Cloning and expression of potential osmoregulatory regulators, guanylin-like peptides and antisecretory factor, in the European eel *Anguilla anguilla*

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A dedication in loving memory of my Dad, Karl Wilson, who encouraged and supported me every step of the way.

Declaration

I, Gillian Dawn Wilson, hereby certify that this thesis, which is approximately 32,500 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

Date 21/2/05..... Signature of candidate

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Finally a huge thanks to my family who encouraged me every step of the way.

Contents

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Abbreviations

ACE	Angiotensin Converting Enzyme
AF	Antisecretory Factor
Ang	Angiotensin
ANP	Atrial Natriuretic Peptide
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BNP	Brain Natriuretic Peptide
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CNP	C-type Natriuretic Peptide
cGMP	Cyclic guanosine monophosphate
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
Cot	Co-transporter
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
E. *coli*	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
FW	Freshwater
GC-A	Guanylate Cyclase A
GC-B	Guanylate Cyclase B
GC-C	Guanylate Cyclase C
GFR	Glomerular filtration rate
5HT	5 Hydroxy Tryptamine (Serotonin)
IPTG	isopropyl _-D-thiogalactopyranoside
MOPS	N-Morpholino -propanesulphonic acid
PAGE	Polyacylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PKA	Protein kinase A
PKG	Protein kinase G
PS	Proximal segment
PVDF	Polyvinylidene difluoride
RACE	Rapid amplification of DNA ends
RAS	Renin Angiotensin System
RNA	Ribonucleic acid
RNase A	Ribonuclease A
RT	Room temperature
SDS	Sodium dodecyl sulphate
STa	*E. coli* heat stable enterotoxin
SW	Seawater
TAE	Tris acetate EDTA buffer
TB	Terrific Broth
TFA	Trifluoroacetic acid
Trx	Thioredoxin
VNP	Ventricular Natriuretic Peptide

Abstract

A member of the guanylin family termed uroguanylin has previously been found to be expressed in the intestine of the European eel (*A. anguilla*). Work in this project has enabled the cloning and sequencing of a further two members of this family which have been termed guanylin and renoguanylin. Homologues of the guanylin genes have also been cloned and sequenced from cod, flounder, plaice, salmon, sculpin, trout and whiting. The teleost guanylin-like peptides were highly homologous to their mammalian orthologues although there was marked divergence in sequences out with the active peptide region which is located at the carboxy terminus of the prohormone. cDNA encoding the three guanylin-like prohormones were inserted into pET 32 Xa/LIC vectors for bacterial expression and then the synthesised prohormones were purified.

Northern blot analysis identified that eel guanylin is only expressed in both the intestine but eel uroguanylin and renoguanylin mRNAs are expressed in the intestine and the kidney. Transfer of FW-acclimated yellow eels to SW for 7 days resulted in a significant increase in mRNA expression of uroguanylin in the intestine. In silver eels transferred to SW there was a significant increase in expression of the three guanylin-like peptides in the intestine after 2 days. In the kidney there was no significant changes in the mRNA expression of renoguanylin and uroguanylin in either yellow or silver eels at all time points measured following transfer to SW.

Antibodies to guanylin, renoguanylin and uroguanylin were synthesised and used for immunohistochemistry and Western blot studies.

The full length complementary DNA (cDNA) of a second hormone termed antisecretory factor (AF) which may be involved in osmoregulation was cloned and sequenced from brain tissue from yellow SW eels. AF displayed high amino acid homology to human and rat AF. Although reverse transcription PCR (RT-PCR) easily amplified AF fragments from the brain no fragments were detected when using gill, intestine or kidney cDNA as templates. It is hypothesised that AF synthesised at an unknown site within the brain may be transported via the bloodstream to the intestine where it may play a role in controlling salt and water levels.

Chapter 1 Introduction

1.1 The Life Cycle of the European Eel (*Anguilla anguilla*)

The European eel, *Anguilla anguilla*, is a catadromous, euryhaline teleost with a unique lifestyle. The cycle begins with spawning of larvae deep in the Sargasso Sea, situated in the Western Atlantic ocean (Feuteun, 2002). The first evidence for this site came from the catches of eel larvae by Johanes Schimdt in the early 1900's (Bertin, 1960). After hatching, these larvae, referred to as leptocephali, migrate to European waters via the Gulf Stream currents. The eels have to drift with these currents as they have no ability to orientate themselves (Wang, *et al.*, 2002) and the journey can take up to two years. After arrival in European coastal waters the leptocephali become more eel-like in appearance but they are transparent and therefore referred to as glass eels. The glass eels move from the seawater (SW) into freshwater (FW) environments of the inland rivers and streams (Bertin, 1960). Once in the lakes and rivers the eels continue to develop and metamorphose into sexually immature yellow eels; this stage in their development is a growth phase that can last from three to thirty years (Rankin, 1993). The final phase in the European eel's life cycle is believed to be endogenously determined (Rankin, 1993) and involves metamorphosis of yellow eels into the sexually mature silver stage, developing either male or female reproductive organs (Bertin, 1960). The silver eels make the catadromous migration downstream to a SW environment where they return to the Sargasso Sea to breed and complete their life cycle (Bertin, 1960).

1.2 Osmoregulation

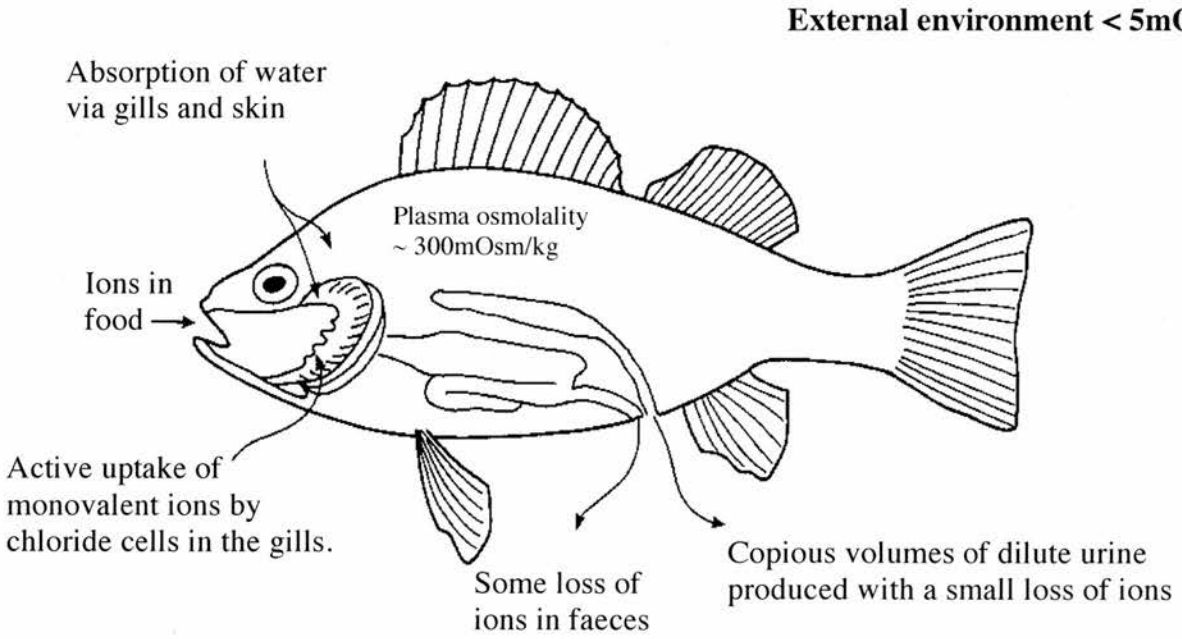
Euryhaline teleosts such as the European eel utilise a number of interdependent physiological mechanisms to maintain their plasma osmolality between 300 and 350 mOsm/kg irrespective of the extremes of environmental salinity encountered during their life cycle. These efficient mechanisms act together to enable osmoregulation, a homeostatic mechanism that maintains internal salt and water levels at optimal concentrations enabling physiological activity of cells.

When in freshwater (< 5 mOsm/kg) the fish are in a hypoosmotic environment. They have to counteract the loss of ions and passive gain of water across permeable body surfaces (McCormick, 2001). The gills are the predominant tissue responsible for over 90% of total body water influx (Jobling, 1995). Scales and large amounts of connective tissue close to the surface of the skin help to

reduce the amount of water influx (Jobling, 1995). To overcome these effects, fish limit their rate of drinking (Utida *et al.*, 1972) and the kidney produces copious volumes of dilute urine which is excreted back into the external environment (Hazon and Balment, 1998). Sodium and chloride ions are efficiently reabsorbed from the glomerular filtrate as it passes along the kidney tubule (Shuttleworth, 1989). These processes are summarised in figure 1.1 (a).

In contrast, when eels inhabit seawater environments (1000 mOsm/kg) they are in a hyperosmotic habitat and the osmoregulatory mechanisms, summarised in figure 1.1 (b) are quite different to those found in FW. There is an osmotic loss of water through the gills (Wilson *et al.*, 1996) and a passive gain of ions (McCormick, 2001). To regain the lost water and to prevent cellular dehydration the eel continuously drinks the SW (Parmalee and Renfro, 1983). Desalination of the ingested SW begins in the oesophagus, which has a low permeability to water but is highly permeable to sodium and chloride ions (Parmalee and Renfro, 1983). Further removal of salt occurs in the intestine where NaCl is actively absorbed by transporters such as the Na-K-2Cl cotransporter located on the luminal membrane of enterocytes (Trischitta *et al.*, 1992). These desalination processes reduce the osmolality of the ingested SW enabling the gut to passively absorb water (Jobling, 1995). The absorbed monovalent ions create a salt load to the body and this excess salt is transported by the blood to the gills where it is excreted by specialised mitochondria-rich cells known as "chloride cells" located in the branchial epithelium of the gills (Wilson *et al.*, 1996). Bicarbonate ions are secreted into the intestine in exchange for chloride ions and recent studies by Wilson *et al.*, (2002) have shown that a high concentration of bicarbonate ions in the intestinal lumen causes the precipitation of divalent cations, such as calcium and magnesium, as carbonates. The precipitate cannot exert an effect on osmotic pressure and therefore secretion of bicarbonate ions promotes further absorption of water from the intestinal lumen (Wilson *et al.*, 2002). The kidney also plays an important role in SW adaptation by producing very low levels of urine, approximately 1 – 2% of the body weight/day thus retaining body water (Jobling, 1995). Unlike mammals where the kidney is the most important tissue for regulation of salt and water concentrations there are three tissues equally

(a) Freshwater



(b) Seawater

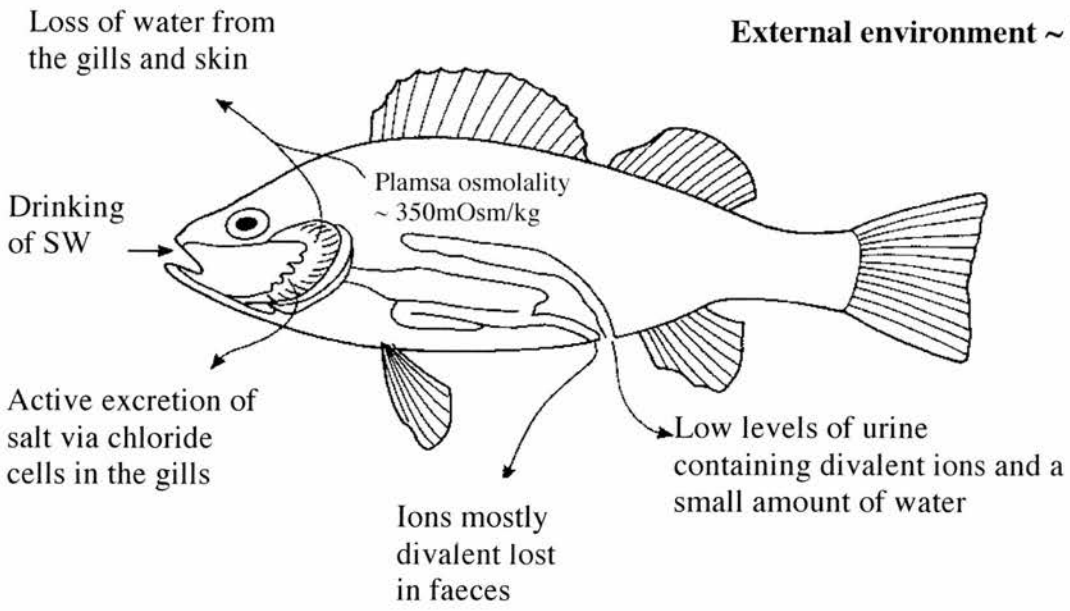


Fig 1.1 A summary of ion exchange in teleost in (a) freshwater and (b) seawater. (Adapted from Jobling, 1995).

involved in osmoregulation in fish, the gills, the kidney and the intestine and the functions of each will be discussed in detail below.

1.2.1 The Gill

The gills are essential for gas exchange, excretion of nitrogenous waste (Girard and Payan, 1980), acid base regulation and osmoregulation (Flik *et al.*, 1997). It is possible for this tissue to carry out such a wide range of functions because of its complex structure. The morphology of the gills is illustrated in figures 1.2 (a) and (b). Gills are composed of four gill arches located on each side of the buccal cavity (Karnaky Jr., 1986). The gill arches contain the efferent and afferent vasculature (shown in figure 2.1 (b)) and also provide skeletal support to hundreds of gill filaments (primary lamellae) which protrude from the gill arches (Karnaky Jr., 1986). The gill filaments are thin, blade-like structures designed to offer minimal resistance to the water current (Hentschel *et al.*, 2000). On the top and bottom of the gill filaments are a number of vertically orientated structures called secondary lamellae, shown in figure 1.2 (b), (Withers, 1992). The secondary lamellae have a thin epithelial layer and have been designed to reduce the water to blood diffusion distance and to maximise the efficiency of gas exchange (Manzon, 2002).

The gill lamellae are composed of a number of cell types including, mucous cells, neuroepithelial cells (Perry, 1997[a]), pillar cells, endothelial cells of blood vessels and two cell types important for respiration and ion exchange, the pavement cells and chloride cells respectively (Pisam *et al.*, 1993). The arrangement of these cells is depicted in figure 1.3 (a).

The respiratory or pavement cells are usually flattened cells (Sardet *et al.*, 1979) and localised in the secondary lamellae (Girard and Payan, 1980). The pavement cells are present in vast numbers and are important for gas exchange as they provide a large surface area suitable for the exchange of oxygen and carbon dioxide. The oxygen is absorbed across these cells into the blood where it is transported to tissues around the body (Jobling, 1995).

Chloride cells were first identified by Keys and Willmer in 1932 and in SW-acclimated teleosts are most abundant in the interlamellar region where the primary and secondary filaments meet (Jobling, 1995). These cells, which are

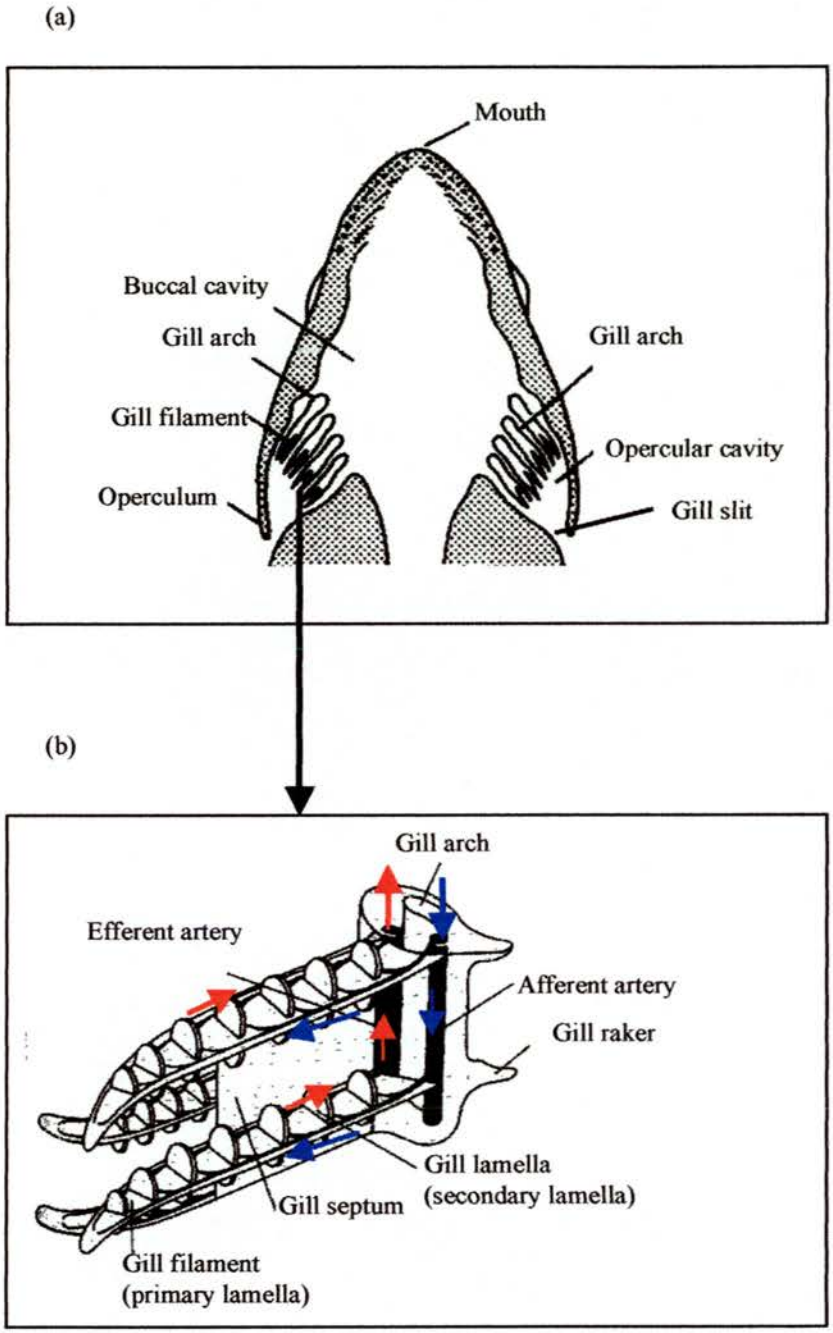
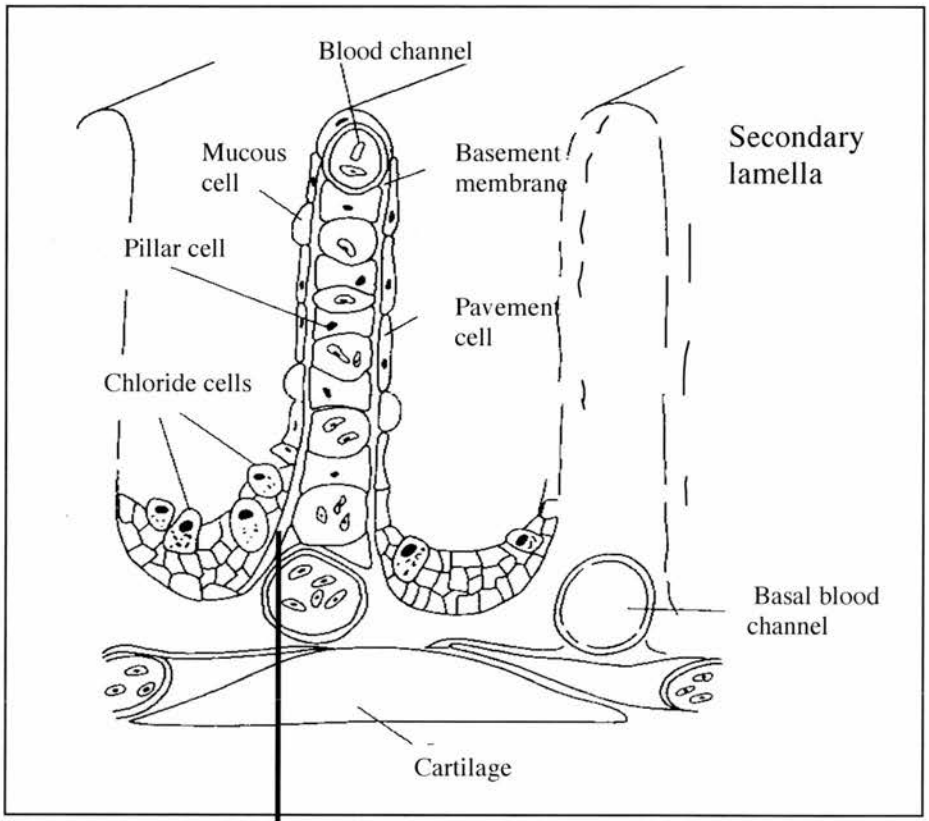


Fig. 1.2 (a) Schematic diagram of a section through a fish head (Pough *et al.*, 1990). (b) Detailed diagram of the structure of gill lamellae (Withers *et al.*, 1992). → indicates the direction of blood flow in the efferent artery, → in the afferent artery.

(a)



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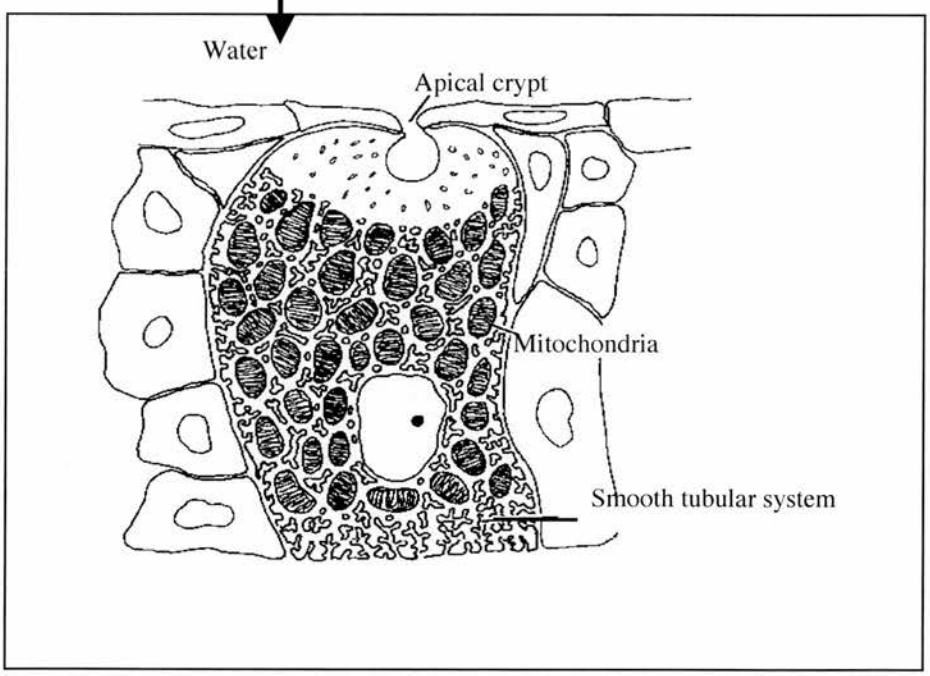


Fig. 1.3 (a) A diagram showing the structure and cellular composition of the secondary lamellae in the teleost gill (Jobling, 1995)
(b) Ultrastructure of the chloride cell (Bone *et al.*, 1999).

depicted in figure 1.3 (a) and (b), have an indented apical membrane which is known as the apical pit or crypt, they possess numerous mitochondria and they have an extensive intra-cytoplasmic membranous tubular system which is continuous with the basolateral membrane (Pisam *et al.*, 1993). There are believed to be two types of chloride cells, α and β (Perry, 1997[a]). The α chloride cells are primarily found in the interlamellar region whereas the β chloride cells are predominately found on the secondary lamellae (Jobling, 1995). The β chloride cells have slight structural differences to the α chloride cells. The β chloride cells are more cuboidal and smaller and do not have an apical pit (Pisam *et al.*, 1987). Studies have shown that an increase in the salinity of the environment induces degeneration of β chloride cells, therefore β chloride cells are only found in FW teleosts (Pisam *et al.*, 1987).

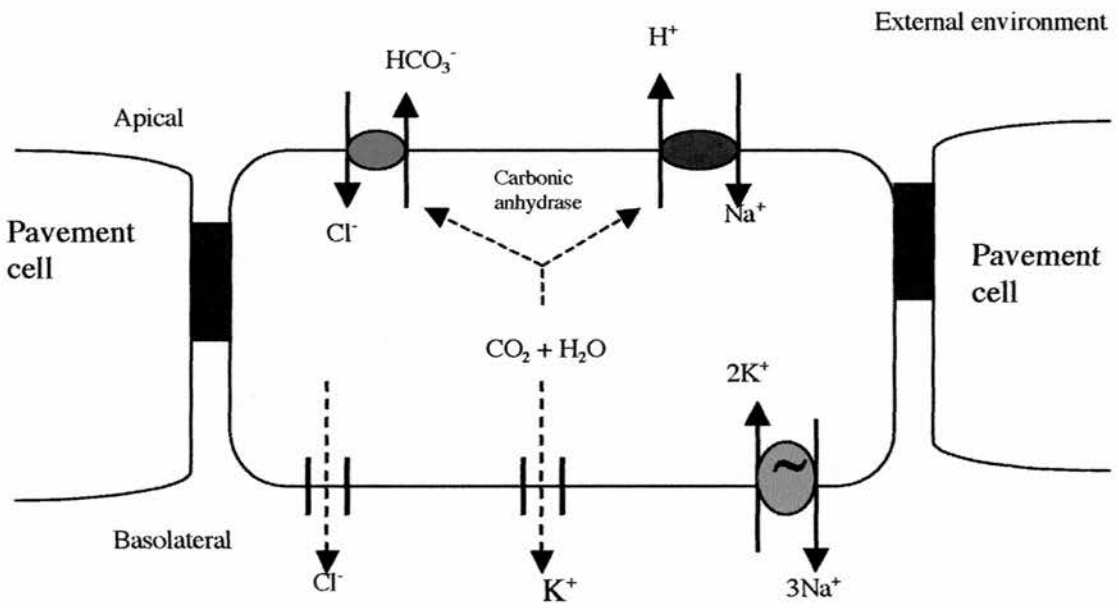
The chloride cells possess a number of ion channels and transporters in their apical and basolateral membranes which are involved in osmoregulation in both FW and SW environments.

In FW, fish are required to retain and absorb ions and the gills perform an essential role in assisting and maintaining a balanced internal ionic environment by absorbing monovalent ions from the external milieu (Bindon *et al.*, 1994). In FW, movement of sodium from the external environment into the body is thought to be driven by an electrochemical gradient supplied by a H^+ - ATPase (V-ATPase) exchanger located in the apical membrane of gill epithelial cells (Perry, 1997[a]). Supplies of H^+ ions for this exchanger come from the hydration of CO_2 by carbonic anhydrase. This hydration also provides ions for the apical Cl^-/HCO_3^- exchanger. In FW sodium enters the gill chloride cells across the apical membrane. Sodium is driven out of the cell into the blood by Na^+, K^+-ATPase located in the basolateral membrane (Thomson *et al.*, 1977). Chloride ions are thought to enter chloride cells from the external environment through a chloride bicarbonate exchanger. Studies on the European eel by Cutler and Cramb (2001) have shown that a Cl^-/HCO_3^- exchanger is expressed in the gills. The chloride ions are thought to move out of the chloride cells in the gills into the bloodstream through a chloride channel in the basolateral membrane, a channel which is thought to be similar to a chloride channel identified in the basolateral membrane of the urinary bladder (Marshall, 1995). These mechanisms are summarised in

figure1.4 (a). In contrast to FW, when in SW, fish need to excrete excess ions back into their habitat and the principal transporters in the α chloride cells involved are shown in figure 1.4 (b). To increase the efficiency of ion transporters and channels involved in osmoregulation in SW there is an increase in both the number and size of α chloride cells and therefore an increase in the number of ion transporters (Girard and Payan, 1980). There are two important ion transport mechanisms situated in the basolateral membrane of chloride cells in SW, the Na^+-K^+-$2Cl^-$ cotransporter (known as NKCC1) and Na^+, K^+ ATPase. NKCC1 cotransporters are localised to the basolateral membrane in chloride cells and expression within the branchial epithelium increases following SW acclimation (Cutler and Cramb, 2002). Studies have suggested that there may be an inwardly rectifying potassium channel, eKir, located on the basolateral membrane of chloride cells which returns the potassium that has entered the cell via NKCC1 to the interstitial body fluids (Suzuki *et al.*, 1999).

