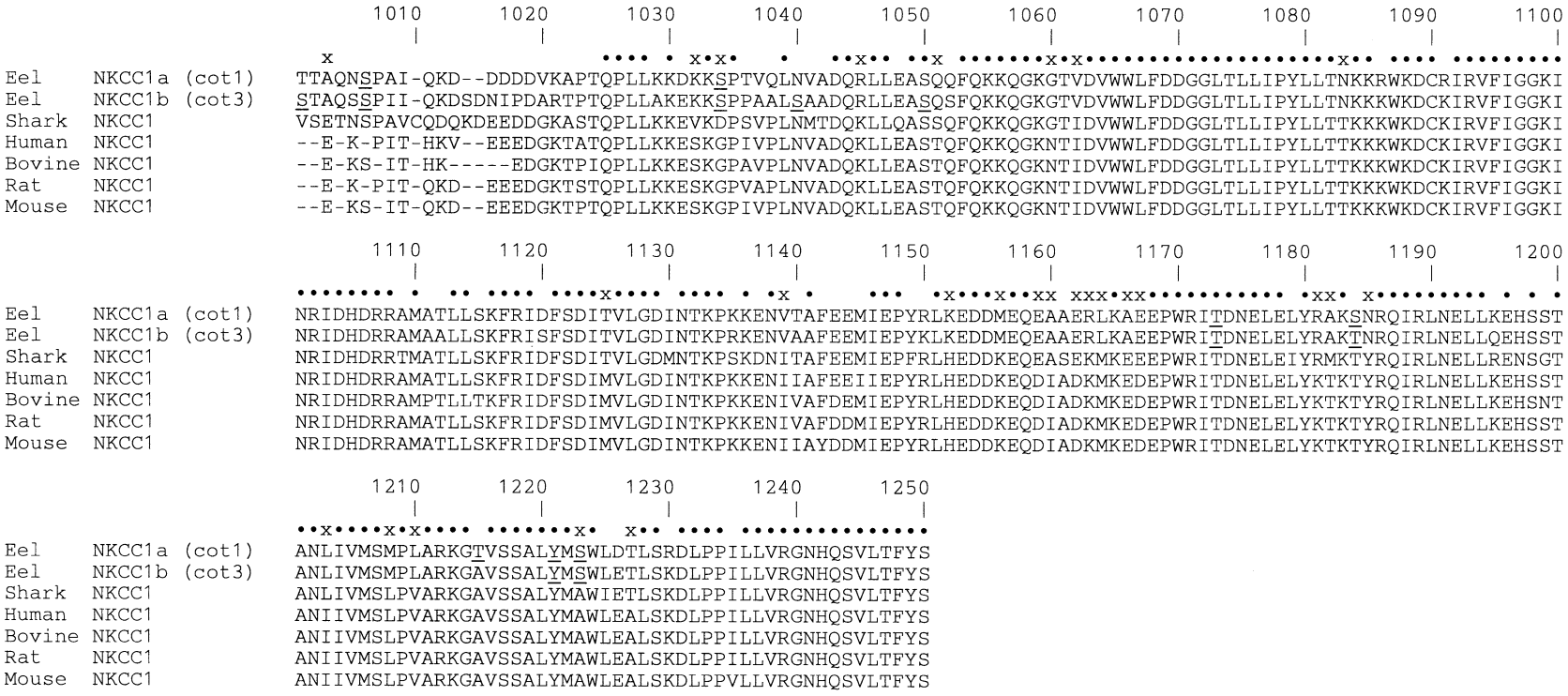


Fig. 1. Amino acid alignment of the eel duplicate NKCC1a and NKCC1b (gill spliceoform) cotransporters (EMBL Accession Nos. AJ486858 and AJ486859, respectively) in comparison to NKCC1 cotransporters from an elasmobranch (EMBL Accession No. U05958 [*Squalus acanthias*]) and also mammalian (EMBL Accession Nos. U30246 [human], BTU70138 [bovine], AF051561 [rat] and NM 009194 [mouse]) species. (–) represent gaps where spaces have been added to improve alignment. (•) represent positions within the alignment which show complete conservation between species and isoforms. X represents position of conserved difference; locations where the eel NKCC1 isoforms have the same aa as each other but where mammalian sequences have a different conserved aa. Text printed in 50% gray are locations of putative N-glycosylation sites within the putative extracellular domains of eel NKCC1s (at positions 580 and 590 of the sequence alignment). Regions with a wavy underline are the putative locations of the transmembrane domains. Amino acids with straight underlines are those within the eel NKCC1 sequences predicted to be potential phosphorylation sites for various cellular protein kinases as determined by NetPhos 2.0 [23].

