CLONING, TISSUE DISTRIBUTION AND CHANGES DURING SALT ADAPTATION OF THREE Na-K-Cl COTRANSPORTER ISOFORMS FROM KILLIFISH, *FUNDULUS HETEROCLITUS*

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Na-K-Cl cotransporters (NKCCs) mediate the electrically neutral transport of Na^+, K^+ and Cl^- ions across cell membranes. The mammalian NKCCs are NKCC1, the secretory isoform, which is present in epithelial and non-epithelial cells, and NKCC2, absorptive isoform, which is found only in thick ascending limb and macula densa of kidney. Recently, a third isoform, NKCC3, was identified in teleosts (*Anguilla Anguilla*, Cutler, C.P., Cramb, G., BBA 1566: 92-103, 2002), and is also apparent in the *Fugu* database). The new isoform, NKCC3, is very similar to NKCC1, suggesting this to be an example of recent gene duplication, a frequent occurrence in teleosts. We have cloned all three NKCCs from killifish, and determined their tissue distribution and changes in levels of mRNA and protein during salt-water adaptation.

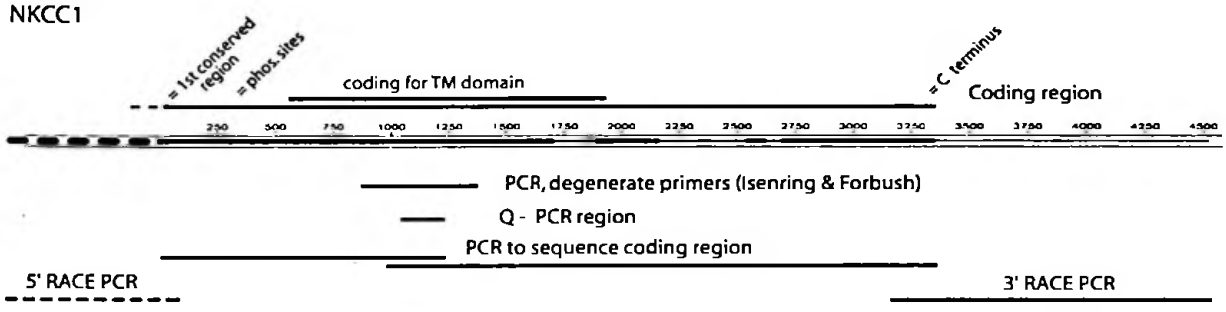

Figure 1. Cloning strategy for NKCCs from killifish tissues.

Cloning of three NKCCs from killifish. Figure 1 shows the cloning strategy for NKCC1; an identical strategy was used for NKCC2 and NKCC3. We have identified three NKCC sequences in killifish using PCR with degenerate primers. Analysis of these fragments clearly indicated that they resulted from three independent NKCC genes. Since then, we have used further PCR and 3'RACE to obtain near full-length clones of all three isoforms from killifish RNAs; although we have not yet succeeded with 5'RACE, we estimate we are within 30 residues of the N-terminus for NKCC1 and NKCC3. Figure 2 illustrates the evolutionary relationship among the NKCCs. The new isoform, NKCC3, is very similar to NKCC1, indicating a late evolutionary split and suggesting that this is

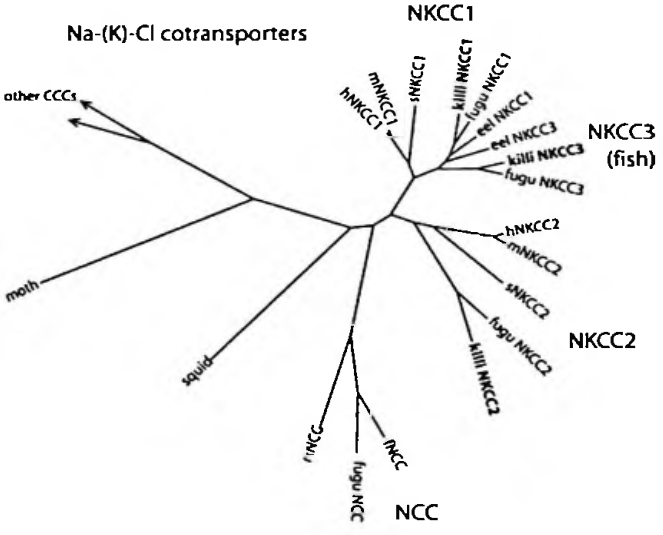

Figure 2. Phylogenic tree of Na-(K)-Cl cotransporters. Alignment and distances obtained from ClustalX.

an example of gene duplication in the teleosts. In fact, it is reasonably clear that the NKCC1-NKCC3 branch point is close to and probably within the teleost division.

