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Branchial expression patterns of claudin isoforms in Atlantic salmon during seawater acclimation and smoltification

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Tipsmark CK, Kiilerich P, Nilsen TO, Ebbesson LO, Stefansson SO, Madsen SS. Branchial expression patterns of claudin isoforms in Atlantic salmon during seawater acclimation and smoltification. *Am J Physiol Regul Integr Comp Physiol* 294: R1563–R1574, 2008. First published March 5, 2008; doi:10.1152/ajpregu.00915.2007.—In euryhaline teleosts, permeability changes in gill epithelia are essential during acclimation to changed salinity. This study examined expression patterns of branchial tight junction proteins called claudins, which are important determinants of ion selectivity and general permeability in epithelia. We identified Atlantic salmon genes belonging to the claudin family by screening expressed sequence tag libraries available at NCBI, and classification was performed with the aid of maximum likelihood analysis. In gill libraries, five isoforms (10e, 27a, 28a, 28b, and 30) were present, and quantitative PCR analysis confirmed tissue-specific expression in gill when compared with kidney, intestine, heart, muscle, brain, and liver. Expression patterns during acclimation of freshwater salmon to seawater (SW) and during the smoltification process were examined. Acclimation to SW reduced the expression of claudin 27a and claudin 30 but had no overall effect on claudin 28a and claudin 28b. In contrast, SW induced a fourfold increase in expression of claudin 10e. In accord, a peak in branchial claudin 10e was observed during smoltification in May, coinciding with optimal SW tolerance. Smoltification induced no significant changes in expression of the other isoforms. This study demonstrates the expression of an array of salmon claudin isoforms and shows that SW acclimation involves inverse regulation, in the gill, of claudin 10e vs. claudin 27a and 30. It is possible that claudin 10e is an important component of cation selective channels, whereas reduction in claudin 27a and 30 may change permeability conditions in favor of the ion secretory mode of the SW gill.

osmoregulation; teleost; tight junction; epithelia; quantitative polymerase chain reaction; expressed sequence tags; *Salmo salar*

TRANSEPITHELIAL ION TRANSPORT is determined by both cellular and paracellular parameters. Cellular ion flux is directly determined by the tissue-specific equipment with channels, exchangers, and actively pumping proteins, the activities of which may be controlled/gated by membrane voltage, membrane tension induced by osmotic phenomena, metabolic status, and cellular signaling compounds. The characteristics of paracellular pathways/pores are mainly governed by the junctional apparatus between neighboring cells. Claudins are membrane proteins, and, as main components of tight junction strands, they are directly involved in determining trans-epithelial resistance (TER) and permeability characteristics (36). It is known from mammalian studies that claudins of neighboring cell membranes interact with each other to create charge- and size-selective paracellular pores (4). Expression of the majority of examined claudin isoforms leads to an increase in TER (4), but there are also examples that claudin expression induces decreased TER due to increased cation permeability (claudin 2: 3; claudin 16: 20), suggesting their involvement in creation of cation selective pores.

In euryhaline fish, the gill epithelium possesses a high degree of plasticity, being able to adapt to the diverse osmoregulatory roles when faced with varying salinities (11). Osmoregulatory corrections require rearrangement of gill chloride cells (15, 16). Most studies aiming to describe the biochemical and molecular basis for osmoregulatory adaptation in the gill have focused on cellular ion transport proteins. From this perspective it emerges that, in most species, induction of specific isozymes of Na^+-K^+-ATPase, Na^+-K^+-$2Cl^-$ cotransporter and cystic fibrosis transmembrane conductance regulator is of fundamental importance to seawater (SW) acclimation (15, 34). In migratory salmonids, development of these homeostatic mechanisms also takes place in the river in advance to and during the downstream journey to the ocean (6, 29). This smoltification process, triggered by the increase in day length in spring, is a physiological preadaptation that prepares the juvenile freshwater (FW) salmon for life in the ocean (6).

In addition to the abundance and activity of ion transport proteins, permeability also contributes to the general transport characteristics of gill epithelia. It has long been known that osmotic permeability of eel gill decreases considerably during SW acclimation (10, 17). The biochemical basis for this change is unknown but may involve downregulation of gill aquaporin 3 expression seen during SW acclimation in eel (9). At the same time as osmotic permeability decreases in SW, the ion conductance increases (10, 26), and tight junction ultrastructure changes (27). A paracellular pathway confined to thin tight junctions that occur in SW between chloride cells and accessory cells is thought to be involved in secretion of sodium (19, 33) and contributes to the relatively high ionic permeability of the gill of SW vs. FW teleosts (17).

There are at least 24 claudin genes in the examined mammalian genomes (38). In Japanese puffer (*Takifugu rubribes*), gene duplication has apparently produced 17 genes closely related to mammalian claudin 3 and 4, and a total array of 56 isoforms have been identified (25). Homologs for some of these are also present in the distantly related zebrafish (*Danio rerio*; see Ref. 21), indicating that duplication occurred early in the evolution of teleosts. Thus the expression of an extensive array of claudins is evident in fish, and, given the crucial

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Table 1. *Primer sequences for mRNA quantification of Atlantic salmon claudin genes by real-time PCR*

Gene	Primer Sequence (5′→3′)	Amplicon Size, bp	Gene Sequence Reference (accession no.)
Claudin 10e	Left: ATCAAGGTGGCCTGGTACTG Right: GACCAGAGCACAGGGAAGTC	95	BK006391
Claudin 27a	Left: GACAGGTATCGTCGGCATCT Right: CCAGCCACAATACAGGCTCT	99	BK006400
Claudin 28a	Left: TGACTGCTCAGGTCATCTGG Right: GGTAAGGCCAGAAGGGAGTC	100	BK006401
Claudin 28b	Left: TTCTACCAGGGCTCCATCAG Right: ATGGGCAGAGCACAGATGAT	107	BK006402
Claudin 30	Left: TGATCATTGGAGGAGGGTTC Right: AACATAGTCCCTGGGTGCTG	104	BK006405
EF-1α	Left: AGAACCATTGAGAAGTTCGAGAAG Right: GCACCCAGGCATACTTGAAAG	71	AF321836

EF, elongation factor.

importance in determining the characteristics of epithelia, a broad examination of gill claudins in euryhaline teleost will be important in understanding the role of tight junction dynamics to osmoregulatory processes.

We therefore: *1*) picked out expressed claudin genes from the salmon transcriptome and classified them according to previously used nomenclature, *2*) confirmed tissue specificity of isoforms found in gill libraries, *3*) examined the expression pattern of five gill isoforms during SW acclimation, and *4*) examined their expression pattern during smoltification. Anadromous Atlantic salmon (*Salmo salar*) was particularly advantageous for this study, because they retain moderate euryhalinity throughout life, undergo smoltification, and are very well represented in the generally available expressed sequence tag (EST) database.