The remainder of both genes were amplified using various PCR-based techniques essentially as described in Ref. [24] with the exception of nested inverse PCR. The first of these two genes is now re-designated NKCC1a here (previously cot1), in order to coincide with the nomenclature now used in other species. The 5′ end of this isoform was obtained using gene-specific primers and the rapid amplification of cDNA ends (RACE) technique, the fragment generated contained a 5′ untranslated region (UTR) of 549 nt. At the 3′ end, an internal overlapping fragment was first cloned using a gene-specific primer and a degenerate (3′ end antisense) primer and the remainder of the open reading frame (ORF) was isolated using nested inverse PCR. Briefly, the nested inverse PCR involved cutting eel gill cDNA with a *Dpn*II restriction enzyme and ligating the ends of the fragments generated together using a T4 DNA ligase reaction. The ligated *Dpn*II fragment containing the 3′ end of NKCC1a was then amplified using two pairs of gene-specific nested primers facing away from each other in order to amplify the region across the (*Dpn*II cut) ligated ends. This fragment then contains additional previously unknown sequence, which in this case, included the ORF's stop codon and some 3′ UTR. The final NKCC1a contiguous cDNA sequence generated (EMBL Accession No. AJ486858) had a 3′ UTR of 37 nt, although the mRNA clearly has a larger 3′ UTR which accounts for its much greater size (see Fig. 2).

For the second gene, now designated NKCC1b (previously cot3) experiments using the original primers had yielded a fragment of 707 nt from the brain (EMBL Accession No. AJ487475). In subsequent experiments, a further pair of degenerate primers were synthesized (modified sense and antisense, Table 1), which took into account sequence information available from newly identified CCCs. These new primers, when used with eel gill mRNA, yielded a further fragment which encoded a larger putative spliceoform of the NKCC1b ORF with an additional 54 nt in comparison to the brain NKCC1b form (the additional aa encoded are at positions 1013–1030 inclusive in the gill spliceoform as shown in Fig. 1). Both 5′ and 3′ RACE techniques were successfully used to generate a complete ORF using gill RNA. Unfortunately, as a result of a homopolymer poly C region in the 3′ UTR, only 23 nt of 3′ UTR was able to be sequenced. The 5′ RACE experiments also generated three fragments with differing UTR sequences which are putative splice variants (EMBL Accession Nos. AJ486860, AJ486861 and AJ486862).

2.4. Northern blot analysis

Northern blotting, nucleic hybridisation and quantification were also performed as in Cutler et al. [24]. The original fragments were used as cDNA probes for Northern blotting. As the NKCC1a and NKCC1b probes did not cross-hybridise with each other's mRNA on Northern blots (Fig. 2), sufficiently high stringency conditions were used to produce specific signals. Quantitative Northern blot data was transformed using $\log_{10}$ to reduce the effects of heteroscedasticity, tested for homogeneity using Bartlett's test, and analysed using ANOVA with Scheffe's *F* or Fisher PLSD post hoc testing of heterogeneous or homogeneous data, respectively.

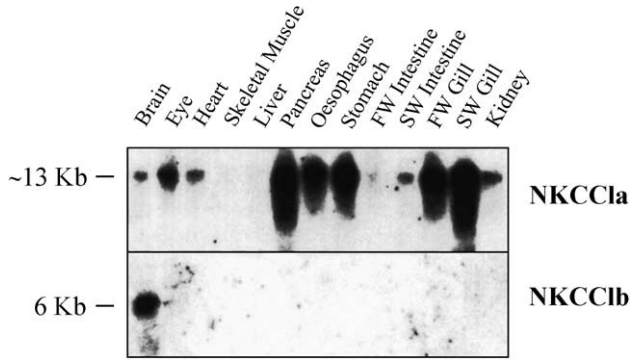


Fig. 2. Northern blots illustrating the tissue distribution of NKCC1a and NKCC1b mRNA expression in total RNA samples (10 μg) from the tissues of SW-acclimated yellow eels, with the exception of samples from FW yellow eels. Blots were exposed to film at −80 °C for 9.6 h (NKCC1a) and 27 h (NKCC1b).

