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AN EXAMINATION OF TWO GENES INVOLVED IN RAINBOW TROUT (*Oncorhynchus mykiss*) SODIUM TRANSPORT

By

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Abstract

This thesis examines the theory that rainbow trout (*Oncorhynchus mykiss*) regulate sodium through an epithelial sodium channel (ENaC) and a proton-ATPase. An attempt was made to clone an ENaC from a rainbow trout cDNA library and from total RNA through nested primers and PCR and RT-PCR resulting in RAPD-type *E. coli* DNA amplification and amplification of trout collagen, however, producing nothing related to ENaC. The second part focuses on the trout proton-ATPase B-subunit (H^+-ATPase) that was used in a Northern hybridization tissue distribution. Phylogenetic analysis showed that the H^+-ATPase is highly similar to other known B-subunits, particularly the brain isoform. H^+-ATPase mRNA quantification attempts to examine the effect of various environmental factors failed due to the use of an improper internal control. Cortisol RIA's showed no change in levels in fish exposed to hypercapnia or reduced ion water but were significantly increased in the cortisol implanted fish.

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AN EXAMINATION OF TWO GENES INVOLVED IN RAINBOW TROUT (*Oncorhynchus mykiss*) SODIUM TRANSPORT.

Summary

This thesis examines the theory that rainbow trout (*Oncorhynchus mykiss*) regulates its ions, specifically sodium, and helps to maintain acid-base homeostasis by way of an epithelial sodium channel and a proton-ATPase.

The first part of this thesis will focus on the attempt to clone an Epithelial Sodium Channel (ENaC) from rainbow trout. The epithelial sodium channel in other organisms is composed of three subunits(α, β and γ) each of which contributes to the proper functioning of the channel. On three different occasions nested primers were designed for use in polymerase chain reaction (PCR) to amplify a portion of the α-subunit. PCR was conducted on a trout gill and kidney complimentary DNA (cDNA) library and on RNA from gill and kidney with reverse transcriptase PCR (RT-PCR). The PCR's resulted in accidental amplification of the positive control DNA, RAPD type *E. coli* DNA amplification, and amplification of trout collagen, however, nothing related to the epithelial sodium channel α-subunit was found.

The second part of the thesis will focus on the proton-ATPase side of the model. Nested-primers were again designed (by S.F.Perry) for use with the cDNA library and produced a product of the expected size. Subsequent sequencing showed that the product was similar to other known proton-ATPase B-subunits, but had not been cloned as of yet. This portion of the B-subunit was used in a Northern hybridization tissue distribution to show high expression of the B-subunit of the proton-ATPase in gill, kidney, intestine, heart, spleen and blood but lower expression in liver and white muscle. The full length derived sequence of the trout proton-ATPase B-subunit was found to be highly similar to other known B-subunits and to be most similar to the vertebrate brain isoforms of the protein.

An attempt was made to quantify the changes in mRNA expression levels of the proton-ATPase B-subunit with changes in environment such as hypercapnia, reduced ion water and cortisol implants. The method of quantification involved a ratio comparison between the amount of the signal seen on a Northern with the proton-ATPase B-subunit and the amount of the signal seen on the same Northern with a control RNA which does not change with treatment. The choice of the 18S rRNA as a control RNA proved to be not useful, as it gave a signal that does not correspond linearly to the amount of total RNA present. Blood samples taken for each of the experiments showed no change in cortisol levels in fish exposed to hypercapnia or reduced ion water but were significantly increased in the cortisol implanted fish when compared to the sham implanted fish.

This thesis shows that the methods used in attempting to clone the α-subunit of the epithelial sodium channel were not sufficient. Two possible causes include that the primers chosen were not appropriately designed and that the material used to initiate the PCR did not have a high enough mRNA concentration for the α-subunit of ENaC. Possible corrections to these problems include the redesign of the primers to include more conserved regions of the protein and the use of mRNA, rather than total RNA, in the PCR reactions. The B-subunit of the proton-ATPase did provide positive evidence for the postulated model of ion regulation/acid-base balance; however, quantification of this mRNA remains to be appropriately worked out.

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So, as I write this at the conclusion of my thesis, I thank you all again. You have shown me that life is not just about research, schoolwork and career, but life is also about living and family.

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Chapter 1

General Introduction to Freshwater Teleost Ion Regulation and Acid-Base Balance.

Epithelial Cell Function

The aquatic environment of a freshwater teleost such as rainbow trout (*Oncorhynchus mykiss*) poses unique problems for ion regulation, acid-base balance and gas transfer, as compared to terrestrial or marine animals. For a fish to live successfully in this environment, it must simultaneously solve all these problems. Water, as a medium for gas transfer, is not highly efficient because oxygen gas does not readily dissolve in it. To compensate for this poor solubility, the fish moves large amounts of water over its respiratory surfaces. The gills are the primary respiratory surfaces in fish and are organised with a superstructure of gill arches from which radiate hundreds of gill filaments. From each of these filaments arise thousands of secondary lamellae that are the true respiratory surfaces of the teleost (Morgan and Tovell, 1973). These secondary lamellae provide a large surface area for water to pass over so that even small amounts of oxygen can be removed from the water. In addition to the large surface area, fish have evolved a counter-current exchange system which causes the blood of the secondary lamellae to flow in the opposite direction of the water. This maximizes the diffusion gradient for oxygen uptake and carbon dioxide excretion and thus optimizes gas transfer.

However, these solutions to the problem of gas transfer, together with cellular metabolism (and the resulting metabolic acidification) and nitrogen excretion cause problems with osmoregulation. Due to its large surface area and counter-current exchange system, the gill is a major organ functioning in body fluid homeostasis; compensating for the diffusive loss of ions due to the large chemical gradients in freshwater. It is also a major site for acid-base regulation as demonstrated by the fact that fish excrete HCO_3^- in exchange for Cl^- and H^+ in exchange for Na^+ (Heisler, 1984; 1986; 1989; 1993; Goss *et al.* 1992; Marshall, 1995; Claiborne, 1998). Acid-base balance has been linked to ion regulation (McDonald *et al.* 1989; Cameron and Iwama, 1989; Wood and Goss, 1990; Wood, 1991; McDonald *et al.* 1991), however, the exact mechanism

of this regulation has not yet been discerned. By some mechanism, the rainbow trout must compensate for an approximate 100 μg/kg/hr loss of Na^+ due to diffusion (Perry and Laurent, 1989; Potts, 1984).

Krogh's Model of Sodium Uptake

Fish live in a hypotonic medium (< 1 mM Na^+), however, they maintain their blood sodium concentration ($[Na]_i$) at about 150 mM (Wood and LeMoigne, 1991) and must, therefore, absorb sodium ions from the water against the chemical gradient to offset the diffusive loss. Two models of sodium regulation in the gills have been proposed to explain this uptake of sodium ions from the water, both of which counter ion uptake with acid excretion. Krogh (1938) suggested the classical model that Na^+ is passively imported in an electro-neutral exchange for ammonium (NH_4^+) (Fig. 1A). However, this theory ran into difficulties as experiments in the field progressed. de Vooys (1968) found that ammonia efflux did not decline in Na^+-free water, suggesting that the two ions were not linked. Kerstetter *et al.* (1970) showed that there was no correlation between ammonia excretion and sodium absorption in trout and suggested that protons (H^+) could substitute for ammonium, maintaining the electro-neutrality. Maetz (1971) suggested that either NH_4^+ or H^+ could be exchanged with Na^+ depending on the relative rates of excretion and the physiological activity of the tissue. Finally, Avella and Bornancin (1989) showed, by maintaining a constant external $[Na^+]$ and varying internal NH_4^+ in an isolated perfused head preparation, that Na^+ import/NH_4^+ excretion is uncoupled. There are systems that function in the manner proposed by Krogh (enzymes known as Na^+/H^+ exchanger's (NHE's) that electro-neutrally exchange sodium and protons/ammonium), however, they are commonly found in tissues that have high levels of sodium outside and high levels of protons inside the cells, such as the proximal tubule of the mammalian kidney. The ionic state of affairs in the trout gill is exactly reversed and thus an NHE type system of exchange is thermodynamically unlikely (Potts, 1994).

Avella and Bornancin's Model of Sodium Uptake

Avella and Bornancin (1989) suggested that neither the concentration of sodium in freshwater nor the concentration of intracellular protons is sufficient to support the first hypothesis of a Na^+/H^+ exchanger and that sodium transport is energized by the proton-ATPase, however, it is, in itself, passive. The membrane potential necessary for electro-neutral exchange can be roughly estimated by taking into account the concentration of some of the ions in the water, from Avella and Bornancin (1989), and estimates of ion concentrations in the gill epithelial cells, from brown trout (*Salmo trutta* L.) (Morgan *et al.* 1994). Assuming water values of 160 μM Na^+, 160 μM Cl^-, 80 μM K^+, pH 8.0 and gill epithelial cell values of 64 mM Na^+, 46 mM Cl^-, 88 mM K^+ and assuming a cellular pH of 7.43 (Wood and LeMoigne, 1991), and by using the Goldman equation and reasoning that activities are equal to concentrations, the membrane potential across the gill epithelium would have to be about -153 mV (inside relative to outside) for the passive flow of sodium into the gill. Estimates of the gill epithelial membrane potential, for the euryhaline European flounder (*Platichthys flesus* L.) (Clarke and Potts, 1998), are around -24 mV and therefore, assuming these figures apply for rainbow trout, Na^+ transport into the gill must be assisted, as it would not occur passively.

The second model (Fig. 1B) was proposed by Evans (1982) and later elaborated by Avella and Bornancin (1989). It suggested that Na^+ transport into the gill epithelium is passive and occurs due to a favourable electrochemical gradient set up by: 1) a Na^+/K^+-ATPase on the basolateral membrane of the gill epithelium, which extrudes Na^+ into the blood and 2) an apically located H^+-ATPase, which expels protons into the water at the expense of ATP. In this model, the influx of Na^+ might be achieved by a localized depolarization of the membrane, such as may be seen by the export of protons by an H^+-ATPase, thereby allowing Na^+ to flow passively down its electrochemical gradient into the gill epithelium

Evidence for the proton-ATPase/sodium channel model of sodium regulation has been mounting

from various areas, including: theoretical (Avella and Bornancin, 1898; Potts, 1994), pharmacological (Lin and Randall, 1991; 1993), biochemical (Lin and Randall, 1993; Kultz and Somero, 1995) and histological (Laurent *et al.* 1994; Lin *et al.* 1994; Sullivan *et al.* 1995; Wilson *et al.* 1997).

The freshwater fish gill has been compared with tight epithelia (Goss *et al.* 1995) that utilize a system similar to that proposed by Avella and Bornancin, such as the turtle urinary bladder (Steinmetz and Anderson, 1982) and frog skin (for a review, see Ehrenfeld and Klein, 1997), and has itself been called a tight epithelium due to the tight junctions between the cell types. These junctions consist of multiple strands of protein, creating desmosomes between the cells, and are therefore resistant to ion loss (Sardet, 1980). The frog skin is a particularly good example of a tight epithelia employing a proton-ATPase/sodium channel system for sodium uptake from freshwater. The frog skin is primarily composed of two cells types that make up the stratum granulosum: granular cells and mitochondria-rich cells. These cells are closely connected by tight junctions constituting a tight epithelia by Sardet's definition (1980). The mitochondria-rich cells contain large apical folds which enhance its surface area similar to the chloride cells of the gill epithelium. In both air and water the frog skin, with its large surface area, encounters problems of osmoregulation similar to that of the fish. To combat ion loss the frog skin has evolved a mechanism for importing sodium by exporting protons that also aids in compensating for metabolic acidosis. The mitochondria-rich cells contain an apical proton-ATPase and an epithelial sodium channel which the basal membrane contains the sodium/potassium-ATPase (Ehrenfeld and Klein, 1997). The H^+-ATPase energizes the apical mem-brane, exporting protons and creating a partial negative electro-chemical gradient down which sodium can passively flow through the epithelial sodium channel. As sodium enters the cell, it is pumped, against its concentration gradient, into the blood by the sodium/potassium-ATPase. Under conditions of chronic acidosis (i.e., NH_4^+ injections), the proton pump has been directly linked to modifications of intracellular pH and consequently

an increase in Na^+ uptake. Thus, the frog skin model links acid-base balance and ion regulation as does the freshwater fish model (Ehrenfeld *et al.* 1990).

The freshwater fish gill also consists primarily of two cell types: chloride (or mitochondria rich) cells and pavement (or respiratory) cells. A debate exists over which of these is the primary participant in ionic regulation. The pavement cells are the most prevalent on the secondary lamellar epithelium and are thought to function primarily in gas exchange, while the chloride cells are located mainly on the primary lamellae and are thought to function mainly in ionic uptake (Goss *et al.* 1995). The chloride cells have numerous mitochondria and therefore have the energy capability to participate in active ion transport. However, a growing body of evidence indicates that the pavement cell may, in fact, be the site of proton-ATPase activity and sodium uptake. During hypercapnia in the brown bullhead (*Ictalurus nebulosus*), the chloride cells become covered by the neighbouring pavement cells and are therefore restrained from direct access to the water (Goss *et al.* 1992). Further, during this respiratory acidosis, a subset of the pavement cells have been shown to increase their numbers of mitochondria and develop numerous apical microplicae to increase their functional surface area (Goss *et al.* 1992). Also during hypercapnia, vesicles presumably bearing proton pumps, that have been localized to the pavement cells, show an increased fusion with the apical membrane (Laurent *et al.* 1994). It was later demonstrated, by both immunocytochemistry and mRNA *in situ* hybridization, that proton pumps are present on the gill epithelium and that the activity of these pumps has also been shown to increase in rainbow trout under hypercapnic conditions (Lin and Randall, 1993; Sullivan *et al.* 1995; Sullivan *et al.* 1996) thereby energizing the epithelia (Lin and Randall, 1995; Perry and Fryer, 1997). This suggests that more pumps are being brought to the apical surface to cope with the pH imbalance by pumping the protons into the water. Lastly, Sullivan *et al.* (1995; 1996), using both the cDNA and a synthetic peptide of the 31 kDa subunit of the bovine proton-ATPase, showed specific localization to the

pavement cell in hypercapnic rainbow trout. If there is an epithelial sodium channel (ENaC) linked to the proton-ATPase, then an increase in proton-ATPase activity should show a corresponding increase in sodium absorption.

Objectives of the Study

Based on the theory that the freshwater fish gill utilizes an epithelial sodium channel and a proton-ATPase to regulate its blood sodium levels and acid-base status, the goals of this project were to use molecular biology to prove the existence of the genes required for the model. This proof would consist of using PCR or RT-PCR technology to clone an epithelial sodium channel subunit and a subunit of the proton-ATPase from rainbow trout. The former was the task of the author while the latter was carried out by Drs. Johnson and Perry at the University of Ottawa. Together, these fragments of the genes were to be used in molecular physiological examination of the tissue distribution and action of the sodium channel and the proton-ATPase. This examination was to include physiological experiments altering the external CO_2 in the aquatic environment, the stress hormone levels of the fish, the ionic composition of the aquatic environment and the internal pH of the fish, followed by a molecular examination, by means of Northern blots, of the cellular effects of these physiological experiments. A further goal of this project was to quantify the mRNA levels of the various genes. Through these means, it was hoped that an understanding of how this fish regulates its day-to-day ion levels and pH balance could be inferred. These clonings were to provide direct molecular evidence for the model of ionic and acid-base balance in the trout proposed by Avella and Bornancin (1989), based on the assumptions that there is an Epithelial Sodium Channel and a Proton-ATPase on the gill epithelium and that these enzymes will control sodium import into the fish while regulating internal pH at the same time. A further assumption is that the Epithelial Sodium Channel present

is of the type seen in the rat, human, chicken, cow and frog, etc. and is composed of subunits which are homologous to those found in the above organisms (see Table 1.1). As well, the proton-ATPase is assumed to be a V-type proton-ATPase and to contain, at least, the B-subunit similar to that found in other organisms (see Table 1.2).

Table 1.1. Epithelial Sodium Channel gene family sequences and their sources.

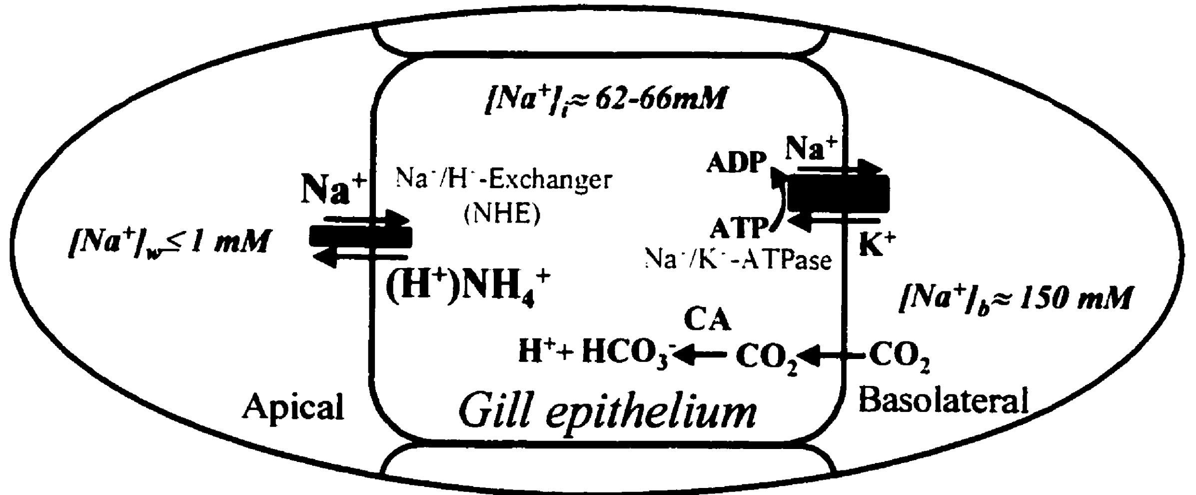
Subunit	Species	GenBank Accession	Reference
α	*Homo sapiens*	L29007, X76180	McDonald *et al.* 1994; Voilley *et al.* 1994
	Bos taurus	U14944	Fuller *et al.* 1995
	Rattus norvegicus	X70497, X70521	Canessa *et al.* 1994; Lingueglia *et al.* 1993
	Gallus gallus	U58475, U62902	Goldstein *et al.* 1996; Killick and Richardson 1997
	Xenopus laevis	U23535	Puoti *et al.* 1995
β	*Homo sapiens*	X87159, L36593	McDonald *et al.* 1995; Voilley *et al.* 1995
	Rattus norvegicus	X77932	Canessa *et al.* 1994
	Xenopus laevis	U25285, Y12000	Puoti *et al.* 1995; Puoti *et al.* 1997
γ	*Homo sapiens*	X87160, L36592	McDonald *et al.* 1995; Voilley *et al.* 1995
	Rattus norvegicus	X77933, X78034	Canessa *et al.* 1994; Lingueglia *et al.* 1994
	Xenopus laevis	U25342, Y12001	Puoti *et al.* 1995; Puoti *et al.* 1997
δ	*Homo sapiens*	U38254	Waldmann *et al.* 1995

Table 1.2. V-Type Proton-ATPase B-subunit gene sequences and their sources.

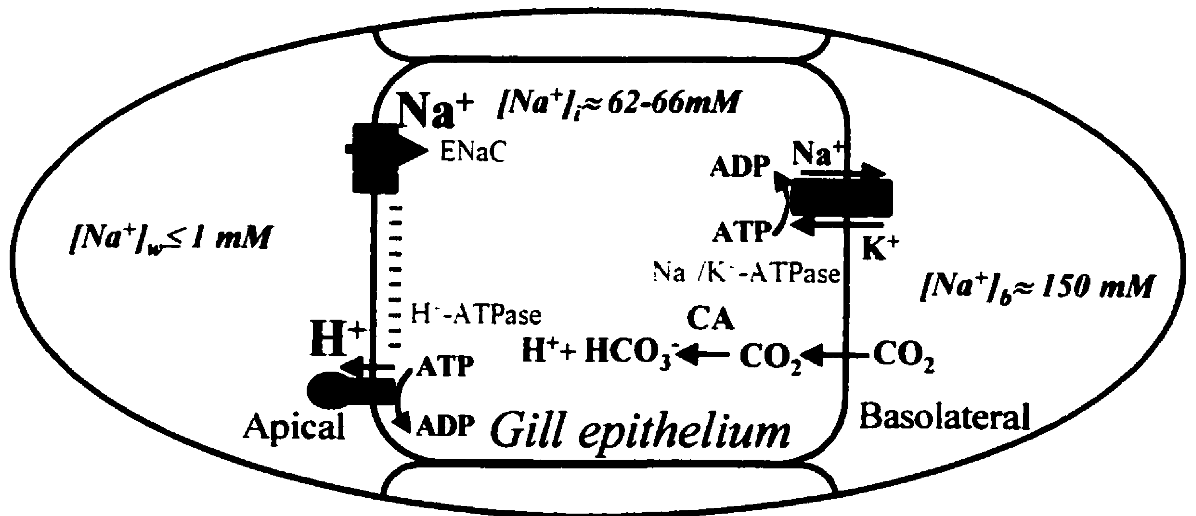
Organism	Subunit	Tissue	Reference
Drosophila melanogaster	B	Malpighian tubules	Davies *et al.* 1996; Bertram and Wessing, 1994
Heliothis virescens	B	midgut and Malpighian tubules	Gill and Ross, 1991
Manduca sexta	B	midgut	Novak *et al.* 1992
Culex pipiens quinquefasciatus	B	midgut and Malpighian tubules	Filippova *et al.* 1998
Anguilla anguilla	B	gas gland	Niederstaetter and Pelster, unpub.
Gallus gallus	B	osteoclast	Bartkiewicz *et al.* 1995
Mus musculus	B	ES-D3 cell line, brain	Laitala-Leinonen *et al.* 1996; Lundberg, unpub.
Bos taurus	B	kidney, brain	Nelson *et al.* 1992
Homo sapiens	B	kidney, brain	Suedhof *et al.* 1989; Bernasconi, 1990

Figure 1.1. Schematic representation of ion transfer processes across the freshwater gill epithelium of fish. A) Krogh and Smith's model: An apical electro-neutral $Na^+/H^+(NH_4^+)$ ion exchanger) on the apical membrane exports protons in exchange for sodium ions while a basolateral Na^+/K^+-ATPase maintains the gradient for sodium. Adenosine Tri-Phosphate (ATP) is hydrolysed to provide the necessary energy. Carbonic Anhydrase (CA) provides protons for the exchange by the hydration of CO_2, which passes directly through the plasma membrane due to its gaseous form, to H^+ and HCO_3^-. Internal and external sodium concentrations are indicated. B) Avella and Bornancin's model of an apically oriented proton-ATPase (H^+-ATPase) which creates a partial negative gradient (red dashes) for the passive inward flow of sodium ions through an epithelial sodium channel (ENaC). A basolateral Na^+/K^+-ATPase exports sodium from the epithelium, thus keeping internal sodium low. Again, ATP is hydrolysed to provide the necessary energy. $[Na^+]_w$ = Sodium ion concentration of the water, $[Na^+]_i$ = Sodium ion concentration of the cellular contents, $[Na^+]_b$ = Sodium ion concentration of the blood.

A.



B.



Chapter 2

Epithelial Sodium Channels in Rainbow Trout (*Oncorhynchus mykiss*): attempted cloning of a subunit.

Introduction

Epithelial Sodium Channels (ENaCs) are defined as proteins on the apical membrane of some tight epithelia that specifically (more or less) transport sodium passively into an epithelial cell (e.g. gill cell in Fig. 1.1B). Pharmacological and physiological tests (reviewed in Garty and Palmer, 1997) have been used to find them in ionic regulating tissues of many different vertebrates including: leech integument (Weber *et al.* 1993), toad skin (Hviid-Larsen *et al.* 1987), turtle urinary bladder (Bentley, 1968), and rat kidney (Brown *et al.* 1989; Duc *et al.* 1994). In all cases, they are differentiated from voltage-gated sodium channels by being relatively insensitive to voltage as pertains to their sodium permeability, meaning that an applied voltage does not make these channels open and conduct sodium. Their sodium conductance is sensitive to blockages by 3,5-diamino-N-(aminoiminomethyl)-6-chloro-pyrazinecarboxamide (amiloride) which acts as a molecular plug to block the ENaC pore (Palmer and Anderson, 1989) and follows the distinct pharmacological blockage profile (most potent to least potent): benzamil > amiloride > ethylisopropyl amiloride (Canessa *et al.* 1995). In contrast, the Na^+/H^+ exchanger exhibits exactly the opposite pharmacological profile. Sodium influx into the trout gill was observed to be blocked by amiloride (Avella and Bornancin, 1989; Perry *et al.* 1981; Perry and Randall, 1981), however, as amiloride blocks the action of both the ENaC and the sodium/proton exchanger (Kleyman and Cragoe, 1990) the results are equivocal between the two theories of Krogh (1938) and Avella and Bornancin (1989) (c.f. Chapter 1).

There are three main types of ENaCs (Palmer, 1992) that can be differentiated by permeability and conductance profiles and sensitivity to amiloride. The Type I ENaC is defined as a highly selective sodium channel which has a permeability ratio of Na^+ vs. K^+ of between 100:1 and 1000:1; however, the selectivity of Na^+ vs. Li^+ is less, on the order of 1.3-1.5:1, indicating that selectivity is based on dehydrated ion size.

Single channel currents of Type I channels are in the range of 0.1-0.5 pA, corresponding to a conductance of approximately 4.9 pS. Mean open and closed times for this type of channel are on the order of 3-4 *s* with a K_i for amiloride of 0.1-1 μM, where K_i is the concentration of the drug required to block 50% of the transepithelial sodium transport. The trout gill might contain an ENaC due to the fact that the $K_{i\,Amiloride}$ for the trout gill has been determined to be approximately 10μM (Avella and Bornancin, 1989). Na^+ concentration seems to affect the binding of amiloride because high luminal concentrations can decrease amiloride affinity (Garty and Benos, 1988). The highly selective sodium channels are akin to the mechanosensitive channels of nematodes (Lingueglia *et al.* 1993; Canessa *et al.* 1994) and there is some evidence that Type I epithelial sodium channels can be activated by mechanical stress (Wills *et al.* 1991).

Type II ENaCs, as defined by Palmer (1992), have: 1) lower permeability ratios of Na^+ to K^+ (between 3-4:1), 2) higher single channel conductance (7-15 pS) and 3) shorter open and closed times ($\leq$ 50 ms). Type III channels are classified as non-selective cationic channels and have a permeability ratio of Na^+ to K^+ ≤ 1.5 and either high (23-28 pS) or low ($\geq$ 3 pS) conductances (Palmer, 1992).

ENaCs are members of a larger gene family, having been found in the invertebrates *Caenorhabditis elegans* and *Helix aspersa*, and the vertebrate lineages of amphibians, birds and mammals, animals which diverged over 650 million years ago (Voilley *et al.* 1997). Members of the gene family include the mechanosensitive channel genes *mec* and *deg*, the α-, β-, γ- and δ-subunit genes of ENaC, Acid sensing ion channel genes, H^+-gated cation channels, neuronal amiloride-sensitive cation channels, brain sodium channels and Phenylalanine-methionine-arginine-phenylalanine-NH_2(FMRF-amide) induced sodium channels (Garty and Palmer, 1997). All of these proteins share some sequence similarity, pointing to a shared common ancestor among them and which may indicate, due to their known diversity, their presence in numerous other species (Voilley *et al.* 1997).

