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Northern blot analysis of the Na^+, K^+-ATPase α-subunit in salmonids

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A 450-bp long fragment of the gene encoding the Na^+, K^+-ATPase α-subunit was PCR (polymerase chain reaction)-amplified from the rainbow trout (*Oncorhynchus mykiss*) genome. The protein coding sequence of the cloned PCR product shared 85 and 73% identity with Na^+, K^+-ATPase α-subunit-encoding sequences of the rat (α_2-isoform) and the teleost species of the white sucker (*Catostomus commersoni*), respectively. Northern blot analysis demonstrated that the fragment hybridized to a major transcript of about 3.7 kb in several salmonid and non-salmonid species. The observed changes in Na^+, K^+-ATPase mRNA levels in osmoregulatory organs of seawater- and freshwater-adapted fish further confirm the specificity of the probe.

Key words: Na^+, K^+-ATPase; α-Subunit; *Oncorhynchus mykiss*; *Catostomus commersoni*.

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Introduction

Sodium- and potassium-activated ATPase (Na^+, K^+-ATPase) is an integral membrane enzyme that performs the ATP-driven transport of Na^+ out of the cell and pumps K^+ into the cell (see review by Horisberger *et al.*, 1991). The enzyme is therefore essential for establishment and maintenance of the Na^+- and K^+-gradient across all animal cell membranes. Marine teleost fish are hyposmotic to the medium and compensate for the osmotic loss of water by drinking seawater (SW). Na^+ absorbed from the intestinal tract, or gained by diffusional influx via the gills, is eliminated by the branchial Na^+, K^+-ATPase (see review by Payan *et al.*, 1984). In fresh water (FW), the diffusional loss of ions and the osmotic influx of water are balanced by reabsorption of ions in the gills, kidney and urinary bladder (Evans, 1979; Avella *et al.*, 1989).

Na^+, K^+-ATPase exists as a heterodimer with an α-subunit responsible for the catalytic activity (Shull *et al.*, 1985), and a β-subunit thought to be of importance for the maturation and transport of the enzyme to the plasma membrane (Geering, 1990). Both subunits exist as several distinct isoforms, and their encoding genes or cDNAs have been characterized in multiple vertebrate species (Shull *et al.*, 1985, 1986, 1989; Takeyasu *et al.*, 1988; Sweadner, 1989; Good *et al.*, 1990; Schønrock *et al.*, 1991) as well as in *Drosophila* (Lebovitz *et al.*, 1989) and in *Artemia* (Baxter-Lowe *et al.*, 1989).

In this study a PCR (polymerase chain reaction)-amplified fragment of the gene encoding the rainbow trout (*Oncorhynchus mykiss*) Na^+, K^+-ATPase α-subunit was cloned. Na^+, K^+-ATPase transcripts were studied in organs of importance for the maintenance of hydromineral balance in both salmonid and non-salmonid species by Northern blot analysis.

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Materials and Methods

Fish

Freshwater-living rainbow trout, brown trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*) were reared in Ewos tanks in a FW hatchery at Sizun, Brittany. Seawater-adapted trout and salmon were collected from populations acclimated to full-strength SW for more than 2 months. Tilapia (*Oreochromis niloticus*) were reared in INRA's laboratory facilities (FW aquarium at 26°C). The cyprinid species *Cottus gobio* and eel (*Anguilla anguilla*) were collected by electro-fishing in a Brittany river and brought alive to the laboratorium at INRA where samples were collected.

PCR amplification

Two PCR primers covering evolutionary conserved domains of the Na^+, K^+-ATPase α-subunit were designed by aligning cDNA sequences of white sucker (*Catostomus commersoni*) (Schønrock *et al.*, 1991), chicken (Takeyasu *et al.*, 1988), sheep (Shull *et al.*, 1985) and human (Shull *et al.*, 1989). The forward primer, A, was placed in a conserved hydrophilic domain between the membrane spanning regions H5 and H6, and the reverse primer, B, was placed in the conserved hydrophobic region H6 (Shull *et al.*, 1985). The sequences of the primers used were (A) 5′ TCCCTGCCATTTCCCTGGCATATGA 3′ and (B) 5′ ATCCA$^{G}/_{C}$CCAG$^{A}/_{G}$GCCTG$^{A}/_{G}$ATCATACC 3′.

DNA extracted from rainbow trout liver was amplified in a 50-μl reaction mixture containing 50 mM KCl, 10 mM Tris–HCl (*p*H 8.4), 1.5 mM $MgCl_2$, 0.001% gelatin, 100 μM of each dNTP, 50 pmol of each primer and 1.25 U Taq polymerase (Cetus). After denaturation of DNA at 94°C for 4 min, the PCR was run for three cycles at 95°C (1 min), 65°C (1 min), 72°C (1 min), followed by 35 cycles at 95, 60 and 72°C. The PCR product was analysed by agarose gel electrophoresis.