2.5. Antibody production and immunohistochemistry

Antibodies were produced as outlined in Cutler et al. [24]. A peptide (NH2-DVKAPTQPLLKKDKK-COOH; extending from aa 1020–1034 in Fig. 1) was chosen from the derived aa sequence of the original NKCC1a cDNA fragment due to its high potential antigenicity [25]. The 15-mer was manufactured as a multiple-antigen peptide (MAP; Severn Biotech., Kidderminster, UK) and used to raise eel NKCC1a polyclonal antibodies in sheep by the National Diagnostics Scotland laboratory (Law Hospital, Carluke, UK). A further isoform-specific antisera was raised against the Na,K-ATPase α1 subunit peptide sequence (NH2-HKNANSEESKHLLV-COOH; at aa positions 492–505; see Ref. [22]) and this was used to identify chloride cells within the branchial epithelium, as the Na,K-ATPase enzyme is well-known to be expressed at high levels in these cells [24]. Immuno-histochemistry was performed using a donkey anti-sheep horseradish peroxidase (HRP)-conjugated secondary antibody and visualised as described previously [24]. Serial cross-sections through the primary filaments and secondary lamellae of the gill were stained with either the NKCC1a antisera or Na,K-ATPase antisera (diluted 1:100). Similarly diluted pre-immune serum was also used as a control on sequential sections (data not shown).

3. Results

3.1. Sequence and structure of NKCC1a and NKCC1b

The derived amino acid sequences of both NKCC1a and NKCC1b are highly homologous to NKCC1 sequen-

Table 2
Percentage amino acid homology of the vertebrate NKCC1 cotransporter sequences in comparison to eel NKCC1a and NKCC1b

Species/Isoform	Eel NKCC1a (cot1)	Eel NKCC1b (cot3)
Eel NKCC1a (cot1)	100	80.4
Eel NKCC1b (cot3)	80.4	100
Shark NKCC1	68.9	68.5
Human NKCC1	71.5	68.1
Bovine NKCC1	70.8	68.0
Rat NKCC1	70.2	68.0
Mouse NKCC1	70.4	67.8

Homologies were calculated using the amino acid alignment in Fig. 1.

ces from other species (Fig. 1), although there are a number of amino acid differences at the N-terminal. The NKCC1a and NKCC1b isoforms are also highly homologous to each other with around 80% amino acid identity over the entire sequence (Table 2). NKCC1a showed greater similarity to mammalian NKCC1s, however, the difference is only of the order of 1–2%. This is also reflected in the increased similarity of NKCC1a to mammalian NKCC1s in comparison to shark NKCC1. NKCC1b has approximately similar levels of homology when compared to mammalian and shark NKCC1s.

After position 225 of the NKCC1 aa alignment, the sequences of NKCC1a and NKCC1b show high levels of conserved structure and homology including the predicted 12 transmembrane domains. In addition, both sequences share two extracellular potential N-glycosylation sites (NKCC1a has another three and NKCC1b another four independent putative N-glycosylation sites) suggesting that like mammalian and shark NKCC1s, eel NKCC1s are glycosylated proteins.

3.2. Messenger RNA expression of eel NKCC1s

Gene-specific probes for NKCC1a and NKCC1b isoforms were used to determine the extent of mRNA expression in various tissues of the eel (Fig. 2). NKCC1a was expressed in a wide range of tissues within the fish as a single mRNA species of approximately 13 kilo bases (kb) (initial size estimates for NKCC1a and NKCC1b mRNAs

