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Branchial expression of an aquaporin 3 (AQP-3) homologue is downregulated in the European eel *Anguilla anguilla* following seawater acclimation

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Summary

A cDNA encoding the homologue of mammalian aquaporin 3 (AQP-3) was isolated by reverse transcription–polymerase chain reaction from the gill of the European eel. The derived amino acid sequence shares 67–70 % homology with other vertebrate AQP-3 homologues. Northern blot analysis revealed two AQP-3-specific mRNA species of 2.4 kb and 7 kb. AQP-3 mRNA is expressed predominantly in the eye, oesophagus, intestine (as found in mammals) and the gill; no expression could be demonstrated in the stomach and only low and sporadic levels in the kidney. Quantitative studies demonstrated that, following the 3-week acclimation of freshwater (FW)-adapted yellow and silver eels to seawater (SW), transcript abundance in the gill was reduced by 76 % and 97 %, respectively. The half time of branchial AQP-3 mRNA downregulation in yellow eels was approximately 10 h, with a maximal 94 % decrease in expression after 2 days in SW (compared to time-matched FW controls). However, in fish acclimated to SW for more than 4 days, the fall in AQP-3 mRNA abundance recovered slightly, such that after 3 weeks, expression was 16 % of that in time-matched FW controls. The potential roles for this aquaporin isoform in water or solute transport in the eel gill are discussed.

Key words: fish aquaporin, teleost, urea, ammonia, carbon dioxide, glycerol, messenger RNA, European eel, *Anguilla anguilla.*

Introduction

A fundamental problem for all aquatic organisms is the control of body fluid composition and osmolality, particularly when faced with a variable external environment (e.g. tidal changes in estuaries or during migration between freshwater and seawater). Despite a wealth of data on the transporters and hormonal regulators of ionic homeostasis in aquatic vertebrates, relatively little is known about the mechanisms controlling water transport in these organisms.

In teleost fish, water transport plays a crucial role in a number of tissues that work together to maintain body fluid homeostasis. In freshwater (FW) fish, water incursion occurs principally across the surface of the gill, which has a large surface area and significant water permeability. This net branchial water influx is counteracted by the relatively high production of dilute urine by the kidney. In marine teleosts, water loss across the gill or in the form of urine is balanced by drinking the seawater (SW). Water and salts are taken up across the gut to maintain body fluid volume with the excess salts excreted across the gills.

In order to study the mechanisms associated with body fluid homeostasis in teleosts, the changes occurring during the FW or SW acclimation of euryhaline species have been extensively investigated. In the European eel (*Anguilla anguilla*) most life stages (including FW ‘yellow’ and migratory ‘silver’ adult eels) are capable of immediate transfer between FW and SW environments (Tsukamoto et al., 1998; Birrell et al., 2000). Mainly due to this physiological plasticity, much of the information available concerning epithelial water transport in fish has been determined in eels.

In teleost fish, a major fraction of water exchanges with the external environment occurs across the gill (Rankin and Bolis, 1984; Kirsch, 1972), underlining the importance of this organ to water balance. The results of a number of experiments have indicated that water transport can take place across the gill epithelium independently of simple diffusion through the lipid membranes or movement through the paracellular pathway. While measurements of branchial diffusional water permeability are similar in FW- and SW-acclimated eels, measurements made in the presence of an osmotic gradient (osmotic water permeability) were between three- and 11-fold higher (depending on temperature) in FW- compared to SW-acclimated fish (Motais and Isaia, 1972). In addition, the osmotic water permeability of FW eels was two- to fourfold higher than the diffusional permeability, which has been taken as evidence that the branchial epithelium of FW eels contains (presumably osmotically activated) ‘water-filled pores’ (Motais et al., 1969; Motais and Isaia, 1972; Isaia, 1972). As the osmotic water permeability of the branchial epithelium in SW eels was similar or lower (particularly at low temperatures) than the diffusional permeability, these factors indicated not

only the lack of pores but also the presence of some unknown water re-absorption system operating against the osmotic gradient (Motais and Isaia, 1972; Motais and Garcia-Romeu, 1972). This water transport pathway may or may not be associated with some some unknown solute-uptake transport system. Further evidence of regulated branchial water transport comes from the acute transfer of eels between FW and SW. Under these conditions, net osmotic water fluxes across the gills are non-symmetrical (rectified) when the osmotic gradient is reversed (Isaia, 1984). In FW fish, gill epithelial cell apical membrane permeability was found to be eightfold higher than that of basal membranes, suggesting that water 'pores' in the epithelium predominate in this membrane (Isaia et al., 1978a).

Adrenaline increases the permeability to water and ions of branchial and opercular epithelia *via* a β-adrenergic receptor pathway (Haywood et al., 1977; Isaia et al., 1978a,b; Isaia, 1979, 1984; Zadunaisky, 1984). The effect was greater in FW- than SW-acclimated fish (Isaia, 1979, 1984). Adrenaline was also found to effect the permeability of both apical and basal membranes of the gill epithelium of FW fish (Isaia et al., 1978a). Although the evidence is controversial (Rankin and Bolis, 1984), the hormone prolactin probably reduces water influxes across FW eel gills (Ogawa, 1974, 1975; Ogasawara and Hirano, 1984), whereas cortisol increases water fluxes (Ogawa, 1975; Rankin and Bolis, 1984) and arginine vasotocin (AVT) is without effect (Rankin and Bolis, 1984).

