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Biochimica et Biophysica Acta 1566 (2002) 16–27

BIOCHIMICA ET BIOPHYSICA ACTA
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Review

Cystic fibrosis transmembrane conductance regulator in teleost fish

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Received 29 May 2002; accepted 22 August 2002

Abstract

The gills and intestinal epithelia of teleost fish express cystic fibrosis transmembrane conductance regulator (CFTR), and utilize this low conductance anion channel in the apical membrane for ion secretion in seawater gill and in the basolateral membrane for ion absorption in freshwater gill. Similarly, in the intestine CFTR is present in the basolateral membrane for intestinal absorption and also in the apical membrane of secreting intestine. The expression of CFTR and the directed trafficking of the protein to the apical or basolateral membrane is salinity-dependent. The CFTR gene has been cloned and sequenced from several teleost species and although all the major elements in the human gene are present, including two nucleotide binding domains that are common to all ATP binding cassette (ABC) transporters, the sequences are divergent compared to shark or human. In euryhaline fish adapting to seawater, CFTR, localized immunocytochemically, redistributes slowly from a basolateral location to the apical membrane while ion secretory capacity increases. The facility with which teleosts regulate CFTR expression and activation during salinity adaptation make this system an appealing model for the expression and trafficking operation of this labile gene product.

Keywords: Gill epithelium; Intestine; Brain; Killifish; Salmon; Tilapia; Fugu; Eel; Protein trafficking; Marine teleost; Ion channel; Amino acid similarity; Expression; ABC cassette protein

1. Introduction

This tribute to Hans Ussing embraces the paradigms he built that have influenced epithelial transport studies for decades. The contributions that have affected studies of anion transport in teleost fish are mainly the practical and theoretical background to allow interpretation of radioisotopic fluxes by open circuit epithelia in "Ussing chambers" [1], the refinement using the short-circuit technique by Ussing and Zerahn [2], and the revelation of a two-step transport where one membrane has a pump and the other a leak for the same ion by Koefoed Johnsen and Ussing [3]. These major features and techniques with a few variations have allowed epithelial Cl^- transport studies to flourish. Even now we use fish skins in Ussing chambers, still a preparation that we find is a particularly effective means of introducing new students to the immediacy and excitement of transport studies.

Cystic fibrosis transmembrane conductance regulator (CFTR) was isolated and sequenced from human in 1989 [4,5] and it was realized soon after that a single amino acid deletion at position 508 (ΔF508) was a sufficient change to produce cystic fibrosis [6]. CFTR is a member of the ATP binding cassette (ABC) proteins (review: Ref. [7]) and characteristically is an anion channel of 7–10 pS conductance [8,9]. There are more than 900 mutations now known that can produce the symptoms of CF [10]. The mutations appear to disrupt not gene expression or channel operation in situ, but rather the successful directed trafficking that inserts CFTR in the apical membrane of airway epithelial cells [11] and other tissues affected [7]. Because of the relevance to CF, much study has been directed to elucidate normal and abnormal trafficking of CFTR (review: Refs. [12–14]). The most recent observations suggest a non-conventional trafficking of CFTR from rough ER to Golgi regulated by coat protein complex II [14]. A CFTR associated ligand (CAL) that has been identified localized to the Golgi also has PDZ domains and may modulate CFTR cell surface expression [15]. Once in the membrane, CFTR activation may involve cytoskeleton and PDZ binding domains [13], but the PDZ binding domain apparently is not

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PII: S0005-2736(02)00584-9

involved in the regulating function of CFTR on epithelial Na^+ channels (EnaC) [16]. There are also promising advances in pharmacological redirection of mistrafficked CFTR protein [17]. Generally, however, mammalian CFTR is expressed and trafficked at low, relatively stable rates.

Teleost fish have highly labile salt transporting systems that rapidly upregulate or downregulate when the animal changes environmental salinity. The processes are well studied (reviews: Refs. [18–20]) and much is known of the regulation of these systems by hormones (review: Ref. [21]), neurotransmitters [22–24] and cell level osmotic cues [25,26]. In 1998 the first full-length cDNA clone of CFTR from teleost fish was identified and opened the field of molecular research of CFTR in teleosts [27]. This review and synthesis will scan what is known thus far on CFTR in teleosts and will point to future avenues of research that may lead to important insights in the regulation and operation of the CFTR anion channel.

2. Evidence for CFTR in teleosts

2.1. Electrophysiology

Low conductance anion channels have been identified in apical membrane of chloride cells in short-term culture isolated from killifish opercular epithelium [28] and in pavement cell primary cultures of seabass gill [29]. In symmetrical 150 mM NaCl at 22 °C, the average single channel conductance is 8.1 + 0.35 pS in killifish and for the sea bass, in 140 mM symmetrical *N*-methyl-D-glucamine–Cl at 20–22 °C, single channel conductance was 8.3 and 11.5 pS during positive and negative voltage clamping, respectively. The killifish channel in excised patches and 150 mM NaCl had no apparent rectification while the seabass channel had a slight tendency to inward rectification. In most cases, as with mammalian CFTR channels, killifish channels deactivated on excision (Table 1; [28]). The sea bass channel instead stayed active on excision and actually increased in the number of active channels when excised patches were depolarized [29]. The killifish channel was demonstrated to be Cl^- selective by shift of the reversal potential with a change in the concentration of Cl from 150 to 40 mM in the bath (cytoplasmic side) [28]. Also, the sea bass channel is active in the presence of only Cl^- as a small permeable ion (i.e. *N*-methyl-D-glucamine is impermermable), thus providing strong evidence for anion selectivity [29]. Sea bass channels are more conductive to Cl^- than to I^- and are rapidly blocked by I^- [29], similar to characteristics shown for mammalian CFTR [9]. While there are slight variations in channel behavior between killifish and sea bass, on balance the two channels are highly similar in their conductance and selectivity. A complete anion selectivity sequence for either channel is still lacking and of particular interest would be the relative permeation by Cl^- and HCO_3^-. This would be important to assess the possible involvement of the channel in bicarbonate fluxes and acid–base balance.

Table 1
Activation by PKA and ATP of low conductance anion channels in excised membrane patches from killifish chloride cells

Treatment	Seals without channels	Seals with channels		Total
		Cell attached	Excised	
Control	39 (54%)	32 (44%)	1 (1.4%)	72 (100%)
PKA + ATP[a]	27 (43%)	23 (51%)	13* (21%)	63 (100%)

[a] 100 U/ml protein kinase A (Sigma) + 0.5 mM ATP (Sigma) added to the bath saline into which seals were excised; methods as per Marshall et al. [28].