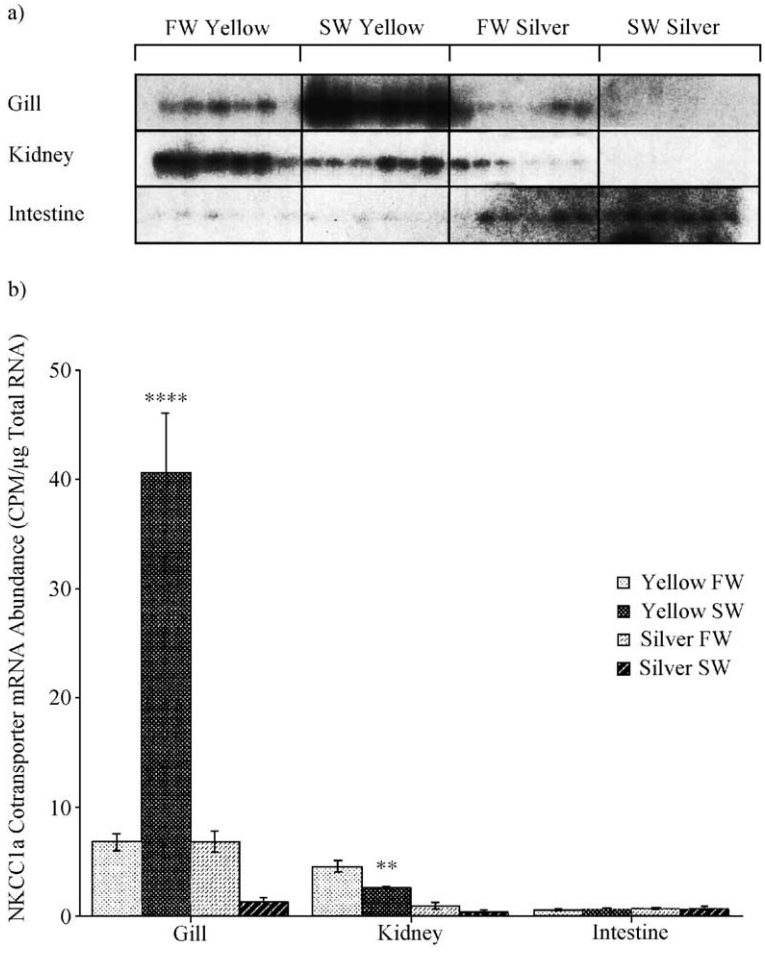


Fig. 3. (a) Northern blots of NKCC1a mRNA expression using total RNA (5 µg) from the gill, kidney and intestine of FW or 3-week acclimated SW yellow or silver eels. (b) Quantification of the levels of NKCC1a mRNA abundance on the Northern blots in panel a. Significant differences between FW and SW (yellow or silver) eels are indicated for each tissue where ** indicated $P<0.01$ and **** indicates $P<0.0001$ using analysis of variance with Scheffe's F post hoc test. Error bars indicate standard error of the mean, where $n=6$ fish.

were revised after the use of higher resolution gels [11]), with highest abundance in pancreas, oesophagus, stomach and gill, with intermediate levels in the eye and low levels in the brain, heart, intestine and kidney. No sign of any NKCC1a mRNA expression appeared to be present in skeletal muscle and liver. NKCC1b expression exhibited a different tissue distribution with a single 6 kb mRNA species found only in the brain (Fig. 2). However, a further blot using 5 μg poly A^+ mRNA (rather than total RNA) from the gills of 7-day SW-acclimated eels indicated that NKCC1b is expressed at extremely low levels in this tissue, as multiple mRNA species with sizes of 3.6, 3.8, 6, 8 and ~ 12 kb (data not shown).

The effect of SW acclimation on NKCC1a mRNA was investigated in both yellow and silver eels (Fig. 3). In the gill of yellow eels, a 5.9-fold increase in NKCC1a mRNA abundance was found 3 weeks after transfer of fish to SW. This was in marked contrast to the situation in silver eels where an 80% decrease in expression was found, although this was not sufficiently different enough to be significant using the Scheffe's *F* post hoc test. In the kidney, there was a 40% decrease in mRNA abundance following salinity acclimation in yellow eels, although the similar 60% decrease found in SW-acclimated silver eels was not significant. In addition, the overall levels of mRNA abundance were significantly lower in silver eel when compared to yellow eel kidney. The time course of changes in gill NKCC1a mRNA abundance during salinity acclimation was further examined in yellow eels (Fig. 4). After 21 days of SW acclimation, the level of NKCC1a mRNA expression showed a significant but lower level (~ 2.5-fold) of increase than in the previous experiment. However, there was also a transient peak in NKCC1a mRNA expression, 2 days after SW transfer where a ~ 2.5-fold increase over time-matched control fish also occurred. However, over the first 4 days, the actual changes in NKCC1a mRNA abundance were masked to some extent by significant increases in expression in the FW to FW-transferred time-matched control eels, which may have been a result of a stress response associated with the transfers. Consequently, the levels of NKCC1a mRNA abundance was 4.3-fold higher in SW eels 2 days post-transfer compared to FW fish at time zero.

Initial experiments indicated low levels of expression of NKCC1a mRNA in the intestine (Fig. 2). Subsequent experiments determined that this expression was confined only to the middle region of the gut with no mRNA detected in anterior or posterior segments (Fig. 5). No significant changes in NKCC1a mRNA expression were seen in midgut sections from yellow or silver eels following SW acclimation.