ENaC was first known as several proteins that together produced an "amiloride-sensitive sodium current" (for a review, see Smith and Benos, 1991). The "amiloride binding protein" which was purified from bovine had a molecular mass of approximately 700 kDa and consisted of six polypeptides with molecular masses of ~315, 150, 95, 71, 55 and 40 kDa (Benos *et al.* 1987). The 315, 150 and 95 kDa subunits are heavily glycosylated (Garty and Benos, 1988) and inhibition of this glycosylation causes a marked decrease in Na^+ absorption (Zamofing *et al.* 1989) indicating a need for the functional channel to be glycosylated either for translocation, assembly or some other unknown activity. There is no clear relationship between the protein subunits listed above and the gene subunits subsequently cloned.

Type I ENaC genes are part of a multigene family consisting of genes such as α, β, γ and δ. They have been cloned in rat (*Rattus norvegicus*), human (*Homo sapiens*), cow (*Bos taurus*), chicken (*Gallus gallus*) and frog (*Xenopus laevis*) where the channel is a trimer of the α-, β- and γ-subunits, each of which have a high degree of sequence similarity to each other (See Table 1.1 for list of references). However, in humans, an additional subunit, δ, has been cloned which assumes the place of α in the trimer (Waldmann *et al.* 1995). The ENaC is the result of a duplication of the α-subunit into the β-/γ-subunits and a further duplication of the β-/γ-subunits into the separate β- and γ-subunits. The δ-subunit also appears to be the result of a duplication of the α-subunit, but separate from and later than the β/γ duplication (Le and Saier, 1996). The β/γ duplication occurred before the divergence of the amphibian and mammalian lineages (Garty and Palmer, 1997; Le and Saier, 1996) as evidenced by the diverse pattern of the lineage today.

Expression cloning of cRNA transcripts resulted in the first ENaC sequence (Canessa *et al.* 1993). In this strategy, Poly-$(A)^+$ RNA was isolated from the colon of rats which had been salt-deprived for one week. Basic cellular biology describes that an mRNA transcript may be translated more than once and thereby produce a significantly higher number of active proteins than corresponds to the number of

transcripts. This implies that if the cell is actively transcribing a low copy number protein, it is likely to have even fewer copies of the transcript in the cell since each transcript can be translated more than once. To increase the number of mRNA transcripts, the rats were salt-deprived and thus forced to create more mRNA transcripts (and then proteins) to increase their salt uptake. The RNA was size fractionated and samples were micro-injected into *Xenopus* oocytes, which have little or no endogenous amiloride-sensitive Na^+ conductive properties (George *et al.* 1989; Palmer *et al.* 1990). The fraction that produced the greatest amiloride-blockable Na^+ current was reverse-transcribed to cDNA and cloned into a library. Pools of clones were then *in vitro* transcribed into cRNA, injected into oocytes and further subdivided based on their ability to produce an amiloride-blockable Na^+ current. Eventually, a single clone encoding the α-subunit of the rat ENaC (α-rENaC) was obtained and sequenced. However, the amount of current produced by the α-rENaC was ≤ 0.1% of the maximal current produced in the intact cell and they concluded that parts of the channel were missing. Canessa *et al.* (1994a) discovered that there were two further subunits necessary for full function of the rENaC, called β- and γ-rENaC, respectively. These subunits were cloned after functional complementation with α-rENaC, which involved injections of cRNA's from α-rENaC and pools of cDNA cRNA's until a maximal amiloride-blockable Na^+ current was produced. The clones contributing to this current (i.e. β- and γ-rENaC) were isolated and sequenced and when co-injected with α-rENaC produced an amiloride-blockable Na^+ current reminiscent of the native channel (Canessa *et al.* 1994a).

The reconstituted "rat-in-oocyte" channel, consisting of α-, β- and γ-rENaC, has a high selectivity of Na^+ over K^+ (it is virtually impermeable to K^+), a 5 pS unitary conductance, is insensitive to membrane potential and is blocked by benzamil (K_i = 0.01 μM) and amiloride (K_i = 0.1 μM) (Canessa *et al.* 1995). These characteristics are similar to the native Type I ENaC's described above. For each subunit there are intracellular NH_2 and COOH termini and a large extracellular loop, as well as two putative transmembrane

domains. Each subunit, in rat, has been shown to be fully N-glycosylated with 6 sites in α, 12 sites in β, and 5 sites in γ, although no physiological role has been determined for the glycosylation (Canessa *et al.* 1994b). However, this glycosylation ties in with the previous protein data (see above) and seems to be necessary for proper activity of the channel. The β-subunit has been proposed to control cell surface expression and channel open probability (Firsov *et al.* 1996; Canessa *et al.* 1994a), however, incorporation of the β- and γ- subunits into the cell membrane is impossible without the α-subunit (Schild *et al.* 1997).

Amiloride-sensitive ENaCs are located only on the apical membrane of the epithelium (Smith *et al.* 1997) and may be maintained in position by cytoskeletal elements. Short actin filaments have been shown to stabilize the activation of rat sodium channel subunits *in vitro* along with the protein Cystic Fibrosis Transmembrane conductance Regulator (CFTR) which is involved in cystic fibrosis. Actin interacts with the α-subunit while CFTR associates with the β- and γ- subunits (Ismailov *et al.* 1997). These data suggest that the α-subunit contains the pore, while the β- and γ-subunits regulate channel activity. Canessa *et al.* (1995) estimate that there are perhaps as few as 10^2-10^3 ENaC's per cell active on the cell membrane.

Lingueglia *et al.* (1993) also cloned the α-subunit from rat colon and called it the Rat Colon Sodium Channel (RCNaCh1). The two DNA sequences are nearly identical to each other and have an identical derived protein sequence. Lingueglia *et al.* (1994) were the first to use a Polymerase Chain Reaction (PCR) based approach to clone an ENaC subunit when they designed degenerate primers based on the derived protein sequences of RCNaCh1, human lung ENaC, Mec4, Mec10 and Deg1, to amplify the γ-subunit (RCNaCh2) cDNA from RNA from the distal colon of a dexamethasone-treated rat. Degenerate primers are DNA primers that are created with multiple nucleotides at some locations, resulting in a pool of similar primers. They can be derived from amino acid sequences in which case, the protein is theoretically reverse translated into DNA and the primer is synthesised using all the possible nucleotides that code for that amino

acid sequence. There are sometimes numerous codons for a particular amino acid due to the degeneracy of the translation system, however, this method of designing primers eliminates the need to know the exact DNA sequence of the target attempting to be amplified.

McDonald *et al.* (1994) cloned the human α-ENaC subunit (α-hENaC) from a human kidney cDNA library. They used PCR to amplify a fragment of α-rENaC and used this fragment to screen the human kidney cDNA library under low-stringency Southern conditions. Southern hybridization is a technique in molecular biology whereby a radioactive DNA probe is hybridized with a DNA sample bound to a membrane. The salt concentration and temperature of the hybridization determine the degree of stringency by influencing the annealing of the probe to the target. The lower the salt or higher the temperature, the more stringent the hybridization and therefore the more alike the two DNA sequences must be. Under high salt and low temperature conditions, two distant DNA sequences can hybridize, as done above. McDonald *et al.* isolated and sequenced a clone that shared significant homology with the rat clone and was able to produce an amiloride-blockable Na^+ current in *Xenopus* oocytes. At the same time, Voilley *et al.* (1994) cloned the lung equivalent of the α-hENaC. They went on to clone the β- and γ- subunits from lung and showed a fully functional human equivalent of rENaC (Voilley *et al.* 1995). They used a portion of α-rENaC (Lingueglia *et al.* 1993) and γ-rENaC (Lingueglia *et al.* 1994) to screen a human lung cDNA library and to purify the β- and γ-hENaC subunits, respectively (Voilley *et al.* 1995). There is some evidence that the human brain may contain a different Type I ENaC that is composed of δ-, β- and γ-subunits where the δ-subunit replaces the traditional α-subunit (Waldmann *et al.* 1995).

Fuller *et al.* (1995) isolated the bovine equivalent of α-ENaC (α-bENaC) by screening a size fractionated cDNA library from bovine renal papillae with α-rENaC. It was found to form a functional chimeric channel when co-expressed with the rat β- and γ- subunits in *Xenopus* oocytes.

In chicken, two α-subunits have been cloned; one from the cochlea (Killick and Richardson, 1997) and one from the intestine (Goldstein *et al.* 1997). The former produced three splice variants, two of which produce identical proteins from different transcripts (2.5 and 3.5 kilobases). The third (3.9 kb) contains a premature stop codon in the open reading frame. A tissue distribution analysis showed that the 2.5 kb transcript is present in cochlea, colon and cartilage, while the 3.5 kb transcript is present in cochlea and colon and the 3.9 kb transcript is found only in the cochlea. There was no expression of this 3.9 kb α-cENaC in the brain, liver, heart, lung or kidney. This evidence implies that cENaC might not be solely for the purpose of sodium uptake in tissues such as lung and kidney, but may serve an auditory function as well. Goldstein *et al.* (1997) isolated a separate α-cENaC clone from chicken intestine and found that its expression was increased when the chickens were kept on a low-salt diet. They used a cDNA library screening technique to isolate the α-cENaC clone, similar to previous researchers, using the coding region for α-rENaC as the probe. They went on to clone part of the β-cENaC as well using a degenerate RT-PCR approach (Goldstein *et al.* 1997). These researchers used Poly(A)$^+$ RNA as the template for the RT, followed by PCR with degenerate primers. As yet, the full length β-cENaC has not been reported in the literature. Interestingly, the two α-cENaC clones are practically identical except for a small variation at the amino-terminus of the protein where Killick and Richardson's clone is slightly longer, possibly reflecting an isoform of the same gene.

As frogs and freshwater fish share a similar environment, it has been hypothesized that they will employ similar solutions to their osmoregulatory problems. Perhaps the most relevant ENaC, concerning the freshwater fish sodium channel model, that has been cloned is from the African clawed frog (*Xenopus laevis*) (Puoti *et al.* 1995; 1997) who used degenerate primers in RT-PCR on Poly(A)$^+$ RNA from aldosterone stimulated A6 cells (a *Xenopus laevis* kidney cell culture line) and identified the α-, β- and γ-

xENaC subunits. To design their degenerate primers, they utilized known sequences from rENaC and from the *mec-4*, *mec-10* and *deg-1* genes of *Caenorhabditis elegans* (Chalfie *et al.* 1993; Chalfie and Wolinsky, 1990; Driscoll and Chalfie, 1991; 1992) which had previously been shown to be similar to the ENaC genes (Canessa *et al.* 1993). In addition, they showed the expression of more than one transcript for the β-xENaC subunit in their Northern blots and proved the existence of isoforms of the β- and γ-xENaC subunits with the cloning of β2- and γ2-xENaC (Puoti *et al.* 1997), which appear nearly identical at the nucleotide level to their previously cloned *Xenopus* counterparts (90 and 92% identity, respectively). However, functionally, the γ2-xENaC, when co-injected with α-xENaC and, β-xENaC produces a lower amiloride-sensitive current, shows a higher affinity for external sodium and a lower affinity for amiloride (Puoti *et al.* 1997) indicating that the γ-subunit regulates the influences of external sodium and that there are different isoforms of the regulating subunit for, as yet, unknown purposes.

The distribution of the ENaC subunits in *Xenopus* is mainly limited to the kidney and lung tissues with weak signals in the stomach and skin, however, the amount of mRNA required for these Northern analyses (20 μg) indicates that the mRNA is present in low copy number (Puoti *et al.* 1995). How the mRNA levels affect the levels of functional channels is unknown. The cloning of the epithelial sodium channel from *Xenopus* provides direct evidence for the model of sodium uptake across the freshwater frog skin as proposed by Ehrenfeld *et al.* (1990).

It was the goal of this research to clone an epithelial sodium channel from rainbow trout (*Oncorhynchus mykiss*) and to thereby discover evidence in support of Avella and Bornancin's theory (1989). Based on the success of the *Xenopus* clonings (Puoti *et al.* 1995; 1997), a degenerate primer PCR approach was designed and executed. The α-subunit was chosen as the focus of the research due to the availability of sequence data for designing the primers.

Materials and Methods

Experimental Animals

Fresh water rainbow trout (*Oncorhynchus mykiss*) were acquired from Linwood Acres Trout farm and kept in large fibreglass tanks supplied with a steady stream of aerated H_2O, made by filtering City of Ottawa tap water through a WaterGroup Industrial Model AAC 60 duplex activated carbon filter, retrofitted for chloramine removal and kept at 15°C. Fish were maintained on a 12h light: 12h dark photoperiod and were fed *ad libitum* on a commercial pelleted fish diet. Fish were not fed during experimentation.

Physiological Treatment of Fish

Rainbow trout were subjected to either 11β,17α,21-Trihydroxy-4 pregnene-3,20-dione 21-hemisuccinate (cortisol) injections or acclimated to soft water. Fish were removed to opaque 100 *l* tanks or perspex boxes prior to experimentation. Cortisol injections were given once a day for seven days at a dose of 4 mg/kg in 0.9% saline to the dorsal muscle of the fish in an attempt to simulate chronic stress conditions under which ENaCs might be upregulated as has been previously seen for the H^+-ATPase counterpart to the model (Lin and Randall, 1993). Trout were acclimated for seven days to reverse osmosis (R.O.) water made with fish water processed through an Ion Pure Reverse Osmosis System (Series 4400) to simulate ion conditions where increased sodium uptake was expected (Avella *et al.*, 1987). Underlying this hypothesis is a presumed increase in expression of the channel (presumably ENaC) to account for this increased uptake. Ion concentrations (i.e. Na^+ and Ca^{+2}) were measured using a Varian 250 Atomic Absorption Spectrophotometer (A.A. Spec.). Typical Na^+ and Ca^+ concentrations in R.O. water are 0.010-0.021 mM and 0.002-0.007 mM, respectively, as compared with normal concentrations of 0.150-0.153 mM

and 0.332-0.376 mM, respectively. Fish were anaesthetized with an overdose of Benzocaine and sacrificed by a blow to the head. Tissues were isolated by dissection, as soon as possible, and instantly frozen in liquid nitrogen.

RNA Isolation

General RNase free techniques, as described by Sambrook *et al.* (1989), were used throughout all RNA procedures. All buffers for RNA experiments were prepared with 0.1% diethyl pyrocarbonate treated H_2O (DEPC) (Sambrook *et al.*, 1989). RNA was isolated from various tissues, previously frozen in liquid nitrogen, using the method of Chomczynski and Sacchi (1987) as modified by Chomczynski and Mackey (1995). Tissues were ground in a Lourdes homogenizer (Model MM-1A) in the presence of 5ml/g tissue Solution D (62.5 g guanidinium isothiocynate, 73 ml DEPC, 4.4 ml of 0.75 M sodium citrate, 6.6 ml of 10% w/v Sarkosyl and 0.3 ml of 2-mercaptoethanol) for three bursts of 20 *s* each separated by 10 *s* pauses. To the homogenate, 1/10 volume of 2.0 M NaOAc, pH 4.0 and ½ vol distilled phenol saturated with DEPC, were added and the mixture was vortexed for 10 *s*. One-fifth volume of chloroform was then added to the mixture and it was again vortexed. The final mixture was transferred into a disposable 17x100 polypropylene test tube and centrifuged at 8,000 RPM for 20 min in a Sorval RC5C centrifuge (SA600 rotor) at 4°C. When the samples were removed from the centrifuge, they had separated into three phases with the RNA being in the aqueous upper phase. The RNA was removed to a new tube with a sterile plugged pippette and the volume transferred was measured. To this volume of RNA, 0.2 vol of isopropanol and 0.2 vol of high salt solution (1.2 M sodium citrate, 0.8 M NaCl) were added and the sample mixed vigorously. The sample was then incubated for 10 min at room temperature, then spun at 8,000 RPM for 15 min and the supernatant discarded The RNA pellet was washed twice with 80% EtOH made with DEPC

and spun again at 8,500 RPM for 10 min. The RNA pellet was dried for 10 min in a vacuum desiccator and resuspended in an appropriate volume of DEPC. The concentration of total RNA was estimated from optical density at 260 nm, assuming that 1 A_{260} is equivalent to 40 ng/µl, using a Pharmacia Gene Quant RNA/DNA calculator (Model 80-2103-98). Quality of the RNA was estimated from both running the RNA on a 1.5% formaldehyde-agarose gel and verifying the presence of the rRNA bands and from the ratio of A_{260}/A_{280} (where a ratio of 2.0 indicates pure RNA) obtained from the Gene Quant. Typical yields of total RNA from liver tissue were 1.7 mg/1 g tissue with an A_{260}/A_{280} ratio of 2.0.

Software

DNA and protein alignments were made using ClustalW (Thompson *et al.* 1994) from sequences downloaded from the National Center for Biotechnology Information's (NCBI) GenBank web site: (http://www.ncbi.nlm.nih.gov/Entrez/). Default parameters were used in all cases.

A commercially available software program, DNAMan (Lynnon BioSoft), was used to analyze primers designed for RT-PCR. The functions of the program were used to check for stem-loop structures within an individual primer, compatible annealing between two primers and GC content of each primer. Stem-loop structures would cause self-annealing within the primer preventing it from participating in the PCR reaction. Modifications to the primers were made, or different primers chosen entirely, if any of these possible problems were severe, in an attempt to avoid mis-priming during RT-PCR.

Distances between DNA sequences were determined using software program MEGA (Kumar *et al.* 1993), which estimated the distance between two sequences by using a DNA alignment of the sequences and comparing the number of identities to the number of differences. Distance was calculated as the number of differences between the two sequences divided by the number of total sites compared.

An Internet based computer program called CODEHOP (Consensus Degenerate Hybrid Oligonucleotide Primers) (Rose *et al.* 1998) was used to scan a protein alignment of the α-ENaC sequences and to suggest primers.

Reagents

Molecular biology and chemical reagents and enzymes were purchased from either Sigma Chemical Co. (St. Louis, MO), Canadian Life Tech (Burlington, ON), New England Biolabs (Mississauga, ON), Amersham Pharmacia Biotech (Oakville, ON), Boehringer Mannheim (Laval, QC), Sangon Ltd. Canada (Scarborough, ON), Promega (Madison, WI, USA) or BDH Chemicals (Toronto, ON). Degenerate PCR primers and CODEHOP designed PCR primers used in the experiments were synthesized by Canadian Life Tech.

cDNA Library

A complementary DNA (cDNA) library, representing 10^6 recombinants with an average size of 1 kb and an amplified titre of 4.5×10^9 pfu/ml, was synthesized by Stratagene (La Jolla, CA) from trout gill and kidney RNA acquired from fish treated with hypercapnia, cortisol injections, soft water treatments and ammonium bicarbonate infusions (Perry *et al.* 2000). The RNA was prepared with Trizol Reagent (Gibco-BRL) as per the manufacturer's instructions.

PCR Optimization

The buffers making up this optimization kit were based on an Invitrogen kit (Catalogue # K1220-01) designed for the same purpose. The different 5X buffers were used to test a variety of pH's (8.5, 9.0, 9.6 and 10.1) and magnesium concentrations (1.5, 2.0, 2.5 and 3.5 mM).

Colony PCR

Individual colonies were plucked from an agar plate with a sterile toothpick and patched onto an agar plate with a grid for identification. The same toothpick was then vortexed in 100 µl ddH_2O to extract remaining bacteria. PCR's were performed with the following ingredients: 1 µl above bacterial dilution, 0.2 mM T7 or M13-forward-24 primer (Life Tech Catalogue #s: 18416-016 and 18257-014, respectively), 0.2 mM M13-reverse-24 primer (Life Tech Catalogue #:18424-010), 0.5 U Taq DNA polymerase, 1XPCR buffer (supplied with the enzyme), 0.2 mM dNTP's (each). The PCR program consisted of: 5 min at 94°C, 30 cycles of (1 min at 94°C, 1 min at 55°C, 1.5 min at 72°C), a 5 min final extension of 72°C and a 4°C sink. The T7, M13-forward-24 and M13-reverse-24 primers are endogenous to the plasmid and will amplify a product of approximately 200 bp without a cloned insert. The purpose of this PCR was to identify, by size comparison on an agarose gel, plasmids with an insert versus those without. PCR products were either directly run on gel, digested with HaeIII or TaqI restriction enzymes or frozen at -20°C for later use. For digestion with enzymes, 7 µl of the PCR reaction was incubated with the appropriate enzyme and buffer to a total volume of 20 µl.

RT-PCR

For RT reactions in Scotland. 2.5 μg total RNA were used according to the manufacturer's instructions with Gibco-BRL Superscript II RT at the prescribed 45°C. PCR reaction conditions were: 1 μl of the cDNA created in the initial RT reaction, 0.2 μM each Primer, 0.2 μM dNTP's, 1X standard PCR buffer, 1.75 U Taq and a PCR cycle of: 30 *s* at 94 °C, 40 cycles of (4 *s* at 94°C, 30 *s* at 55°C, 1 min at 72°C), 1 hr final extension at 72°C and a sink of 4°C.

RT-PCR in Ottawa followed the above procedure for the RT, but used different conditions for the PCR, as suggested by Dr. Vance Trudeau, consisting of: 1.5 mM Mg^{+2}, 10 mM dNTP's and 1 μM each primer. The PCR program consisted of: 5 min at 94°C, 10 cycles of (1 min at 94°C, 1 min at 48°C, 1.5 min at 72°C), 35 cycles of (1 min at 94°C, 1 min at 53°C, 1.5 min at 72°C), and a final extension of 72°C for 5 min and a 4°C sink.

Southern Transfer and Hybridization

After gels had been photographed, they were denatured for 20 min in 1.5 M NaCl and 0.5 M NaOH. They were then neutralized twice for 20 min in 3 M NaOAc, pH 5.5, and transferred overnight by capillary action to Hybond N membrane (Amersham). Blots were cross-linked for 5 min with a UV lamp and pre-hybridized with 10 ml/100 cm^2 of membrane of 6 X Standard Saline Citrate (1XSSC=0.15 M NaCl, 0.015 M NaCitrate), 0.05 M NaP_i, 5 mM EDTA, 5 X Denhardt's, 0.2% SDS, 200 μg/ml single stranded salmon sperm DNA at 65°C in a hybridization oven.

Probes for Southern hybridization were made using α^{32}P-dCTP (Amersham) multiprime labelling of plasmid and gel purified DNA restriction fragments. Plasmids were isolated with Wizard Preps (Promega) and DNA fragments were gel purified by spin column through a 0.22 μm filter. 25-50 ng of DNA was

incubated with random primer extension buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.4 mg/ml BSA (NEB), 2.5 U/ml Hexamers (Pharmacia), pH 7.4), deoxynucleotide mixture (5 mM Tris-HCl, 20 μM dATP, 20 μM dGTP, 20 μM dTTP), large fragment DNA polymerase I (Klenow enzyme) (NEB), and the radiolabelled nucleotide for 1 h at room temperature in a total volume of 30 μl. The reaction was stopped by the addition of 100 μl of stop buffer (50 mM Tris-HCl, 10 mM EDTA, 150 mM NaCl, 0.1% SDS) and the solution was centrifuged through a Sephadex G50 spin column stoppered with glass wool to eliminate unincorporated radiolabelled nucleotide. The probe was counted using a Packard 2000CA TriCarb liquid scintillation counter, and ~ 4×10^6 counts per minute (CPM) were used in each hybridization.

The pre-hybridization solution was removed and 5 ml/100 cm^2 of the same solution was added along with the radioactive probe. Hybridization was carried out at 65°C overnight in a hybridization oven. The radioactive solution was then decanted and the blots were washed with an SSC and SDS solution. The concentration of salt (SSC) and temperature used depended on the degree of stringency required for the wash. The less salt, the more stringent the wash. The higher the temperature, the more stringent the wash. Typical washers were very stringent and consisted of 2 X SSC and 0.2 % SDS. Radioactive blots were exposed to Kodak XAR or BIOMAX film at -80°C with an intensifying screen.

Colony Lifts

ICN Biotrans membrane was cut to fit and placed on chilled bacterial agar petri dishes covering the colonies on their surface. A needle was used to penetrate the membrane and the agar plate to mark the orientation of the colonies relative to both. The membranes were lifted, after approximately 30-45 *s* and transferred colony side up to 0.4 M NaOH for 5 min, followed by 0.5 M TrisHCl, pH 8.0 for 5 min and 2 X SSC for 5 min. Lastly, the membranes were cross-linked for 5 min with a UV lamp. Hybridizations were

carried out as per the Southern hybridizations listed above. Plates were re-incubated at 37°C for several hours to regenerate the colonies that had been lifted from the plate. The orientation markings were used to track a specific colony once the hybridization had been confirmed.

cDNA clones

The α, β, γ-rENaC (Canessa *et al.* 1993) and α, β, γ-xENaC (Puoti *et al.* 1995) cDNA clones were kindly provided by B.C. Rossier of the Université de Lausanne, Switzerland. The plasmids (pSport I and pBlueScript II KS, respectively) containing the cDNA clones were transformed into *E. coli* DH5αF' using the Hanahan transformation procedure (Hanahan, 1985). The transformed plasmids were recovered using Promega's Wizard prep kit.

Sequencing

Plasmids containing cloned sequences were recovered from their host bacteria using Promega's Wizard prep kit and sequenced using the Thermosequenase 5 PrimerCycle Kit (Amersham Pharmacia Biotech). Sequences were analysed on a LiCor 4200L automated DNA sequencer by Canadian Molecular Research Services (Ottawa, ON).

Results

Based on calculations by Canessa *et al.* (1995), that there are 10^2 to 10^3 ENaCs per cell, and experimental data by Puoti et al. (1995), that 20 μg mRNA was needed for a visible signal on a Northern, it was presumed that the sodium channel protein would be present in small numbers on the trout gill. It was for these reasons that a PCR based technique was employed in order to amplify mRNA transcripts for the α-subunit of the epithelial sodium channel. Table 2.1 is a summary of the primers used, their sequences and their position in the protein relative to α-xENaC.

Primer Design

The goal of this research was to clone a subunit of the epithelial sodium channel from rainbow trout. The approach chosen began by examining known α-ENaC protein sequences (see Table 1.1 for a list) as downloaded from the NCBI's GenBank and aligned using ClustalW (Thompson *et al.* 1994) (See Appendix A for alignment). The alignments were then examined for conserved regions that would be appropriate for primer design. These conserved regions were then theoretically reverse-translated from protein to DNA and completely degenerate primers were designed for use in PCR or RT-PCR. The term "completely degenerate" refers to the incorporation of every possible nucleotide in the synthesis of the primer to encode the amino acid desired. This results in a pool of primers that are similar to each other, but not exactly alike and covers all the possible DNA sequences of the target, assuming the target amino acid sequence matches that used to design the primers. This procedure allows for the primers to ignore the codon bias of a species, however, it decreases the number of exact primers being used in the PCR by increasing the number of possible primers available for use in the PCR.

A successful primer design will take several factors into account including conservation, degeneracy, length and self-complementarity. First and foremost, the sequence must be conserved between the aligned proteins so that a predicted protein sequence in the unknown protein may be discerned. A major assumption of this approach is that the target sequence has a similar amino acid sequence to that of the known sequences in the region of the primer. Second, assuming that multiple areas of conservation are present, it is wise to choose a region with amino acids that are coded for by fewer codons (i.e. less degenerate). For example, if there is a choice to avoid a serine, S, and design a primer with a methionine, M, instead, this would be preferred. Serine has six codons while methionine has only one and thus less combinations of nucleotides result in the primer with methionine. Third, the length of the primer should be between 18 and 30 nt. If the primer is shorter, the chance of annealing is reduced and if the primer is longer the chance of mis-priming is increased. Lastly, self-complementarity in the primer should be avoided. A stem-loop structure within the primer, formed by self-complementarity among the bases of the primer, will prevent proper annealing of the primer to the target and thus, inhibit its activity. As well, complementarity between primer pairs should be avoided to prevent the formation of "primer-dimers" which interfere with proper PCR amplification (Innis and Gelfand, 1990).