Na^+, K^+ ATPase is a major consumer of ATP within the cell (Marshall and Bryson, 1998) and studies have shown that there is an upregulation in expression of Na^+, K^+ ATPase following acclimation to SW (Cutler *et al.*, 1996; Marshall and Bryson, 1998). Na^+, K^+ ATPase creates a Na^+ gradient which results in the movement of chloride ions from the blood via the NKCC1 cotransporter into the cell. Once in the chloride cell these chloride ions diffuse through apically located anion channels to the external environment (Marshall, 1995). The principle chloride channel located in the apical membrane is thought to be the teleost homologue of the mammalian cystic fibrosis transmembrane conductance regulator (CFTR) which has been shown to be expressed at high levels in the gills of the killifish (Singer *et al.*, 1998) and eel (Cutler *et al.*, 1996). In SW there are tight junctions present between pavement cells and chloride cells which permits little passage of electrolytes but between chloride cells and accessory cells there are leaky tight junctions (Sardet *et al.*, 1979). Sodium ions pass through these leaky tight junctions to the seawater medium. The extrusion of Cl^- through the CFTR chloride channel creates a transepithelial potential which causes movement of Na^+ ions into the external environment.

(a) Freshwater



(b) Seawater

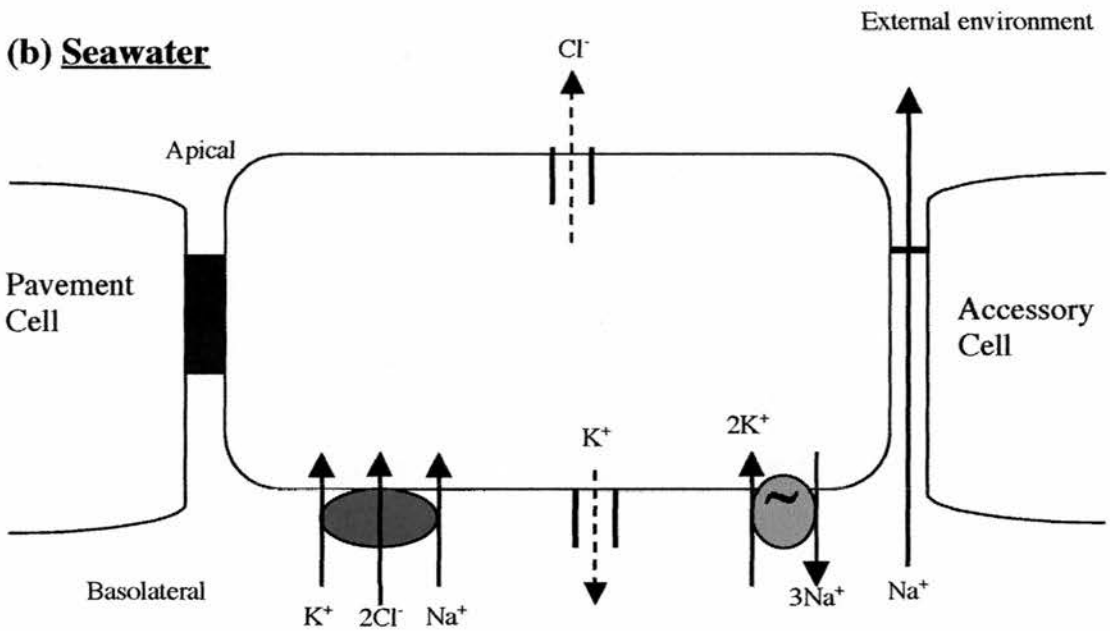


Fig.1.4. A summary of the ion transporters present in chloride cells in the gills in (a) freshwater and (b) seawater (Adapted from Marshall, W.J. 1995). Key: solid arrows indicate active transport and co transport pathways and dashed arrows indicate ion channels. Solid blocks between cells are tight junctions.

1.2.2 The Renal System

In teleosts with elongated bodies such as the European eel the kidney is an extended symmetrical organ located along the ventral surface of the vertebral column adjacent to the body cavity above the swim bladder (Hentschel *et al.*, 2000). The teleost kidney can be divided into two regions, the anterior or head kidney and the posterior or renal kidney. It is believed that the anterior region is important for producing red blood cells, a function carried out by the hematopoietic bone marrow in higher vertebrates (Hentschel *et al.*, 2000). The posterior region contains numerous nephrons, which filter the blood and enable the kidney to regulate water and ion concentrations and remove metabolic waste products such as ammonia (Pough *et al.*, 1990). At the beginning of the nephron is the Bowmans capsule which contains the glomerulus, which is the filter through which the blood plasma passes. The glomerulus filters the blood and the composition of the filtrate is similar to that of the blood plasma minus the high molecular weight proteins (Jobling, 1995). The glomerulus is connected to the filtering tubules via a ciliated neck region (Withers, 1992) and the cilia are thought to assist in moving the filtrate into the proximal tubule (Bone *et al.*, 1999).

The kidneys of teleosts are quite varied, a number of species such *as Lophius piscatorius* are aglomerular possessing no glomerulus and therefore the plasma is not filtered. Fish with aglomerular kidneys have a low urine flow rate and the urine is formed by solute secretion and passive water cotransport from the tubules into the urine (Withers, 1992). Other teleosts have a glomerular kidney and there are differences in the make up of the nephrons of freshwater and seawater fish as illustrated in figure 1.5. In FW, teleosts need to retain monovalent ions and excrete copious volumes of hypoosmotic urine. Large volumes of fluid are filtered through the glomerulus at a rate of approximately $4ml.kg^{-1}h^{-1}$. The filtrate passes through the neck segment into the proximal tubule, which is characterised by the presence of a brush border (Hentschel *et al.*, 1989). In FW, the proximal tubule is comprised of two segments, called PS I and PS II. PS I is believed to be where organic solutes like glucose and amino acids are reabsorbed via sodium-dependent transport mechanisms localised at the

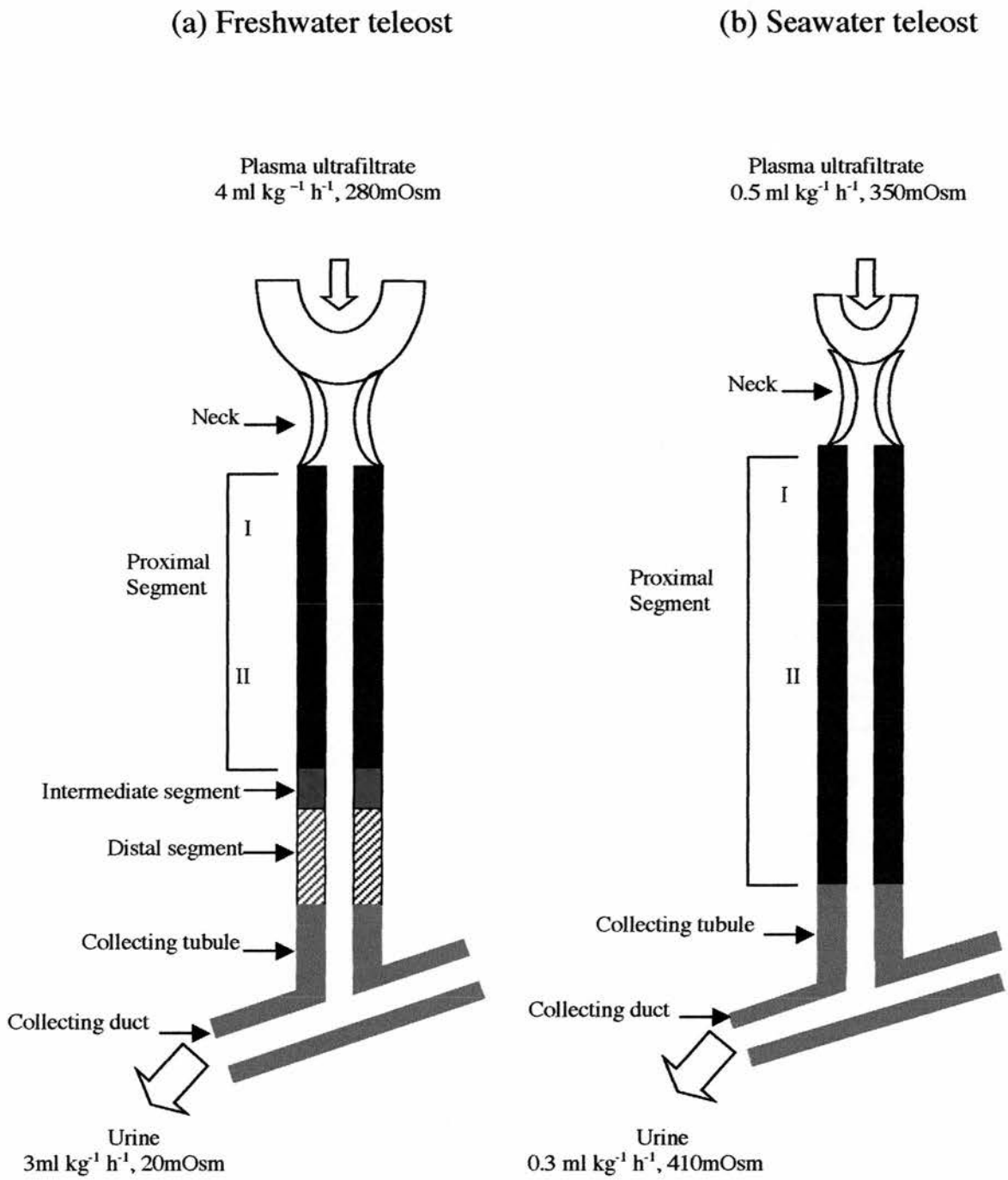


Fig. 1.5. Schematic structure of the kidney nephron in (a) freshwater and (b) seawater teleosts. Indicated are the major morphological segments, the plasma osmolality and glomerular filtration rate and the urine osmolality and flow rate (Adapted from Withers, 1992).

luminal brush border (Hentschel *et al.*, 1989) and where divalent cations and urea are secreted (Withers, 1992). PS II is the largest segment of the nephron (Hentschel *et al.*, 1989) and has both reabsorptive and secretory roles (Withers, 1992). The fluid passes from PS II into the intermediate segment. The cilia in the intermediate segment are thought to mix the divalent ions secreted in PS II with the tubular fluid and prevent the precipitation of the divalent ions (Hentschel *et al.*, 1989). The fluid is rapidly moved from the intermediate segment into the distal segment to minimise fluid reabsorption when fish are in FW (Withers, 1992). Unlike mammals there is no loop of Henle and therefore no countercurrent arrangement of tubule segments in the distal region, instead the tubular fluid is progressively diluted along this segment (Hentschel *et al.*, 1989) as monovalent ions (Na^+ and Cl^-) are reabsorbed. NaCl is absorbed from the lumen of the distal segment into cells via a Na-K-Cl cotransport system located in the apical membrane (Hentschel *et al.*, 1989). From studies in mammals and lower vertebrates such as dogfish and trout, the active transport of NaCl from the distal segment is powered by the activity of the Na^+, K^+ ATPase, located in the basolateral cell membrane (Hentschel *et al.*, 1989). Sodium ions are actively transported out of the cell in exchange for K^+ ions and then Cl^- ions follow the Na^+ ions out of the cell across the basolateral membrane through chloride channels. Further absorption of ions from the tubular fluid occurs in the collecting tubule and collecting duct. The fluid is then transferred along the collecting duct to the urinary bladder, an essential component of the teleost renal system (Bone *et al.*, 1999). The bladder is able to absorb some sodium and chloride ions that remain in the urine but the absorption of water is minimal because the bladder epithelia has a low permeability to water in FW (Utida *et al.*, 1972). The final urine produced is extremely dilute and only contains low concentrations of inorganic ions (Jobling, 1995).

Unlike teleosts in FW, when fish are in a marine environment they need to conserve water and eliminate ions. To achieve this the diameter of the glomerulus is decreased compared to that seen in FW, the schematic structure of the kidney in SW teleosts is shown in figure 1.5(b). The alteration of the size of the glomerulus has been suggested to occur via the action of vasoactive hormones (Hentschel *et al.*, 1989). The glomerular filtration rate (GFR) is reduced to approximately 0.5 $ml.kg^{-1}h^{-1}$, which is about 1/10 of the GFR of FW

fish (Brown *et al.*, 1980). Studies in single nephrons in trout showed that at least 45% of the nephrons were actively filtering in FW but only 5% of the nephron appeared active in seawater (Bone *et al.*, 1999). In SW the proximal tubule is important for recovering filtered substances from the urine and in PS II divalent ions are secreted from cells into the lumen (Hentschel *et al.*, 1989). The rate of production of the urine is very low, approximately 5 – 10 % of the rate of drinking (Jobling, 1995). Urine production is only 0.3 $ml.kg^{-1}h^{-1}$ and the urine passes from the kidney into the urinary bladder and is then excreted into the external environment.

1.2.3 The Gastrointestinal Tract

The intestine is essential for nutrient absorption and is extremely important for teleost osmoregulation, especially when fish are in a SW environment (Baldisserotto *et al.*, 1994). The gastrointestinal tract of the eel is composed of a single folded layer of epithelial cells (Bicho *et al.*, 1999). Studies on the winter flounder have shown that the intestine lacks crypts and the columnar epithelial cells are morphologically uniform from the crest to the base of its mucosal folds (Rao *et al.*, 1984).

When a fish moves from FW to SW it immediately begins to drink the external medium (Hentschel et al, 2000). Ions from the imbibed SW are absorbed across the oesophageal epithelia, which have a high permeability to Na^+ and Cl^- ions (Parmalee and Renfro, 1983) and both ions and water are absorbed across the intestine to compensate for the loss of water (Utida *et al.*, 1972). NaCl is actively absorbed across the enterocytes in the intestine and then water osmotically across the apical membrane (Trischitta *et al.*, 1992, Wilson *et al.*, 1996). Water can accompany ions through the ion channels or it can move through protein channels called aquaporins in either basolateral or apical membranes and there may also be an extracellular pathway across tight junctions.

Located within the apical brush border membrane is a homologue of the absorptive isoform of the Na^+-K^+-$2Cl^-$ cotransporter (NKCC2 or cot 2), the primary transporter responsible for the absorption of NaCl from the intestinal lumen (Cutler and Cramb, 2001). The cot 2 isoform is a member of the diuretic sensitive chloride/cation cotransporter gene family and studies have demonstrated that this transporter is predominately located in the anterior and

mid regions of the intestine (Cutler and Cramb, 2001). It is thought that the activity of Na^+-K^+-$2Cl^-$ in the intestine is stimulated by phosphorylation and activity of intracellular kinase cascades are thought to be responsible for covalently modifying the cotransporter (Barrett and Keely, 2000). The electroneutral Na^+-K^+-$2Cl^-$ symport allows two chloride ions to enter the cell for every sodium and potassium ion and Na^+-K^+-$2Cl^-$ works in parallel with a Ba^{2+} inhibitable K^+ conductance channel which recycles the potassium that has entered the cell back into the intestinal lumen (Trischitta *et al.*, 1992). Potassium ions can also exit on the basolateral side through basolateral K^+ channels (Marvão *et al.*, 1994). Cot 2 is the major isoform present in the eel intestine but NKCC1, a secretive isoform of the cotransporter, is also located within the epithelial cells in the eel intestine possibly on the basolateral membrane (Cutler *et al.*, 1996). It is thought that NKCC1 is associated with luminal fluid secretion for digestive processes (Cutler and Cramb, 2001). On the basolateral membrane there are a number of important ion channels and transporters. There is a Na^+-Cl^--HCO_3^- antiporter located on the basolateral membrane (Bicho *et al.*, 1996) which provides a mechanism for secretion in the intestine and this may also be important for acid base balance in SW teleosts (Wilson *et al.*, 1996). The Na^+-Cl^--HCO_3^- antiporter works by removing sodium and bicarbonate ions from the blood into the cell in exchange for chloride ions which are removed from the intracellular medium into the blood. The bicarbonate ions are excreted from the cells into the intestinal lumen in exchange for chloride ions by a Cl^-/HCO_3^- transporter located on the apical membrane (Wilson *et al.*, 2002).

A K^+-Cl^- cotransporter transfers potassium from the cell to the serosa and this electrochemical gradient drives the movement of chloride ions out of the cell (Marvão *et al.*, 1994). An important enzyme located in the basolateral membrane is the Na^+, K^+ ATPase, which is implicated in having a major role in absorption of salt and water. Na^+, K^+ ATPase is a heterodimer composed of an α and a β subunit and distinct isoforms of each subunit have been characterised in teleosts (Cutler *et al.*, 1995[a,b]; Barrett and Keely, 2000). It is thought that the β subunit is important for the insertion of the $\alpha\beta$ complex into the basolateral membrane and

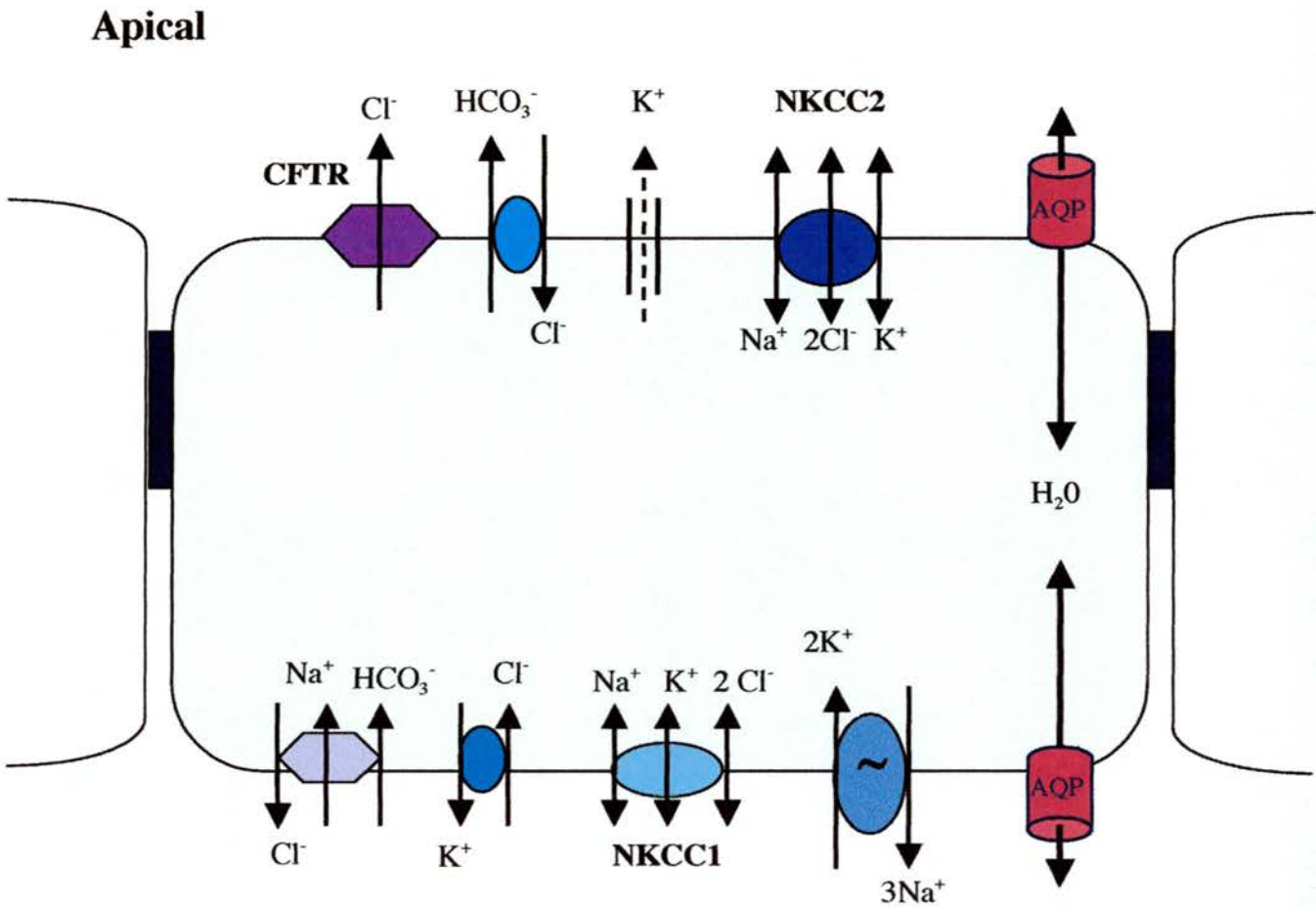


Fig. 1.6. A summary of the transporters thought to be present in enterocytes in the intestine of fish adapted to seawater. (Adapted from Marvão *et al.*, 1994).

ensuring that the αβ complex is stable in the membrane (Skou and Esmann, 1992; Barrett and Keely, 2000). The α subunit is responsible for the catalytic activity of Na^+, K^+ ATPase (Kisen *et al.*, 1994). The Na^+, K^+ ATPase pump transports three intracellular Na^+ ions out of the cell in exchange for two extracellular K^+ ions and for each cycle of the pump this action uses up one molecule of ATP. The various transporters such as NKCC1 and NKCC2, Na^+, K^+ ATPase and other transporters are shown in figure 1.6. As previously mentioned water can accompany ions through the ion channels but it can also move through aquaporin water channels. At least ten aquaporins have been identified in

humans (Borgnia *et al.*, 1999). Aquaporins are related to a functionally undefined family of membrane channel proteins including major intrinsic protein (MIP; van Os *et al.*, 2000). Aquaporins exist in the membrane as tetramers and each subunit has six bilayer spanning domains comprising two diversely symmetrical structures to form the aqueous pore (Borgnia *et al.*, 1999). Water flow through aquaporins is bidirectional and the direction is determined by the osmotic gradient (Borgnia *et al.*, 1999). The exact physiological roles of aquaporins are unknown. From studies looking at the expression of aquaporins in the eel it is known that there is a high level of expression of the AQP1 isoform in the intestine of SW adapted eels compared to eels adapted to FW (Cutler and Cramb, 2001) suggesting that AQP1 may play a role in osmoregulation in the intestine of eels, at least when in a SW environment.

A summary of the possible ion and water channels and ion transporters involved in osmoregulation in fish adapted to SW is shown in figure 1.6.

1.3 The Endocrine System and the Regulation of Osmoregulation in Teleosts.

The endocrine system is a collection of glands that secrete hormones that act as extracellular chemical messengers. Hormones are released into the bloodstream to be transported to tissues where they can exert their effects by binding to specific receptors. Some hormones also have paracrine actions and mediate their effects by diffusing short distances from the cell of release to neighbouring cells within the same tissue (Withers, 1992).

Studies in a variety of fish species have shown that a number of these chemical messengers play an important role in osmoregulation. A number of hormones, for example prolactin and cortisol, participate in osmoregulation by having regulatory effects on various ion transporters and cotransporters involved in osmoregulation in the gill, the intestine and the kidney. A selection of the hormones involved in osmoregulation are discussed below.

1.4 Cortisol

Studies have found that cortisol, secreted from the adrenal cortex in mammals or the interrenal gland in teleosts, is the dominant mineralocorticoid in fish (Hazon and Balment, 1998), and is especially important for osmoregulation in SW fish (Wong *et al.*, 2001). Cortisol acts on a number of tissues involved in osmoregulation, including the gill, gut and kidney (Wong *et al.*, 2001) and the main functions of this hormone in teleosts are ion regulation and energy metabolism (Hentschel *et al.*, 2000). It is known that the concentration of cortisol in the plasma increases as euryhaline teleosts such as salmonids and eels are transferred from freshwater to seawater (Hazon and Balment, 1998). Data suggests that when eels enter into brackish water there is a burst of cortisol secretion which is believed to play an important role in preparing the fish for migration into 100% seawater (Forrest *et al.*, 1973). During periods where the levels of cortisol are elevated there is a profound increase in the number and size of chloride cells located in the gill and an increase in Na^+, K^+ ATPase activities (Wong *et al.*, 2001), the driving force for monovalent ion transport in chloride cells (Hazon and Balment, 1998). In the intestine cortisol also causes an increase in the activity of Na^+, K^+ ATPase in epithelial cells and this mediates an increase in intestinal fluid secretion (Hazon and Balment, 1998). Overall in teleosts, secretion of cortisol results in increased ion and water absorption in the intestine and extrusion of salt in the gill (Hazon and Balment, 1998). Once an increase in the level of Na^+, K^+ ATPase activity in the gut and gill have been established in euryhaline teleosts in seawater the levels of cortisol are decreased to levels similar to those found in FW fish (Forrest *et al.*, 1973).

1.5 Renin Angiotensin System (RAS)

The renin angiotensin system (RAS) is an enzyme-activated system which helps to regulate extracellular electrolyte and fluid balance (Tierney *et al.*, 1995[b]). This biochemical cascade begins with the cleavage of a glycoprotein, produced in the liver and released into the blood, called angiotensinogen, by the enzyme renin. Renin, stored in the juxtaglomerular cells of the kidney in mammals, acts locally and systemically to cleave angiotensinogen into the decapeptide angiotensin I (Ang I; Tierney *et al.*, 1995[a]). Ang I is then converted to the octapeptide angiotensin II (Ang II) by the angiotensin converting enzyme (ACE). Ang II is believed to be the most active peptide component of this system (Balment *et al.*, 2003).

In mammals the RAS is responsible for regulating blood pressure, body fluid homeostasis and adrenocortical activity (Tierney *et al.*, 1995[b]; Hazon and Balment 1998). In teleosts the RAS plays a fundamental role in osmoregulation (Carrick *et al.*, 1983). Ang II has been shown to be a potent dipsogen in SW-adapted animals (Balment *et al.*, 2003) and experiments have shown that exogenous Ang II increases blood pressure and the glomerular filtration rate (Malvin *et al.*, 1980). In SW, teleosts maintain their body fluids hypo-osmotic to their environment which they achieve by drinking large volumes of water to replace the osmotic loss that occurs primarily across the gills (Tierney *et al.*, 1995). In SW the fish endure volume depletion and dehydration which stimulate the RAS and this may play a role in the sustained drinking observed in teleost fish (Carrick *et al.*, 1983). This idea was further supported by observations that in fish transferred from FW to SW the drinking rate could be attenuated by blockade of the RAS by inhibiting angiotensin converting enzyme which converts Ang I to Ang II (Beasley *et al.*, 1986).

The basic structure of Ang II is highly conserved throughout the vertebrate groups (Tierney *et al.*, 1995[b]) with only a small number of amino acid changes between Ang II peptides from teleosts and mammals. Most mammalian Ang I peptides have an aspartic acid at position 1 and isoleucine at position 5. In comparison, in teleosts studied to date, there is an asparagine at position 1 and valine at position 5 (Balment *et al.*, 2003). There are also various substitutions at position 9. These replacements are believed to increase the vasopressor potency of Ang I in the eel (Rankin, 1993).

1.6 Prolactin

Prolactin, a hormone produced in the adenohypophysis is a member of a protein family that includes growth hormone and teleostean somatolactin (Manzon, 2002). Like in mammals, prolactin from teleosts is synthesised as a prohormone but in teleosts the active signal peptide is only 23 – 24 amino acids in length, in mammals the prolactin signal peptide is 28 amino acids long (Manzon, 2002). Another difference between prolactin peptides from mammals and teleosts are the number of disulphide bonds. Mammalian prolactin has three disulphide bridges, one at the N terminal, one in the middle and one at the C terminal of the protein. In contrast prolactin peptides from teleosts only have two disulphide bridges, lacking the mammalian N terminal disulphide bond (Manzon, 2002). The effects of prolactin are mediated by a prolactin receptor (PRLR), a member of the class I cytokine receptor superfamily that includes receptors for interleukins and growth hormone (Manzon, 2002).

In mammals, prolactin is important for a number of functions including growth, development, metabolism and prolactin is involved in stimulating milk production in mammary glands (Manzon, 2002). In teleosts prolactin plays an important role in FW adaptation (Seale *et al.*, 2002) which was first observed by Pickford *et al.*, (1959) in hypophysectomized killifish (*Fundulus heteroclitus*). Normally hypophysectomized fish would be unable to survive in freshwater but after an injection of prolactin these fish were able to survive. These experiments suggested that prolactin had a role in regulating salt and water levels in FW. Experiments have shown that prolactin brings about an increase in plasma ion concentrations and decreases water uptake in osmoregulatory organs such as the gills and the intestine (Manzon, 2002). In the gills the β chloride cells are only present in FW-adapted teleosts. Studies have found that following injections of prolactin to SW-adapted tilapia, β chloride cells, normally absent in fish in SW, re-appeared (Pisam *et al.*, 1993). This data suggests that prolactin may control the abundance of β chloride cells in the gills of FW adapted teleosts (Manzon 2002). Prolactin also acts on the mucous cells of the gills to increase mucous secretion and this may contribute to the regulation of ion and water balance in FW fish as this could possibly impede the movement of ions in and out of the gills (Manzon, 2002).