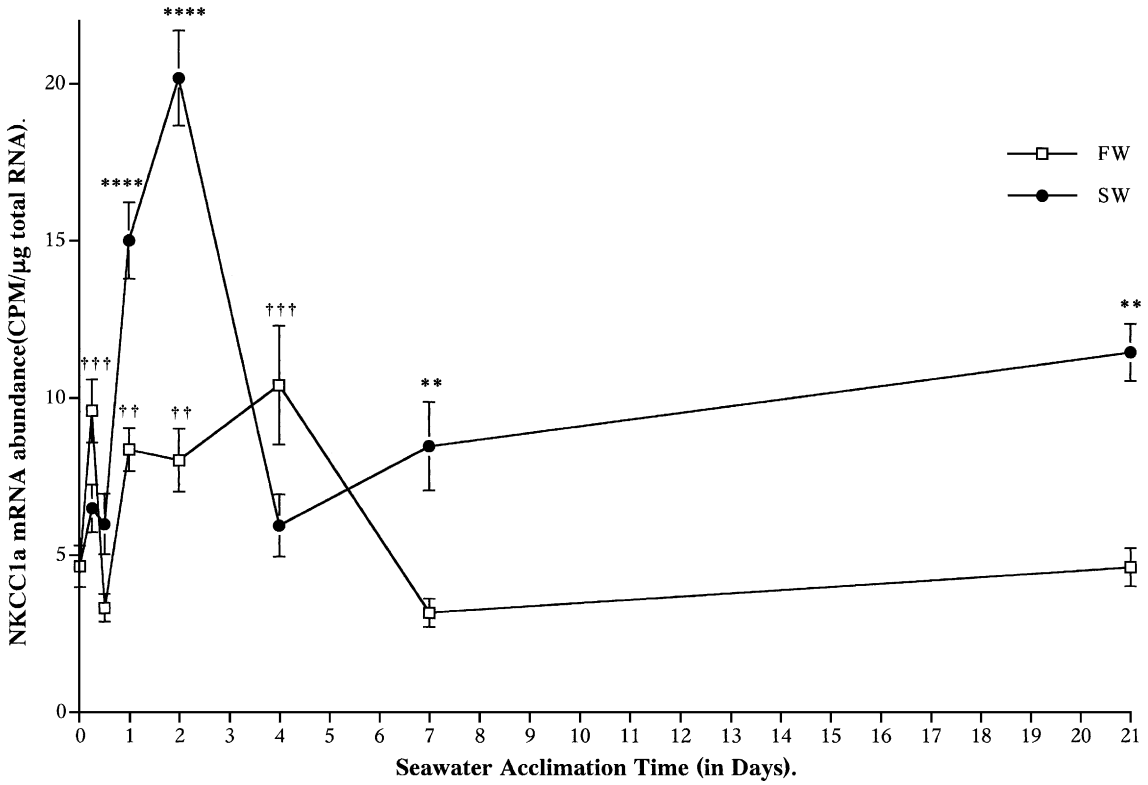


Fig. 4. Quantification of Northern blots showing the levels of branchial NKCC1a mRNA abundance during the time course of acclimation of yellow eels from FW to SW and to control fish similarly transferred from FW to FW. Significant differences between FW/FW or FW/SW eels and fish at the initial time 0 point are indicated where ** or †† indicates $P<0.01$, ††† indicates $P<0.001$, **** indicates $P<0.0001$ using analysis of variance with Fishers PLSD post hoc test. At each time point, the levels of NKCC1a mRNA abundance was significantly different to that in FW time-matched controls. Error bars indicate standard error of the mean, where $n=6$ fish.

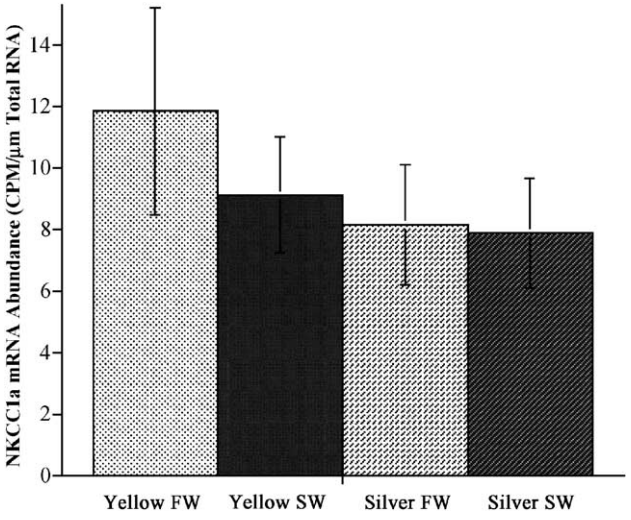


Fig. 5. Quantification of Northern blots showing the levels of NKCC1a mRNA abundance in the middle region of the intestine of FW or 3-week acclimated SW yellow or silver eels. Error bars indicate standard error of the mean, where $n = 6$ fish.