MATERIALS AND METHODS

Fish and Sampling

FW-to-SW transfer experiment. For examination of the temporal development in gene expression during SW acclimation, one-year-old Atlantic salmon (Ätran stock) was obtained in early January, 2006, from *The Danish Centre for Wild Salmon* (Randers, Denmark; http://www.vildlaks.dk/). Fish were kept in FW fiberglass tanks and exposed to natural variation in temperature and daylight conditions. In September, postsmolts were moved to indoor FW tanks at 14°C at a 12:12-h light-dark cycle and, in October, either transferred to 28 ppt SW or sham transferred to FW. Eight fish from each group were sampled at 12 h, 24 h, 3 days, and 7 days after transfer, and 8 fish were sampled at 0 h as a control group.

Tissue expression experiment. For examination of tissue mRNA expression, the same Ätran salmon stock was kept in FW at the Odense Campus until sampling in January 2007. The fish were fed ad libitum three times a week with pelleted trout feed. When sampled, fish were stunned with a blow to the head, and blood was collected with a heparinized syringe from the caudal vessels after which the fish was killed. Gill, kidney, anterior intestine, heart muscle, skeletal muscle, brain, and liver tissues were dissected and, when needed, trimmed free of cartilage, fat, and connective tissue and thereafter instantly frozen in liquid nitrogen until further processing.

Smoltification experiment. Gill claudin expression was monitored during the parr-smolt development in an anadromous strain of Atlantic salmon (River Vosso; South-Western Norway). Sampling proce-

Table 2. *Claudin genes detected in the transcriptome of Atlantic salmon alongside their amino acid similarity with Japanese puffer, zebrafish and human claudin isoforms*

Gene	Salmon Reference (TPA accession no.)	Tissue Expression (cDNA sources)	Claudin Gene	Similarity (%)	E Value (tblastn)
Claudin 3a	BK006381	Mixed	CldnH-Dr	80/89 (89)	2e-83
			Cldn3aTru	81/89 (91)	7e-104
			Cldn3Hs	80/89 (89)	1e-80
Claudin 3b	BK006382	Kidney, head kidney, mixed	CldnC-Dr	19/22 (86)	3e-11
			Cldn3bTru	32/46 (69)	3e-14
			Cldn6Hs	27/46 (69)	0.006
Claudin 3c	BK006383	Mixed	CldnH-Dr	41/65 (63)	1e-25
			Cldn3cTru	44/70 (62)	1e-33
			Cldn4Hs	33/46 (71)	8e-25
Claudin 5a	BK006384	Brain, mixed	Cldn5Dr (NM_213274)	185/210 (88)	4e-104
			Cldn5aTru	198/212 (93)	2e-118
			Cldn4Hs	159/209 (76)	4e-71
Claudin 5b	BK006385	Thyroid	Cldn5Dr (NM_213274)	85/10 (85)	1e-35
			Cldn5bTru	88/99 (88)	2e-40
			Cldn4Hs	68/85 (80)	6e-20
Claudin 6	BK006386	Mixed	CldnJ-Dr	123/145 (84)	5e-67
			Cldn6Tru	132/145 (91)	4e-80
			Cldn4Hs	112/162 (69)	3e-51

Continued

Table 2.—*Continued*

Gene	Salmon Reference (TPA accession no.)	Tissue Expression (cDNA sources)	Claudin Gene	Similarity (%)	E Value (tblastn)
Claudin 7	BK006387	Reproductive system, thyroid, thymus, skin, mixed	Cldn7Dr	158/178 (88)	9e-95
			Cldn7bTru	48/48 (100)	1e-76
			Cldn7Hs	136/181 (75)	9e-72
Claudin 10b	BK006388	Mixed	Cldn10Dr	163/181 (90)	9e-101
			Cldn10bTru	68/72 (94)	3e-102
			Cldn10Hs	169/188 (89)	9e-100
Claudin 10c	BK006389	Thyroid	Cldn10cDr (XM_688085)	161/203 (79)	5e-77
			Cldn10cTru	74/79 (93)	5e-96
			Cldn10Hs	126/173 (73)	9e-52
Claudin 10d	BK006390	Thyroid	Cldn10dDr (XM_687354)	152/196 (77)	1e-85
			Cldn10dTru	72/79 (91)	2e-95
			Cldn10Hs	118/175 (67)	5e-55
Claudin 10e	BK006391	Gill, thyroid	Cldn10eDr (XM_678711)	121/159 (76)	7e-67
			Cldn10eTru	76/77 (98)	7e-98
			Cldn10Hs	118/175 (67)	5e-55
Claudin 12	BK006392	Kidney	Cldn12Dr	104/129 (84)	8e-78
			Cldn12Tru	71/78 (91)	3e-83
			Cldn12Hs	79/124 (63)	1e-48
Claudin 13	BK006393	Thyroid	CldnH-Dr	29/51 (56)	3e-05
			Cldn13Tru	88/115 (76)	6e-53
			Cldn4Hs	25/47 (53)	8e-06
Claudin 14	BK006394	Mixed	CldnC-Dr	42/54 (77)	4e-16
			Cldn2Tru	45/54 (83)	8e-20
			Cldn14Hs	52/54 (96)	7e-27
Claudin 15	BK006395	Gastrointestinal tract, mixed	Cldn15Dr	186/225 (82)	2e-102
			Cldn15bTru	68/76 (89)	7e-81
			Cldn15Hs	140/189 (74)	1e-66
Claudin 19	BK006396	Brain	Cldn19Dr (NM_001017736)	107/110 (97)	4e-69
			Cldn19Tru	62/63 (98)	7e-68
			Cldn19Hs	107/110 (96)	3e-64
Claudin 20a	BK006397	Mixed	Cldn7Dr	77/115 (66)	2e-30
			Cldn20aTru	113/116 (97)	3e-72
			Cldn20Hs	96/116 (82)	4e-50
Claudin 25a	BK006398	Gastrointestinal tract, spleen	Cldn10Dr	49/64 (76)	6e-24
			Cldn25Tru	28/39 (71)	2e-27
			Cldn15Hs	38/54 (70)	1e-19
Claudin 25b	BK006399	Gastrointestinal tract, mixed	Cldn25Dr (NM_31771)	67/97 (69)	2e-55
			Cldn25Tru	67/73 (91)	2e-55
			Cldn15Hs	58/85 (68)	4e-42
Claudin 27a	BK006400	Gill, thyroid, mixed	CldnB-Dr	134/152 (88)	2e-67
			Cldn27aTru	146/160 (91)	5e-79
			Cldn4Hs	136/152 (89)	2e-66
Claudin 28a	BK006401	Gill, thyroid, thymus, mixed	CldnE-Dr	182/205 (88)	7e-97
			Cldn28aTru	176/189 (93)	4e-108
			Cldn4Hs	162/190 (85)	2e-79
Claudin 28b	BK006402	Gill, thyroid, thymus	CldnE-Dr	69/76 (90)	4e-61
			Cldn28bTru	74/81 (91)	9e-66
			Cldn3Hs	70/75 (93)	6e-54
Claudin 29a1	BK006403	Reproductive system	CldnD-Dr	89/101 (88)	1e-88
			Cldn29aTru	91/101 (90)	7e-92
			Cldn4Hs	83/101 (82)	4e-76
Claudin 29a2	BK006404	Reproductive system	CldnB-Dr	78/88 (88)	1e-78
			Cldn29aTru	83/87 (95)	7e-91
			Cldn6Hs	77/87 (88)	6e-73
Claudin 30	BK006405	Gill, thyroid, thymus, mixed	CldnA-Dr	167/189 (88)	3e-99
			Cldn30cTru	168/191 (87)	1e-103
			Cldn4Hs	161/190 (84)	2e-88
Claudin 31	BK006406	Brain, head, mixed	Cldn31Dr (NM_001003464)	112/145 (77)	4e-28
			Cldn31Tru	116/144 (80)	8e-34
			Cldn6Hs	66/94 (70)	2e-16

Cldn, claudin; Tru, Japanese puffer caludin isoform; Dr, zebrafish caludin isoform; Hs, human claudin isoform. The expect (E) value describes the number of hits expected to occur by chance; therefore, a low E value indicates a reliable match. Obvious zebrafish orthologs that are unnamed are shown with reference numbers in parentheses. The proposed salmonid nomenclature is based on the matching known isoforms and the nodes in the maximum likelihood tree.