1.7 Atrial Natriuretic Peptides (ANP)

Atrial natriuretic peptide (ANP) is a cardiac hormone belonging to a family of natriuretic peptides which includes brain natriuretic peptide (BNP), C type natriuretic peptide (CNP) and in teleosts, ventricular natriuretic peptide (VNP). VNP is more homologous to mammalian ANP than mammalian BNP (Rankin, 1993). ANP is produced by the central nervous system, the heart and the adrenal medullary cells in mammals (M^{c}Kendry *et al.*, 1999). In mammals the circulating form of this peptide is a 28 amino acid C terminal fragment of a larger 126 amino acid prohormone (Evans, 1990). The prohormone is synthesised by ventricular and atrial cardiocytes and stored within specific secretory vesicles (Bianchi *et al.*, 1989). The actions of ANP in mammals are widespread and include diuresis and natriuresis (Ando *et al.*, 1992), which are mediated by increasing the glomerular filtration rate and decreasing tubular reabsorption of water (Evans, 1990). ANP also inhibits the secretion of aldosterone and renin (Bianchi *et al.*, 1989) and reduces blood pressure by causing a decrease in vascular resistance (O'Grady *et al.*, 1985). In teleosts, ANP is recognised as a powerful antidipsogen and suppresses the intestinal absorption of sodium and water (Takei and Tsuchida, 2000). Recent studies by Kaiya and Takei (1996) have revealed that secretion of ANP and VNP in the eel is regulated by osmotic and volaemic mechanisms, although unlike mammals the osmotic mechanisms are more important in the eel. The study found that there was a significant increase in levels of plasma ANP in eels following an injection of a hypertonic solution but increased plasma volume only caused a small increase in the levels of ANP (Kaiya and Takei, 1996). ANP has been shown to be beneficial in the adaptation of the eel to a SW environment. In SW, eels drink copious volumes of water which causes an increase in blood volume and changes in plasma osmolality which are thought to be a trigger for the secretion of ANP. In the eel intestine ANP acts to decrease the blood volume and correct the plasma osmolality by stimulating the excretion of sodium ions and inhibiting the absorption of water (Tsuchida and Takei, 1998). ANP elicits its actions by activating the transmembrane guanylate cyclase type A receptors (Carrithers *et al.*, 2000[a,b]) and this causes an increase in intracellular cyclic guanosine monophosphate (cGMP). Experiments by O'Grady *et al.*, (1985) showed that

ANP causes a two-fold increase in cGMP in the intestinal mucosa of flounder. The increase in cGMP subsequently mediates electrolyte and fluid transport in cells by activating protein kinases, such as protein kinase G, which modulate the phosphorylation state of ion channels located in the apical membrane of epithelial cells resulting in the secretion of sodium and chloride ions (Carrithers *et al.*, 2000[a,b]).

1.8 Guanylin

The guanylin peptides have been relatively well characterised in mammals but their role (if any) in teleosts is unknown. From mammalian studies it is known that guanylin is a small, 15 amino acid, heat stable endogenous peptide first isolated from the rat jejunum (Currie *et al.*, 1992) and is the founder member of a small family of related peptides. Other members of this family include uroguanylin, which was first isolated from the urine of opossums (Hamra *et al.*, 1996) and has recently been found to be expressed in the intestine of the European eel (Comrie *et al.*, 2001[a]). Another member of the guanylin peptide family, which has only been found in opossums, is lymphoguanylin (Forte *et al.*, 1999[a]). A number of *E. coli* heat stable peptides (ST) found in enteric bacteria are structurally similar to the guanylin peptides (Forte and Currie, 1995). A distinctive feature of this family is the number of intramolecular disulphide bonds formed between cysteine residues and the peptides have been classified according to the number of intramolecular disulphide bonds that they possess (Forte, 1999[b]) as shown in figure 1.7. Lymphoguanylin has three cysteine residues and one disulphide bond (Forte *et al.*, 1999[a]), guanylin and uroguanylin, from both mammals and the European eel, have four cysteine residues and two disulphide bonds (Forte *et al.*, 1999[a]; Comrie *et al.*, 2001[a]), while the STs have six cysteine residues and three disulphide bonds (De Sauvage *et al.*, 1992). ST is more potent at activating intestinal GC-C receptors than the endogenous members of the guanylin family. It is thought that this is due to the third disulphide bond because reduction of the disulphide bridges reduced the ability of ST to bind to receptors (Lucas *et al.*, 2000). The correct formation of the

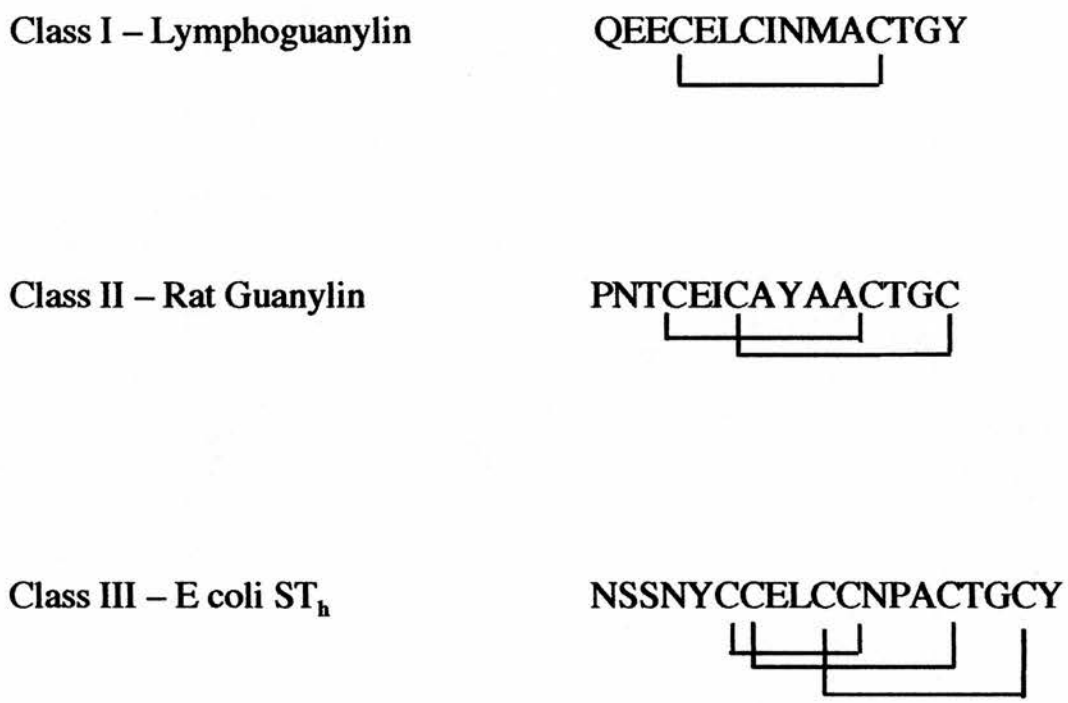


Fig 1.7 The classes of the guanylin-like peptides (adapted from Forte, 1999[b]).

disulphide bonds, such as 1-3 and 2-4 in guanylin, is essential for biological activity of the peptides (Lucas *et al.*, 2000).

1.8.1 Actions of the Guanylin family in mammals

Understanding of the mechanism of action of guanylin and uroguanylin came from work on the exogenous application of the bacterial peptide STa (Forte *et al.*, 2000[a]). STa inducing bacteria elicit secretory diarrhoea (Forte and Currie, 1995) by stimulating intestinal fluid secretion within the colon above the capacity to reabsorb the lost fluid (Forte *et al.*, 2000[a]). In mammals STa has been shown to activate guanylate cyclase type C (GC-C), a receptor located within the apical membranes of enterocytes which line the large and small intestine (Forte *et al.*, 2000[b]). Studies using T84 cells have demonstrated that guanylin competes with STa for binding sites suggesting that both activate a common receptor (Forte *et al.*, 1992).

In mammals, guanylin and uroguanylin are released both into the intestinal lumen and into the bloodstream in the form of larger precursor molecules, known as proguanylin and prouroguanylin respectively (Lauber *et al.*, 2002). The sequences of both pre-proguanylin and pre-prouroguanylin from the opossum have been determined (Hamra *et al.*, 1996[a]). A study by Forte (1999[b]) has suggested that in opossum pre-prouroguanylin there is a cleavage site between a serine residue and a valine residue (highlighted in figure 1.8) which cleaves the preprohormone removing what is known as the 'signal peptide' from the N terminal of the prohormone. The valine residue is conserved in a number of mammalian pre-proguanylin and pre-prouroguanylin peptides (highlighted in figure 1.8) and cleavage at this site may be necessary for posttranslational processing of the pre-prohormones to yield mature prohormones (Forte, 1999[b]). The bioactive peptide is located at the C terminal end of the precursor and is between 15 and 16 amino acids in length, for example, human uroguanylin is 16 amino acids in length but rat guanylin is only comprised of 15 amino acids (Forte *et al.*, 1999[a], Carrithers *et al.*, 2001[a]).

From experiments conducted using the intestinal T84 cell line, guanylin is known to function optimally under basic conditions (Fan *et al.*, 1997) but uroguanylin is more active at an acidic pH (Whitaker *et al.*, 1997). The pH

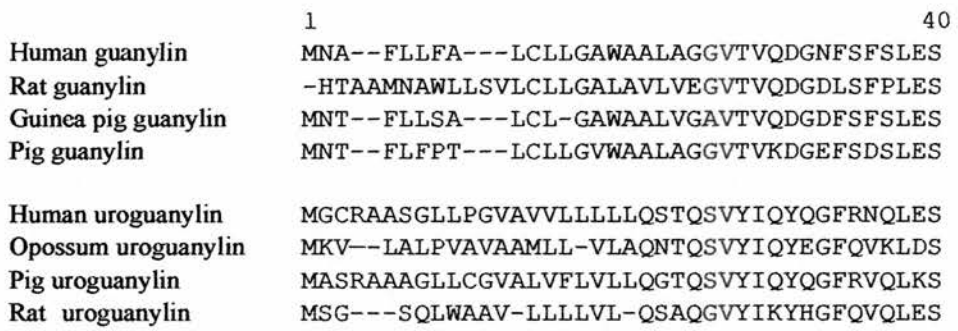


Fig. 1.8 Comparison of amino acids 1 – 40 of the N terminal region of the preprohormone of mammalian guanylin and uroguanylin peptides (Hamra *et al.*, 1996[a]). A potential cleavage site to cleave the preprohormone to yield the mature prohormone is highlighted in blue.

conditions are thought to influence the conformational state of the peptide (Hamra *et al.*, 1996[a]). It has been suggested that human uroguanylins preference for an acidic environment is due to a pair of acidic amino acids found at the NH_2 terminus of the active peptide, highlighted in figure 1.9 (Forte, 2003). It is thought that this pair of acidic amino acids contribute to the maintenance of high affinity and potency of uroguanylin under conditions of acidic pH (Forte, 2003). From a number of studies in mammals it is known that the main site of synthesis of guanylin and uroguanylin is the small intestine (Whitaker *et al.*, 1997; Nakazato, 2001) and levels of mRNA for these two peptides have been demonstrated to be as abundant as many highly expressed housekeeping genes (Forte *et al.*, 2000[a]). Using antibodies raised against guanylin Cetin *et al.*, (1994) showed that in the guinea pig intestine, guanylin is located in enterochromaffin cells which are unique enterocytes that can secrete peptides and other cellular products into the intestinal lumen (Forte *et al.*, 2000[b]). The guanylin family of peptides are known to be important mediators of an intra-intestinal paracrine signalling system (Comrie *et al.*, 2001[a]) which participates in the regulation of salt and water levels in mammals (Nakazato, 2001). Proguanylin is released from enterochromaffin cells into the intestinal mucosa (Fan *et al.*, 1997). In the intestinal lumen the prohormones are converted to the active peptide by luminal proteases and then the active guanylin peptides act in the intestine to stimulate transepithelial secretion of chloride and bicarbonate anions. The movement of these anions provides the driving force for the secretion of sodium ions and water into the intestinal lumen as explained in section 1.8.3 (Forte, 1999[b]). A study by Comrie *et al.*, (2001[a]) found an increase in the mRNA expression of uroguanylin in the eel intestine following transfer to SW. These results suggest that uroguanylin may play a role in osmoregulation.

1.8.2 Guanylate Cyclase C (GC-C)

The guanylate cyclase family is comprised of two forms, the soluble and the particulate enzymes (Kita *et al.*, 1994). The soluble enzymes are heterodimers that serve as receptors for the membrane-permeable signalling molecule, nitric oxide (Krause *et al.*, 1994). In mammals seven particulate guanylate receptors known as GC-A to GC-G have been identified (Wedel and Garbers, 1997; Lucas

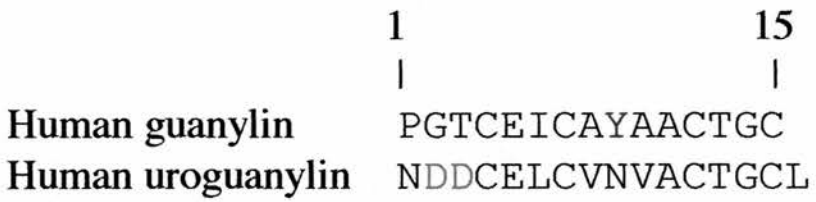


Fig. 1.9 Comparison of the active peptide structures of human guanylin and uroguanylin (Kita *et al.*, 1993). Highlighted in blue is a tyrosine residue only present in the guanylin peptide which results in guanylin being inactivated by chymotrypsin-like proteases and highlighted in red are the acidic amino acid residues of uroguanylin thought to be important for pH sensitivity.

et al., 2000) and these enzymes are thought to serve as cell surface receptors for various endogenous peptides (Forte, 1999[b]). The first member of the particulate family to be identified was the transmembrane receptor for the natriuretic peptides ANP and BNP called GC-A and subsequently the identification of GC-B a receptor for CNP (Schulz *et al.*, 1990). Subsequently, from studies on heat stable enterotoxins, the intestinal membrane guanylate cyclase, GC-C, was identified (Krause *et al.*, 1994, Hidaka *et al.*, 1998).

In the European eel the mRNA for GC-C is approximately 4.8kb in size (Comrie *et al.*, 2001[b]) and the gene for GC-C has been evolutionarily conserved in a wide range of animals (Wedel and Garbers, 1998). Particulate guanylate cyclases are composed of four major signature domains (Wedel and Garbers, 1998). There is an extracellular NH_2 terminal region that provides the ligand binding domain (London *et al.*, 1997) and the primary amino acid sequences of the extracellular domains between GC-A, GC-B and GC-C have been compared and found to be highly divergent (Schulz *et al.*, 1990). The next section of this receptor is a single hydrophobic domain that forms an α helix, which locates the particulate guanylyl cyclases (GC-A – GC-G) to the plasma membrane lipid bilayer (Lucas *et al.*, 2000). Guanylate cyclases contain two intracellular domains (Kita *et al.*, 1994) and between these two regions there is an intracellular hinge region (Wedel and Garbers, 1998). One of the intracellular domains is a well conserved catalytic region located at the carboxyl terminal (Bhandari *et al.*, 2001). The second intracellular domain is a protein kinase-like region which is thought to regulate the catalytic activity of guanylate cyclase receptors (Bhandari *et al.*, 2001). Both of the intracellular regions are highly conserved between GC-A, GC-B and GC-C (Schulz *et al.*, 1990). A comparison of the eel GC-C amino acid sequence of the hinge region indicates that it is more than 80% homologous to GC-C in other species, including amphibians and mammals (Comrie *et al.*, 2001[b]).

In mammals, GC-C is predominately expressed throughout the mucosa in the brush border membrane of intestinal cells (Potthast *et al.*, 2001) and renal proximal tubules (Hamra *et al.*, 1997). The GC-C receptor is thought to only be located on the apical surface of epithelial cells as experiments found that guanylin was only able to induce secretion of chloride when applied to the apical surface of T84 cells (Forte and Currie, 1995).

Guanylin, uroguanylin and STa bind to the extracellular domain of the apically located GC-C receptor (Fonteles *et al.*, 1998) resulting in the secretion of fluid and electrolytes into the intestinal lumen (Cohen *et al.*, 1995). Studies have found that although guanylin and uroguanylin are structurally related to the heat-stable enterotoxins they are less potent activators of GC-C than STa (Whitaker *et al.*, 1997). The importance of GC-C as the receptor linked to guanylin and fluid secretion has been demonstrated in GC- C knock out mice. These mice had no detectable fluid secretion response to STa (Forte *et al.*, 2000[b]) and guanylate cyclase activity in the intestinal mucosa was approximately 16 fold higher in the wild type mice (Wedel and Garbers, 1997).

1.8.3 Guanylin/Guanylate Cyclase Signalling System

Upon activation of GC-C there is an increase in the intracellular levels of cGMP, a second messenger molecule first discovered in 1963 (Wedel and Garbers, 1998) and subsequent stimulation of protein kinase G activity (Forte and Currie, 1995). Protein kinase G (PKG) is found at high levels in the intestinal mucosa (Li and Goy, 1993) and is a key enzyme that is found in chloride secreting cells (Forte *et al.*, 1992). The predominant chloride-secreting channel located in the apical membrane (Sheppard and Welsh, 1999) is the cystic fibrosis transmembrane conductance regulator (CFTR); first isolated and sequenced from humans in 1989 (Riordan *et al.*, 1989). The importance of CFTR to intestinal function has been shown in transgenic mice with the CFTR gene disabled. These mice exhibit reduced intestinal electrolyte secretion in response to uroguanylin (Forte *et al.*, 2000[b]). CFTR is a member of a large family of proteins known as the ATP binding cassette (ABC) proteins (Forte *et al.*, 2000[b]) and comprises two nucleotide binding folds which bind and hydrolyse ATP and twelve membrane spanning domains presumed to form the pore of the channel (Barrett and Keely, 2000). Phosphorylation of CFTR by PKG results in the opening of the channel. Opening and closing of the CFTR chloride channel is tightly controlled by the covalent modification of the regulatory domain and hydrolysis of ATP in the nucleotide binding domains (Barrett and Keely, 2000). The large regulatory domain contains a number of potential sites for phosphorylation of protein kinases such as PKA and PKG (Barrett and Keely, 2000). Chloride ions are secreted through the CFTR chloride channel into the intestinal lumen (Fan *et al.*,

1997) and the transcellular chloride secretion is the driving force for the paracellular movement of sodium ions through leaky tight junctions (Forte and Hamra, 1996), as shown in figure 1.10. This results in sodium chloride accumulating in the lumen of the intestine and provides the osmotic basis for the movement of water (Barrett and Keely, 2000) either through leaky tight junctions or through aquaporin water channels as shown in figure 1.10.

The cascade of reactions, which arise from guanylin-like peptides binding to GC-C, are summarised in figure 1.10. In mammals this movement of ions and water is essential for the regulation of ion secretion into the intestinal lumen during digestion (Fan *et al.*, 1997) but guanylin and uroguanylin have also been found in the blood plasma and this suggested that these peptides regulate water and ion levels in tissues other than the intestine. Experiments on isolated perfused kidneys found that uroguanylin elicited diuretic, natriuretic and kaliuretic actions (Forte *et al.*, 2000[b]). Proguanylin has also been detected in the plasma from rats uroguanylin may be involved in an endocrine axis connecting the intestine and the kidney (Forte *et al.*, 2000[b]).

1.9 Antisecretory Factor; a potential osmoregulatory hormone in teleosts

Antisecretory factor (AF) is a heat-labile, 41kDa (Björck *et al.*, 2000) acidic protein (Hansen and Skadhauge, 1995; Lange *et al.*, 1987[a]) which is synthesised and stored in the pituitary gland (Johansson *et al.*, 1995). Studies have demonstrated that AF has a lectin-like affinity to certain polysaccharides as it interacts with agarose and dextran gels (Lönnroth and Lange, 1986). In the intestine AF acts as a potent inhibitor of the effects of cholera toxin from *Vibrio cholera*, toxin A from *Clostridium difficile* and heat-labile enterotoxins form *Escherichia coli* (*E. coli*) (Lönnroth and Lange, 1986). The existence of this endogenous hormonal system suggests that AF might function to antagonise the guanylin peptides actions in the mammalian gut. Therefore if teleosts also express an AF-like hormone it is possible that this signalling system may also be involved in osmoregulation.

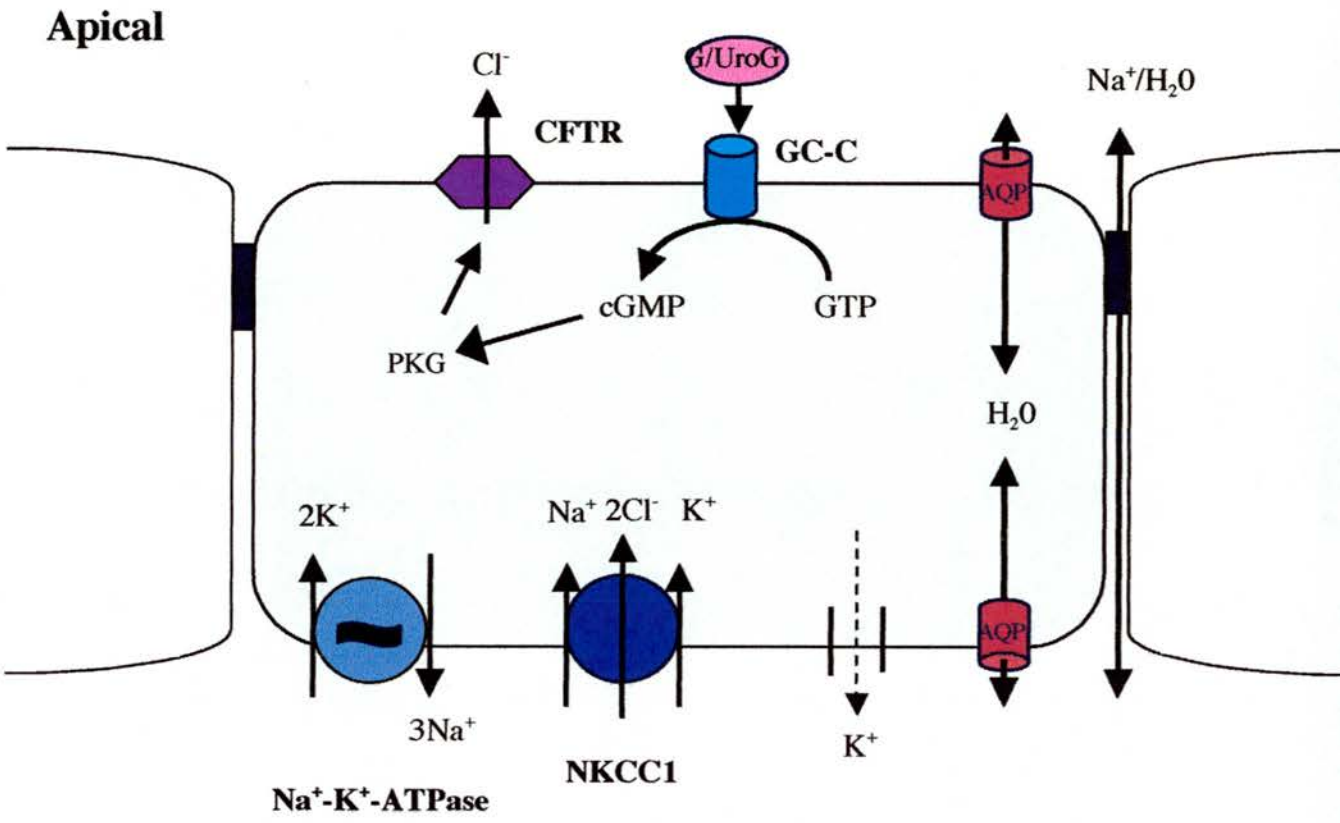


Fig. 1.10 The mechanism of action of guanylin or uroguanylin in enterocytes located in the intestine (Adapted from Forte and Hamra, 1996).

1.9.1 The actions of cholera toxin

Vibrio cholera, *Clostridium difficile* and *E. coli* are bacteria which produce and release toxins (Guerrant *et al.*, 1999). These toxins act in the intestine and cause diarrhoea due to massive salt and water secretion (Lencer *et al.*, 1999). These effects result in dehydration and low blood volume and in children under the age of five in third world countries diarrhoea is a leading cause of death (Lencer *et al.*, 1999). Cholera toxin is composed of two subunits, an A subunit, comprised of two major domains A1 and A2, and a pentameric B subunit (Lencer *et al.*, 1999). It is the A subunit which triggers a cascade of events which results in an extreme loss of fluid and ions in the form of diarrhoea in mammals (Lundgren and Jodal, 1997). For cholera toxin to become biologically active it must gain access into the epithelial cells which line the lumen of the intestine. To achieve this the B subunits of cholera toxin binds with high affinity and specificity to ganglioside GM1, a specific cell surface lipid, and then the A subunit of cholera toxin is incorporated into the cell by endocytosis and vesicular transport (Lencer *et al.*, 1999). Once present inside the cell the A subunit of cholera toxin catalyses the ADP ribosylation of the regulatory G protein Gsα (Lencer *et al.*, 1999). ADP ribosylation inactivates the intrinsic GTPase activity of the G protein (Lencer *et al.*, 1999). Therefore Gsα-GTP is not hydrolysed and this action locks the Gsα G protein in the "on" position and leads to prolonged activation of adenylyl cyclase and results in an increased cellular concentration of cAMP (Lundgren and Jodal, 1997). The elevated levels of cAMP in the cell activate protein kinase A (PKA) which phosphorylates CFTR. Activation of CFTR causes an increase in the secretion of chloride ions into the intestinal lumen (Guerrant *et al.*, 1999).

1.9.2 Actions of AF in mammals

A small group of seven amino acids VCHSKTR (amino acids 36 – 42) located near the amino terminal of the prohormone (shown in figure 1.11) have been found to be essential for the activity of AF (Johansson *et al.*, 1997[a]). Mutated proteins which do not contain this short sequence are inactive and unable to inhibit intestinal ion secretion (Johansson *et al.*, 1997[a]). Another important feature of the amino acid sequence of AF is that there are only four cysteine

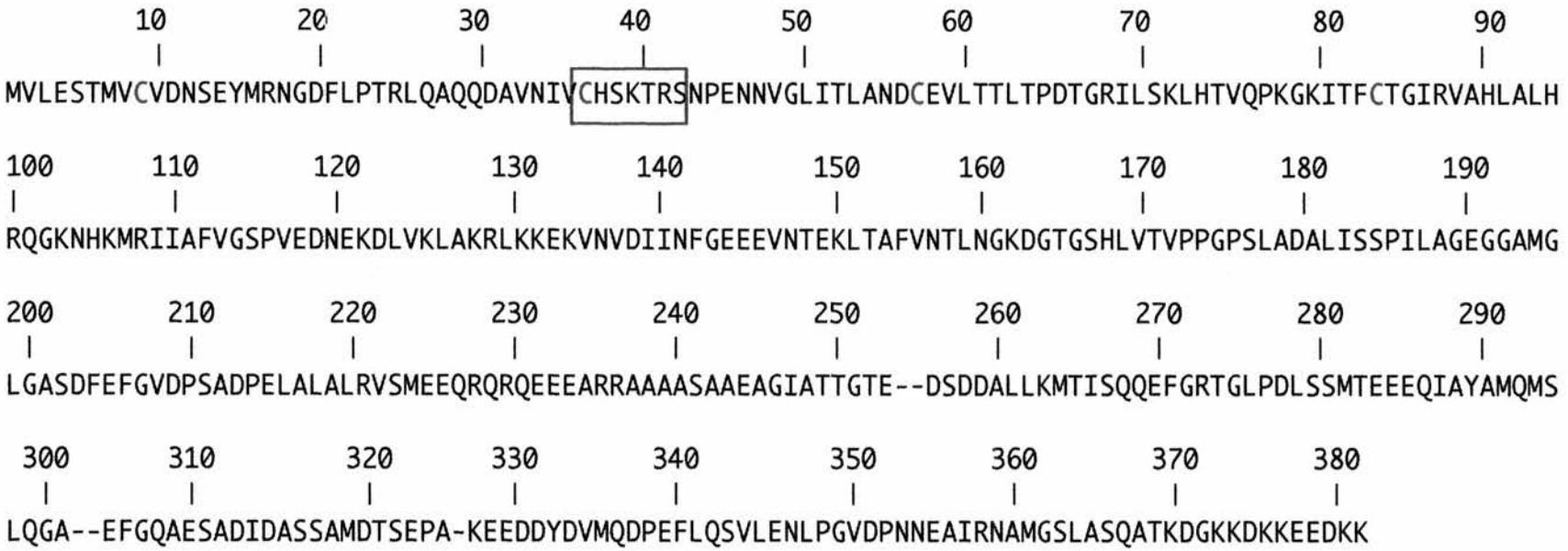


Fig. 1.11 The amino acid sequence of human AF (Johansson *et al* 1995). The boxed region highlights the amino acid sequence which is essential for biological activity of AF and the four cysteine residues also thought to be important for AF activity (Johansson *et al* 1997) are highlighted in blue.

residues in the whole sequence and they are all situated within the first 85 amino acids (highlighted in figure 1.11). It is possible that disulphide bridges may form between these four residues and this appears to be important for the activity of AF (Johansson *et al.*, 1997[a]). The formation of disulphide bonds and the seven amino acids mentioned above are important for AF to inhibit fluid secretion (1997[a]) found that AF is extremely potent at inhibiting the actions of cholera toxin. The study found that only picomolar concentrations (10^{-12} mol/kg body weight) of AF were required to produce half-maximal inhibition of the secretory effects induced by cholera toxin in rats and pigs.

