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Regulation of expression of two aquaporin homologs in the intestine of the European eel: effects of seawater acclimation and cortisol treatment

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Martinez, Anne-Sophie, Christopher P. Cutler, Gillian D. Wilson, Claire Phillips, Neil Hazon, and Gordon Cramb. Regulation of expression of two aquaporin homologs in the intestine of the European eel: effects of seawater acclimation and cortisol treatment. *Am J Physiol Regul Integr Comp Physiol* 288: R1733–R1743, 2005. First published January 13, 2005; doi:10.1152/ajpregu.00747.2004.—Complementary DNAs encoding homologs of the mammalian aquaglyceroporins (termed AQPe) and aquaporin-1 isoforms (termed AQP1) were isolated from the European eel. The AQP amino acid sequences share 35–54% identity with other known human AQPs. Although AQPe mRNA expression was approximately equivalent along the entire length of the gut, AQP1 expression was the highest in the posterior/rectal segment. Seawater (SW) acclimation increased AQP1 mRNA abundance by 5- and 17-fold in the anterior, 14- and 23-fold in the mid-, and 9- and 7-fold in the posterior/rectal gut regions of yellow and silver eels, respectively. SW acclimation had an effect on AQPe mRNA expression only in the midintestine of silver eels, where a small but significant 1.7-fold increase in abundance was measured. Western blots using an eel AQP1-specific antibody identified the presence of a major immunoreactive 28-kDa protein, primarily within the posterior/rectal segment. A 3-wk SW transfer induced an increase in AQP1 protein abundance in all intestinal segments, with the posterior/rectal region still expressing protein levels ~40- and 8-fold higher than the anterior and midsegments, respectively. Strong AQP1 immunofluorescence was detected within the vascular endothelium in both freshwater (FW)- and SW-acclimated eels and in the epithelial apical brush border in the posterior/rectal gut regions of SW-acclimated eels. Cortisol infusion into FW eels had no effect on intestinal AQPe mRNA expression but induced increases in AQP1 mRNA and protein levels. These results provide evidence for the presence of a SW-induced and steroid-regulated AQP water channel pathway within the intestine of the European eel.

Anguilla anguilla; gastrointestinal tract; AQP1; AQPe; corticosteroid

WHEN FACED WITH EXTERNAL ENVIRONMENTS of varying salinity, euryhaline teleosts, such as the European eel (*Anguilla anguilla*), have a fundamental osmoregulatory problem: the maintenance of their body fluid composition and osmolality. Osmoregulation is achieved by linked ion and water transport in the gill, kidney, gastrointestinal tract, and urinary bladder (15). The intestine is a major osmoregulatory organ in euryhaline teleost fish, especially when acclimated to seawater (SW). When transferred to the marine environment, euryhaline fish such as the European eel increase their drinking rate by >10-fold and the ingested SW is mainly desalinated, first within the esophagus, possibly by passive ion diffusion, and thereafter along the entire length of the intestine, by active transport of monovalent ions into the blood. The subsequent water absorption is considered to take place in the intestine by osmotic mechanisms after active absorption of the monovalent ions, the "solute-linked water flow" pathway (52). The magnitude of water fluxes across various parts of the intestine has also been determined, with highest levels occurring in the midregion followed in descending order by the posterior and anterior intestine and rectum (5, 9).

Although intestinal salt and water transporters are central to the maintenance of body fluid homeostasis in euryhaline fish, especially during the course of adaptation to different salinities, there is currently a lack of information on the molecular mechanisms by which water is transported across intestinal epithelia. Aquaporins (AQPs) are a family of structurally related membrane proteins that have been identified in mammals to function as water channels (11) involved in fluid transport within various organs, including the gastrointestinal tract (10, 11, 24, 37, 43). To date, eight AQP isoforms (AQP1, -3–5, -7–10) have been found to be expressed in the gastrointestinal tract in mammals (24, 37, 43). Seven AQPs have been described so far in teleost fish, five of which [three homologs of mammalian AQP1, a homolog of AQP3, plus a novel aquaporin called sbAQP from sea bream (*Sparus auratus*)] have been reported to be expressed within the gastrointestinal tract (10, 15, 16, 41, 51). Expression of these and other teleost isoforms are also reported in the gill, kidney, and lens (15, 16, 31, 51, 61). The finding that AQPs are expressed at high levels within the teleost gill, gut, and kidney suggests a physiological role for these water channels in osmoregulation in both freshwater (FW) and marine environments (11, 41). As SW acclimation is associated with increases in drinking and water uptake across the gastrointestinal tract, a role for AQPs in water absorption in the teleost intestine is a distinct possibility.

Cortisol is the major corticosteroid found in euryhaline teleost fish, with release from the interrenal gland being stimulated as fish are transferred into SW (25). Cortisol is often referred to as a "SW-adapting" hormone because it is heavily implicated in the ability of fish to maintain water and electrolyte balance when in the SW environment (45). The physiological actions of this hormone within the gastrointestinal tract of FW-acclimated fish include increases in the ionic permeability of the esophagus and intestine with concomitant upregulation of intestinal fluid absorption (25), enabling the fish to hypo-osmoregulate after moving to a hypersaline environment (32).

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As part of our investigations into the mechanisms of water transport within euryhaline teleosts, we report the cloning of two eel AQP isoforms (termed AQPe and AQP1) that are homologs of known mammalian aquaporins. In addition, we have examined the effects of SW acclimation and cortisol treatment on the expression of these isoforms within the intestine at two different adult life stages of the eel. Experiments were conducted with both sexually immature "yellow" eels, which are indigenous to the FW waterways throughout Europe, and sexually maturing "silver" eels, which have undergone a series of morphological and physiological changes before migration to SW to breed (56). Although eels at both developmental stages can successfully adapt to SW, previous studies indicated that salinity-induced changes in gene expression may not always be equivalent in yellow and silver eels (16, 18, 19), possibly reflecting preadaptive changes that are confined only to the migratory silver eels.

MATERIALS AND METHODS

Fish. Adult FW sexually immature yellow and sexually maturing migratory silver eels were obtained from local suppliers in Inverness, Blairgowrie, and Kelso, Scotland and transferred to laboratory aquariums at the Gatty Marine Laboratory. Unfed eels were maintained at ambient temperature (5–14°C) in aerated natural FW (0–10 mosmol/kg) before experiments and then acclimated to SW (960–1,020 mosmol/kg) (or FW for controls) for periods up to 21 days before use in experiments. A 12:12-h light-dark photoperiod was maintained. Fish were decapitated and pithed before removal of tissues.

Cortisol infusion and RIA. Silver and yellow FW-acclimated eels were anesthetized (48 ppm MS-222; 3-aminobenzoic acid ethyl ester), weighed, and implanted with Alzet mini osmotic pumps (model 1003D) releasing vehicle alone (a 30% solution of 2-hydroxypropyl-β-cyclodextrin; Fluka, Gillingham, UK; control animals) or cortisol dissolved in the vehicle (10 mg cortisol·kg wet wt fish^{-1}·pump^{-1} = rate of delivery of cortisol 15 μg·kg wet wt^{-1}·h^{-1}). After 8 days, blood was collected and centrifuged and the resulting plasma was frozen and stored at −80°C until analysis. Plasma concentrations were measured by RIA using a sheep anti-cortisol antibody (National Diagnostics) essentially as described by Van Anholt et al. (58).