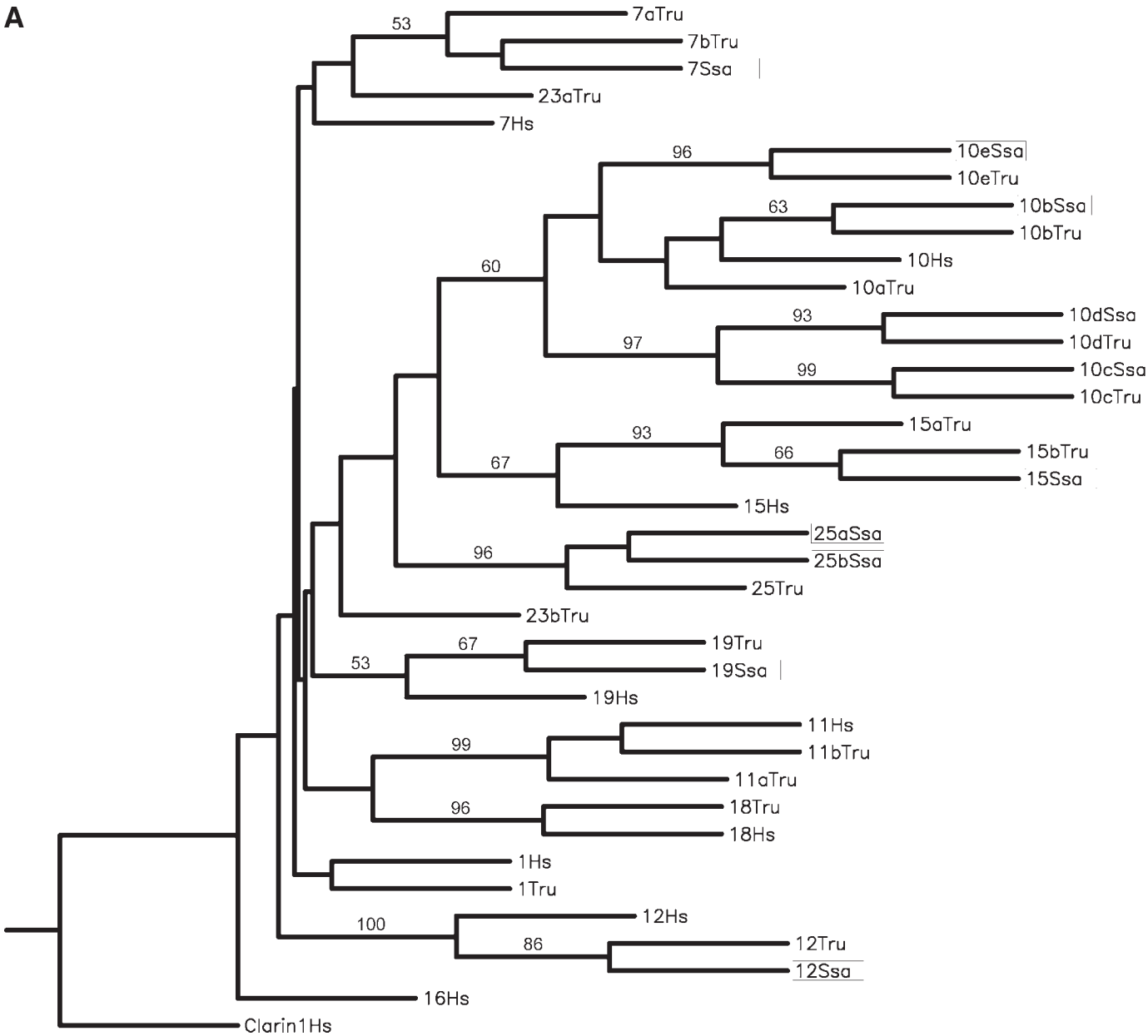

Fig. 1. The phylogenetic tree is based on maximum-likelihood analysis of amino acid sequences of human [*Homo sapiens* (Hs)], Japanese pufferfish [*Takifugu rubripes* (Tru)], and Atlantic salmon [*Salmo salar* (Ssa)] claudins. The numbers of the claudins are shown with a suffix to signify the species and salmon isoforms are framed. For clarity, claudin genes were first separated into two groups of related proteins based on initial phylogenetic analysis, and trees were created separately for the two groups (*A* and *B*). Nos. represent bootstrap values in percent of 100 replicates. Human clarin 1 was used as out group.

dure, tissue processing, and experimental details have all been described in detail previously (29). Gill samples were analyzed from FW fish sampled on the following dates: February 26, April 15, May 15, and June 18. In addition, gills were analyzed in smolts kept in SW for 96 h on May 20 and June 22.

Experimental protocols in the tissue expression experiment and FW-to-SW transfer experiment were approved by the Danish Animal Experiments Inspectorate in accordance with The European convention for the protection of vertebrate animals used for experiments and other scientific purposes (no. 86/609/EØF). The smoltification experiments described were approved by the local responsible laboratory animal science specialist under the surveillance of the Norwegian Animal Research Authority and registered by the Authority.

Plasma Chloride Analysis

Levels of chloride ions were measured in 10-μl undiluted plasma samples by coulometric titration (Radiometer, Copenhagen, Denmark).