* $P < 0.001$ Fisher exact test.

2.2. Molecular biology of teleost CFTR genes

The first teleost CFTR homologue was cloned from the gill of seawater-adapted killifish *Fundulus heteroclitus* [27]. This full-length cDNA clone has a single long open reading frame of 1503 amino acids. At the nucleotide level the kfCFTR coding sequence is 62.2% identical to human CFTR and only 59% identity at the amino acid level, making it the most divergent homologue yet cloned. As expected, the R-domain is the least conserved domain while the nucleotide-binding domains show the highest homology. Expression of the kfCFTR homologue is highly tissue-specific, with a major 7.5-kb mRNA species present in gill, opercular epithelium, intestine and (to a lesser extent) brain of seawater adapted killifish. A second smaller mRNA species of 5.5 kb is also present in these tissues and may represent an alternatively spliced form. Following abrupt seawater exposure of freshwater-adapted killifish, mRNA levels have been shown to rise ninefold by 24 h and gradually subside to approximately threefold after 28 days in seawater [27]. Protein expression patterns indicate a single 175-kDa band to an antibody for the carboxy terminus of CFTR [30], intermediate between mRNA expression with two forms (160 and 210 kDa) present in both gill and intestine. Killifish CFTR protein appears to be expressed predominately in chloride cells of the gills and opercular epithelium rather than in other cell types (see below).

Subsequent to the cloning of a killifish CFTR, two additional teleost homologues have been become available. The Atlantic salmon is the second teleost from which CFTR has been cloned and is unique in that two isoforms have been identified [31]. Like the killifish, the Atlantic salmon is euryhaline and provides an additional important model to examine the molecular nature of seawater adaptation. This is the first example of an organism which expresses more than one CFTR isoform. These two isoforms share 93% sequence identity at the nucleotide level and are 95% identical at the amino acid level. A splice variant of Atlantic salmon CFTR I has also been discovered lacking exon 12, a region normally containing a highly conserved Walker B motif within nucleotide binding domain 1. The functional importance of each of these isoforms has yet to be eluci-

dated. Studies of Atlantic salmon smolts abruptly exposed to seawater demonstrate unique expression patterns of CFTR I and II, with CFTR I showing sustained threefold increases in mRNA levels after 24 h while CFTR II showing transient lower-fold increases [32].

The third teleost species from which a CFTR homologue has been cloned is the fugu (puffer fish) *Takifugu rubripes* [33]. It is the only example of a teleost CFTR homologue for which the full genomic sequence is known. In contrast to the human CFTR genomic sequence, the fugu CFTR is approximately 10-fold smaller. The region of the fugu genome containing CFTR shows conservation of synteny with the human genome. Overall sequence analysis suggests conservation of intron/exon boundaries between fugu and human CFTR. Likewise, partial sequencing of killifish CFTR intron–exon genomic structure confirms this finding [34]. Fugu is the only stenohaline marine teleost from which a CFTR homologue has been cloned and thus provides the basis of investigations to compare genomic regulatory elements between it and the two euryhaline teleost CFTR homologues. Fig. 1 summarizes the phylogenetic relationships between the amino acid sequences of the teleost CFTR homologues described here and several non-teleost homologues. The percentage amino acid sequence identity between CFTR homologues is summarized in Fig. 2.

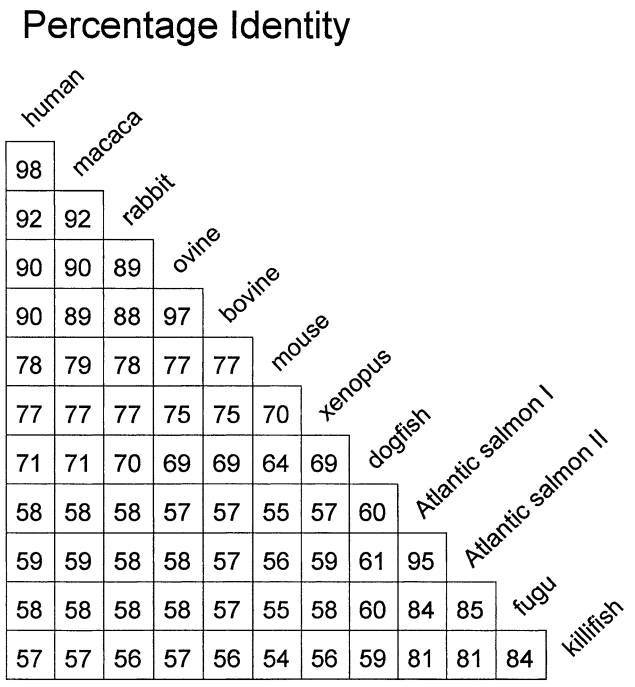

Fig. 2. Percentage amino acid sequence identity between CFTR homologues. (See Fig. 1 for the CFTR sequences used).

2.3. Pharmacology

Diphenylanine-2-carboxylate (DPC), 5-nitro-2-(3-phenylproylamino) benzoate (NPPB) and glibenclamide are blockers for CFTR-like channels [8]. Glibenclamide also blocks the outward rectifying Cl^- channel [35] so the specificity is not absolute. However, unlike some other anion channels (especially ClC type), CFTR channels are insensitive to the disulfonic stilbene DIDS (e.g. Ref. [36]). The killifish opercular epithelial short-circuit current [28] and the mudskipper skin [37] are inhibited by DPC. In the