The branchial transport/permeability of the gill to urea also parallels changes in water transport. Depending on the diet, eels excrete between 23–42 % of their nitrogenous waste as urea (Engin and Carter, 2001) and fish acclimated to FW have a threefold higher branchial urea clearance rate than SW eels (Masoni and Payan, 1974). Adrenaline at 10^{-6} mol l^{-1} increased both urea and water permeability by around 100 % (Haywood et al., 1977; Isaia et al., 1978b), with higher doses (10^{-5} mol l^{-1}) causing much larger increases (400–440 %) in branchial urea efflux (Bergman et al., 1974; Sorenson and Fromm, 1976). Studies measuring unidirectional urea fluxes suggest that gill urea transport occurs *via* passive diffusion rather than by facilitated transport processes such as those involving UT type, urea transporters (Wright et al., 1995; Wright and Land, 1998).

At the initiation of this study, the existence of aquaporins had not been demonstrated in any fish species, despite having been identified in many other organisms ranging from bacteria to mammals. However, it seemed possible that these proteins, which are known water- and urea-transporter/channels, might represent the branchial 'pores' suggested to be present in previous reports. Aspects of aquaporin structure, function and regulation have been reviewed extensively (see Hamann et al., 1998; Borgnia et al., 1999; Ma and Verkman, 1999; Marples et al., 1999; van Os et al., 2000). In mammals the aquaporins are a large gene family, currently with 12 members that are related by amino acid homology and genomic structure, which have been grouped into three broad subfamilies. These include the 'water-selective' aquaporins, comprising AQPs 0–2 and AQPs 4–6 (although AQP-0 is also permeable to glycerol; Ishibashi et al., 2000), the glycero-aquaporin group, which contains AQPs 3, 7 and 9, which are permeable to water and/or glycerol and urea (Echevarria et al., 1996; Yang and Verkman, 1997; Ishibashi et al., 1997, 1998), and finally AQPs 8, X1 and X2, which are an anomalous group of channels with lower amino acid homology where AQP 8 is permeable to water and/or urea, AQP X1 to water but not glycerol, and the properties of AQP X2 are unknown (Ishibashi et al., 2000).

Many of the mammalian aquaporins such as AQP-3 are expressed in renal tissues, where much research has been focussed. However, in addition, AQP-3 has also been shown to be expressed in other tissues such as the eye, digestive, respiratory and urinary tracts as well as in the bladder, spleen, skin, epidermis and in erythrocytes (Hamann et al., 1998; Borgnia et al., 1999; Matsuzaki et al., 1999, 2000). AQP-3 is expressed on the basolateral surface of epithelial cells, particularly in tissues interfacing with the external environment, where it may act to prevent cell dehydration. In these tissues, expression may be controlled by extracellular fluid osmolality (Ecelbarger et al., 1995; Terris et al., 1996; Ishibashi et al., 1997; Matsuzaki et al., 1999, 2000). Several hormones have also been implicated in AQP-3 regulation: both anti-diuretic hormone (ADH) and corticosteroids may be involved in the long-term regulation of expression of AQP-3 protein abundance (Terris et al., 1996; Tanaka et al., 1997). The characteristics of body fluid homeostasis in teleost fish, and in particular water and urea transport, suggested the existence of a water and/or urea transport pathway across the eel branchial epithelium. This putative transport pathway, probably involving one or more transporters, is likely to be of importance in the control of water balance and/or urea excretion. This study set out to investigate whether members of the aquaporin gene family are expressed in aquatic organisms such as the eel. The expression of these putative water transporters was investigated in both indigenous freshwater 'yellow' and migratory 'silver' eels to determine whether developmental maturation prior to seawater exposure had any regulatory affect on gene expression.

Materials and methods

Fish

Adult FW 'yellow' and migratory 'silver' eels were obtained from local suppliers in Inverness, Blairgowrie and Kelso, and transferred to laboratory aquariums at the Gatty Marine Laboratory, where they were maintained on a 12 h:12 h light:dark cycle in FW before experimentation. Eels settled in tanks for 2–4 days and then were acclimated to SW (or FW for controls) for periods up to 21 days before use in experiments. Fish were not fed and kept at ambient temperature (5–10 °C).

Total RNA extraction

RNA used for cloning experiments was extracted by a modified LiCl procedure as described in Cutler et al. (1995).

RNA for northern blotting experiments was extracted by a modification of the Chomczynski and Sacchi method (Chomczynski and Sacchi, 1987) as described in Cutler et al. (2000). Messenger RNA was purified for reverse transcriptase-polymerase chain reaction (RT-PCR) experiments as previously described (Cutler et al., 1995).