Cloning and sequencing of PCR product

The PCR-amplified fragment was phenol-extracted prior to digestion with *Eco*RI and *Bam*HI and cloned into *p*GEM-3Zf(+) vector (Promega). The cloned PCR product was sequenced by the dideoxy method using a multiwell mitrotitre plate DNA sequencing system with T7 DNA polymerase (Amersham, Bucks, U.K.) including SP6 and T7 primers.

Isolation of mRNA—Northern blot analysis

Total RNA was prepared by the guanidium thiocyanate method (Chomczynski and Sacchi, 1987), and mRNA was purified using oligo(dT) 9-trisacryl column (IBF Biotechnics). For Northern blot analysis 10 μg of mRNA were denatured and electrophoresed on a 1% agarose gel containing formaldehyde (0.66 mol/l). The mRNA was transferred to a nylon filter by capillary action in 20 × SSC (1 × SSC = 0.15 mol/l NaCl, 0.015 mol/l Na–citrate, *p*H 7.0).

The filters were hybridized for 16 hr at 42°C with the ^{32}P-labelled PCR product (Multiprime DNA Labelling Kit, Amersham). The hybridization buffer contained 50% v/v formamide, 5 × SSC, 5 × Denhardt's solution, 0.1% (w/v) sodium dodecyl sulphate (SDS) and 0.1 mg denatured calf thymus DNA. The filters were washed three times in 2 × SSC/0.1% SDS at 22°C for 5 min each time, and then twice in 0.1 × SSC/0.1% SDS at 50°C for 15 min each time. Na^+, K^+-ATPase transcripts were visualized by autoradiography (Amersham Hyperfilm) for 3 days at −70°C. The filters were then dehybridized and rehybridized with a rainbow trout actin probe (Pakdel *et al.*, 1989) as a control of quantity, and autoradiographed for 3 hr at 22°C.

Results and Discussion

Whereas the attempts to PCR-amplify a cDNA fragment of the rainbow trout Na^+, K^+-ATPase α-subunit were unsuccessful, a genomic fragment of approximately 450 bp was amplified and cloned. Since the design of the two PCR primers was based on conserved sequences localized in exons 18 and 19 (Shull *et al.*, 1989), the resulting product included an intron sequence of about 300 bp. The remaining protein coding sequence shared a 73% identity with the white sucker Na^+, K^+-ATPase α-subunit-encoding sequence (Schønrock *et al.*, 1991) (Fig. 1). Due to the high incidence of silent mutations, however, only two of the 28 deduced amino acid residues in this region were substituted. Alignment of the region with cDNA sequences encoding the rat Na^+, K^+-ATPase α_1, α_2 (Fig. 1) and α_3 isoforms (Shull *et al.*, 1986) revealed 79, 85 and 81% identity, respectively.

The major Na^+, K^+-ATPase transcript of about 3.7 kb in rainbow trout is somewhat shorter than the prominent mRNA species of 4.15 kb isolated from the white sucker brain (Schønrock *et al.*, 1991). The discrepancy could be due to the presence of different isoforms of the α-subunit in the examined organs, which would be consistent with the reported organ-specific expression of multiple isoforms in higher vertebrates (Lingrel, 1992). Hence, the additional faint band of 3.8 kb in the white sucker brain might be the homologue to the major mRNA species in the osmoregulatory-important organs of the examined teleosts.

```
rainbow trout   1GCA GCC GAG AGT GAC ATC ATG AAG CGT C.. ... ... ... CCC CAC CAG
                1ala ala glu ser asp ile met lys arg _x_ _x_ _x_ _x_ pro his gln
                                                    gln pro arg asn    lys thr
white sucker     --T --T --- --- --T --- --- --- A-A CAG CCC AGA AAC --G A-A ACA

rat (α2)         --G --T --- --C --- --- --- --- A-G CAG CCA CGG AAC T-- C-G AC-


rainbow trout  49GAC AAG CTG GTG AAC GAG AGG CTC ATC AGC ATC GCC TAC GGA CAA ATC G
               17asp lys leu val asn glu arg leu ile ser ile ala tyr gly gln ile
white sucker     --- --A T-A --- --T --A --A --T --T --- --T --G --T --- --- --A -

rat (α2)         --- --- --- --- --- --- --- --T --- --- --G --T --- --- --G --- -
```

Fig. 1. Nucleotide and deduced amino acid sequence of the PCR-amplified exon region of the rainbow trout Na⁺, K⁺-ATPase α-subunit-encoding gene. Dots represent nucleotides which could not be unambiguously determined by the dideoxy method, and Xs denote undetermined residues. For comparison the sequence is aligned with the α-subunit nucleotide and deduced amino acid sequences of the white sucker (Schønrock *et al.*, 1991). The nucleotide sequence of the rat α_2-isoform (Shull *et al.*, 1986) is also included. Hyphens represent nucleotides identical to the upper sequence.