Tissue distribution of three NKCCs in killifish. We have used quantitative PCR (Q-PCR), western blots, and immunofluorescence microscopy to examine the tissue distribution of NKCC1, NKCC2 and NKCC3 in killifish. Table 1 summarizes results obtained from the Q-PCR experiments. We find that NKCC1 is most abundant in the gill, where it is the only easily detectable isoform. This transporter is present in the extensively infolded basolateral membrane of the chloride cells in the gill epithelium. Brain has high levels of NKCC3, with little NKCC1. Kidney expresses high levels of both NKCC2 and NKCC3, with a low level of NKCC1, in contrast to mammalian kidney in which only NKCC2 is abundantly present. Killifish gut expresses very high levels of only NKCC2. Immunofluorescence microscopy dramatically demonstrates that the NKCC2 protein is restricted to the apical (brush-border) membrane of the gut epithelium (Figure 3). These results are in excellent agreement with previous reports of NKCC in the apical membrane of intestinal epithelium of winter flounder (Suvitayavat, W. et al., Am. J. Physiol. Cell Physiol. 267:C375-C384, 1994) and European eel (Lionetto, M.G. et al., Cell. Physiol. Biochem. 11: 41-54, 2000).

Table 1. Summary of Q-PCR experiments showing NKCC1, NKCC2 and NKCC3 isoform abundance in killifish tissues. n.d.- not detected, n.t.- not tested.

	NKCC1	NKCC2	NKCC3
Gill	++++	n.d.	n.d.
Brain	+	n.d.	++++
Gut	n.d.	++++	n.d.
Kidney	+	+++	+++
Liver	+	++	n.d.
Sk.muscle	+	n.d.	+

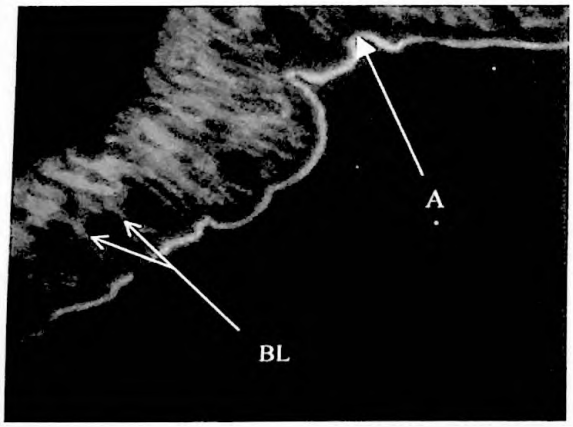

Figure 3. Immunofluorescence of gut epithelium. 100 x magnification. A Apical membrane labeled with anti-NKCC antibody. BL Basolateral membrane labeled with anti-NaK pump antibody.

Changes of NKCC mRNA and protein levels during salt adaptation. Estuarine fish, such as *Fundulus heteroclitus*, must tolerate a wide range of salinity, keeping their plasma osmolality relatively constant. We found that adaptation of killifish to water of high salinity induced a very large increase in the level of NKCC1 mRNA in the killifish gill as has been previously found for NKCC1 protein in killifish (Flemmer, A. et al., Bull. MDIBL, 38:80-2, 1999) and salmon (Pelis. R.M. et al., Am. J. Physiol. Regulatory Integrative Comp. Physiol. 280:R1844-R1852, 2001). In these studies we have also found that whereas there are only small, if any, changes in NKCC levels in the kidney during this process, there is a large increase in NKCC2 expression in the gut, seen for both RNA and protein (not shown). These changes can be appreciated in the context of a model in which teleosts adapt to the plasma-sea water osmotic imbalance by drinking sea water: water is absorbed through the gut epithelium by accompanying NKCC2-mediated salt uptake; the excess salt is extruded against an osmotic gradient by NKCC1-mediated transport in the gills. This work was supported by NIH DK 47661 and NIEHS P30-ES 3828.