3.3. Branchial NKCC1a immunohistochemistry

The NKCC1a polyclonal antiserum was used in immunohistochemistry to localise this protein in gill sections (Fig. 6; pre-immune serum gave no similar staining, data not shown). In cross-sections through the gill of SW eels, staining was seen in large cells within the primary filamental epithelium. Similar sequential sections through the gill filament, stained with the Na,K-ATPase α1 subunit polyclonal antibody also stained large cells at the same position within the section. As Na,K-ATPase is well known to occur at very high levels in chloride cells (for example see Ref. [24]), staining in the same cell type as Na,K-ATPase with the NKCC1a-specific antibody suggests that the expression of the NKCC1a protein predominates within chloride cells. In addition, there was also further general NKCC1a staining of epithelial cells, particularly within the secondary lamellae; staining with the Na,K-ATPase antibody in this region was of much lower intensity, suggesting that the expression of NKCC1a is somewhat more evenly distributed in gill epithelial cells than is the expression of the Na,K-ATPase α1 subunit.

4. Discussion

For some time, it has been acknowledged that the genomes of certain teleost fish species underwent an ancient duplication in comparison to the tetrapod genome [26,27], with the result that around 20% of genes in zebrafish (*Danio rerio*) and 10% of genes in pufferfish (*Fugu rubripes*) have additional paralogues or isoforms without comparable orthologues in higher vertebrates [28]. There is some indication that this is a teleost-wide phenomenon which includes the eel [12]. The presence of two copies of the NKCC1 cotransporter therefore adds another small piece of evidence in support of this theory. The availability of further sequence data from diverse vertebrate groups such as the eel, offers an opportunity to improve the identification of conserved residues within the amino acid sequence, which may be critical to the regulation or function of the protein (Fig. 1). The relatively high homology of the eel NKCC1 aa sequences in comparison to other species, however, limits their usefulness in this regard (Table 2). The presence of the two eel NKCC1 isoforms does at least allow an analysis of unique motifs which are conserved between eel NKCC1 isoforms but are different to the conserved residues at the equivalent positions in mammalian NKCC1s. On this basis, there are 107 conserved aa differences between the eel isoforms and mammalian sequences, but most of these are single isolated residues. However, there are three motifs in predicted intracellular domains which may have particular significance. These are (1) the GLG motif at position 666 of the alignment which is AFQ in mammalian sequences; (2) the aa region FKDLA/TNDQV from position 861 of the alignment which is MKEMSIDQA in mammalian sequences; and (3) the aa region MEQEAAERLKAE from position 1156 of the alignment which is KEQDIADKMKED in mammalian sequences. These conserved differences may well be responsible for structural, functional or regulatory differences subsequently found between eel and mammalian NKCC1s, and are therefore worthwhile targets for future mutagenic or chimeric (eel/mammalian NKCC1 chimeras) studies.

NKCC1a mRNA is expressed in a wide variety of eel tissues, with high levels of abundance in the pancreas, oesophagus, stomach and gill, with some also present in kidney, intestine, heart and brain and this tissue distribution is similar to mammalian NKCC1 (Fig. 2) [19,29]. One obvious difference between eel NKCC1a and the expression of NKCC1 in other species is the relatively large size of the mRNA which in mammals, sharks and amphibians is around 6.5–7.5 kb [19,20,29,30] but in eels is estimated to be around 13 kb. Most of the increased size of the eel NKCC1a mRNA is likely to be due to a long 3′ UTR, which may be as large as 9 kb in eel NKCC1a. The NKCC1b brain mRNA, which is estimated to be 6 kb, is much more similar in size to NKCC1 counterparts in other species, but again some of the minor NKCC1b transcripts found in eel gill are estimated to be almost as large as eel NKCC1a (results not shown). Five differently sized NKCC1b mRNAs were isolated form the gill and the existence of such a large number is so far unique to this isoform, although a second smaller NKCC1 transcript has been reported in other species (5.1–5.2 kb) [19,20]. Another difference between eel NKCC1a and NKCC1 expression in other species is the relatively low level of mRNA abundance in eel brain. This of course may be explained by the presence of the second isoform, NKCC1b, in this tissue (Fig. 2). The somewhat offsetting level of expression of NKCC1a and NKCC1b in

the brain, suggests that the functions of these two isoforms may have subdivided the function of NKCC1 in non-duplicated species, as has been shown for other duplicate isoforms in teleost fish [28].

