Synthesis of gill Na+ -K+ -ATPase in Atlantic salmon smolts: differences in alpha-mRNA and alpha-protein levels

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Synthesis of gill Na^+-K^+-ATPase in Atlantic salmon smolts: differences in α-mRNA and α-protein levels

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D'Cotta, Helena, Claudiane Valotaire, Florence Le Gac, and Patrick Prunet. Synthesis of gill Na^+-K^+-ATPase in Atlantic salmon smolts: differences in α-mRNA and α-protein levels. *Am. J. Physiol. Regulatory Integrative Comp. Physiol.* 278: R101–R110, 2000.—Several parameters were analyzed to determine the mechanisms responsible for the enhancement of the gill Na^+-K^+-ATPase activity of Atlantic salmon smolts. A major α-subunit transcript of 3.7 kb was revealed by Northern blot in both parr and smolt gills when hybridized with two distinct cDNA probes. The α-mRNA abundance demonstrated an increase to maximal levels in smolts at an early stage of the parr-smolt transformation. This was followed by a gradual rise in α-protein levels, revealed by Western blots with specific antibodies and by an increase in gill Na^+-K^+-ATPase hydrolytic activity, both only reaching maximum levels a month later, at the peak of the transformation process. Parr fish experienced a decrease in α-mRNA abundance and had basal levels of α-protein and enzyme activity. Measurement of the binding of [^{3}H]ouabain to Na^+-K^+-ATPase was characterized in smolts and parr gill membranes showing more than a twofold elevation in smolts and was of high affinity in both groups (dissociation constant = 20–23 nM). Modulation of the enzyme due to increased salinity was also observed in seawater-transferred smolts, as demonstrated by an increase in α-mRNA levels after 24 h with a rise in Na^+-K^+-ATPase activity occurring only after 11 days. No qualitative change in α-expression was revealed at either the mRNA or protein level. Immunological identification of the α-protein was performed with polyclonal antibodies directed against the rat α-specific isoforms, revealing that parr, freshwater, and seawater smolts have an α_3-like isoform. This study shows that the increase in Na^+-K^+-ATPase activity in smolt gills depends first on an increase in the α-mRNA expression and is followed by a slower rise in α-protein abundance that eventually leads to a higher synthesis of Na^+-K^+ pumps.

sodium pump; salmonids; smoltification; [^{3}H]ouabain binding; transcript

MIGRATING SALMONIDS are particular euryhaline species in which the alterations for ocean life are preparative, precede seawater entrance, and take place in freshwater during the developmental process of the parr transformation into a smolt (or smoltification). This transient stage is a complex physiological shift accompanied by both behavioral and metabolic modifications controlled by a multitude of endocrine factors (3, 15). Preparatory mechanisms in osmoregulatory functions are specially accentuated at the gill level and are associated with alterations in gill epithelia (2, 21). These fish face diffusional ion loss and water entry in freshwater requiring ion absorption. The process is reversed in seawater, where the excretion of salts becomes necessary to compensate for dehydration and salt accumulation. During smoltification, the fish undergo an increase in both size and number of branchial Cl^- cells (19, 34), an enhancement of the Cl^- cell tubular network, which consists of invaginations of the basolateral membrane (35), and an increase in branchial Na^+-K^+-ATPase activity (13, 21, 37, 48). The Cl^- cell membrane and tubular system are the main location of the gill Na^+-K^+-ATPase (16, 19), now commonly accepted as being the primary mechanism involved in ion output of seawater fish (11, 43).

Studies concerning the Na^+-K^+-ATPase performed in higher vertebrates have demonstrated that the enzyme comprises an α- and β-subunit. The α-polypeptide carries the catalytic and ion transport properties, whereas the β-subunit appears to modulate protein maturation and the enzyme translocation to the plasma membrane (12, 25). Heterogeneity of the α-subunit with various isoforms in higher vertebrates has been demonstrated (41, 42). These α-isoforms show different affinities for Na^+ and K^+, varying sensitivity to the inhibitor ouabain (44), and are present in different tissues at certain developmental stages (14, 26). In fish, studies have been centered mainly on some of the biochemical enzyme properties and changes in enzyme hydrolytic activity (2, 13, 32). The presence of different α-isoforms still deserves further clarification. Indeed, cloning studies of teleost α-subunits have only recently been initiated (8, 40, 46), with the identification of a cDNA of an α_1-isoform and only part of an α_3-cDNA having been found in the euryhaline eel (6, 7).

Although numerous studies have evaluated Na^+-K^+-ATPase activity changes in different euryhaline species, performed either after salinity changes or during the parr-smolt transformation in salmonid species (2, 28), little information exists on the actual mechanisms responsible for this enhancement. In a preliminary study conducted in Atlantic salmon *Salmo salar*, we determined that a quantitative difference in the expression of a 3.7-kb α-mRNA found between parr and smolts was one of the molecular mechanisms respon-

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sible for the increase in gill Na^+-K^+-ATPase activity (8). This has also been reported in masu smolts *Onchorynchus masou*, although qualitative changes were due to 3.3- and 5-kb transcripts (46). Salinity has also been observed to modify the enzyme, exerting an even more substantial rise in activity. In tilapia *Oreochromis mossambicus*, a salinity-dependent stimulation of the α-protein levels has recently been described (23). Despite these reports, the time course and mechanisms leading to the rise in gill Na^+-K^+-ATPase activity are still unclear.

The present study was performed to evaluate exhaustively the mechanisms and the time course leading to the large increase in gill Na^+-K^+-ATPase hydrolytic activity during the parr-smolt transformation in freshwater. Changes in the α-mRNA levels, α-protein variations, and the number of enzyme units were analyzed. Effects of increased salinity on these parameters were also examined in smolts after seawater transfer, focussing on short- and long-term modulation of the enzyme. Furthermore, modifications of α-isoforms were investigated using a more variable cDNA probe, and protein qualitative changes were examined with rat α-isoform-specific antibodies.

MATERIALS AND METHODS

Rearing and Sampling Conditions

Atlantic salmon belonged to a Norwegian strain (Sundansøra/Matre) and were reared at the Drennec fish hatchery in Sizun (Brittany, France). The fish were raised under a natural photoperiod in indoor tanks using well-spring water until the age of 3 mo, after which they were transferred to outdoor 1.8-m^3 circular tanks supplied with Drennec river water. They remained in these tanks for 1 yr until the process of smoltification took place. The fish were fed a daily ratio (dependent on their weight and water temperature) of commercial feed (Aqualim). Average rearing temperatures oscillated between 7°C in the coldest month (February) to 18°C in July and August. According to the bimodal growth of salmon, the fish were divided into two groups in early March, consisting of parr fish (with a slow growth rate that will not undergo smoltification that year) and future smolts (rapid-growing fish). Both groups were subjected to the same environmental conditions. Two experiments were performed on these fish.