The specificity of the rainbow trout Na⁺, K⁺-ATPase probe was further confirmed by Northern blot analysis of isolated mRNA from gills, gut, kidney and urinary bladder of several salmonid species. Following three weeks of adaptation to SW the gills (Fig. 2) and gut (Fig. 3) of rainbow trout displayed increased Na⁺, K⁺-ATPase mRNA levels, without any change in the control actin mRNA signals (data not shown). Correspondingly, the gills of SW-living brown trout and adult Atlantic salmon were also characterized by a prominent mRNA species of about 3.7 kb (Fig. 2). The results are in agreement with the documented increase in the specific Na⁺, K⁺-ATPase activity of these tissues, which is associated with the augmented active transport of Na⁺ across gills and intestinal mucosa in hyperosmotic medium (Jampol and Epstein, 1970; Collie and Bern, 1982; Payan *et al.*, 1984; Borgatti *et al.*, 1992).

Whereas marine glomerular teleosts suffer a water shortage and excrete a scanty urine isotonic to plasma, the excess water that enters the body of FW fish is eliminated as a profuse hypotonic urine. The FW adaptation involves increased glomerular filtration rate and increased reabsorption of filtrated Na⁺ by the kidneys and the urinary bladder (Evans, 1979; Curtis and Wood, 1991). *In vitro* studies demonstrated substantial mucosal to serosal Na⁺ transport in the isolated urinary bladder from FW trout (Fossat and Lahlou, 1979; Harvey and Lahlou, 1986), and the adaptation to SW induced a significant decrease in Na⁺, K⁺-ATPase activity of the trout urinary bladder (Fossat *et al.*, 1974). Consistently, Northern blot analysis of the urinary bladder of rainbow trout in FW indicated high Na⁺, K⁺-ATPase activity. There was, however, no difference in renal levels of this transcript, nor of the control actin transcripts (data not shown), in FW- and

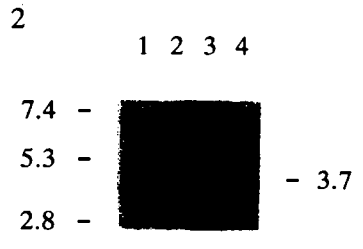

Fig. 2. Northern blot analysis of Na⁺, K⁺-ATPase α-subunit transcripts in the gills from: lane 1, FW-living rainbow trout; lane 2, SW-adapted rainbow trout; lane 3, SW-living brown trout; and lane 4, SW-living Atlantic salmon. Molecular markers are shown to the left.

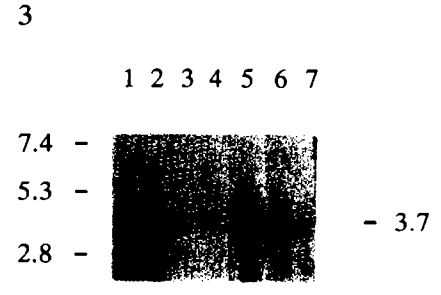

Fig. 3. Northern blot analysis of Na⁺, K⁺-ATPase α-subunit transcripts from: lane 1, gut of FW-living rainbow trout; lane 2, gut of SW-adapted rainbow trout; lane 3, kidney of FW-living rainbow trout; lane 4, kidney of SW-adapted rainbow trout; lane 5, urinary bladder of FW-living rainbow trout; lane 6, liver of FW-living rainbow trout; and lane 7, liver of SW-adapted rainbow trout. Molecular markers are shown to the left.

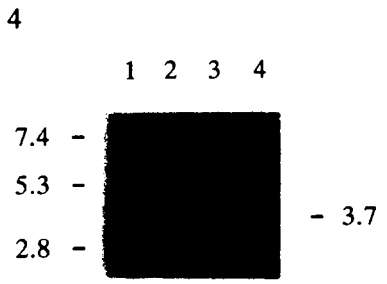

Fig. 4. Northern blot analysis of Na^+, K^+-ATPase α-subunit transcripts from the gills of: lane 1, FW-living tilapia; lane 2, SW-living Atlantic salmon; lane 3, FW-living *Cottus gobio*; and lane 4, FW-living eel. Molecular markers are shown to the left.

SW-living brown trout (Fig. 3). Conflicting results in kidney function during parr–smolt transformation have been reported (Holmes and Stainer, 1966; Eddy and Talbot, 1985) and McCormick *et al.* (1989) found no evidence for developmental changes in renal Na^+, K^+-ATPase activity of Atlantic salmon during smoltification. Further studies will be needed in order to explain this discrepancy. No difference in hepatic Na^+, K^+-ATPase mRNA levels was observed between FW- and SW-adapted rainbow trout (Fig. 3).

Northern blot analysis of the gills of FW-living tilapia and eel at the yellow stage demonstrated a prominent transcript of 3.7 kb (Fig. 4). The strong signal suggests both a high level of gene expression and regions of high sequence identity of the Na^+, K^+-ATPase α-subunits between the trout and the two non-salmonid species. A faint band of a similar length was also identified in the gills of the FW stenohyaline cyprinid *Cottus gobio* (Fig. 4).

In summary, sequence comparison and Northern blot analysis demonstrated that the cloned PCR product functions as a specific probe in expression studies of the Na^+, K^+-ATPase α-subunit-encoding gene in at least the salmonid species. The possible existence of multiple isoforms in lower vertebrates that might be expressed, in a development- and tissue-specific manner should, however, be elucidated before drawing further conclusions.

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