As expression of NKCC1b was minimal in tissues such as the gill, further experiments concentrated on NKCC1a expression in the major osmoregulatory tissues. SW-acclimation had a marked but somewhat variable effect on NKCC1a mRNA expression in the gill, with up to a 6-fold increase in expression in yellow eels but with no change or perhaps even a decrease in expression in silver eels (Fig. 3). This suggests that when eels are unexpectedly faced with SW acclimation, as would naturally be the case for FW yellow eels, NKCC1a expression was induced, presumably to allow elevated ion secretion in the gills. However, in developmentally metamorphosed silver eels which are caught during their natural migration from freshwater to the sea, it appears likely that with respect to branchial ion transport, these fish (and specifically their levels of NKCC1a mRNA expression) are pre-acclimated for survival in the marine environment and therefore elevated levels of NKCC1a mRNA abundance were not necessary. It is also possible that some other transporter or transport mechanism may be induced, which negates the need for up-regulation of NKCC1a in silver eels when in SW. This data is consistent with studies in Atlantic salmon where the level of gill NKCC protein increased 2.5-fold following SW acclimation in parr (which do not naturally migrate to SW). Gill NKCC protein levels were also transiently increased 3.3-fold in the gill during smoltification, prior to natural SW migration [10]. In a similar fashion to the increase in NKCC1a mRNA expression in 3-week SW-acclimated yellow eels, a 4-fold increase in phosphorylated NKCC protein was also seen in gills of SW-acclimated killifish (compared to FW controls, [8]).The time course of SW acclimation of yellow eels, indicates that not only are there higher levels of NKCC1a mRNA in long-term, 3 week acclimated yellow eels, but also there was a significant transient increase within 1 or 2 days (Fig. 4). NKCC1a mRNA levels were however not significantly elevated in the short-term, 6 h post-transfer. Flemmer and Forbush [8] have reported a 3-fold increase in the levels of phosphorylated gill NKCC protein in the killifish 1 h post-transfer to SW, and this may reflect an acute regulatory response to activate NKCC1 in teleost gill in a similar fashion to other species [1].

From the immunohistochemical study using a NKCC1a-specific polyclonal antibody, it is clear that NKCC1a is predominately expressed in chloride cells within the branchial epithelium (Fig. 6), although low levels of immunoreactivity were also found in other parts of the gill epithelium, particularly the secondary lamellae. A number of studies have previously reported the presence of NKCC protein within the chloride cells of teleost gill [8–10], however, in none of these studies was the type of NKCC isoform identified. This is principally due to the fact that the antibody utilised in at least two of these studies was the T4 antibody raised against the C-terminal 310 aa of human NKCC1, which is known to have affinity for both NKCC1 and NKCC2 isoforms in higher vertebrates, although the affinity to the closely related sodium chloride cotransporter,

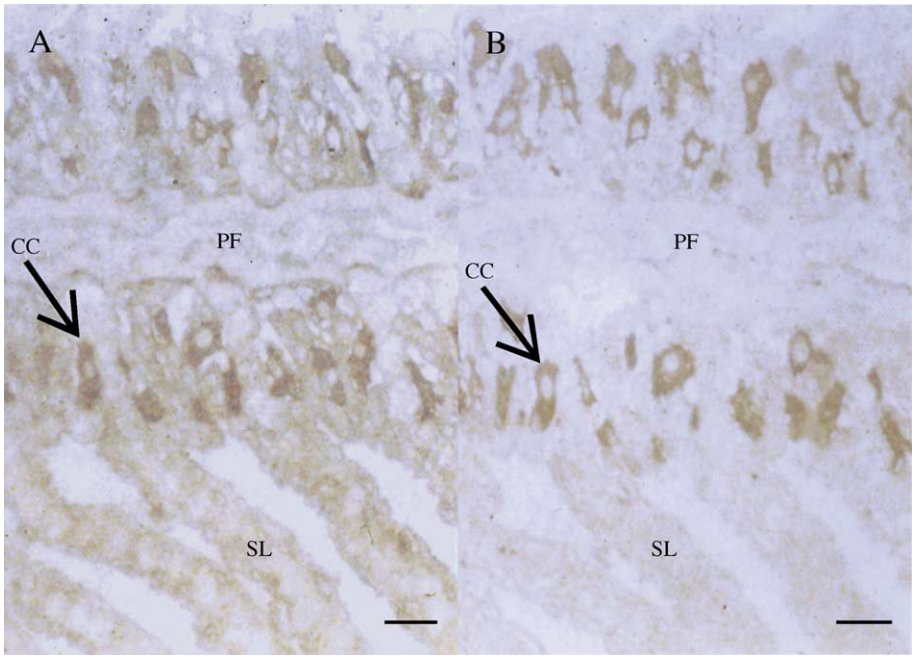


Fig. 6. Immunohistochemical detection of NKCC1a in the gill of SW-acclimated yellow eels. Serial cross-section through the primary filament (PF) and the secondary lamellae (SL) of the gill were incubated with (A) a primary eel NKCC1a-specific polyclonal antisera and (B) a primary eel Na,K-ATPase α1 subunit-specific polyclonal antisera. An example of a large Na,K-ATPase-rich chloride cell is also indicated (CC). Sections incubated with pre-immune serum for both proteins showed no staining.