Primer Group 1: RT-PCR

Fully degenerate primers (Table 2.1) were designed around the conserved derived amino acid regions of the α-ENaC: NTTIHGA (Primer 1), YGNCYTFN (Primer 2) and QWSLWFG (Primer 3) (Fig. 2.1a). These primers were arranged in a semi-nested configuration where Primer 2 is internal to Primers 1 and 3.

A similar arrangement was designed for the β-subunit of ENaC (Fig.2.1B) and consisted of Primers 4, 5 and 6 where Primer 5 is internal to Primers 4 and 6 and can only be used in conjunction with Primer 6.

While in Scotland, at the University of St. Andrews in Dr. Gordon Cramb's lab, Primers 2 and 3 and 5 and 6, having expected sizes of 773 and 953 bp, respectively, were used in RT-PCR on total RNA from cortisol and soft water treated gill and kidney tissues of rainbow trout. In RT-PCR reactions using cDNA created in the initial RT reaction these primers failed to produce results in a consistent manner and it was suggested to use the primers CC-for and CC-rev, designed by Dr. Chris Cutler, on the same RNA (having expected sizes of 735 bp for α-ENaC, 780 bp for β-ENaC and 780 bp for γ-ENaC, based upon amino acid alignments). CC-for and CC-rev had been designed around two conserved regions in the ENaC family to amplify all epithelial sodium channel subunits simultaneously (Fig. 2.2). Reaction conditions were identical to the above except for the use of the primers in 5 μM concentrations as recommended by Dr. Cutler.

These primers gave rise to consistent products of approximately 800-850 bp that were in the expected size range, as well as smaller products of ~ 600 bp. Controls for the reactions consisted of single primer controls, a negative control of no DNA and a positive control, to ensure the PCR worked, of P-type ATPase primers (supplied by Dr. Cramb). Single primer controls consisted of the same components as a normal PCR reaction except for the inclusion of only one of the two primers to test for amplification by a single primer acting in both the forward and reverse directions. A negative control of no DNA consisted of exactly the same components as a normal PCR reaction except for the exclusion of any input DNA from the reaction to test for DNA contamination of the primers. All double-primer products appeared to be different than those acquired with controls of only one primer even after two rounds of amplification (using the product of the first round as seed for the second) (Fig. 2.3) and regardless of the RNA originating from gill or kidney. These were, therefore, considered likely candidates for clones of a trout ENaC and were cloned into pCR II.1 (Invitrogen) resulting in 162 colonies that were screened for appropriately sized cloned fragments. Of these, the 105 larger positives (800-850 bp) were processed for further analysis and were

amplified by colony PCR (cf. Chapter 2, Materials and Methods). The products were digested with TaqI and HaeIII restriction enzymes, in an attempt to distinguish duplicate clones, and grouped into eleven different classes based on the digest pattern.

These eleven clones were sequenced and screened against GenBank using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.* 1990) available on the NCBI web site. The option BLASTX was used to translate the DNA sequences to the possible six frames and scan the protein database, because it was assumed that any sequence cloned would be a coding sequence and the translation to protein sequence eliminates degeneracy at the 3rd position of the codon. Sequence analysis, as of January 2000, revealed only sparse random matches to the database but no significant correlations with any sequences in GenBank. Sequence analysis of the smaller products (samples 117-162), after similar digestion and grouping, also failed to identify any significant matches in GenBank (data not shown).

Primer Group 2: cDNA library amplification

Following the lack of results with the previous primers sets, the design of the primers was re-examined. It was decided that a semi-nested approach to PCR amplification, as before, was appropriate, and that more combinations of primers should be constructed to increase the chance of a product. Four primer sets were designed, consisting of five primers (Fig. 2.4), and defined as follows: 1) FG(M/L)MYWQF & EPAFMDDG, 2) YGNCYTFN & EPAFMDDG, 3) YGNCYTFN & Puoti, 4) FG(M/L)MYWQF & NFTYCNGY (See Table 2.1 for sequences) with expected product sizes, based on the α-xENaC protein sequence, of 798, 192, 375 and 626 bp, respectively. The Puoti primer was modified from that used by Puoti *et al.* (1995) as their reverse primer. Although this primer does not meet the typical requirements of a "good" primer, as described in the Materials and Methods, because of a lack of conservation among all

the subunits, it was synthesized and tested according to the logic that it had been used successfully before and might work again. The primer YGNCYTFN and its compliment NFTYCNGY are highly conserved and low degeneracy primers used by all previous PCR-based clonings (cf. Chapter 2, Introduction). The other two primers, FG(M/L)MYWQF and EPAFMDDG are conserved regions among the α-subunits and were created with as little degeneracy as possible.

These primers were used in two sets of PCR experiments on a gill and kidney cDNA library that had been constructed in such a way to maximize the possibility of expression of ENaC and Proton-ATPase subunits (see Perry *et al.* 2000). The cDNA library was used instead of RNA and RT-PCR to minimize expense and simplify the number of experiments required by leaving out the RT step of the reaction. The use of the cDNA library had the added advantage of having no introns in the RNA sequence and therefore it was more reliable to estimate the size of expected products. A series of experiments was designed and implemented to titrate the annealing temperature, buffer composition (using the optimization kit), the various primer sets and the amount of the library required.

In the first set of experiments, 4 µl of the library was used as the template DNA, α-xENaC plasmid was used as a positive control for PCR and temperature titrations were conducted from 42-57°C, in five degree intervals. Initially, buffers with pHs of 8.5, 9.0, 9.6 and 10.1 and magnesium concentrations of 2.5 and 3.5 mM were used to test the optimal pH of the PCR reaction. Cycling was the same as per colony PCR and at first only the first three primer sets were attempted. Single primer controls were included, however, only buffer H (a pH 9.0, 3.5 mM Mg^{+2} buffer) was used in order to simplify the controls. Results of this series are displayed in Fig.2.5A-D and show a large number of bands with all primer sets except primer set 3 (YGNCYTFN & Puoti). No specific band appears at the expected size with any of the buffers or temperatures, however, as a general trend, more bands appear with the higher Mg^{+2} buffers and the lower

annealing temperatures. This would be expected since Mg^{+2} stabilizes primer binding to template and lower annealing temperatures mean less specific binding of the primer to the template. This coincides with the expected pattern of decreased bands with increased annealing temperature due to failure of primers to anneal to the DNA and decreased bands with decreased Mg^{+2} due to the stabilizing affect that Mg^{+2} has on primer annealing and polymerase activity.

As no specific bands were apparent, the next set of experiments was to attempt nested PCR where DNA from the first PCR was used as the template DNA in the second PCR, in order to try to isolate a specific band of the expected size. Primer set 4 (FG(M/L)MYWQF & NFTYCNGY) reactions (52°C annealing, 30 cycles, 0.2 μM dNTP's, 0.05 U Taq, either Buffer C, D, G, H, K, L, O, or P, 5 μM each primer) were inoculated with Primer set 1 (FG(M/L)MYWQF & EPAFMDDG) DNA that had been produced in the exact same manner as above but with different primers. Single primer controls from previous reactions were also re-inoculated and a new single primer control for the NFTYCNGY primer was added. The reproducible results (Fig. 2.6) were encouraging and Southern blots of all the PCR reactions were created to check for hybridization with the *Xenopus* α-ENaC clone (cf. Chapter 2, Materials and Methods). It was theorized, as discussed in the Introduction, that stringent positive hybridization would be a good indication that the *Xenopus* and trout clones were similar. Stringent positive hybridization did occur and DNA from the reaction Buffer O, Primer Set 4, Second amplification, was cloned into the plasmid pCR 2.1. Since hybridization was observed with PCR products from every buffer, a random sample was chosen to be cloned. Colonies were screened by colony lift, followed by hybridization with α-xENaC, since the previous hybridization had worked so well. Plasmids from positive colonies were isolated and sequenced, and when searched against the NCBI database identified as the α-subunit of the *Xenopus* Epithelial Sodium Channel, indicating contamination of PCR materials with the positive control DNA.

The hypothesis concerning the *Xenopus* α-ENaC clone and hybridization was re-examined with a measurement of the nucleotide distance between α-xENaC and the rest of the ENaCs. The known α-ENaCs are found in three classes of vertebrates: amphibians, birds and mammals, with most of the data being on mammalian sequences. It was calculated, using MEGA and the sequences DNA sequences available from GenBank, that while the rat and cow sequences are 82% identical, the frog sequence is only 59% identical to rat, 60% identical to chicken and 61% identical to cow. This means that the *Xenopus* sequence is around 40% different from the mammalian and avian sequences and thus should not cross-hybridize with them under stringent Southern conditions, as used above, because it would be too different. There is no reason to suspect that the trout sequence will be any closer to the *Xenopus* sequence than that *Xenopus* sequence is to the mammalian sequences and therefore, there is no reason to suspect that they will cross-hybridize. This does not mean that degenerate primers cannot be used to amplify the α-ENaC of trout, but it does mean that hybridization will not be able to be used to screen colonies of clones. Thus, any cross-hybridization detected is most probably contamination as we have seen here.

A contamination test of the primers was designed whereby the primers were used with no input DNA in the exact same manner as outlined above. Reaction mixtures from the first reaction, although not showing any products on the gel, were used to initiate a second reaction using the nested primer set, as before. This was equivalent to performing a buffer optimization on negative controls and, as would be expected if contamination of the primers was an issue, bands of the expected size did appear. Therefore, the conclusion was drawn that the primers had been contaminated with the α-xENaC positive control, the only possible source of the DNA (data not shown).

New primers were ordered to replace the contaminated ones, all materials related to the first series of PCR's were discarded and a second series of temperature (45-60°C, in 5° increments) and buffer (C, D,

G, H, K, L, O, P) titrations were begun. Single primer controls were again instituted to ensure products were not the result of a single primer acting in both directions. A positive control for PCR other than α-xENaC (i.e. the B subunit of the H^+-ATPase) was used to avoid contamination of the experimental samples with the control samples, by aerosol or other means, and steps were taken to re-sterilize any remaining tips and tubes after use. Nested PCR was again employed using Primer Set 1 (FG(M/L)MYWQF & EPAFMDDG) for the first reaction and Primer Sets 1 and 4 (FG(M/L)MYWQF & NFTYCNGY) for the second reaction (seeded with DNA from the first).

A single random phage was isolated from the library and used as a control for randomly amplified products. This phage was carried through the same buffers and reactions as the cDNA library and the two sets of reactions were compared for bands that differ. Both 2 μl and 4 μl of the library and the random phage were used in the PCR's to see if the amount of template DNA used made any difference in the product profile, however, no clear distinction was present. A fraction corresponding to the expected size of approximately 600 bp was isolated from a 55 °C, 30 cycle PCR with Buffer K and Primer Set 4 (inoculated with same from 1st reaction with Primer Set 1) (Fig. 2.7) and spun through a 0.22 μm Millipore filter. The resulting eluate was then used in a like PCR to amplify enough product for cloning. The product was cloned into pCR2.1 with a TA cloning kit from Invitrogen. Colony PCR and subsequent digestion with TaqI and HaeIII identified 17 clones for sequencing. All 17 clones were identified with high probability as sequences already in the database, however, these sequences were transmembrane proteins of *E. coli* and not the sodium channel as previously hoped (Table 2.2). On further examination of the sequence results, the FG(M/L)MYWQF primer was seen at both ends of the sequence, indicating that this primer alone contributed to the amplification of the products by acting as both the forward and the reverse primer.

To develop a screening method to try to find *bona fide* clones, an experimental PCR was conducted

where only the FG(M/L)MYWQF primer was used (Fig. 2.8A). This screening worked regardless of the source of the DNA being a culture, a boiling lysis (Berghammer and Auer, 1993) of a culture or a plasmid prep (Fig. 2.8B). Using this screening, other fractions like that above were screened for single primer clones saving time in sequencing and evaluation. Unfortunately, no products that were not single primer clones were discovered although some 136 additional clones were screened.

Primer Group 3: RT-PCR Revisited

Having failed once again to amplify a portion of the epithelial sodium channel, it was decided to make one more attempt at RT-PCR, with several modifications from the first attempt. Firstly, the primers would be designed for a specific subunit, thereby eliminating part of the degeneracy present in the first set of RT primers (CC-for and CC-rev, Table 2.1). Secondly, the primers would not be designed as fully degenerate, as in past attempts, but would utilize the program Consensus Degenerate Hybrid Oligonucleotide Primers (CODEHOP) (Rose *et al.* 1998) to design the primers. This program and its creators proposed a revolutionary way to design primers for distantly related sequences such as ENaC. Based on the distance data described above, it was decided that trout ENaC was possibly as far from *Xenopus* ENaC as *Xenopus* ENaC was from mammalian ENaCs and that if this was the case, conventional primer design would create primers that were too degenerate to be useful. With increasing degeneracy comes increasing numbers of primers in the pool and decreasing concentration of each specific primer. Thus, even if a specific primer was to anneal to the proper sequence, its concentration within the reaction might not be great enough to properly amplify the sequence.

CODEHOP avoids this problem by dividing the design of the primer into two parts, a 5' consensus clamp (18-25 bp) and a 3' degenerate core (11-12 bp) for each primer. The 3' degenerate core is where

polymerization begins and must be bound directly to the template DNA. It is, therefore, designed to be fully degenerate. The 5' consensus clamp acts as an anchor for the 3' degenerate core and according to Rose *et al.* (1998) has more flexibility in its exact binding to the template DNA. Their evidence suggests that even a mismatched consensus clamp act well enough as long as the 3' degenerate core is directly attached. According to this theory, the primer is chosen from a conserved protein region, as before, however, only the 3' core is made fully degenerate and the 5' consensus clamp is a result of the consensus sequence of the protein alignment. Codon bias by the organism is taken into account by the program so that only one codon appears in the 5' consensus clamp.

Again, primers were designed in a semi-nested configuration (Fig. 2.9) around the conserved α-ENaC protein regions of PMYGNCYTFND, HDRNEPAFMDD, EPAFMDDGG, and WGYCYYKLQAE (See Table 2.1 for sequence and Appendix A for alignment). Once the regions were identified, based upon their sequence conservation, the portion of the alignment containing the conserved regions was entered into the CODEHOP program. The program calculated all possible primers based on the input regions and gave a score for each primer based upon degeneracy, consensus sequence in the clamp region, and melting temperature.

With these factors in mind, a primer for each region was chosen and synthesized. In the case of the EPAFMDD region two primers were made, one forward and one reverse, so that each might act as a semi-nested primer in different primer sets. The previous region of FG(M/L)MYWQF was eliminated because of its propensity to amplify single primer products. As well, the RT primers were developed from regions closer to the COOH end of the protein to increase the possibility of amplifying a portion of the transcript. The previous design had been located towards the 5' end of the mRNA transcript and perhaps this had contributed to its failure to produce results. The nature of RT or cDNA library construction is such that the

3' end of the gene is more likely to get cloned than the 5' end due to the use of a oligo-(dT) primer to bind to the Poly(A)$^{-}$ mRNA tail and initiate reverse transcription. Thus, this primer design included areas of conserved protein sequence closer to the COOH terminus. Primers in putative transmembrane regions, as identified in the literature (Puoti *et al.* 1995), were avoided and any primers with stem-loop (self-complementary) structures were modified to eliminate these. As well, primer sets were tested for formation of primer-dimers by checking for annealing between primers. Three primer sets were possible: 1) RT-ENaC-for1 & RT-ENaC-rev1, 2) RT-ENaC-for1 & RT-ENaC-rev2 and 3) RT-ENaC-for2 & RT-ENaC-rev2 (See Table 2.1) having expected product sizes of 206, 495 and 329 bp, respectively.

These primers were optimized, in a manner similar to that outlined above. Using Primer set 3 on trout gill cDNA from soft water and cortisol treated animals, a prominent band of about 475 bp was identified that was not present in single primer control reactions run at the same time (Fig. 2.10). Although this band was larger than that expected, it was excised from the gel and cloned into a TA cloning kit as per the manufacturer's instructions. Numerous colonies resulted and were screened using colony PCR for products of the correct size (Fig. 2.11A). Colonies of the correct size were further screened with Primer Set 3 to ensure that they were, in fact, a result of the intended cloning (Fig. 2.11B). Four colonies resulted in products of expected size and plasmids from these colonies were sent for sequencing. Sequence analysis, by BLASTX search of GenBank, characterized all of these clones as belonging to trout or chicken collagen clones (Table 2.3). Examination of the sequences failed to reveal the primers used in the PCR, even though these primers are expected to be seen. No DNA alignment is possible with the collagen DNA from the BLASTX results (i.e. AB008374 - alpha 3 type I collagen from *Oncorhynchus mykiss*) and the sequences examined here. It is unknown how these collagen-like clones were amplified with primers designed for the α-subunit of the Epithelial Sodium Channel, nor why the primers are not visible within the sequence.

Table 2.1. Primers designed for PCR and RT-PCR based on protein alignments of ENaC subunit sequences. Primers are grouped by subunit except in the case of CC-for and CC-rev which were designed as general ENaC subunit primers. Sequences are listed in standard IUPAC codes: A=deoxyadenine, C=deoxycytosine, G=deoxyguanidine, T=deoxythymidine, i=deoxyinosine, B=C+T+G, D=A+T+G, H=A+C+T, K=T+G, M=A+C, N=A+C+T+G, R=A+G, S=C+G, V=A+C+G, W=A+T, Y=C+T. The incorporation of more than one IUPAC code in the synthesis of the primer is indicated by parentheses. Amino acid positions refer to the position within the α-xENaC protein sequence (GenBank Accession U23535). * Designed by Dr. Chris Cutler, University of St. Andrews and used with permission.

Subunit	Date Created	Primer Name	DNA Sequence	Protein Sequence and A.A. Position	Direction
α	19/02/97	Primer 1	5'-AAYACSACCATCCAYGGNGC-3'	NTTIHGA (29-35)	FOR
	19/02/97	Primer 2	5'-TAYGGRAAYTGCTAYACYTTY-3'	YGNCYTFN (261-268)	FOR
	19/02/97	Primer 3	5'-CCRAACCASAGRCTCCACTG-3'	GFWLSWQ (518-512)	REV
	35472	FG(M/L)MYWQF	5'-TTYGGNHTNATGTAYTGGCARTTY-3'	FG(M/L)MYWQF (59-66)	FOR
	35472	YGNCYTFN	5'-TAYGGNAAYTGYTAYACNTTYAA-3'	YGNCYTFN (261-268)	FOR
	35472	NFTYCNGY	5'-TTRAANGTRTARCARTTNCCRTA-3'	NFTYCNGY (268-261)	REV
	35472	Puoti	5'-CATRTTYTCYTGRAARCA-3'	MNEQFC (385-380)	REV
	35472	EPAFMDD	5'-RTCRTCCATRAANGCNGGYTC-3'	DDMFAPE (325-319)	REV
	11/1998	RT-ENaC-for1	5'-CCATGTACGGCAACTGCTACACNTTYAAYSA-3'	PMYGNCYTFND (259-269)	FOR
	11/1998	RT-ENaC-for2	5'-CCACGGTCAGAACGAGCCTGCATTYATGGAYGA-3'	HDRNEPAFMDD (315-325)	FOR
	11/1998	RT-ENaC-rev1	5'-GCCGCCATCATCCATGAANGCNGGYTCNT-3'	GGDDMFAPE (327-319)	REV
	11/1998	RT-ENaC-rev2	5'-CGACCTGCAGCTTGTAGTAGCARTRNCCCCA-3'	EAQLKYYCYGW (424-414)	REV
α/β*	1997	CC-for	5'-GTTTWYTGYV(i/c)BAAYAC(i/c)AMCAY(i/c)CAYGG-3'	(F/Y)C(S/N/D/L/M)NT(T/N)(I/T)H(26-33)	FOR
α/β*	1997	CC-rev	5'-GTTTRAARRTRATRCARTT(i/c)CCRTA-3'	(Y/H)GNCY(T/I)FN (268-261)	REV
β	19/02/97	Primer 4	5'-AACACCAACACHCAYGGCCCC-3'	NTNTHGP (38-44)	FOR
	19/02/97	Primer 5	5'-GGMAACTGYTACATCTTYAAC-3'	YGNCYIFN (288-295)	FOR
	19/02/97	Primer 6	5'-GGTGTGRGCCTCCACCAGCTC-3'	THAEVLE (606-600)	REV

Table 2.2. BLASTX search result of single primer product clones from FG(M/L)MYWQF. Probability of a random match is calculated by BLAST and indicates the chance that the sequence searched with matches something in the database by accident. The lower the probability, the more likely the match is true. Values lower than 1×10^{-10} are generally considered valid matches, although no hard and fast numbers are agreed upon by the literature.

Clone	GenBank Accession# & Description of Related Seqeunce	Probability of Random Match
36621	M14641 - *E. Coli* Membrane-associated Protein	1.00×10^{-48}
36626	AE000177 - *E. Coli* Hypothetical 34.7 Kd Protein	4.00×10^{-82}
36628	M88701 - *E. coli* protein ThiF	3.00×10^{-39}
4-20f	D90832 - *E. coli* Excinuclease ABC Subunit C	2.00×10^{-73}
36644	D90709 - *E. coli* Potassium-transporting ATPase	1.00×10^{-32}
4-32	X13463 - *E. coli* Glucitol Operon Repressor	4.00×10^{-53}
4-36	D90709 - *E. coli* Potassium-transporting ATPase	7.00×10^{-42}
4-45	P14283 - *E. coli* ORF	1.00×10^{-66}
4-46	P07028 - *E. coli* Excinuclease ABC Subunit c	e^{-117}
4-48	Y10545 - *E. coli* fused-ccdB	7.00×10^{-05}
4-50	Y10545 - *E. coli* fused-ccdB	3.00×10^{-18}
4-54	P14283 - *E. coli* ORF	2.00×10^{-68}
4-58	M88701 - *E. coli* protein ThiF	2.00×10^{-46}
4-66a	U00039 - *E. coli* hypothetical 45.5 kD protein	2.00×10^{-34}
4-67a	U00039 - *E. coli* hypothetical 45.5 kD protein	4.00×10^{-37}
4-68a	U00039 - *E. coli* hypothetical 45.5 kD protein	2.00×10^{-33}
4-70b	U00039 - *E. coli* hypothetical 45.5 kD protein	4.00×10^{-37}

Table 2.3. BLASTX search results of RT-PCR products from Primer Set 3. Probability of match is defined in Table 2.2. Clones were sequenced in the forward and the reverse direction and overlapped whenever possible. This was not possible for MB1 and MB2, due to uncertainties in the sequence, and so the forward and reverse sequences were BLASTed separately, as displayed here.

Clone	GenBank Accession# & Description of Related Seqeunce	Probability of Random Match
mb1	Forward: AB008374 - alpha 3 type I collagen (*Oncorhynchus mykiss*)	9.00×10^{-04}
	Reverse: A57032 - collagen alpha 1(II) chain - (*Gallus gallus*)	2.00×10^{-04}
mb2	Forward: AB008374 - alpha 3 type I collagen (*Oncorhynchus mykiss*)	4.00×10^{-22}
	Reverse: AB008374 - alpha 3 type I collagen (*Oncorhynchus mykiss*)	4.00×10^{-10}
mb3	AB008374 - alpha 3 type I collagen (*Oncorhynchus mykiss*)	2.00×10^{-42}
mb4	AB008374 - alpha 3 type I collagen (*Oncorhynchus mykiss*)	1.00×10^{-47}

Fig. 2.1. Schematic of primer design and direction of amplification for the initial attempt at primer design. Primer names are indicated. The direction of the arrows indicates the direction of the polymerization with Taq polymerase, assuming the primers bind properly and a product is amplified. Primers are shown in relation to the *Xenopus* ENaC subunit, although all known ENaC subunits were used in the determination of the primers. Primer sequences are listed in Table 2.1. A) Primers for amplification of the α-ENaC subunit. B) Primers for the amplification of the β-ENaC subunit.

A

Primer 1 →

α-xENaC MTKEEKNEKEALIEFFSSYRELFEFFCSNTTIHGAIRLVCSRRNRMKTAFWLVLFLVTFGLMYWQFGLLFGQYFSYPVSINLNVNSDKLPFPAVTVCTLN 100

PYRYKAIQNDLQELDKETQRTLYELYKYNSTGVQGWIPNNQRVKRDRAGLPYLLELLPPGSETHRVSRSVIEEELQVKRREWNIGFKLCNETGGDCFYQT 200

Primer 2 →

YTSGVDAIREWYRFHYINILARVPQEAAIDGEQLENFIFACRFNEESCTKANYSSFHHAIYGNCYTFNQNQSDQSNLWSSSMPGIKNGLTLVLRTEQHDY 300

IPLLSSVAGARVLVHGHKEPAFMDDNGFNIPPGMETSIGMKKETINRLGGKYSDCSEDGSDVDVKNLFQSEYTEQVCVRSCFQAAMVARCGCGYAFYPLS 400

PGDQYCDYNKHKSWGHCYYKLIIEFTSNKLGCFTKCRKPCLVSEYQLTAGYSKWPNRVSQDWVLHTLSRQYNLTDRNGIAKLNIYFEELNYKTILESPTI 500

NMAMLLSLLGSQWSLWFGSSVLSVVEMLELVIDFVIIGVMILLHRYYYKKANEGEETTVVPTPAPAFADLEQQVPHIPRGDLSQRQISVVADITPPPAYE 600

← Primer 3

SLDLRSVGTLSSRSSSMRSNRSYYEENGGRRN 632

B

Primer 4 →

β-xENaC MIHGEMKRLKRYFTRALHRIQKGPGYTYKELLVWFCDNTNTHGPKRIIKEGPKKRVMWFILTLVFAGLVFWQWGVLILTYLSYGVSVSLSIGFKTMEFPA 100

VTLCNANPFKYSRVKPLLKELDELVATALDRIQFSSQNQGNTFTHNNQTRQNVTLDPALWNHIPLVVIDETDPRNPIIHNIFDNNAVYSKNSSIRNSSED 200

Primer 5 →

QTSYSQRYKVAMKLCTNNNTQCVYRNFTSGVQALREWYLLQLSSIFSNVPLSGRIDMGFKAEDLILTCLFGGQPCSYRNFTHIYDADYGNCYIFNWGQEG 300

ENTMSSANPGADFGLKLVLDIEQGEYLPFLQTTAAARLILHQQRSFPFVKDLGIYAMPGTETSISVLVDQLEHMEAPYSSCTVNGSDIPVQNLYAEFNSS 400

YSIQSCLRSCYQEEMVKTCKCAHYQYPLPNGSEYCTNMKHPDWVPCYYSLRDSVAIRENCISLCQQPCNDTHYKMVISMADWPSAGAEDWIFHVLSYEKD 500

SSHNITVNRNGIVRLNIYFQEFNYRSISESEATNVVWLLSNLGGQFGFWMGGSVLCIIEFGEIIIDCMWITILKFLAWSRNRRQRRKRPQYSDPPPTVSE 600

LVEAHTNSGFQHDDGDHVPVDIPGTPPPNYDSLRVNTAEPVSSDEEN 647

← Primer 6

Fig. 2.2. Schematic of primer design and direction of amplification for primers CC-for and CC-rev, designed by Dr. Chris Cutler at the University of St. Andrews, Scotland, for the amplification of all ENaC subunits. The direction of the arrows indicates the direction of the polymerization with Taq polymerase, assuming the primers bind properly and a product is amplified. Primers are shown in relation to the *Xenopus* α-ENaC subunit, although all known ENaC subunits were used in the determination of the primers. Primer sequences are listed in Table 2.1.