Cloning and sequencing

As no information was available on the possible existence of any aquaporins present in fish, primers used for amplification were designed based on the available information in mammalian species. As the presence of direct counterparts to mammalian aquaporins could not be guaranteed, a more general approach to primer design had to be taken for the amplification of fish aquaporins. Unfortunately, the amino acid (aa) homology between mammalian aquaporins is not very high, which further led to compromises in primer design, and consequently the primers used could not encompass the complete range of degeneracy found within the sequence data. Amino acids are referred to by the single-letter nomenclature. Within aquaporins a major site of relatively high conservation is around the NPA motifs thought to be associated with the channel pore (Bill et al., 2000). The synthetic degenerate primers (MWG Biotech AG, Ebersberg, Germany), corresponded to approximately 6 aa upstream and up to 2 aa downstream of the NPA motif for the sense primer and 1 aa upstream and up to 7 aa downstream of the NPA motif for the anti-sense primer. Primers were designed to be relatively long (34–35mers) to allow a certain amount of mismatching to be permitted whilst maintaining a reasonably high annealing temperature (see Table 1).

RT-PCR using the degenerate primers (Table 1) was performed on a single strand cDNA template prepared using 5 μg of mRNA from the gill of 7-day SW-acclimated yellow eels, as previously described (Cutler et al., 1995). PCR was performed using a hot-start technique with an initial 2 min incubation at 92 °C, followed by 40 cycles of 94 °C for 1 min, 53 °C for 30 s and 72 °C for 30 s, with a final incubation of 72 °C for 10 min. Reactions of 20 μl were produced with separate additions of primers (4 μmol l^{-1} final concentration) and template (0.5 μl undiluted reverse transcriptase reaction mix), and initiated with the addition of the master mix (to give final concentrations of 50 mmol l^{-1} KCl, 1.5 mmol l^{-1} $MgCl_2$ 10 mmol l^{-1} Tris-HCl, pH 9.0, 200 μmol l^{-1} dNTPs and 1.5 units/20 μl Taq DNA polymerase). DNA fragments within PCR reactions were then separated by Tris-acetate-EDTA agarose gel electrophoresis (Sambrook et al., 1989) and bands of interest purified using Geneclean II (Bio101, Carlsbad, California).

Further AQP-3 5′- and 3′-RACE (Rapid Amplification of cDNA Ends) DNA fragments were produced from SW-acclimated yellow eel gill mRNA and a Marathon cDNA amplification kit (Clontech, Basingstoke, UK) as described previously (Cutler et al., 2000). 5′-RACE products were produced in nested PCR reactions using eel AQP-3-specific antisense primers 1 and then 2 (Table 1) in conjunction with the Marathon kit nested primers. PCR fragments generated by the degenerate primers or by 5′-RACE amplification were cloned into an Original TA Cloning Kit (Invitrogen, Leek, The Netherlands) and were sequenced from colony PCR-amplified fragments using a Big Dye Terminator sequencing kit (Perkin Elmer Biosystems, Warrington, UK) as described previously (Cutler et al., 2000). The 3′-RACE nested amplifications generated a series of faint bands with varying length. Cloning of the largest fragment proved unsuccessful. DNA from pooled, multiple, nested 3′-RACE PCR amplifications (using Sense primer 3 then 4) was therefore purified directly using Geneclean II and partially sequenced (Cutler et al., 2000) using a further nested specific primer (Sense primer 2). In order to sequence the reverse strand a further specific primer (3′-RACE Antisense 1) was synthesised using the initial sequence data obtained.

Table 1. *Primer sequences used for the amplification of eel AQP-3 cDNA fragments*

	Amino acid sequence
Degenerate primers*	
Sense	GTTTSAGYGGXGSYCAYXYSAAYCCXGCXGTSAC
Antisense	GTTTAYXGCXGRRSCXAAXKAXCKRGCWGGRTTVA
5′-RACE primers	
Antisense 1	GTTTCCATTGAGGAGGGTCAGATGAT
Antisense 2	GTTTGCCGAAGTCCCACATTGC
3′-RACE primers	
Sense 1	GTTTGCCTGTCCATGGGCTTCAA
Sense 2	GTTTGTGGTGCTGGTCATCGG
Sense 3	GCACGGCGGCACTGATC
Sense 4	CGATCGTGGACCCTTACAACAA
Antisense 1	GGTGGAAAGCCTGGATTGTG

Amino acids are identified by the single letter code.
*Using IUPAC codes for wobbled bases and where X=inosine/cytidine wobbles.
RACE, rapid amplification of cDNA ends.

Northern blotting and analysis

Northern blots were performed as described previously (Cutler et al., 2000). The probe used for northern analysis was a colony PCR-amplified plasmid insert of the original fragment of AQP-3 produced with the degenerate sense and antisense primers. The amount of total RNA present in each lane of the gel was determined before blotting using ethidium bromide staining of rRNA quantified by a gel documentation and analysis system (Syngene, Cambridge, UK). Quantified values were used to adjust the final radioactive signals obtained per μg total RNA, from the hybridisation. Quantitative analysis of radiolabelled AQP-3 DNA probes hybridising to blots was determined by electronic autoradiography using an Instant Imager (Canberra Packard, Meriden, CT). Statistical analysis was performed using StatView 4.01 software (Abacus Concepts, Berkeley, CA, USA). To reduce potential problems with heteroscedasticity, the data was $\log_{10}$-transformed to reduce the heterogeneity of variances. The data were analysed using ANOVA with levels of significance determined using Scheffe's *F*-test.