Total RNA extraction. Intestinal epithelial RNA was extracted from epithelial scrapes of the intestine either by a modification of the LiCl procedure (19) or by a modification of the Chomczynski and Sacchi method (13) as described in Cutler et al. (20). RNA used in the expression studies was extracted from epithelial scrapes of whole intestine or three equal-length segments of the intestinal epithelium (anterior, mid-, and posterior/rectal regions). mRNA used for the initial cloning was purified from intestine and/or kidney total RNA by affinity adsorption on immobilized oligo(dT) cellulose as described in Cutler et al. (19).

Cloning and sequencing. Initial fragments for all AQPs were amplified by RT-PCR using synthetic sense and antisense degenerate primers as previously described by Cutler and Cramb (17): 5′- and 3′-rapid amplification of cDNA ends (RACE) DNA fragments were produced from 3-wk SW-acclimated silver eel kidney and intestine mRNAs with a Marathon cDNA amplification kit (Clontech, Basingstoke, UK) as described previously (20). 5′-RACE products were produced in nested PCR reactions using eel AQP1- or AQPe-specific antisense *primers 1* and then *2* in conjunction with the Marathon kit nested AP1 and AP2 primers. 3′-RACE products were obtained by the same procedure, using eel AQP1- or AQPe-specific sense *primers 1* and then *2* in conjunction with the Marathon kit AP1 and AP2 primers. PCR fragments amplified with the degenerate primers or by 5′- and 3′-RACE amplification were cloned and sequenced as described in Cutler et al. (20).

Northern blotting and analysis. Northern blotting and quantitative determination of transcript abundance were performed essentially as described previously (20). The probes used for Northern blot analysis were colony PCR-amplified plasmid inserts of the original fragments of AQP1 (387 bp) and AQPe (445 bp) produced with the degenerate sense and antisense primers. Probes (25 ng cDNA) were radiolabeled to specific activities between 2 and 2.6 dpm/μg DNA with [α-^{32}P]dCTP (6,000 Ci/mmol) and a Megaprime DNA labeling kit (Amersham Biosciences, Little Chalfont, UK) according to the manufacturer's instructions and purified by size exclusion chromatography on a Sephadex G50 column before use in the hybridizations. After stringent washing protocols, the specific radioactivity hybridizing to the blots was quantified by electronic autoradiography using an Instant Imager (Canberra Packard, Meriden, CT) and individual mRNA abundance was expressed as counts per minute per microgram of total RNA after first correcting for the relative amounts of total RNA loaded for each sample by quantification of the combined 18S and 28S ribosomal RNAs in each lane, as described previously (20). The blots were finally exposed for varying times to X-ray film (Kodak BioMax MS) at −80°C and developed for autoradiography.

AQP1 antibody production and purification. Commercially prepared (Pepceuticals, Leicester, UK) AQP1 antibodies were raised against a 15-amino acid COOH-terminal peptide (VNGPDDVPAVEMSSK) as described previously (41). Anti-AQP1 antibodies were affinity purified from the antiserum by affinity adsorption to the peptide antigen cross-linked to activated thiol Sepharose 4B, as instructed by the manufacturer (Amersham Biosciences). The antiserum (20 ml) was continually mixed with 4 ml of the peptide-conjugated beads for 40 min at room temperature, cooled to 4°C, and poured into a column. The eluted serum was collected, and the beads were washed with 5 volumes of ice-cold wash buffer (50 mM Tris, 0.5 M NaCl, 1 mM EDTA, pH 7.5). The bound AQP1 antibodies were eluted from the column with 15 ml of ice-cold 0.1 M glycine-HCl, pH 2.5, and 1.2 ml fractions were collected into 0.3 ml of 1 M Tris pH 8.0. Fractions containing antibody (as determined by SDS-PAGE; Ref. 39) were pooled together, aliquoted, and stored at −20°C until use for Western blotting and immunohistochemistry.

Western blotting. The anterior, mid- and posterior/rectal segments of the intestine were quickly removed from silver FW-acclimated and 3-wk SW-acclimated eels. The epithelial tissue was scraped from the underlying smooth muscle, and plasma membrane fractions were prepared by discontinuous sucrose density gradient centrifugation as described previously (41). Protein concentrations were determined by the method of Bradford (12), and Western blotting was conducted with standard techniques (23). In brief, membrane samples (up to 100 μg protein) were solubilized and denatured by addition of an equal volume of sample preparation buffer (125 mM Tris·HCl, 4.6% SDS, 1.4 M β-mercaptoethanol, 20% glycerol, 0.01% bromophenol blue, pH 6.8) and incubated at 100°C for 15 min, and the proteins were separated by SDS-PAGE with 10% acrylamide gels (39). Proteins were electroblotted onto polyvinylidene difluoride membranes and immediately processed for immunodetection at room temperature as previously described by Lignot et al. (41). The dilutions of the purified primary antibody and the secondary antibody (alkaline phosphatase-conjugated donkey anti-rabbit; Sigma) were 1:100 and 1:10,000, respectively. The control blots were incubated with the immune serum preincubated at 4°C with 50 μg/ml of the peptide antigen (peptide-negated antiserum). The level of immunoreactivity was measured as integrated peak areas (arbitrary units) with an image capture and analysis system (Genesnap/Genetools image analysis system, Syngene, Cambridge, UK).

Immunofluorescence light microscopy. Immunohistochemical localization of AQP1 was performed on intestinal and rectal samples removed from silver eels as previously described by Lignot et al. (41). The dilutions of the purified primary antibody and the secondary antibody [FITC-conjugated donkey anti-rabbit IgG (H&L); Jackson Immunoresearch, West Baltimore, MD] were 1:25 and 1:200, respec-

tively. The control sections were incubated with the immune serum preincubated overnight at 4°C with 50 μg/ml of the peptide antigen (peptide-negated antiserum).

Statistical analysis. The data were analyzed with StatView 4.01 software (Abacus Concepts, Berkeley, CA), using ANOVA with levels of significance determined by Fisher's post hoc test.

RESULTS

Nucleotide and amino acid sequences of eel AQP1 and AQPe. The amplified eel AQP1 cDNA sequence comprises 1,189 bp containing 24 bp of a 5′ untranslated region (UTR), 789 bp of an open reading frame (ORF), and 376 bp of a 3′ UTR; the amplified eel AQPe cDNA comprises 1,377 bp containing 79 bp of a 5′ UTR, 954 bp of an ORF, and 344 bp of a 3′ UTR. The AQP1 and AQPe cDNAs encode proteins with 263 and 318 amino acids (aa), respectively (Fig. 1) with calculated molecular masses of 27.5 and 34.1 kDa.

The eel AQP1 protein shared highest levels of amino acid identity with the Japanese eel AQP1 sequence (94%; Ref. 10) and another European eel aquaporin isoform that has been termed AQP1-duplicate (69%; Ref. 16) (Fig. 1*A*). With respect to mammals, highest amino acid identity was found compared with the AQP1 isoforms including the human AQP1 protein (54%), with lower identities to other isoforms, e.g., human AQP0, -2, -4, -5, and -6 (between 35 and 40%) (Fig. 1*A*). The eel AQPe protein shared highest amino acid identity with novel aquaporins recently isolated from the sea bream (*S. auratus*) (67%; Ref. 51) and *Xenopus laevis* (58%; Ref. 62), with reduced homologies compared with teleostean or mammalian AQP3s including the eel (46%), zebrafish (44%), and human (45%) isoforms and slightly lower identity to other AQP isoforms, e.g., human AQP7, -9, and -10 (40–42%) (Fig. 1*B*).