Parr-smolt transformation study. Monthly samplings commenced in late October 1993 and lasted until July 1994, whereas bimonthly samplings were performed from early March to late May. As a result of a low parr availability, this group was only sampled in March and April. Additional parr and smolt fish were sampled during the smoltification period of 1998 for [^{3}H]ouabain binding studies. Timing of the parr-smolt transformation was determined by evaluating the increase in branchial Na^+-K^+-ATPase activity and morphological alterations (loss of melanin parr marks, silvery coating, darkening of fin margins, and streamline appearance).

Seawater transfer and freshwater comparison of smolts. At peak time of smoltification (April), 130 smolts were transported from the Drennec hatchery to the Rennes fish facilities and were acclimated for 1 wk before beginning the experiment. On April 21, one-half of the smolts (of ~77 g) was transferred to 31‰ salinity seawater prepared with artificial salt (Instant Ocean), and the other one-half was maintained in freshwater for comparison studies. Fish were sampled (n = 9) 1, 3, 12, and 24 h after seawater transfer and at 4, 11, and 25 days after seawater transfer. Sampling conditions were basically the same in both experiments. Before each sampling, the fish were fasted for 24 h. At sampling, the fish were anesthetized with ethylene glycol monophenyl ether (0.3 ml/l; Merck) and then killed by a blow on the head. Blood was obtained from the dorsal vein with heparinized syringes, and plasma was collected after centrifugation. The plasma was stored at −20°C until analysis. The first pair of gill arches was excised for Na^+-K^+-ATPase activity measurements, and the remaining were sampled for microsome preparations or for RNA extraction. The arches were quickly frozen in liquid nitrogen and kept at −70°C until the day of preparation. In all cases, gill filaments were collected per fish except in the parr sampled for the analysis of [^{3}H]ouabain binding, where the gills of four individuals were pooled.

Plasma Analysis of the Freshwater-Seawater Experiment

Plasma osmolarity was monitored by analyzing Na^+ and Cl^- levels, which were performed in duplicate using 20-μl aliquots. For Na^+ measurements, the plasma was diluted 1:3,000 times and then measured on an atomic absorption spectophotometer (Varian). Cl^- plasma was analyzed using a colorimetric method on a CL 900 automatic chlorometer. In both cases, values are given as milliequivalents per liter.

Na^+-K^+-ATPase Hydrolytic Activity

Enzyme hydrolytic activity was measured by determining the liberated P_i on a semicrude preparation of gill tissue (8). The tissue was resuspended in the homogenization buffer (0.3 M sucrose, 20 mM Na_2EDTA, and 100 mM imidazole; pH 7.1) after thawing, on the day of the enzyme assay. Two centrifugations were performed at 2,000 *g* for 7 and 6 min, the last after 0.1% sodium deoxycholate was added to the homogenization buffer when the pellet was resuspended. Activity measurements were performed in a 0°C ice bath unless indicated otherwise. For the Na^+-K^+-ATPase activity measurements, 20 μl of enzyme preparation were added to 500 μl activity solution (either *A* or *B*, see below), together with 100 μl of ATP (33 mM), and this mix was then incubated at 37°C for 10 min. Liberated P_i was measured after adding 3 ml of a (1:1) mix of Lubrol (10 g/l)-molybdate (10 g/l with 10% H_2SO_4). A 30-min incubation at 20°C was performed before reading at 405 nm. Na^+-K^+-ATPase activity was calculated from the difference in total activity using a solution without ouabain (*A solution* = 23 mM $MgCl_2 \cdot 6H_2O$, 155 mM NaCl, 75 mM KCl, and 115 mM imidazole; pH 7) and the activity obtained in separate tubes containing 0.576 mM ouabain (*B solution*) in the above buffer. The total protein content was measured using the Bio-Rad protein dye with BSA as standard.

Gill Microsome Preparation

Gill microsomes were purified by differential centrifugation just a couple of days before analysis because of better conservation of the Na^+-K^+-ATPase activity in unprepared samples. Gill filaments were scraped off the cartilage with a scalpel and immersed in 4 ml cold homogenization buffer (H: 0.3 M sucrose, 20 mM Na_2EDTA, and 100 mM imidazole, pH 7.1, with 1 mM phenylmethylsulfonyl fluoride). All of the preparative steps were performed at 4°C. The tissue was homogenized with a tissumizer at low speed for 6 s. The tissue was filtered through hybridization buffer-soaked gauze, the volume was increased to 17 ml, and the tissue was centrifuged at 8,000 *g* for 10 min. The supernatant (S1) was recuperated, the pellet was resuspended in hybridization buffer using a Douce homogenizer, and the volume was brought to 10 ml and recentrifuged at 8,000 *g* for another 10

min to recuperate all possible Na^+-K^+-ATPase present in the nuclei and mitochondria pellet. The supernatant from this centrifugation was combined with S1, and this was then centrifuged at 100,000 *g* (Rotor R55 38) for 1 h. The pellet obtained was resuspended in hybridization buffer, adjusted to 15 ml, and centrifuged again at 100,000 *g* for 1 h. The final pellet of purified gill microsomes was resuspended in 0.3 M sucrose, 2 mM Na_2EDTA, and 50 mM imidazole (pH 7.1) in 0.6–1.5 ml depending on pellet size (~4 μg/μl) and was stored at −70°C. Enrichment of the microsome preparation was assessed by measurement of the Na^+-K^+-ATPase activity and was compared with the starting crude homogenate and each centrifuged fraction.

Northern and Dot-Blot Analysis

Gill total RNA was extracted following the procedure published (5). To detect qualitative changes of the α-subunit, RNA was analyzed by Northern blots. Denatured total RNA samples (15 μg) were fractionated on 1% agarose-formaldehyde gels and transferred by capillary transfer to nylon membranes (Pall) using a solution of 20× SSC buffer (1× SSC = 0.15 M NaCl, 15 mM sodium citrate; pH 7). To quantify changes in the α-mRNA amounts, dot blots were performed according to the procedure previously published (38), applying 5 μg of denatured total RNA to a nylon membrane using a manifold system. The membranes were probed with two different cDNA fragments encoding for the rainbow trout α-subunit: αt-T20, a cDNA (672 bp) corresponding to the coding region containing the putative H-5 and H-6 conserved transmembrane domains (8), and αt-EL, a cDNA (1,122 bp) corresponding to a more variable region containing the regions of the H-1 to H-4 transmembrane domains (D'Cotta, Valotaire, and Prunet, unpublished observation). The DNA were ^{32}P labeled by the multiprime DNA labeling kit (Amersham). Hybridization of the Northern and dot blots was performed overnight at 42°C in 50% (vol/vol) formamide, 5× SSC, Denhardt's solution, 0.1% (wt/vol) SDS, and 0.1 mg denatured calf thymus DNA. The membranes were washed four times in 2× SSC-0.1% SDS at 20°C for 5 min each wash and then three times in 0.1× SSC-0.1% SDS at 50°C for 15 min each wash. All samples belonging to a same experiment were analyzed on the same blots and therefore had equivalent exposure times (48–72 h). After autoradiographs of the Na^+-K^+-ATPase α-subunit were obtained, the membranes were dehybridated with 10 mM $NaPO_4$, pH 6.5, and 50% formamide for 1 h at 65°C and then were washed for 30 min at 20°C in 2× SSC and 0.1% SDS. They were then rehybridized with a rainbow trout β-actin probe (33). Relative intensities of the bands were measured with a densitometer.