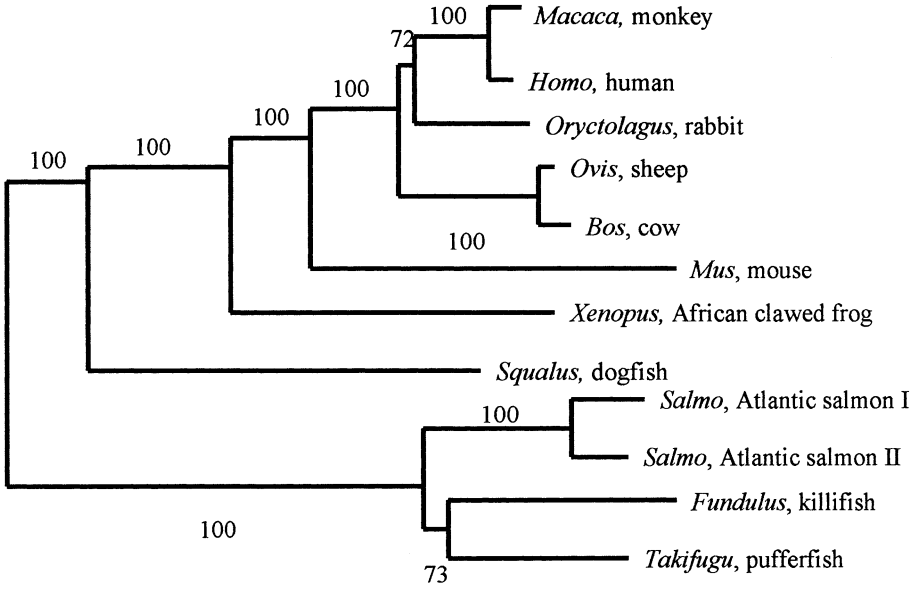

Fig. 1. Phylogenetic relationships between CFTR proteins. Complete amino acid sequences were analyzed using Clustal W. A neighbour-joining tree was obtained using the MEGA phylogenetic analysis software package. The results (%) of 1000 bootstrap replications are shown adjacent to the branches. Note that the teleosts form a strongly supported monophyletic group more divergent from the mammals than the dogfish shark. (Accession No. of CFTR sequences used for analysis: *Macaca*, AF13753; *Homo*, M28668; *Oryctolagus*, AF189720; *Ovis*, U20418; *Bos* M76128; *Mus*, M69298; *Xenopus*, X65256; *Squalus*, M83785; *Salmo-I*, AF155237; *Salmo-II*, AF161070; *Fundulus*, AF000271; *Takifugu*, AJ271361).

killifish opercular epithelium, NPPB blocked Cl^- secretion with maximal inhibition of approximately 90% at 1.0 mM with DPC being slightly less effective at the same concentrations [28]. As well, DPC blocks AVT-stimulated transepithelial Cl^- secretion in sea bass gill cell cultures [38]. In excised membrane patches from sea bass cultures, 1.0 mM DPC and 0.1 mM NPPB reversibly inhibited single channel activity. DIDS (1.0 mM) was ineffective in inhibiting channel activity in isolated membrane patches [38] and failed to inhibit Cl^- secretion in whole epithelia [28]. Thus, the blockade of single channel and whole epithelial currents is in agreement with the existence of a CFTR type anion channel in teleosts.

2.4. Activation by cAMP and protein kinase A (PKA)

Mammalian CFTR is activated by phosphorylation from PKA that in turn is stimulated by cAMP, and this phosphorylation is necessary for activation and sustained operation of the channel [8,39]. The progressive phosphorylation of the two nucleotide binding domains by PKA increase the number of active channels and their open probability in a complex kinetic interaction that is still to be elucidated [7].

Sea bass excised patches with few copies of the channel were activated by addition of ATP (2 mM) and PKA (0.1 μM) and multiple channels appeared in a few minutes [29]. Killifish opercular membranes increase whole epithelial Cl^- secretion by cAMP augmentation [22,26,40,41], and addition of cAMP + IBMX to the bathing solution of cultured opercular epithelium significantly increased the number of active 8-pS channels in cell attached patches [28]. PKA and ATP addition significantly increased open probability of channels in excised patches from 0.45 to 0.62 [29]. PKA (100–200 U/ml) and ATP (0.5 mM), added to the bath of eight cultured opercular epithelial preparations, significantly increased the percentage of channels that remained active after excision, from 1.4% to 21% of patches (Table 1; Marshall, unpublished), indicating sustained activation by PKA and ATP. Thus, there is strong evidence for activation of the channel by PKA via cAMP. Such activation is another diagnostic feature of CFTR channels [8,39].

2.5. Immunocytochemistry

The availability of antibodies directed against human CFTR has allowed initial investigations of CFTR in fish. In