Quantitative expression of AQP1 and AQPe mRNA in FW- and SW-acclimated yellow and silver eel intestine. Quantitative Northern blots (Figs. 2) were used to investigate AQP1 and AQPe mRNA expression in the anterior, mid-, and posterior/rectal intestinal segments of FW- and 3-wk SW-acclimated yellow and silver eels. Although AQP1 exhibited variable levels of expression between individual members of all fish groups, the silver eels consistently exhibited higher AQP1 abundance in all gut segments, averaging 2.5-fold greater than the expression in the yellow eels (results not shown). In both yellow and silver eel groups the highest AQP1 mRNA abundance was found in the posterior/rectal intestinal segments, with expression in the FW-acclimated groups being 10- to 20-fold higher than either anterior or midsegments (Figs. 2*A* and 3*A*). In contrast, there was no significant difference in AQPe mRNA expression between intestinal regions or between yellow and silver eels (Figs. 2*B* and 3*B*). Three weeks after SW transfer, AQP1 mRNA expression increased significantly by ~6-, 14-, and 9-fold in yellow eels and by 17-, 23-, and 7-fold in silver eels, in the anterior, mid-, and posterior/rectal intestinal segments, respectively (Figs. 2*A* and 3*A*). In contrast, AQPe mRNA expression only exhibited a small but statistically significant 1.7-fold increase in the midintestine of the silver eels after SW transfer (Figs. 2*B* and 3*B*).

Quantitative expression of AQP1 protein in FW- and SW-acclimated silver eel intestine. The expression of the eel AQP1 protein isoform was characterized within the intestine of silver eels with a specific antibody prepared as described in MATERIALS AND METHODS. Western blotting using this antibody and purified plasma membrane fractions from both FW- and SW-acclimated silver eels resulted in the appearance of an immunoreactive band of ~28 kDa, primarily in the posterior/rectal segments of the intestine. Even with very high protein loadings (>100 μg) on the gel, the immunoreactive signals from the mid- and anterior segments of the intestine were very faint or even sometimes undetectable in protein extracts, especially in FW-acclimated animals (Figs. 4 and 5). After SW acclimation for 21 days, AQP1 protein levels were increased in all segments, with expression some 44-fold higher in the midintestinal segment and 9-fold higher in the posterior/rectal intestinal segment (Figs. 4 and 5). The immunoreactive 28-kDa band was not detected on blots where the AQP1 antibody was preincubated overnight at 4°C in the presence of 50 μg/ml of the antigenic peptide (results not shown).

Cellular localization of AQP1 protein in silver eels by immunofluorescence light microscopy. With the eel AQP1 antibody, specific immunofluorescence was detected within the endothelial cells of the blood vessels distributed within the muscle layers and the submucosa in both FW- and SW-acclimated silver eels (Fig. 6, *E–H*). Blood vessels associated with immunoreactivity were found throughout the entire length of the intestine, although they were more frequent and exhibited stronger immunofluorescence in the mid- compared with the anterior sections (Fig. 6, *E* and *F*) and in SW- compared with FW-acclimated eels (Fig. 6, *G* and *H*). In the SW-acclimated eels, an intense immunofluorescence for AQP1 was also detected in the apical brush border of the epithelial cells immediately before and after an intestinal sphincter that marks the terminal portion of the posterior intestine and the start of the rectum (an intestinal region no more than 1 cm long) (Fig. 6, *A–C*). The immunofluorescence was stronger and more regular in the epithelia of the posterior intestine (Fig. 6, *A* and *B*) than in epithelial cells within the rectum (Fig. 6*C*). In direct contrast, no AQP1 immunofluorescence was observed in any intestinal segments from FW-acclimated eels (Fig. 6, *E* and *F*), even sections within the posterior/rectal area. In both FW- and SW-acclimated eel intestine, negative controls using the peptide-negated AQP1 antiserum showed no positive immunofluorescence, with only nonspecific autofluorescence being restricted to red blood cells (Fig. 6*D*).

Cortisol plasma levels. Plasma cortisol levels in FW-acclimated fish were 8.7 ± 2.7 ng/ml in silver and 7.4 ± 2.5 ng/ml in yellow eels. Long-term (3 wk) SW-acclimated eels exhibited similar cortisol concentrations of 8.6 ± 2.4 and 10.5 ± 4.2 ng/ml for yellow and silver eels, respectively; however, elevated levels averaging 20.4 ± 7.8 and 18.4 ± 7.2 ng/ml were measured for yellow and silver eels 2 days after acute FW-SW transfer. In the implanted animals (see MATERIALS AND METHODS), after 8 days, plasma cortisol levels had increased approximately fourfold from average values for vehicle-implanted fish of 8.8 ± 1.7 and 9.6 ± 2.2 ng/ml to 33.4 ± 4.9 and 35.8 ± 5.3 ng/ml for cortisol-implanted yellow and silver eels, respectively.

Effect of cortisol on AQP1 and AQPe mRNA expression in yellow and silver eel intestine. Quantitative Northern blots (Fig. 7) were used to investigate AQP1 and AQPe mRNA expression within the whole intestine of control (vehicle treated) and cortisol-treated FW-acclimated yellow and silver eels. As in previous experiments, AQP1 exhibited a variable expression profile between individual members in all fish