CC-For →

α-xENaC MTKEEKNEKEALIEFFSSYRELFEFFCSNTTIHGAIRLVCSRRNRMKTAFWLVLFLVTFGLMYWQFGLLFGQYFSYPVSINLNVNSDKLPFPAVTVCTLN 100

PYRYKAIQNDLQELDKETQRTLYELYKYNSTGVQGWIPNNQRVKRDRAGLPYLLELLPPGSETHRVSRSVIEEELQVKRREWNIGFKLCNETGGDCFYQT 200

YTSGVDAIREWYRFHYINILARVPQEAAIDGEQLENFIFACRFNEESCTKANYSSFHHAIYGNCYTFNQNQSDQSNLWSSSMPGIKNGLTLVLRTEQHDY 300

← CC-rev

IPLLSSVAGARVLVHGHKEPAFMDDNGFNIPPGMETSIGMKKETINRLGGKYSDCSEDGSDVDVKNLFQSEYTEQVCVRSCFQAAMVARCGCGYAFYPLS 400

PGDQYCDYNKHKSWGHCYYKLIIEFTSNKLGCFTKCRKPCLVSEYQLTAGYSKWPNRVSQDWVLHTLSRQYNLTDRNGIAKLNIYFEELNYKTILESPTI 500

NMAMLLSLLGSQWSLWFGSSVLSVVEMLELVIDFVIIGVMILLHRYYYKKANEGEETTVVPTPAPAFADLEQQVPHIPRGDLSQRQISVVADITPPPAYE 600

SLDLRSVGTLSSRSSSMRSNRSYYEENGGRRN 632

Fig. 2.3. RT-PCR results using CC-for and CC-rev. RT reactions, using 2.5 μg total RNA, were conducted according to the manufacturer's instructions with Gibco-BRL Superscript II RT at the prescribed 45 °C. PCR's were carried out using conditions of: 1 μl of the cDNA created in the initial RT reaction, 0.2 μM each Primer, 0.2 μM dNTP's, 1X standard PCR buffer, 1.75 U Taq and 55 °C annealing for 40 cycles (4 *s* at 94 °C, 30 *s* at 55 °C, 1 min at 72 °C). The second round of PCR's was initiated using 0.5 μl of the first as input DNA while the other variables were maintained constant. Size of marker bands is indicated. Larger products (~800-850 bp) are indicated by a blue arrow and smaller products (~600 bp) by a green arrow.

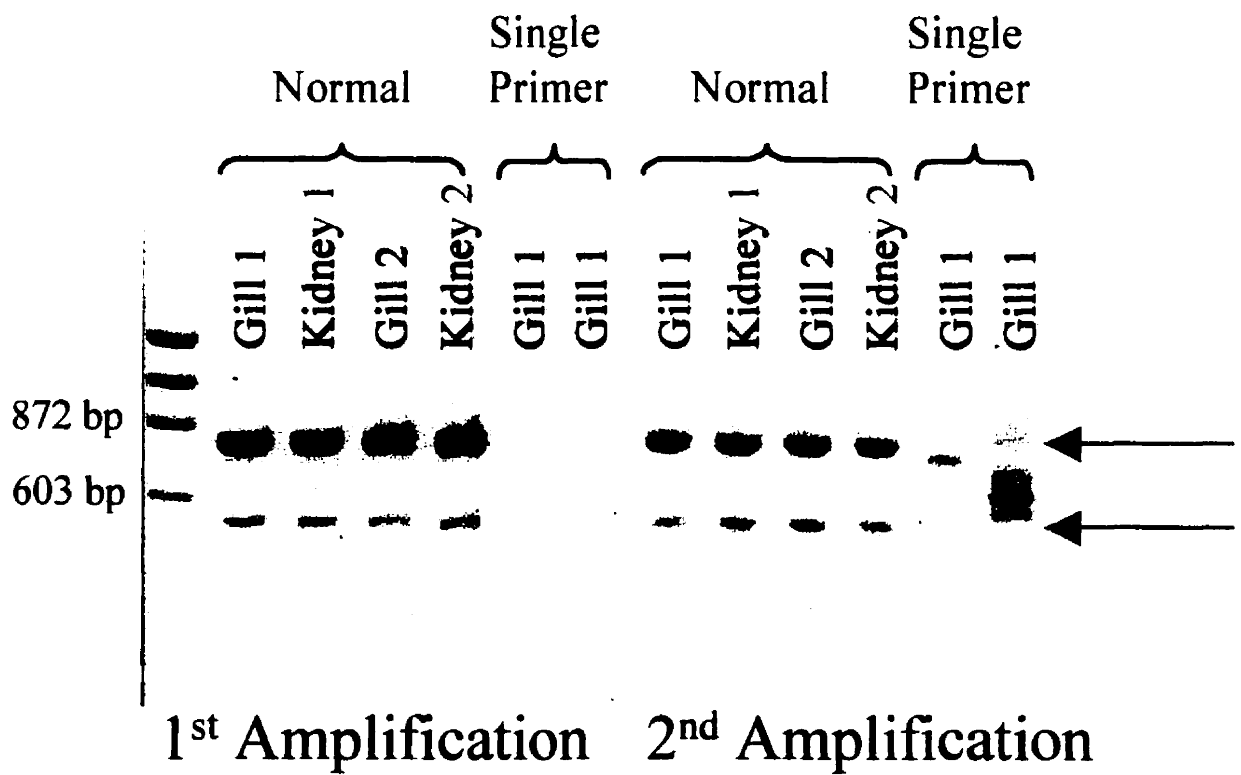

Normal
Single Primer
Normal
Single Primer
Gill 1
Kidney 1
Gill 2
Kidney 2
Gill 1
Gill 1
Gill 1
Kidney 1
Gill 2
Kidney 2
Gill 1
Gill 1
872 bp
603 bp
1st Amplification
2nd Amplification

Fig. 2.4. Schematic of primer design and direction of amplification for the nested PCR on the cDNA library. Primer names are indicated. The direction of the arrows indicates the direction of the polymerization with Taq polymerase, assuming the primers bind properly and a product is amplified. A double-headed arrow indicates that a primer was made in both directions. Primers are shown in relation to the *Xenopus* α-ENaC subunit, although all known α-ENaC subunits were used in the determination of the primers. Primer sequences are listed in Table 2.1.

FG(M/L)MYWQF →

α-xENaC MTKEEKNEKEALIEFFSSYRELFEFFCSNTTIHGAIRLVCSRRNRMKTAFWLVLFLVTFGLMYWQFGLLFGQYFSYPVSINLNVNSDKLPFPAVTVCTLN 100

PYRYKAIQNDLQELDKETQRTLYELYKYNSTGVQGWIPNNQRVKRDRAGLPYLLELLPPGSETHRVSRSVIEEELQVKRREWNIGFKLCNETGGDCFYQT 200

YGNCYTFN ↔

YTSGVDAIREWYRFHYINILARVPQEAAIDGEQLENFIFACRFNEESCTKANYSSFHHAIYGNCYTFNQNQSDQSNLWSSSMPGIKNGLTLVLRTEQHDY 300

IPLLSSVAGARVLVHGHKEPAFMDDNGFNIPPGMETSIGMKKETINRLGGKYSDCSEDGSDVDVKNLFQSEYTEQVCVRSCFQAAMVARCGCGYAFYPLS 400

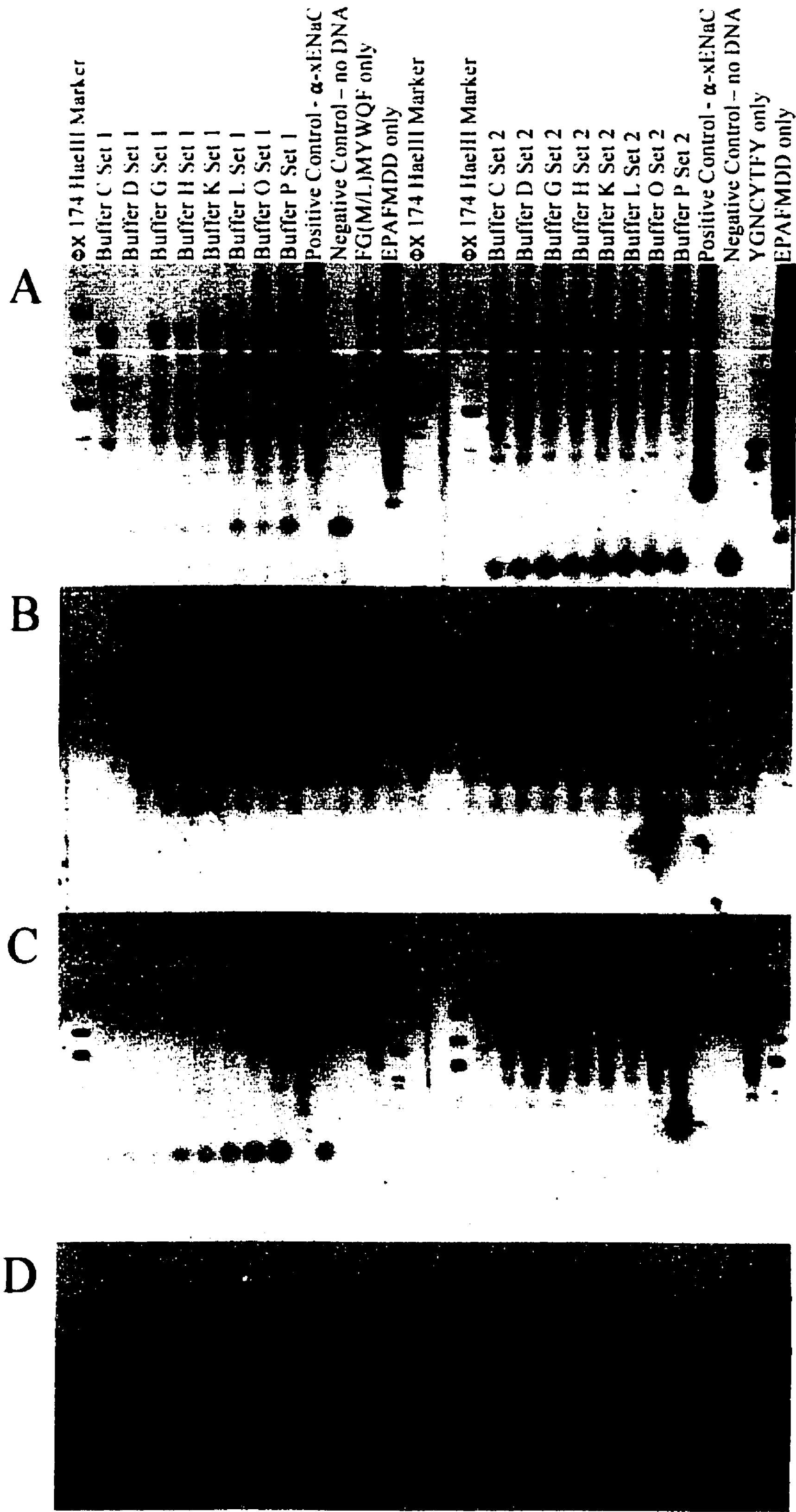
← EPAFMDD ← Puoti

PGDQYCDYNKHKSWGHCYYKLIIEFTSNKLGCFTKCRKPCLVSEYQLTAGYSKWPNRVSQDWVLHTLSRQYNLTDRNGIAKLNIYFEELNYKTILESPTI 500

NMAMLLSLLGSQWSLWFGSSVLSVVEMLELVIDFVIIGVMILLHRYYYKKANEGEETTVVPTPAPAFADLEQQVPHIPRGDLSQRQISVVADITPPPAYE 600

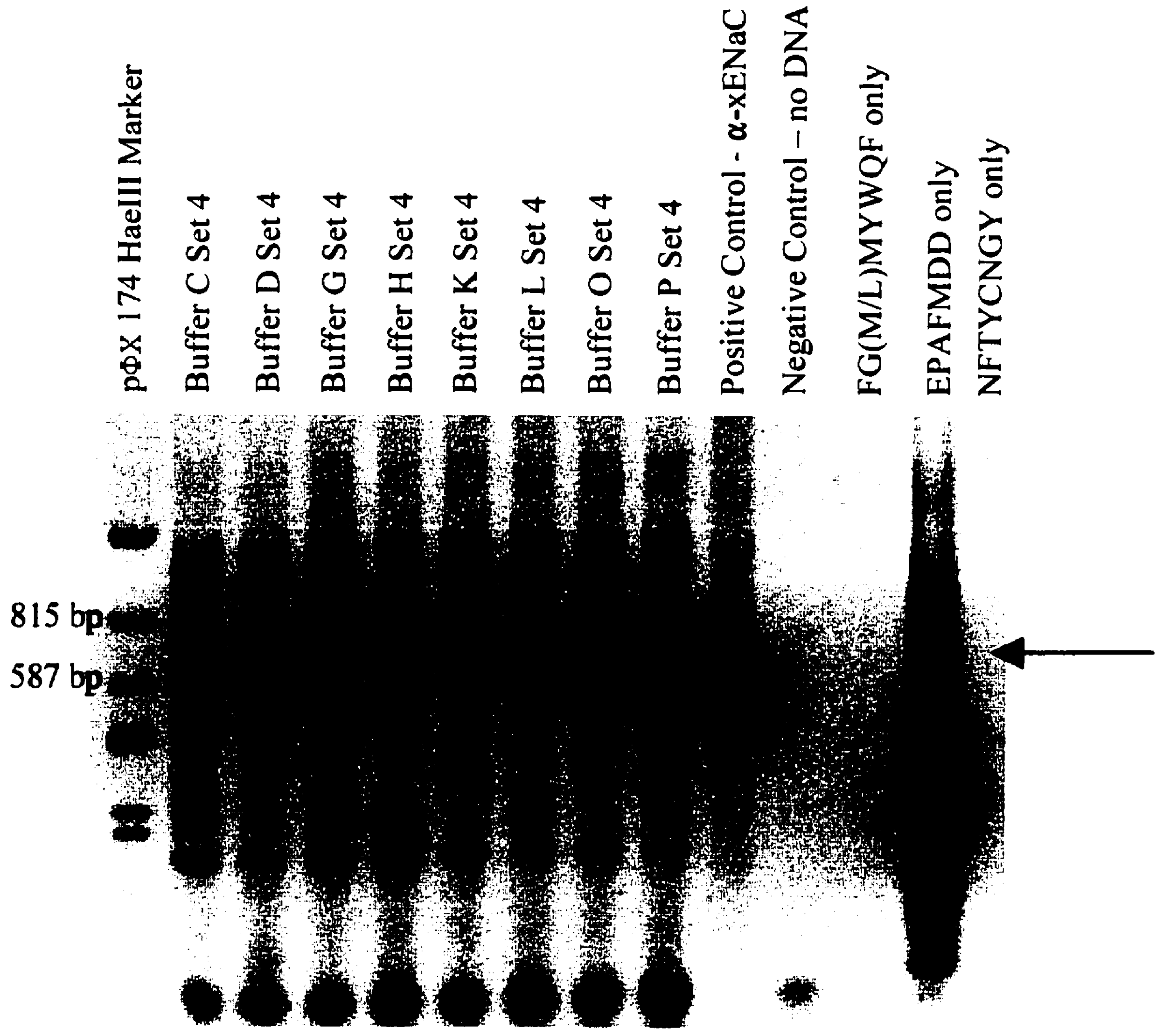
SLDLRSVGTLSSRSSSMRSNRSYYEENGGRRN 632

Fig. 2.5. Temperature/Buffer Series of PCR on the cDNA Library. Buffers C, D, G, H, K, L, O and P. Temperatures 42°C (A), 47°C (B), 52°C (C) and 57°C (D). Products from Primer Sets 1 (FG(M/L)MYWQF & EPAFMDDG) and 2 (YGNCYTFN & EPAFMDDG) are illustrated. Primer Set 3 (YGNCYTFN & Puoti), which produced no products, was omitted for the sake of clarity. All four temperature series reactions are arranged in the same order. Reactions were carried out as follows: 4 *s* at 94°C, 30 *s* at 42, 47, 52, or 57°C, 1 min at 72°C for 30 cycles in 0.2 μM dNTP's, 0.05 U Taq, either Buffer C, D, G, H, K, L, O, or P, and 5 μM each primer using 4μl of the library as template.



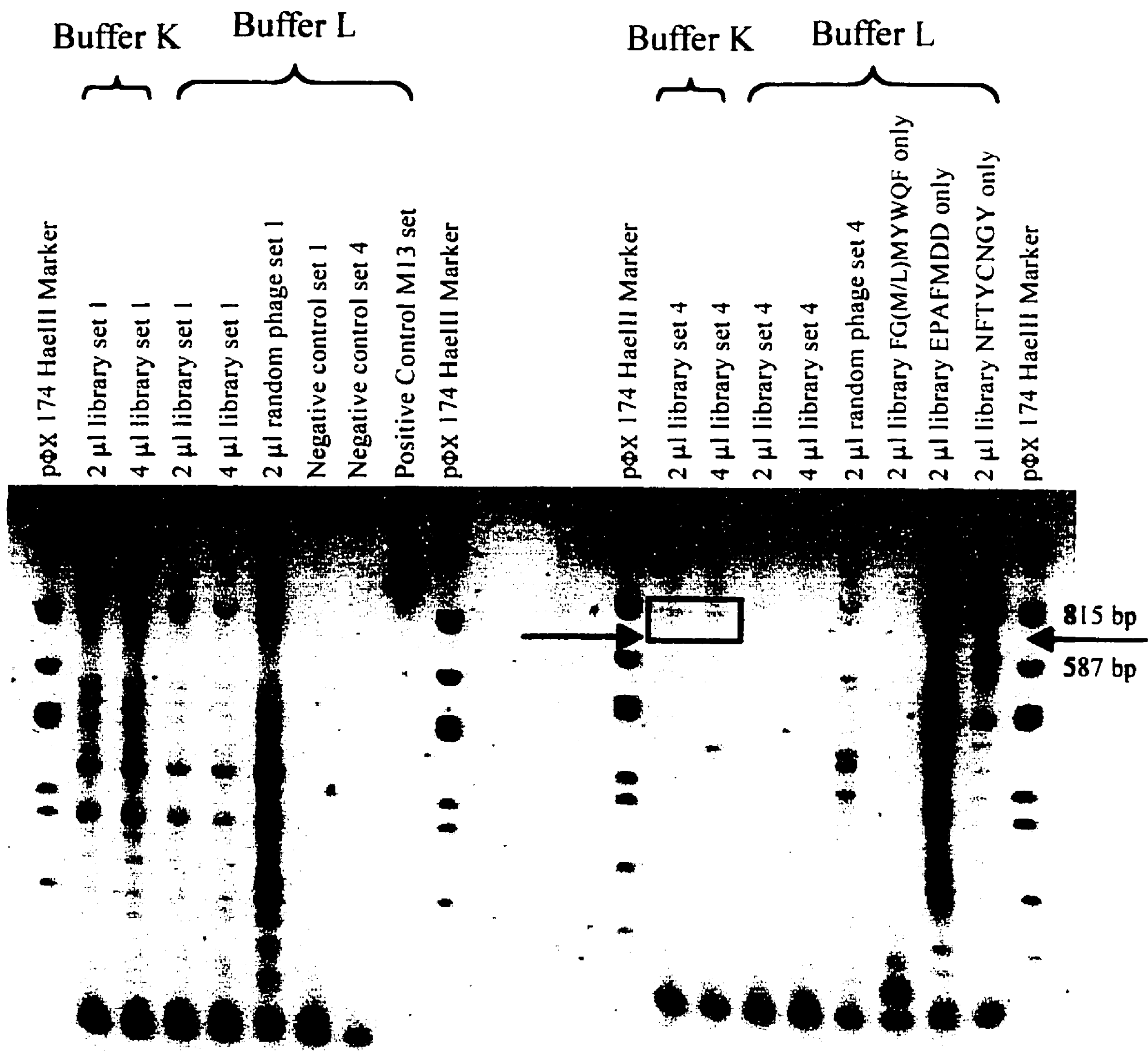
A
B
C
D
ΦX 174 HaeIII Marker
Buffer C Set 1
Buffer D Set 1
Buffer G Set 1
Buffer H Set 1
Buffer K Set 1
Buffer L Set 1
Buffer O Set 1
Buffer P Set 1
Positive Control - α-xENaC
Negative Control – no DNA
FG(M/L)MYWQF only
EPAFMDD only
ΦX 174 HaeIII Marker
ΦX 174 HaeIII Marker
Buffer C Set 2
Buffer D Set 2
Buffer G Set 2
Buffer H Set 2
Buffer K Set 2
Buffer L Set 2
Buffer O Set 2
Buffer P Set 2
Positive Control - α-xENaC
Negative Control – no DNA
YGNCYTFY only
EPAFMDD only

Fig. 2.6. Results of semi-nested PCR on the cDNA library with a 52°C annealing temperature and using Primer Set 4 (FG(M/L)MYWQF & NFTYCNGY) and inoculated from a similar reaction with Primer Set 1(FG(M/L)MYWQF & EPAFMDD). Reactions were carried out as follows: 4 *s* at 94°C, 30 *s* at 52°C, 1 min at 72°C for 30 cycles in 0.2 μM dNTP's, 0.05 U Taq, either Buffer C, D, G, H, K, L, O, or P, and 5 μM each primer using 4μl of the library as template for the first amplification and 0.5 μl of that reaction to initiate the second. Size of marker bands is indicated. The arrow indicates the approximate position of the expected product (~626 bp).



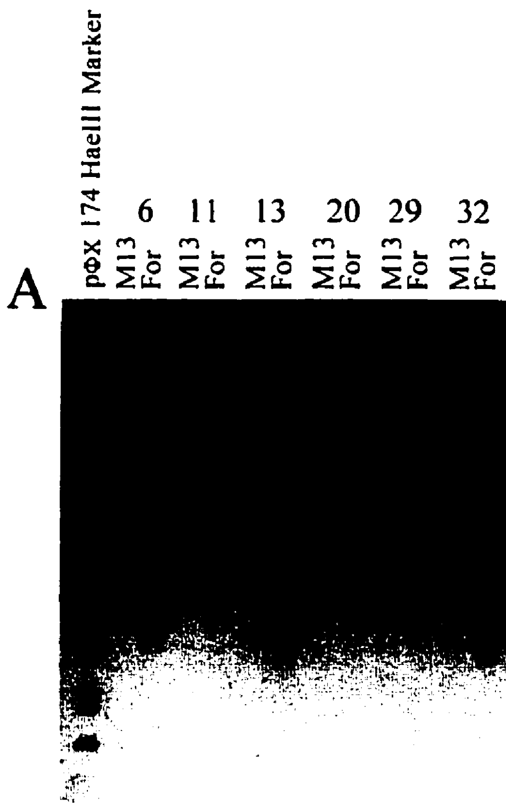
pΦX 174 HaeIII Marker
Buffer C Set 4
Buffer D Set 4
Buffer G Set 4
Buffer H Set 4
Buffer K Set 4
Buffer L Set 4
Buffer O Set 4
Buffer P Set 4
Positive Control - α-xENaC
Negative Control – no DNA
FG(M/L)MYWQF only
EPAFMDD only
NFTYCNGY only
815 bp
587 bp

Fig. 2.7. Results of PCR with Primer Sets 1 and 4 on the cDNA library after elimination of contaminating α-xENaC. Reaction annealing temperature is 55°C for 30 cycles. All other variables are as listed above. The expected product size for the reaction was 626 bp and its approximate position is indicated by arrows. The positive control is pSFP1 (the fragment of the B subunit of the H^+-ATPase) in plasmid form. The negative control consists of a normal reaction with water substituted for DNA. The fraction isolated for cloning is indicated by the box. Size of marker bands is indicated.



Buffer K
Buffer L
Buffer K
Buffer L
pΦX 174 HaeIII Marker
2 µl library set 1
4 µl library set 1
2 µl library set 1
4 µl library set 1
2 µl random phage set 1
Negative control set 1
Negative control set 4
Positive Control M13 set
pΦX 174 HaeIII Marker
pΦX 174 HaeIII Marker
2 µl library set 4
4 µl library set 4
2 µl library set 4
4 µl library set 4
2 µl random phage set 4
2 µl library FG(M/L)MYWQF only
2 µl library EPAFMDD only
2 µl library NFTYCNGY only
pΦX 174 HaeIII Marker
815 bp
587 bp

Fig. 2.8. Results of single primer screening of possible ENaC clones with FG(M/L)MYWQF primer. Colonies were screened by colony PCR. Those that showed clones of approximately the correct size were then re-amplified by PCR using only the single primer FG(M/L)MYWQF. A) Alternating PCR's of M13 primers (M13) and FG(M/L)MYWQF only PCR's (For) on plasmid preparations. Note that all of these products are able to be amplified using both means, thus indicating that the clones are single primer products in nature. B) Screening of cultures, boiling lysis preps of cultures or plasmid preps of cultures using the same method as described above. All three methods of plasmid amplification work equally well, thus allowing for quick screening of single primer clones by colony PCR.


A
pΦX 174 HaeIII Marker
M13 For 6
M13 For 11
M13 For 13
M13 For 20
M13 For 29
M13 For 32

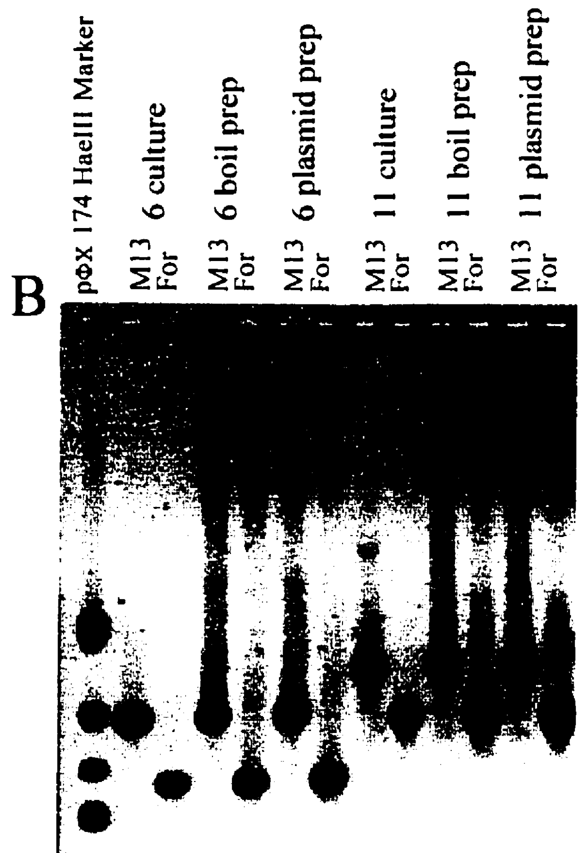

B
pΦX 174 HaeIII Marker
M13 For 6 culture
M13 For 6 boil prep
M13 For 6 plasmid prep
M13 For 11 culture
M13 For 11 boil prep
M13 For 11 plasmid prep

Fig. 2.9. CODEHOP primers. Schematic of primer design and direction of amplification for the nested PCR on the cDNA library. Primer names are indicated. The direction of the arrows indicates the direction of the polymerization with Taq polymerase, assuming the primers bind properly and a product is amplified. A double-headed arrow indicates that a primer was synthesized in both directions. Primers are shown in relation to the *Xenopus* α-ENaC subunit, although all known α-ENaC subunits were used in the determination of the primers. Primer sequences are listed in Table 2.1.

```
α-xENaC   MTKEEKNEKEALIEFFSSYRELFEFFCSNTTIHGAIRLVCSRRNRMKTAFWLVLFLVTFGLMYWQFGLLFGQYFSYPVSINLNVNSDKLPFPAVTVCTLN   100

          PYRYKAIQNDLQELDKETQRTLYELYKYNSTGVQGWIPNNQRVKRDRAGLPYLLELLPPGSETHRVSRSVIEEELQVKRREWNIGFKLCNETGGDCFYQT   200
                                                                            RT-ENaC-for1 ──────►
          YTSGVDAIREWYRFHYINILARVPQEAAIDGEQLENFIFACRFNEESCTKANYSSFHHAIYGNCYTFNQNQSDQSNLWSSSMPGIKNGLTLVLRTEQHDY   300

          IPLLSSVAGARVLVHGHKEPAFMDDNGFNIPPGMETSIGMKKETINRLGGKYSDCSEDGSDVDVKNLFQSEYTEQVCVRSCFQAAMVARCGCGYAFYPLS   400
                                ◄────► RT-ENaC-for2, rev1
          PGDQYCDYNKHKSWGHCYYKLIIEFTSNKLGCFTKCRKPCLVSEYQLTAGYSKWPNRVSQDWVLHTLSRQYNLTDRNGIAKLNIYFEELNYKTILESPTI   500
                             ◄────── RT-ENaC-rev2
          NMAMLLSLLGSQWSLWFGSSVLSVVEMLELVIDFVIIGVMILLHRYYYKKANEGEETTVVPTPAPAFADLEQQVPHIPRGDLSQRQISVVADITPPPAYE   600

          SLDLRSVGTLSSRSSSMRSNRSYYEENGGRRN   632
```

Fig. 2.10. Result of RT-PCR on cortisol and soft water treated gill total RNA with RT Primer Set 3 (RT-ENaC-for2 and RT-ENaC-rev2). The reaction consisted of two separate steps, the first of which was 10 cycles at 48 °C annealing temperature and the second was 35 cycles at 53 °C. The band of interest, which was slightly larger than predicted but only present in samples with both primers, is marked by a box. This band was excised from the gel and the DNA cloned.