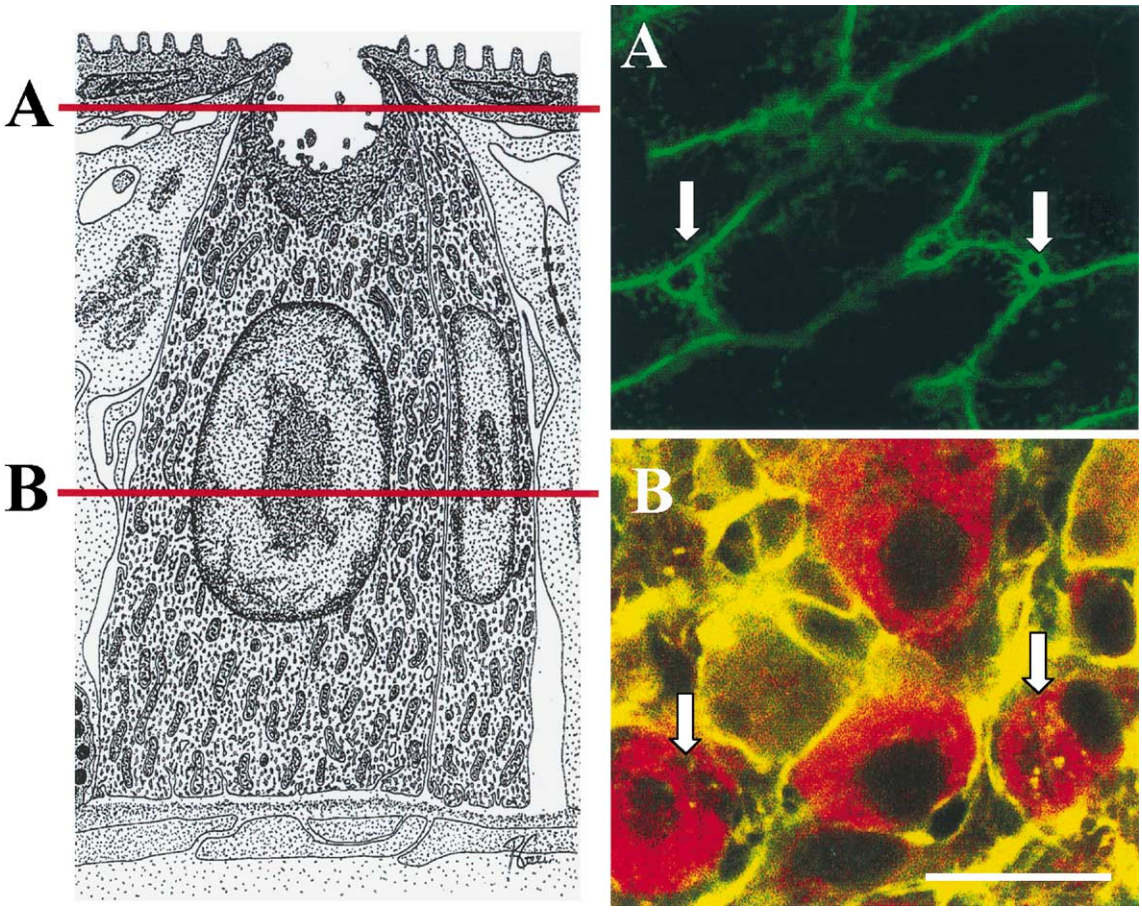

Fig. 3. Confocal laser scanning images of killifish opercular epithelium. (A) Phalloidin staining of F-actin rings at the plane of the apical crypt of mitochondria rich cells. (B) Same frame but focused at the plane of the nuclei showing strong mitochondrial staining (Mitotracker Red) but little actin staining in the chloride cells. (Adapted from Daborn et al. [77]). Bar is 20 μm.

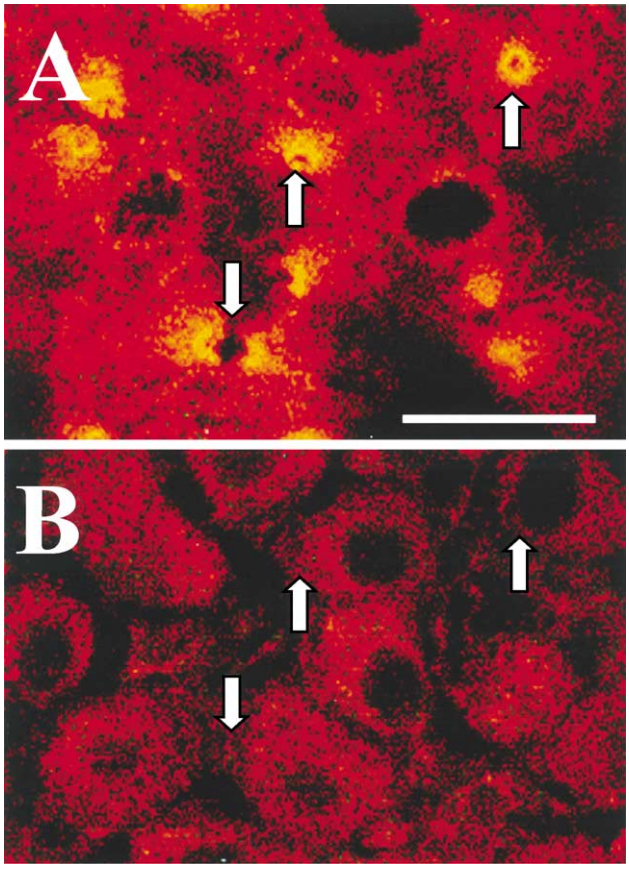

Fig. 4. Immunohistochemical staining of CFTR in chloride cells from seawater killifish using the monoclonal anti hCFTR carboxy terminus, a sequence shared between human and killifish. (A) At the plane of the apical crypt, there is strong CFTR staining in the apical crypt, following the ring shape of the crypt. Yellow color is positive for CFTR (Oregon Green) and for mitochondria (Mitotracker Red). (B) Same frame, but at the plane of the nuclei where Mitotracker Red stains mitochondria but there is no CFTR immunostaining. (Adapted from Marshall et al. [43]). Bar is 20 μm.

the case of mudskipper (*Periophthalmodon schlosseri*) adapted to brackish water or seawater, and using a monoclonal anti hCFTR antibody (Neomarkers Inc.), Wilson et al. [42] localized CFTR immunofluorescence to crescent- or cup-shaped portions of the apical side of mitochondria-rich cells in the interlamellar spaces of the gill. These preparations were formalin-fixed, paraffin-embedded and sectioned to observe the cells in profile. The controls lacking primary antibody had no immunofluorescence. Thus, CFTR appeared to be restricted to the apical membrane in the apical crypts of chloride cells from seawater-adapted mudskippers [42]. The seawater-adapted killifish opercular epithelium is rich in chloride cells (Fig. 3) and the apical membrane of these cells form apical crypts that are structurally supported by F-actin (Fig. 3A,B). These cells also showed CFTR immunofluorescence restricted to the apical membrane [43]. In this case, the epitope of the antibody directed against the carboxy terminus is the same as that in the human CFTR (in both cases, the carboxy terminus is −dtrl) so there is less probability of nonspecific binding. In optical sections collected 3–5 μm below the surface, CFTR immunofluorescence appeared as 1–2 μm diameter rings (Fig. 4A,B), consistent with an apical membrane distribution of CFTR in apical crypts of chloride cells. There was no detectable immunofluorescence deeper in the tissue and none in control sections lacking primary antibody (Fig. 4B) [41]. In seawater killifish gill, CFTR colocalizes with the chaperonin HSP60 (a 60-kDa heat shock protein) in chloride cells of gill epithelial Cl^- cells [44], suggestive of involvement of HSP60 in CFTR post processing. Hence, in seawater-adapted teleosts CFTR is restricted to the apical membranes of mitochondria-rich Cl^- secreting cells.