Results

Nucleotide and amino acid sequences of eel AQP-3

The eel AQP cDNA sequence contained an open reading frame of 295 aa (Fig. 1) giving the putative translated protein a molecular mass of 32 kDa. The protein shares highest levels of amino acid homology with *Xenopus* AQP-3 (69.9 %), with marginally lower homology to AQP-3s from the higher mammals (67–69 %). The eel AQP cDNA also exhibited low amino acid homology (37–42 %) to other members of the (mammalian) glycero-aquaporin sub-group (AQP-7 and 9), with further reduced homologies to other mammalian aquaporins. In addition to the open reading frame, the cDNA also included 38 nucleotides (nts) of 5′ untranslated and 382 nts of 3′ untranslated sequence (see EMBL accession no. AJ319533). As only partial sequencing of the multiple 3′-RACE products obtained was possible, the eel AQP-3 mRNA contains a considerable amount of additional 3′ untranslated sequence, probably in excess of 1 kb.

Although vertebrate AQP-3 aa sequences are on average reasonably well conserved, the level of conservation shows considerable heterogeneity along the aa chain. The most highly conserved regions of the eel AQP-3 homologue in comparison to AQP-3 sequences from other species occur around the first putative membrane spanning domain (aa 20–50) and a broad region encompassing the end of the extracellular loop C (Borgnia et al., 1999) through the fifth putative membrane spanning domain and through most of (conserved second NPA motif containing) loop E (aa 160–230). A number of small conserved motifs are also distributed throughout the protein. Notable regions of low conservation include a region from aa 6–16 within the cytoplasmic N-terminal tail, a region in the middle of the extracellular loop C (aa 127–142), and the C-terminal tail including the putative sixth transmembrane domain (from aa 237 to the end). The C-terminal tail also contains an insertion of 4 aa in comparison to other AQP-3 sequences.

Tissue distribution of mRNA expression

Northern blots (Fig. 2) indicated that high levels of expression were present in the gill of FW eels, where two

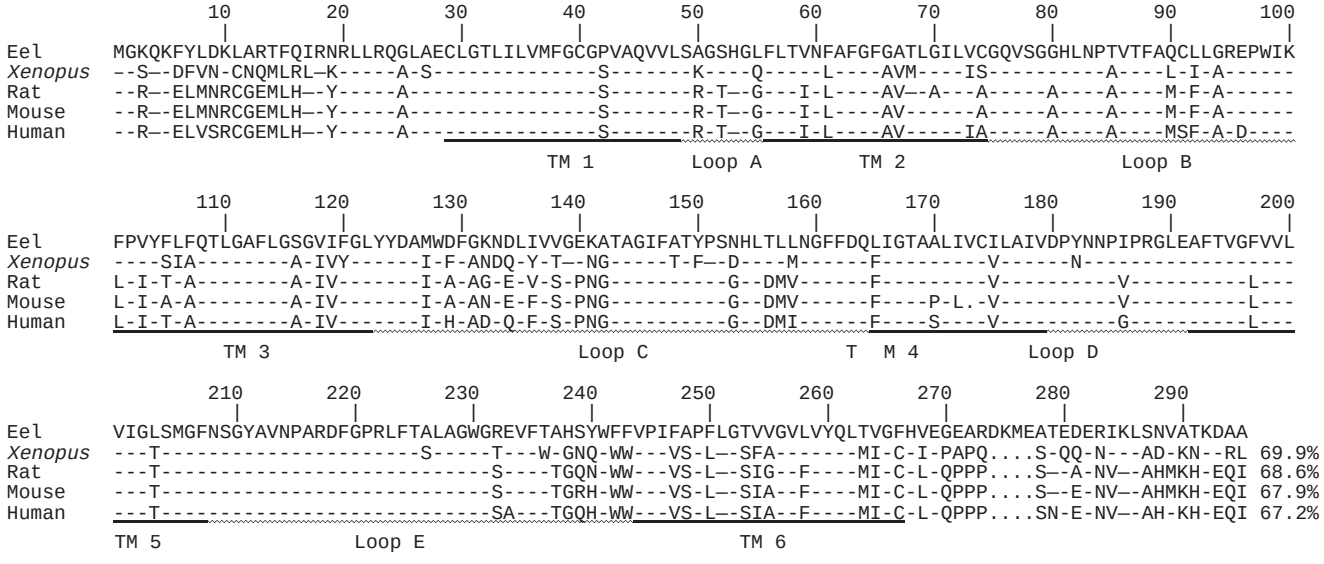

Fig. 1. Alignment of derived AQP3 amino acid sequences from the eel in comparison to those from other species. Dashes indicate identical residues, periods indicate spaces introduced to give an optimal alignment, bold underlinings indicate the approximate locations of membrane spanning domains (TM 1-6: Kyte and Dolittle, 1982) and wavy underlinings indicate the location of inter-transmembrane loops (A–E; Borgnia et al., 1999). Percentages represent the amino acid homology of each sequence to that of the eel. Sequences were aligned using GeneJockey II software (Biosoft). Dashes, underlining and periods were added after alignment. The EMBL/Swiss prot accession numbers for sequences were as follows: Eel (AJ319533), Xenopus (AJ131847), Rat (L35108), Mouse (AF104416) and Human (AB001325).