It has been suggested that the signal to the pituitary gland to initiate the synthesis and secretion of AF may be triggered by serotonin, histamine or cAMP, all agents whose levels have been found to be elevated in the serum of cholera patients (Lange *et al.*, 1987[a]). These agents are thought to initiate a nerve reflex in the intestinal mucosa which is transmitted to the central nervous system and the pituitary gland where AF is released into the bloodstream (Johansson *et al.*, 1995). It is uncertain how AF inhibits the actions of cholera toxin. One hypothesis is that AF may downregulate the activity of adenylyl cyclase and this would subsequently decrease the intracellular levels of cAMP (Lange *et al.*, 1987[a+b]). Alternatively AF may inhibit ion channels in the epithelial membrane which would result in the suppression of salt and water transport (Lange *et al.*, 1987[a+b]) but further studies are required to completely understand the mechanism of how AF suppresses the activity of bacterial agents such as cholera toxin.

1.10 Research Aims

From studies in both mammals and teleosts it is known that transepithelial ion and water transport is a complex process that is regulated by an array of hormones. One important mechanism of regulation in mammals is the guanylin/GC-C signalling system which is known to participate in the control of intestinal salt and water levels. A second and novel hormone is antisecretory factor (AF). In mammals it is known that AF counteracts the extreme loss of water and electrolytes caused by infectious bacterial agents such as cholera toxin. The aim of this research was to determine if similar guanylin and AF systems were present and possibly involved in osmoregulation in teleost fish. In this study the European eel (*A. anguilla*) is used as the primary model species. The

European eel is an excellent model system as this fish is able to survive in a wide range of salinities from FW (< 5 mOsm/kg) to twice concentrated SW (2000 mOsm/kg). A uroguanylin homologue from the European eel has previously been cloned by Comrie *et al.*, (2001[a]) but the first objective of this project was to establish if other guanylin-like peptides were present in the intestine of a range of teleost fish using the polymerase chain reaction and then to sequence these fragments to determine which members of the guanylin family were present. By obtaining the full DNA sequence the homology between the species could be determined and potential cleavage sites of the prohormone to yield the active peptide identified. Cloning and sequencing of the guanylin-like peptides in a range of teleost fish may identify regions of homology that be different in teleost fish compared to regions of homology in mammals. The same techniques were used to determine whether AF was expressed in *A. anguilla*.

In SW, teleost fish are known to drink copious volumes of their external medium and it has been hypothesised that the guanylin/GC-C signalling system may prevent sudden changes in blood volume and plasma ion levels and help the fish adapt to a SW environment. To investigate this hypothesis Northern hybridisation was used to characterise the effect of salinity adaptation on the expression of guanylin-like peptide mRNAs in both yellow and silver eels.

The third aim of this project was to raise antibodies to the eel guanylin-like peptides. The full DNA sequences of the guanylin-like peptides were used to identify key regions for the development of the peptide specific antibodies. These antibodies were used for immunological studies to assess the production of recombinant guanylin peptides in bacteria and to determine the cellular location and quantify any changes in abundance as eels acclimatise to different salinities. The full length cDNA fragments of guanylin-like peptides were also used to synthesise recombinant guanylin-like peptides and the antibodies were used to determine if the recombinant guanylin-like peptides had been produced successfully.

Chapter 2 Materials and Methods

Unless otherwise stated all chemicals were Analar® grade supplied by Sigma Aldrich Ltd, Poole, Dorset or from VWR Laboratory Supplies, Poole, Dorset. Addresses for suppliers of materials and equipment are found in Appendix 1 and the sequences of the synthetic oligonucleotides (MWG Biotech) and those supplied with commercial kits are listed in Appendix 2.

2.1 Animals

Eels were obtained from private suppliers in Blairgowrie, Perthshire. Fish were kept without feeding under a 12h light/dark cycle in tanks of either freshwater or seawater in the Gatty marine laboratories at St Andrews University. The eels were acclimated for a minimum of 3 weeks at either salinity before use unless otherwise stated. For the experiments sexually immature yellow eels and sexually mature silver eels were used and the fish were decapitated and pithed before removal of tissues.

Other teleosts used were obtained from the Gatty Marine Laboratories, St Andrews University and frog tissue was kindly donated by Dr Sommerville, St Andrews University.

2.2 Extraction of RNA from tissues

Total RNA was isolated from all tissues using a method adapted from the high salt precipitation method of Chomczyski and Mackay (1987). The tissues to be investigated were rapidly removed from the fish and homogenised using a Polytron PT 10 homogeniser (Kinematica Ltd.) in 1:10 (wt/vol). Solution D (4 M guanidinium isothiocyanate, 25 mM sodium citrate, 0.5% (v/v) sarkosyl and 50 mM 2-mercaptoethanol). Homogenates were either processed immediately or stored at –20°C. Following homogenisation the total RNA was extracted by sequential addition of 0.1 vol. 2 M sodium acetate (pH 4.0), 0.5 vol. water saturated phenol and 0.2 vol. of bromochloropropane. Tubes were vortexed between the addition of each solution and then centrifuged at 3954 g for 30 min at 4°C in a Beckman J6-MC centrifuge, 4.2 rotor (Beckman Instruments Inc.). The upper aqueous phase was transferred to a fresh tube and 0.2 vol. of propan-2-ol and 0.2 vol. of high salt buffer (1.2 M NaCl, 0.8 M sodium citrate, pH 7.0), were added sequentially with vortexing. This solution was incubated at room temperature (RT) for 10 min then centrifuged at 3954g for 30 min at RT. The

supernatant was removed and the pellet washed twice in 80% ethanol and centrifuged as above, the tube was inverted and the pellet left to dry at room temperature. The pellet was resuspended in MilliQ®H_2O (20 – 30 µl/g tissue) and diluted samples (1:100) were prepared and the absorbance determined at 260 nm and 280 nm (Phillips PU 8620 UV/VIS/NIR spectrophotometer) to estimate both the purity and concentration of the sample. The concentration of RNA in each sample was calculated by: -

1 A_{260} unit = 40 ng RNA/µl

∴ (absorbance reading at A260 x 40) x (dilution factor) = ng RNA/µl

RNA samples extracted from each tissue were run on denaturing formaldehyde gels as detailed below and stained with ethidium bromide to ensure that no degradation of the RNA had occurred.

2.3 Preparation of RNA samples for gel electrophoresis

Total RNA samples (5 µg) were diluted in 23 µl sample denaturing buffer; 1 x MOPS (20 mM 3- [N-Morpholino]-propanesulphonic acid, 8mM sodium acetate, and 1mM EDTA pH 7.8), 50% (v/v) formamide and 6.7% (v/v) formaldehyde. The samples were denatured at 65°C for 15 minutes then snap cooled on ice and centrifuged for 30s at 20,000g (Eppendorf centrifuge 5417C, Helena Bioscience). After addition of 0.1 volumes of loading dye (0.025% bromophenol blue, 0.025% xylene cyanol and 50% glycerol, all w/v) the sample was loaded onto to a 1.2 % (w/v) agarose/formaldehyde gel as detailed in the next section.

2.4 RNA denaturing agarose gel electrophoresis

Denaturing agarose gels were prepared by boiling 1.2 g agarose (Biogene Ltd.) in 70 ml of MilliQ®H_2O plus 10 ml 10 x MOPS buffer (200 mM 3- [N-Morpholino]-propanesulphonic acid, 80 mM sodium acetate, and 10 mM EDTA pH 7.8) until dissolved. The mixture was cooled to approximately 55°C then 18 ml of 37 % formaldehyde was added to give a final formaldehyde concentration of 6.7% (v/v). The mixture was poured into a suitable gel rig and an appropriately sized comb was added before the gel was left to set. The RNA

samples (prepared as above) were loaded into the wells and electrophoresis conducted at 10 V/cm for 5 minutes and then 5 V/cm for 1h in 1x MOPS buffer. After electrophoresis the gel was washed in three changes of MilliQ®H_2O for 1h and then stained with ethidium bromide solution (1 μg/ml) for 30 min, and destained in Milli-Q® H_2O for 1h as before. The gel was viewed on an UV transilluminator (UVT-20M Trans-luminator, Herolab). The relative amount of RNA present in each sample was determined using the combined intensity levels of the 18S and 28S ribosomal RNA bands which were quantified using Gene Snap and Gene Tools computer software (Syngene).

2.5 Synthesis of cDNA from RNA

Synthesis of cDNA was carried out by reverse transcription using total RNA extracted from a variety of tissues from the European eel and the intestine from various other teleost species. Complementary DNA was synthesised by first mixing together 2.5 μg of total RNA and 1 μl 10 μM oligo dT primer (Pharmacia Ltd) in a total volume of 5 μl. The sample was incubated at 70°C on a thermal cycler (Techne Progene, Techne Ltd.) for 10 min, then snap-cooled on ice for 2 min and the sample briefly centrifuged (Eppendorf centrifuge 5417C, Helena Bioscience). Sequentially added to the sample was 2 μl 5x reaction buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$ and 50 mM DTT, pH 8.3; Promega) and 1 μl 10 mM dNTPs (deoxynucleotide triphosphates - 10 mM each of, dATP, dTTP, dCTP and dGTP; Promega). The sample was mixed gently and placed in the thermal cycler at 45°C for 2 min. To this was added 1 μl of Moloney-Murine Leukaemia Virus reverse transcriptase (M-MLV RT) enzyme (200 U/μl; Promega), an RNA dependent DNA polymerase. The sample was mixed and incubated at 45°C for 2-5 h. The synthesised cDNA was stored at –20°C until required.

2.6 Amplification of mRNA using Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

PCR is a technique that enables amplification of any specific fragment of DNA using synthetic oligonucleotides which correspond to the nucleotide sequences flanking the DNA region of interest. For this study degenerate primers were designed to the most homologous regions of guanylin-like peptides or

antisecretory factor proteins based on the known amino acid sequences from all species submitted to the Genbank. Stock degenerate primers were made up to a concentration of 100 μM, and specific homologous primers were diluted to 4 μM for use in the PCR reaction. A PCR reaction comprises three important steps; firstly denaturation to separate the double stranded DNA into two single strands which then act as templates for the synthesis of new DNA. The second step provides an optimal temperature for the sense and antisense primers to anneal to each strand of the DNA. And the final step, usually at 72°C, allows Taq DNA polymerase to elongate each primer by creating a reverse copy of each single stranded template (Hames *et al.*, 1997). These three stages make up one cycle in a PCR reaction and it is repeated for 40 cycles. The repeated cycles generate a large amount of cDNA product, the length of which is determined by the positions of the sense and antisense primers. The components for a PCR reaction comprise, 1 μl sense primer (4 or 100 μM), 1 μl antisense primer (4 or 100 μM) and 0.5 μl cDNA (template) which are mixed together in a 0.2 ml PCR tube along with 2 μl 10x Taq DNA polymerase buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0, 15 mM $MgCl_2$; Amersham Pharmacia Biotech Ltd.), 0.4μl 10 mM dNTPs, 15 μl Milli-Q® H_2O (deionised water) and 0.125 – 0.25 μl of Taq DNA polymerase (0.03125 units/μl; Biogene). The PCR thermal cycler (Techne Progene, Techne Ltd, Cambridge UK) was set with the following parameters: -

Section	**No. of Cycles**	**Temperature (°C)**	**Time**
1	1	92	2min
2	40	94	5s
		55 – 60 (varies with primers)	20s
		72	1min. extension for every 1kb fragment
3	1	72	15min

When the PCR programme was complete, the samples were centrifuged at 20,000g for 10 s and 0.1 volumes of sample preparation solution (50% glycerol, 0.025% bromophenol blue and 0.025% xylene blue; all w/v) added before the

samples were loaded onto a 1% agarose gel containing 1 μg/ml ethidium bromide (section 2.9) to detect the presence of amplified fragments.

2.7 Marathon cDNA Synthesis

The 5' and 3' end of the cDNAs were amplified by RACE (rapid amplification of cDNA ends) reactions using Clontech's Marathon cDNA amplification kit.

2.7.1 First Strand Synthesis

To synthesise the first strand of the Marathon™ cDNA up to 5 μg of total RNA, 1 μl of phosphorylated Smart oligo (Appendix 2; which binds to the 3' end of the CAP site at the 5' end of the mRNA and serves as an extended template for reverse transcription of full length cDNAs) and 1 μl of 10 μM Clontech cDNA synthesis primer (Appendix 2) were mixed and incubated at 70°C for 10 min, then snap cooled on ice. To this solution was added, sequentially, 1 μl 10 mM dNTPs and 2 μl 5x first strand buffer (250 mM Tris pH 8.5, 40 mM $MgCl_2$ and 150 mM KCl), mixed and incubated at 45°C for 3 min. Either 1 μl of M-MLV reverse transcriptase (200 U/μl; Promega) or 1 μl of Superscript II RNase H^- reverse transcriptase (200 U/μl; Gibco BRL) was added and the sample incubated at 45°C for 2 h.

2.7.2 Second Strand Synthesis

To all of the first strand reaction was added 48.8 μl MilliQ®H_2O, 16 μl of 5x second strand buffer (500 mM KCl, 50 mM $(NH_4)_2SO_4$, 25 mM $MgCl_2$, 0.75 mM β-NAD, 100 mM Tris pH 7.5 and 0.25 mg/ml BSA), 1.6 μl 10 mM dNTPs and 4 μl 2^{nd} strand enzyme cocktail (DNA Polymerase I, 6 U/μl, *E. coli* DNA ligase, 1.2 U/μl and RNase H 0.25 U/μl), the contents mixed and incubated at 16°C for 1.5 h. After incubation 2 μl of T4 DNA ligase (5 U/μl) was added and the sample incubated at 16°C for 45 min. To terminate the second strand synthesis reaction, 4 μl 0.2 M EDTA/2 mg/ml glycogen mix was added before extraction with 100 μl phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v, pH 7.5), the mixture vortexed and centrifuged at 20,000g for 10 min to separate phases. The upper aqueous phase was transferred to a fresh tube and 0.5 volume of 4 M ammonium acetate and 2.5 volumes of 95% ethanol were added, the contents vortexed then

centrifuged at 20,000g for 10 min and the supernatant discarded. The pellet was overlaid with 300 µl 80% ethanol and centrifuged at 20,000g for 10 min, the supernatant was discarded and the pellet dried then resuspended in 5 µl MilliQ®H_2O.

2.7.3 Adapter Ligation

Sequentially added to the cDNA was 2 µl Marathon cDNA adapter (10 µM; Appendix 2), 2 µl 5x DNA ligation buffer (250 mM Tris-HCl pH 7.8, 50 mM $MgCl_2$, 5 mM DTT, 5 mM ATP and 25 % (w/v) polyethylene glycol) and 1 µl T4 DNA ligase (400 U/µl), the contents mixed and incubated at 16°C overnight before heating to 70°C for 5 min to denature the ligase. The Marathon cDNA was diluted 1:50 in Tricine-EDTA buffer (0.1 mM EDTA and 10 mM Tricine-KOH pH 8.5) prior to use.

2.8 Rapid amplification of cDNA ends (RACE)

RACE reactions are completed in two stages, in the first part of the reaction 0.5 µl of Marathon cDNA (diluted 1:50), 1 µl of the Marathon kit AP1 primer (Appendix 2) and 1 µl of the gene specific 3'or 5' RACE 1 primer (Appendix 2) were mixed with 2 µl 10x PCR Taq DNA polymerase buffer, 0.4 µl dNTPs (10 mM), 15 µl Milli-Q® H_2O and 0.25 µl of the enzyme Taq DNA polymerase (0.03125 units/µl) and placed in the PCR thermal cycler and the parameters in section 2.6 were followed. To increase the specificity of the product amplified from this reaction using the RACE 1 primers a nested reaction using a second gene specific RACE primer was carried out. The product from the first RACE reaction was used as the template for the nested reaction. The template (0.5 µl) was mixed with 1 µl gene specific 3' or 5' RACE 2 primer (Appendix 2) and 1 µl of the Marathon kit AP2 primer (Appendix 2) and 2 µl 10x Taq DNA polymerase buffer, 0.4 µl dNTPs (10 mM), 15 µl Milli-Q® H_2O and 0.25 µl of the enzyme Taq DNA polymerase (0.03125 units/µl) and placed in the PCR thermal cycler and the parameters in section 2.6 were followed. Products of the second PCR reaction were electrophoresed on a 1% agarose gel, as detailed in section 2.9. Any bands produced by the first and second PCR amplifications would differ in size by the same number of base pairs that separate the two gene

specific primers and the nucleotides between the AP1 and AP2 primer sites. The bands were purified, cloned and then sequenced (sections 2.10, 2.11 and 2.13 respectively) to confirm the identity of the fragment.

2.9 Non-Denaturing Agarose Gel Electrophoresis

Agarose gels (1%) were prepared by boiling 1% (w/v) agarose in 1X TAE (40 mM Tris acetate and 10 mM EDTA, pH 8.0) until vigorous foaming had subsided. The agarose was cooled to ~45 - 50°C then ethidium bromide (10 mg/ml) was added to give a final concentration of 1 μg/ml of gel and the mixture poured into a suitable gel cast (Scie-Plas, UK) containing the appropriate comb and the gel left to set. A 100 bp DNA stepladder size marker (500 μg/ml; NEB Biolabs) was prepared (1 μl marker + 19 μl 1x TAE) and 2 μl of sample preparation solution (0.025% bromophenol blue, 0.025% xylene cyanol, 50% glycerol, all w/v) added to the ladder and samples. Electrophoresis was carried out at 5 V/cm for 20 – 30 min. After electrophoresis was complete the gel was viewed on an UV transilluminator and an image captured on a CCD camera using Gene Snap software (Syngene).

2.10 Purification of DNA Fragments

A number of identical PCR reactions were carried out and the DNA fragments excised from the gel and pooled together to ensure that there was sufficient material present for the purification process. Glassmilk (400 μl; Gene Clean Spin kit, Bio 101, Anachem Ltd.) was added and the contents were mixed and heated at 55°C for 5 minutes, placed on ice for 5 min, then centrifuged briefly. The supernatant was removed and discarded and 0.5 ml New Wash (Gene Clean Spin kit, Anachem Ltd.) added and the pellet re-suspended before pelleting the glass beads as above. The wash step was repeated prior to the addition of 15 μl of Milli-Q®H_2O, preheated to 55°C, to resuspend the glass beads which were heated to 55°C for 2 min. The sample was centrifuged at 20,000g for 1 min, and the supernatant transferred to a filter tube which was centrifuged at 20,000g for 2 min. A further 15 μl of water was added to the pellet and the subsequent steps followed as before. Absolute alcohol (75 μl) and 3 μl 3 M Na acetate pH 4.2 were added to the filtrate, mixed and left to allow the DNA to precipitate

overnight at –80°C. The samples were centrifuged at 33,300g in a Beckmann J2-MC centrifuge, 18.1 rotor (Beckmann Instruments Inc.), 4°C for 1 h and the supernatant removed. The pellet was washed with 200 µl 70% alcohol and re-centrifuged as above for 20 min. The supernatant was removed and the pellet air-dried at RT under vacuum.

2.11 Cloning of DNA Fragments

2.11.1 Preparation of LB Agar Plates

Two 10 cm bacterial culture plates were prepared for each sample. For each plate 20 ml of Ezmix™ LB agar (35.6 g/l; Sigma) was prepared and sterilised by autoclaving at 110°C for 20 min. The agar was then cooled to 50 – 55°C and kanamycin antibiotic added to give a final concentration of 50 µg/ml. Kanamycin was used as the pCR 4 TOPO® vector used in the cloning contains the kanamycin resistant gene (Figure 2.1) and therefore only cells transformed with this vector will be able to survive and grow on the agar plates. The mixture was poured into culture plates and allowed to set.

2.11.2 Cloning Reaction

To the dried gel extracted DNA fragment 3 µl MillQ® H_2O was added and a 0.5 µl aliquot electrophoresed on a 1% agarose gel to quantify the product from the Geneclean® process (section 2.10). Between 0.5 and 3 µl DNA fragment, 1 µl Salt Solution (1.2 M NaCl, 0.06 M $MgCl_2$; TOPO® PCR cloning kit, Invitrogen) and 1 µl TOPO® PCR 4 vector (10 ng/µl plasmid DNA) were mixed and incubated at room temperature for 15 min (Figure 2.1, Invitrogen). A sample of the TOPO® cloning reaction (2 µl) was added to a vial of One Shot® chemically competent cells (Invitrogen), gently mixed and incubated on ice for 30 min. The cells were heat shocked at 42°C for 30s and immediately transferred to ice for 2 min before addition of 200 µl of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) and incubation with rotation at 37°C for 1 h. A sample of cell suspension (50-100 µl) was spread on each agar plate, and the plates incubated at 37°C overnight. Only cells containing the vector with a DNA insert should grow and form bacterial colonies. The pCR 4 TOPO® vector (Figure 2.1) possesses a ccdB gene which is

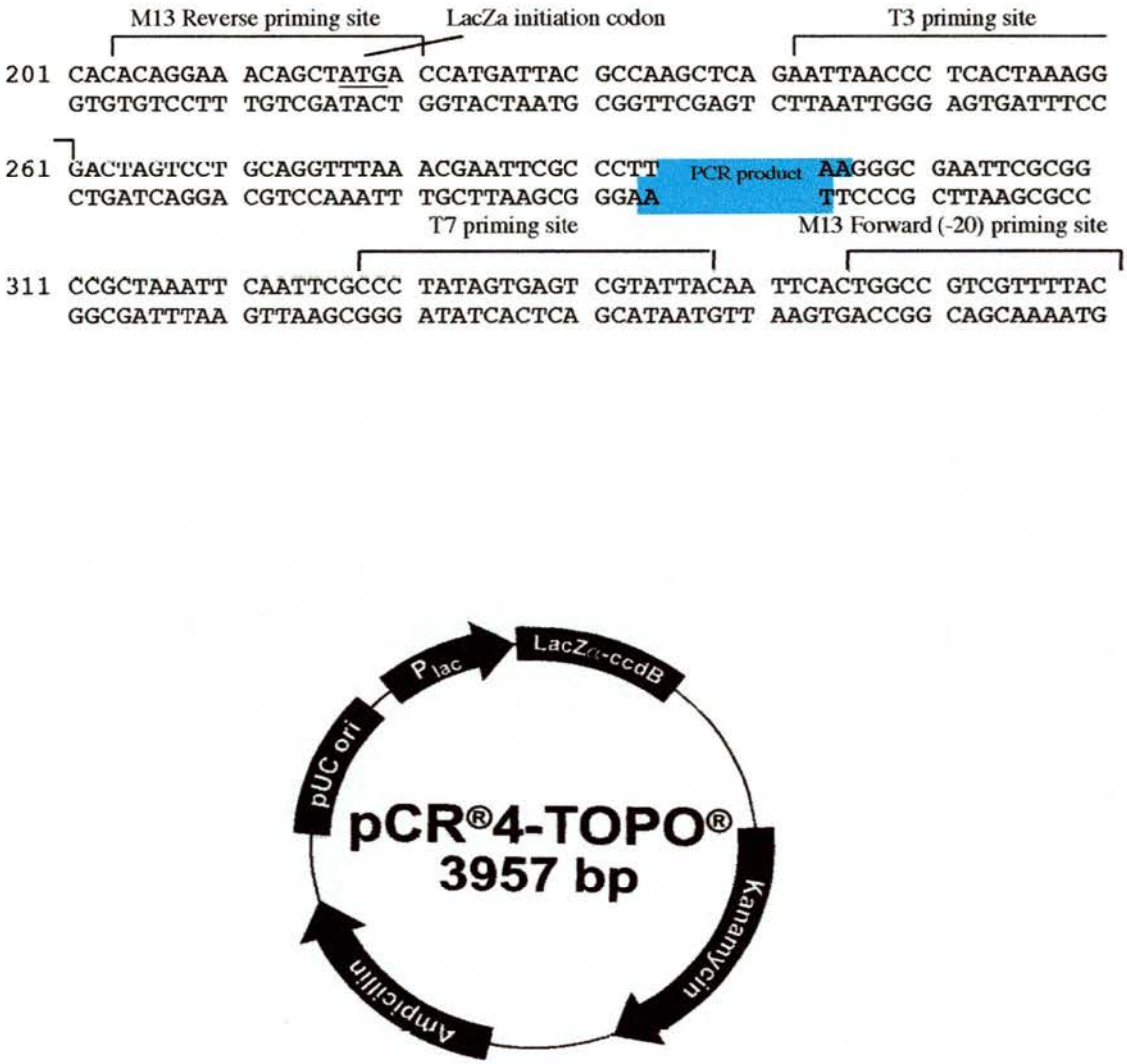


Fig 2.1 Diagrammatic representation of theVector map of pCR®4-TOPO® with cloning site and M13 primer sites. Taken form Invitrogen TOPO cloning manual (version J).

lethal to *E. coli*. The ccdB gene is fused to the carboxy terminus of the Lac Zα fragment. If a PCR product is ligated into the vector the PCR product disrupts the production of the Lac Zα-ccdB gene fusion.

Therefore cells into which the vector containing the PCR product have been inserted are able to survive as the ccdB gene has been inactivated, without insertion of the PCR product the ccdB gene remains active and is lethal to the host cells. The vector also possesses resistance to the antibiotic kanamycin, which is present in the agar of the bacterial culture plates, and only cells containing the vector are able to survive in this environment.

211.3 Selection of Colonies

Colonies were selected using a sterilised metal loop and used to inoculate 1 ml Terrific Broth (47.6 g/l; Sigma) containing 0.8% glycerol (v/v) and 50 μg/ml kanamycin antibiotic. The Terrific Broth provides the cells with the required salts for optimum growth and the presence of kanamycin ensures only bacteria transfected with the kanamycin resistance plasmid will grow. The cell suspension was incubated at 37°C with rotation for 24 h and a 50 μl sample was taken and centrifuged at 20,000g (Eppendorf centrifuge 5417C, Helena Bioscience) for 1 min and the pellet of cells resuspended in 400 μl MillQ®H_2O.

2.11.4 Colony PCR

Colony PCR was conducted by addition of 0.5 μl of the resuspended cells, 1 μl M13 forward primer (4 μM; Appendix 2) and 1 μl M13 reverse primer (4 μM; Appendix 2), 17.525 μl PCR cocktail (comprising 15 μl MilliQ®H_2O, 2 μl 10x Taq buffer, 0.4 μl 10 mM dNTP and 0.125 μl DNA Taq polymerase, 0.03125 U/μl) which were mixed and placed in the thermal cycler (Thermal cycler, TechneProgene, Techne Ltd) set with the following parameters: -

Section	No. of Cycles	Temperature (°C)	Time
1	1	94	2min
2	40	94	5s
		58	20s
		72	1min extension for every 1kb fragment
3	1	72	15min

When the PCR programme was complete, the samples were briefly centrifuged then loaded onto an ethidium bromide containing non-denaturing agarose gel (section 2.9) to detect positive colonies. Three positive colonies were selected and the colony PCR repeated. The amplified fragments were purified directly from the PCR reaction using the Quickstep™ 2 PCR purification kit (Edge Biosystems). SOPE™ (solid phase oligo/protein elimination) resin (4 µl) was added to 20 µl of the PCR reaction products and mixed. This SOPE™ resin which binds all non incorporated nucleotides and primers, enzymes, ssDNA and proteins present in the sample was then added to a PERFORMA® DTR gel filtration cartridge and centrifuged at 800g for 3 min. By passing the amplified DNA fragment through the PERFORMA® DTR gel filtration cartridge any buffers, dNTPs, Taq polymerase, primers and salts are bound to the SOPE™ resin are removed from the sample ensuring that the cDNA is highly purified prior to sequencing.