Immunocytochemical identification of CFTR has also been performed recently in freshwater adapted killifish (Fig. 5A,B) with the result that CFTR appears to be present on the basolateral membrane of Cl^- and of pavement cells in the opercular epithelium, while the highly fluorescent apical distribution (typical of SW) is absent [41]. In animals

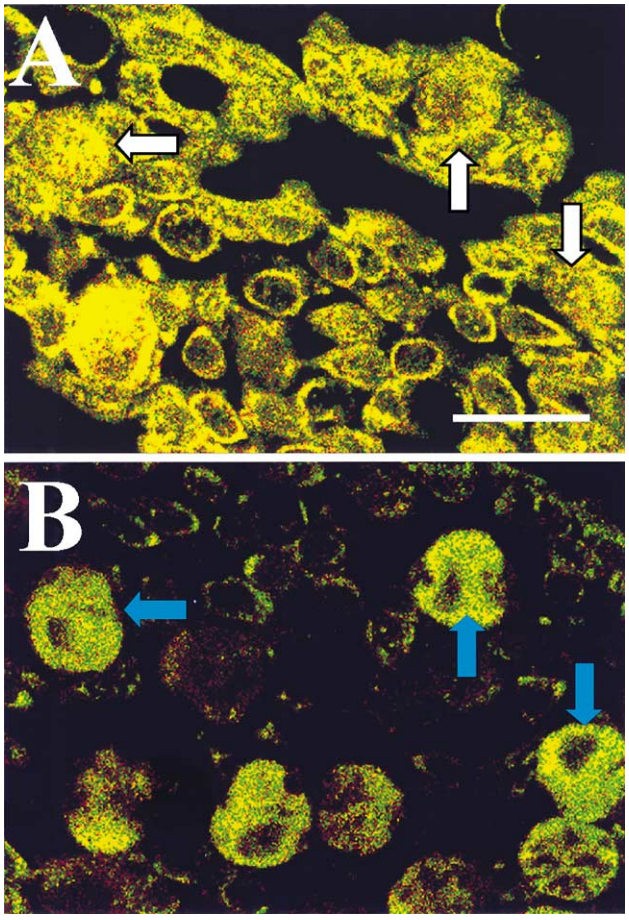

Fig. 5. Freshwater-adapted killifish immunocytochemical staining for CFTR; methods as for Fig. 4. In freshwater acclimated animals, (A) at the plane of the apical crypts there is widespread staining for CFTR in pavement cells but no obvious ring staining of apical membranes of chloride cells. (B) In the same frame, but at the plane of the nuclei, there is strong staining for mitotracker and for CFTR. The CFTR is likely located not in the cytosol, but on the tubular system that is contiguous with the basolateral membrane of these cells. (Adapted from Marshall et al. [43]). Bar is 20 μm.

transferred to seawater for 24 and 48 h, the immunocytochemical picture included a punctate CFTR concentration near the apical crypt while low levels of diffuse CFTR are also present, suggesting the presence of CFTR in apical and basolateral membranes of Cl^- cells during this transition [43]. The basolateral distribution of CFTR in mitochondria-rich cells and pavement cells of freshwater teleosts is consistent with the involvement of CFTR anion channels in NaCl uptake.

3. Role and regulation of CFTR

3.1. Seawater gills

The apical location by immunocytochemistry is appropriate for involvement of CFTR in Cl^- secretion by seawater fish. The upregulation of CFTR expression relative to actin to levels ninefold higher than in freshwater controls [27]. Further, the activation of low conductance anion channels by cAMP in patch clamp studies [28] and in *Xenopus laevis* oocytes expressing kfCFTR mRNA [27] associates the gene to the operation of the channel in situ in seawater chloride cells. While the involvement of CFTR in ion secretion by marine teleost chloride cells in opercular skin [43] and gill [42] is clear, it is perhaps premature to assign the exclusive role to CFTR, as other anion channels, notably the ClC family, have not been examined in teleosts.

Stimulation of CFTR in situ appears to be via cAMP and phosphorylation by PKA. In intact epithelia, cAMP is augmented by isoproterenol [26] and single channels in excised patches are activated by PKA + ATP in sea bass [29] and in *Fundulus* (Table 1). Thus, short-term activation of Cl^- secretion by hormones mediated by cAMP may be acting through CFTR phosphorylation. Notably, these could include urotensen I [45], glucagon [46], VIP [46], PGE_2 [38,47], AVT [38] and, pharmacologically, the action of isoproterenol on these Cl^- secreting epithelia [26,38,40].

Upregulation of CFTR expression in more protracted acclimation to seawater is associated with the interrenal steroid cortisol, inasmuch as plasma cortisol levels rise dramatically to 170 ng/ml at one hour after salinity transfer [48,49] shortly before CFTR expression increases at 8 h [49]. This association requires consolidation but the presence of a putative glucocorticoid binding site consensus sequence in the upstream proximal promoter region of the killifish CFTR gene [34] suggests regulation of CFTR transcription involving cortisol as an important component. In Atlantic salmon (*Salmo salar*) smolts exposed to seawater, the CFTR I mRNA levels in gill tissue rise and stay elevated relative to β-actin expression, while the mRNA levels of CFTR II are generally lower than for CFTR I and have a transient expression peak at approximately 24 h after transfer to seawater [32]. A separate study has demonstrated that intraperitoneal injections of cortisol significantly elevated gill CFTR I expression while having no impact on CFTR II expression of smolts held in freshwater [50]. Thus, there is evidence in salmonids for differential regulation of CFTR I and CFTR II isoforms.

The other major seawater osmoregulatory factors are growth hormone (GH) and associated growth factors such as insulin-like growth factor (IGF-1). GH and IGF-1 appear to act synergistically to enhance seawater adaptation in salmonid fish [21,51,52]. The general growth promoting actions of these hormones could likely produce more transporting cells and would enhance expression of any gene otherwise cued for upregulation. Hence, GH and IGF would likely augment cortisol-induced upregulation of CFTR during seawater adaptation. It is tempting to speculate that GH and IGF-1 may be associated with the increase in chloride cell size and density on adaptation to high salinities that has been observed for several species (*F. heteroclitus* [53]; *Oreochromis mossambicus* [54]; *Gillichthys mirabilis* [55]; *S. salar* [56]). If so, then the major role for cortisol may be to upregulate ion transporter expression in preexisting and newly formed chloride cells.

There is much information involving inhibitory effects on Cl^- secretion by α_2 adrenoceptors, activated by clonidine and blocked by yohimbine [22,23,38,40]. The inhibition of Cl^- secretion by α_2 adrenoceptors is activated by neural stimulation, as demonstrated by stimulation of the trigeminal nerve in a nerve–epithelium combined in vitro preparation [24]. The α_2 adrenergic response is mediated via inositol tris phosphate [24] and is mimicked by calcium ionophores such as ionomycin [23,24]. However, it is not clear that these adrenergic effects directly involve CFTR. Instead, these actions could be directed either to the NKCC basolateral cotransporter or the barium-sensitive K^+ channel [19], both of which are necessary for Cl^- secretion to continue.