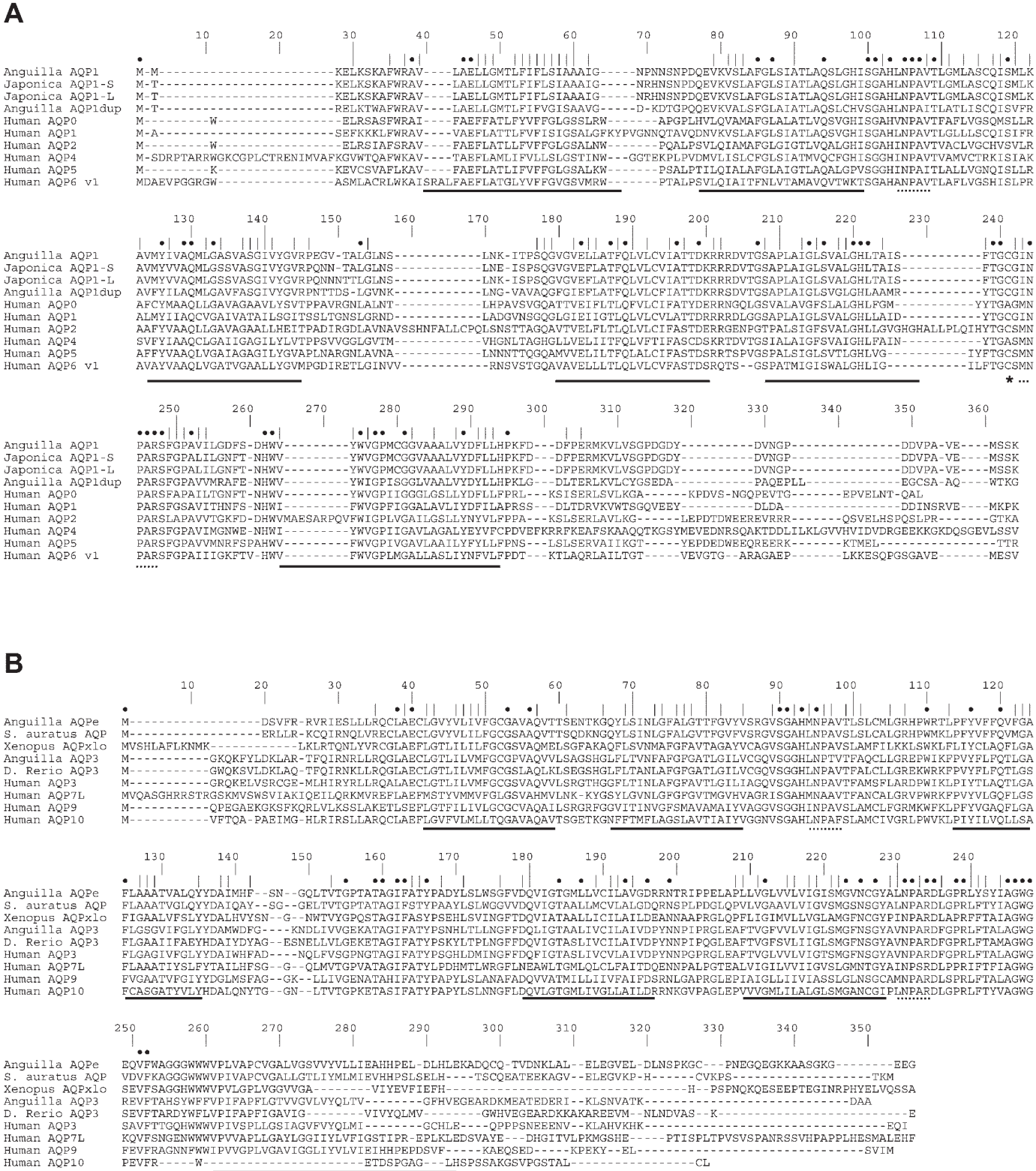

Fig. 1. Alignment of aquaporin (AQP)1 (*A*)- and AQPe (*B*)-derived amino acid sequences from the eel compared with those from other species. Symbols in the alignment are as follows: dots, conserved amino acid; vertical lines, amino acid with conserved similarity; hyphens, spaces introduced to give an optimal alignment; dotted underlining, NPA motif; asterisk, potential mercury binding site; solid underlining, approximate locations of membrane-spanning domains [TM 1–6; Kyte and Dolittle (38), Borgnia et al. (11)]. Sequences were aligned with GeneJockey II software (Biosoft). The EMBL/Swiss prot accession numbers for sequences were as follows: European eel AQP1 (AJ564420), European eel AQP1dup (AJ564421), Japanese eel AQP1S (AB094502) and AQP1L (AB094501), human AQP0 (BC074913), human AQP1 (BC022486), human AQP2 (BC042496), human AQP4 (BC022286), human AQP5 (BC032946), human AQP6 v1 (NM001652), eel AQPe (AJ784153), *Sparus auratus* AQP (AY363261), *Xenopus laevis* AQPxlo (AY120934), eel AQP3 (AJ319533), *Danio rerio* AQP3 (BC044188), human AQP3 (BC013566), human AQP7L (BC062701), human AQP9 (BC026258), human AQP10 (BC069607).

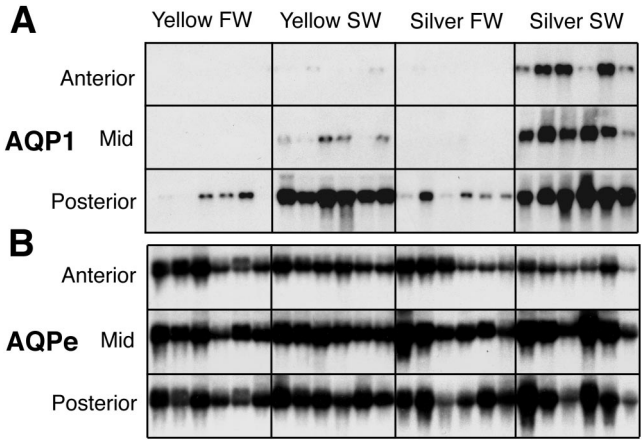

Fig. 2. Northern blot using total RNA (10 μg) showing the expression of AQP1 (*A*) and AQPe (*B*) mRNA in the intestine of freshwater (FW)-acclimated and 3-wk seawater (SW)-acclimated yellow and silver eels ($n = 6$ from each group). RNA sizes (kb) were estimated from RNA standards (not shown). The autoradiographs were exposed for 1 h 10 min for AQP1 and for 7 h 05 min for AQPe, at −80°C.

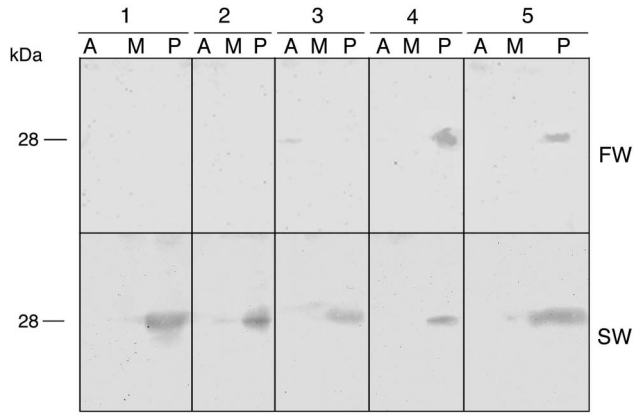

Fig. 4. Western blot analysis of AQP1 in tissues from the anterior, mid- and posterior/rectal intestine of silver eels maintained in FW or acclimated for 3 wk to a SW environment. The blot shows AQP1 immunoreactivity in plasma membrane fractions isolated from the intestine of FW-acclimated eels (*top*) and SW-acclimated eels (*bottom*) in the anterior (A), mid- (M), and posterior/rectal (P) segments of the organ. Controls included the use of peptide-negated antiserum in both FW- and SW-acclimated animals, which showed no bands (results not shown). *Lanes 1–5*, protein samples from 1 fish in each experimental group. The position of the immunoreactive band is indicated on *left*.

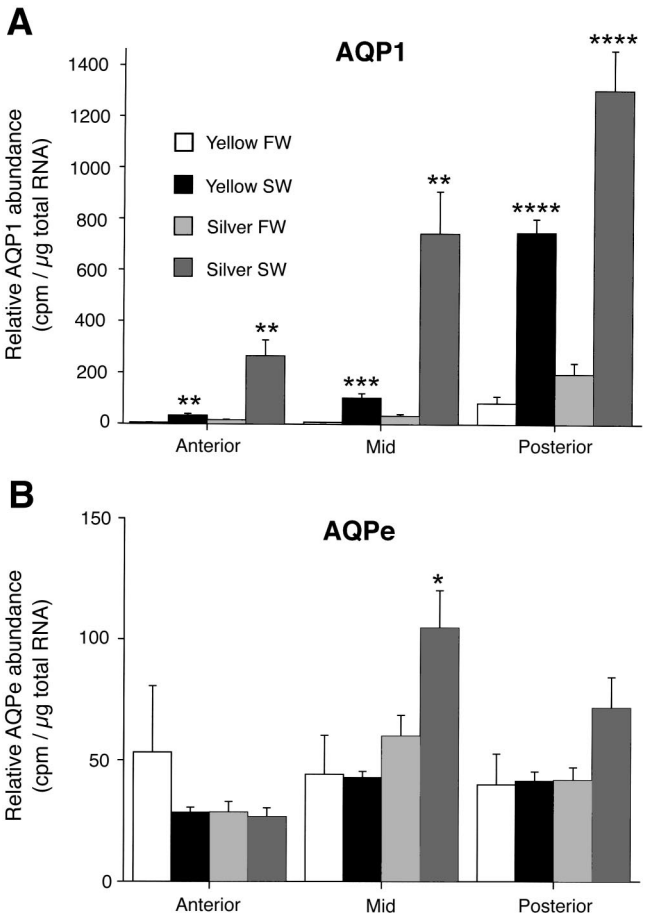

Fig. 3. Quantification of the radiolabeled AQP1 (*A*) and AQPe (*B*) DNA probe bound to Northern blots of intestinal RNA samples isolated from FW-acclimated and 3-wk SW-acclimated yellow and silver eels (as shown in Fig. 2). Values are means ± SE ($n = 6$). Not significant, $P > 0.05$. *$P < 0.05$, **$P < 0.01$; ***$P < 0.001$, ****$P < 0.0001$ (values for FW to SW comparisons).

groups (Figs. 7*A* and 8*A*). Cortisol infusion in FW-acclimated yellow eels resulted in *1*) a significant 49-fold increase in AQP1 mRNA expression (Figs. 7*A* and 8*A*) and *2*) no significant change in AQPe mRNA expression (Figs. 7*B* and 8*B*). Moreover, cortisol infusion had no significant effect on the expression of mRNA of either AQP in the FW-acclimated silver eels (Figs. 7 and 8).