2.12 Quantification of DNA Samples

After purification, 2 µl of the DNA sample was added to 18 µl 1x TAE and 3 µl of sample preparation solution and loaded onto a 1% agarose gel with 0.5 µl and 1 µl of 100 bp DNA ladder (NEB) or Phi x174 HAE III DNA quantification marker (equivalent to 100 ng DNA; NEB). After electrophoresis the gel was viewed on an UV transilluminator, an image captured using a CCD camera and bands analysed using Gene Tools and Gene Snap computer software (Syngene) to determine the concentration of DNA present in each sample.

2.13 DNA Sequencing

DNA sequencing was conducted using the Applied Biosystems ABI Prism Big Dye Terminator Sequencing kit. This sequencing method is an adaptation of the dideoxy chain termination technique described by Sanger *et al.*, 1977. The technique works by the incorporation of a fluorescein donor dye linked to a different rhodamine acceptor dye in each dideoxy nucleotide (ddNTP). This results in a different emission wavelength for the fluorescence for each of the four nucleotides A, G, T and C. When the fluorescent labelled ddNTP has been incorporated into the DNA chain during PCR it terminates chain elongation preventing the addition of further nucleotides. After the sequencing reaction the sample is electrophoresed on a polyacrylamide gel with all fragments separated on the basis of size and the terminated nucleotide fragments are detected as they pass the fluorescent analyser and depicted as different coloured bands representative of each nucleotide on an electrophoretogram (Figure 2.2). After quantification of the DNA sample to be sequenced, 40 ng of the template was used in the sequencing reaction with 1 µl of 4 µM specific vector primer (either T7 or T3; Appendix 2) and 1 µl terminator ready reaction mix. BigDye™ terminator ready reaction mix contains A-Dye terminator labelled with dichloro[R6G], C-Dye terminator labelled with dichloro[TAMRA], G-Dye terminator labelled with dichloro[R110], T-Dye terminator labelled with dichloro[ROX], deoxynucleoside triphosphates, AmpliTaq DNA Polymerase, $MgCl_2$ and Tris-HCl buffer pH 9.0, (ABI Prism, PE Biosystems). The sequencing reaction mixture was placed in the thermal cycler programmed with the following parameters: -

Section	No. of cycles	Temperature (°C) (Rate of change of temperature is 1°C/s)	Time
1	25	96	10 s
		50	5 s
		60	4 min
2	1	4	15 min – 24 h

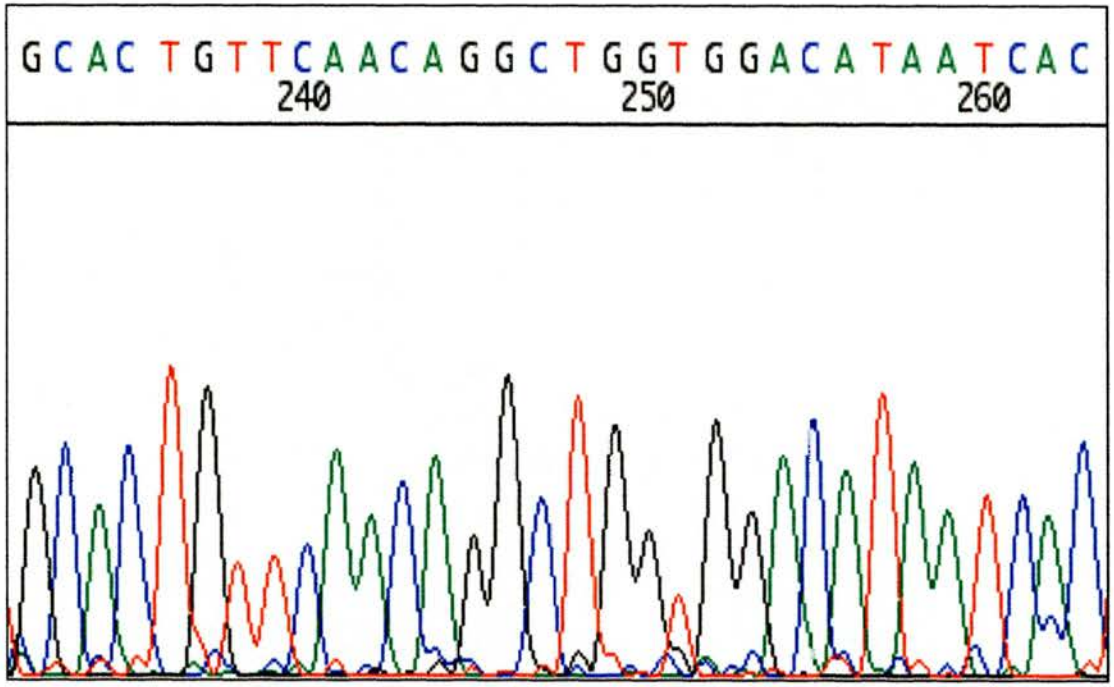


Fig. 2.2 An example of an electrophoretogram from a DNA sequencing reaction. Each of the four nucleotides is colour coded, G – black, C – blue, A – green, T – red.

Upon completion, 15 µl isopropanol was added to each sample, mixed and incubated at RT for 20 min, centrifuged at 20, 000g for 20 min (Eppendorf centrifuge 5417C, Helena Bioscience) and the supernatant discarded. The pellet was washed with 400 µl 70% ethanol and then centrifuged as before. The pellet was left to dry before sending to the Protein and Nucleic Acid Chemistry Laboratory (PNACL), Leicester University, where the sample was electrophoresed on a polyacrylamide gel and bands detected using a fluorescent analyser. The results were analysed with GeneJockey II software (Biosoft).

2.14 Northern blotting and analysis

The Northern blotting technique is used to detect and quantify specific transcripts within total RNA samples which had been size-separated by electrophoresis on denaturing agarose gels (section 2.4). The technique allows detection of specific RNA molecules which are complementary to the radiolabelled DNA probe used for detection. Northern blotting was used to determine in which tissues mRNAs for the guanylin-like peptides were located and the technique was also used to study any effects of FW/SW transfer had on mRNA expression in tissues from both yellow and silver eels.

2.14.1 Preparation of gel and cassette for electroblotting

Total RNA samples were prepared and quantified as described in section 2.3. Samples containing 5 – 10 µg of total RNA were electrophoresed on a denaturing agarose gel (section 2.4) and then stained in ethidium bromide (1 µg/ml) as detailed in section 2.4. The gel was viewed on an UV transilluminator and an image captured using a CCD camera and Gene Snap software (Syngene). and the relative amount of RNA in each sample determined using the combined intensity levels of the 18S and 28S RNA bands quantified using Gene tools software (Syngene). A cassette comprised of Scotbrite™ pads, Whatman® 3MM filter paper, Zetaprobe™ membrane (Biorad laboratories Ltd.) and the RNA gel was assembled (as shown in Figure 2.3) under ice-cold 1x TAE buffer. The assembled cassette was loaded into a tank containing ice cold 1x TAE and the gel was transferred onto the Zetaprobe™ membrane at a constant current of 0.25 amps using plate electrodes overnight (BioRad 250/2.5 power supply, BioRad

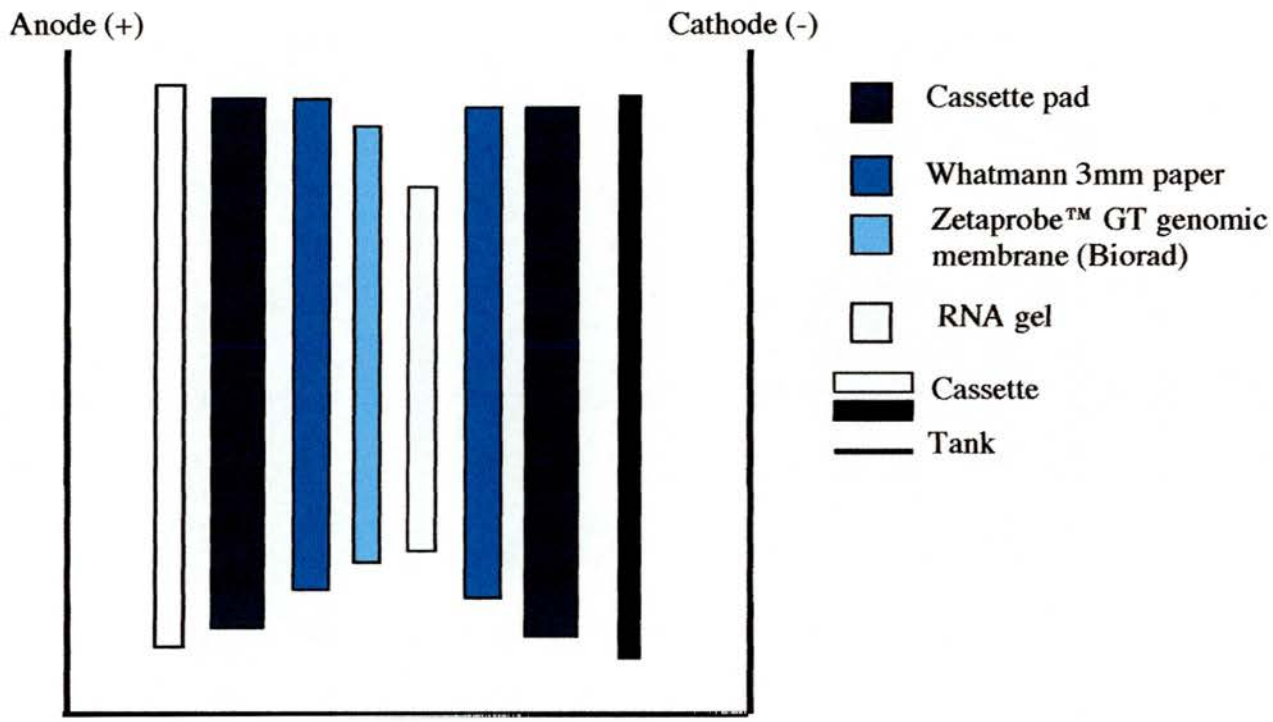


Fig 2.3 Diagrammatic view of assembly of the Northern blotting cassette.

Lab. Ltd.). After blotting was completed the cassette was carefully opened and the position of the sample wells on the agarose gel were marked on the membrane. The membrane was then placed between two sheets of Whatman® 3MM filter paper and placed in the vacuum oven for 10 min at room temperature to dry. When the membrane was dry the RNA samples were covalently linked to the nylon membrane by irradiating with ultra violet light (L 1500 UV Crosslinker, Spectronics Corporation). The membrane was stored at –20°C until required.

2.14.2 (i) Preparation of the radioactive probe

Cloned PCR product (30 μg) in a volume of 24.5 μl was added to 10 μl 5x labelling buffer (250 mM Tris-HCl (pH 8.0), 25 mM $MgCl_2$, 10 mM DTT) and 26 U/ml random nucleotides; Promega) and boiled for 5 min then snap cooled on ice. The mixture was briefly centrifuged before the addition of 5 μl 200 μM dNTPs (without dCTP), 5 μl 0.4 mg/ml BSA, 0.5 μl Klenow enzyme (5 U/μl; NEB) and 5 μl γ[^{32}P] dCTP (6000 Ci/μmol; Amersham Pharmacia Biotech) which was mixed gently and incubated at 37°C for 15 – 30 min before centrifuging as before. In this procedure random nonamer primers bind to the cDNA product and in the presence of the Klenow enzyme, γ[^{32}P] dCTP unlabelled dNTPs radioactive ^{32}P is incorporated into probes of varying length which are subsequently used in hybridisation reactions. Non-incorporated nucleotides were removed by exclusion column chromatography as detailed below.

2.14.2 (ii) Removal of non-incorporated nucleotides

Siliconised glass wool and glass beads were used to plug the bottom of a siliconised 2 ml glass column. Sephadex G50, pre-swollen in water and degassed in a vacuum oven for 15 min, was added to the column leaving sufficient space at the top to add the sample and for water to elute the sample from the column. The product from the radiolabelling reaction was added and washed into the column with water. The column was eluted with water and when the radioactive sample percolated through the Sephadex to near the bottom of the column (detected using a Geiger counter) 16 serial samples (4 drops/tube) were collected. A 1 μl sample was taken from each fraction collected and added to

Pony scintillation vials containing 4.5 ml MilliQ®H_2O. Using a scintillation counter (Packard TRI-CARB 1600TR counter, Packard Ltd.) the radioactivity present in each sample was determined. The percentage radioactivity that was incorporated into the cDNA probe was estimated as follows: -

$$\frac{\text{Total radioactivity (cpm) in first 5 tubes}}{\text{Total of radioactivity (cpm) from all samples}} \times 100 = \% \text{ incorporation}$$

From the readings the products from the leading peak which had the highest activity were selected. These samples contained the radiolabelled DNA probe and only labelled probes with a minimum level of 50% incorporation were used. The non-incorporated dNTP fractions containing $\gamma[^{32}P]$ dCTP which gave a second peak in the readings were discarded.

2.14.3 Hybridisation and washing of Membranes

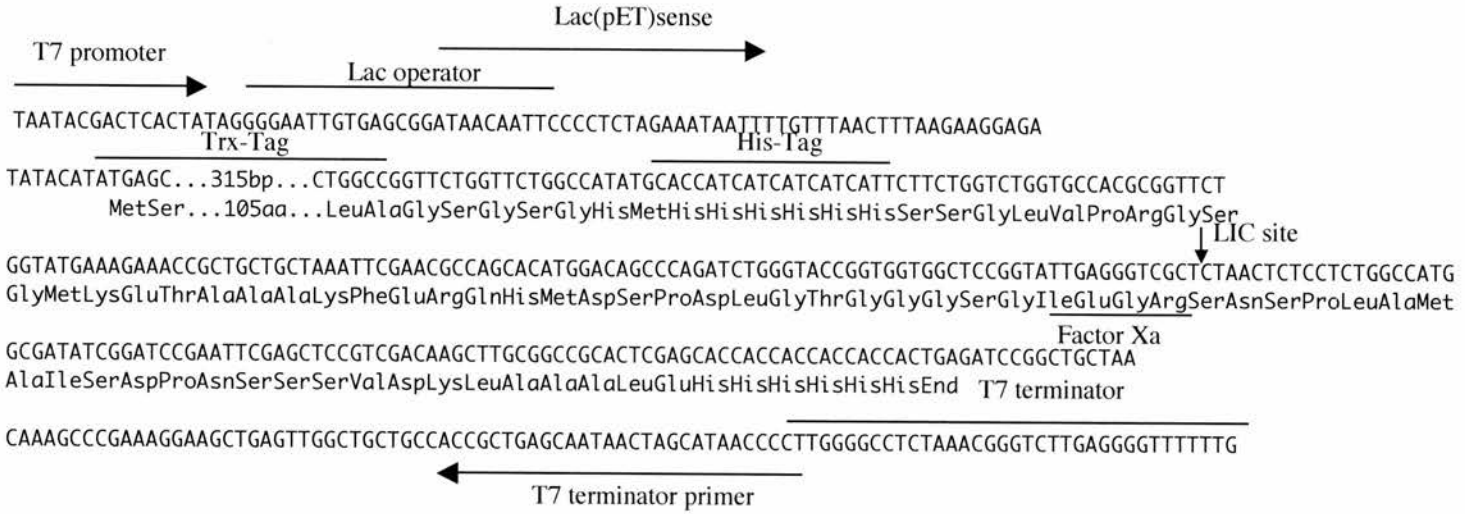
The fractions collected for use as the probe for Northern blotting were combined and boiled for 15 min, cooled rapidly on ice for 5 min and then centrifuged briefly to collect all of the sample at the bottom of the tube. The radiolabelled probe was added to 15 ml of hybridisation solution (Ultrahyb™ buffer; Ambion Inc.) which had previously been incubated at 47°C for at least 4 h with the membrane in a hybridisation oven (Techne Hybridisor HB-1D). The pre-hybridisation process blocked any non-specific binding sites to ensure that the radiolabelled DNA probe only bound to homologous mRNA crosslinked to the membrane. The membranes were incubated with the radioactive probe at 47°C overnight in a hybridisation oven with continuous rotation. After overnight incubation the Ultrahyb™ buffer containing the radioactive probe was poured off and discarded. The blots were washed in 30 ml of Wash 1 (1% SDS and 1 x SSC; 0.15 M NaCl and 15 mM sodium citrate pH 7.0) at 47°C for 20 min. This was discarded and then 30 ml of Wash 2 (0.2 x SSC, 0.1% SDS) for 20 min at 47°C and finally with 30 ml of Wash 3 (0.1x SSC, 0.1% SDS) for 30 min. The membranes were removed, sealed in polythene and analysed by electronic autoradiography using an Instant Imager (Canberra Packard, Meridan, CA). The imager collected the radioactive signal intensity of each sample on the blot, if the background levels of radioactivity were still high then the membrane was washed

again in wash solutions 2 and 3. The data were adjusted to account for variations in RNA loading on the gel as determined by the intensity of the ethidium bromide stained 28S and 18S RNA bands (section 2.4). The correction factor took into account any sample that may have been lost during preparation of the samples or when loading the denaturing agarose gel. Autoradiographs were also taken by exposing the blot to X-ray film (Kodak Biomax™ film) within a Kodak X-omatic cassette with intensifying screens which was left at –80°C for various times depending on levels of radioactivity hybridising to the blot. The X-ray film was developed with Kodak GBX developer and fixer solutions (Sigma).

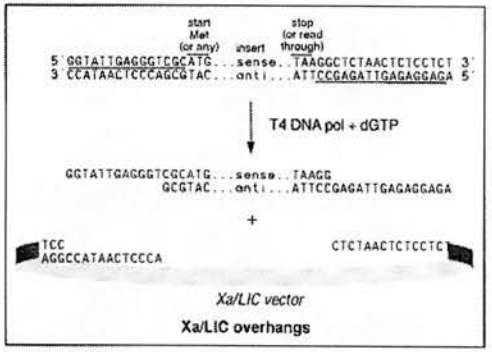
After probing the blots were cleaned by boiling in 0.1% SDS for 10 min then analysed on the Instant imager to ensure the radiolabelled probe had been removed.

2.15 Bacterial Expression of Guanylin-like Peptides

The pET expression system, developed by Studier and Moffatt in 1986, allows for the rapid production of a large quantity of recombinant proteins in *E coli*. The vector selected for the expression of the prohormone was pET 32 Xa/LIC (figure 2.4; Novagen). The pET 32 Xa/LIC vector results in the production of an N terminal fusion protein comprised of thioredoxin (Trx), which helps to solubilise the protein of interest, and a hexa histidine tag found between Trx and the protein of interest. The pET expression system contains a number of mechanisms to control protein expression and these control systems are shown in figure 2.5. The host cells are lysogenic for a fragment of the phage DE3 and this fragment is inserted into the *E. coli* chromosome of the host cell. The DE3 fragment contains the lac I gene, the lac UV5 promoter, the start of the lac Z gene (β galactosidase) and the T7 RNA polymerase gene. The expression of T7 RNA polymerase is induced by the addition of a synthetic inducer isopropyl β-D-thiogalactopyranoside (IPTG). T7 RNA polymerase is very selective for the T7 promoter situated upstream (5') of the target gene in the pET vector and the T7 promoter activates the T7 RNA polymerase to initiate transcription of the target gene. The introduction of IPTG into the cell expression system relieves the inhibition of the lac promoter and T7 promoter by the lac repressor binding and the lac operater site. *E. coli* RNA polymerase is responsible for expressing *E. coli* genes but the expression of these genes is suppressed due to the activity of



Primer sequence extensions required for Xa/LIC compatibility are underlined in the diagram below.



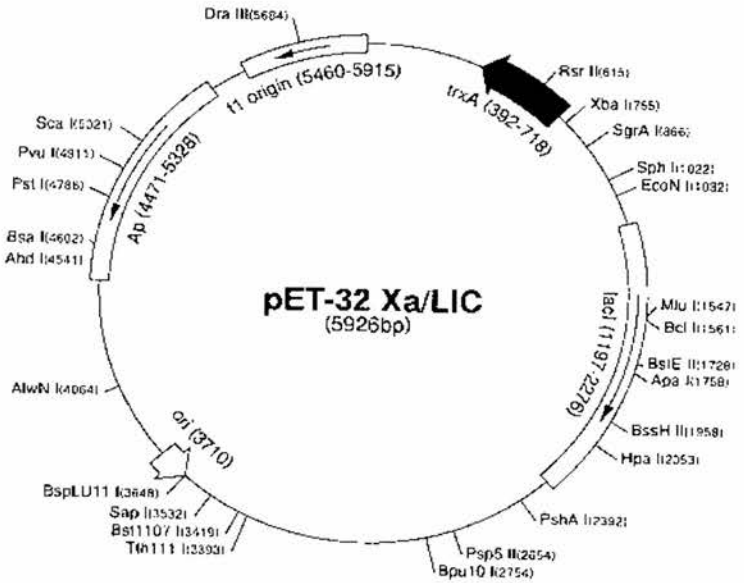


Fig 2.4 Diagrammatic representation of the vector map of pET 32Xa/LIC vector. Taken form the Novagen pET cloning manual.

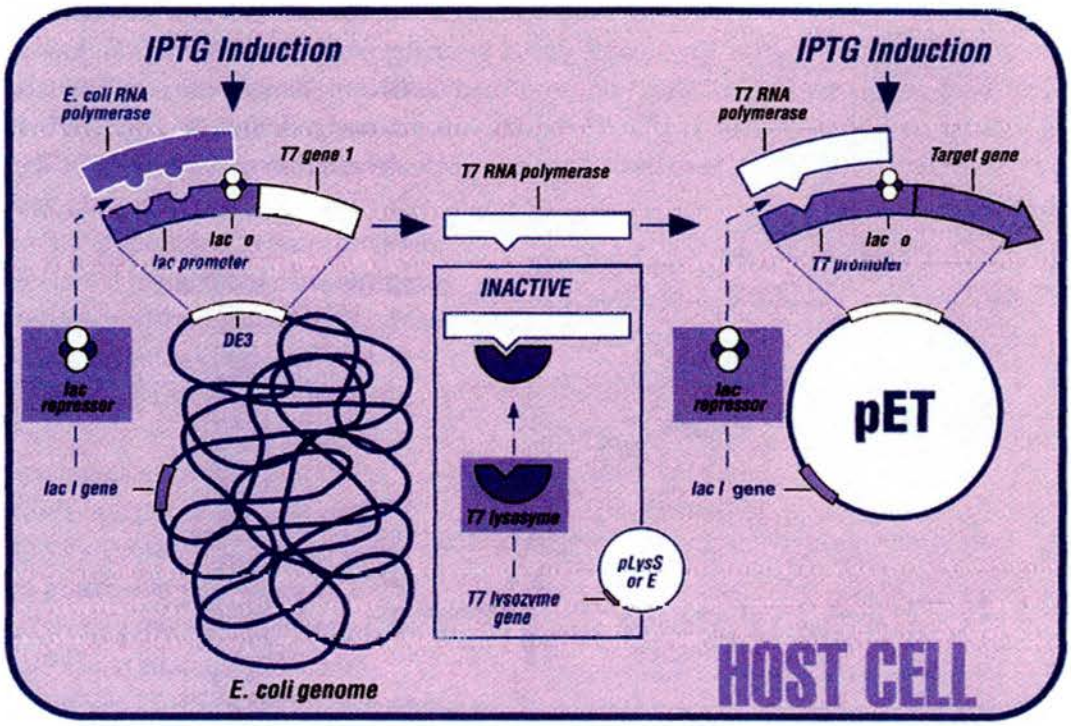


Fig 2.5 Control elements of the pET expression system (Novagen)

T7 RNA polymerase which is approximately five times greater than that of *E. coli* RNA polymerase. This means that the genes controlled by T7 promoters are amplified and expressed in large quantities. Low levels of the T7 RNA polymerase gene are also transcribed in the absence of IPTG but this activity is controlled by the lac I gene, found in the *E. coli* chromosome or in the pET vector. The lac I gene expresses a lac repressor which binds to the lac promoter and inhibits transcription of the T7 gene. To further control the activity of T7 RNA polymerase the host carries pLysS, a plasmid that encodes T7 lysozyme, a natural inhibitor of T7 RNA polymerase.

2.15.1 Ligation of DNA into pET 32 Xa/LIC Vector

DNA fragments encoding the prohormone sequence of eel guanylin, renoguanylin and uroguanylin were amplified using guanylin/renoguanylin/uroguanylin specific pET 32 Xa/LIC sense and antisense primers (Appendix 2) as described in section 2.6. The amplified sequences included a PreScission™ protease site within the sense primer which would allow the production of the prohormones from the synthesised fusion proteins by digestion with the PreScission™ protease enzyme Amersham Biosciences; section 2.15.6 (ii)).

The PCR amplified proguanylin/prorenoguanylin/prouroguanylin fragment (20 ng) was mixed with 1 µl 10x T4 DNA polymerase buffer, 1 µl 25 mM dGTP, 0.5 µl 100 mM DTT, 0.2 µl T4 DNA polymerase (2.5 U/µl) and water to a final volume of 10 µl and incubated at room temperature for 30 min. The T4 DNA polymerase has 3' to 5' exonuclease activity and removes nucleotides from one strand of the DNA fragment until it encounters guanosine nucleotides which are replaced by dGTP (deoxyguanosine 5'-triphosphate) present in the digest mix. This enzyme digest ensures that the ends of the DNA fragments have specific vector-compatible overhangs, shown in figure 2.6. The vector has a specific 12 base single stranded overhang upstream of the insert and a specific 15 base pair overhang downstream of the insert (shown in figure 2.6). This allows direction dependent insertion of amplified fragments into the vector. The sample was then incubated at 75°C for 20 min to inactivate the T4 DNA polymerase enzyme. To insert the DNA fragment into the pET 32 Xa/LIC vector, 2 µl of the product from the T4 DNA polymerase enzyme digest was mixed with 1 µl pET 32

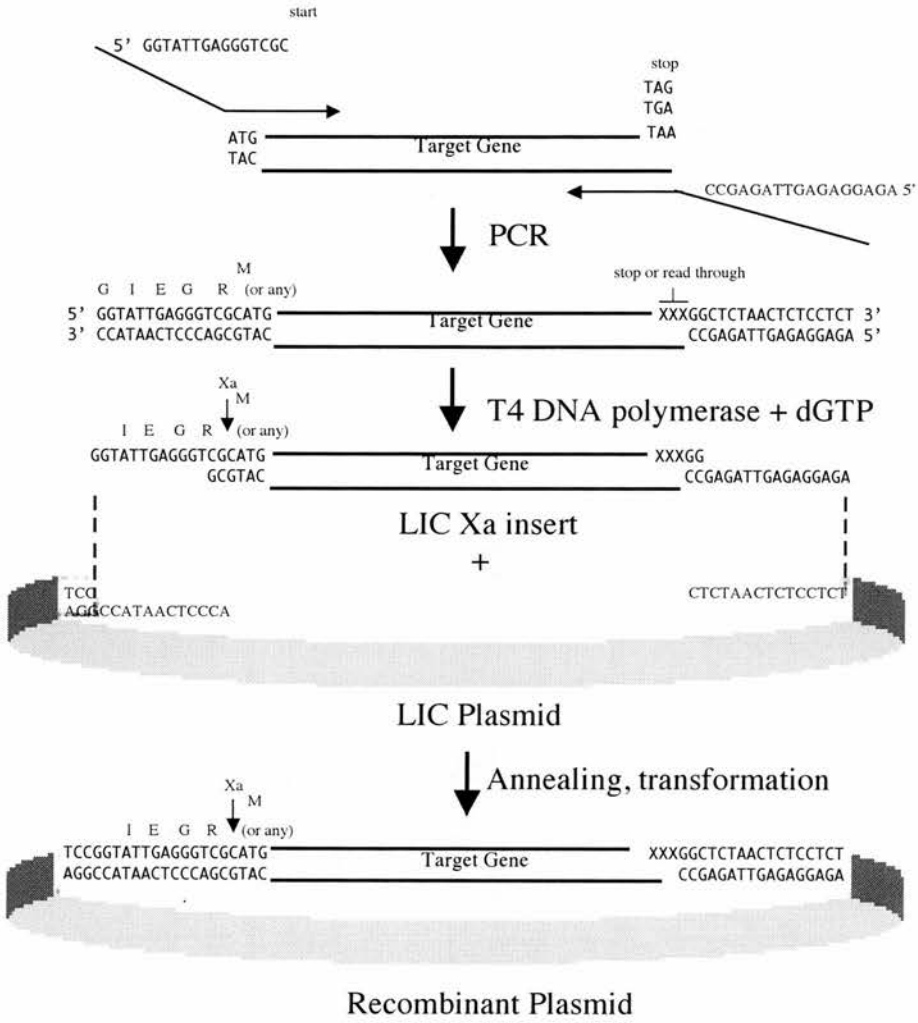


Fig. 2.6 Diagram of the Xa/LIC method explaining how the target gene is inserted into the pET 32 Xa/LIC vector (Adapted from Novagen Xa/LIC cloning kit manual). After amplification with primers including the indicated 5' LIC extensions, the PCR insert is treated with LIC qualified T4 DNA Polymerase (+dGTP), annealed to Novagen's linearized vector, and transformed into competent *E. coli* (Novagen).