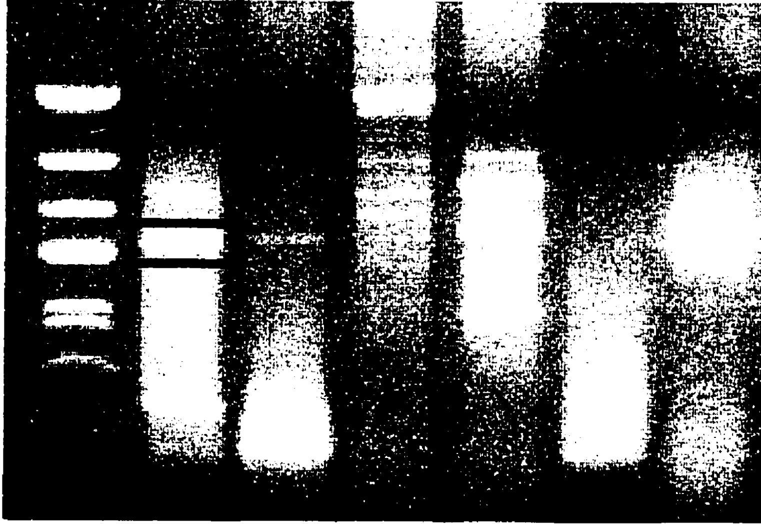
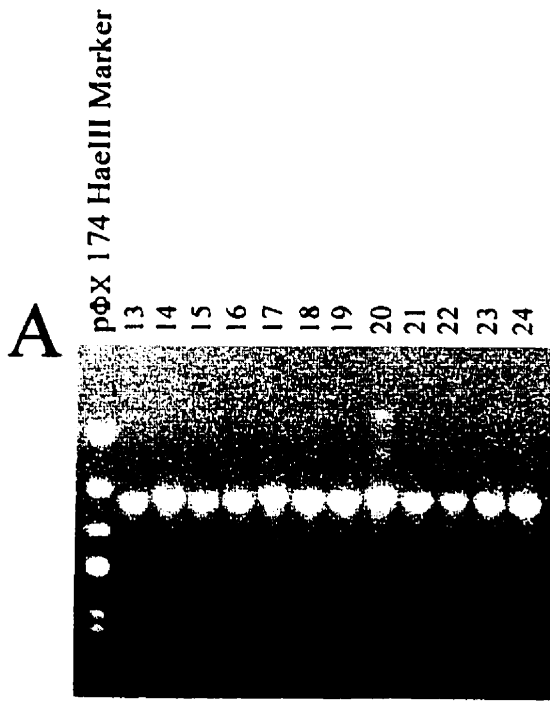
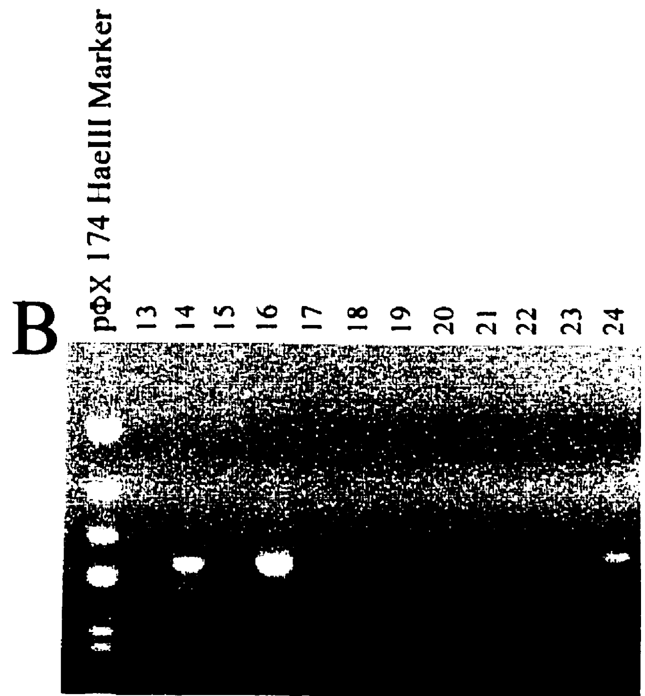

pΦX 174 HaeIII Marker
Cortisol cDNA, Set III
Soft H_2O cDNA, Set III
Soft H_2O cDNA, RT-for2 only
Soft H_2O cDNA, RT-rev2 only
No cDNA, Set III
Soft H_2O cDNA, β-actin primers

Fig. 2.11. Colony PCR and RT-PCR primer screening of clones generated by RT-PCR. A) Colony PCR screening of some clones generated by RT-PCR with RT-ENaC-for2 and RT-ENaC-rev2 as outlined in Fig. 2.10. B) Screening of the same clones in A) with the RT-ENaC primers before sending them off for sequencing. Only three of these twelve clones (14, 16 and 24) were created with the RT-ENaC primers. A fourth clone, that was also sequenced, is not shown here.


A
pΦX 174 HaeIII Marker
13
14
15
16
17
18
19
20
21
22
23
24


B
pΦX 174 HaeIII Marker
13
14
15
16
17
18
19
20
21
22
23
24

Discussion

The original goal of this project was to clone a portion of the epithelial sodium channel from rainbow trout to add support to Avella and Bornancin's theory (1989) of how trout regulate their whole body sodium content. Several attempts were made to clone subunits of this channel, however, a concentrated effort was made to clone the α-subunit since the most sequence data are available for this subunit and it was thus considered the easiest to clone.

Other research groups (cf. Chapter 2, Introduction) had cloned subunits of the channel by expression cloning and physiological tests. Essentially, all the mRNA in a tissue was cloned into a cDNA library, pooled and *in vitro* transcribed. The resulting cRNA pools were then injected into *Xenopus* oocytes, having little or no endogenous sodium channel activity themselves, and the oocyte was assayed for a change in sodium uptake rates and sensitivity of these rates to amiloride. The pool of cRNA which caused this change was then further subdivided and the procedure repeated until a single clone was isolated. This method is technically challenging in that it requires knowledge of cDNA library synthesis and manipulation, micro-injection and experience in measuring electrical potential changes across an oocyte.

A different method was used by other research groups (cf. Chapter 2, Introduction) and could be employed only after the first method had produced some sequence data about the channels. The basic plan was to use freely available sequence data to design primers to amplify a portion of a channel subunit sequence *via* PCR or RT-PCR. With RT-PCR even the smallest amount of a specific sequence is theoretically able to be amplified. This was an especially important requirement for the cloning of ENaC considering the estimated low numbers of active ENaC channels on a cell membrane (as noted in the Introduction).

With a full understanding of these techniques, the protein structure of the α-ENaC was examined by creation of a protein alignment of available cloned sequences (Appendix A). The alignment was scanned visually for regions that were conserved among all of the organisms examined. These regions would be used to design the primers based on the premise that a conserved region in several organisms is likely to be conserved in an unknown organism, like rainbow trout. Sub-regions were chosen which contained the most number of least degenerate amino acids. Primers were then designed, except in the case of the RT-ENaC-for and -rev primers, that were fully degenerate; that is, they contained all possible nucleotide sequences coding for the given amino acid.

Over the course of the experiment, three groups of primers were designed, each of which was an improvement upon the previous. The three groups of primers were: 1) Primer 1, Primer 2 and Primer 3, 2) FG(M/L)MYWQF, YGNCYTFN, NFTYCNGY, Puoti and EPAFMDD and 3) RT-ENaC-for1, RT-ENaC-for2, RT-ENaC-rev1 and RT-ENaC-rev2 (as described in Table 2.1). Each of these groups of primers was used to some degree in an attempt to amplify a portion of the ENaC from trout and each entailed certain faults which impeded their function. All of the primers were to be used in either RT and then PCR or direct PCR on a cDNA library. The use of a cDNA library, or RNA, assumes that the tissue source used to create the library, or perform the RT, contains the expressed mRNA of the gene for ENaC.

Primer Group 1

The first group of primers was never fully tested in either RT or library PCR. Although this primer set was taken over to Scotland where it was thought it would be tested in RT-PCR, it was quickly abandoned, without full testing, in favor of CC-for and CC-rev, designed by Dr. Chris Cutler

of the University of St. Andrews, because his primers appeared initially to provide the fragments of DNA desired (Fig. 2.3). Unfortunately, these fragments did not turn out to be the epithelial sodium channel, nor anything recognizable by the GenBank database (last examined Jan. 2000), and in the meantime Primer group 1 had been set aside.

The experiments in this thesis dealt solely with total RNA and perhaps that was an error. Since less than 2% of total RNA is mRNA and the transcript being looked for is thought to be expressed at a low level, it might increase the chance of having a transcript in the reaction by beginning with mRNA. As well, these RT-experiments worked solely with an oligo-(dT) primer for the RT and did not examine the possibility of using a specific primer. Since all mRNA transcripts have a Poly(A)$^{+}$ tail, the use of an oligo-(dT) primer is non-specific. For the amplification to work then, the primers must bind to the correct specific transcript despite the large number of non-specific transcripts in the mix. The use of a specific primer in the RT reduces this "sorting" by only allowing reverse transcription of transcripts to which the primer binds. This translates to a smaller number of cDNA transcripts for the primers to "sort" through during the amplification process.

Lastly, this set of primers (Primer 1, Primer 2 and Primer 3) were designed to be used as nested primers, meaning that for example, Primer 1 would be used in conjunction with Primer 3 for one PCR reaction and that a small portion of the DNA amplified from that reaction would be used to initiate a second reaction with Primers 2 and 3. The theory is that a non-specific product may be generated by the first reaction, however, it is likely that a specific product was generated as well. Non-specific products might be generated by the PCR because the primers were designed to be degenerate in the hopes that they would pick up a sodium channel sequence. However, in that design is a certain amount of error because only one, of the hundreds of combinations of primers in the primer pool, will be the

exact match to the trout epithelial sodium channel.

For example, in the case of the combination of Primers 2 and 3, there are 512 separate primers in the pool. Primer 2 is 64 fold degenerate (2 fold degenerate in 6 positions) and Primer 3 is 8 fold degenerate (2 fold degenerate in 3 positions). Only two of those 512 primers will exactly match the trout epithelial sodium channel, if any, while the other 510 primers are subject to priming any other piece of DNA that they might match and thus create non-specific products in the reaction. Although a perfect match is not required for amplification, the less exact the match, the less likely amplification is to occur. This method further assumes that the unknown sequence (e.g. trout) matches the known sequences in the location of the primer.

The introduction of a second set of primers allows the isolation of those specific products by altering the previous amplification pattern in favor of the desired product. The primer set was never tested as designed due to the fact that the experiment only employed Primers 2 and 3 (cf. Chapter 2, Results). Therefore, a small number of specific products may have been created by the reaction but have remained hidden under a large amount of non-specific product waiting for the second, nested, reaction to bring it to the surface.

To fully examine these primers they would have to be used in RT-PCR experiments on rainbow trout RNA under a variety of conditions. Several variables would have to be tested including: annealing temperature of the primers to the template, magnesium concentrations, primer concentration and pH of the reactions. Also to be tested would be the amount of cDNA, synthesized in the RT, to be included in the PCR, the amount of RNA to include in the RT reaction and whether it would be better to make cDNA from total RNA or from mRNA.

CC-for and CC-rev Primer Amplifications

The use of primers CC-for and CC-rev constituted a significant portion of these experiments and their failure to perform as desired deserves some discussion. These primers, designed by Dr. Cutler, were intended to amplify any or all of the ENaC subunits and were inherently degenerate to a large degree. It is perhaps this degeneracy which contributed most to their failure to work, however, it should be noted that this primer overlaps the same region as Primer 1 in Primer Group 1 and that both of these are contained within the putative transmembrane domain of ENaC.

It should also be noted that these primers were not fully optimized and so their inability to produce results is by no means a definitive condemnation of their design. To fully test these primers, optimizations of buffer, annealing temperature, primer concentration and initial quantity of cDNA should be performed. These tests were not done because initial experiments (Fig. 2.3) produced results that appeared to be significant.

The idea of designing degenerate primers to amplify any or all of the ENaC subunits is not, in and of itself, flawed but the use of these primers should be coupled with a second PCR utilizing primers that are more specific to a single subunit. As a last point, these primers may have failed because their location within the transcript is primarily towards the 5' end. Their successful use assumes extension of the RT enzyme from the Poly(A)$^{+}$ tail nearly to the translation initiation point. The 3' untranslated region is variable and its exact length, in rainbow trout, is unknown. In the α-ENaC sequences in Table 1.1, for example, it ranges from 242 - 1397 bp and may, therefore, be kilobases in length. If this is the case for trout, placement of the primer sets towards the 5' end of the transcript requires a large, continuous RT product; a requirement that might not be realistic.

Primer Group 2

This primer group was designed to be more versatile than the first by including more primers and allowing more nested sets. It also included primers that had been shown to perform for others (YGNCYTFN and Puoti were utilized, with 42°C annealing temperatures, by Puoti *et al.* 1995) in the hopes that they would function again. This particular experiment was plagued by contamination of the α-xENaC positive control. The use of this cDNA as a control is perhaps the greatest fault of this experiment and if it was to be repeated this should definitely be rectified. The difficulty with using this cDNA as a positive control is that its sequence exactly matches one primer in each of the pools of primers because it was used to construct the degenerate primers. Thus a small amount of contamination with the plasmid, through aerosols or any other means, taints the entire experiment, as was seen here. For future reference, this plasmid, or any others of the type, should not be used within the same laboratory. In fact, there is no need for these clones until a trout clone exists and experiments such as the creation of a trout/*Xenopus* hybrid channel in *Xenopus* oocytes are desired to be carried out.

The use of this primer group was especially educational concerning transmembrane domains and the desire to avoid them while designing primers. Transmembrane domains are regions of proteins that pass through the plasma membrane of the cell and by their nature tend to be extremely hydrophobic, which limits their amino acid composition primarily to the hydrophobic amino acids. The primer FG(M/L)MYWQF lies within the transmembrane domain, as does Primer 1 from the first primer group, and although it was designed to specifically amplify ENaC sequences the results here show that it also amplifies *E. coli* sequences. In fact, it amplifies *E. coli* sequences by acting as a primer in both directions, a technique known as the Random Amplification of Polymorphic DNA

(RAPD). The source of the *E. coli* DNA amplified is from the cDNA library and is produced by the lysis of the cells as the phage grows. It is impossible to completely eliminate the contaminating *E. coli* DNA and normally this would not be necessary, except that the primer used in the PCR matched the contaminating DNA and may have interfered with proper amplification of the ENaC desired. Further, in at least one case shown here, GLMYWQ of the FG(M/L)MYWQF primer aligns by BLASTX with GMQSWQ of *E. coli* Membrane-associated Protein UIDC Precursor (data not shown), another transmembrane protein like ENaC (See Table 2.2). This shows that at least two transmembrane proteins share similar sequence in their transmembrane domains.

Although this experiment did test various annealing temperatures, buffer magnesium concentrations and pH's, as well as testing the amount of the library required and using a random phage to try to eliminate background bands, the experiment was never fully completed. Among the experiments not done was a complete analysis, given the variables of temperature, buffer, etc., of nested PCR with the various primer sets. Only the combination of PCR with Primer Set 1 (FG(M/L)MYWQF & EPAFMDD) followed by Primer Set 4 (FG(M/L)MYWQF & NFTYCNGY) was ever fully examined. Considering the nature of the construction of a cDNA library, as stated above, it seems slightly insufficient, in hindsight, to test with nested PCR a primer set located nearer to the 5' end than the 3' end. Also considering what is now known about the FG(M/L)MYWQF primer, it would be interesting to know what would result from a buffer and annealing temperature titration and nested PCR experiment with Primer Sets 1 and 2 (YGNCYTFN and EPAFMDD) or 1 and 3 (YGNCYTFN and Puoti). Further, although this primer was never designed, it might be interesting to look at using EPAFMDD as the forward primer in conjunction with Puoti as the reverse primer. All three of these combinations of primers are located more towards the 3' end of the

transcript which may increase the possibility of an amplification product.

Primer Group 3: RT-PCR Revisited

With the knowledge that the cDNA library had contaminating *E. coli* DNA that was being amplified by Primer Group 2, it was decided to return to RT-PCR as a means of avoiding any DNA other than that desired. The primers (RT-ENaC-for1, RT-ENaC-for2, RT-ENac-rev1, RT-ENaC-rev2) were positioned within the 3' end of the coding sequence in an attempt to avoid the possible problems outlined above. They were not designed as fully degenerate but instead utilized the rational of the CODEHOP program. The logic for not being fully degenerate was that possibly the degeneracy of previous primers was aiding the amplification of non-specific products and that the specific primers in the primer pool that matched the trout ENaC clone were being made ineffectual by the sheer numbers of the other primers. It was hoped that by following the rational of CODEHOP (Rose *et al.* 1998), the 3' degenerate core would anneal to the trout ENaC cDNA but that the 5' consensus clamp would prevent rampant degeneracy of the entire primer and would, in fact, aid in later rounds of amplification.

There were three primer sets within this group: 1) RT-ENaC-for1 & RT-ENaC-rev1, 2) RT-ENaC-for1 & RT-ENaC-rev2 and 3) RT-ENaC-for2 & RT-ENaC-rev2. The combination of RT-ENaC-for2 & RT-ENac-rev1 was not used as they are essentially the same primer in opposite directions and would not result in a product. The objective of this experiment was to use primer set 2 in an initial PCR followed by primer sets 1 and/or 3. However, as a starting point, each of the primer sets was attempted individually on cDNA created by RT with an oligo-(dT) primer. The annealing temperature cycles used (10 cycles at 48 °C, followed by 35 cycles at 53 °C) were suggested by Dr.

Vance Trudeau at the University of Ottawa and are the result of his experience in amplifying low expression level mRNA's for proteins such as glutamate dehydrogenase (GAD) from a multitude of piscine organisms.

Primer set 3 produced a band, slightly larger than expected, that was not present in the single primer controls but was present in the cortisol treated gill cDNA. This band was pursued because, although a size was predicted from the available amino acid sequences of other organisms, the expected size was not an absolute. Lack of time prevented a proper titration of PCR conditions, as outlined above, as should have been done.

The fact that this band turned out to be a product relating to trout collagen may be a direct result of the use of oligo-(dT) as the primer for the initial RT. Using this primer, all mRNAs within the tissue were reverse translated and available for the PCR reaction. Although the primers used in the reaction cannot be found in the sequences analyzed, for reasons unknown, it is possible that the low temperature of the PCRs contributed to mis-priming. The CODEHOP primers are designed to amplify specifically in the first few rounds by the inclusion of their 3' degenerate cores. However, if these degenerate cores mis-prime and produce false products, the 5' consensus clamp will continue to allow those mis-primed products to be amplified. The intended purpose of the consensus clamp is to allow amplification in the later stages of the PCR even if all the primers matching the original sequence have been used up in previous rounds (Rose *et al.* 1998). As such, the primers may have functioned exactly as intended except for the initial stage where they mis-primed.

As a suggestion for revision of this experiment, the primer used in the initial RT, using Poly(A)$^+$ RNA, should be a degenerate primer based on the ENaC sequences and the annealing temperature used in the PCR should be higher to avoid mis-priming events. Even using a degenerate primer in the

initial RT will eliminate a large amount of unrelated mRNAs. The annealing temperature of the PCR reaction should be increased since the primers were designed to have annealing temperatures between 57 and 62°C, perhaps decreasing mis-priming events.

Future Work: Redesigning the Experiment

The lack of results in this Chapter does not illustrate the inability to ever isolate an ENaC from rainbow trout, but instead demonstrates experience that can only come from the learning process. The physiological evidence that an Epithelial Sodium Channel exists in rainbow trout (see Introduction) is compelling and the inability to isolate one in these experiments does not invalidate the evidence for its presence.

With the information gathered in this thesis, concerning how not to design the experiment, the experiment could be reinvented and hopefully produce results. There are, however, several improvements to be made in the design and execution of the experiment. First, a set of primers should be designed that is located towards the 3' end of the coding sequence. These primers should be designed to be used as semi- or fully nested and should not be contained within the putative transmembrane domains. There is no evidence to date that CODEHOP-type primers are any better than fully degenerate primers and this represents another variable to be tested. These primers should also be designed to allow an increase in the annealing temperature and to ensure no self-complementarity (stem-loop structures) nor complementarities between primers. The fact remains that the CODEHOP primers designed in this experiment have yet to be fully tested due to time constraints and may still produce positive results. Once again, it is suggested to concentrate on the α-subunit of ENaC because the most sequence data is available for this subunit.

Secondly, the general lab technique should be improved and may include such measures as allocating all components of the PCR(e.g. dNTP's, Mg^{+2}, water, primers, cDNA) into small samples that can be used once and then discarded and re-autoclaving all tips and tubes that remain unused after a PCR is set up.

In any PCR reactions carried out, single primer controls, negative (no DNA) controls, and positive controls should be included. The positive control ensures that the PCR worked and should consist of a completely different sample and primer set than the experimental to prevent cross-contamination. In carrying out positive controls for PCR, cDNA, from one of the sequences used to create the primers, should be avoided entirely. These cDNAs will certainly be amplified by the primers and may possibly contaminate the components of the PCR by aerosol or other means. Negative controls, using the same primers as the experimental, ensure that no contaminating DNA has entered the reaction. Negative controls for each primer set should be employed regularly. Single primer controls will help differentiate single primer products from the expected double primer products and thus help eliminate false positives.

Lastly, it is suggested to use Poly(A)$^{+}$ mRNA along with a specific RT primer in the initial RT reaction. These two modifications should: 1) increase the number of cDNA molecules synthesized by increasing the proportion of mRNA and 2) increase the number of ENaC cDNA molecules synthesized by increasing the proportion of those cDNAs to total cDNA.

When these suggestions are coupled together with buffer magnesium and pH titrations, as well as titration of the annealing temperature, and the use of nested or semi-nested primers, a product of the correct size should become apparent. Once a product of the expected size appears and is located only in the double primers and no contamination of the controls is seen, this product should be cloned

and sequenced. If the product is related to Epithelial Sodium Channels by BLASTX search, but is not so closely related as to be exactly the same as a previous clone, the ENaC from trout may be declared cloned and the full length cloning from the cDNA library may be initiated.

The portion cloned may be used to screen the cDNA library in a manner similar to colony lifts and Southern hybridization. The full length clone, once isolated, can be *in vitro* transcribed along with the β and γ subunits from either rat or *Xenopus*, and micro-injected into *Xenopus* oocytes. The physical properties of the trout ENaC can then be measured and compared to physiological data concerning sodium uptake in the intact rainbow trout thus adding molecular evidence to support Avella and Bornancin's theory.

Chapter 3

Northern Hybridization Analysis and mRNA Quantification of the V-type H^+-ATPase B-subunit in Rainbow trout (*Oncorhynchus mykiss*)

Introduction

A Proton-translocating-Adenosine-Tri-Phosphatase (H^+-ATPase) constitutes the second part of the model of sodium uptake from freshwater as described by Evans (1982) and Avella and Bornancin (1989). The three types of proton-ATPases (P-Type, V-Type and F-Type) are differentiated by their location, mode of operation, inhibitors and function; however, they all serve to move protons across a charged cellular membrane (Penderson and Carafoli, 1987).

The plasma membrane type (P-Type) ATPase consists of two subunits, operates by way of a phospho-enzyme intermediate and is inhibited by Vanadate, VO_3^-, which acts as a phosphate analogue in their phospho-enzyme complex (Penderson and Carafoli, 1987). It serves as a housekeeping enzyme to regulate intra-organellar and intra-cellular pH (Nelson, 1992).

Neither the mitochondrial-ATPases (F-Type) nor the vacuolar type (V-Type) ATPases utilize a phospho-enzyme intermediate and are probably derived from a common ancestor (Forgac, 1989). F-type ATPases are inhibited by sodium azide and are composed of 18 subunits while the V-Type ATPase is inhibited by bafilomycin A_1/ concanamycin A and is composed of 16 subunits. The F-Type ATPases are located only on the inner mitochondrial membrane and synthesize ATP by turning the electrochemical energy of protons falling down their concentration gradient into the chemical energy of ATP.

The V-Type H^+-ATPase (V-ATPase) performs the same function as the P-Type ATPase and operates by converting ATP to ADP + P_i and coupling the released energy to the translocation of approximately 3 protons (Al-Awqati and Dixon, 1982). V-ATPases are found on the plasma membranes of various acid secreting or ion-regulating epithelia such as the insect midgut and

Malpighian tubules, freshwater teleost and elasmobranch gills, amphibian skin and urinary bladder, reptile urinary bladder, rat epididymis, bovine corneal epithelium, and mammalian kidney and osteoclasts (See Table 1.2 for a list of references).

The V-ATPase is composed of two sectors, a V_0 membrane bound sector and a V_1 catalytic sector. The V_1 sector is made up of 6 proteins labelled A-F and having molecular masses of 68, 57, 44, 28, 26 and 14 kDa. The stoichiometry is such that 3A:3B:1C:1D:1E:1F make up one V_1 sector (Arai *et al.* 1988). The A-subunit is the catalytic subunit while the B-subunit is regulatory. Both together contain the ATP binding site (for a review, see Nelson and Klionsky, 1996). The V_0 membrane bound sector is composed of several subunits but its exact composition has not yet been fully determined. It is primarily made up of the proteolipid *c* subunits which have been shown to be the proton-translocating subunits. Additional subunits of the V_0 sector are: *a*, M16, M115, M45 and M39 (Nelson and Klionsky, 1996).