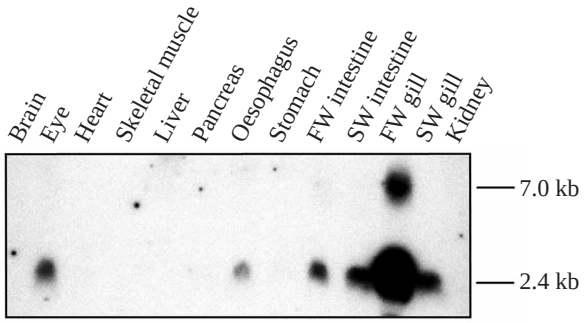

Fig. 2. Northern blot using total RNA (10 µg) showing the tissue distribution of AQP3 mRNA expression in a seawater (SW)-acclimated yellow eel, with the exception of RNA samples from a freshwater (FW) yellow eel where labelled. RNA sizes (kb) were estimated from RNA standards (not shown).

distinct mRNA transcripts were evident: a major 2.4 kb band and a minor 7 kb band, which probably represents an immature precursor or extended 3′-spliceoform of the mature mRNA. Much lower levels of expression were present in SW-acclimated eel gill, in the intestine of both FW and SW-acclimated eels, and in the oesophagus and eye of a SW-acclimated eel. Expression was notably absent in the kidney (SW-acclimated eel) as well as in other tissues.

Quantitative mRNA expression in yellow and silver eel gill and intestine

Quantitative northern blots were used to investigate possible changes in expression in the three major osmoregulatory organs, namely the gill, intestine and kidney of both FW or 21-day SW-acclimated yellow and silver eels (Fig. 3). In the kidney, long autoradiographic exposures (32 h) were required to visualise bands in FW yellow and silver eel RNA samples (data not shown). However, the presence of signals was not consistent between samples. In the intestinal RNA samples, there was no significant difference in expression between FW- or SW-acclimated yellow or silver eels (Fig. 4).

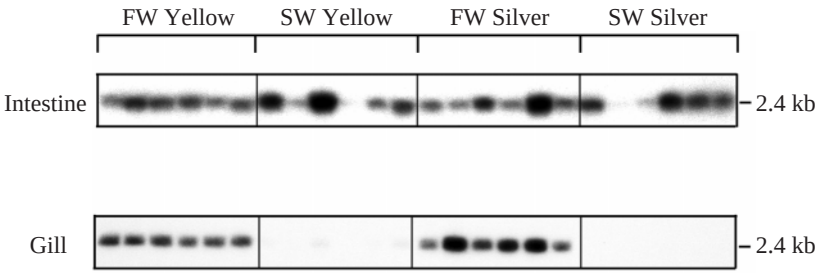

Fig. 3. Northern blots using total RNA (10 µg) showing the expression of AQP3 mRNA in the gill and intestine of freshwater (FW)- and 3-week seawater (SW)-acclimated yellow and silver eels. Each sample on the blot was taken from an individual fish such that N=6 for each group. RNA sizes (kb) were estimated from RNA standards (not shown). The gill blot autoradiograph was exposed for 3 h at –80 °C and the intestine blot, 32 h at –80 °C. The 7 kb band seen in the tissue blot in Fig. 2 was not visible on autoradiographs in this figure, although it was included in the quantification results (Fig. 4).

In the gill, significant differences were found in AQP-3 mRNA expression both between yellow and silver eels and following transfer from FW to SW environments. FW silver eels exhibited a 60 % increase in AQP-3 mRNA expression over the FW yellow eels. 3 weeks after transfer to SW there was a marked reduction in AQP-3 expression in both groups of fish, with a much larger decrease in silver (97 %) compared to yellow (76 %) eels. As a result, there was still a sixfold higher level of AQP-3 expression in the yellow compared to the silver SW groups at this time point.

Time course of changes in mRNA expression in yellow eels

The acclimation of yellow eels to SW resulted in a decrease in AQP-3 mRNA expression in the gill that occurred with a half time of approx. 10 h (Figs 5, 6). AQP-3 expression levels became significantly reduced 1 day after transfer, compared to the FW to FW transferred control group. AQP-3 mRNA abundance reached a minimum 2 days post SW transfer, where the level of expression was reduced by 94 % (compared to the FW time-matched control). After 7 days, the level of AQP-3 mRNA expression in SW-transferred fish partially recovered, with transcript abundance increasing significantly compared to the 2- or 4-day (SW) expression levels. Consequently, by the end of the time course (21 days), the level of expression in SW-transferred fish was only reduced by 84 % (compared to FW controls). There was also an indication that AQP-3 mRNA expression levels in the FW control fish increased slightly over the time course, although only the 6 hour time point was significantly different to those at 1 and 21 days.