During seawater acclimation, there is immunocytochemical evidence that CFTR redistributes by intracellular trafficking in the first 48 h after transfer to seawater [43]. CFTR starts in the basolateral membrane of chloride cells in freshwater and moves progressively from lower parts of the cells to the apical crypt [43]. Intracellular vesicular trafficking has been documented in chloride cells of toadfish [57] but it is as yet unclear how CFTR is redistributed and what proportion is de novo synthesized CFTR [27] or the actual relocation of preexisting channel protein.

The trafficking of hCFTR has received much attention because in the disease, this process is interrupted and there is some controversy as to which cell types have a cAMP-triggered rapid trafficking of vesicles containing hCFTR to the apical membrane of cultured human epithelial cells (e.g. Ref. [58]). In many cases, cAMP simply activates channels already in situ in the plasma membrane (e.g. Ref. [59]). Phosphorylation of CFTR by PKA in the ER seems not to impede the ability of the protein to mature and avoid proteolysis [60]. The slow redistribution of hCFTR over 48 h in teleost fish is not likely a cAMP-mediated event, rather it would seem to be mediated by more complex processes including cytoskeletal elements. CFTR is well

known to associate with actin and disruption of actin cross-linking prevents normal activation of channels by cAMP [61]. Unlike the human system that generally has low constant levels of hCFTR expression, the teleost chloride cells in the gill and opercular epithelia have high levels of CFTR expression. There is a convenient means of triggering the upregulation and movement of the protein, i.e. transfer of the animals to a hypertonic medium such as seawater. The induction of CFTR expression and the slow trafficking to the apical membrane could be a better model than is acute cAMP-mediated CFTR trafficking for the chronic problem in CF disease of failure of newly synthesized protein to move to the apical membrane. The immunocytochemical colocalization of kfCFTR with the chaperonin HSP-60 [44] is a promising first step in the study of CFTR post processing and trafficking.

3.2. Freshwater teleost gills

Compared to the well-accepted model of seawater gill function in teleost generally and of CFTR involvement in particular, very little is known of the role of CFTR anion channels in freshwater ion uptake by the gill epithelium. The model for NaCl uptake involves a V-type H^+ ATPase as the main source of a large electrochemical driving force that favors Na^+ uptake across the apical membrane, proposed by Lin and Randall [62] (reviews: Refs. [19,20]). In the same model, Cl^- uptake at the apical membrane is proposed to be in exchange for Cl^-, helped by a local acidification that is predicted to reduce HCO_3^- activities in the outside boundary layer [19,20,62]. In this way, Cl^- may accumulate sufficiently in the cytosol to allow passive conductive Cl^- flow across the basolateral membrane. Whereas in trout the V-type H^+ ATPase is localized to the apical membrane [62], in the euryhaline elasmobranch, *Dasyatis sabina* adapted to freshwater, the enzyme is localized instead to the basolateral membrane [63], a novel finding that may necessitate a reconsideration of the model. The August Krogh model of NaCl uptake by freshwater animals has generally assumed the existence of a high Cl^- conductance at the basolateral membrane [64] but the identity of the Cl^- conductance is unknown.

There is baseline low level expression of kfCFTR in freshwater killifish gills [27,49] that is approximately 1/4 that of static marine fish levels and much less than the peak that occurs 8–48 h after entry into seawater. Further, there is immunocytochemical evidence for a basolateral located kfCFTR in pavement cells and chloride cells of freshwater opercular epithelium (Fig. 5A,B) [43], based on a monoclonal antibody directed against the carboxy terminus of hCFTR that is identically conserved in kfCFTR. Other cells in the freshwater opercular epithelium (i.e. granular cells and undifferentiated cells) were not positive for kfCFTR. Hence, chloride and pavement cells of skin and gill (at least in killifish) contain basolateral CFTR that could account fully for Cl^- conductance at this location or contribute to anion conductance and regulation of other basal anion channels [44], such as the outward rectifying and ClC-K anion channels. As yet, CFTR is the only anion channel positively identified in the basolateral membrane.

The physiological role for CFTR in freshwater fish may be involvement in Cl^- uptake. The freshwater opercular membrane actively transports Cl^- in the uptake direction by the Ussing flux ratio criterion [1] but, in vitro, these membranes still have a net loss of Cl^- [65]. Similar results were obtained independently for *Tilapia* opercular epithelium [66]. Thus far, there is no pharmacological evidence to support involvement of CFTR in NaCl uptake by the freshwater gill epithelium.

Mammalian CFTR is known to be permeable to HCO_3^- and HCO_3^- may block Cl^- conductance in CFTR channels in pancreatic duct cells [67]. Thus, there is potentially a role for CFTR in acid–base equilibrium across the basolateral side of chloride cells, if CFTR is present in large amounts and activated. The only evidence regarding regulation of anion channels in freshwater is that Cl^- uptake is modestly increased across the isolated freshwater opercular epithelium by cAMP and IBMX [65] and this could represent CFTR or other cAMP-activated anion channels.

Thus, there is a potential interaction between acid–base balance (HCO_3^- activity) and Cl^- transport via basolateral CFTR channels in the gills of freshwater fish. Of particular interest and value now would be the elucidation of the regulatory cues that direct CFTR transporter to the apical membrane of seawater chloride cells, and yet to the basolateral membrane of freshwater chloride cells.