Extraction of RNA and cDNA Synthesis

For the tissue expression study, total RNA was extracted by the TRIzol procedure (Invitrogen, Carlsbad, CA) using maximally 100 mg tissue/ml TRIzol according to the manufacturer's recommendation. Total RNA concentrations were determined by measuring the absorbance at 260 nm (A_{260}) in duplicate with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

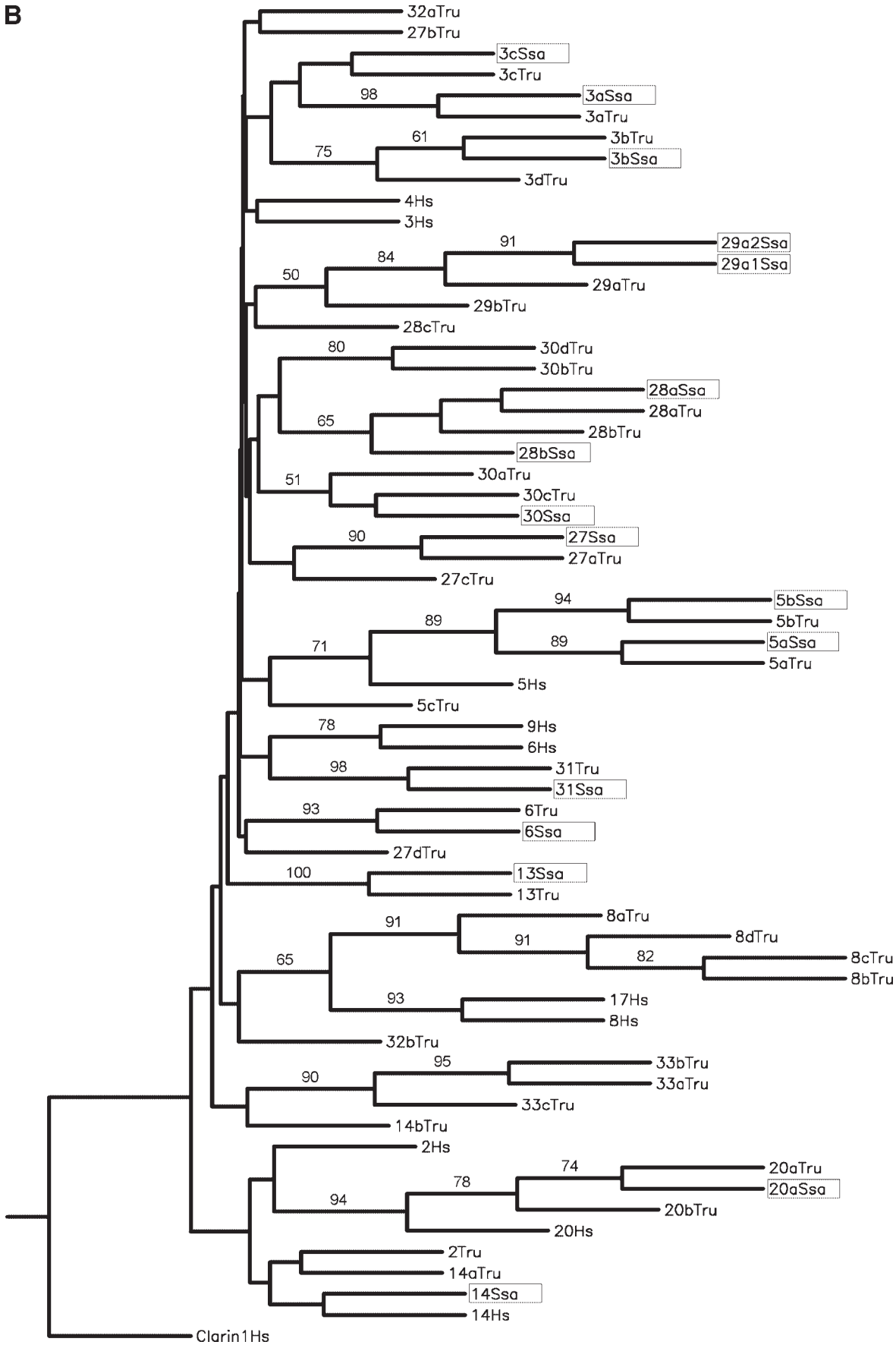

Fig. 1.—*Continued*

Purity (A_{260}/absorbance at 280 nm) ranged from 1.8 to 2.0. RNA (1 μg) was treated with 1 unit RQ1 DNase (Promega, Madison, WI) for 40 min at 37°C in a total volume of 10 μl followed by 10 min at 65°C to inactivate RQ1 DNase. Single-stranded cDNA was synthesized by reverse transcription carried out on 1 μg DNase-treated RNA using 0.5 μg oligo(dT)$_{12–18}$ primers (Invitrogen) and 200 units Superscript II reverse transcriptase (Invitrogen) for 1 h at 42°C in a reaction volume of 25 μl. At the end, the reaction mixture was heated to 70°C for 10 min and then kept at −20°C until used. The procedures for the SW acclimation experiment were similar except that total RNA was purified using the GenElute Mammalian Total RNA kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's recommendations, and reverse transcription was carried out with 200 units Moloney murine leukemia virus reverse transcriptase (Invitrogen) for 1 h at 37°C in the presence of 40 units of RNAguard (GE Healthcare, Bio-Sciences, Little Chalfont, UK) in a total volume of 25 μl. At the end, the cDNA was diluted with 50 μl milliQ H_2O. Extraction of RNA and cDNA synthesis in the smoltification experiment have been described previously (29).

Claudin Nucleotide Sequences

Sequences were obtained from GenBank (5), and, although a few salmon claudins are already annotated, most were located by similarity search. We used the bioinformatics tools available at the NCBI (www.ncbi.nlm.nih.gov) and ExPASy proteomics server of the Swiss Institute of Bioinformatics at http://www.expasy.org/sprot/ (13). The main search tool was BLAST (1), and we used the known teleost claudins in zebrafish and Japanese puffer to search the salmonid EST database. Multiple alignments were performed with ClustalW server at the European Bioinformatics Institute at http://www.ebi.ac.uk/clustalw (14), and consensus sequences were generated in Jalview 2.2 (8). Deduced consensus sequences and suggested nomenclature were submitted to the Third Party Annotation database (accession no. BK006381-BK006406).

Sequences representing the particular salmon isoforms are indicated here by UniGene and/or accession no. in brackets: Claudin 3a (Ssa.11584), Claudin 3b (Ssa.2717), Claudin 3c (Ssa.22769), Claudin 5a (Ssa.11001), Claudin 5b (EG871842), Claudin 6 (DY693877, DY722048), Claudin 7 (Ssa.30991, Ssa.37124), Claudin 10b (Ssa.27249), Claudin 10c (Ssa.24472), Claudin 10d (Ssa.29181), Claudin 10e (Ssa.26547), Claudin 12 (BG934502), Claudin 13 (EG869816), Claudin 14 (Ssa.23046), Claudin 15 (Ssa.3182, Ssa.32678), Claudin 19 (EG647816), Claudin 20a (DW577098), Claudin 25a (Ssa.3205), Claudin 25b (Ssa.3557, Ssa.34951), Claudin 27a (Ssa.7721), Claudin 28a (Ssa.31966), Claudin 28b (Ssa.36697, DW180135), Claudin 29a1 (Ssa.4781), Claudin 29a2 (Ssa.5418), Claudin 30 (Ssa.4482), and Claudin 31 (Ssa.8281).

Primers

Primer3 software (32) was used to design primers detecting the deduced sequences and checked using NetPrimer software (Premier Biosoft International). Primer sequences are listed in Table 1. They were tested for nonspecific product amplification and primer-dimer formation by analysis of melting curve and agarose gel verification of amplicon size.