Protein Separation and Quantification

Gill microsomal proteins (30 μg) were separated on either 5 or 7.5% SDS-polyacrylamide minigels after denaturing in a sample buffer containing 150 mM dithiothreitol and heating at 65°C for 5 min. Proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore) using a 39 mM glycine, 48 mM Tris·HCl, 0.0375% SDS transfer buffer in a semi-dry system. Transfer efficiency was assessed by staining the gel with Coomassie blue and by membrane staining with Ponceau red. Membranes were blocked with 5% instant dry milk and 2% pork serum in Tris-buffered saline (TBS: 20 mM Tris, pH 8, 0.137 mM NaCl with 0.1% Tween 20) for 1 h at 20°C. Membranes were subsequently incubated with either specific α-isoform polyclonal antibodies (1:100) or monoclonal α_5-IgG directed against chicken α-subunit (1:10,000), diluted in blocking solution, and incubated overnight at 4°C. The polyclonal antibodies used were site directed and isoform specific and were derived from oligopeptides; the antibodies were kindly provided by Dr. T. A. Pressley (Texas Tech University, Lubbock, TX). They corresponded to sequence regions of rat specific α-isoforms. These rabbit antibodies were as follows: LEAVE, a generic antibody; NASE, an α_1-specific antibody; HERED, an α_2-specific antibody; and TED, α_3-specific antibody. The monoclonal antibody developed by Dr. D. M. Fambrough was obtained from the Developmental Studies Hybridoma Bank maintained by The University of Iowa Department of Biological Sciences (Iowa City, IA; NO1-HD-7–3263 from the National Institute of Child Health and Human Development). Blots incubated with polyclonal antibodies were washed (3 × 15 min) and incubated for 1 h at 37°C with goat anti-rabbit IgG horseradish peroxidase conjugate (1:8,000; Sigma). Color detection was obtained using 1% *o*-dianisidine prepared in acetonitrile with 1.66% solution (1 M imidazole, pH 7.5) and in 0.05% (vol/vol) Tween 20 plus 0.066% H_2O_2. Blots incubated with monoclonal antibody were washed six times for 5 min in TBS and incubated with horseradish peroxidase-conjugated antimouse IgG (Dako) diluted at 1:12,000 for 1 h at 20°C. After thorough washings (7 × 5 min; 2 × 10 min), the bound antibodies were visualized by chemiluminescence with the enhanced chemiluminescence (ECL) system (Amersham) and with exposure to Kodak BioMax MR1 film. Quantification of the α-protein was performed on samples from the parr-smolt experiment by immunoblots. Ten micrograms of gill microsome proteins were deposited per well, probed with the monoclonal antibodies, and visualized with the ECL procedure described above. Total protein concentrations ranging from 3 to 25 μg were tested and showed a linear relationship with dot intensity. Signal intensity was quantified using the Bio-Rad image analyzer, with multiple exposures of the films. We established an arbitrary value of one for the protein concentrations obtained for the first sampling date (December 14, 1993).

[^{3}H]ouabain Binding

Determination of the number of Na^+-K^+-ATPase units was performed on parr and smolt gill microsomes sampled in 1998 by measuring [^{3}H]ouabain binding in the presence of vanadate to block the pump in the phosphorylated configuration. Maximal binding (B_{max}) was assessed using a concentration range of 1 nM-2 μM [^{3}H]ouabain (initially 15 Ci/mmol, ~65 μM, diluted to 1.5 Ci/mmol with unlabeled ouabain; NEN). Total binding was determined in a buffer containing 120 mM NaCl, 3 mM $MgSO_4$, 3 mM Na_2HPO_4, 10 mM Tris, pH 7, and 1 mM Na_3VO_4 in a volume of 200 μl. Nonspecific binding was assessed in the presence of 1,000× excess unlabeled ouabain and 30 mM KCl. The specific binding was obtained by subtracting the nonspecific binding from the total binding and was normalized to milligrams of total microsomal protein. A pool of parr samples (n = 4) was necessary per measurement due to reduced tissue size. Four measurements performed in duplicate were performed for each fish group. Incubation time required to reach equilibrium at 37°C was assessed in initial experiments. Equilibrium was established after 30 min and remained stable for >2 h. Therefore, B_{max} analysis was performed by incubating samples for 60 min at 37°C. A preincubation step of solutions was carried out for 5 min at 37°C, after which gill microsome samples (~50 μg/20 μl) were added. At the end of the binding incubation, samples were placed on ice, diluted with 1 ml ice-cold total binding buffer, and filtered under vacuum on 0.22-μm GSWP filters (Millipore) using a Millipore filtering apparatus. Filters were then rinsed three times with 1 ml of the same ice-cold buffer,

placed in Filtercount scintillation liquid (Packard), and digested for ~24 h before counting.

Statistics

Data are given as means ± SE. ANOVA was performed for multiple comparisons, and significant differences ($P < 0.05$) found between means were further analyzed using the follow-up Tukey test. When only two comparisons were done, Student's *t*-test was used.

RESULTS

Parr-Smolt Transformation

Na^+-K^+-ATPase activity and determination of Na^+-K^+ pump number in gills. The Na^+-K^+-ATPase hydrolytic activity was measured in semicrude gill preparations of parr and smolts throughout the parr-to-smolt transformation. These two groups were sampled and divided according to their growth rate, as described in MATERIALS AND METHODS. Gill Na^+-K^+-ATPase activity remained low in both of these groups before smoltification occurred (Fig. 1), with a constant level ranging between 5 and 9 μmol $P_i \cdot mg\ protein^{-1} \cdot h^{-1}$. In the smolt group, activity levels started rising gradually on March 8 (30.32 ± 3) and were nearly fourfold higher than those observed in the parr (8.25 ± 1). Gill enzyme activity continued increasing in smolts, reaching peak levels on April 20 (57.3 ± 4.7) after which it declined to significantly reduced levels (44.4 ± 3.6) on June 18 and dropped even further to near basal amounts (19.3 ± 1.8) in July. In the parr fish, no fluctuations in activity were noticed, with levels remaining stable in the range of 4–8 μmol $P_i \cdot mg\ protein^{-1} \cdot h^{-1}$. As judged by the different morphological parameters and by the gill Na^+-K^+-ATPase activity curve, we estimate that the rapid-growing mode fish (smolts) went through the parr-smolt transformation between March 23 and May 18.