The proton ATPase (either V- or P-Type) is electrogenic (Steinmetz and Anderson, 1982; Ehrenfeld *et al.* 1985) and can, therefore, be used to create the electrochemical gradient needed for sodium transport in freshwater fish (Lin and Randall, 1995). Pumps can be added or removed from the plasma membrane according to various environmental stimuli, such as hypercapnia (Brown, 1989; Laurent *et al.* 1994; Schwartz and Al-Awqati, 1985; Stetson, 1989). The localization of V-ATPases on the freshwater fish gill (Lin and Randall, 1991; 1993; Laurent *et al.* 1994; Sullivan *et al.* 1995;1996) provides strong support for the proton-ATPase/sodium channel model.

The goals of this Chapter are to show molecular evidence of V-ATPase B-subunit gene expression in the RNA of rainbow trout and to determine if differential regulation of this subunit exists under physiological stress by attempting to quantify the level of expression.

Materials and Methods

Surgical Procedures/Tissue Isolations

Fish were anaesthetized in 1:12,000 (weight/volume) benzocaine (ethyl-*p*-aminiobenzoate) and after cessation of breathing movement were transferred to a surgical table where the gills were irrigated with the same anaesthetic solution. A small opening was made just posterior to the anus and just ventral to the lateral line. The caudal vein and artery were exposed and cannulated with indwelling polyethylene cannulae (Clay-Adam PE 50; ID=0.58 mm, OD=0.97 mm), after the basic method of Soivio *et al.* (1975). After stitching the wound, the fish were revived and allowed to recover for 24 h in opaque acrylic (perspex) boxes that were supplied with flowing water.

For tissue isolations, fish were sacrificed by a blow to the head. They were dissected with a ventral incision just posterior to the pectoral fins through the muscle wall. The cut was extended, while care was taken not to tear the heart, from the anus to the opercula. A small incision was made in the ventricle through which a catheter was inserted. The catheter was advanced through the ventricle into the bulbous arteriosus and clamped at the junction of the bulbous and the ventricle. The fish were then perfused, through the catheter, with at least 240 ml of ice cold 0.9% NaCl and the required tissues were dissected out in the order gills, kidney and liver. Upon isolation, the tissues were immediately frozen in liquid nitrogen.

Physiological Treatment of Fish

Cortisol implants were made by interperitoneal injections of 0.2 ml/100g of 75 mg/ml cortisol in coconut oil for a target dosage of 150 mg/kg, while controls were implanted with equivalent

volumes of pure coconut oil. Both groups of fish were kept in the 100 *l* tanks for a period of 4 days. Hypercapnia was carried out by exposing cannulated fish to 1% CO_2 in air for 1, 2 and 3 hours. The water suppling the boxes was equilibrated with CO_2 mixed with air, using a gas mixing pump and a gas equilibrium column, making an approximate P_{CO_2} of 7.5 torr. Blood and water variables were measured during the period of the hypercapnia and captured using the data acquisition program Acknowledge™. Reduced ion stress was simulated by the mixing of regular fish water with R.O. water to achieve a level of approximately 0.04 mM sodium, as measured by the AA Spec (cf. Chapter 2, Materials and Methods). This was modified from previous protocols calling for pure R.O. due to the fact that the stress was to great for survival of the fish. Fish were exposed in perspex boxes to 6 hr, 12 hr, 24 hr, 48 hr and 72 hr of reduced ion water (approximately 0.05 mM Na^+, 0.007 mM K^+ and 0.125 mM $^{Ca+2}$).

RadioImmuno Assay (RIA)

The ImmunoChem Coated Tube Cortisol ^{125}I RIA kit (ICN, Costa Mesa, CA) was used, according to the manufacturer's instructions, to measure cortisol levels in plasma samples. Blood was taken from fish by a caudal puncture and micro-centrifuged to pellet the red blood cells. The plasma was removed to a new tube and fast frozen in liquid nitrogen for later analysis.

RNA Gel Electrophoresis and Northern Transfer

RNA was isolated as described in the Materials and Methods of Chapter 2. RNA samples in DEPC H_2O were incubated with RNA loading buffer (1X MOPS {0.02 M 3-(N-Morpholino)Propanesulphonic Acid, 0.005 M Sodium Acetate, 0.8 mM Na_2^+EDTA, pH 7.0}, 50% v/v

deionized formamide, 2.1 M formaldehyde, 0.6% v/v glycerol, 0.7 mM EDTA, bromophenol blue and Ethidium bromide for visualization) at 65°C for 20 min prior to loading on the gel. Samples were loaded onto formaldehyde/agarose gels (1X MOPS, 1.5% w/v agarose, 5% v/v formaldehyde) and run in 1X MOPS buffer for approximately 4 hr at 100 V. The gels were photographed using a Polaroid MP-4 Land camera and Kodak 667 film. Gels were washed 2X20 min in ddH_2O, 1X20 min in 20XSSC and blotted onto Genescreen⁺ membrane (NEN Life Sciences) using capillary action and 20XSSC (Sambrook *et al.* 1989).

Slot Blots

Samples to be loaded on the slot blot apparatus (Schleicher & Schuell, Minifold II) were denatured with 50% v/v formamide, 2.2 M formaldehyde, 20 mM MOPS, 5 mM NaOAc and 1 mM EDTA at a ratio of 40 µl denaturation solution per 20 µg total RNA. The solution was heated to 65°C for 10 min and quick chilled on ice. It was then diluted with an equal volume of 20XSSC to make a final concentration of 10XSSC. Serial dilutions were made with equal volumes of 10XSSC so that the concentration of total RNA in the sample decreased by half with each dilution (i.e. 20 µg, 10 µg, 5 µg, etc.). Duplicate concentrations were loaded on each membrane. The Minifold was loaded with Genescreen⁺ membrane and GB002 filter paper (Schleicher & Schuell), all pre-soaked in 10XSSC. Vacuum was applied and the wells of the Minifold were rinsed with 10XSSC followed by addition of the denatured RNA solution and lastly the wells were rinsed again with 10XSSC. The wells were allowed to dry under vacuum pressure for 10 min following the final rinse and upon removal, the blots were cross-linked with a UV lamp for 5 min. Northern hybridization proceeded as described below.

Northern Hybridization

Probes for Northern hybridization were prepared in the same manner as for Southern hybridization (See Chapter 2, Materials and Methods). Probes consisted of an 810 bp portion the V-ATPase B-subunit from rainbow trout originally cloned by PCR amplification of the cDNA library (GenBank Accession # AAF140022) and the 18S rRNA cDNA clone from *Oncorhynchus kisutch* (Hervio *et al.* 1997).

Northern hybridizations were carried out as per standard conditions (Sambrook *et al.* 1989). 4 ml of hybridization mixture (5 X Standard Saline Citrate (SSC), 50 mM sodium phosphate buffer, 5 mM EDTA, 5 X Denhardt's solution, 0.15-0.25 mg/ml ssDNA, 48% v/v deionized formamide, 0.1% SDS) per 100 cm^2 of membrane was incubated with the blot for 4 hr at 65°C in a TekStar Hybridization oven (Model H010115). The pre-hybridization mixture was poured off and 2 ml of hybridization mixture/100 cm^2 of membrane, with the appropriate amount of probe (approximately $4x10^6$ CPM), was added to the blot and incubated over night at 65°C. The hybridization mixture was poured off and the blot washed four times at 65°C with 2 X SSC and 0.1% SDS for 15 min each followed by two washes with 0.1-0.5 X SSC and 0.1% SDS for 15 min each. The blots were then exposed to x-ray film (Kodak BioMax or XAR) for varying times at -80°C with an intensifying screen.

Results

The goal of this portion of the research was to examine the expression and characteristics of the B-subunit of the trout V-ATPase as cloned by Drs. Steve Perry and Douglas Johnson, of the University of Ottawa (Perry *et al.* 2000). Northern hybridization and mRNA quantification were used to examine the expression of the subunit, while sequence analysis was used to partially characterize its relationship to B-subunits in other organisms.

Northern Hybridization

RNA from various tissues of untreated rainbow trout was isolated, run on a gel and transferred to Genescreen* membrane, as stated in the Materials and Methods. The blot was probed with the 810 bp sequence of the B-subunit, exposed to film and followed by subsequent exposure to the 18S rRNA probe. The two images were superimposed upon one another to create the image in Fig. 3.1. The purpose of the dual exposure was to estimate, to a certain degree, equal loading among the samples and to assist in estimating the size of the transcript. As a required housekeeping transcript, the 18S rRNA is presumed not to change in expression level between treatment. If two samples were seen to be radically different in their 18S rRNA signal, the gel and blot were reconstructed and the process repeated. The V-ATPase B-subunit is seen to be expressed in all tissues examined, but is particularly strong in gill, kidney (anterior and posterior), heart and spleen while being reduced in the intestine and much reduced in the liver, blood and white muscle tissues. The large amount of expression in the gill and kidney may be related to their known capacity as ion-regulating tissues.

mRNA Quantification

From the Northern blot performed, it was unclear as to the level of expression of the V-ATPase mRNA. Further, it was desired to examine the effects of various physiological stresses on this expression. Therefore, a series of experiments was designed to quantify the level of mRNA expression *via* slot blot analysis and to test changes in this expression level with exposure of the fish to respiratory acidosis, simulated chronic stress and reduced ion stress.

Twelve treatment groups were designed with six fish per treatment group. Two groups were assigned as controls and consisted of fish kept in perspex boxes overnight after surgery (Box) and fish drawn directly from the large holding tanks without additional stress (Tank). A hypercapnic treatment composed the respiratory stress component of the experiment and the treatment groups included fish exposed to one, two or three hours of hypercapnia. Chronic stress was simulated with interperitoneal coconut oil implants (Cortisol) and controlled by sham implants (Sham). Reduced ion stress was simulated by the mixing of regular fish water with R.O. water and maintaining the fish in the mixed water for 6, 12, 24, 48 or 72 hours. This mixing was accomplished in a period of < 15 min over which ion levels were reduced from their normal levels of 0.156 mM Na^+, 0.02 mM K^+ and 0.37 mM Ca^{+2} to 0.05 mM Na^+, 0.007 mM K^+ and 0.125 mM Ca^{+2}, a reduction of approximately 66% (Fig. 3.2).

Cortisol levels for all treatments were measured, from blood samples taken with a caudal puncture, using a cortisol RIA kit (Fig. 3.3). All treatments show no change in basal cortisol levels, despite manipulation, except for the cortisol implant treatment. While the basal levels of cortisol in these fish are high, indicating error in accuracy (perhaps due to overextended sampling time) the precision of the results is assured by their consistency.

Gill, kidney and liver samples were isolated from all fish in the twelve treatments described above and fast frozen in liquid nitrogen for later RNA extraction. Gill and kidney were isolated because of their high level of expression of the V-ATPase (as seen in Fig. 3.1) and the theorized change in that expression with treatment due to their role in ion regulation. Liver was isolated as a control tissue as the level of expression was not expected to change with treatment. RNA samples from the three tissues and various treatments were run on a slot blot apparatus allowing the entire RNA sample to be drawn into a small area. The slot blots were probed with the V-ATPase and the amount of signal in each slot was scanned with a BioRad GS-525 Molecular Imaging System Phosphorimager and analyzed using the Molecular Analyst software (BioRad). The same blots were then re-probed with the 18S and rescanned with the phosphorimager. It was presumed that any residual V-ATPase signal would be masked by the much greater 18S signal. The object of using the two scans in concert was to correct for unequal loading of the slot blots by dividing the intensity of the V-ATPase signal by the intensity of the 18S signal. In this manner, ratios would be produced for every sample that would normalize each result with an internal standard. Unfortunately, the 18S signal does not appear as a linear response to the amount of RNA present on the slot blot and therefore, cannot be used as the internal standard (Fig.3.4).

Sequence Analysis

Using the NCBI GenBank database, a BLASTX search (Altschul *et al.* 1990) of the full length derived protein sequence of the V-ATPase (Perry *et al.* 2000) produced over 500 matches with B-Subunit proteins from vertebrates, invertebrates, plants and fungi all with random match expected scores of $< 1 \times 10^{-21}$. For clarity, representative sequences of the vertebrates and invertebrates were

chosen and aligned with ClustalW (Thompson *et al.* 1994) (Appendix B). The trout V-ATPase B-subunit protein sequence shows remarkable conservation with other known B-subunits; between 85 % identity with *Ascidia sydneinsis* Samea and 95% identity with *Anguilla anguilla.*

A phylogenetic tree (Fig. 3.5) was created using Phylogeny Inference Package (PHYLIP) (Felsensetin, 1993) and 19 of the sequences representing vertebrates, invertebrates, plants and fungi. PHYLIP uses several programs to infer the phylogeny of the given sequences and the user may choose options based on parsimony or distance, molecular clock or no molecular clock, rooted and unrooted trees and more. The phylogeny of the sequences was constructed with as few assumed variables as possible and included no assumption of a molecular clock, unrooted trees (no outgroup defined) and distance calculations between sequences. The tree was bootstrapped to measure of the confidence in each of the nodes. Bootstrapping is an accepted statistical measurement and involves sampling with replacement a known alignment and testing the number of times that a node appears in a tree. The more often that node appears, the more confidence can be had that the node is true (Felsensetin, 1993). These numbers are then transposed to the constructed tree, thus producing the final figure. The results show that the trout sequence groups with other vertebrate sequences and particularly with the brain isoforms of the B-subunit. The fact that it did not group with the kidney isoforms may suggest the presence of more than one isoform in rainbow trout.

Fig. 3.1. Tissue distribution of V-ATPase B-Subunit mRNA in perfused tissues as determined by Northern hybridization with the homologous 810 bp sequence of the V-ATPase and subsequent hybridization with the 18S rRNA cDNA sequence. The two autoradiographs are superimposed and the positions of the two transcripts are indicated by arrows. 20 μg total RNA is loaded in each well, as determined by OD.

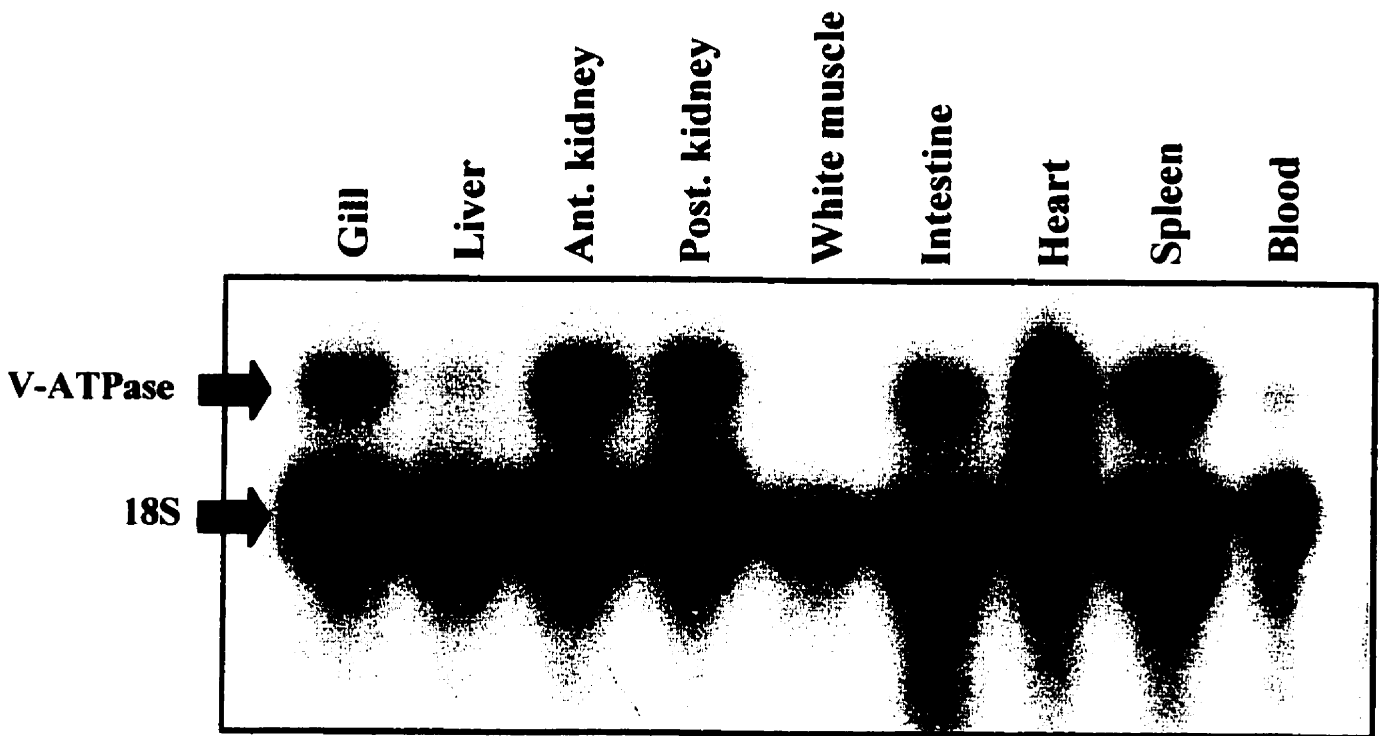
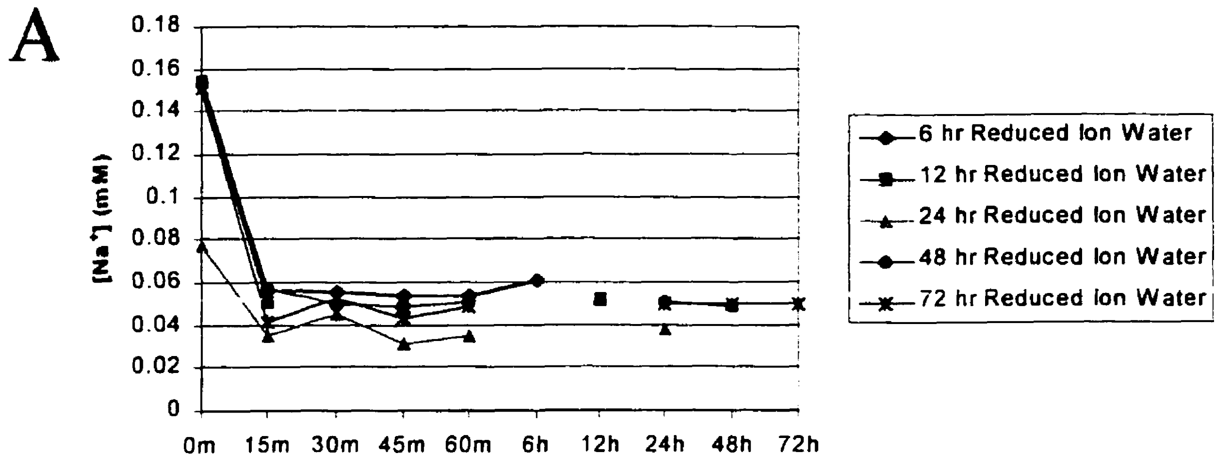
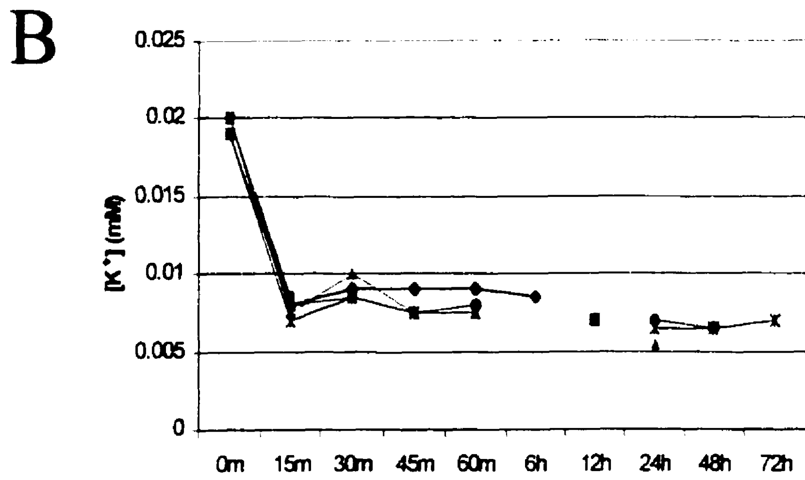

Gill
Liver
Ant. kidney
Post. kidney
White muscle
Intestine
Heart
Spleen
Blood
V-ATPase
18S

Fig. 3.2. Graphs of ion levels in reduced ion level water over time, as determined by flame emission spectrophotometetry. A) Sodium levels over time for all treatment groups. B) Potassium levels over time for all treatment groups. C) Calcium levels over time for all treatment groups. Samples were taken from 0-60 min in 15 min internals and then every 24 hours for all treatments except the 12 hr time course where only a 0, 15 min and 12 hr sample was taken. One sample per time period was taken and thus no statistical results are possible.

A



B



C

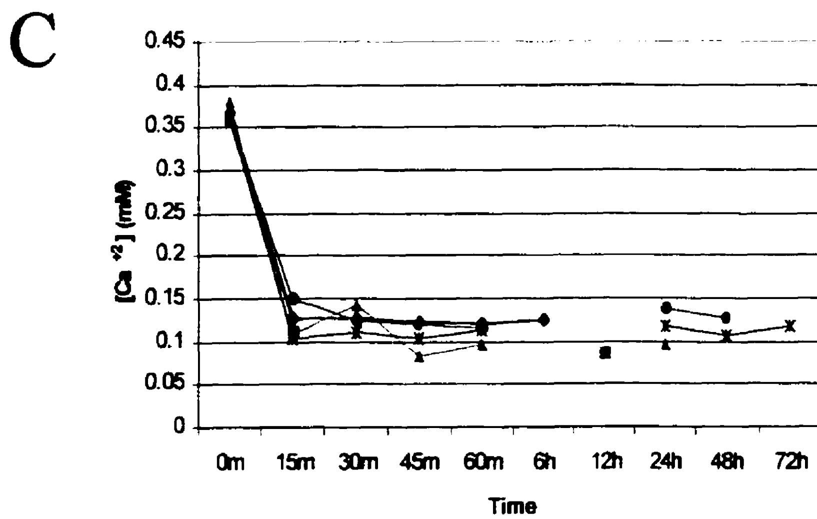
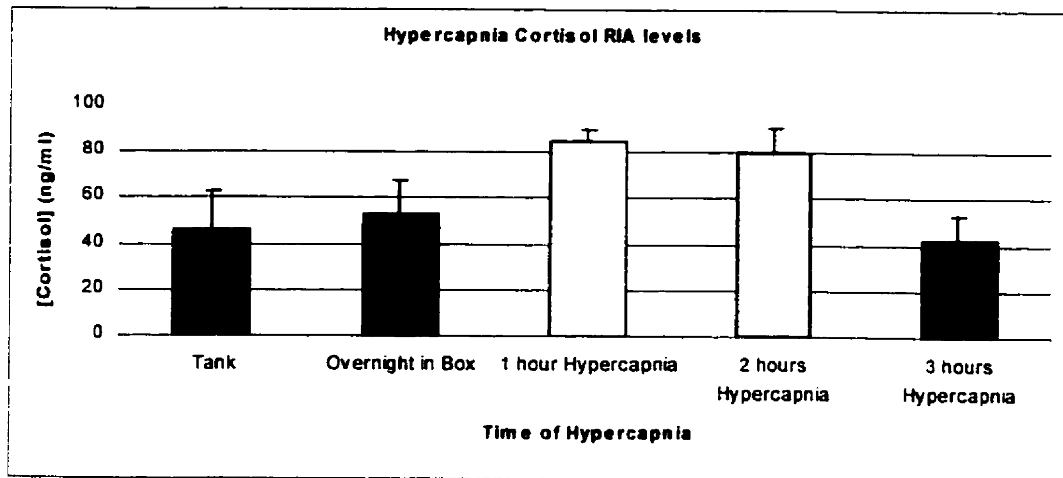


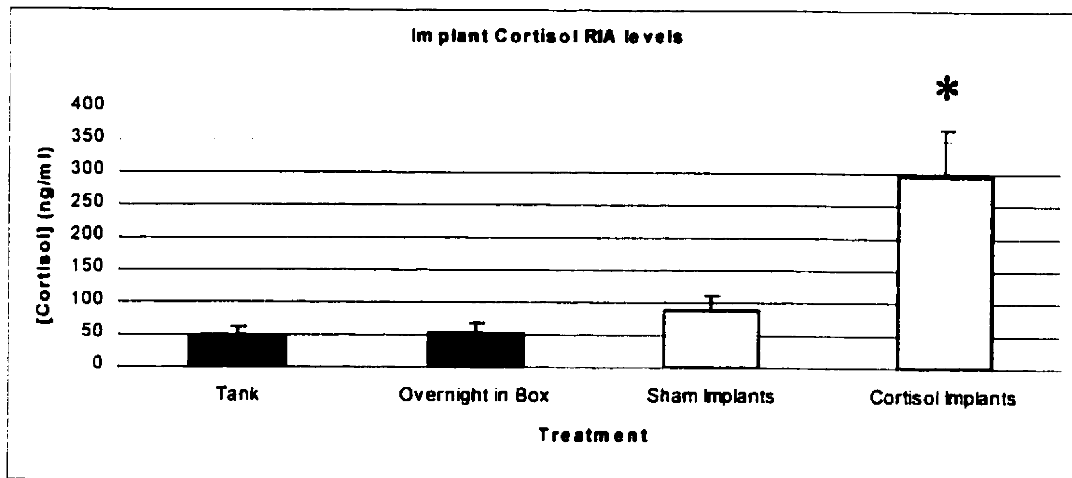
Fig. 3.3. Cortisol levels in treatment groups. A) Hypercapnic Fish, B) Cortisol Implanted Fish and C) Reduced Ion Water Exposed Fish. Samples were taken and measured as indicated in Materials and Methods, Chapter 3. Radioactivity was measured with a Packard Gamma Counter. All treatments have an n=6 (except Tank, n=4) and standard errors of the mean are indicated by the error bars. Data from Box and Tank Samples in A) were re-used in B) and C) to conserve the number of fish required in the experiment.

* Indicates that a One-Way ANOVA (Student-Newman-Keuls Method) using Sigma Stat 2.03™ determined a statistical difference ($P < 0.05$) between the Cortisol Implanted Fish and the Tank, Overnight in Box and Sham Implanted treatments. No significant difference exists in any of the other treatment groups.

A.



B.



C.