Quantitative PCR

Quantitative real-time PCR (QPCR) analysis using SYBR Green detection was carried out on a Mx3000p instrument (Stratagene, La Jolla, CA) using standard software settings with adaptive baseline for background detection, moving average, and amplification-based threshold settings with a built-in FAM/SYBR filter (excitation wavelength: 492 nm; emission wavelength: 516 nm). Reactions were carried out with 1 μl cDNA (40 ng RNA or, in the SW challenge experiment, 13 ng RNA), 150 nM forward and reverse primer, and 12.5 μl 2× SYBR Green JumpStart (Sigma) in a total volume of 25 μl. Cycling conditions were as follows: 95°C for 30 s and 60°C for 60 s in 40 cycles. Melting curve analysis was carried out consistently with 30 s for each 1°C interval from 55°C to 95°C. For each primer set, cDNA was diluted 2, 4, 8, and 16 times and analyzed by QPCR to establish amplification efficiency.

The amplification efficiency for each primer set was used for calculation of relative copy numbers of individual target genes. For normalization of gene expression, elongation factor 1α (EF-1α) was used in accordance with Olsvik and coworkers (30). Relative copy numbers of the target genes were calculated as $E_a^{\Delta C_t}$, where C_t is the threshold cycle number and E_a is the amplification efficiency (31). Normalized units were obtained by dividing the relative copy number of claudin genes with the relative copy number of EF-1α. The EF-1α levels are presented as $10^6 \times$ copy number.

Phylogenetic Analysis

All amino acid sequences were obtained from GenBank. All human and Japanese puffer claudin isoforms were included with the deduced predicted salmon proteins in the phylogenetic analysis. Based on the distant relationship of claudins to another four-transmembrane-domain protein, clarin-1 (1), this protein was used as an out group in the analysis.

The predicted amino acid sequences were aligned using ClustalW. Maximum likelihood analysis was used for the phylogenetic tree construction. A total of 100 bootstraps was used to test the consistency of the grouping within the tree. The maximum likelihood consensus tree was generated using SEQBOOT, PROML, and CONSENSE, all programs in the PHYLIP package (12).

Statistics

Tissue expression of claudin isoforms was analyzed by Kruskal-Wallis nonparametric one-way ANOVA followed by the Mann-Whitney *U*-test. Testing for overall differences between FW and SW groups in the SW challenge and smoltification experiments was done with two-way ANOVA. When the interaction between factors was significant, this was followed by Bonferroni's test. The time course of gene expression during smoltification in FW-residing salmon was analyzed by one-way ANOVA followed by Tukey's post hoc test. A significance level of $P < 0.05$ was chosen. All tests were performed using Simstat (version 2.5.5 for Windows, by Provalis Research, Montreal, QC, Canada).

RESULTS

In the current study, we detected 26 expressed claudin isoforms in the transcriptome of Atlantic salmon. They were classified according to their similarity with previously named isoforms in the zebrafish Japanese puffer and human genome (Table 2). The phylogenetic relationship between the salmonid and nonsalmonid claudins was examined by constructing a phylogenetic tree based on the coding sequences of Japanese pufferfish and human claudins (Fig. 1). All salmon claudins clustered with the corresponding puffer isoforms with bootstrap values in most cases exceeding 70. The nodes of the tree (Fig. 1) do not definitely group salmon claudin 28a and 28b to its homolog in Japanese pufferfish, and naming is based on highest homology as determined by the BLAST (Table 2).

Salmon claudin 29a1 and 29a2 are both most similar to Japanese pufferfish claudin 29a, but, since they are clearly different, they were named as an a1 and an a2 isoform. Two salmon homologs of Japanese pufferfish claudin 25 were named 25a and 25b. Homologies in Table 2 propose that zebrafish claudin 10 is homologous to claudin 10b in Japanese pufferfish, and homologs of the remaining claudin 10 subforms were detected among unnamed zebrafish claudins.

Sequences coding specific isoforms can often be traced to their tissue of origin (Table 2), and in gill libraries claudin 10e, 27a, 28a, 28b, and 30 were found. Using specific primers (Table 1), we analyzed their expression pattern in a series of tissues (gill, kidney, intestine, heart, skeletal muscle, brain, and liver). In all cases, expression in the gill was several orders of magnitude greater than in any other examined tissue (Fig. 2). The normalization gene, EF-1α, was expressed at a similar level in all tissues (Fig. 2*F*).

Transfer of FW salmon to SW induced an increase in plasma chloride after 24 h and a return to control levels after 7 days (Fig. 3*A*). The expression of gill claudin 10e mRNA increased fourfold after transfer to SW (Fig. 3*B*). By contrast, an overall decrease was seen in the expression of claudin 27a and claudin 30 in response to SW (Fig. 3, *C* and *F*). SW had no overall effect on claudin 28a and claudin 28b, but an initial decrease in the latter was observed after 24 h (Fig. 3, *D* and *E*). No significant changes in the normalization gene, EF-1α, were observed in the SW transfer experiment (Table 3).

In a separate experiment, gill claudin expression was examined during smoltification from February until the end of June. As reported by Nilsen and coworkers (29), fish of the Vosso stock went through smoltification during spring. Their development peaked in May, based on a surge in gill Na^+-K^+-ATPase activity concurrent with optimal SW tolerance (not shown here). Gill claudin 10e expression increased fourfold in May compared with February, and this surge was followed by a decrease in June (Fig. 4*A*). Claudin 27a, 28a, 28b, and 30 were unchanged during the months examined (Fig. 4). For clarity, SW-transferred groups in May and June are shown separately in Fig. 5 with the corresponding FW groups. Ninety-six hours in SW had no effect on claudin 10e in May but induced increased levels in June (Fig. 5). In June, 96 h in SW reduced the transcript level of claudin 27a (Fig. 5). A trend toward decreased expression of claudin 30 in the 96-h SW group was observed (SW factor P value = 0.09). Also in the smoltification experiment, no significant changes in the normalization gene were observed (Table 4).

DISCUSSION

Claudins are membrane proteins involved in the formation of tight junctions, and expression of specific isoforms is quite variable among tissues (38). The proteins form pores with specific permeation preferences concerning molecule charge and size (4). The expression of claudins in the teleost gill and