Because the increase in gill Na^+-K^+-ATPase activity seen in smolts could be the result of higher pumping activity and/or due to an increase in enzyme synthesis, we performed a quantification of the number of Na^+-K^+ pump units. Quantification was estimated by [^{3}H]ouabain binding at saturation on gill microsomes from both parr and smolt fish analyzed during the smoltification peak (Fig. 2*A*). Assessment of binding, when analyzed by Scatchard plot linearization, indicated apparent dissociation constant values (K_d) of 23.4 ± 2.7 and 20.8 ± 5.7 nM in smolts and parr, respectively. Estimation of B_{max} levels indicated significantly higher ouabain binding in smolts, with values of 75 ± 13.3 pmol ouabain bound/mg (Fig. 2*B*) compared with parr fish (33.4 ± 4.7 pmol ouabain bound/mg protein). In these fish, gill Na^+-K^+-ATPase activity obtained in 1998 was similar to levels shown in Fig. 1, with high values of 30.3 ± 3.2 μmol $P_i \cdot mg\ protein^{-1} \cdot h^{-1}$ in smolts and significantly lower amounts of 10.2 ± 2 in parr fish. Although enzyme activity was measured in semicrude gill preparations while [^{3}H]ouabain binding was performed on microsomes, a rough turnover rate of the pump can nevertheless be estimated by dividing the maximum hydrolytic activity by B_{max}. Salmon gill Na^+-K^+ pump showed a turnover rate of ~6,000 cycles/min, which is in the range of mammalian pumps (20, 30).

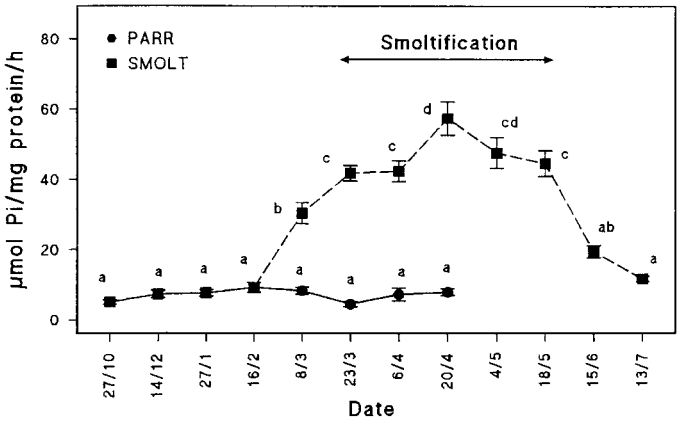

Fig. 1. Changes in gill Na^+-K^+-ATPase activity throughout the parr-smolt transformation. Values are means ± SE (n = 11 or 12 fish). Groups with different letters indicate significant difference ($P < 0.05$).

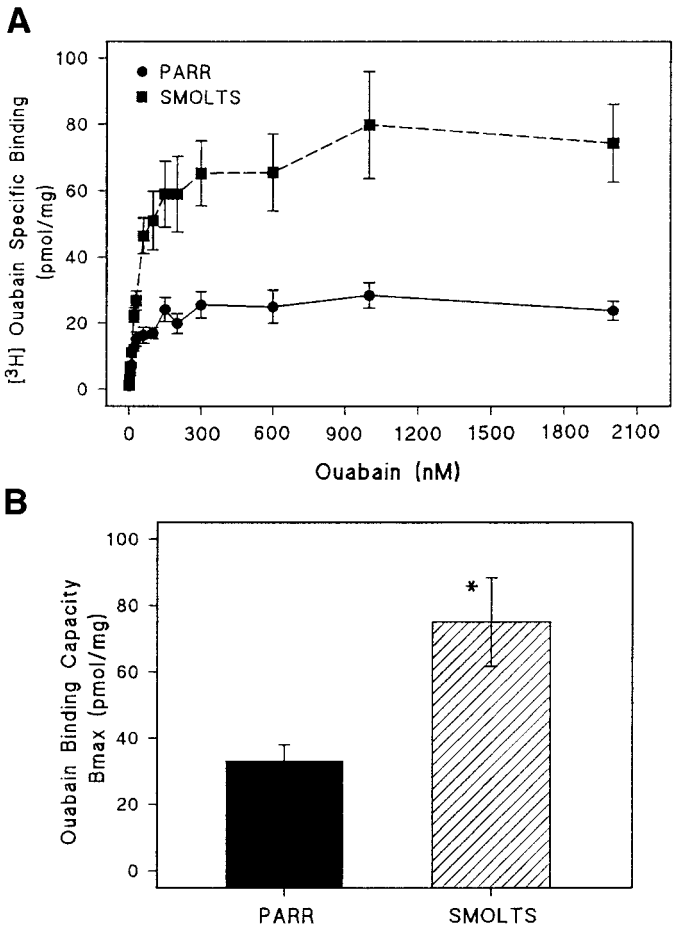

Fig. 2. *A*: saturation curve of [^{3}H]ouabain specific binding to parr and smolt gill microsomes. Each data point represents the mean ± SE of 4 different measurements performed either on 4 smolt fish or on a pool of 4 parr fish. *B*: ouabain maximal binding (B_{max}) values (pmol ouabain bound/mg microsomal protein) calculated for parr and smolt gills; data represent the concentration of Na^+-K^+-ATPase units in the gill tissue. *Significant difference between parr and smolt ($P < 0.05$).

mRNA analysis. Possible modifications in the expression of the mRNA encoding for the α-subunit were conducted by a time course study throughout the parr-smolt transformation. For this, we selected the most pertinent sampling dates during the smoltification cycle, taking into account the Na^+-K^+-ATPase activity changes. No qualitative changes in α-mRNA expression were detected. Our Northern blot analysis gave similar results, whether we used the conserved α-subunit cDNA probe (αt-T20) or the more divergent cDNA (αt-EL; Fig. 3). These cDNAs hybridized strongly to a 3.7-kb mRNA in both parr and smolt samples. In smolts from April, two additional α-mRNAs were clearly revealed of ~5.4 and 6.1 kb. They were also visualized in parr fish in April, but were much fainter. These bands were also seen in postsmolts of June and July (data not shown) but again were fainter than those of smolts in April, which clearly suggests that they are not related to the parr-smolt transformation and could be integrated in the total quantification of the hybridization signal obtained using the α-subunit cDNA probe. Relative abundance of the α-subunit mRNAs was determined by dot-blot analysis, normalizing against actin mRNA. The initial sample point was on December 14 (0.92 ± 0.04). Thereafter, the fish were separated into parr and smolts, as described previously. By March 8, marked differences in α-mRNA amounts were established between parr and smolt groups (Fig. 4). In parr fish, the α-mRNA levels went through a twofold reduction, already partly appreciable on March 8 (0.78 ± 0.13) but being significant (0.48 ± 0.06) on April 6 and remaining at this steady state until April 20. The contrary, however, was observed for smolt fish in which