Effect of cortisol on AQP1 protein expression in silver eel intestine. Western blotting (Fig. 9) was performed with the same antibody as before and purified epithelial cell membrane fractions from the intestine of FW-acclimated silver eels implanted with pumps releasing vehicle (controls) or vehicle plus cortisol. Quantification of immunoblots determined that al-

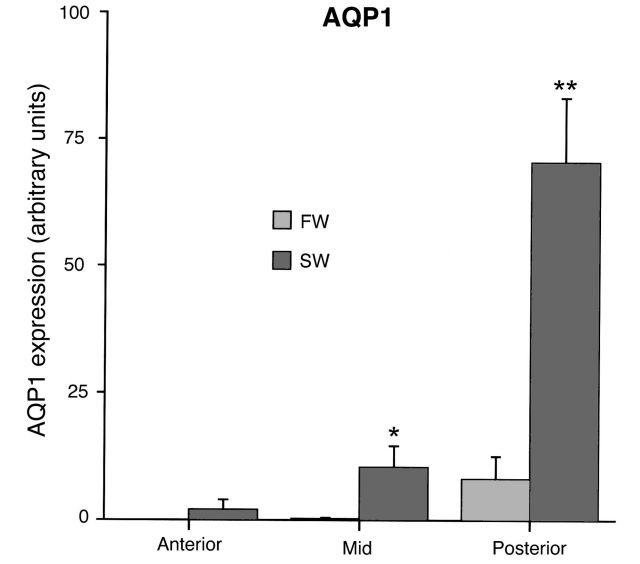

Fig. 5. Quantitative analysis of intestinal AQP1 protein expression in FW and 3-wk SW-acclimated silver eels. Values are means ± SE ($n = 3$). Not significant, $P > 0.05$. *$P < 0.05$, **$P < 0.01$ (values for FW to SW comparisons).

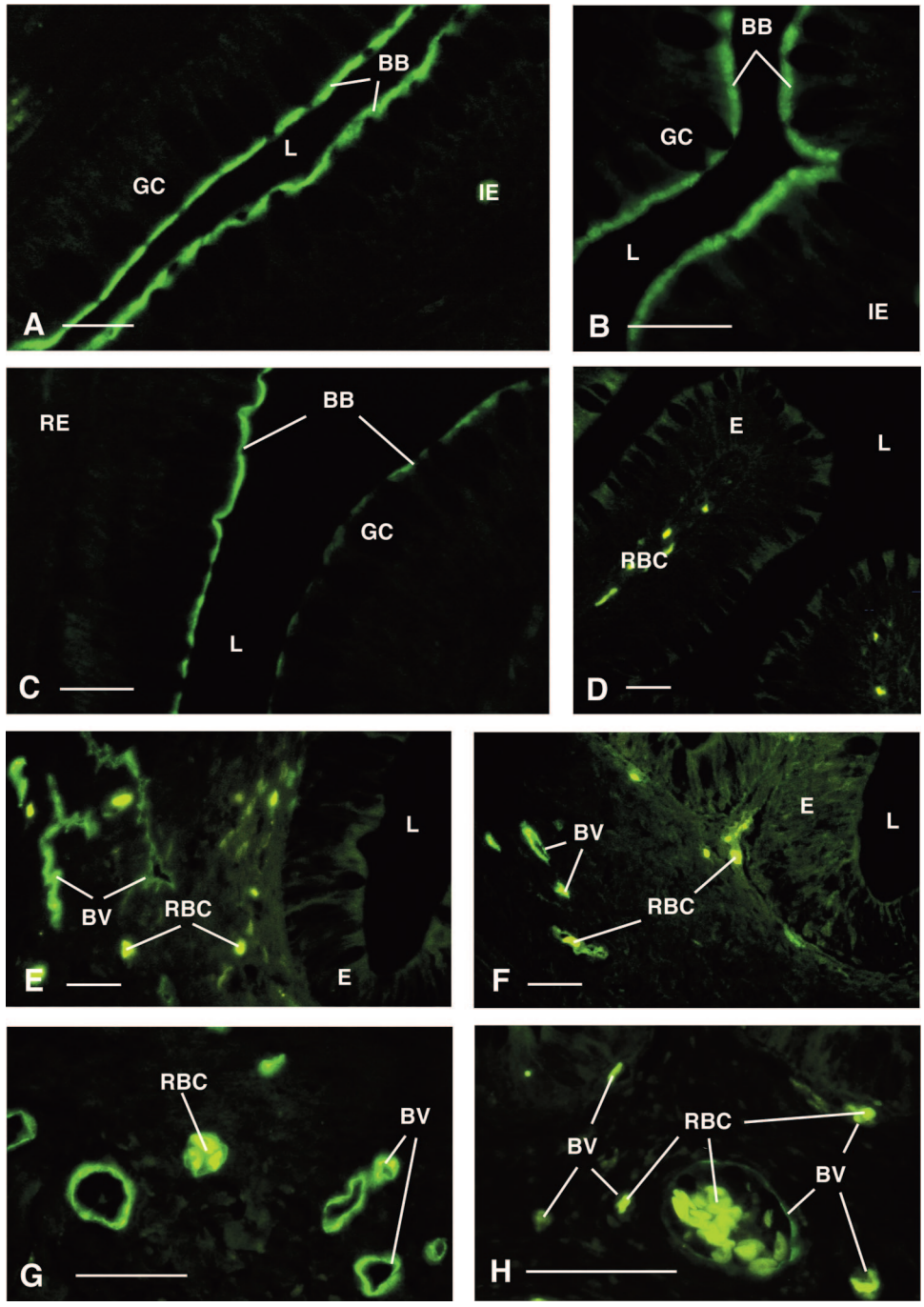

Fig. 6. Immunolocalization of AQP1 in the intestinal and the rectal epithelia of silver eels. Typical immunofluorescence of the blood vessels of the submucosa: SW-acclimated eel midintestine (*E*); SW-acclimated eel anterior intestine (*F*); SW- and FW-acclimated eel posterior intestine (*G* and *H*, respectively); and posterior intestinal and rectal epithelium of SW-acclimated silver eels (posterior intestine, *A* and *B*; rectum, *C*). Control sections were incubated with the peptide-negated antiserum (*D*). BB, brush border; BV, blood vessel; E, epithelium; GC, goblet cell; IE, intestinal epithelium; L, lumen; RBC, red blood cell; RE: rectal epithelium. Bars, 25 μm.

though cortisol infusion did not appear to upregulate levels of AQP1 mRNA, there was a significant fourfold increase in the expression of AQP1 protein throughout the whole intestine (Fig. 10).