Xa/LIC vector (50 ng/μl) and incubated at RT for 5 min. EDTA (25 mM, 1 μl) was added and the mixture incubated at RT for a further 5 min. The vector and the DNA fragment have compatible ends which enables them to anneal together. The sample was cloned into Top 10 cells (Invitrogen), a non protein expressing cell line as described in section 2.11. Colony PCR was carried out using Lac pET sense and T7 terminator primers (Appendix 2) to ensure the colonies contained a DNA insert. Three colonies containing inserts were sequenced (section 2.13) to ensure no errors had been incorporated.

2.15.2 Plasmid DNA purification by the small scale alkaline lysis method

This technique enabled the DNA fragments which had been inserted into the pET 32 Xa/LIC vector to be isolated from a non-expressing host cell line before insertion into a bacterial host suitable for protein expression. A 50 μl aliquot of pET 32 Xa/LIC vector containing the DNA fragments in Top 10 cells was added to 1 ml of Terrific broth (47.6 g/l containing glycerol 8 ml/l; Sigma) containing carbenicillin (50 μg/ml) and incubated at 37°C for 3 h with continuous rotation. This sample was used to seed 50 ml of Terrific Broth (TB) containing carbenicillin (50 μg/ml) and incubated as before. To harvest the pET 32 Xa/LIC vector containing the DNA fragment 50 ml of cell suspension was centrifuged at 3480 g in a Beckman J6-MC centrifuge, rotor 4.2 (Beckman Instruments Inc.) for 10 min at 4°C and the supernatant removed and discarded. The bacterial pellet was resuspended in 2 ml of Solution I (50 mM glucose, 25 mM Tris HCl pH 8.0, 10 mM EDTA) and transferred to a 15 ml Corex® tube and 4 ml of freshly prepared Solution II (0.2 M NaOH, 1% SDS) was added and the contents mixed by inversion of the tube 5 – 6 times. After mixing, 3 ml of ice-cold Solution III (5 M potassium acetate, 11.5% glacial acetic acid) was added and the contents vortexed. The sample was incubated on ice for 3 – 5 min, then centrifuged at 12,000 g (J2-MC Beckmann JA 13.1) for 5 min at 4°C. Isopropanol (1 volume) was added to the supernatant and left on ice for 1 h to precipitate the double stranded DNA before centrifugation, as detailed above, for 40 min. The pellet was washed with 2 ml of 70% ethanol and centrifuged as before at 4°C for 15 min. The supernatant was removed and the pellet air-dried under vacuum. The pellet was resuspended in 200 μl 10 mM Tris, 1 mM EDTA pH 8.0 (T.E) buffer and 40 μl of 12 M LiCl (masks the negative charges of RNA allowing the RNA

to precipitate) added before centrifugation as above for 30 min. Isopropanol (1 volume) was added to the supernatant and left on ice for 30 min then centrifuged as above for 30 min. The pellet was washed with 70% ethanol and re-centrifuged at 4°C for 15 min. The supernatant was discarded and the pellet dried under vacuum then resuspended in 100 µl 10 mM Tris, 100 mM NaCl pH 7.4 (T.N) buffer. Ribonuclease A (RNase A; 5 µl, 10 mg/ml) was added to the sample to digest any remaining RNA which would contaminate the DNA. The digest was incubated at 37°C for 30 min before addition of 200 µl 25:24:1 (v/v/v) phenol/chloroform/isoamylalcohol mix to the digest and centrifugation at 12,000 g for 5 min at RT. The upper phase was transferred to a new tube and 100 µl of chloroform/isoamylalcohol (24:1) was added, mixed and re-centrifuged at 12,000 g for 2 min at RT. The DNA was precipitated by addition of 0.1 volumes of 3 M Na acetate (pH 5.2) and 2 volumes of absolute ethanol and incubated overnight at –20°C. The sample was centrifuged at 12,000 g for 20 min at RT and the pellet was washed in 3 ml of 70% ethanol and centrifuged as before. The pellet was air-dried under vacuum then resuspended in 30 µl of MillQ®H_2O.

2.15.3 Electroporation of Origami Cells and Transformation of Fragments

The purified vector containing the sequenced insert was transfected into Origami B pLys S electrocompetent cells (50 µl; Novagen) by electroporation (Electroporator, Eppendorf) at 1600V. SOC medium (400 µl of 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) was added to the cell suspension and incubated at 37°C for 1 h on a rotating mixer. After incubation, 50 µl of the cell suspension was spread on an agar plate (section 2.11.1) which contained the antibiotic ampicillin or carbenicillin (50 µg/ml; Sigma). The pET 32 Xa/LIC vector carries the β-lactamase gene and hence induces bacterial resistance to penicillin antibiotics such as ampicillin and carbenicillin, therefore only cells transformed with this vector will grow on the agar plates.

2.15.4 Expression of guanylin-like peptide prohormones

A 4 l stock of TB (47.6 g/l containing 0.8% glycerol (v/v)) was sterilised by autoclaving then a cocktail of antibiotics was added (final concentrations were 50 µg/ml carbenicillin, 15 µg/ml kanamycin, 12.5 µg/ml tetracycline and 34 µg/ml

chloramphenicol). The Origami B host cells (Novagen) are resistant to chloramphenicol due to the presence of the pLysS plasmid and the Origami B cells have been mutated in two positions within the host genome. The first is the gene for the enzyme thioredoxin reductase and the second is glutathione reductase. The mutations render these enzymes/genes inactive and therefore will increase the likelihood of disulphide bond formation of the transfected protein in the host cytosol. The mutations are selected by incorporation of the antibiotic resistance genes for kanamycin (thiroredoxin reductase) and tetracycline (glutathione reductase) in the host genome. The pET 32 Xa/LIC vector also carries resistance to ampicillin. Therefore only Origami B cells transformed with the pET 32 Xa/LIC vector will be able to survive and grow in this ampicillin containing medium. Cells transformed with the vector containing the DNA fragment encoding the prohormone sequences were used to seed 1 ml of TB and incubated at 37°C for 2 h. The cell suspension was then transferred to a flask containing 50 ml of TB and incubated at 37°C overnight with continuous rotation. After overnight incubation, the cells were added to 500 ml of TB, mixed and a 1 ml sample was taken to determine the absorbance reading at 600 nm (Phillips PU 8620 UV/VIS/NIR spectrophotometer). The remaining TB culture was incubated at 37°C for 2 – 3 h in an orbital shaker at 180 rpm until an absorbance (A_{600nm}) of 0.6 was obtained then 5 ml 100 mM IPTG was added to the remaining culture and incubated at 30°C for a further 2.5 – 3 h. The culture was placed on ice for 5 - 10 min then centrifuged for 15 min at 4°C at 4,000 g (Beckman J-21B centrifuge, JA10 rotor; Beckman Instruments Inc). The cells were resuspended twice in 500 ml ice cold water and centrifuged for 15 min as before. The pellet was resuspended in 50 ml ice cold water and centrifuged at 3954 g (Beckman J6-MC centrifuge, 4.2 rotor) for 15 min at 4°C. The bacterial pellet was stored at -20°C until required.

2.15.5 Protein isolation from the bacterial host cell

The cell pellets (section 2.15.4) were thawed on ice then resuspended in 5 ml (per 500 ml culture) of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) containing the following cocktail of protease inhibitors, amastatin (1 μM), aprotinin (7.77 trypsin inhibitor units/ml), leupeptin (10 μM), bestatin (5 μg/ml), E64 (5 μM), pepstatin (1 μM), phenylmethylsulfonylfluoride

(PMSF; 1 mM), phenanthroline (200 μM) and phosphoramidone (1 μM). Lysozyme was added to the suspension to a concentration of 1 mg/ml and incubated on ice with mixing for 30 min. The suspension was sonicated six times for 10 s at 10 s intervals keeping on ice throughout. Triton X 100 was added to a final concentration of 0.01 % and the suspension vortexed briefly. The suspension was then freeze-thawed in liquid nitrogen three times, 50 μl of 1 M $MgCl_2$ and 50 μl 1 mg/ml DNase A were added and the suspension mixed and incubated at RT with rotation for 10 min. The lysate was centrifuged at 12,000g (J2-MC Beckmann J-A13.1) for 30 min at 4°C and the supernatant retrieved.

2.15.6 (i) Protein purification - Metal chelation chromatography

Upstream of the DNA fragment inserted into the pET 32 Xa/LIC vector is a thioredoxin fusion protein. This larger fusion protein helps to maintain the solubility of the protein and is important for purification. A hexa his-tag sequence is incorporated into the vector between the thioredoxin protein and the protein of interest. The hexa his-tag is used for purification of the fusion protein as the six sequential histidine residues can chelate nickel ions. The supernatant (~ 5 ml) retrieved after protein extraction from the bacterial host cell (section 2.15.5) was mixed with 4 ml of Ni-NTA bead slurry suspension (50% beads in 30% ethanol; Novagen) and incubated at 4°C with continuous rotation for 2 h. The suspension was poured into a 10 ml glass column and the beads allowed to settle. The extract was allowed to flow through the column and the eluent was collected. The column was washed sequentially with 10 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole, pH 8.0), 4 – 5 ml wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0) and then the bound His-tagged protein was eluted with 10 – 15 ml of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole, pH 8.0) and collected in 1 ml fractions. After collection the column was washed with 10 ml of lysis buffer and the beads collected and stored at 4°C for future use. The fractionated samples were electrophoresed by SDS PAGE (50 μl sample diluted 1:1 with 2x gel loading buffer) as described in section 2.18 and proteins detected using Coomassie blue as detailed in section 2.18.2. Alternatively guanylin-like peptide prohormones were also detected by Western blotting (section 2.20).

2.15.6 (ii) Protein purification – PreScission™ protease enzyme digest

The second stage in the purification of the protein of interest was to remove the amino terminal thioredoxin hexa his-tag from the fusion protein. Adjacent to the amino terminal of the prohormone the amino acid sequence, Leu-Phe-Gln-Gly-Pro, was incorporated into the fusion protein and these amino acids are recognised by PreScission™ protease (Amersham Biosciences). This protease is synthesised as a GST fusion protein and cleaves between the glutamine and glycine residues of the Leu-Phe-Gln-Gly-Pro sequence leaving a Gly-Pro sequence at the amino terminal of the prohormone. The fractions containing the expressed fusion protein were pooled together and dialysed for 24 h at 4°C against two changes of 50 mM Tris, 150 mM NaCl and 1 mM EDTA, pH 7. The sample was retrieved from the dialysis tubing and the protein concentration of the sample determined by the Bradford assay (section 2.16). The dialysed samples were stored at 4°C.

The dialysed protein sample (50 μg) was incubated at 4°C with 2 units (2 U/μl) of the PreScission™ protease enzyme and buffer (50 mM Tris, 150 mM NaCl and 1 mM EDTA, pH 7) to a final volume of 400 μl. Aliquots (50 μl) of the digest were collected at time 0 and after 1 h, 2 h, 4 h, 8 h, 12 h and 24 h. After 24 h the digest was mixed with 1.5 ml Ni-NTA beads (Novagen) and incubated at 4°C with continuous rotation for 2 h. The digest/bead mixture was poured into a 5 ml column and the eluent (which contains the prohormone of interest cleaved from the amino terminal of the thioredoxin fusion protein) was collected. The fractions were sampled (50 μl aliquots diluted 1:1 in 2x gel loading buffer) and electrophoresed by SDS PAGE (section 2.18). The samples containing the prohormone were then put through a GST column (Amersham Bioscience) to remove the PreScission™ protease, a GST fusion protein. The collected prohormone was assessed for protein by the Bradfords assay (section 2.16) then dialysed again against water to remove the imidazole buffer before aliquoting and freeze drying.

2.16. Extraction of endogenous peptides from the eel intestine

2.16.1 Extraction of endogenous peptides using a sucrose gradient

Intestinal tissue was taken from a FW yellow eel and the epithelial mucosa was scraped from the muscle layer using a microscope slide and homogenised in 10 ml of ice cold 25 mM Hepes, 0.25 M sucrose, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4 containing 1 mM PMSF (phenyl methyl sulfonyl fluoride) using a Polytron PT 10 homogeniser (Kinematica Ltd.). The homogenate was filtered through four layers of muslin gauze, placed in an ultraclear Beckman centrifuge tube and diluted with two volumes of 25 mM Hepes, 70% (w/v) sucrose, pH 7.4 and the contents mixed. The diluted homogenate was overlaid with 5 ml of 25 mM Hepes, 46 % (w/v) sucrose, pH 7.4, then 5 ml of 25 mM Hepes, 40 % (w/v) sucrose, pH 7.4 and finally overlaid with 2 - 3 ml of 25 mM Hepes, 8 % (w/v) sucrose, pH 7.4. The tubes were centrifuged at 100,000g for 2.5 h at 4°C using a Beckman L7 ultracentrifuge and a SW 28 rotor. After centrifugation the membranes banding between each sucrose layer were collected and diluted to 40 ml in 25 mM Hepes and 0.25 M sucrose, pH 7.4 buffer and centrifuged at 30,000g (Beckmann J2-MC JA 20) for 30 min at 4°C. The supernatants were discarded and the pellets resuspended in 0.5 – 1 ml 25 mM Hepes and 0.25 M sucrose pH 7.4, aliquoted and stored at –20°C. The protein concentration of each fraction was assessed by the method of Bradford (section 2.17).

2.16.2 Extraction of endogenous peptides using C_8 Sep Pak Cartridges

Intestinal tissue was taken from FW yellow eels and the epithelial mucosa was scraped from the muscle layer using a microscope slide and the material was divided into two. One half of the intestinal tissue was homogenised in 3 ml (w/v) of ice cold 25 mM Hepes, 0.25 M sucrose, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4 containing 1 mM PMSF (phenyl methyl sulfonyl fluoride) using a Polytron PT 10 homogeniser (Kinematica Ltd.). The second half of the intestinal tissue was boiled in 3 ml (w/v) 1 M acetic acid for 10 min before homogenisation with the Polytron PT10 homogeniser. The two extracts were centrifuged at 20, 000g at 4°C for 45 min and the supernatants collected and acidified with HCl to a pH of less than 3 before addition to C_8 Sep-Pak cartridges. Unbound and weakly bound material was washed through the columns with two 3 ml washes of 0.1 % trifluoroacetic acid (TFA). Tightly bound proteins were eluted with 2 ml of 60 %

acetonitrile, 0.1 % TFA. The eluted samples were freeze dried and resuspended in 1 x gel loading buffer and electrophoresed by SDS PAGE (section 2.18).

2.17 Bradford protein assay

The assay was carried out using a 96 well micro titre plate and OD changes determined by a plate reader. A series of bovine serum albumin (BSA) standards, 2000 - 20 μg/ml, were made from a 2 mg/ml BSA stock. The standards were added to the plate in duplicate (20 μl sample/well) along with various dilutions of the membrane samples. To each well was added 200 μl of Bradford's Reagent (100 mg/l Coomassie brilliant blue G, 110 ml/l orthophosphoric acid and 55 ml/l 96% ethanol). The plate was read at 650 nm using an ELISA plate reader and the protein concentration of each sample calculated.

2.18 SDS Polyacrylamide gel electrophoresis (PAGE)

SDS PAGE is a technique that separates proteins by their molecular weight. The proteins are dissolved in the presence of both SDS and β mercaptoethanol. SDS masks any charges of the proteins and disrupts hydrophobic bonds and β mercaptoethanol reduces any intra or inter molecular disulphide bonds which therefore lead to the unfolding of the proteins and thus ensures that the proteins separate according to their molecular weight. The dissolved proteins are electrophoresed on a gel made of two components, a stacking gel and a running or separating gel. In the stacking gel the proteins are concentrated into a very thin line with the proteins with low molecular weights at the bottom of the stack and proteins with a high molecular weight at the top of the stack. In the running gel the proteins become unstacked and are separated out according to their intrinsic size. Large molecular weight proteins remain in the upper regions of the separating gel while low molecular weight proteins are able to migrate more easily through the gel matrix and are located in the lower half of the running gel. The SDS PAGE technique was used to electrophorese the endogenous membrane proteins (described in section 2.16) and for the proteins synthesised by the bacterial expression system (section 2.15).

A 12 % acrylamide separating gel was prepared as detailed in Appendix 3 then a 4 % stacking gel (Appendix 3) was prepared and poured on top of the separating gel and an appropriate comb inserted and the gel left to polymerise.

2.18.1 Preparation and electrophoresis of protein samples

The protein samples (100 μg) were diluted 1:1 with 2x gel loading buffer (62.5 mM Tris HCl pH 6.8, 2 % SDS, 10 % v/v glycerol, 0.005 % bromophenol blue w/v and 5 % β mercaptoethanol) and boiled for 15 min, cooled to room temperature and then centrifuged at 20,000g for 5 min. The supernatants and 20 μl of SeeBlue® Plus2 pre-stained molecular weight standards (Invitrogen) were loaded onto the gel. The gel was placed into a suitable gel tank and the upper and lower chambers were filled with 1x running buffer (25 mM Tris, 200 mM glycine and 0.1% SDS, pH 8.3) which was continuously circulated and cooled by tap water. The gel was electrophoresed at a constant current of 25 mAmp/gel for 4 – 5 hours. Upon completion of electrophoresis the gel was either stained with Coomassie blue (see section 2.18.3) or transferred onto a PVDF membrane (section 2.18.4) in preparation for Western blotting (sections 2.19.2 and 2.19.3).

2.18.2 Coomassie blue staining of PAGE gels

When the gel had been electrophoresed it was incubated at room temperature with constant shaking in a solution of Coomassie brilliant blue stain (0.1% Coomassie Brilliant Blue R-250, 50% methanol and 10% glacial acetic acid) for 1 h. The gel was then destained by washing with constant shaking in four changes of destain I solution (50% methanol and 10% glacial acetic acid) for 2 h and then with 1 – 2 washes of destain II solution (10% methanol and 10% glacial acetic acid) for 3 h. The gel was viewed on a white light box and the image captured using Gene Snap software (Syngene).

2.18.3 Transfer of proteins to PVDF membrane

To enable the proteins to be identified with antibodies which had been raised against specific peptide sequences the proteins were transferred from the gel to a polyvinylidene difluoride membrane (PVDF; Amersham Pharmacia). The PVDF membrane was prepared by soaking consecutively in methanol for 5 min then MilliQ®H_2O for 5 min and finally in 1 x transfer buffer (48 mM Tris, 1 M glycine, pH 9.2) for 10 min. A cassette was placed in 1x transfer buffer with two Scotbrite™ pads and two sheets of Whatman® 3MM paper and the cassette was assembled as previously described for Northern blotting in section 2.14.1

The assembled cassette was loaded into a transfer tank containing 1x transfer buffer constantly cooled to 4 – 5 °C and the proteins were transferred from the gel to the membrane overnight at 30V (power supply, BioRad 250/2.5 (BioRad Lab. Ltd.). After transfer the membrane was dried under vacuum and sealed in polythene and stored at –80°C until required for Western blotting.

2.19 Synthesis of antibodies to guanylin-like peptide prohormones

Sequences of 15 amino acids were selected from the known prohormone sequences of guanylin (ESVKQLKDLMDSDLC) renoguanylin (KLEELMGVDMTVKQC) and uroguanylin (MSTPHPPNLGSHAVC) as shown in figure 2.7. These regions were selected as there was no significant homology between these amino acids rather than the amino acids within the active peptide region where there is high homology between guanylin, renoguanylin and uroguanylin. Therefore the antibodies should not cross react with the other guanylin-like peptides.Peptide production and antibody synthesis was carried out commercially by Sigma Genosys. The peptides were synthesised and HPLC purified then approximately 2 – 3 mg of the peptide was conjugated by cysteine coupling to the carrier protein, key lympet hemocyanin (KLH). Antibodies were raised in two rabbits for each peptide with six immunisations and five bleeds taken per animal over a 77 day period. Prior to the immunisation protocol pre-immune sera (5 ml from each rabbit) were collected and screened by Western blot analysis (section 2.20) to ensure low background immunoreactivity of the preimmune serum to the separated eel peptides. The rabbits were immunised with antigen mixed in complete Freunds adjuvant with further injections of peptide, made up in incomplete Freunds adjuvant, at two week intervals. The protocol which was used for antibody production is shown below: -

Day 0	Antigen injection	**Day 49**	Bleed
Day 14	Antigen injection	**Day 56**	Antigen injection
Day 28	Antigen injection	**Day 63**	Bleed
Day 35	Bleed	**Day 70**	Antigen injection
Day 42	Antigen injection	**Day 77**	Bleed

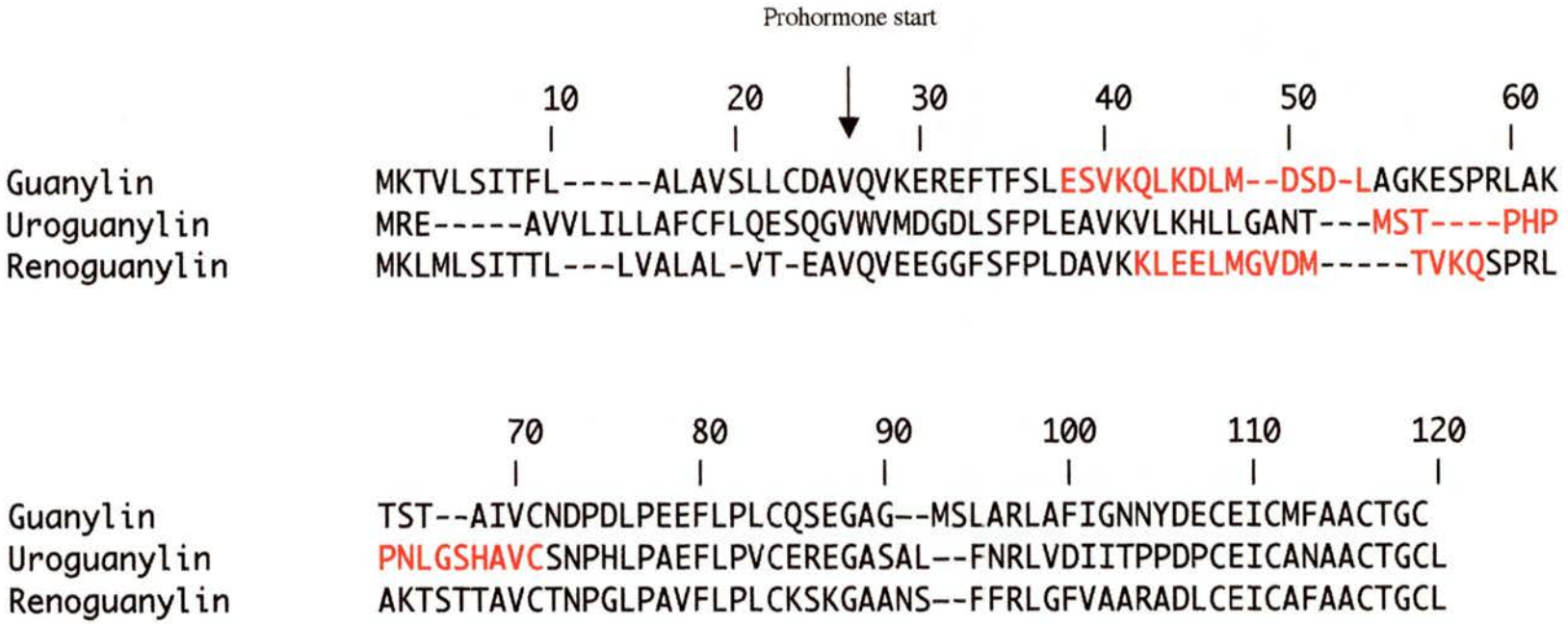


Fig 2.7 The amino acid sequences for preproguanylin, preprorenoguanylin and preprouroguanylin. The sequences of the peptides which were used to synthesise the antibodies are highlighted in red.

2.19.1 Purification of antibodies

The peptide anti-sera was purified by affinity chromatography by reversibly coupling the synthetic peptide antigens through thiol groups to Sepharose™ 4B (Amersham Biosciences) via a glutathione spacer arm. The activated thiol Sepharose™ 4B (1 g) was swollen in 200 ml of buffer (50 mM Tris, 0.5 M NaCl and 1 mM EDTA, pH 7.5) for 15 min at RT. The swollen activated thiol Sepharose™ 4B was collected on a sintered glass filter then washed with 100 ml of the same buffer. The majority of the buffer was removed and the activated thiol Sepharose™ 4B added to 2.5mg of peptide dissolved in 5 ml elution buffer. The resin/peptide mixture was mixed together at RT for 2 h then at 4°C overnight. The resin/peptide mixture was washed six times in 50 ml of buffer, collecting the resin by centrifugation each time at 284g in the Beckman J6 4.2 rotor before addition of 10 ml of antiserum and then mixed together at RT with continuous rotation for 45 min. The mixture was poured into a 10 ml column and the supernatant was allowed to flow through and was collected. The resin was washed with 10 volumes of elution buffer (50 mM Tris, 0.5 M NaCl and 1 mM EDTA, pH 7.5) to elute any unbound material. To release the antibody from the column, 10 ml of 0.1 M glycine pH 2.5 was passed through the column and 10x 1 ml fractions were collected into 0.3 ml of 1 M Tris HCl pH 8.0. The fractions containing the antibody were determined by SDS PAGE (section 2.18).

2.20 Western Blotting

The membrane (section 2.16) was brought to room temperature (if stored at –20°C) and soaked in methanol for 5 min then rinsed in water for 5 – 10 min and then incubated in Solution 1 (5% non-fat milk in PBS) for 1 h at RT to block non-specific binding sites. The membrane was then incubated with the primary antibody (diluted 1/10 - 1/100) in Solution 2 (1% non-fat milk and 0.5% Tween 20 in PBS) for 90 min at RT. Following washes (3 x 15 min) in Solution 3 (0.2% Tween 20 in PBS) to remove non-bound primary antibody the membrane was incubated for 90 min with an alkaline phosphatase-conjugated secondary antibody (Jackson Immuno Research) diluted 1/10,000 in Solution 4 (0.5% Tween 20 in Solution 1) then the membrane was washed as before in Solution 3 (3 x 15 min) and then in two changes of PBS for 30 min then rinsed in water for 5 min. To detect binding of the secondary antibody the membrane was stained

with Western Blue® stabilised substrate (Promega) for 5 min followed by washing in water to halt the alkaline phosphatase reaction.

2.21 Immunofluorescent Light Microscopy

2.21.1 Fixation of Tissues

Tissue samples from the intestine or the kidney of the European eel were fixed in 4% paraformaldehyde for 24 h at RT then rinsed in 70% ethanol (3 x 100 ml) over 48 h at RT. The samples were dehydrated by placing in a series of graded alcohol solutions (70 – 100 %) over a 4 h period then submerged in chloroform for 24 h. After dehydration the tissues were placed into three changes of paraffin wax at 60°C for 3 h then placed in a mould and left to set at RT overnight. Sections (3 – 5 μm) were cut on a Leitz Wetzlar microtome and collected on poly-L-lysine coated slides. The paraffin was removed from the tissues by rinsing the slides in two 5 min changes of Histochoice clearing agent (Sigma) and the slides were then placed in a series of graded ethanol solutions (from 100% to 50%), 5 min in each solution, to rehydrate the tissues.