Discussion

The eel AQP-3 represents the first aquaporin homologue to be cloned from fish. The eel has the most divergent AQP-3 sequence (67–70 % aa homology) of any species reported to date. As a result, it provides additional evolutionary information about the conservation or otherwise of functional groups or motifs within the protein, which may be useful for future mutagenesis studies.

One of the more interesting amino acid sequence features is the loss of the single putative N-linked glycosylation site in AQP-3s from other species, where this motif is NGT in other species (at position 141–143), but is KAT in eel AQP-3. This suggests that the eel AQP-3 may not be glycosylated. The eel KAT motif is also identical to that found in mouse AQP-7 at the same alignment position (see EMBL accession no. AB010100). Despite the loss of this site, eel AQP-3 does have another putative glycosylation site, although this is the normally conserved first NPA amino acid motif in loop B, which is thought to form part of the water pore of aquaporin within the membrane (Borgnia et al., 1999). In eel AQP-3, the normally conserved alanine residue (in mammalian AQPs) within

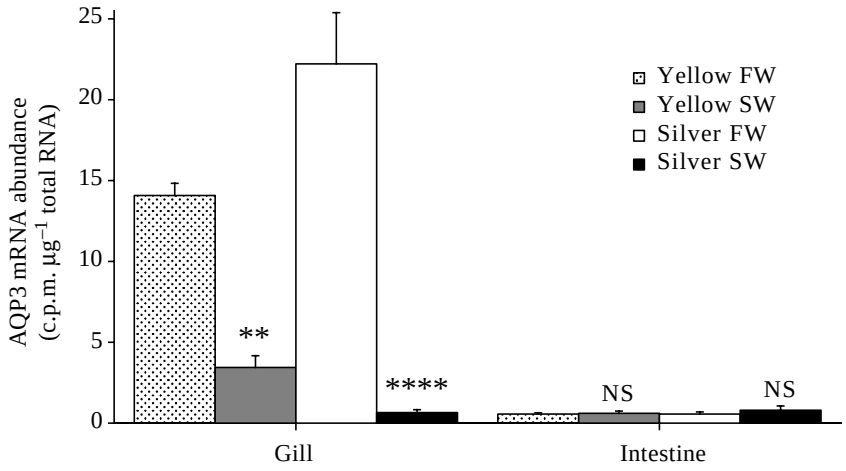

Fig. 4. Quantification of the radiolabelled AQP3 DNA probe bound to northern blots of gill and intestinal RNA samples isolated from freshwater (FW)- and 3-week seawater (SW)-acclimated yellow or silver eels (as shown in Fig. 3). Values are means ± S.E.M. NS, not significant $P>0.05$; **$P<0.01$; ****$P<0.0001$; these values indicate FW to SW comparisons, where $N=6$ samples from different fish in each group.

this NPA site is changed to a threonine, creating the potential glycosylation site, although the central role of this motif in aquaporin channel function suggests that this site is unlikely to be glycosylated. In mammals the only similar non-conservation of NPA motifs occurs within the second NPA motif region of AQP-7, where the normally conserved alanine is replaced by a serine (an aa chemically similar to threonine). The yeast Fps1p channel also has a similar alanine–serine replacement within the first NPA motif site (Bill et al., 2000) as in eel AQP-3. Replacement of the alanine of the first NPA motif of the *E. coli* glycero-aquaporin homologue (GlpF) with a serine has been shown to reduce the glycerol transport of this channel (Bill et al., 2000). While the range of molecules transported by eel AQP-3 and their inherent permeabilities are currently unknown, this information suggests that the channel characteristics may well be somewhat different from the AQP-3 homologues of other species.

The cysteine residue in mammalian AQP-1 (equivalent to the position of aa 211 in eel AQP-3) is thought to be responsible for the mercury sensitivity of aquaporins, although a cysteine at this position is absent in mammalian AQP-0 (MIP), AQP-3, AQP-4 (MIWC or mercury-insensitive water channel) and AQP-7 (Borgnia et al., 1999). In eel AQP-3, in common with mammalian AQP-3s and AQP-7s, a tyrosine is substituted at this site, suggesting that if the eel AQP-3 (and others) is sensitive to mercurial reagents, this probably is a result of interactions at some other site.