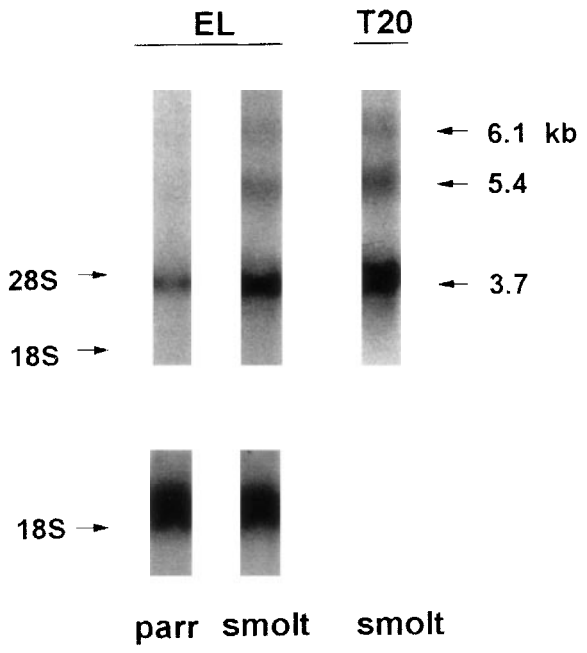

Fig. 3. Representative Northern blot analysis of total RNA from parr and smolt Atlantic salmon gill tissue, hybridized with either the rainbow trout α-subunit EL cDNA (1,122 bp) or the T20 cDNA (672 bp) in two identical Northern blots. Positions of ribosomal RNA are indicated on *left*. Positions of mRNA detected are indicated on *right*. Actin levels are shown at *bottom*.

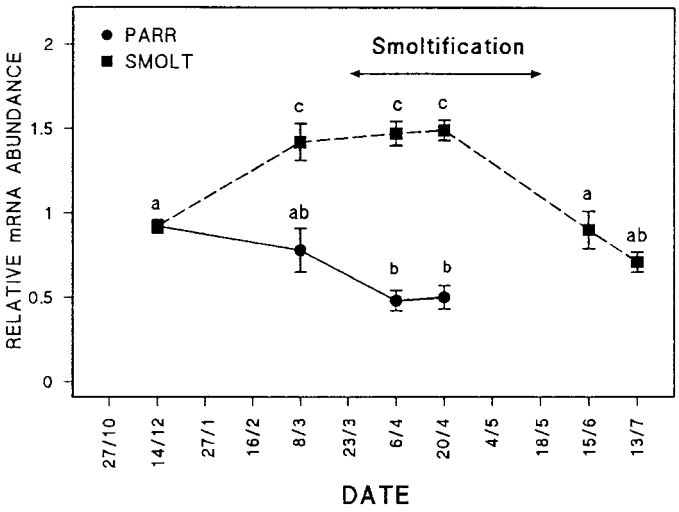

Fig. 4. Changes in relative abundance of Na^+-K^+-ATPase α-subunit mRNA vs. actin mRNA ratios at various dates throughout parr-smolt transformation. Total RNA (5 μg) was deposited. Values are means ± SE (n = 4 or 5 fish). Different letters indicate significant difference ($P < 0.05$).

α-subunit mRNA levels experienced a 1.5-fold increase on March 8 (1.42 ± 0.11) and stayed elevated at these maximum levels during the whole smoltification period. Amounts started declining on April 20, reaching low levels on June 15 similar to those found initially in December and decreasing further to amounts resembling those of parr fish on March 8.

Immunological identification and quantification of α-subunit proteins. To test for the presence of different α-isoforms in salmon gills, gill membranes were separated by Western blots and probed with specific α-subunit antibodies. We used rat antibodies that have been previously shown to detect fish α-subunit (36). Replicate Western blots containing parr, smolt, freshwater-adapted, and seawater-adapted smolt gill microsomes were prepared. The blots were stained with the α_1-, α_2-, α_3-, and α-generic polyclonal antibodies. Salmon α-subunit was observed to be of ~101 kDa, with an electrophoretic band revealed by the generic α-LEAVE antibody (Fig. 5*A*). The wider 101-kDa band in smolts and seawater smolts suggests a greater quantity of α-subunit than that present in parr and freshwater smolts. Probing with the monoclonal antibody also revealed a large band of approximately the same molecular weight (Fig. 6). Interestingly, probing of the membranes with the α_1- and α_2-antibodies revealed no band in any of the samples (Fig. 5*C*), but the specific α_3-TED antibody did stain a polypeptide band (Fig. 5*D*). With the use of the LEAVE antibody, no change was seen in the size of the ~101-kDa seawater α-protein. A similar band was visualized when probing both seawater and freshwater retained smolts with the α_3-antibody. Incubation with the other α-specific antibodies did not reveal the appearance of any other size band or a change in α-isoform type.

Quantification of band intensity was performed on dot blots using the monoclonal α_5-antibody after verifying the specificity of the antibody on Western blots (Fig.

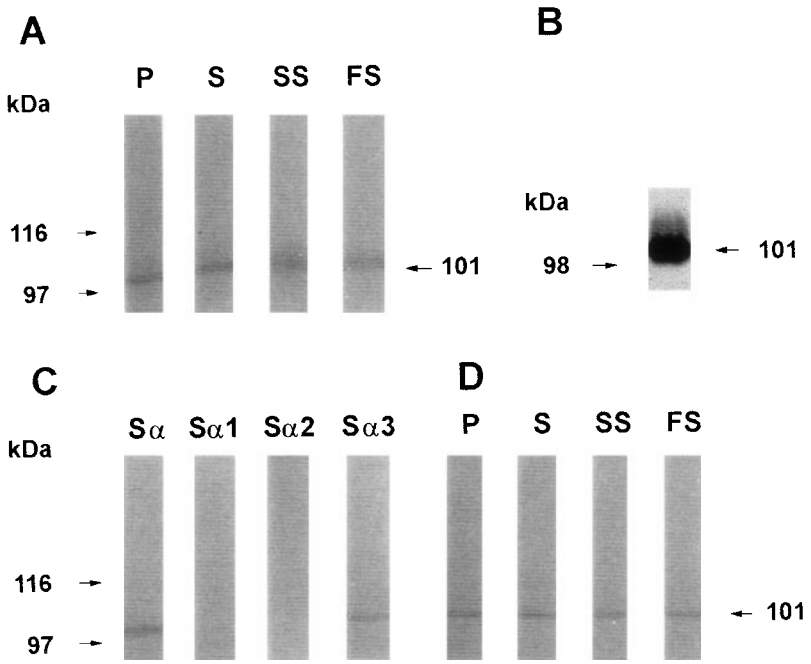

Fig. 5. Western blot analysis of α-subunits. *A*: probed with the generic α-antibody (LEAVE) on salmon parr (P), smolt (S), 25 days seawater smolt (SS), and 25 days freshwater smolt (FS) gill membranes. *B*: gill membranes of smolts probed with α_5-monoclonal antibody separated on a 7.5% SDS-PAGE. *C*: gill membranes of smolt fish incubated with α (LEAVE)-, α_1 (NASE)-, α_2 (HERED)-, and α_3 (TED)-antibodies. *D*: gill membranes of parr, smolt, seawater smolt, and freshwater smolt incubated with α_3 (TED)-antibody.