2.21.2 Immunolabelling

To permeabilise the tissues the slides were pre-incubated in Solution A (0.01% Tween 20, 150 mM NaCl in 10 mM phosphate buffer pH 7.3) for 10 min at RT. The slides were transferred to Solution B (50 mM NH_4Cl in 20 mM PBS pH 7.3) for 5 min at RT to reduce background associated with free aldehyde groups of the fixative. The sections were washed in 1% BSA in 20 mM PBS pH 7.3 (BSA/PBS) for 5 min before transfer to Solution C (1% BSA, 0.1% gelatin in 20 mM PBS pH 7.3) at RT for 10 min to block any non-specific binding sites. Primary antibody diluted 1:5 to 1:100 in BSA/PBS was added to each slide and allowed to incubate at RT for 2 h in a wet chamber. After incubation with the primary antibody the slides were washed in BSA/PBS (3 x 15 min) to remove any excess primary antibody and the sections incubated in the wet chamber for 1 h at RT in 100 μl of FITC-conjugated anti-rabbit secondary antibody (1:200 dilution in BSA/PBS). Incubation of the secondary antibody was carried out in the dark to reduce degradation of the fluorescence. Following washes in BSA/PBS (3 x 15 min) the sections were mounted with anti-bleaching mounting

medium (Sigma). The sections were examined with a fluorescent microscope (Leitz Dialux 20 coupled to a Ploempak 1-Lambda lamp) equipped with the appropriate filter set (450-490 nm band pass excitation filter) and a phase contrast device. Images were captured using a Wild MPS 45 photoautomat camera and controller.

Chapter 3 Results

Cloning and expression of guanylin-like peptides from teleost fish.

3.1. RT-PCR Amplification and cloning of guanylin-like cDNAs from the European eel and other teleost fish

Using the degenerate primer pair, guanylin sense 5 and guanylin antisense 5 (Appendix 2) fragments of ~ 230 bp were amplified from cDNA synthesised from intestinal RNA of various teleost fish including *Anguilla anguilla* (eel), *Gadus morhua* (cod), *Platichthys flesus* (flounder), *Pleuornectus platessa* (plaice), *Salmo salar* (salmon), *Myxocephalus scorpius* (sculpin), *Salmo gairdneri* (rainbow trout) and *Merlangus merlangus* (whiting). Figure 3.1 shows the results of the amplifications after the samples were electrophoresed on a 1% agarose gel as described in section 2.9. The fragments shown in figure 3.1 were purified, cloned and sequenced as described in sections 2.10, 2.11 and 2.13 respectively. After successful cloning a number of colonies were selected from each transformation and colony PCR (as described in section 2.11.4) was carried out to confirm the presence of an insert. Figure 3.2 shows an example of an ethidium bromide stained agarose gel of the cloned guanylin fragment from the European eel. The fragment is approximately 350 bp as it contains regions of the vector spanned by the M13 forward and reverse primers (Appendix 2). Three positive clones were sequenced for each fragment cloned (as explained in section 2.13).

Using the sequences of guanylin obtained after amplification of cDNA using guanylin sense 5 and antisense 5 primers, RACE primers were designed for each species which would amplify the 5' and the 3' ends of each guanylin-like peptide cDNAs (Appendix 2). Nested RACE reactions were carried out for each species using cDNA synthesised using the Clontech Marathon™ technique (section 2.8) and the AP1 primer (Appendix 2) and a specific RACE 1 primer (Appendix 2). The product of this reaction was used as a template and re-amplified with the AP2 primer (Appendix 2) and the specific RACE 2 primer (Appendix 2). Figure 3.3 shows 5' and 3' RACE fragments of guanylin amplified from cDNAs synthesised from the intestine of the European eel. The 5' RACE fragment was ~ 350bp and the 3' RACE product was ~ 800bp. The fragments were purified, cloned and sequenced.

3.2 Sequencing of guanylin-like cDNAs from the European eel and other teleost fish

Following sequencing of all clones in both sense and antisense directions three guanylin-like peptides were found in the European eel. By comparing the sequences with known sequences of guanylin peptides from mammals and other teleosts (using GeneJockey II software) the three peptides were designated uroguanylin (as previously cloned and sequenced by Comrie *et al.*, (2001[a]), guanylin and renoguanylin. Both renoguanylin and uroguanylin were successfully amplified using the degenerate primer pair sense and antisense 5 (Appendix 2). However, eel guanylin was amplified using a primer (Appendix 2) based on the reported sequence of guanylin reported in the Japanese eel (Yuge *et al.*, 2003). The interleaved amino acid and nucleotide sequences of guanylin and renoguanylin are shown in figure 3.4 and 3.5 respectively. From the sequence data obtained from the other fish species both guanylin and uroguanylin were amplified from cod and flounder the only teleosts other than *A. anguilla* from which more than one guanylin-like peptide was amplified. Only the sequence for flounder uroguanylin has been shown (Figure 3.7) because the 5' and 3' ends of flounder guanylin have not been successfully amplified to date. The amino acid sequences of the guanylin-like peptide preprohormones from all the teleosts studied, eel, cod, flounder, plaice, salmon, sculpin and trout and an elasmobranch fish the bullshark and an amphibian, xenopus are compared. The peptides which are most guanylin-like are compared in figure 3.6, the uroguanylin-like peptides in figure 3.7 and the renoguanylin peptides, for which there is only the Japanese and European eels, compared in figure 3.8. In each figure the putative active peptide situated at the C-terminal is highlighted. The percentage homologies (the percentage of amino acids identical for the peptides following optimum sequence alignment using GeneJockey II software) between the guanylin-like peptides from teleosts and guanylin and uroguanylin from humans are presented in table 3.1.

3.3 Size of guanylin-like peptide mRNAs

Northern blot analysis was carried out as described in section 2.14 to determine the size of guanylin-like mRNAs present in the intestine of each species. The

resulting autoradiographs are shown in figure 3.9. From studies in mammals guanylin mRNA is known to be approximately 1 kb and a fragment of this size was observed in all species. Additional, larger mRNAs ranging from 2 kb – 6 kb present in Northern blots for eel, cod, flounder and sculpin, may represent immature mRNA precursors.

3.4 Tissue expression of guanylin-like peptides

The guanylin-like peptides were all amplified and cloned using RNA isolated from the intestine. Northern blot analysis was carried out to determine which other tissues expressed guanylin and renoguanylin. Investigations into the tissue distribution of uroguanylin mRNA in yellow eels was previously carried out by Comrie *et al.*, (2001[a]). Comrie *et al.*, (2001[a]) found a major 1 kb fragment and a much less intense 2 kb fragment of uroguanylin were expressed in intestinal samples from both FW- and SW- acclimated yellow eels. Very low levels of expression were also observed in the kidney of FW and SW yellow eels.

Total RNA (5 μg) extracted from heart, white skeletal muscle, liver, oesophagus, gill, intestine and kidney of 3-week SW-acclimated yellow eels and the gill intestine and kidney from FW-acclimated yellow eels was electrophoresed and hybridised (as described in section 2.14) using γ [^{32}P] labelled cDNA probes for guanylin and renoguanylin. The guanylin cDNA probe hybridised to a single guanylin mRNA species of approximately 1 kb in the intestine from both FW- and SW-acclimated fish and with a low signal also being detected in FW kidney (Figure 3.10). The Northern blots shown in figure 3.11 indicated the presence of a renoguanylin mRNA species of approximately 1 kb in the intestine and kidney of both FW- and SW-acclimated fish. Higher molecular weight fragments were present and are more apparent in figure 5.7 (a) (See chapter 5). These fragments probably represent immature mRNA precursors of renoguanylin.

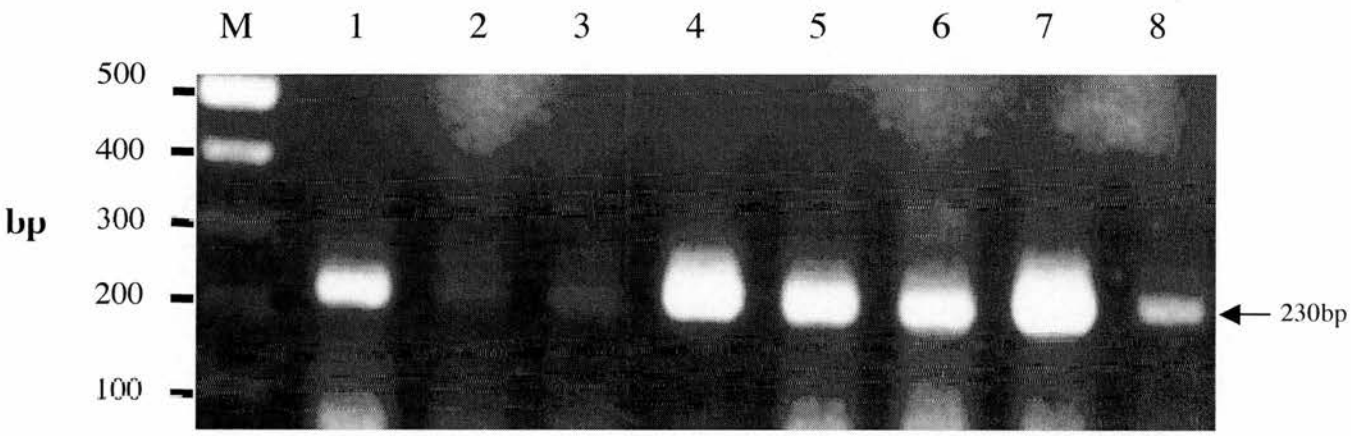


Fig. 3.1. Results of RT – PCR using degenerate guanylin primers (Appendix 2) with template cDNA synthesised from the intestine from various teleosts. Lanes from left to right are M – 100 bp DNA ladder (NEB), and PCR amplified products from 1 – eel, 2 – cod, 3 – flounder, 4 – plaice, 5 – salmon, 6 – sculpin, 7 – trout and 8 – whiting.

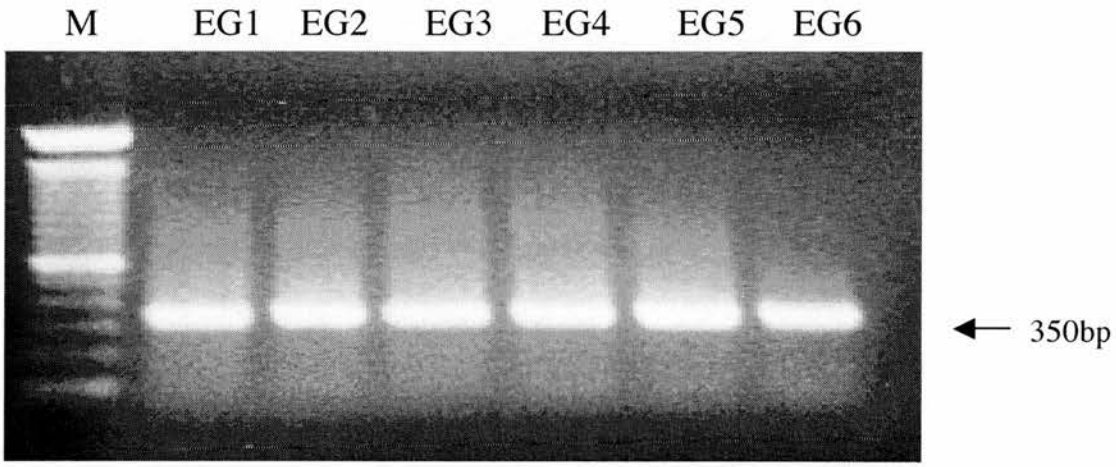


Fig 3.2. Example of a colony PCR of eel guanylin fragments inserted into the TOPO® PCR 4 vector (Invitrogen). The colonies, EG1 – EG6 were amplified using M13 forward and reverse primers and three of these positive colonies were purified and sequenced. M – 100 bp DNA ladder.

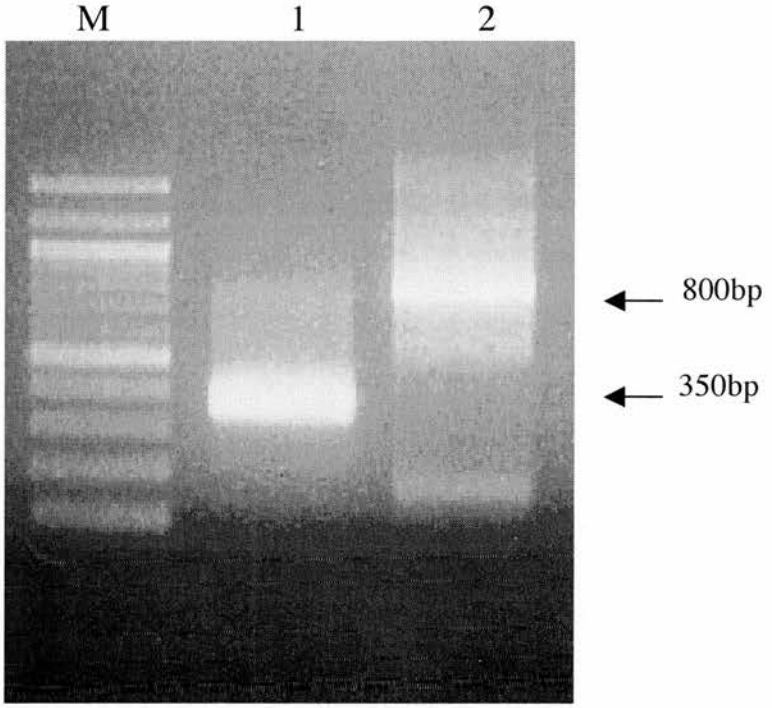


Fig. 3.3 Results of 5'RACE-PCR and 3'RACE-PCR using Marathon™ cDNA synthesised from the intestine of yellow SW-acclimated eels. M indicates the 100 bp DNA ladder (NEB). Lane 1 is amplification of the 5' RACE fragment after a nested reaction first with gene specific 5' RACE 1 and the kit AP1 primers and then gene specific 5' RACE 2 and the kit AP2 primer. Lane 2 is amplification of the 3' RACE fragment after a nested PCR first with gene specific 3'RACE 1 and the kit AP1 primers and then with gene specific 3' RACE 2 and the kit AP2 primers.

```
  1                          ACAGGAACACTTTCACAGACAGACGAGACCAGACCTCAGTCAAAGACCAAGACCAAG
    Met Lys Thr Val Leu Ser Ile Thr Phe Leu Ala Leu Ala Val Ser Leu Leu Cys Asp Ala     20
 58 ATG AAG ACC GTG CTC AGC ATC ACT TTC CTC GCC CTG GCT GTG AGC CTG CTC TGC GAT GCC
    Val Gln Val Lys Glu Arg Glu Phe Thr Phe Ser Leu Glu Ser Val Lys Gln Leu Lys Asp     40
118 GTC CAG GTC AAA GAA AGA GAG TTC ACA TTC TCA CTT GAG TCT GTG AAA CAG CTT AAG GAC
    Leu Met Asp Ser Asp Leu Ala Gly Lys Glu Ser Pro Arg Leu Ala Lys Thr Ser Thr Ala     60
178 CTG ATG GAC AGC GAC CTG GCG GGA AAG GAG AGC CCT CGC CTG GCC AAG ACC AGT ACT GCA
    Ile Val Cys Asn Asp Pro Asp Leu Pro Glu Glu Phe Leu Pro Leu Cys Gln Ser Glu Gly     80
238 ATC GTG TGC AAT GAC CCT GAC TTA CCT GAG GAG TTC CTG CCC TTG TGT CAG AGC GAA GGA
    Ala Gly Met Ser Leu Ala Arg Leu Ala Phe Ile Gly Asn Asn Tyr Asp Glu Cys Glu Ile    100
298 GCT GGC ATG TCC CTC GCC AGG CTG GCA TTT ATT GGC AAC AAC TAC GAT GAA TGT GAG ATC
    Cys Met Phe Ala Ala Cys Thr Gly Cys Stop                                         109
358 TGC ATG TTT GCT GCC TGC ACC GGT TGC TAA ACCAAGGACCAGCAAGGATCGGCAGGCAGATCTCGAGTG
427 ATTTCTGCATTTCTGTATTCTCTCAGTTACATTTCGTTTCTGTCTTACTTGTTATTAAGACCGCACAATTCCTCCTCAA
506 GTTCACTATATGGAAAAAGGCAGCTGGTGGCAGAGGGAAAATATGATATCTTCAATCTTTCTAAATAGGCTAAATGAAA
585 TAACAATGCATTAAATAATAACAGAAAATATGCGTACTACTACCACTATTACGGCTAATAATAATATAATGAATAATAA
664 TGGATTTGCCTATTTCATTTTGTTTTGCTGTGTATCAAGGATATTTTCACATTATATCATGTGAACAACTACGTTGAAA
743 CTCATCCTGTCACCTTGGCTTCAAAGCTTGCTATTTAACCGCAAAAAGTTTTTATTATTATTATTTTATTATATTTTCA
822 TTATGTTTATCCTTGTTATGTTACTGGTTCAGTTTAAGCCTTTTACTTATTTAACAGAGTGACCTTTTTATAAGAATGT
901 TTTTGTTGTTGAGCTATACTTACATATGTATTCATTTTGGAGGTGTTTTTTTTTTTCCATTCATAAAAACTGAATAAAA
980 GTTTTTCATTCTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

Fig 3.4. The interleaved nucleotide and derived amino acid sequence of preproguanylin from *Anguilla anguilla*. The possible signal sequence is highlighted in red and the putative active guanylin peptide is highlighted in blue.