The tissue distribution of eel AQP-3 was very similar to that found for AQP-3 in mammals, where it is primarily expressed in tissues exposed to the external environment such as the eye, respiratory tract (equivalent to the teleost gill) and digestive tract (Borgnia et al., 1999; Hamann et al., 1998; Matsuzaki et al., 1999, 2000). However, there are one or two notable exceptions between the tissue distribution of eel and mammalian AQP-3. Firstly, eel AQP-3 was only expressed in the kidney at a low level and in an inconsistent fashion between individual fish, whereas in mammals the kidney is a major site of AQP-3 expression, where it is found in abundance in renal collecting duct principal cells (Borgnia et al., 1999). The lack of a consistent or high level of renal AQP-3 expression increases the likelihood of the presence of other aquaporins in eel kidney, and this may include the presence of a duplicate copy of AQP-3, as has been demonstrated for other ion transporters in the eel (Cutler et al., 2000). Although there was no evidence for the presence of AQP-3 mRNA in the eel stomach, a tissue that exhibits significant levels of expression in mammals (Matsuzaki et al., 2000), AQP-3 mRNA was easily detected in other parts of the eel gastrointestinal tract.

The presence of AQP-3 mRNA in the oesophagus is of special interest as there is thought to be little net water flux across this epithelium in SW-acclimated eels that are drinking the hyperosmotic seawater environment (Hirano and Mayer-Gostan, 1976). Despite this report, a high unidirectional water flux has been reported to occur across the flounder oesophagus when in SW (Parmelee and Renfro, 1983). Taken together, this suggests that while there may be no significant net flow of water across this epithelium, there may be water transport mechanisms present that counteract the osmotic loss of water to the hyperosmotic SW in the lumen. Since it is unlikely that there is a fundamental difference in the physiological function of eel and flounder oesophagus, AQP-3 could play an active

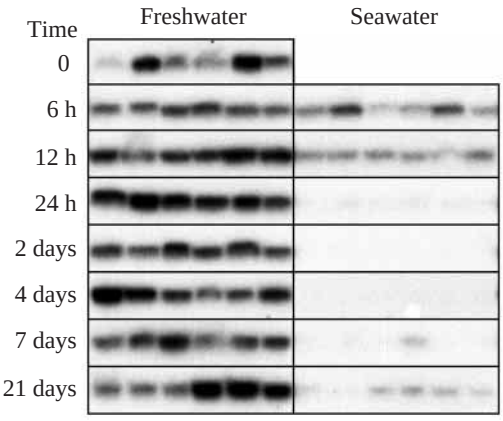

Fig. 5. Northern blots using total RNA (15 μg) showing the time course of changes in gill AQP3 mRNA expression during the seawater (SW) acclimation of yellow eels. Times indicate the period for either freshwater (FW) to SW acclimation or for FW to FW transfer of control eels. Time 0 fish were taken directly from the stock tank. The 7 kb band seen in the tissue blot (Fig. 2) was not visible on autoradiographs in this figure, although it was included in the quantification results (Fig. 6).

role in both species by providing a conduit for serosal (basolateral) transport of water into the epithelia, thus preventing cellular dehydration, as has been previously suggested (Matsuzaki et al., 1999, 2000).

In the quantitative expression studies, FW to SW transfer induced no change in the AQP-3 mRNA levels found in intestinal samples taken from either yellow or silver eels. This suggested that AQP-3 was unlikely to play any role in the absorption of water that occurred following the drinking response in marine teleosts. Subsequent to these experiments more recent immunohistochemical studies have indicated that AQP-3 is not expressed in intestinal enterocytes but, is in fact localised to discrete structures, possibly macrophages, within the intestinal epithelium of both FW and SW eels (Lignot et al., 2002). These results further support our contention that AQP-3 has little role in water absorption across the intestinal epithelium.

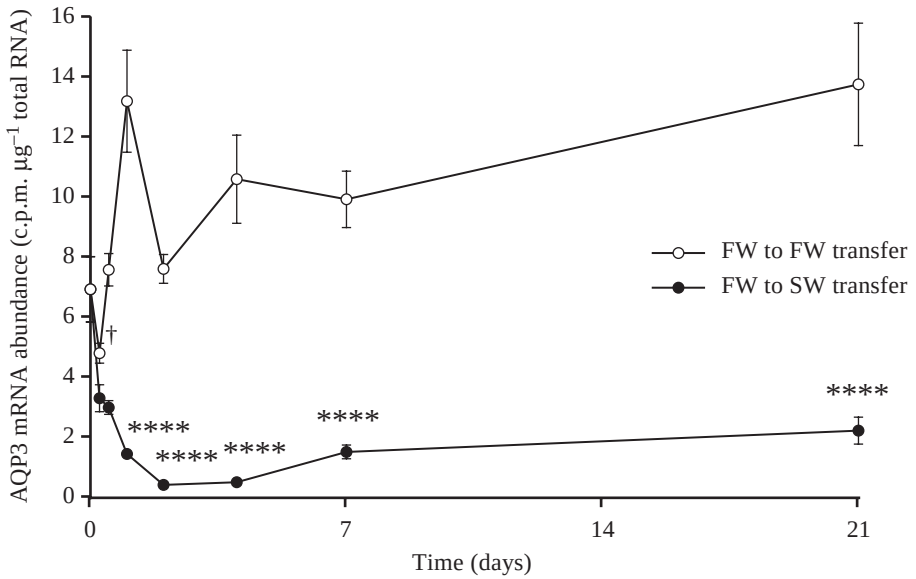

Fig. 6. Quantification of the radiolabelled AQP3 DNA probe bound to the northern blot of yellow eel gill RNA samples following FW to FW and FW to SW transfer. FW, freshwater; SW, seawater. Values are means ± S.E.M. ****P<0.0001; these values refer to FW to SW comparisons for each time point, where N=6 samples from different fish in each group. The 6 hour control time point was significantly different from FW to FW controls at 1 day (P=0.049) and 21 days (P=0.038).