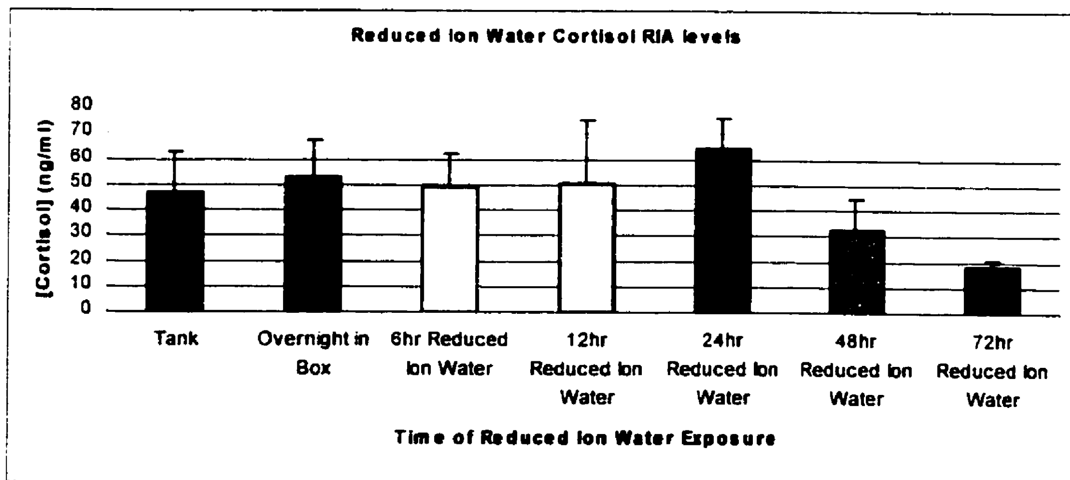


Fig. 3.4. Amount of radioactive signal present, as determined by phosphorimaging, in a given tissue sample vs. the amount of total RNA present. Gill and liver RNA are from trout Tank samples and Soybean nodule RNA is from *Glycine max* nodules. Serial dilutions were made from 20 μg to 0.625 μg and duplicate samples were loaded on one membrane. Points on the graph are means of the duplicate samples and therefore statistical analysis was impossible. Soybean RNA was included as a control for non-specific hybridization and was shown to cross-react with the 18S rRNA clone, but not with the V-ATPase clone. It is included here as evidence of the lack of a linear signal to sample ratio, contrary to what was expected. If the response of the 18S hybridization was linear, then the ~3600 counts per minute (cpm) gill sample at 20 μg loaded would change to 900 cpm with 5 μg loaded instead of the ~2000 cpm signal seen here.

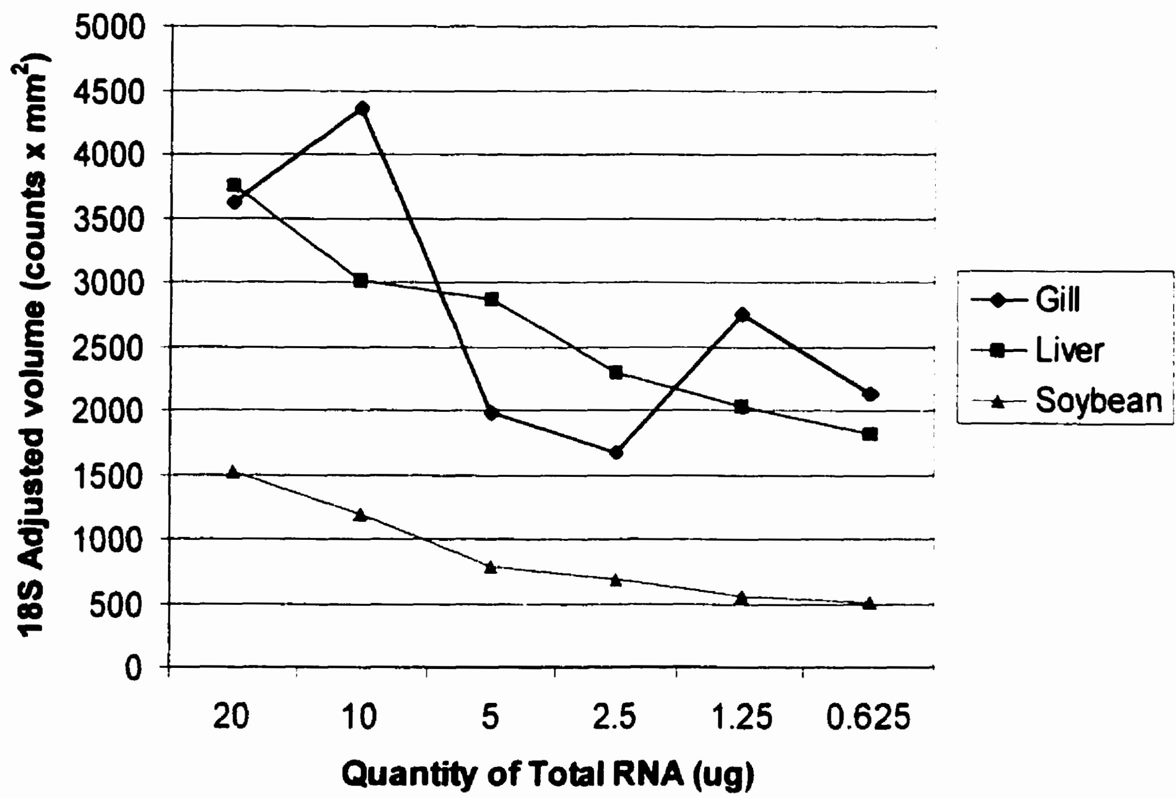
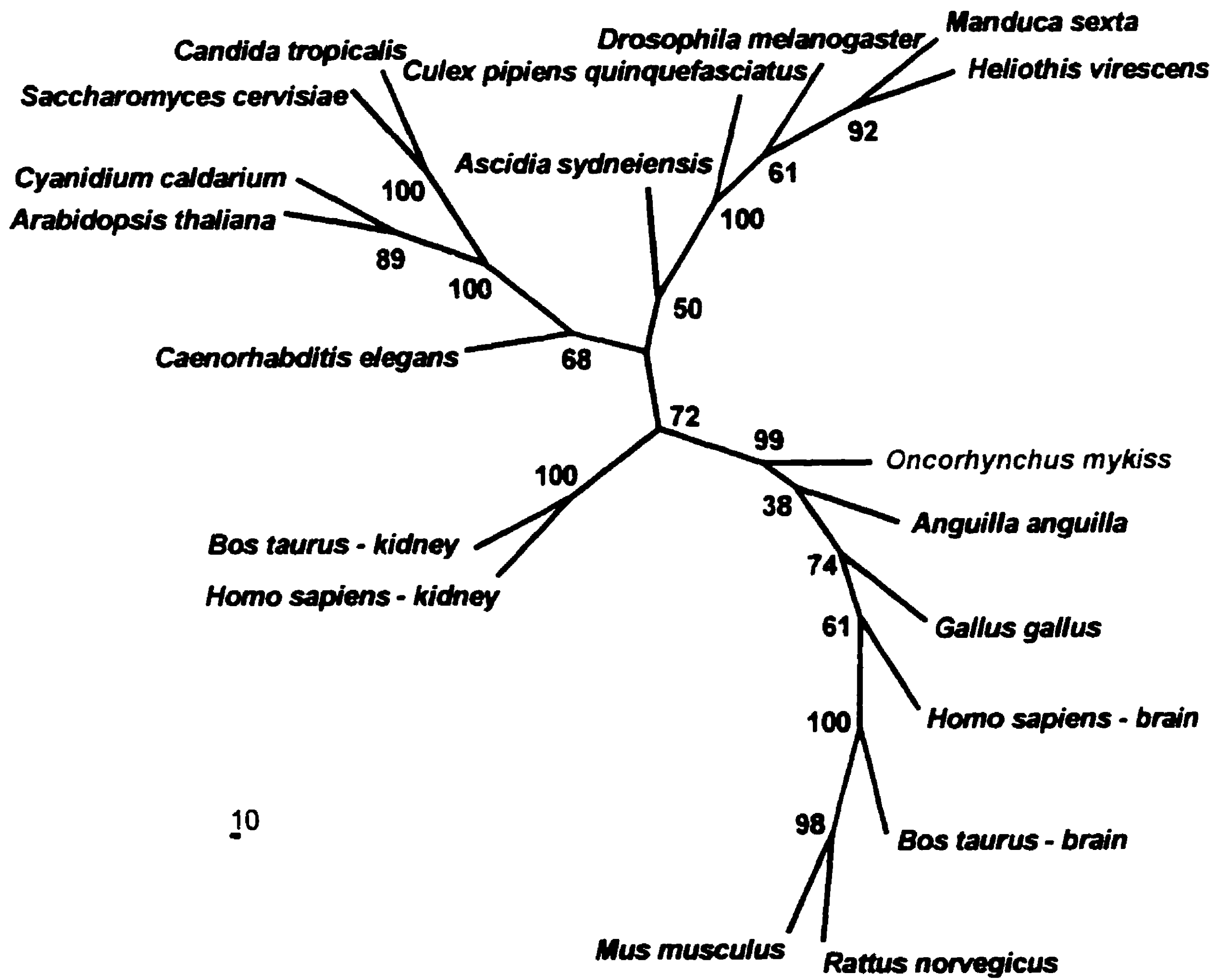

5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
18S Adjusted volume (counts x mm²)
20
10
5
2.5
1.25
0.625
Quantity of Total RNA (ug)
Gill
Liver
Soybean

Fig. 3.5. Phylogenetic tree of V-ATPase B-subunit constructed using ProtDist using the "PAM-Dayhoff" matrix followed by the Fitch algorithm to find the most likely tree. The tree was bootstrapped with Seqboot using 10 jumbles per data set and 100 multiple data sets for a total of 1000 trees. Bootstrapped values, indicated at the nodes of the tree, are a measure of the confidence in that node and are considered significant if the value is over 50. The tree was drawn by Treeview (Page, 1996) and labels were manipulated in PowerPoint2000. The sequences used in the tree (listed by GenBank nucleotide Accession number) are: *Manduca sexta* (X64353), *Drosophila melanogaster* (X67839), *Anguilla anguilla* (AF099743), *Gallus gallus* (U20766), *Bos taurus* (M88691-brain isoform), *Homo sapiens* (M25809-brain isoform), *Oncorhynchus mykiss* (AF140022), *Culex pipiens quinquefasciatus* (AF037468), *Ascidia sydneiensis* (AB016484), *Heliothis virescens* (S61797), *Caenorhabditis elegans* (U41015), *Candida tropicalis* (X54875), *Saccharomyces cerevisiae* (J04450), *Arabidopsis thaliana* (J04185), *Cyanidium caldarium* (U17101), *Mus musculus* (Y12634), *Homo sapiens* (M60346-kidney isoform), *Bos taurus* (M88690-kidney isoform) and *Rattus norvegicus* (Y12635). The rainbow trout branch is highlighted in purple. The scale bar indicates distances in units of the fraction of amino acids differing between two sequences.


Candida tropicalis
Saccharomyces cervisiae
Cyanidium caldarium
Arabidopsis thaliana
Caenorhabditis elegans
Culex pipiens quinquefasciatus
Drosophila melanogaster
Manduca sexta
Heliothis virescens
Ascidia sydneiensis
Oncorhynchus mykiss
Anguilla anguilla
Gallus gallus
Homo sapiens - brain
Bos taurus - brain
Mus musculus
Rattus norvegicus
Bos taurus - kidney
Homo sapiens - kidney
100
89
100
68
50
100
61
92
72
99
38
74
61
100
98
100
10

Discussion

The goals of this project were to examine changes in the steady state expression level of the V-ATPase B-subunit caused by various physiological treatments, designed to elicit a response, and to examine the relatedness of the trout V-ATPase B-subunit to other known sequences.

Quantification of Expression Level Changes

The initial attempts at examining expression level were made using luminance (Perry *et al.* 2000), however, film was found to not provide a linear response to the signal over a wide range and many exposures were required. This limited the ability to process large numbers of samples and the alternatives, quantitative RT-PCR or slot blot analysis were considered. Slot blots were chosen for speed and ease of execution.

Slot blots are a simple method to ensure that the entire RNA sample is transferred to the membrane thus avoiding the problems of capillary transfer. When followed by Northern hybridization and exposure to a phosphorimager, an accurate estimate of the total radioactive signal in a slot is achieved. One pitfall of this, or any technique, is that small pipetting or concentration errors will alter the amount of signal seen and produce a false difference between samples. This can be corrected for by re-hybridizing the blot with a second probe that is constitutively expressed in all tissues, such as the 18S rRNA clone. When the radioactive signal from the first probe is divided by the radioactive signal from the 18S probe, a correction will be made that normalizes each sample with itself.

For reasons unknown, the 18S signal seen on the slot blots was not consistent with a linear response between amount of RNA loaded and radioactive signal seen. This inconsistency cannot be

due to pipetting error alone because an examination of Fig. 3.4 shows positive and negative increases in signal for the gill which are too great to be explained by this reason alone. Estimating the slope of the lines between the points, errors of up to a 10 fold addition of too much or too little RNA would have to be made to account for these observations. The possibility that the readings on the phosphorimager were inaccurate could be examined by counting each of the slots in a liquid scintillation beta counter.

An alternate hypothesis may have to do with the relative proportions of the two samples being examined. The mathematical manipulation of the V-ATPase and 18S signals (V-ATPase/18S), used to standardize two V-ATPase signals, may be providing false differences in V-ATPase sample signals by reason of the difference in order of magnitude between the V-ATPase and 18S signals. It is generally excepted that, all of the mRNA of a cell composes about 2% of the total RNA and of the remaining 98%, 18S and 28S rRNA make up about 90% with tRNAs and other rRNAs composing the rest. If the assumption is made that the 18S rRNA composes about half of the 90% listed above and if 20 μg of total RNA (9 μg 18S rRNA) are loaded on one slot and 10 μg total RNA (4.5 μg 18S rRNA) are loaded on another slot, it would be expected that half of the signal seen in the 20 μg sample would be seen in the 10 μg sample. However, this is not the case with the 18S (see Fig. 3.4) and may be related to the large amount of rRNA and the relatively small amount of a specific mRNA present in the sample. Even if the assumption is made that the V-ATPase mRNA composes 1% of the total mRNA, a sum that would be considered large, then the calculation makes the V-ATPase out to be 0.02% of total RNA a difference with 18S of about 2250 fold.

A better solution would be to use a comparison RNA that is expressed, if not at the same level then at least, in the same order of magnitude. One suggestion might be to use β-actin from rainbow

trout and in this manner achieve homologous mRNA signals for both of the probes. It remains to be determined if β-actin is constitutively expressed and does not change under physiological experimentation of the type done here. Another suggestion would be to use mRNA in the slot blots.

Once the expected linear response to each probe is seen, then examination of the hypercapnic, cortisol implanted and reduced ion water samples may be undertaken. With six samples per treatment group, statistical analysis of the molecular data can be performed and significant conclusions can be drawn as to the changes in steady-state expression levels of the V-ATPase B-subunit with physiological stress.

The cortisol RIA results (Fig. 3.3) show that, aside from the intended cortisol implants, none of the physiological treatments elicited a stress response as indicated by a rise in cortisol levels. Normal basal cortisol levels for a trout are around 5 ng/ml (Gamperl *et al.* 1994) and the levels shown here are consistently higher (between 18 and 84 ng/ml). The higher basal levels seen here may be due to over handling during sampling, however, the fact that they are consistent attests to the lack of an increase due to the physiological treatment.

Phylogeny of Rainbow Trout V-ATPase

It was known before the creation of the phylogeny that, at least in humans and cow, two isoforms of the B-subunit existed: a kidney and a brain isoform. The kidney isoform is located only within the kidney (Sudhof *et al.* 1989; Nelson *et al.* 1992) while the brain isoform is seen in all tissues examined (Puopolo *et al.* 1992). The two isoforms are largely identical except for variations in the amino and carboxy termini (Nelson *et al.* 1992). The examination of the relatedness of the rainbow trout V-ATPase B-subunit to other B-subunits produced results indicating that the trout B-subunit was

most closely related to the brain isoform. The fact that the trout B-subunit trees with the brain isoform when isolated from a gill/kidney cDNA library may indicate the presence of two isoforms in this organism as well. It remains to be determined if a second isoform is found in rainbow trout since to date they have only been found in mammals.

The tree is grouped into branches radiating from a central point and, as expected, the major "trunks" are composed of vertebrates, invertebrates and plants and fungi. For reasons unknown, the *C. elegans* clone "trees" with the plants and fungi, however, this may have to do with its ancestral nature as it is considered one of the more primitive invertebrates. The vertebrate sequences are divided into the brain and kidney isoform branches.

Chapter 4

General Discussion of Trout ENaC cloning and V-ATPase Expression

This thesis was composed to two separate molecular projects that were linked through a physiological model (Fig. 1.1). In both cases, molecular evidence was sought to confirm physiological experiments. In the case of the Epithelial Sodium Channel, the goal was to clone the α-subunit using PCR and then to examine its physiological characteristics. The V-ATPase had been cloned by Drs. Perry and Johnson and the object of the study was to examine its steady state expression levels under various physiological stresses.

ENaC Cloning

Cloning of the α-ENaC from rainbow trout was unsuccessful for a variety of reasons, not the least of which was inexperience. The experiment was plagued with problems of contamination by another ENaC subunit, being used as a positive control for proper function of the PCR reactions. Improper primer design also contributed to the lack of success due to the use of a region in the transmembrane domain. However, also contributing to the lack of success was incomplete testing of all the primer sets. It is still possible that a piece of the sodium channel subunit may be amplified, from the cDNA library or through RT-PCR, with the primer sets designed. Particularly interesting would be to examine the action of the primers NFTYCNGY and EPAFMDD (in the forward direction) with the Puoti primer in a second round of PCR that had been inoculated with DNA from a first round of PCR with the Primers FG(M/L)MYWQF (or another primer) and Puoti. Even though FG(M/L)MYWQF produced *E. coli* fragments, it almost surely produced ENaC products as well. However, two rounds of PCR with FG(M/L)MYWQF may have masked any ENaC fragments amplified. The issue then becomes how to separate the two different types of fragments and this may be possible with nested PCR, as suggested above.

Another interesting possibility is to re-examine RT-PCR and to begin with mRNA. Once mRNA is isolated from total RNA, the concentration of each mRNA transcript is increased relative to the amount of RNA added to the initial RT reaction. Considering that Puoti *et al.* (1995) needed to load 20 μg of mRNA on a gel to achieve a Northern blot that was visible and that Canessa *et al.* (1995) has calculated the number of ENaC proteins, and presumably mRNA transcripts, to be lower than most proteins, the next phase of using mRNA in RT-PCR may be required. Coupled with this step could be the use of a specific primer for RT, perhaps the RT-ENaC-rev2 or a similar primer, as opposed to using a oligo-(dT) primer to initiate the reaction. This would again increase the number of transcripts related to ENaC relative to the total number of transcripts and thereby increase the chance of achieving a product related to ENaC.

Lastly, there is one other possibility to consider. A BLASTP (protein database) search of GenBank with the *Xenopus* α-ENaC (Table 4.1) revealed the only other known sodium channel sequence from a fish, that of the *Fugu rubripes* (puffer fish) sodium channel 2 sequence (G.R. Riboldi Tunnicliffe, unpublished). Sodium channel 2 sequences have been shown to be similar in characteristics and sequence to epithelial sodium channels (Garcia-Añoveros *et al.* 1997), however, there is no data on the function of sodium channel 2 in *Fugu*. The sodium channel 2 sequences of *Fugu* appear to be equally related to the *C. elegans* MEC and DEG proteins and to the ENaCs of vertebrates (Fig. 4.1). There are several examples of proteins related to MEC and DEG found in vertebrates that are not ENaCs and the *Fugu* sequence appears to be one of these. The possibility exists that trout does not utilize an ENaC, as previously thought, and instead uses another type of sodium channel, perhaps similar to the *Fugu* sodium channel 2. More research should be done into the various acid sensing ion channels, proton-gated sodium channels and mechanosensitive sodium

channels as to their characteristics and mode of action. Should they prove to function in a manner that is consistent with the model proposed by Avella and Bornancin (1989) and supported by the physiological evidence of sodium uptake in rainbow trout, the possibility should be examined that trout uses this type of a channel instead of an ENaC. If this is the case, then the design of the primers needs to be re-examined and take into account these new protein sequences.

V-ATPase Quantification

The attempt made to quantify changes in the steady-state expression level of the mRNA (mRNA levels) for the V-ATPase made a good beginning. Some new internal standard needs to be found and perhaps β-actin will perform as desired. The physiological treatment and RNA preparations of tissues of those treatments have been completed. All that remains is to create slot blots of those treatments and to perform the hybridizations and phosphorimager scans. The data acquired from these scans will provide accurate ratios, assuming the internal standard responds in a linear fashion, that can be used to draw conclusions about the effect of the treatments at a molecular level.

The experiment was designed to test what is the "sensor" for increased transcription of the V-ATPase in trout. If the "trigger" is external acid-sensors, implying that more acid is entering the fish and needs to be exported, then an increase mRNA levels will be seen in the hypercapnia treated fish, provided that the time of exposure is sufficient to elicit the response. One, two or three hours may not be sufficient and longer periods may have to be examined in a similar fashion. If, however, the trigger is ion loss, the reduced ion water exposed fish will show a corresponding increase in mRNA levels for the V-ATPase. Again, this assumes the exposure times are sufficient and that the response is not so quickly turned on and off as to be lost between, for example, the six and twelve hours samples. If the

sensor is a general stress sensor, then perhaps the cortisol implanted fish will show an increase in mRNA levels that is not present in the sham implanted fish. Lastly, all three of these experiments may each elicit a similar increase in mRNA levels.

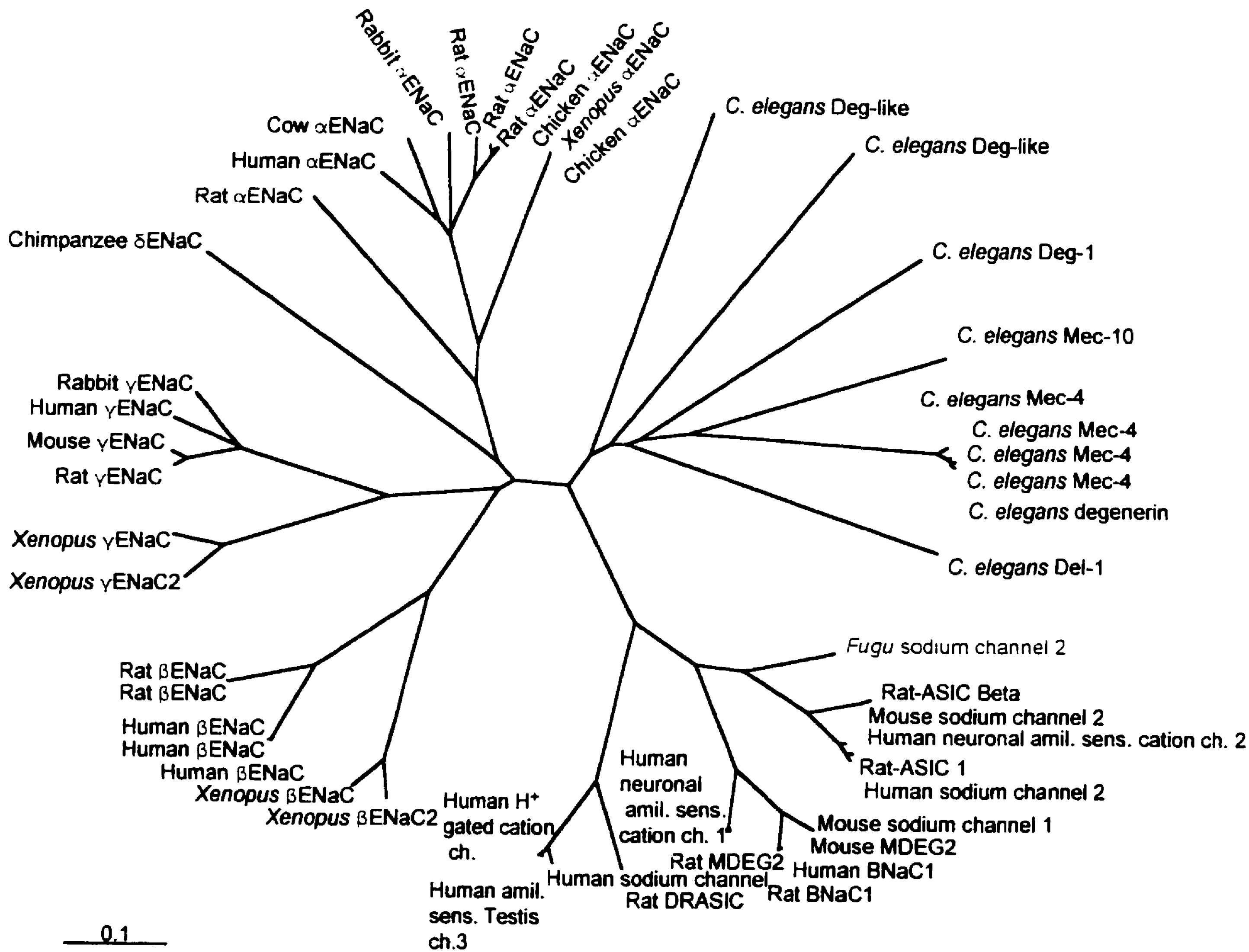
One experiment not included here that would be interesting is to see if fish exposed to base infusion, i.e. NH_4^+, will experience a decrease in mRNA levels for the V-ATPase in an attempt to retain protons within their cells.

By these experiments, it is hoped to gain a greater understanding of the physiological cause and molecular effects in rainbow trout. These experiments are designed to answer physiological questions but to use molecular tools in doing so. It is becoming clearer from the literature that the traditional physiological experiments are not answering the detailed questions being asked and that molecular tools are being more frequently employed. It was these type of questions that drove this research in the direction that it took and it is hoped that it will continue along the path outlined.

Table 4.1. GenBank Protein Accessions used in constructing phylogenetic tree of sodium channel sequences.

Accession#	Description	Organism
2734871	Sodium Channel 2	*Fugu*
3445468	ASIC-beta	rat
2500842	Proton Gated Cation Channel ASIC1	rat
4501863	Amiloride-sensitive Cation Channel2-neuronal	human
1871168	Sodium Channel2	human
2815275	Proton-gated Cation Channels Modulatory Subunit MDEG2	mouse
2500840	Amiloride-sensitive Brain Sodium Channel BNAC1	human
2815277	Proton-gated Cation Channels Modulatory Subunit MDEG2	rat
2500841	Amiloride-sensitive Brain Sodium Channel BNAC1	rat
4501861	Amiloride-sensitive Cation Channel1-neuronal	human
3702836	Proton-gated Cation Channel Subunit	human
4757710	Amiloride-sensitive Cation Channel 3-testis	human
2352949	Proton Gated Cation Channel DRASIC	rat
3097314	Sodium Channel	human
1872467	Sodium Channel 1	mouse
1871172	Sodium Channel 2	mouse
3024579	Amiloride-sensitive Sodium Channel Beta-2-subunit	*Xenopus*
1710871	Amiloride-sensitive Sodium Channel Beta-subunit	*Xenopus*
462589	Mechanosensory Protein 10 (Degenerin MEC-10)	*C. elegans*
4506815	Sodium Channel-nonvoltage-gated 1-alpha	human
1706358	Degenerin DEG-1	*C. elegans*
3023629	Degenerin-like Protein ZK770.1 in Chromosome I	*C. elegans*
4469399	Epithelial Sodium Channel Alpha Subunit	mouse
102528	Mechanosensory Protein 4	*C. elegans*
2135081	Epithelial Sodium Channel Beta Subunit	human
2507405	Mechanosensory Protein 4 (Degenerin MEC-4)	*C. elegans*
1326256	Mechanosensory Protein 4	*C. elegans*
1345441	Degenerin	*C. elegans*
4741594	EnaCB Amiloride Sensitive Sodium Channel Beta Subunit	human
2500839	Mechanosensory Protein 4 (Degenerin MEC-4)	*C. elegans*
4158224	Epithelial Sodium Channel Alpha Subunit	rabbit
1710869	Amiloride-sensitive Sodium Channel Alpha-subunit	*Xenopus*
2500838	Degenerin DEL-1	*C. elegans*
4506817	Sodium Channel-nonvoltage-gated 1-beta	human
458846	Epithelial Sodium Channel Alpha Subunit	rat
631885	Sodium Transport Protein Alpha Chain	rat
632251	Sodium Transport Protein Beta Chain	rat
2143976	Sodium Channel Protein-Amiloride-Sensitive	rat
458848	Epithelial Sodium Channel Beta Subunit	rat
2500845	Degenerin-like Protein C41C4.5 IN Chromosome II	*C. elegans*
2851470	Amiloride-sensitive Sodium Channel Alpha-subunit	cow
2500837	Amiloride-sensitive Sodium Channel Alpha-subunit	chicken
1465753	Na+ Channel Alpha Subunit	chicken
3024580	Amiloride-sensitive Sodium Channel Gamma-2-subunit	*Xenopus*
1710874	Amiloride-sensitive Sodium Channel Gamma-subunit	*Xenopus*
2828217	Amiloride-sensitive Sodium Channel Gamma-subunit	rat
845512	Epithelial Sodium Channel Gamma Subunit	human
2724058	Epithelial Sodium Channel Delta Subunit	chimpanzee
4469403	Epithelial Sodium Channel Gamma Subunit	mouse
4158228	Epithelial Sodium Channel Gamma Subunit	rabbit

Fig. 4.1. Neighbour Joining Phylogenetic tree, constructed using Protdist, of sequences similar to α-xENaC according to a GenBank BLASTP search. Protein sequences for each of the subunits were aligned with ClustalW (Thompson *et al.* 1994) and the tree was constructed with PHYLIP (Felsenstein, 1993). Neighbour Joining was chosen, despite its less accurate results, due to the large number of sequences and the time and computing power required for a Fitch analysis. The scale bar indicates distances in units of fraction of amino acids differing between two sequences. The *Fugu* sequence is highlighted in purple. See Table 4.1 for GenBank accession numbers of the sequences used.