DISCUSSION

The eel AQP1 and AQPe amino acid sequences revealed homologies with an expanding group of homotetrameric proteins originally designated the "MIP family" (after the first protein described, the major intrinsic protein of the lens) and now also called the "aquaporin family." In the eel, two members of this family, an AQP1 homolog and a unique aquaglyceroporin homolog called AQPe, were amplified and cloned, with each isoform exhibiting a number of structural features characteristic of known aquaporins, e.g., amino acid sequences were consistent with proteins comprising six transmembrane domains in which the first three domains are homologous to the second three, suggesting an evolutionary internal duplication event (14). Sequence alignments of eel and human AQPs show several highly conserved motifs including the [S/A]E[F/L/C][L/M] sequence (60) near the NH_2 terminal of the protein (aa 45, Fig. 1*A*; aa 39, Fig. 1*B*). The predicted eel AQP molecular size of 27.5 kDa for AQP1 and 34.1 kDa for AQPe are consistent with the monomer size range for mammalian AQPs

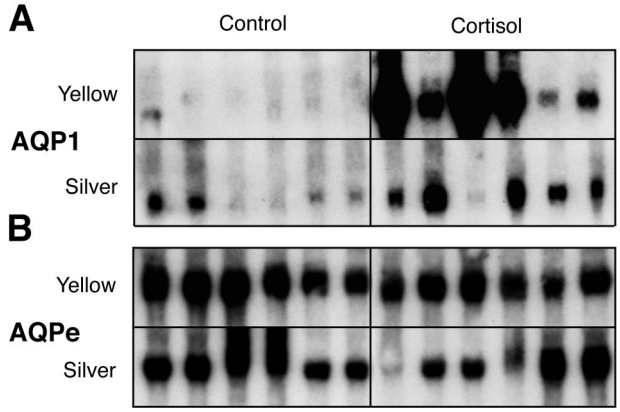

Fig. 7. Northern blot using total RNA (10 μg) showing the expression of AQP1 (*A*) and AQPe (*B*) mRNA in the intestine of vehicle- and cortisol-treated yellow and silver eels ($n = 6$ for each group). RNA sizes (kb) were estimated from RNA standards (not shown). The blot autoradiographs were exposed for 1 h 00 min for AQP1 and for 4 h 50 min for AQPe, at −80°C.

(11, 60). The high percentage of amino acid identity of the AQP1 and AQPe eel isoform sequences with human AQPs (between 35 and 54%) compares well with the values determined across all known mammalian AQPs, which range from 19 to 52% (60). Within the mammalian AQP family, two subgroups have been defined: the orthodox "water-selective" AQPs and the aquaglyceroporins, which can also transport glycerol and other larger solutes in addition to water (11). The eel AQP1 sequence shows some specific characteristics of the first group, with the eel AQPe sequence more similar to the aquaglyceroporins. For example, the NP[A/S]RD motif (aa 231, Fig. 1*B*) in the aquaglyceroporin sequences is replaced by a NPARS motif in the orthodox AQP sequences (aa 244, Fig. 1*A*; Ref. 11). Likewise, sequence alignments of the orthodox AQPs show the highly conserved motif, HW[V/I] [F/Y]WXGP, which is present near the COOH terminal of the eel AQP1 protein (aa 262, Fig. 1*A*) and absent in AQPe and all aquaglyceroporins (Fig. 1*B*; Ref. 60). A cysteine residue responsible for the mercury sensitivity of the AQPs, present in the orthodox AQPs except AQP0 and AQP4 (MIWC, or mercury-insensitive water channel; aa 241, Fig. 1*A*) is replaced by a tyrosine or isoleucine in the majority of aquaglyceroporin sequences including AQPe (aa 226, Fig. 1*B*; Refs. 11, 17). This suggests that if the eel AQPe is sensitive to mercurial reagents, this is probably a result of interactions at some other site.

Results from the quantitative expression studies indicate that AQP1 and AQPe mRNAs were expressed at different levels within the intestine: in extracts of whole intestine epithelial scrapes, AQP1 was consistently more abundant, with expression being at least sixfold higher than AQPe (results not shown). This is contradictory to the findings in the mammalian gastrointestinal tract, where at least eight AQP isoforms are expressed (AQP1, -3–5, -7–10) and where AQP1 expression is only a minor component with abundance limited to the capillary and lymphatic endothelia (11, 24, 37, 43, 50). In the eel intestine, AQP1 mRNA expression was consistently higher in the silver eels compared with the yellow eels irrespective of the acclimation salinity, suggesting that unknown developmental changes in the physiology of the migratory silver eels may be responsible for the specific upregulation of intestinal AQP1. In both yellow and silver eels AQP1 mRNA expression increased when moving from anterior to mid- to posterior/rectal intestinal segments with an approximate ratio of 1:2:15 in both FW-acclimated silver or yellow eels and 1:3:5 or 1:3:20 in SW-acclimated silver and yellow eels, respectively. These results are somewhat contradictory to the reports in the literature, which indicate that the highest levels of water flux within the teleost intestine occur in the midregion, followed in descending order by the posterior and anterior intestine and finally the rectum (4, 9). These anomalies could be explained by the presence of some other functional AQPs, such as AQPe, or by unknown AQP-independent mechanisms, operating in the midregion of the intestine. Expression levels of AQPe mRNA in the three intestinal segments were not significantly different in either FW-acclimated yellow or silver eels, with the exception that a small increase in expression was found in the midsections of SW-acclimated silver eels.

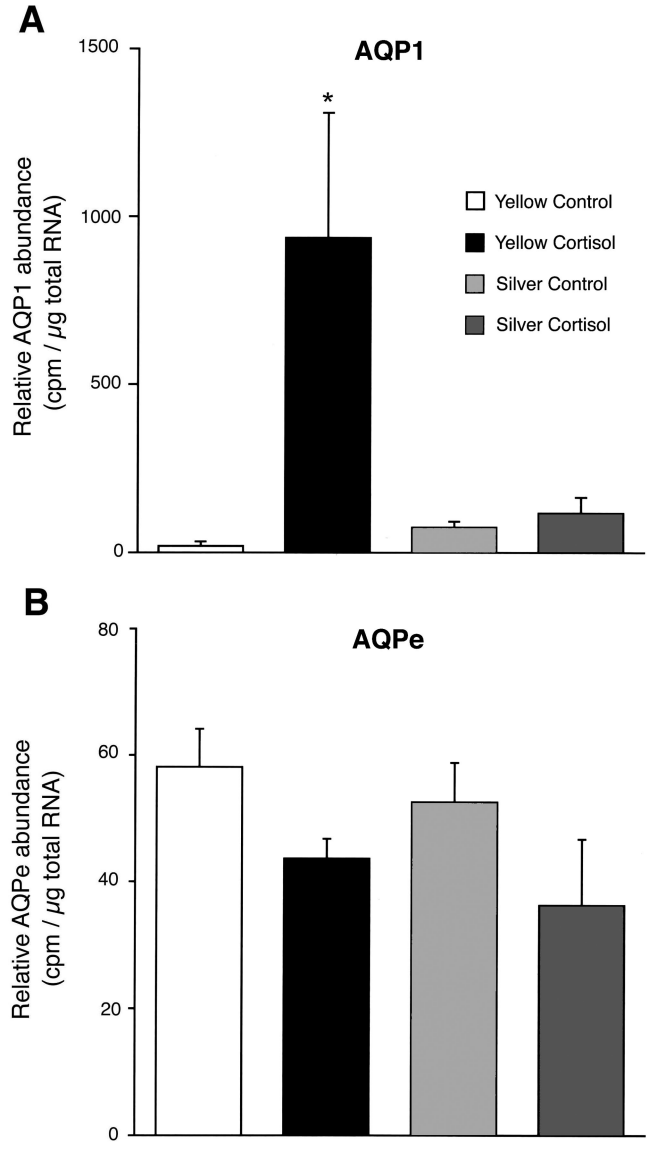

Fig. 8. Quantification of the radiolabeled AQP1 (*A*) and AQPe (*B*) DNA probe bound to Northern blots of intestinal RNA samples isolated from vehicle- and cortisol-treated yellow and silver eels (as shown in Fig. 7). Values are means ± SE ($n = 6$). Not significant, $P > 0.05$. *$P < 0.05$ (values for control to cortisol treated comparisons).