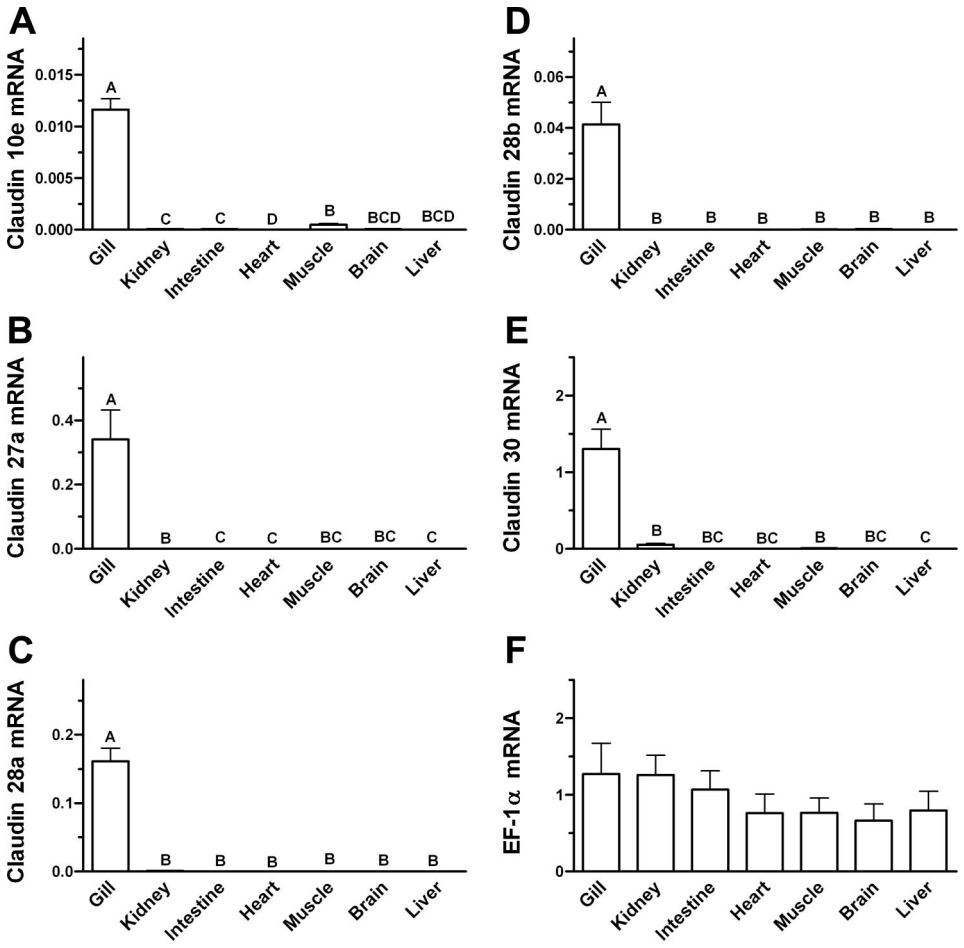

Fig. 2. Expression of claudin 10e (*A*), claudin 27a (*B*), claudin 28a (*C*), claudin 28b (*D*), claudin 30 (*E*), and elongation factor (EF)-1α (*F*) mRNA in gill, kidney, intestine, heart, skeletal muscle, brain, and liver tissue from freshwater (FW) salmon. Expression of target genes is relative to expression of EF-1α. Groups marked with different letters are significantly different, and values are means ± SE (n = 6).

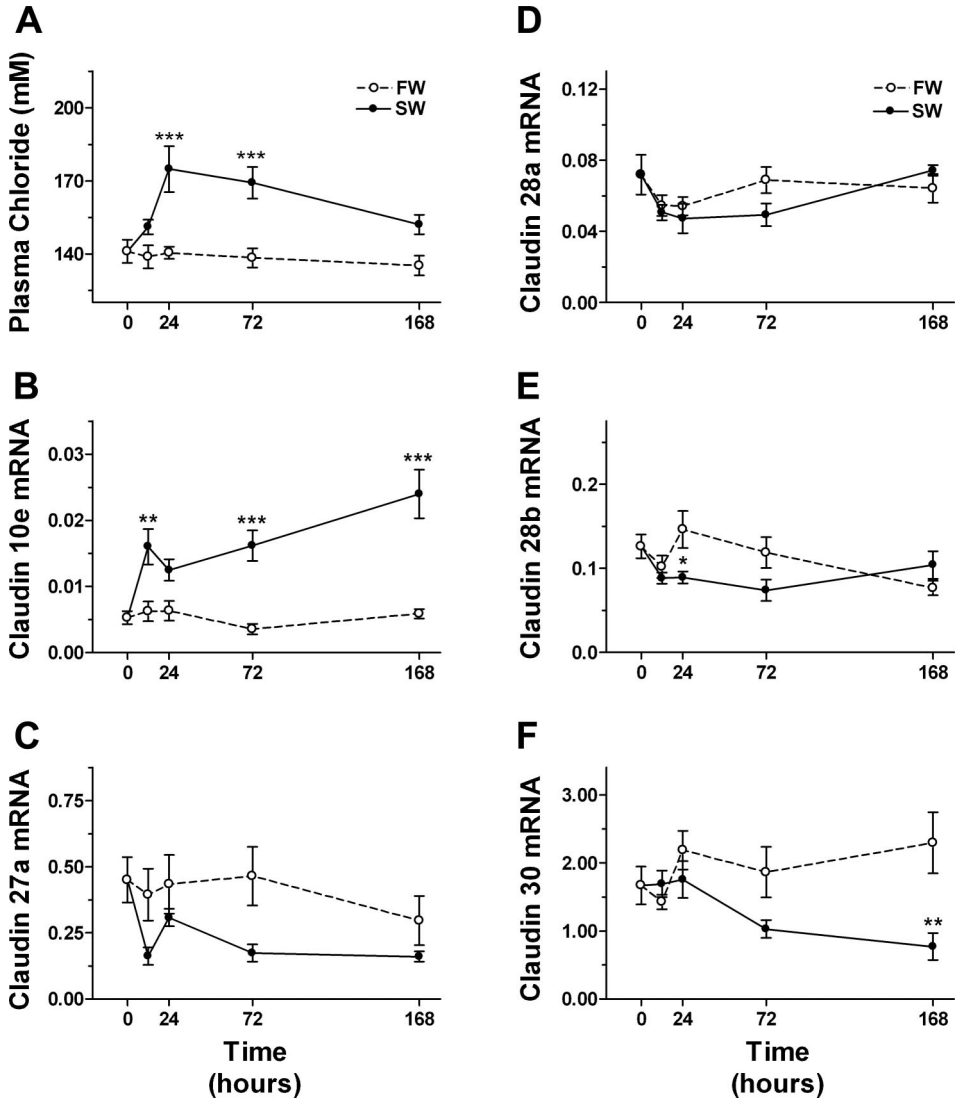

Fig. 3. Effects of FW to seawater (SW) transfer on plasma chloride (*A*) and claudin 10e mRNA (*B*), claudin 27a mRNA (*C*), claudin 28a mRNA (*D*), claudin 28b mRNA (*E*), and claudin 30 mRNA (*F*) in the gill. FW-acclimated fish were transferred to SW (●; SW challenged) or FW (○; sham control) at *time 0*. Overall, SW increased plasma chloride and claudin 10e (*A* and *B*; $P < 0.001$) but decreased claudin 27a (C; $P < 0.01$) and claudin 30 (*F*; $P < 0.01$). Overall, SW had no significant effect on claudin 28a and 28b (*A* and *B*; $P > 0.05$). Post hoc comparisons where made when the interaction between the two factors was significant. Expression of target genes is relative to expression of EF-1α. *Significant effects compared with sham controls (*$P <$ 0.05, **$P < 0.01$, and ***$P < 0.001$). Values are means ± SE ($n = 8$).

its dynamics during salinity acclimation of euryhaline fishes are for the most part unknown, even though it is clear that major permeability changes occur in gill epithelia during salinity acclimations (10). The present study therefore reviewed the array of claudins found in the known salmon transcriptome and examined the expression patterns of gill isoforms during acclimation to SW and the smoltification process.