NCC, was not determined [31]. Of the amino acids which are conserved between human NKCC1 and NKCC2 within the C-terminal fragment (which are likely to form the epitope for this cross-reacting antibody), around 65% are also conserved within NCC, suggesting that the T4 antibody may also bind NCC isoforms. Within eel gill, as well as NKCC1a and NKCC1b, an NCC isoform can also be detected by RT-PCR (data not shown) and therefore it is possible that the T4 antibody used across species may be detecting one or a number of CCC isoforms in the teleost gill. However, the relative abundance of CCC isoform mRNAs in eel gill, together with the localisation experiments in this study, suggest that studies using the T4 antibody on teleost gill sections are likely to be detecting a NKCC1a-like isoform.

In the kidney, there was a decrease in NKCC1a mRNA expression in both yellow and silver eels when acclimated to SW (Fig. 3). In teleost kidney, a proportion of tubular fluid is known to be produced by fluid secretion in the proximal tubule using a similar ion transport mechanism to that found in chloride cells [32]. Marine teleosts are known to have a reduced number of relatively small glomeruli with reduced urine flow rates compared to FW fish [33]. While there are a number of possible explanations, the changes in eel kidney NKCC1a mRNA expression, suggests that ion secretion involving the NKCC1a cotransporter in eel kidney is reduced following SW acclimation and this may be partly responsible for the lower urine flow rates found in marine fish including euryhaline species acclimated to SW.

In the intestine, only low amounts of NKCC1a mRNA expression were found in yellow or silver eels and no change in these levels were seen following SW acclimation (Fig. 3). When expression was investigated in three segments of the intestine, detectable levels of NKCC1a mRNA were only present in the middle region of the intestine, but again, no change in expression was observed following SW acclimation (Fig. 5). This suggests that NKCC1a is not involved in the absorption of ions across the intestine following the normal marine teleost osmoregulatory drinking response. Ion absorption in this tissue is probably related to the presence of NKCC2 isoforms [12]. The presence of the NKCC1a secretory cotransporter in the middle segment of the intestine suggests that this region may have an ion secretory capacity, as has also been demonstrated in killifish intestine [11–13]. However, the presence of NKCC1a expression in the middle intestine of the eel apparently contrasts with killifish, where fluid secretion occurred in the posterior intestinal segment [13]. In anatomical terms, it is likely that a portion of the eel middle intestinal section is equivalent to the cephalad portion of the killifish posterior intestinal segment (although the morphology of eel intestine is somewhat variable). The localisation of NKCC to the apical/brush border membranes rather than the basolateral membranes of killifish intestinal surface epithelial cells (again using the T4 antibody, [31]) probably relates to the presence of NKCC2 absorptive cotransporter isoforms or possibly an NCC isoform also present in this segment; at least if killifish shows similar CCC expression patterns to the eel [12]. Although the NKCC1 cotransporter can be localised to apical membranes in specialised tissues such as the choroid plexus, it has always otherwise been shown to have a basolateral membrane cellular localisation [1]. Unfortunately, the NKCC1a-specific antibody was not sensitive enough to detect the NKCC1a protein in eel intestinal sections.

In summary, two cDNA isoforms of the NKCC1 secretory cotransporter have been isolated from the European eel. This again supports the possibility that an additional genome duplication event occurred during the evolution of the ray-finned fish lineage. The mRNA expression of the NKCC1a isoform was widely distributed in the tissues of the eel, in a similar fashion to mammals, whereas NKCC1b was expressed mainly in the brain. Following SW acclimation, NKCC1a gill mRNA expression was up-regulated transiently after 2 days but also in the longer term in yellow eels. NKCC1a expression was localised mainly to the chloride cells within the branchial epithelium of SW acclimated yellow eels. Gill NKCC1a expression levels remain unchanged or may even be reduced in silver eels prior to SW migration. This suggests that either levels of protein are pre-acclimated to those required for SW survival (negating the need for a rise in NKCC1a mRNA levels) or that the function of this isoform is taken over by some other unknown transporter or transport system. NKCC1a mRNA abundance was also decreased in the kidney following SW acclimation and this may reflect reduced tubular ion/fluid secretion concomitant with reduced urine flow rates, which are known to occur following SW acclimation. NKCC1a is also expressed at low levels in the middle intestine which may indicate the presence of an ion secretion mechanism in this segment of the gut.

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