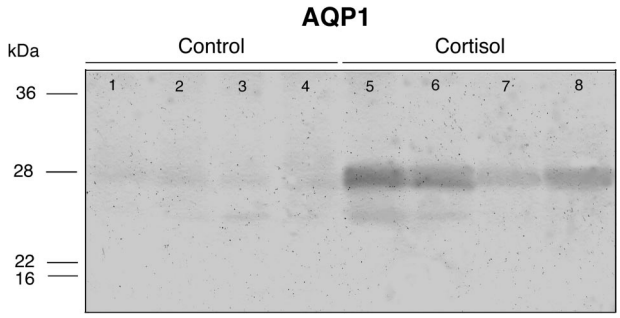

Fig. 9. Western blot analysis of AQP1 in the intestine of vehicle- and cortisol-treated silver eels. The blot shows AQP1 immunoreactivity in plasma membrane fractions isolated from the intestine of vehicle (control; *lanes 1–4*)- and cortisol (*lanes 5–8*)-treated silver eels. Individual lanes represent protein samples from 1 fish in each experimental group. The position of the immunoreactive band is indicated on *left*.

In the euryhaline fish intestine, increased water absorption is observed after increasing the external salinity (44). Imbibed SW is first partially desalinated in the esophagus before passing through the stomach and entering the intestine. The absorption of water across the intestinal epithelium takes place with the concomitant uptake of salts. Water transport across the teleost intestine is generally considered to occur by two processes *1*) the luminal to serosal secondary-active transport of water also known as the "solute-linked water flow," which is linked to "confined regions" of high osmolality created by active ion/solute absorption; and *2*) passive or "general bulk flow" of water across the epithelia, which follows the immediate osmotic gradient between the lumen and blood (52). The secondary-active water transport component, which may occur through both trans- and paracellular pathways, has been investigated in a number of studies and is tightly coupled to the absorption of Cl^- and Na^+ and/or K^+ (3–7, 52, 53). The capacity for ion absorption (especially Cl^-) and solute-linked water flux within the whole intestine were found to increase with acclimation to increasing salinities, with 3- to 3.4-fold increases reported after SW acclimation (3, 52, 53, 57). It is also interesting to note that the passive osmotic water permeability of the intestine also increases by 2- to 6-fold after SW acclimation (3, 52). The functional roles played by AQP1 and AQPe in the intestine, which may account for these changes in water permeability, remain to be determined.

Although variable AQP1 expression levels were observed between individual fish, FW to SW transfer induced statistically significant increases in *1*) mRNA transcript abundance in anterior, mid-, and posterior/rectal intestinal sections of both yellow and silver eels and *2*) protein expression in the mid- and posterior/rectal intestinal regions of silver eels. In direct contrast, SW transfer had no effect on the expression of AQPe mRNA, with the possible exception of the midintestine of silver eels where a small, but significant, 1.7-fold increase in mRNA abundance was measured. Including this report, six homologs of mammalian AQP isoforms have now been reported to be present within the gastrointestinal tract of fish; AQP1, AQP1dup, AQPe, and AQP3 in the European eel (16, 17, 41), AQP1 in the Japanese eel (10), and sbAQP in the sea bream (51). The AQP1 isoform isolated from the European eel shares 94% amino acid homology with two forms of AQP1 reported to be present in the Japanese eel (10). As found with the European eel, AQP1 mRNA expression in the intestine of the Japanese eel is markedly increased after SW acclimation, an effect that is accompanied by a 2.5-fold increase in water absorption in the posterior section of the gut (10). The strong AQP1 expression and its increase after SW transfer suggest that this water channel may play a prominent role in mechanisms associated with water absorption, particularly toward the posterior end of the intestine.

The immunohistochemical detection of AQP1 in intestinal epithelial cells further supports the idea of a transcellular pathway for intestinal water absorption that is upregulated after SW acclimation. However, the intense immunoreactivity for AQP1 was limited to only a small segment of the posterior intestine, immediately before and just after the intestinal sphincter that delineates the end of the posterior intestine and the start of the rectum. In distinct contrast, immunoreactivity was absent in the epithelial cells from all other regions of the intestine of SW-acclimated eels and also from all epithelial cells within the intestine and rectum of FW-acclimated eels. If this is indeed the case, then this would suggest that although AQP1 may act as a water entry site in intestinal epithelial cells in SW-acclimated fish, transepithelial water transport via AQP1 is confined to this small section of the intestine. The immunohistochemical results suggest that the low levels of both AQP1 mRNA and protein abundance found in the anterior and midsections of the intestine may reflect AQP1 expression within blood vessel endothelial cells, which is also upregulated after SW acclimation. Alternatively, it is possible that low levels of expression of AQP1 within the apical brush border of epithelial cells of the anterior and midsegments are below the detection limits of the antibody. In the Japanese eel, although epithelial cell immunoreactivity predominated in the posterior segments, very weak immunoreactivity was also reported within the brush border of cells in the anterior intestine (10). These results may help explain why earlier functional studies reported low water permeability and slow responses to osmotically driven water transport in intestinal brush border vesicles of eels, therefore hypothesizing the absence of water channels in the apical surface of eel enterocytes (1, 2). If AQP1 is the

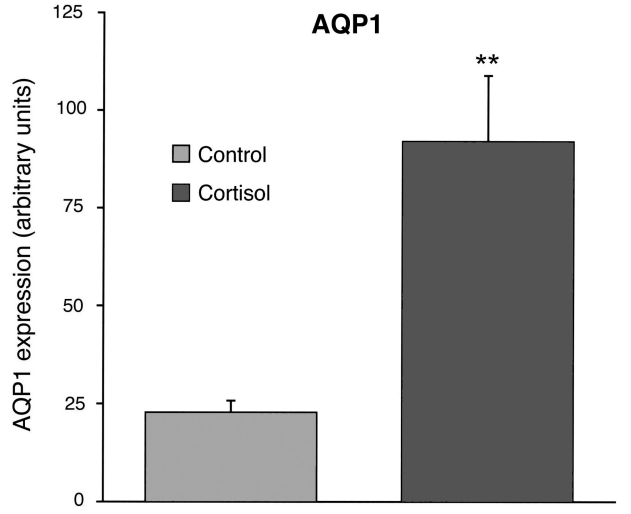

Fig. 10. Quantitative analysis of intestinal AQP1 protein expression in vehicle- and cortisol-treated silver eels. Values are means ± SE ($n = 4$). $^{**}P < 0.01$ (values for control to cortisol treated comparisons).