Rabbit αENaC
Rat αENaC
Rat αENaC
Rat αENaC
Chicken αENaC
Xenopus αENaC
Chicken αENaC
Cow αENaC
Human αENaC
Rat αENaC
Chimpanzee δENaC
C. elegans Deg-like
C. elegans Deg-like
C. elegans Deg-1
C. elegans Mec-10
C. elegans Mec-4
C. elegans Mec-4
C. elegans Mec-4
C. elegans Mec-4
C. elegans degenerin
C. elegans Del-1
Rabbit γENaC
Human γENaC
Mouse γENaC
Rat γENaC
Xenopus γENaC
Xenopus γENaC2
Fugu sodium channel 2
Rat-ASIC Beta
Mouse sodium channel 2
Human neuronal amil. sens. cation ch. 2
Rat-ASIC 1
Human sodium channel 2
Mouse sodium channel 1
Mouse MDEG2
Human BNaC1
Rat BNaC1
Rat MDEG2
Human neuronal amil. sens. cation ch. 1
Human sodium channel
Rat DRASIC
Human H+ gated cation ch.
Human amil. sens. Testis ch.3
Rat βENaC
Rat βENaC
Human βENaC
Human βENaC
Human βENaC
Xenopus βENaC
Xenopus βENaC2
0.1

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Appendix A

CLUSTAL W(1.60) α-ENaC multiple sequence alignment.
Regions where primers were designed are highlighted in bold text.
Primer CC-for and CC-rev, as designed by Dr. Cutler, are highlighted in italics.
See Table 2.1 for details on primer sequence.
* indicates identity among all sequences, . indicates similarity.

```
Chicken    ------------------------------MGTASRGGSVKAEKMPEGEKTRQCKQETE-
Cow        ---------------------------------------------MKGDKPEEPGPGPEP
Chicken    --------------------------------------------MPEGEKTRQCKQETE-
Rat        MLDHTRAPELNIDLDLHASNSPKGSMKGNQFKEQDPCPPQPMQGLGKGDKREEQGLGPEP
Frog       ------------------------------------------------------------
Human      -------------------------MEGNKLEEQDSSPPQSTPGLMKGNKREEQGLGPEP


Chicken    --QQQKEDEREGLIEFYGSYQDVFQFFCSNTTIHGAIRLVCSKKNKMKTAFWSVLFILTF
Cow        SGPPPPTEEEEALLEFHRSYRELFEFFCNNTTIHGAIRLVCSQHNRMKTVFWAVLWLCTF
Chicken    --QQQKEDEREGLIEFYGSYQELFQFFCSNTTIHGAIRLVCSKKNKMKTAFWSVLFILTF
Rat        SAPRQPTEEEEALIEFHRSYRELFQFFCNNTTIHGAIRLVCSKHNRMKTAFWAVLWLCTF
Frog       -MTKEEKNEKEALIEFFSSYRELFEFFCSNTTIHGAIRLVCSRRNRMKTAFWLVLFLVTF
Human      AAPQQPTAEEEALIEFHRSYRELFEFFCNNTTIHGAIRLVCSQHNRMKTAFWAVLWLCTF
                   * * *.**   **...*.*** **************..*.*** ** **  . **

Chicken    GLMYWQFGILYREYFSYPVNLNLNLNSDRLTFPAVTLCTLNPYRYSAIRKKLDELDQITH
Cow        GMMYWQFGQLFGEYFSYPVSLNINLNSDKLVFPAVSICTLNPYRYKEIQEELEELDRITE
Chicken    GLMYWQFGILYREYFSYPVNLNLNLNSDRLTFPAVTLCTLNPYRYSAIRKKLDELDQITH
Rat        GMMYWQFALLFEEYLSYPVSLNINLNSDKLVFPAVTVCTLNPYRYTEIKEELEELDRITE
Frog       GLMYWQFGLLFGQYFSYPVSINLNVNSDKLPFPAVTVCTLNPYRYKAIQNDLQELDKETQ
Human      GMMYWQFGLLFGEYFSYPVSLNINLNSDKLVFPAVTICTLNPYRYPEIKEELEELDRITE
           *.*****   *. .* ****  .*.*.***.* ****..********  *.   * ***. *

Chicken    QTLLDLYDYNMSLARSDGSAQFSHRRTSRSLLHHVQRHPLR------RQKRDNLVSLPEN
Cow        QTLFDLYKYNSSKTLVAHARSR--RDLREPLPHPLQRLPVPAPPHAARGVRRAGSSMRDN
Chicken    QTLLDLYDYNMSLARSDGSAQFSHRRTSRSLLHHVQRHPLR------RQKRDNLVSLPEN
Rat        QTLFDLYKYNSSYTRQAGARRRSSRDLLGAFPHPLQRLRTPPPPYSGRTARSGSSSVRDN
Frog       RTLYELYKYNSTGVQGWIPNNQRVKRDRAGLPYLLELLPPG------SETHRVSRSVIEE
Human      QTLFDLYKYSSFTTLVAGSRSR--RDLRGTLPHPLQRLRVPPPPHGARRARSVASSLRDN
           .** .** *           .         ..           .    *. .

Chicken    SPSVDKNDWKIGFVLCSENNEDCFHQTYSSGVDAVREWYSFHYINILAQ--MPDAKDLDE
Cow        NPQVNRKDWKIGFQLCNQNKSDCFYQTYSSGVDAVREWYRFHYINILSRRRQDTSPSLEE
Chicken    SPSVDKNDWKIGFVLCSENNEDCFHQTYSSGVDAVREWYSFHYINILAQ--MPDAKDLDE
Rat        NPQVDRKDWKIGFQLCNQNKSDCFYQTYSSGVDAVREWYRFHYINILSR-LSDTSPALEE
Frog       ELQVKRREWNIGFKLCNETGGDCFYQTYTSGVDAIREWYRFHYINILAR--VPQEAAIDG
Human      NPQVDWKDWKIGFQLCNQNKSDCFYQTYSSGVDAVREWYRFHYINILSR-LPETLPSLEE
              *   .* *** **  ..  *** ***.*****.**** *******..         ..

Chicken    SDFENFIYACRFNEATCDKANYTHFHHPLYGNCYTFND---NSSSLWTSSLPGINNGLSL
Cow        DVLGKFIFTCRFNQDSCNEANYSHFHHPMYGNCYTFNDK--NSSNLWMSSMPGVNNGLSL
Chicken    SDFENFIYACRFNEATCDKANYTHFHHPLYGNCYTFND---NSSSLWTSSLPGINNGLSL
Rat        EALGNFIFTCRFNQAPCNQANYSKFHHPMYGNCYTFNDK--NNSNLWMSSMPGVNNGLSL
Frog       EQLENFIFACRFNEESCTKANYSSFHHAIYGNCYTFNQNQSDQSNLWSSSMPGIKNGLTL
Human      DTLGNFIFACRFNQVSCNQANYSHFHHPMYGNCYTFNDK--NNSNLWMSSMPGINNGLSL
              **..****.  *  ***. ***  .*********      * ** **.**. ***.*
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```
Chicken    VVRTEQNDFIPLLSTVTGARVMVHDQNEPAFMDDGGFNVRPGIETSISMRKEMTERLGGS
Cow        TLRTEQNDFIPLLSTVTGARVMVHERDEPAFMDDAGFNLRPGVETSISMSKEAVDRLGGD
Chicken    VVRTEQNDFIPLLSTVTGARVMVHDQNEPAFMDDGGFNVRPGIETSISMRKEMTERLGGS
Rat        TLRTEQNDFIPLLSTVTGARVMVHGQDEPAFMDDGGFNLRPGVETSISMRKEALDSLGGN
Frog       VLRTEQHDYIPLLSSVAGARVLVHGHKEPAFMDDNGFNIPPGMETSIGMKKETINRLGGK
Human      MLRAEQNDFIPLLSTVTGARVMVHGQDEPAFMDDGGFNLRPGVETSISMRKETLDRLGGD
            .*.**.*.*****.*.****.** . ******* ***. **.**** * **     ***

Chicken    YSDCTEDGSDVPVQNLYSSRYTEQVCIRSCFQLNMVKRCSCAYYFYPLPDGAEYCDYTKH
Cow        YGDCTKNGSEVPVENLYNTKYTQQVCIHSCFQESMIKECGCAYIFYPRPDGVEFCDYRKH
Chicken    YSDCTEDGSDVPVQNLYSSRYTEQVCIRSCFQLNMVKRCSCAYYFYPLPDGAEYCDYTKH
Rat        YGDCTENGSDVPVKNLYPSKYTQQVCIHSCFQENMIKKCGCAYIFYPKPKGVEFCDYRKQ
Frog       YSDCSEDGSDVDVKNLFQSEYTEQVCVRSCFQAAMVARCGCYAFYPLSPGDQYCDYNKH
Human      YGDCTKNGSDVPVENLYPSKYTQQVCIHSCFQESMIKECGCAYIFYPRPQNVEYCDYRKH
           * **.  **.* * **. . **.***..****  *.  * * * ***    . ..*** *.

Chicken    VAWGYCYYKLLAEFKADVLGCFHKCRKPCKMTEYQLSAGYSRWPSAVSEDWVFYMLSQQN
Cow        NSWGYCYYKLQDAFSSDRLGCFTKCRKPCSVTIYKLSASYSQWPSATSQDWVFQMLSRQN
Chicken    VAWGYCYYKLLAEFKADVLGCFHKCRKPCKMTEYQLSAGYSRWPSAVSEDWVFYMLSQQN
Rat        SSWGYCYYKLQGAFSLDSLGCFSKCRKPCSVINYKLSAGYSRWPSVKSQDWIFEMLSLQN
Frog       KSWGHCYYKLIIEFTSNKLGCFTKCRKPCLVSEYQLTAGYSKWPNRVSQDWVLHTLSRQY
Human      SSWGYCYYKLQVDFSSDHLGCFTKCRKPCSVTSYQLSAGYSRWPSVTSQEWVFQMLSRQN
            .** *****   *     **** ****** .  *.*.* **.**    *..*.    ** *

Chicken    KYNITSKRNGVAKVNIFFEEWNYKTNGESPAFTVVTLLSQLGNQWSLWFGSSVLSVMELA
Cow        NYTIKNKRDGVAKLNIFFKELNYKSNSESPSVTMVTLLSNLGSQWSLWFGSSVLSVVEMA
Chicken    KYNITSKRNGVAKVNIFFEEWNYKTNGESPAFTVVTLLSQLGNQWSLWFGSSVLSVMELA
Rat        NYTINNKRNGVAKLNIFFKELNYKTNSESPSVTMVSLLSNLGSQWSLWFGSSVLSVVEMA
Frog       NLTD---RNGIAKLNIYFEELNYKTILESPTINMAMLLSLLGSQWSLWFGSSVLSVVEML
Human      NYTVNNKRNGVAKVNIFFKELNYKTNSESPSVTMVTLLSNLGSQWSLWFGSSVLSVVEMA
            .      * *.**.**.* * ***.  ***.  ***. ..  *** ** *************.*.

Chicken    ELILDFTVITFILAFRWFRSKQWHSSPAPPPNSHDNTAFQDEAS--GLDAPHRFT--VE-
Cow        ELIIDLLVITFLMLLRRFRSRYWS--PGRGGKGTQEVASTPAASLPSSFCPHPAFFSSSP
Chicken    ELILDFTVITFILAFRWFRSKRWHSSPAPPPNSHDNTAFQDEAS--GLDAPHRFT--VE-
Rat        DVIFDLLVITLLMLLRRFRSRYWS--PGRGARGAREVASTPASSFPSRFCPHPTSPPPSL
Frog       ELVIDFVIIGVMILLHRYYYKKAN----EGEETTVVPTPAPAFADLEQQVPHIPRGDLSQ
Human      ELVFDLLVIMFLMLLRRFRSRYWS--PGRGGRGAQEVASTLASSPPSHFCPHPMSLSLSQ
           ... *  .*  ..  .  .  .                  .       .      **

Chicken    ---------AVVTTLPSYNSLEPCG-PSKDGETGL-E--------------
Cow        PDPAISP--ALSAPPPAYATLGPHPAPSGLAEASTSAHAPGEP--------
Chicken    ---------AVVTTLPSYNSLEPCG-PSKDGETGLE---------------
Rat        PQQGMTPPLALTAPPPAYATLGPSAPPLDSAAPDCSACALAAL--------
Frog       RQ---ISVVADITPPPAYESLDLRSVGTLSSRSSSMRSNRSYYEENGGRRN
Human      PGPAPSP--ALTAPPPAYATLGPRPSPGGSAGASSSTCPLGGP--------
                     *   .   *.*  .*
```

Appendix B

Clustal W Protein alignment of predicted trout (*Oncorhynchus mykiss*) V-ATPase B-subunit (AF140022) against selected vertebrate and invertebrate sequences listed by GenBank nucleotide accession number: *Ascidia sydneiensis* Samea (AB016484), *Manduca sexta* (X64354), *Drosophila melanogaster* (X67839), *Anguilla anguilla* (AF099743), *Gallus gallus* (U20766), *Mus musculus* (Y12634), *Rattus norvegicus* (Y12635), *Bos taurus* (M88690) and *Homo sapiens* (M25809).

* indicates identity among all sequences, . indicates similarity.

```
AB016484    ------MATTLDMAQLS-----------AAKEHALAVSRDYISQPRLTYQTVSGVNGPLV
X64354      ------MAKTLSAAQAN-------------KEHVLAVSRDFISQPRLTYKTVSGVNGPLV
X67839      ----------MNAQQAQ-------------REHVLAVSRDFISQPRLTYKTVSGVNGPLV
AF099743    -MAMKAIRGIVDGAMTELSSAVSGTKAAMTREHVQAVSRDYISQPRLTYKTVSGVNGPLV
U20766      MAAVAAARRMVNGAGP----------GG-AREQAAALTRDFLSQPRLTYKTVSGVNGPLV
Y12634      -MALRAMRGIVNGAAPELPVPTG-GPMAGAREQALAVSRNYLSQPRLTYKTVSGVNGPLV
Y12635      -MALRAMRGIVNGAAPELPVPTG-GPMAGAREQALAVSRNYLSQPRLTYKTVSGVNGPLV
M88690      -MALRAMRGIVNGAAPELPVPTS-G-LAGSREQALAVSRNYLSQPRLTYKTVSGVNGPLV
M25809      -MEIDSRPGGLPGSSCN---------LGAAREHMQAVTRNYITHPRVTYRTVCSVNGPLV
Trout       HEVNLTMATLVGNRTMDINGP------AAARTHTQAVTRNYISQPRLTYSTVSGVNGPLV
                      .                   . .  *..* ....**.** **. ******

AB016484    ILEKVKFAKFAEIVTLNLADGTQRSGQVLEVSGDKAVVQVFEGTSGIDAKHTTCEFTGDI
X64354      ILDEVKFPKFSEIVQLKLADGTHRSGQVLEVSGSKAVVQVFEGTSGIDAKNTLCEFTGDI
X67839      ILDEVKFPKFAEIVQLRLADGTVRSGQVLEVSGSKAVVQVFEGTSGIDAKNTLCEFTGDI
AF099743    ILDHVKFPRYAEIVHLTLPDGTKRSGQVLEVSGSKAVVQVFEGTSGIDAQKTSCEFTGDI
U20766      IT---------------------------------------------------SCEFTGDI
Y12634      ILDHVKFPRYAEIVHLTLPDGTKRSGQVLEVSGSKAVVQVFEGTSGIDAKKTSCEFTGDI
Y12635      ILDHVKFPRYAEIVHLTLPDGTKRSGQVLEVSGSKAVVQVFEGTSGIDAKKTSCEFTGDI
M88690      ILDHVKFPRYAEIVHLTLPDGTKRSGQVLEVSGSKAVVQVFEGTSGIDAKKTSCEFTGDI
M25809      VLDRVKFAQYAEIVHFTLPDGTQRSGQVLEVAGTKAIVQVFEGTSGIDARKTTCEFTGDI
Trout       ILDNVKFPRYAEIVHLTLPDGTRRSGQVLEVIGTKAVVQVFEGTSGIDAKKTACEFTGDI
            .                                                    *******

AB016484    LRIPTSEDMLGRIFNGSGKPIDKGPSVLPEDYLDIQGQPINPKSRIYPEEMIQTGISAID
X64354      LRTPVSEDMLGRVFNGSGKPIDKGPPILAEDFLDIQGQPINPWSRIYPEEMIQTGISAID
X67839      LRTPVSEDMLGRVFNGSGKPIDKGPPILAEDFLDIQGQPINPWSRIYPEEMIQTGISAID
AF099743    LRTPVSEDMLGRVFNGSGKPIDRGPMVLAEDYLDIMGQPINPQCRIYPEEMIQTGISAID
U20766      LRTPVSEDMLGRVFNGSGKPIDRGPAVLAEDFLDIMGQPINPQCRIYPEEMIQTGISAID
Y12634      LRTPVSEDMLGRVFNGSGKPIDRGPVVLAEDFLDIMGQPINPQCRIYPEEMIQTGISAID
Y12635      LRTPVSEDMLGRVFNGSGKPIDRGPVVLAEDFLDIMGQPINPQCRIYPEEMIQTGISAID
M88690      LRTPVSEDMLGRVFNGSGKPIDRGPVVLAEDFLDIMGQPINPHFRIYPEEMIQTGISAID
M25809      LRTPVSEDMLGRVFNGSGKPIDKGPVVMAEDFLDINGQPINPHSRIYPEEMIQTGISPID
Trout       LRTPVSEDMLGRVFNGSGKPIDRGPTVLAEDYLDIMGQPINPQCRIYPEEMIQTGISAID
            ** ● *******.*********.** .. **.*** ******  ************* **

AB016484    TMNSIARGQKIPIFSANGLPHNEIAAQICRQGGLVKLPDKDVMDSHEDNFAIVFAAMGVN
X64354      VMNSIARGQKIPIFSAAGLPHNEIAAQICRQAGLVKIPGKSVLDDHEDNFAIVFAAMGVN
X67839      VMNSIARGQKIPIFSAAGLPHNEIAAQICRQAGLVKLPGKSVLDDHTDNFAIVFAAMGVN
AF099743    GMNSIARGQKIPIFSAAGLPHNEIAAQICRQAGLVKK-SKDVMDYSADNFAIVFAAMGVN
U20766      GMNSIARGQKIPIFSAAGLPHNEIAAQICRQAGLVKK-SKDVMDYSEENFAIVFAAMGVN
Y12634      GMNSIARGQKIPIFSAAGLPHNEIAAQICRQAGLVKK-SKDVVDYSEENFAIVFAAMGVN
Y12635      GMNSIARGQKIPIFSAAGLPHNEIAAQICRQAGLVKK-SKDVVDYSEENFAIVFAAMGVN
M88690      GMNSIARGQKIPIFSAAGLPHNEIAAQICRQAGLVKK-SKDVVDYSEENFAIVFAAMGVN
M25809      VMNSIARGQKIPIFSAAGLPHNEIAAQICRQAGLVKK-SKAVLDYHDDNFAIVFAAMGVN
Trout       GMNSIARGQKIPIFSAAGLPHNEIAAQICRQAGLVKK-SKDVMDYSDDNFAIVFAAMGVN
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AB016484    MEAARFFKSDFEQHGSMDNVCLFLNLANDPTIERIITPRIALTTAEFLAYQCEKHVLVIL
X64354      METARFFKQDFEENGSMENVCLFLNLANDPTIERIITPRLALTAAEFLAYQCEKHVLVIL
X67839      METARFFKQDFEENGSMENVCLFLNLANDPTIERIITPRLALTAAEFLAYQCEKHVLVIL
AF099743    METARFFKSDFEENGSMDNVCLFLNLANDPTIERIITPRLALTTAEFLAYQCEKHVLVIL
U20766      METARFFKSDFEENGSMDNVCLFLNLANDPTIERIITPRLALTTAEFLAYQCEKHVLVIL
Y12634      METARFFKSDFEENGSMDNVCLFLNLANDPTIERIITPRLALTTAEFLAYQCEKHVLVIL
Y12635      METARFFKSDFEENGSMDNVCLFLNLANDPTIERIITPRLALTTAEFLAYQCEKHVLVIL
M88690      METARFFKSDFEENGSMDNVCLFLNLANDPTIERIITPRLALTTAEFLAYQCEKHVLVIL
M25809      METARFFKSDFEQNGTMGNVCLFLNLANDPTIERIITPRLALTTAEFLAYQCEKHVLVIL
Trout       METARFFKSDFEENGSMDNVCLFLNLANDPTIERIITPRLALTSAEYLAYQCEKHVLVIL
            **.***** ***..*.* *********************.***.**.*************

AB016484    TDMSSYAEALREVSAAREEVPGRRGFPGYMYTDLATIYERAGRVNGRNGSITQIPILTMP
X64354      TDMSSYAEALREVSAAREEVPGRRGFPGYMYTDLATIYERAGRVEGRNGSITQIPILTMP
X67839      TDMSSYAEALREVSAAREEVPGRRGFPGYMYTDLATIYERAGRVEGRNGSITQIPILTMP
AF099743    TDMSSYAEALREVSAAREEVPGRRGFPGYMYTDLATIYERAGRVEGRNGSITQIPILTMP
U20766      TDMSSYAEALREVSAAREEVPGRRGFPGYMYTDLATIYERAGRVEGRNGSITQIPILTMP
Y12634      TDMSSYAEALREVSAAREEVPGRRGFPGYMYTDLATIYERAGRVEGRNGSITQIPILTMP
Y12635      TDMSSYAEALREVSAAREEVPGRRGFPGYMYTDLATIYERAGRVEGRNGSITQIPILTMP
M88690      TDMSSYAEALREVSAAREEVPGRRGFPGYMYTDLATIYERAGRVEGRNGSITQIPILTMP
M25809      TDMSSYAEALREVSAAREEVPGRRGFPGYMYTDLATIYERAGRVEGRGGSITQIPILTMP
Trout       TDMSSYAEALREVSAAREEVPGRRGFPGYMYTDLATIYERAGRVEGRNGSITQIPILTMP
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AB016484    NDDITHPIPDLTGYITEGQIYIDRQLHNRQIYPPINVLPSLSRLMKSAIGEGMTRKDHSD
X64354      NDDITHPIPDLTGYITEGQIYVDRQLHNRQIYPPVNVLPSLSRLMKSAIGEGMTRKDHSD
X67839      NDDITHPIPDLTGYITEGQIYVDRQLHNRQIYPPVNVLPSLSRLMKSAIGEGMTRKDHSD
AF099743    NDDITHPIPDLTGYITEGQIYVERQLHNRQIYPPINVLPSLSRLMKSAIGEGMTRKDHAD
U20766      NDDITHPIPDLTGYITEGQIYVDRQLHNRQIYPPINVLPSLSRLMKSAIGEGMTRKDHAD
Y12634      NDDITHPIPDLTGYITEGQIYVDRQLHNRQIYPPINVLPSLSRLMKSAIGEGMTRKDHAD
Y12635      NDDITHPIPDLTGYITEGQIYVDRQLHNRQIYPPINVLPSLSRLMKSAIGEGMTRKDHAD
M88690      NDDITHPIPDLTGYITEGQIYVDRQLHNRQIYPPINVLPSLSRLMKSAIGEGMTRKDHAD
M25809      NDDITHPIPDLTGFITEGQIYVDRQLHNRQIYPPINVLPSLSRLMKSAIGEGMTRKDHGD
Trout       NDDITHPIPDLTGYITEGQVYVDRQLHNRQIYPPINVLPSLSRLMKSAIGEGMTRKDHAD
            *************.*****.*..***********.*********************** *

AB016484    VSNQLYANYAIGKDVQAMKAVVGEEALTADDMLYEFLGKFEKTFIAQGPYENRSVFDSL
X64354      VSNQLYACYAIGKDVQAMKAVVGEEALTPDDLLYEFLTKFEKNFITQGNYENRTVFESL
X67839      VSNQLYACYAIGKDVQAMKAVVGEEALTPDDLLYEFLTKFEKNFISQGNYENRTVFESL
AF099743    VSNQLYACYAIGKDVQAMKAVVGEEALTSDDLLYEFLQKFEKNFIAQGAYENRTVFETL
U20766      VSNQLYACYAIGKDVQAMKAVVGEEALTSDDLLYEFLQKFEKNFIAQGPYENRTVYETL
Y12634      VSNQLYACYAIGKDVQAMKAVVGEEALTSDDLLYEFLQKFEKNFITQGPYENRTVYETL
Y12635      VSNQLYACYAIGKDVQAMKAVVGEEALTSDDLLYEFLQKFEKNFITQGPYENRTVYETL
M88690      VSNQLYACYAIGKDVQAMKAVVGEEALTSDDLLYEFLQKFEKNFITQGPYENRTVYETL
M25809      VSNQLYACYAIGKDVQAMKAVVGEEALTSEDLLYEFLQKFEKNFINQGPYENRSMFESL
Trout       VSNQLYACYAIGKDVQAMKAVVGEEALTSDDLLYEFLQKFEKNFIAQGPYDNRTVYETL
            ******* ********************.**.***** ****.** ** *.**.....*

AB016484    NIGWELLRIFPKEMLKRIPRNVINEYYPRKKVPSAQDKQEDQKEEK-
X64354      DIGWQLLRIFPKEMLKRIPASILAEFYPRDSRH--------------
X67839      DIGWQLLRIFPKEMLKRIPASILAEFYPRDSRH--------------
AF099743    DIGWQLLRIFPKEMLKRIPQSTLAEFYPRDSKH--------------
U20766      DIGWQLLRIFPKEMLKRIPQTTLAEFYPRDSTAKH------------
Y12634      DIGWQLLRIFPKEMLKRIPQSTLSEFYPRDSAKH-------------
Y12635      DIGWQLLRIFPKEMLKRIPQSTLSEFYPRDSAKH-------------
M88690      DIGWQLLRIFPKEMLKRIPQSTLSEFYPRDSAKH-------------
M25809      DLSWKLLRIFPKEMLKRIPQAVIDEFYSREGRLQDLAPDTAL-----
Trout       DIGWQLLRIFPKEMLKRIPQSTLAEFYPRESAARH------------
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