3.3. Teleost intestine

The posterior intestine of teleosts is well known to absorb NaCl and fluid involving a bumetanide-sensitive cotransporter in the apical membrane and anion channels in the basolateral membrane, reviewed by Loretz [68]. Marine teleosts drink seawater, and absorb salts and fluid across the esophageal and intestinal epithelia that have high osmotic permeability. There also is considerable bicarbonate secretion in the posterior intestine that produces precipitated carbonate salts of metals [69]. In freshwater animals, NaCl is also absorbed to maintain ionic homeostasis but there is little fluid movement, as osmotic permeability is low. A voltage-sensitive, high conductance anion channel was identified in goby intestine [70] and could account for Cl^- uptake. Killifish CFTR is expressed in posterior intestine at levels at least as high as in gill tissue, as detected by Northern blot analysis [27]. In mammalian intestine, CFTR channels are likely involved in cAMP/cGMP and Ca^{2+}-activated NaCl secretion [71]. The uptake of NaCl and fluid normally present in seawater animals can be rapidly reversed to NaCl and fluid secretion by application of secretagogues cAMP, IBMX and ionomycin, [30], an action blocked by lumenal DPC (Fig. 6). Immunocytochemical evidence suggests kfCFTR distribution in intestine in most enterocytes is basal

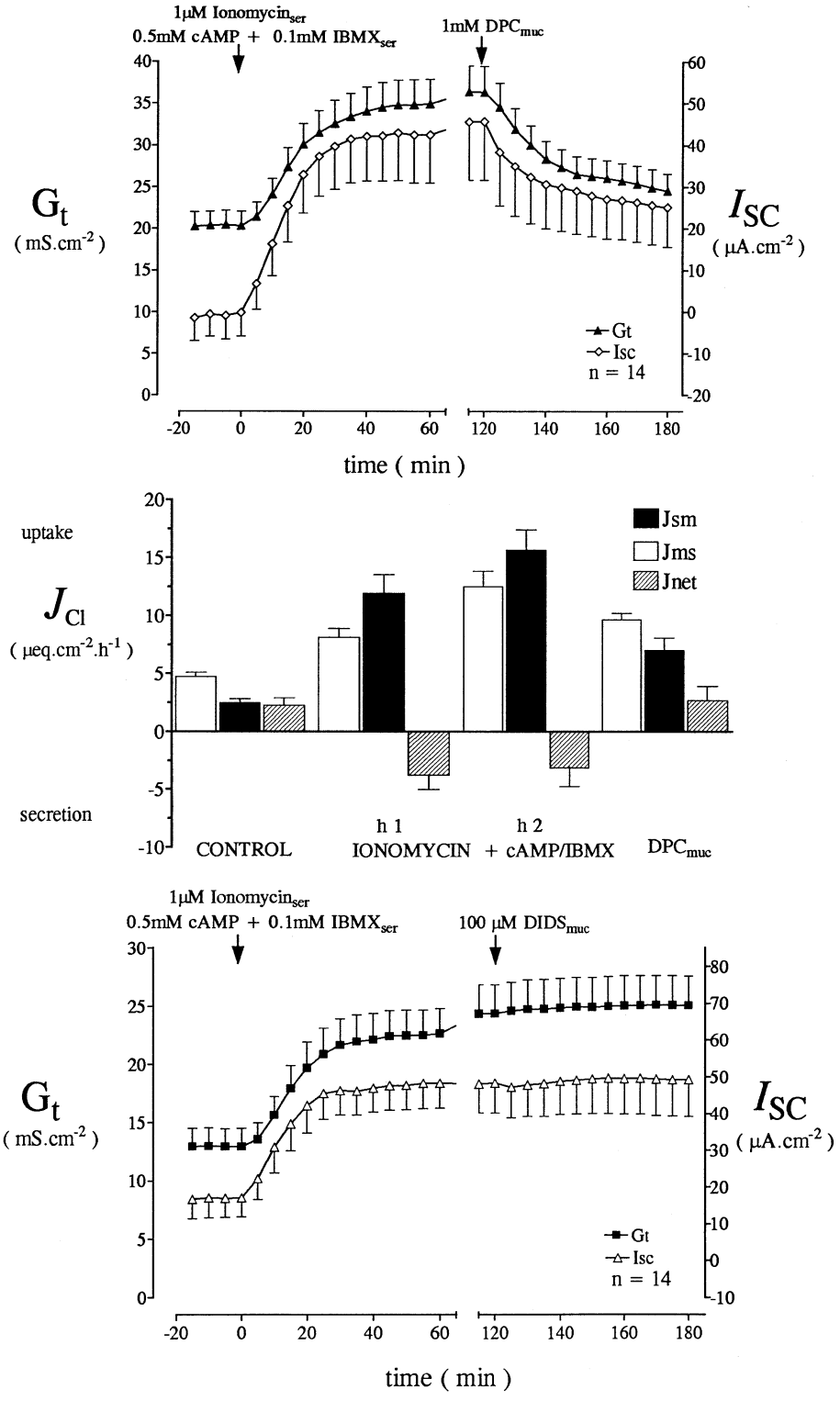

Fig. 6. Seawater killifish posterior intestine can be made to secrete NaCl and fluid if stimulated by db-cAMP and ionomycin, an efficient calcium ionophore. Upper panel: The combination of IBMX and db-cAMP increased shortcircuit current (Isc) and epithelial conductance (Gt), but addition of diphenylamine-2-carboxylate (DPC) 1.0 mM to the lumen side decreased both Isc and Gt. Middle panel: Net Cl^- movement (by radioisotope fluxes) was in the uptake direction initially but turned to secretion with the addition of db-cAMP and IBMX. Addition of DPC to the lumenal side decreased both unidirectional Cl^- fluxes and restored the Cl^- net flux in the uptake direction. Lower panel: In contrast to the DPC effect, intestine pieces that had been stimulated with db-cAMP and ionomycin were unaffected by application of 1.0 mM DIDS to the lumenal side. Thus, Cl^- (and fluid) secretion by killifish intestine can be inhibited by the anion channel blocker DPC, applied to the lumenal side. (Adapted from Ref. [30]).

(Fig. 7) [30] but in a minority of cells (approximately 20%) CFTR is also present in the apical membrane.

The stimulation of secretion by cAMP and IBMX is consistent with PKA activation of CFTR. Also, the inhibition by apically added DPC is suggestive of a CFTR-like anion channel. Mammalian colon can be made secretory by augmentation of cAMP alone, as part of enterotoxin-driven secretory diarrhea, and the process involves activation of CFTR in the apical membrane [72,73]. Thus, there are major similarities between the operation of teleost posterior intestine and mammalian colon.

It is unclear why the teleost intestine requires both cAMP and calcium pathways to activate secretion, while the mammalian intestine only requires cAMP. It could be that the natural pathway for secretion activation in both tissues instead is via cGMP mediation and protein kinase G, as is suggested below.