Quantitative northern blots indicated that there was a major downregulation in AQP-3 mRNA expression in the branchial epithelium after SW-transfer. 3 weeks after SW transfer, AQP-3 mRNA abundance had decreased by 76% in yellow eels and 97% in silver eels compared to FW control fish. These differences in branchial AQP-3 expression between FW- and SW-acclimated eels correlates well with the three- to 11-fold higher levels of osmotic water permeability found in the gills of FW fish (Motais and Isaia, 1972). There is also an additional correlation between AQP-3 expression and the threefold higher levels of branchial urea clearance rates found in FW compared to SW eels (Masoni and Payan, 1974), indicating that AQP-3 may be involved in either water and/or urea transport.

It is still not clear why water (and urea) transport across the gills is much higher in FW- than SW-acclimated fish, and what role an aquaporin channel such as AQP-3 might play. There is no reason to believe that the respiratory requirements of FW or SW eels should be markedly different, so changes in water permeability are unlikely to relate to respiratory gas transfer. One possible explanation for the higher osmotic water permeability of FW eels is that, as a result of differences in expression of one or more apically located solute transporters, there is an increase in water uptake across the apical membrane into the branchial epithelial cells. As AQP-3 has universally been shown to have a basolateral localisation in mammalian epithelia, it is possible that the eel AQP-3 may act as a conduit in the basolateral membrane for the release of water to the serosal fluid and the prevention of cell swelling (Cutler and Cramb, 2000; Matsuzaki et al., 2000).

One pathway that may exist in branchial surface epithelial cells is the putative 'bulk fluid flow' ion transport mechanism thought to operate through the tubular system of chloride cells (Isaia, 1984). Here, AQP-3 would be most likely to act as a conduit for water into or out of the tubular system. Links between ion and water transport have already been established in mammals, where AQP-3 is thought to be regulated by the cystic fibrosis transmembrane conductance regulator (CFTR; Schreiber et al., 2000). However, as the 'bulk flow' mechanism is associated with ion transport in marine teleosts, it is likely to be less, rather than more, active in FW eels.

Finally, the possibility also exists that water transport is just the consequence of some primary role of AQP-3 such as the transport of some other solute(s) molecule. One obvious possibility is that AQP-3 is essential for urea excretion across the gills. This would correlate well with the parallels between water and urea permeability/clearance across the gill (Motais and Isaia, 1972; Masoni and Payan, 1974) as well as their regulation by adrenaline (Bergman et al., 1974; Sorenson and Fromm, 1976; Haywood et al., 1977; Isaia et al., 1978; Isaia, 1979, 1984; Zadunaisky, 1984). Another potential substrate for the AQP-3 transporter is ammonia. Ammonia, a more abundant nitrogenous waste product than urea in many fish, is also excreted across the gills, and certain plant (Cooper et al., 2000) and animal (Nakhoul et al., 2001) aquaporins have been shown to transport this compound. FW fish are known to excrete a higher percentage of total body ammonia across the gills than marine teleosts (Wilkie, 1997). This may be because SW fish can excrete much of their nitrogen waste as ammonium ions

through ion transport mechanisms in the gill (Wilkie, 1997). Another molecule that might additionally be transported by eel AQP-3 is CO_2. Several studies have shown that the transport of CO_2 can be facilitated by mammalian AQP-1 (Cooper and Boron, 1998; Nakhoul et al., 1998; Prasad et al., 1998; Yang et al., 2000). As is the case for oxygen uptake, it would seem unlikely from a respiratory standpoint that a significantly greater branchial permeability for CO_2 would exist in FW than SW fish. However, it could be argued that there is a need for additional controls of body fluid pH while in FW environments. If the latter is indeed the case, changes in environmental pH should have profound effects on the expression of branchial AQP-3 in the eel. Recently it has been suggested that mammalian AQP-3 but not AQPs 0, 1, 2, 4 or 5 may be gated by H^+ ions (Zeuthen and Klaerke, 1999). Whether this mechanism operates in teleosts for the regulation of water permeability or body fluid pH awaits further investigation.

Possible clues to the principle role of AQP-3 in the eel gill may reside in studies that reveal the cellular location of this transporter in the branchial and intestinal epithelium. The accompanying paper (Lignot et al., 2002) presents some initial immunohistochemical studies which go some way to answering these questions.

Although this paper has taken a significant first step in improving our understanding of the processes that underlie water transport in fish, much remains to be determined. Future studies will need to determine the range of solutes that can be transported by eel AQP-3 and also to focus on the hormonal regulation that underpins the changes in AQP-3 expression and action during salinity acclimation.

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