5*B*). In smolts, protein abundance gradually increased twofold on March 8 and continued rising significantly until the parr-smolt transformation was complete, achieving more than a threefold increase compared with initial values, and the α-subunit abundance subsequently declined in this group. In parr fish, as with the Na^+-K^+-ATPase activity levels, the α-protein abundance did not change, remaining constant throughout the dates tested.

Freshwater-Seawater Transfer of Smolts

To determine possible modifications of the gill Na^+-K^+-ATPase due to salinity, smolt fish were transferred during the peak of smoltification (20 April) to 31‰ salinity seawater and were maintained at this salinity until the end of the experiment. In these fish, plasma Na^+ and Cl^- levels rose above those of freshwater smolts (Fig. 7), but this disequilibrium of the hydromineral balance was transient, indicating that these fish were clearly smolts and were capable of osmoregulating in seawater.

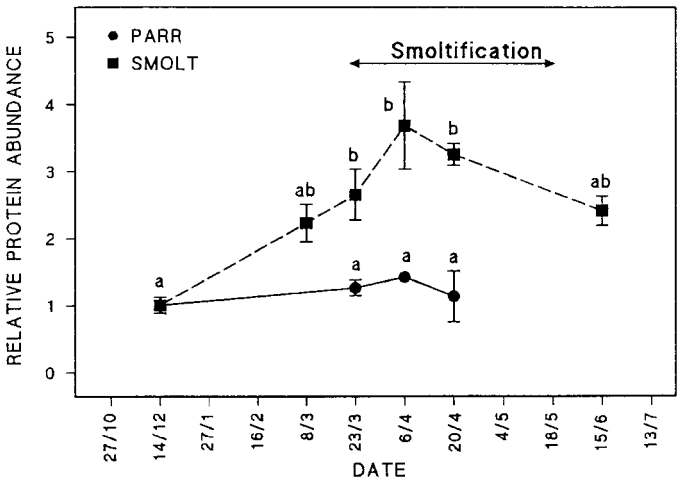

Fig. 6. Changes in relative protein abundance of the Na^+-K^+-ATPase α-subunit at various dates throughout the parr-smolt transformation. Relative value of 1 was designated to data from 14/12. Values are means ± SE (n = 4 fish). Different letters indicate significant difference ($P < 0.05$).

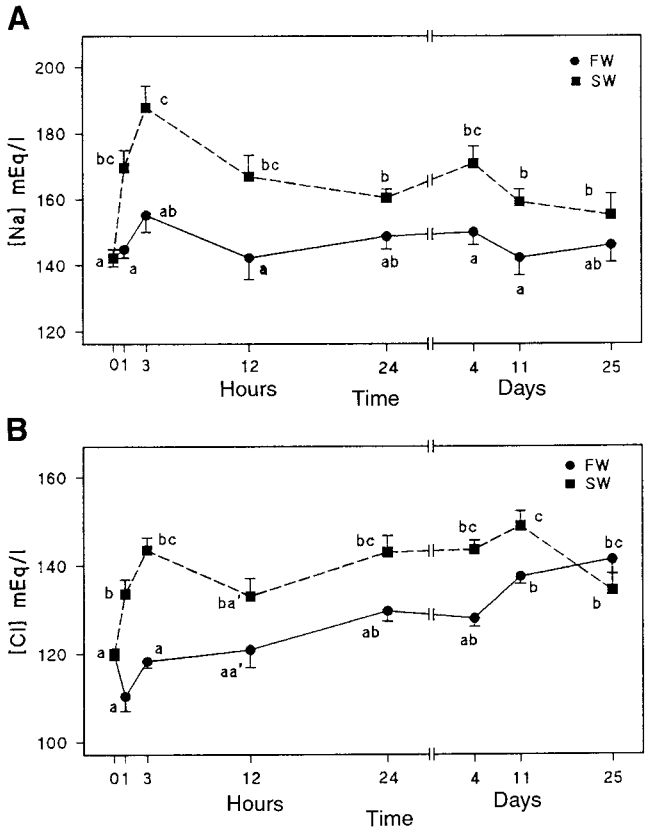

Fig. 7. Changes in plasma ion levels. *A*: Na^+ levels of seawater-transferred smolts and of freshwater smolts. *B*: values for Cl^- plasma levels of the same fish. Values are means ± SE (n = 8 fish). Brackets denote concentration. Different letters indicate significant difference ($P < 0.05$).

α-mRNA abundance and Na+-K+-ATPase hydrolytic activity. The abundance of α-subunit mRNA began to rise by 3 h (Fig. 8*A*), reaching nearly a 1.8-fold increase at 24 h when compared with the freshwater fish and the amounts detected for smolts before seawater transfer. Abundance of α-mRNA declined thereafter to values very close to those of freshwater fish on *day 11* and experienced a slight, although not significant, increase on *day 25*.

Branchial Na^+-K^+-ATPase activity of smolts (46.2 ± 4.3) upon seawater transfer showed an increase on *day 4* in seawater smolts and reached significant values (69.23 ± 12.6) on *day 11* (Fig. 8*B*). Moreover, these values were found to be significantly different from those of freshwater smolts. The enzyme activity declined thereafter in both seawater and freshwater smolts.

DISCUSSION

Abundant literature exists concerning the increase of the gill Na^+-K^+-ATPase activity experienced by euryhaline fish upon entering seawater (28). In salmon, these activity changes increase severalfold and constitute a preadaptation occurring when the fish undergo the parr-smolt transformation (3). Nevertheless, the molecular mechanisms responsible for the rise in enzyme activity experienced by smolts are not understood. By simultaneously analyzing the accumulation of the α-mRNA, the abundance of α-protein, and the number of Na^+-K^+-ATPase units and enzyme activity, we have been able to evaluate the time course of some of the mechanisms implicated. We have shown an early rise in α-mRNA expression and a later increase in α-protein levels, leading to higher biosynthesis of the gill enzyme. A comparable modulation of the Na^+-K^+-ATPase also appears to occur in smolts after their transfer to seawater.