only isoform present within the apical surface of eel intestinal epithelial cells, as a consequence of its restricted distribution and dependence on SW acclimation for expression, then perhaps it is not surprising that a low water permeability was reported in brush border membrane vesicles prepared from the entire intestine of fish maintained in 50% SW (1). In mammals, although several AQP isoforms are expressed in the gastrointestinal tract, none has been localized to within the apical membrane of the enterocytes (43). Thus AQPs do not appear to be the primary pathway for water absorption in the mammalian intestine. However, it should be noted that if AQP1 expression is also limited to specific regions within the mammalian intestine, it is possible that these putative specialized enterocytes have gone undetected in previous studies. Although the role(s) of AQP1 in water absorption in anterior and midgut regions remains to be established, AQP1 appears to have a second role within the intestine of the European eel. It is clear that expression of AQP1 is much higher within the endothelial cells of blood vessels of the lamina propria throughout the intestine in both FW- and SW-acclimated fish than within the epithelial cells of the anterior and midintestine, a finding that is in agreement with the expression profile of this AQP in the intestine of the Japanese eel (10). Although not quantitatively assessed, AQP1 immunofluorescence associated with blood vessels was consistently more intense and abundant in intestinal sections taken from SW-acclimated fish. Indeed, it is possible that the increases in mRNA and protein abundance found in the anterior and midsections of the intestine may be solely attributable to increases in endothelial AQP1 expression. In mammals, AQP1 expression in vascular endothelial cells has also been reported in various tissues (47, 48, 59) including the intestine (37, 43).

Expression studies indicate that the eel AQPe isoform may also play an important role in water absorption and/or secretion within all intestinal segments. However, as the expression of AQPe mRNA within the intestine does not change markedly after FW-SW transfer, it is unlikely that this protein has a major osmoregulatory role in this tissue. This is in direct contrast to the kidney, the other major site of expression of this isoform, where AQPe expression is significantly reduced after SW acclimation (unpublished observations). Comparison of the amino acid sequence of AQPe with other AQPs suggests that this is a novel protein that is more related to the aquaglyceroporin subgroup within the AQP family and may therefore have an alternate role to the transport of water. The closest homolog to AQPe is the recently reported sea bream AQP, designated sbAQP, which shares 67% identity with the eel isoform at the amino acid level (51). The sbAQP isoform is expressed mainly within a subset of kidney tubules, within cells of the primary lamellae of the gill consistent with Cl^- cells, and within cells localized to the lamina propria and between the two smooth muscle layers within the gut (51). As no immunoreactivity was observed within the intestinal epithelial cells, this would support the hypothesis that sea bream sbAQP, like eel AQPe, is not involved in transepithelial water absorption in the intestine. Although the expression of sbAQP in the kidney and intestine is consistent with what is found in the eel for AQPe, Northern blot analyses failed to detect any AQPe expression in the eel branchial epithelium (unpublished observations), where an AQP3 homolog is the most abundant isoform (17, 41). Northern blot analysis of mid- and posterior intestinal extracts from silver eels exhibited two AQPe transcripts of 2 and 7.5 kb. The larger 7.5-kb transcript, which possibly reflects an immature precursor or spliceoform, was much reduced or completely lacking in the anterior segments of silver eels and in all intestinal segments of yellow eels. The nature of this larger transcript awaits further study. The potential roles for AQPe in the eel are unclear at present. As this isoform is a putative member of the aquaglyceroporin family, it is possible that function could also be associated with the cellular transport of small polar solutes such as urea and glycerol. The *Xenopus* oocyte paralog, AQPxlo, has recently been shown to transport both of these solutes in addition to water (62), indicating that these novel isoforms may indeed be the evolutionary ancestors of one or more members of the mammalian aquaglyceroporin family. However, the physiological role of AQPe within the eel intestine awaits future immunohistochemical localization and functional expression analyses.

Other results in this study suggest that cortisol, at plasma concentrations similar to or slightly higher than those reported during FW-SW acclimation, induces an upregulation of AQP1 gene expression in the yellow eel intestine similar to that found after SW acclimation. However, the FW-acclimated silver eels appeared to be less responsive to cortisol infusion and, although small increases in AQP1 mRNA expression were found in some fish, full analyses of all of the data indicated that there was no statistically significant change in AQP1 mRNA abundance after cortisol treatment. For unknown reasons, large variations in levels of AQP1 expression were again found between animals within each group. It is possible that the high variability found, particularly with the silver eels, may have masked any small effects of the steroid on AQP1 mRNA abundance. Although no significant increases in AQP1 mRNA expression were found in silver eels, cortisol treatment induced a significant fourfold increase in AQP1 protein abundance in the intestine. These results implicate cortisol as a transcriptional and/or translational regulator of intestinal AQP1 expression during SW acclimation. As suggested by Hirano and Utida (28), it is highly likely that various hormonal factors other than cortisol may also be involved in the enhanced rate of water absorption in SW eels, and this is possibly also true of AQP1 mRNA upregulation. There was no significant change in intestinal AQPe mRNA levels in either yellow or silver eels after cortisol treatment. These results suggest that regulatory pathways controlling AQPe expression within the intestine are insensitive to cortisol and the steroid is unlikely to be responsible for the small increases in AQPe mRNA expression found in the midintestine after SW acclimation.

In the higher vertebrates, aldosterone is the major mineralocorticoid stimulating ion and water absorption in various species, including the toad and human colon and the rat jejunum (30). The presence of aldosterone in fish blood has long been debated, and cortisol, the major corticosteroid released by the interrenal gland in both FW and SW fish, is known to have mineralocorticoid as well as glucocorticoid actions (25). The significance of a pituitary-interrenal axis in relation to fish SW adaptation has been intensively investigated by several investigators (26, 27, 29). Cortisol has largely been identified as a SW-adapting hormone, primarily promoting a number of SW-adapting functions (25). When administered to FW-acclimated fish, the steroid has been shown to induce epithelial features similar to those of SW fish such as *1*) Cl^- cell proliferation and

differentiation, with associated increases in the Na^+,K^+-ATPase activity (25, 49); and *2*) an increase in the permeability of ions in the esophagus (24).

The effects of corticosteroids on ion and intestinal water absorption in fish have been mainly studied in eels (8, 21, 22, 27, 28, 30, 57). Among the corticosteroids, only cortisol, hydrocortisone, and cortisol acetate seem to have an augmentive effect on the water movement in the isolated intestine, whereas cortisone acetate, corticosterone, progesterone, aldosterone, and deoxycorticosterone acetate had little effect (21, 27, 40). Cortisol, when injected into FW eels, produces changes in intestinal water movement similar to those occurring after transfer of fish to SW by inducing features such as increasing the permeability of the intestine with commensurate increases in the net absorption of monovalent ions and water and in the development of serosa-negative electrical potential difference (originally interpreted as evidence for a Cl^- pump) (27, 28, 30, 57).

This study is the first to investigate the effects of corticosteroids on the expression of aquaporins in teleost fish and suggests that cortisol mediates an upregulation in the expression of the eel homolog of AQP1 that may account for the previously observed actions of this steroid on water absorption in the eel intestine. The few reported studies regarding the effect of corticosteroids on AQP expression have only concerned mammalian AQPs, with the steroids upregulating expression in the kidney (33, 34, 63), the lung (35, 36, 42, 55), the peritoneal epithelium (54), and red blood cells (46). The actions of the corticosteroids on AQP expression in mammals are consistent with the finding presented in this manuscript that cortisol induces upregulation of at least one AQP isoform in the intestine of the European eel. These results provide new information that will help elucidate the role and mechanisms of action of corticosteroids on water transport in the intestine of vertebrates in general and of eels in particular, especially in association with migration to the SW environment.

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GRANTS

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