We identified 26 claudin isoforms from the very extensive EST database available from Atlantic salmon (>400,000 sequences), including libraries from many tissues. Although it is important to be aware that deep branching in the phylogenetic tree (Fig. 1) has only little meaning, the terminal grouping was significant in assigning a nomenclature, when combined with similarity analysis (Table 2). Because some claudins may be expressed in tissues or developmental stages not represented or at very low levels, we expect this list to be partial. However, the genes present in the represented tissue libraries are likely to be of significance in the specific tissue since they are picked up in the transcriptome. Therefore, we chose to focus on gill expression of claudins identified specifically in gill libraries, however, not excluding a possible role of other isoforms in this tissue.

The mRNA of the five isoforms found in the gill libraries are regulated differentially in response to salinity and during smoltification, and probably these expressional changes precede changes in epithelial protein abundance. A highly significant overall stimulation of claudin 10e and inhibition of claudin 27a

Table 3. *Levels of EF-1α mRNA in samples from SW transfer experiment (see Fig. 3)*

Hours After Transfer	Salinity	EF-1α (relative copy no.)
0	FW	5.09±0.48
12	FW	4.53±0.37
	SW	4.81±0.41
24	FW	4.48±0.62
	SW	5.07±0.49
72	FW	4.57±0.36
	SW	5.42±0.54
168	FW	4.15±0.47
	SW	4.52±0.45

Values are means ± SE; $n = 8$ experiments. FW, freshwater; SW, seawater. No significant differences were observed.

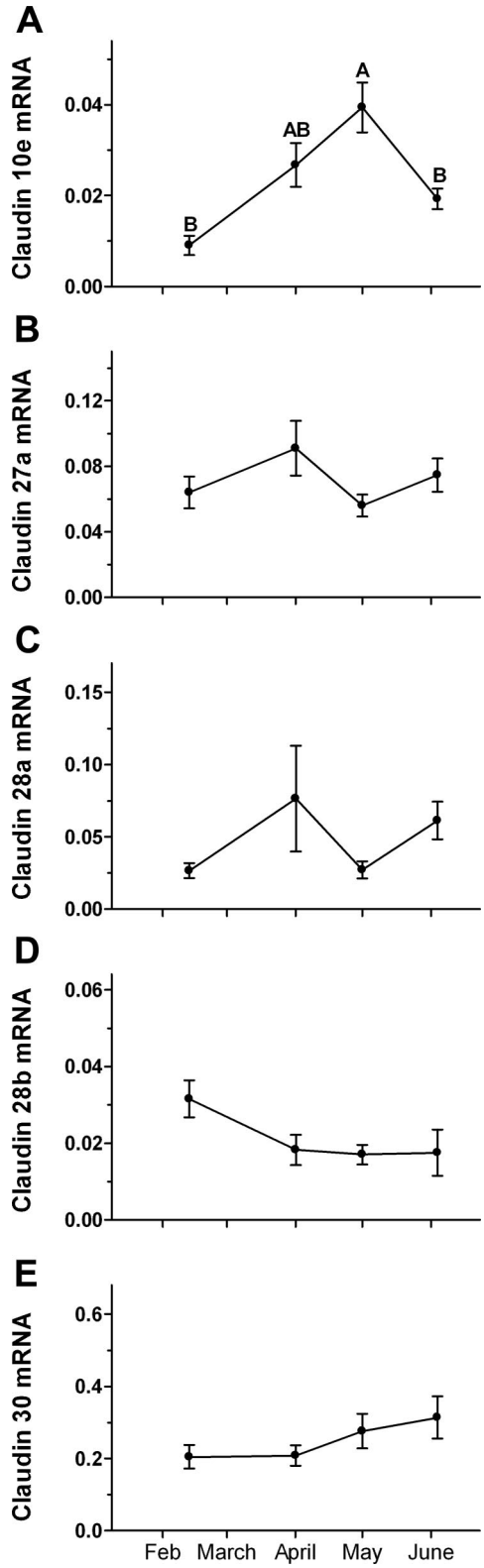

Fig. 4. Expression of claudin 10e (*A*), claudin 27a (*B*), claudin 28a (*C*), claudin 28b (*D*), and claudin 30 (*E*) mRNA in salmon gills during the smoltification period. Expression of target genes is relative to expression of EF-1α. Different letters indicate significant difference between groups. Values are means ± SE ($n = 8$).

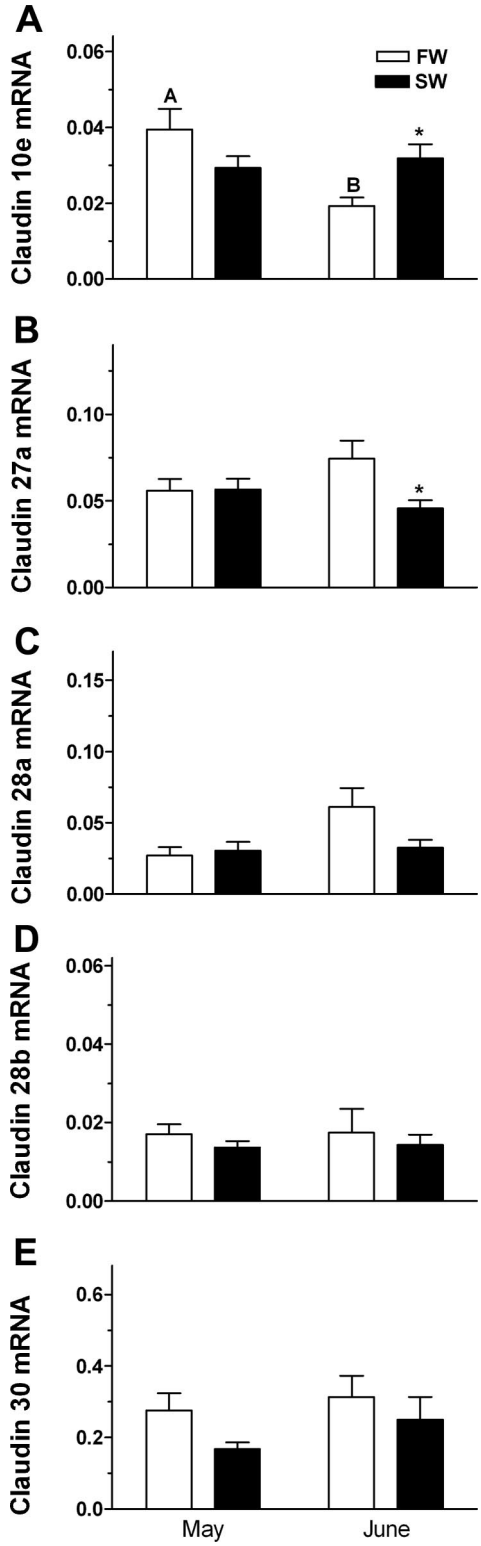

Fig. 5. Expression of claudin 10e (*A*), claudin 27a (*B*), claudin 28a (*C*), claudin 28b (*D*), and claudin 30 (*E*) mRNA in salmon gills in May and June. FW-residing fish (○) were transferred to SW (●) for 96 h. Different letters indicate significant difference between FW groups as also seen in Fig. 4. Post hoc comparisons where made when the interaction between the two factors was significant. Expression of target genes is relative to the expression of EF-1α. *Significant effects compared with sham controls ($P < 0.05$). Values are means ± SE ($n = 8$).