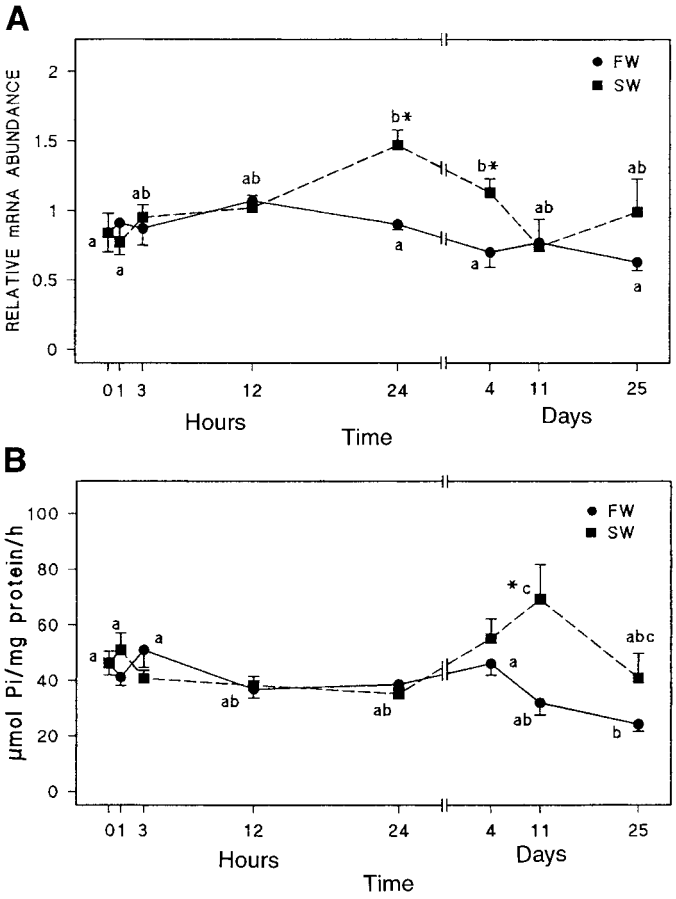

Fig. 8. *A*: changes in relative α-subunit mRNA abundance in smolts transferred to seawater compared with freshwater-retained smolts. Values are means ± SE (n = 4 or 5 fish). *B*: changes in gill Na^+-K^+-ATPase activity of seawater-transferred smolts and freshwater-retained smolts. Data are means ± SE (n = 8 fish). Different letters indicate significant difference ($P < 0.05$). * Significant difference found between freshwater and seawater fish.

Similar to studies concerning other salmonid presmolts (4, 21, 48), our Atlantic salmon had low gill Na^+-K^+-ATPase hydrolytic activities, with levels gradually increasing as smoltification advanced. Enzyme activities rose substantially and, during the peak moment of smoltification, achieved a sevenfold increase in smolts compared with parr fish. During this period, we estimated the number of pumps present by [^{3}H]ouabain binding (30) by taking into consideration that there is one ouabain binding site per Na^+-K^+ pump and by using vanadate to facilitate the binding. Our saturation experiments determined that smolts possessed a 2.4-fold higher [^{3}H]ouabain binding than parr fish with no modification in the apparent K_d values, indicating that in smolts there is a higher synthesis in the number of Na^+-K^+ pumps. Thus increased pump biosynthesis is at least one of the events leading to the enhanced Na^+-K^+-ATPase activity found in smolt gills. Nevertheless, it may not necessarily be the only modulation occurring. On the basis of the maximum hydrolytic activity obtained in our semi-crude preparations and the number of ouabain binding sites measured on microsomes, we estimated a rough turnover pump rate of 6,000 cycles/min, which proved to be similar for both parr and smolt fish. This suggests that a higher synthesis of Na^+-K^+ pumps occurs in smolts but that it is not accompanied by an increase in turnover rate, at least not at this peak moment of smoltification. Values measured for gill Na^+-K^+ pump units are comparable to those obtained in other fish species upon seawater exposure. Ouabain binding in mullet *Mugil cephalus* was of 6–7 pmol/mg (17), whereas in eels a 3.1-fold increase in enzyme activity was associated wish a parallel increase in [^{3}H]ouabain binding sites (39).

The increase in the number of gill Na^+-K^+ pumps found in smolts points toward higher transcriptional rates and translation of the enzyme polypeptides. This appears to be the case for the α-subunit because our data revealed an increase in a 3.7-kb α-mRNA abundance, accompanied by a rise in the relative amounts of α-protein. However, it is difficult to pinpoint at what moment the cascade of events leading to a higher enzyme activity occurs. We have established that the α-mRNA rose 1.5-fold at an early stage of the parr-to-smolt transformation. Threshold maximum levels were reached immediately and remained at this steady state until the end of the smoltification period. The increase in α-mRNA abundance could, however, involve a higher α-gene expression and/or lower degradation rates. Sta-

bility of the elevated smolt α-mRNA amounts may be brought about by a transcriptional regulation at these two levels. Higher amounts of α-mRNAs were also observed in masu salmon smolts (46), although they corresponded to 3.3- and 5-kb transcripts rather than to a 3.7-kb transcript.

A twofold increase in α-protein abundance was observed in smolts, but this rise was gradual and in discordance with the α-mRNA accumulation. This gradual rise suggests that either a higher percentage of α-protein matures at a later stage of smoltification or that a higher translation rate occurs at this stage. Whether one or both of these parameters lead to the slow rise in α-protein detected in smolts is not clear. Nevertheless, changes in relative amounts of the α-protein were parallel to those of the Na^+-K^+-ATPase hydrolytic activity. The distinct parameters of α-mRNA, α-protein, and enzyme activity analyzed for both parr and smolts were not coordinated, either in their amounts or in the time changes. This discrepancy would suggest a two-stage modulation of the salmon enzyme occurring, the first modulation possibly acting on the α-gene expression seen at the transcriptional level and the second regulation acting at a posttranscriptional stage, apparently affecting the α-translation, which eventually influences functional Na^+-K^+ pumps. However, other factors may be contributing to the delay between reaching maximum α-mRNA levels and in reaching maximum α-protein levels. It may take some time to achieve the new steady state showing the new increased rate of synthesis in view of the constant turnover rate of existing gill Na^+-K^+-ATPase. The Na^+-K^+-ATPase is a multisubunit enzyme and becomes functional when the αβ-complex is formed (12). The activity can therefore be mediated by several mechanisms acting at various stages (10). Different rates of α-mRNA transcription and translation have been detected in mammals, not only for the α-subunit but also for the β-subunit (1, 22). In muscle cells, a fivefold increase was found for α_2-mRNA after 3,5,3′-triiodothyronine injection, but only a threefold rise was found in α-protein (1). In contrast, the β-subunit mRNA increased nearly 4-fold, and its protein increased 2-fold, whereas the activity was estimated to be only 0.5-fold higher. Na^+-K^+-ATPase activity is also regulated by the β-subunit, since the β-protein influences the stability of the α-polypeptide (10, 12). In fact, overproduction of the α-protein is rate limited by the β-protein, since assembly of the αβ-complexes is necessary for functionality, and, therefore, the β-subunit also mediates the number of pumps formed (24, 29). This means that the activity changes detected in the present study are not only under the influence of posttranscriptional α-protein changes but, as in higher vertebrates, are probably modulated by a more complex mechanism involving, in particular, the β-subunit. In the current study, attempts to evaluate β-protein levels were performed with two different antibodies, but both resulted in high amounts of nonspecific binding.