Guanylin, a naturally occurring secretagogue that acts in vertebrate (mouse) intestine, via cGMP, to augment Cl^- and HCO_3^- secretion via CFTR channels [72], has been cloned and sequenced from European eel (*Anguilla anguilla*) [74]. In mammals, guanylin receptors on the apical surface of enterocytes link via guanylate cyclase, protein kinase G and CFTR channels to augment secretion [72]. These receptors are the targets for bacterial enterotoxins that have higher affinity for the receptors than guanylin itself [75]. In some marine teleosts, HCO_3^- is secreted and lumenal HCO_3^- concentrations are very high, sufficient for metals to precipitate as carbonate salts [69]. Thus, CFTR could contribute to normal intestinal NaCl absorption, normal HCO_3^- secretion mediated by guanylin and in enterotoxin-driven secretory diarrhea.

3.4. CFTR in other teleost tissues

The expression of CFTR in fish brain [27] suggests a possible function for the protein in brain function. Mammalian brain CFTR is apparently not associated with epithelial

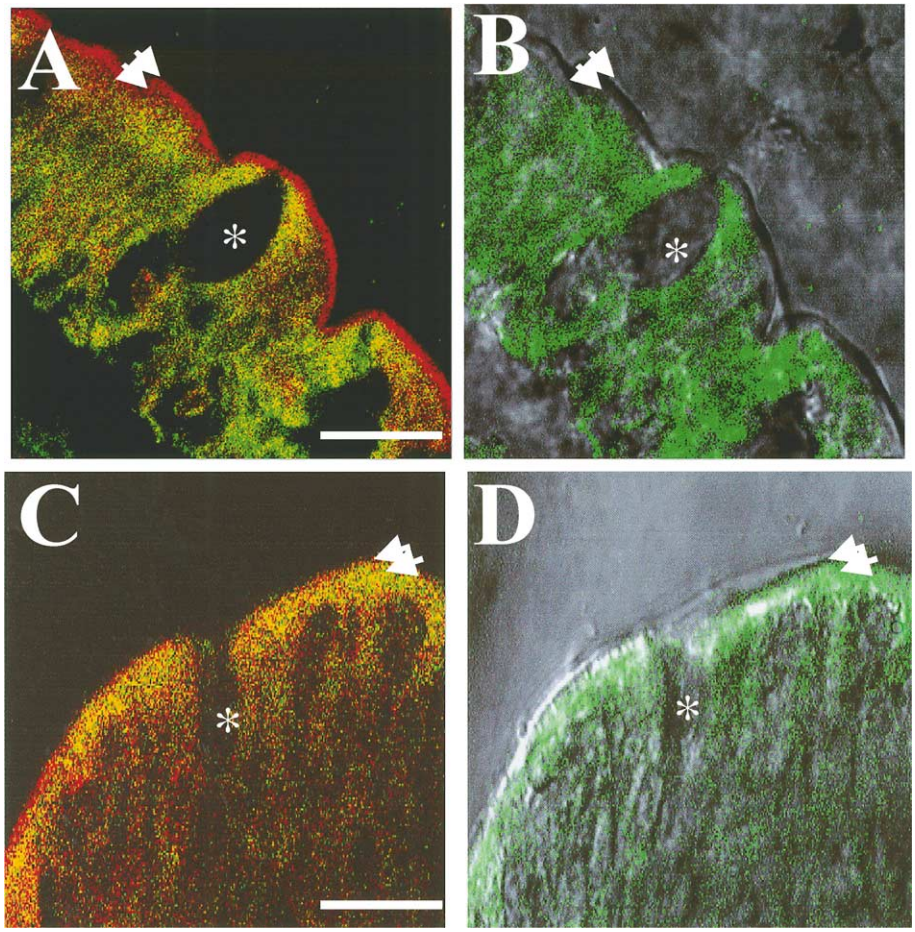

Fig. 7. Immunocytochemical staining of frozen and fixed sections of killifish posterior intestine for CFTR using same antibody as in Figs. 3 and 4. (A) CFTR staining with Oregon green, mitochondria with Mitotracker red; combination is yellow. In most sections (approximately 80%) CFTR immunofluorescence is limited to the basal portions of the enterocytes and is absent from the brush border (between the arrows), here nonspecifically stained with mitotracker (added post fixation) indicating a absorptive type cell. Mucous cells (*) were also negative for CFTR and mitochondria. (B) Same frame as for (A) but with green immunofluorescence overlain with bright field. (C and D) In some sections (ca. 20%), there is also CFTR staining of the brush border for CFTR (green immunofluorescence between the arrows), indicative of a secretory type cells. CFTR, thus, is likely contributing to NaCl absorption and, when conditions are right, also to intestinal secretion. (Adapted from Ref. [30]). Bar is 20 μm.

transport in choroid plexus. Instead, CFTR is involved in GnRH release from the hypothalamus [76] and CFTR mutations thus often produce developmental aberrations in reproductive systems. It would be very interesting to examine teleost GnRH neurons to trace the phylogenetic association of CFTR with reproductive function.

Mammalian pancreas and, particularly, pancreatic duct have CFTR and it is involved in alkaline (HCO_3^-) secretion [67]. However, in *F. heteroclitus* this is unusual as it lacks a stomach and thus there is little need for alkali secretion by pancreas, although pancreas and gallbladder have not been specifically examined for CFTR expression. Other tissues tested by Northern blot analysis for expression of CFTR (kidney, ovary, testis, eye, heart, spleen and liver) were negative for CFTR-like mRNA [27].

3.5. Future directions

Killifish, salmon, eel and fugu CFTR genes have been identified and all, except eel, have complete coding sequences known. The function and tissue distribution of CFTR is consistent with an important role in ion and water balance in fish. The regulation of CFTR at the genomic and post expression stages can be studied easily in teleost fish where transport systems are easily accessible (opercular epithelium and intestine) and upregulation of expression is easily induced. This is in contrast to elasmobranchs that, for most species, do not change its environmental salinity and thus has relatively static transporter expression. The compact nature of the certain teleost genomes, especially the fugu, and the presence of two CFTR isoforms in the tetraploid Atlantic salmon provide unique opportunities to examine both gene and protein regulation not present in mammalian homologues. Thus, the euryhaline teleost is an ideal model to study regulation of vertebrate CFTR expression and the post-processing and trafficking of the channel into apical membranes of seawater chloride cells and basal membranes of freshwater chloride cells.

Acknowledgements

We thank C. Cutler, E. Hoffman and J. Wilson for illuminating discussions; J. Ehrenfeld for review; and NSERC, The Canadian Foundation for Innovation, and Atlantic Canada Opportunities Agency for financial support.

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