Table 4. *Levels of EF-1α mRNA in samples from smoltification experiment (see Figs. 4 and 5)*

Date of Sampling	EF-1α (relative copy no.)
February 26	0.72±0.14
April 15	0.92±0.21
May 15	0.74±0.20
May 19	1.29±0.39
June 18	0.43±0.10
June 22	0.57±0.19

Values are means ± SE; $n = 8$ experiments. No significant differences were observed.

and 30 induced by SW transfer indicate a specific role of these isoforms in the transformation between FW- and SW-type gill epithelia. On the other hand, no overall effect of SW was observed for claudin 28a and 28b, suggesting a general significance at both salinities. SW induces a transient drop in claudin 28b expression, indicating that this claudin may be modulated during the early transition phase. The patterns of change of claudin isoforms 10e and 27a during smoltification are in good agreement with the above changes and support their involvement in transforming the gill from a hyper- to a hypoosmoregulatory organ. The elevated level of claudin 10e expression at the peak smolt stage and its reactivation by SW transfer in June, where the FW-expression level had regressed, both support a role in hypoosmoregulation. On the contrary, claudin 27a is at a low level and unresponsive to SW transfer in peak smolts in May; however, in regressing FW smolts in June, it further declines upon SW transfer, similar to what was found in the SW transfer experiment. The inverse dynamics of these isoforms reinforce the current conception of smoltification as being preparatory to SW transition.

The regulation of claudin 27a observed in the present study in response to SW is consistent with a recent study in eel gill where claudin 27a expression is higher in long-term acclimated FW fish vs. SW fish (18). Another study by Boutet et al. (7) also addressed claudin expression in a euryhaline teleost. They sequenced a partial claudin 3-like gene in the European seabass, which in teleosts is most similar to claudin 8d in Japanese puffer (25). They found higher gill mRNA expression of this claudin in fish from the open sea (35 ppt; 11°C) than in those living in a lagoon where salinity fluctuates (3.5–41 ppt) and temperature is lower (5°C). Whether this gene responds to temperature, salinity, or both is uncertain, but the study raises the possibility that this claudin might be upregulated during long-term SW residence. We did not identify a claudin 8 isoform in the available salmon transcriptome, but this does not preclude the presence of one in the salmonid gill. In a recent study on Mozambique tilapia, we found that claudin 28a and claudin 30 increased following FW entry (35), an observation that, in part, parallels our observations in salmon.

It is important to keep in mind that epithelial permeability to ions and water may change independently. The solvent drag model, describing how paracellular water carries along dissolved solutes, is widely accepted even though direct evidence for water-permeable tight junctions is missing (see Ref. 23). Thus it remains controversial whether tight junctions allow any significant passage of water, and there are studies failing to document any paracellular water movement (22). In gill epithelia, total water permeability decreases in SW (17). Conversely, ion conductance increases (10, 26), and thinner "leaky type" tight junctions are formed between neighboring chloride cells and chloride cells or accessory cells (33). Thus the simultaneous thinner tight junctions and decreased water permeability may suggest that, in the gill, water transport through tight junctions is minor compared with the transcellular component (via aquaporins).

Our examination of the dynamics in expression of gill claudins during SW acclimation showed a very significant inhibitory effect of SW on both claudin 27a and claudin 30. This suggests that a decline in claudin 27a and claudin 30 is important in transforming the gill to an ion-secreting epithelium that is more "leaky" to ions and includes a paracellular pathway for passive extrusion of Na^+. Conversely, the higher claudin 27a and 30 expression in FW gill may be important in the epithelial tightening needed to reduce ion permeability, as is seen in fish residing in FW environments (27). Interestingly, salmon claudin 27a and 30 are both very similar to human claudin 4, with expected values of 4e-60 and 3e-78, respectively. In mammalian kidney, claudin 4 strongly increases TER (36) which, based on similarity, further supports that the role of claudin 27a and 30 in the salmon gill is to tighten the epithelium. In contrast to these findings, claudin 10e is strongly stimulated by SW transfer and, in addition, during the climax of smoltification and thus peak SW tolerance in the FW-residing smolt. In mammalian kidney, variants of claudin 10 form low-resistance pores, and claudin 10b is more selective for cations than claudin 10a (37). Claudin 2 and 16 also increase ion permeability in canine kidney cell cultures (3, 20), possibly through formation of cation-selective pores. Because a trans-junctional Na^+ pathway between chloride and accessory cells is critical to ion extrusion in the SW gill, teleost claudin 10e may be involved in formation of similar cation-selective pores. This is not clear from the present observations, but the data certainly suggest a central role in the acquisition of SW tolerance. During the smoltification process, the attainment of SW tolerance is not accompanied by depressed expression of claudin 27a and claudin 30, as seen following abrupt SW transfer. This probably reflects the need of an intact tight FW-type epithelium in the smolt when still residing in FW. On the other hand, the isoform associated with SW acclimation, claudin 10e, is strongly upregulated during the peak of smoltification and before SW entry, suggesting a critical role for this in the preparation process. It is possible that rapid reorganization of tight junctions then follow SW transfer of the fully developed smolt. In such a scenario, acute deactivation of claudin 27a and claudin 30 may be accompanied by activation or recruitment to the cell membrane of claudin 10e proteins. Interestingly, acute regulation of claudins by phosphorylation and removal of claudins from the tight junction by endocytosis may play a role in regulation of the paracellular barrier in mammals (38). For example, in mammalian kidney, aldosterone rapidly induces claudin 4 phosphorylation on threonine residues and thereby increases epithelia permeability (25).

In conclusion, an extensive array of expressed claudin genes is present in the Atlantic salmon transcriptome, and five are apparent gill isoforms. The expression pattern in the gill of these claudins during acclimation or preadaptation to SW is diverse. The simultaneous decrease in claudin 27a and 30 and

increase in claudin 10e during acclimation from FW to SW suggest that they have specific functional roles and may bestow vital characteristics to the epithelial tight junctions in these diverse environments.

Perspectives and Significance

Gill permeability and its dynamics during hyper- to hypo-osmoregulatory transitions have only been investigated minimally, and its molecular basis is largely unknown. The present identification of expressed claudin proteins in the transcriptome of a euryhaline teleost thus represents a first contribution to open this novel area of research in fish osmoregulatory physiology. The study illustrates the diversity of claudin isoforms expressed in the gill and their differential regulation in response to both salinity and photoperiodic variation. The use of the Atlantic salmon gill as a model thus gives a unique opportunity to study the dynamics of claudin function both in response to acute acclimation and developmental preparation to move from FW to SW. It will be important, in future studies, to analyze protein expression and localization in the gill and to identify the functional barrier and/or transport characteristics of individual claudin proteins.

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