An additional explanation leading to the change in hydrolytic activity could be the presence or appearance of new α-isoforms associated with the parr-smolt transformation. Cutler et al.'s (6, 7) work in eels showed the presence of an α_1-isoform, and they partly sequenced a putative α_3-isoform. We have not evidenced the presence of α-isoforms in the current study. Even though different transcript sizes were revealed in Northern blot, none, except a 3.7-kb band, could be associated to the parr-smolt transformation. Ura et al. (46) have shown the presence of three different α-RNA transcripts of 3.3, 3.7, and 5 kb, with an homogeneous α-cDNA in wild masu salmon *Oncorhynchus masou* using poly(A)$^+$ RNA. Interestingly, they detect higher levels of both the 3.3- and 5-kb mRNA but no change in the 3.7-kb during smoltification. These findings differ considerably from ours and could be due to species differences. Strong developmental distinctions exist between Atlantic and masu salmon, and their smoltification processes can not be readily compared with masu salmon entering seawater at a much earlier age (18). In the current work, we did not possess α-isoform-specific cDNAs, but it seemed plausible that the use of a different probe containing some regions known to be more variable in higher vertebrate α-isoforms (41) could put into evidence different α-isoform mRNAs. Therefore, it is possible that more than one α-isoform mRNA is confined in the 3.7-kb size band, similar to the 3.7-kb transcripts of both α_1 and α_3 found in rat brain (31).

Identification of the α-subunit type and presence of possible α-isoform proteins was also tested by using polyclonal antibodies directed against rat α-isoforms (36). The analysis yielded an electrophoretic band of ~101 kDa in all gill samples tested and only gave positive staining when incubated with the α_3-specific antibody. This suggests that salmon gills have an α-subunit of the α_3-like type. Specific staining of the α_3-antibody was also obtained in catfish brain and gills, and, as with our findings in salmon, there was no detection of an α_1- or α_2-like isoform (36). Recently, Lee et al. (23) analyzed α-protein amounts in freshwater- and seawater-adapted tilapia fish using different α-specific antibodies and found distinctions in seawater in the ratio of α_1- and α_3-like isoforms. Although we have used the same α_2-antibody as Lee et al. (23), different α_1- and α_3-antibodies were used. The most plausible explanation for these discrepancies is that the specificity of the rat antibodies to salmon gills is different from that of tilapia gill tissue.

In the present study, we were also interested in the effects of salinity on the α-mRNA abundance in smolt fish. An enhancement of the enzyme activity was seen; this enhancement showed that external NaCl acted as a stimulus. However, we have determined that it was not at the level of the pumping mechanism but that it acted on the α-gene expression. This was evidenced by an elevation in α-mRNA abundance preceding the rise in smolt activity after entering seawater. The time lapse between these two processes is nevertheless considerable, 24 h for the rise in α-mRNA vs. 4–11 days for the enzyme activity increase. This discrepancy can perhaps be attributed to some posttranscriptional regu-

lation, similar to that depicted during the parr-smolt transformation. Although no measurement of the number of pumps was performed after seawater exposure, it seems plausible that a higher synthesis of pumps occurs. Seawater transfer of smolts could increase levels to similar values observed in mullet (17) and eels (39) upon seawater transfer. Alternatively, no new synthesis occurs, but a recruitment of smolt latent pumps could be taking place. Because measurements of both Na^+-K^+-ATPase activity and of ouabain units have been performed in gill homogenates, it is important to clarify that the amount of ouabain bound represents total αβ-units present in the homogenized tissue, although they may not necessarily be located in the plasma membrane. Na^+-K^+-ATPase present in the gill is involved in both Na^+ and Cl^- extrusion in seawater fish (47). Increased enzyme activity is not apparently required until excess salt loading of seawater occurs and may even be detrimental for smolts in freshwater. Therefore, an increase in the number of pumps could be preparative, perhaps remaining in intercellular compartments and only becoming active upon seawater entrance. Recruitment of latent pumps has been shown to be an activation mechanism present in different mammalian organs (9).

A multitude of endocrine factors control the parr-smolt transformation (3, 15, 37). Among these, enhanced growth hormone (GH) levels are commonly reported in the plasma of smolts (37), with levels increasing further after seawater entrance (4, 45). In addition, GH treatment has been shown to stimulate the α-mRNA abundance (27). In our study, the rise in α-transcript and subsequently Na^+-K^+-ATPase activity may be brought about by higher plasma GH levels after increased salinity. The additional rise in enzyme activity after seawater entry has been shown numerous times, although smolts in freshwater have already undergone the morphological cell alterations and elevation in activity necessary for seawater hypoosmoregulation (3, 34). In the present study, smolts adjusted their plasma ions rapidly upon entering seawater. Therefore, the additional increase in activity stems from the requirements of higher ion translocation across the cell.

In conclusion, the present study has demonstrated for the first time some of the molecular pathways by which the gill Na^+-K^+-ATPase activity is regulated between parr and smolt Atlantic salmon. We have determined that synthesis of new pump units is responsible for the enhanced activity seen in smolts. In smolts, there is a stimulus causing increased α-mRNA abundance, whereas in parr the opposite is suggested, with perhaps an inhibitory mechanism causing a decline in the α-mRNA expression. In addition, a slower enhancement in the α-protein levels occurs, eventually causing an increase in the Na^+-K^+-ATPase activity. In parr, the change in α-mRNA amounts is followed by a stabilization in both the α-protein and enzyme activity. In postsmolts, both regulatory mechanisms are acting, leading to a decrease in α-mRNA, α-protein, and Na^+-K^+-ATPase activity. Increased water salinity is a stimulus affecting both mechanisms leading to α-mRNA abundance and enzyme activity.

Perspectives

The reason for the time lag found in the current study between salmon gill α-mRNA abundance and both α-protein and enzyme activity needs further clarification. If two modulating pathways are taking place, as our data seem to indicate, complementary studies concerning the endocrine factors involved would greatly clarify the process leading to Na^+-K^+-ATPase activity variations between parr and smolt fish.

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