```
  1                      GTCACAAACAGGAAGACAAGACAAAAAGCTATATCAAAAGCGAACCACTCACAGGAGAAG
    Met Lys Leu Met Leu Ser Ile Thr Thr Leu Leu Val Ala Leu Ala Leu Val Thr Glu Ala     20
 61 ATG AAG CTG ATG CTG TCT ATC ACG ACC CTG CTT GTA GCT CTC GCT CTG GTC ACC GAA GCC
    Val Gln Val Glu Glu Gly Gly Phe Ser Phe Pro Leu Asp Ala Val Lys Lys Leu Glu Glu     40
121 GTC CAA GTG GAA GAA GGA GGC TTC AGC TTC CCA CTT GAT GCA GTG AAA AAA CTC GAA GAG
    Leu Met Gly Val Asp Met Thr Val Lys Gln Ser Pro Arg Leu Ala Lys Thr Ser Thr Thr     60
181 CTC ATG GGG GTT GAC ATG ACA GTG AAA CAG AGC CCT CGC CTG GCA AAG ACC AGT ACT ACG
    Ala Val Cys Thr Asn Pro Gly Leu Pro Ala Val Phe Leu Pro Leu Cys Lys Ser Lys Gly     80
241 GCC GTC TGT ACC AAC CCA GGC CTG CCA GCT GTG TTC TTG CCC CTC TGC AAG AGC AAA GGA
    Ala Ala Asn Ser Phe Phe Arg Leu Gly Phe Val Ala Ala Arg Ala Asp Leu Cys Glu Ile    100
301 GCG GCC AAT TCT TTC TTC AGA CTG GGT TTT GTG GCA GCC CGA GCA GAC CTC TGT GAA ATC
    Cys Ala Phe Ala Ala Cys Thr Gly Cys Leu Stop                                      110
361 TGT GCG TTC GCT GCC TGC ACT GGG TGC CTC TGA AAGTCCTGATCCTGACTGACTGTCACACCCCCCCC
429 CGAGTTTTCATTTCTCCGTTCTTTAAATTAATTTTTAAATTAGCGCACAATTATCTGAGATTTCCTGATAANAATNAGG
508 GAGAAAATATATGCTTTTTGTTTTTCCTTGTTCATTTTTTAATATCATTTATTAATGCGAACATTTGTTTGGATTTGTA
587 TTGTTTCATGTTTAATTTAATACTCCTATACAAAATCCAACCTTTTACATTGGAATAATGTGCTGAACGTGCGAAAGAA
666 ATGTTTTTNTTATTTTTATTTTTCTATTGTACTGTTACTTTGGCTGAATTTAGCTATTATATGCTATTTAATGGTTCTT
745 AATTTTGTGTTGTTATCATTGGCTTCCAGAGGCCCAAAAGGTAGCTACTTTATATTTAAAATAGCCTTTTTTTATATTT
824 TTGTAAATACAGTATTAGTATTGGGGCCTGTTTTAACTTGGTACAATGCCGTTTCTTGGTTCTTCATCATTTTAATAAA
903 ATGCTTTAAAAAAAAAAAAAAAAAAAAAAAAAA
```

Fig 3.5 The interleaved nucleotide and amino acid sequence of preprorenoguanylin from *Anguilla anguilla*. The possible signal sequence is highlighted in red and the putative active renoguanylin peptide is highlighted in blue.

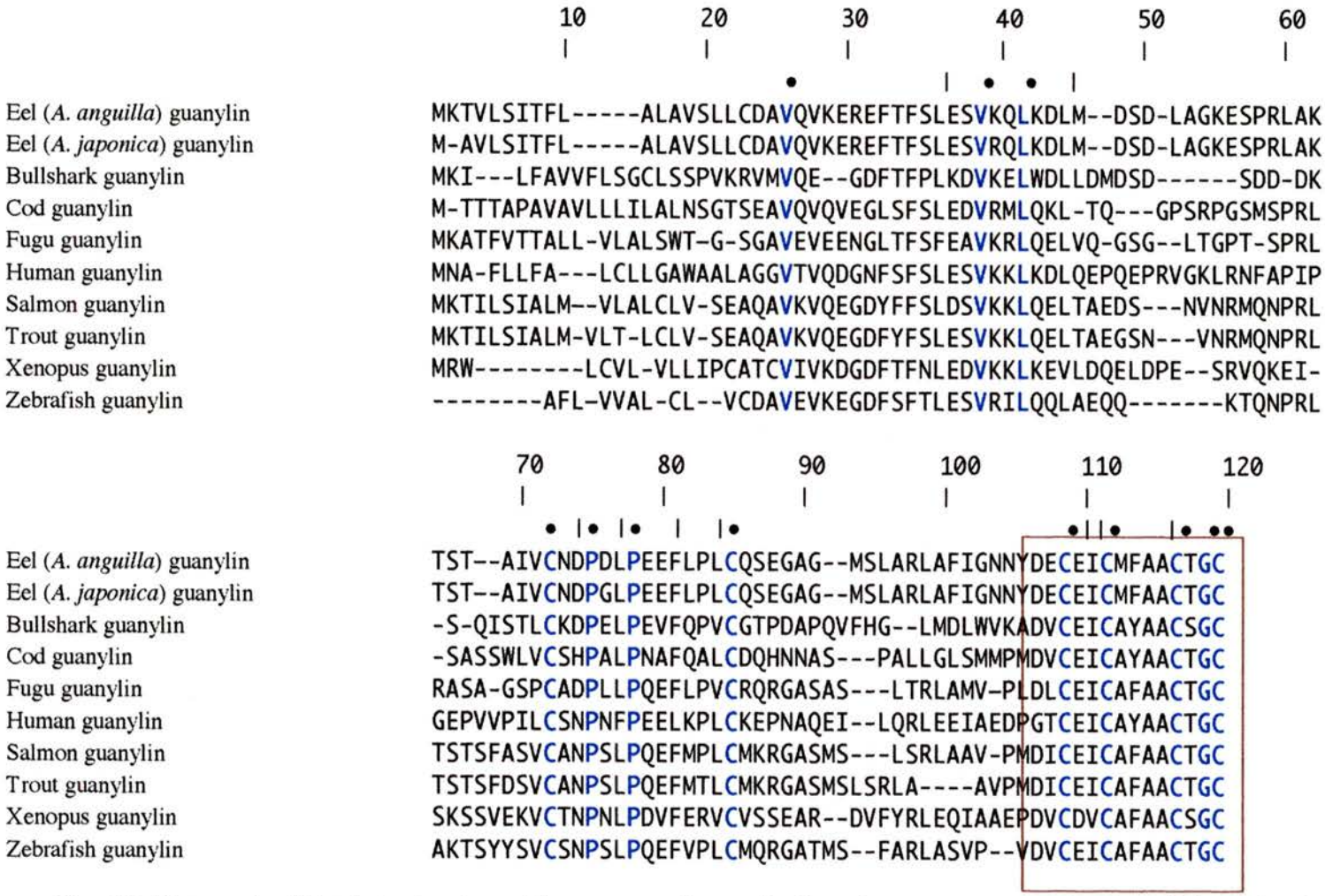


Fig. 3.6 Alignments of the derived amino acid sequences of guanylin, from human, xenopus and various fish species. Symbols in the alignment are as follows: • indicated where there is a conserved amino acid (also highlighted in blue); | indicates an amino acid position with conserved similarity and, – indicates spaces introduced to optimise alignment. The boxed region highlights the putative active peptide. The EBI/GenBank Accession Nos., where available, are as follows: for zebrafish (*Danio rerio*) AW018969, human (*Homo sapiens*) M97496 and *A. japonica* AB080640.

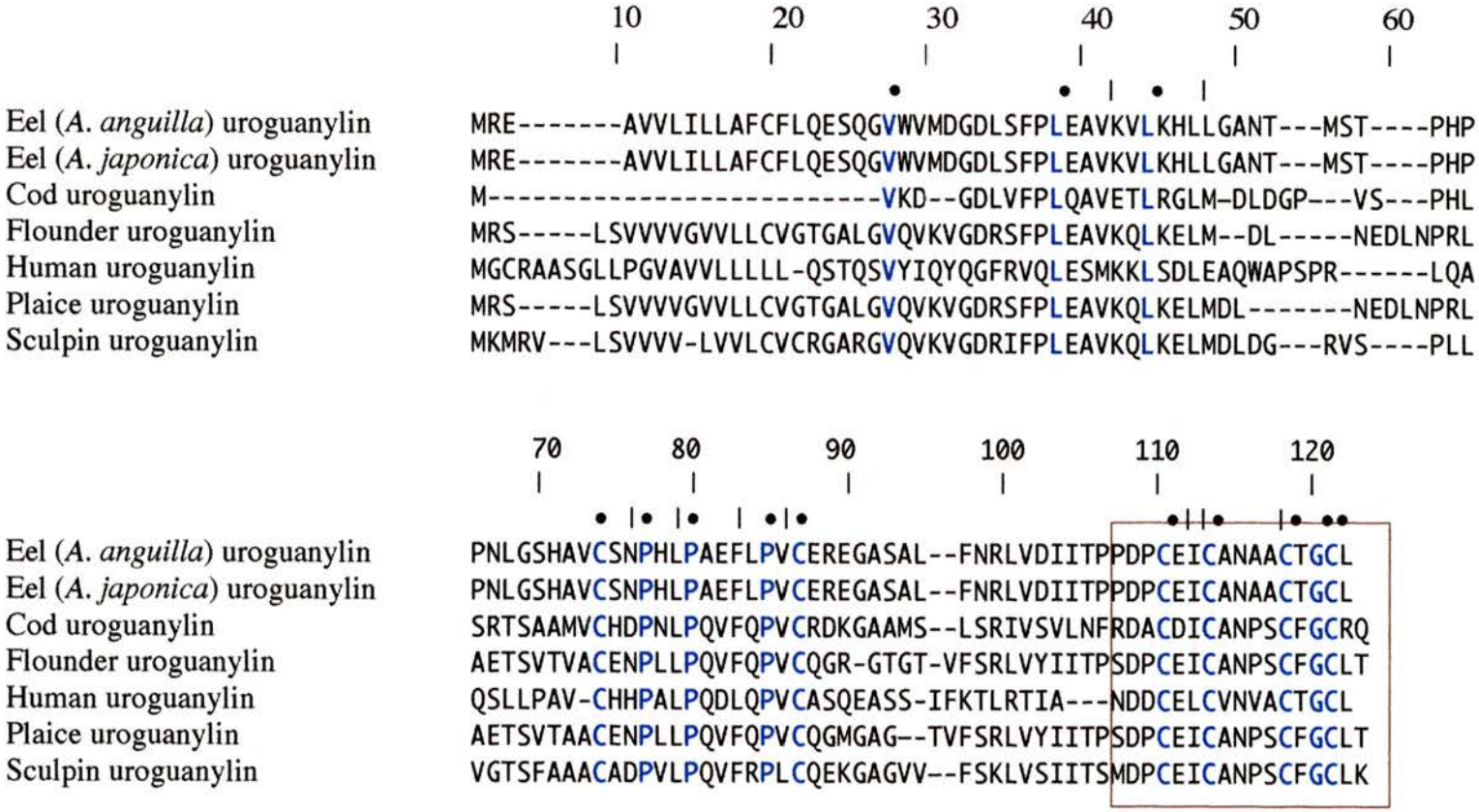


Fig. 3.7 Alignments of the derived amino acid sequences of uroguanylin, from human and various fish species. Symbols in the alignment are as follows: • indicated where there is a conserved amino acid (also highlighted in blue); | indicates an amino acid position with conserved similarity and, – indicates spaces introduced to optimise alignment. The boxed region highlights the putative active peptide. The EBI/GenBank Accession Nos., where available, are as follows: human (*Homo sapiens*) U34279 and A. japonica AB080642.

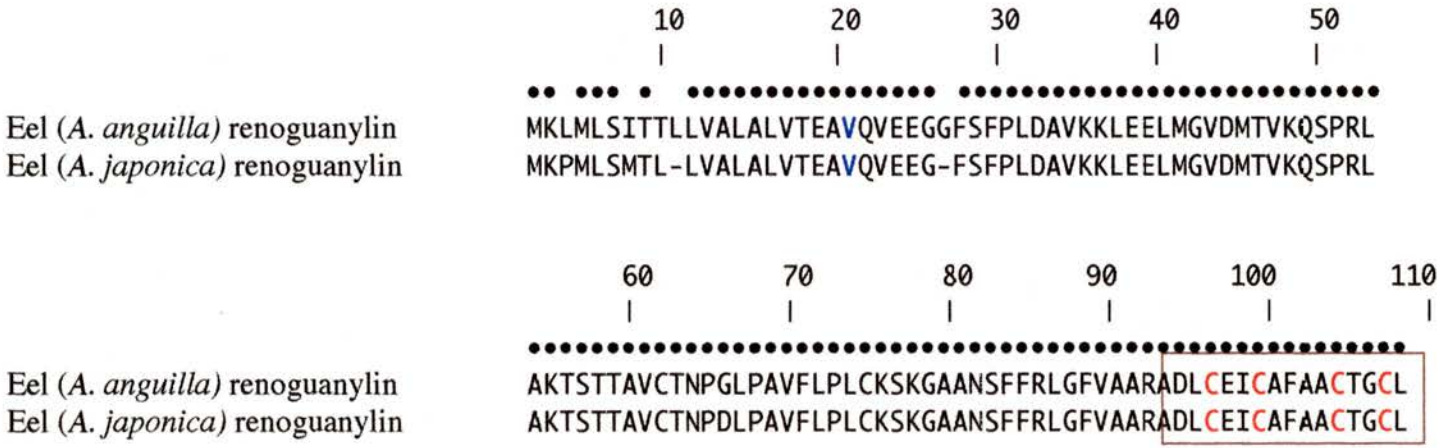


Fig. 3.8 Alignments of the derived amino acid sequences of renoguanylin from *A. anguilla* and *A. japonica* Symbols in the alignment are as follows: • indicated where there is a conserved amino acid and – indicates spaces introduced to optimise alignment. Highlighted in blue is the cleavage site of the prohormone and highlighted in red are the four cysteine residues which form disulphide bonds which are important for biological activity of the active peptide. The boxed region highlights the putative active peptide. The EBI/GenBank Accession No. for *A. japonica* AB080641.

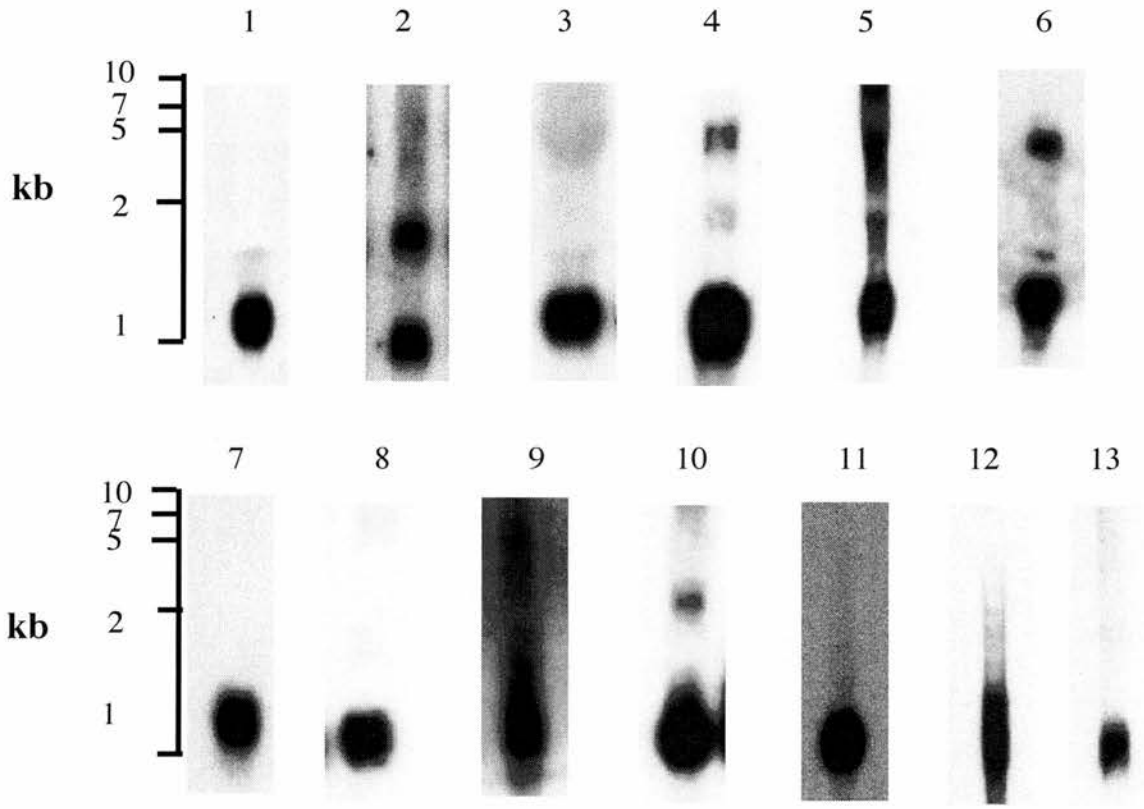


Fig 3.9 Autoradiographs of Northern blots indicating the expression of guanylin, renoguanylin and uroguanylin mRNA transcripts in total RNA (5 μg) samples from various teleosts, the Bullshark (an elasmobranch fish) and the frog *Xenopus laevis* (an amphibian species). Unless otherwise stated the mRNA fragment is guanylin. 1 – eel, 2 – eel uroguanylin, 3 – eel renoguanylin, 4 – cod , 5 – cod uroguanylin, 6 – flounder, 7 – plaice, 8 – salmon, 9 – sculpin, 10 – trout, 11 – whiting, 12 – bullshark, 13 – frog.

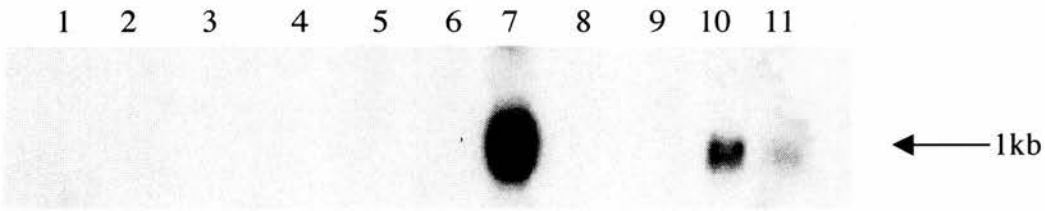


Fig. 3.10 Northern blot showing the differential tissue expression of guanylin mRNA in yellow eels acclimated for three weeks to SW (lanes 1 – 8) and yellow eels maintained in FW (lanes 9 – 11). For each sample 5 μg of total RNA was loaded onto the agarose gel (section 2.14). Lanes 1 – 11 from left to right are; 1 – heart, 2 – white skeletal, 3 – liver, 4 – stomach, 5 – oesophagus, 6 – gill, 7 – intestine, 8 – kidney, 9 – gill, 10 – intestine and 11 – kidney.

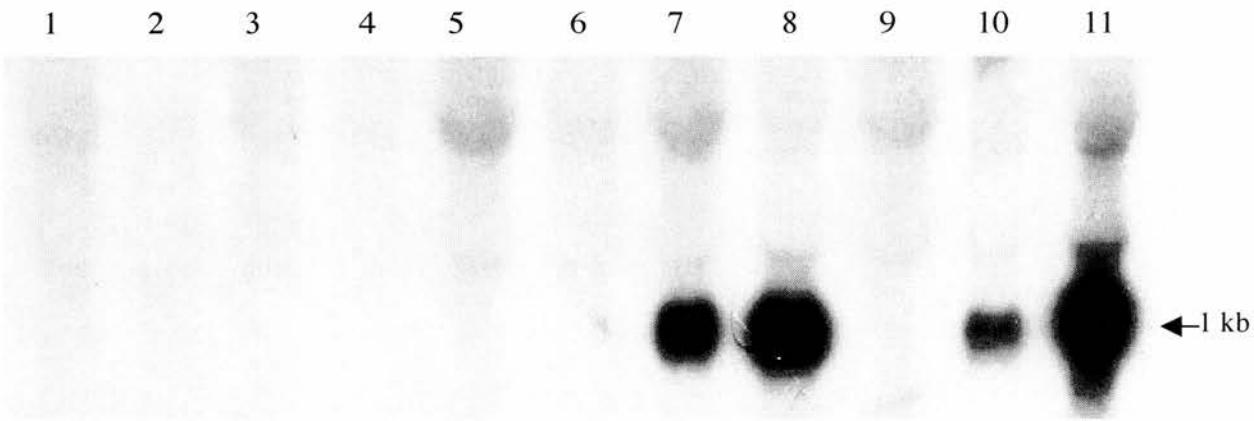


Fig. 3.11 Northern blot showing the differential tissue expression of renoguanylin mRNA in yellow eels acclimated for three weeks to SW (lanes 1 – 8) and yellow eels maintained in FW (lanes 9 – 11). For each sample 5 μg of total RNA was loaded onto the agarose gel. Lanes 1 – 11 from left to right are; 1 – heart, 2 – white skeletal muscle, 3 – liver, 4 – stomach, 5 – oesophagus, 6 – gill, 7 – intestine, 8 – kidney, 9 – gill, 10 – intestine, 11 – kidney.

Human guanylin														
25	Human uroguanylin													
35.5	25	*A.anguilla* guanylin												
38	30	33	*A. anguilla* uroguanylin											
34	24	47	43	*A. anguilla* renoguanylin										
36	26	97.5	33	47	*A. japonica* guanylin									
37	30	34	98	43	34	*A. japonica* uroguanylin								
34	24	45	43	94	43	42	*A. japonica* renoguanylin							
31	27	31	34	38	31	34	38	Cod guanylin						
25	28	30	38	40	31	37	40	25	Cod uroguanylin					
32	26	36	47	44	34	47	27	32	41	Flounder uroguanylin				
31	25	35	48	44	36	37	43	33	40	89	Plaice uroguanylin			
35	30	43	38	51	41	37	50	39	35	38	37	Salmon guanylin		
30	25	38	47	46	37	46	45	31	43	60	63	43	Sculpin uroguanylin	
35	27	44	34	46	35.5	34	45	37	32	37	37	82	37	Trout guanylin

Table 3.1 Percentage amino acid sequence homology between the preprohormone of human guanylin and uroguanylin and a range of teleost guanylin like peptides.

Chapter 4 Discussion

Cloning and expression of guanylin-like peptides from teleost Fish.

4.1 Guanylin-like peptides

Guanylin is the founder member of a small family of peptides that was first isolated from the rat jejunum by Currie *et al.*, (1992). Other members of the guanylin family in mammals include uroguanylin, first discovered in opossum urine by Forte and Hamra (1996) and lymphoguanylin isolated from the spleen of opossums by Forte *et al.*, (1999). These peptides are responsible for the stimulation of fluid secretion in a number of tissues, but especially the intestine and the kidney. Guanylin and uroguanylin peptides are released into the intestinal lumen and into the bloodstream in the form of larger precursor molecules known as proguanylin and prouroguanylin respectively (Lauber *et al.*, 2002) with the active 15 – 17 amino acid peptides being located at the carboxy terminal of the prohormones (Forte *et al* 1999, Carrithers *et al.*, 2001[a]). From studies in mammals, it is known that guanylin-like peptides bind to, and activate a guanylate cyclase (GC-C) receptor located on the apical membranes of epithelial cells which line the large and small intestine. Activation of GC-C results in an increase in the intracellular levels of the second messenger, cGMP (Wedel and Garbers, 1998). The postulated intracellular events leading to the secretory response in the intestine include the activation of protein kinase G by cGMP which results with the phosphorylation and activation of cytic fibrosis transmembrane conductance regulator (CFTR) which functions as a chloride channel and is present in the apical membrane (Forte and Currie, 1995; Forte *et al.*, 2000[b]). This series of steps culminates in the transepithelial movement of chloride ions, first through the basolateral $Na^+/K^+/Cl^-$ cotransporter and then via the activated CFTR chloride channel into the lumen of the intestine (Forte, 1999). This is followed by Na^+ ions via the paracellular pathway, and then by water presumably both via transcellular and paracellular pathways. In mammals the secretion of fluid and ions is important for digestion. However, it is hypothesised that guanylin-like peptides may show osmoregulatory roles in teleost fish. Before determining if guanylin is involved in osmoregulation the presence of guanylin-like peptides in teleost fish had to be confirmed.

Previous studies in this laboratory (Comrie *et al.*, 2001[a]) resulted in the cloning of a guanylin-like peptide from the European eel. Although this was originally called eel

"guanylin" subsequent studies have shown that this peptide was in fact uroguanylin. This current study identified a further two other guanylin-like peptides, eel guanylin itself and a novel peptide which has subsequently been called renoguanylin. During the period of this work these three teleost guanylin-like peptides (guanylin, uroguanylin and renoguanylin) were also cloned and sequenced from the Japanese eel (*Anguilla japonica*) by Yuge *et al.*, (2003). The results from the European eel and the Japanese eel show that three guanylin-like peptides are present in eels, however there is no evidence that these three guanylin-like peptides, guanylin, renoguanylin and uroguanylin are also expressed in other teleosts.

In this study RT-PCR experiments using degenerate sense and antisense primers confirmed that guanylin-like prohormones were present in a number of teleost species (figure 3.1) including *A. anguilla*, *Platichthys flesus* and *Salmo salar*. The fragments generated were cloned and sequenced and the presence of guanylin mRNA in the teleosts was subsequently confirmed by Northern blot analysis.

4.2 Sequences of guanylin-like peptides

In mammals members of the guanylin family have been categorised into classes I, II or III depending on the number of disulphide bonds which are present within the active peptide (Forte, 1999). Mammalian guanylin and uroguanylin fall into class II as they have four cysteine residues forming two disulphide bonds in the active peptides. Hidaka *et al.*, (1998) have found that the correct disulphide bond arrangement must form to enable the peptide to be active. These bonds are between cysteines 1 and 3 and cysteines 2 and 4 in the peptide sequence (see figure 1.7). All the guanylin-like peptides cloned from teleost fish are also class II, as four cysteine residues are conserved at the same positions as those from mammalian species (see figures 3.6, 3.7 and 3.8).

The greatest homology between the guanylin preprohormones from mammalian species and fish species is found within the active peptide region and there are distinct differences between guanylin and uroguanylin peptides from all species. The guanylin active peptide is 15 amino acids in length but the active peptide region of uroguanylin is comprised of 15 - 17 amino acids depending on the species. The

additional one or two amino acids at the C terminal may serve to stabilise the active conformation of uroguanylin after hydrolysis from the prohormone (Comrie *et al.*, 2001[a]). Within the active region of the peptide, at position 9 in the amino acid sequence, guanylin peptides have a hydrophobic phenylalanine or tyrosine residue while all uroguanylin peptides possess an asparagine residue, as shown in figure 4.1. In mammals, it is thought that due to this amino acid substitution uroguanylin peptides are more resistant to hydrolysis by extracellular peptidases such as chymotrypsin (Joo *et al.*, 1998). These proteases, found at high concentrations in the renal tubules (Forte and Hamra, 1996), hydrolyse peptide bonds on the COOH terminal side of aromatic residues such as phenylalanine or tyrosine present in guanylin (Forte *et al.*, 2000[b]). These differences within the active region of guanylin and uroguanylin may be functionally significant and ensure that there is not excess renal secretion of fluid and ions as only uroguanylin has been found to be active in the kidney. These structural and functional differences in guanylin-like peptides may also be prevalent in teleost fish. The peptide length and the presence of F, Y or N at position 9 in the active peptide region were used to determine whether the sequences from teleost fish were more guanylin-like or more uroguanylin-like.

The European eel was found to express three guanylin-like peptides, guanylin, uroguanylin and renoguanylin. The three preprohormones from the European eel were more than 90% homologous to the corresponding peptide precursors in *Anguilla japonica*. Cod, flounder, salmon and rainbow trout all express a protein homologous to mammalian proguanylin. The active peptides of these sequences were composed of 15 amino acids and contained a tyrosine or phenylalanine amino acid residue at position 9. A second proguanylin-like cDNA was found in cod and flounder with similar proguanylin-like cDNAs amplified from plaice and sculpin, which were more homologous to the prouroguanylin sequence from human and *A. japonica*. The putative active peptide regions comprised 16 or 17 amino acids due to C terminal extensions with all peptides having an asparagine residue at position 9. It is possible that all teleosts express both guanylin and uroguanylin and maybe even renoguanylin, a member of the guanylin family currently only found in members of the *Anguilla* family. Further studies using different primers, designed using the

sequences of renoguanylin from *A. anguilla* and *A. japonica* are required to elucidate if renoguanylin homologues are also expressed in other teleost fish. Another member of the guanylin family, lymphoguanylin appears to only be found in the marsupial, the American opossum, and perhaps like lymphoguanylin, renoguanylin may be unique to the *Anguilla* family. The renoguanylin and lymphoguanylin peptides are structurally dissimilar. Lymphoguanylin contains only one disulphide and is mainly expressed in lymphoid issues (Forte *et al.*, 1999[a]) whereas renoguanylin has two disulphide bonds and is abundantly expressed in the kidney and the intestine. Renoguanylin is structurally similar to guanylin as it has a phenylalanine residue present at position 9 but unlike guanylin, renoguanylin has an extra amino acid present at the C terminal. Unfortunately there was insufficient time to investigate whether renoguanylin or lymphoguanylin were present in other teleost species.

The percentage homologies between human and teleost preproguanylin and preprouroguanylin amino acid sequences are shown in table 3.1. The homology between mammalian and teleost guanylin peptides was found to be very low at both nucleotide and amino acid levels, with identities ranging between 25 and 37%. Higher identities between the proteins were found within the active peptide region shown in figure 4.1. High homology between guanylin or uroguanylin preprohormones only occurred between closely related species. For example salmon and trout, which are from the superorder Salmoniformes, the amino acid sequences of the two guanylin preprohormones were 82% homologous. The amino acid sequences of the preprouroguanylin-like peptides from plaice and flounder, two teleost species from the order Pleuronectidae, were 89% homologous. In contrast the amino acid sequences of preproguanylin, from the European eel and salmon were only 43% homologous. High homology between amino acid sequences was also observed between the two members of the *Anguilla* family, *Anguilla anguilla* and *Anguilla japonica*. For example between the two guanylin peptides there is 98% homology. Overall from the data in table 3.1 the general trend was that there is low homology between the amino acid sequences of preproguanylin, preprouroguanylin and preprorenoguanylin across all species especially at the amino terminal and high

homology only occurred between the same members of the guanylin family from closely related species.

Although most homology between the amino acid sequences of guanylin-like peptides occurs at the carboxy terminal there are a few amino acids conserved throughout the prohormones. The NH_2 terminus of the preprohormone of opossum guanylin was sequenced in a study by Hamra *et al.*, (1996[a]) and this study identified a cleavage site (between residue 26, serine and amino acid 27, valine) for post-translational processing to produce a mature prohormone. The mature proguanylin from rats is produced by hydrolysis of peptide bonds between glycine and valine (residues 26 and 27) and human prouroguanylin by hydrolysis between serine and valine residues (residues 26 – 27; Forte *et al.*, 2000[a,b]), highlighted in blue in figure 4.2. In all of the amino acid sequences of teleost preproguanylins and preprouroguanylins a valine residue at position 27 has been conserved. This suggests that like the prohormones of guanylin and uroguanylin peptides in mammals, the cleavage site to yield the mature prohormone is also at this site in teleosts. In a number of guanylin and uroguanylin sequences there is an additional alanine/glycine and valine pairing (highlighted in red in figure 4.2) upstream from the aforementioned cleavage site. Although from work on rat and human guanylin and uroguanylin prohormones it is known that the start of the prohormone is after the aforementioned cleavage site (highlighted in blue in figure 4.2) but it may be possible that the preprohormone is cut twice, once at one of the additional alanine/ glycine valine sites and then cleaved again at the alanine/ glycine valine conserved at positions 26 and 27 in all of the guanylin-like peptides before a mature prohormone is formed. Further work is required to confirm whether or not the additional alanine/glycine and valine pairing sites are additional cleavage sites.

A second region of partial homology found at the N terminus of the prohormone of guanylin and uroguanylin is a sequence of 10 amino acids (residues 38 – 47). A study by Hamra *et al.*, (1996[a]) on opossum prouroguanylin found that this sequence, LDSVKKLDEL, was highly conserved when compared to the same region in human proguanylin (LESVKKLKDL). They concluded that the conservation of this structural motif in guanylin and uroguanylin precursors indicated that it might have

a biological function. In another study by Li *et al.*, (1997) it was hypothesised that this structural motif may be important for folding and processing of the prohormone to the active peptide. A similar motif has been conserved in all teleost peptides which have been cloned and sequenced (highlighted in green in figure 4.2) but further research is required to completely understand the importance of this structural motif.

In addition to the four cysteine residues within the active peptide region at the C terminal of the prohormone two additional cysteine residues are conserved in all of the guanylin-like peptides cloned from all species. It is possible that these cysteine residues may form a third disulphide bond within the prohormone in addition to the well characterised disulphide bonds which are formed within the active peptide. Also conserved near the C terminal of the prohormone are two proline residues. These four conserved amino acids within the prohormone sequence (2 cysteines, 2 prolines) may be involved in the folding of the prohormone especially as proline residues are thought to cause turns in amino acid chains.

The site of cleavage of the precursor to form the active guanylin peptide, located at the carboxy terminal, has still to be fully elucidated. From studies on human proguanylin it was suggested that cleavage of the prohormone occurred between the amide bond formed between an aspartic acid and a proline residue (amino acids 106 and 107, figure 3.6) to produce an active guanylin peptide which was 15 amino acids in length (Schulz *et al.*, 1999). An aspartic acid residue has been conserved in all of the teleost guanylin-like peptides (highlighted in red in figure 4.1) but it is two amino acids downstream of the aspartic residue of human guanylin which has been proposed as a cleavage site. If this aspartic residue is a cleavage site then this would make the active peptide region of all teleost guanylins 13 amino acids in length, eel uroguanylin and renoguanylin 15 amino acids in length and all other teleost uroguanylins 16 amino acids in length. More research is required to confirm the exact location of the cleavage site of the prohormone and from this information the exact length of the active peptide region can be confirmed.

A further difference between mammalian guanylin and uroguanylin peptides is the optimum pH at which they stimulate secretion. Studies measuring the levels of

cGMP produced by T84 cells, when uroguanylin or guanylin were added to the medium, found that the potency of uroguanylin remained high when the pH of the growth medium was acidic (pH 5.5). When the pH of the growth medium was raised to 7.5 the effectiveness of uroguanylin decreased and the potency of guanylin increased (Fan *et al.*, 1997; Forte, 2003). In mammals, guanylin and uroguanylin are important for regulating intestinal fluid and ion secretion during digestion. It is thought that these two peptides have evolved to function optimally under different pH conditions because the pH of the intestine of mammals changes during digestion (Fan *et al.*, 1997). When the chyme containing HCl enters the duodenum from the stomach the intestinal lumen and surface mucosa are acidified and it is thought that under these conditions uroguanylin is the most effective agonist for the receptor, GC-C. Gradually the intestinal lumen becomes alkalinised due to the secretion of HCO_3^- ions and in these conditions guanylin would become the more potent agonist of GC-C (Fan *et al.*, 1997; Forte, 2003). It has been suggested that human uroguanylins preference for an acidic environment is due to two acidic amino acids found at the NH_2 terminus of the active peptide (underlined in figure 4.1; Forte, 1999[b]). This pair of acidic amino acids were found to confer high potency and efficacy upon uroguanylin in the activation of cGMP production via GC-C receptors on the surface of T84 intestinal cells under conditions of high mucosal acidity (Forte, 1999[b]). However only one acidic amino acid (D) is present in teleost uroguanylins and this is followed by a proline residue at a position usually occupied by the acidic D or E in mammals. Eel guanylin does possess two acidic residues, D and E at the amino terminus of the active peptide but no other teleost guanylin possesses these two residues. Initially research is required to establish whether teleost guanylins and uroguanylins are sensitive to different pH conditions. If they are sensitive to pH then further work is required to establish what benefit it would have to teleosts. Research is also required to establish which properties of guanylin and uroguanylin determine whether the peptide is more potent in acidic or alkaline conditions.

4.3 Tissue expression of guanylin-like peptides

Northern blots using RNA isolated from various tissues from the European eel were used to determine the tissue-specific expression of the two new guanylin-like peptides, guanylin and renoguanylin in *A. anguilla*. Previous research on uroguanylin (referred to as guanylin in the literature) by Comrie *et al.*, (2001[a]) found that the mRNA for this peptide was present in both eel intestine and to a lesser extent in the kidney. This is in agreement with the expression of uroguanylin in mammals, such as the rat (Nakazato *et al.*, 1998). Northern blot analysis confirmed that guanylin mRNA (Figure 3.10) was only detectable in the eel intestine. This is similar to the expression of mammalian guanylin mRNA which is predominately located in the intestinal tract, mainly in the ileum and colon (Kita *et al.*, 1994). In mammals it is known that guanylin and uroguanylin are predominately synthesised in the small intestine (Whitaker *et al.*, 1997, Nakazato 2001). In the lumen of the intestine the prohormones are cleaved to produce the active peptide (see section 4.2) which binds to an apically located GC-C receptor and initiates a series of steps resulting in the secretion of water and ions into the intestinal lumen (Forte *et al.*, 2000[a]). The prohormones are released into the bloodstream and although levels of circulating proguanylin (biologically active guanylin has not been detected in the plasma) are considerably higher than those of prouroguanylin and biologically active uroguanylin only uroguanylin is detected in the urine (Forte *et al.*, 2000[a]). Proguanylin is only detected in the urine when there is renal failure (Forte *et al.*, 2000[a]). Under normal conditions when the kidneys are healthy what happens to guanylin, why is it not detected in the urine? Proguanylin, prouroguanylin and biologically active uroguanylin are filtered through the glomerulus but proguanylin in the filtrate is hydrolysed and inactivated by chymotrypsin-like proteases located on the brush border of cells lining the proximal renal tubules (Hamra *et al.*, 1996; Forte *et al.*, 2000[a]). Guanylin peptides and their prohormones are believed to be susceptible to these endoproteases because they have either a phenylalanine or tyrosine residue present at position 9 in the active peptide (highlighted in blue in figure 4.1). These amino acids have an aromatic acid side chain which allows chymotrypsin to hydrolyse the peptide bond between tyrosine or phenylalanine and

alanine (Hamra *et al* 1996, Forte, 2003). Uroguanylin is not susceptible to chymotrypsin-like proteases as there is an asparagine residue at position 9 (highlighted in blue in figure 4.1) in the active peptide sequence rather than the hydrophobic phenylalanine or tyrosine residues. Uroguanylin is also expressed in the kidney and studies have suggested that prouroguanylin produced in the kidney (Fan *et al.*, 1997) can be converted by proteases in the lumen of the kidney to active uroguanylin which stimulates natriuresis, kaliuresis and diuresis (Fonteles *et al.*, 1998). Because uroguanylin is expressed in the intestine and the kidney and it is also found in the blood it has been hypothesised that uroguanylin is part of an endocrine axis connecting the digestive tract and the kidney and this may form an important physiological mechanism regulating salt and water homeostasis (Forte *et al.*, 2000[a,b]; Potthast *et al.*, 2001). Uroguanylin and renoguanylin may also play similar roles in osmoregulation in teleosts as these peptides are also expressed in both the intestine and the kidney.

In comparison to guanylin and uroguanylin, renoguanylin the third guanylin-like peptide in *A. anguilla* (figure 3.13), is predominately expressed in the kidney, hence its name, and to a lesser degree but still at significant levels in the intestine. This data correlates with studies of renoguanylin expression in *Anguilla japonica* by Yuge *et al.*, (2003). Renoguanylin has a phenylalanine residue conserved at the same position as the phenylalanine residue in guanylin (highlighted in blue in figure 4.1). As discussed previously the phenylalanine residue in guanylin makes this peptide susceptible to hydrolysis by chymotrypsin-like proteases in the kidney lumen. So why is renoguanylin in the kidney not hydrolysed by these proteases? It is possible that the additional residues at the carboxy terminal of renoguanylin may stabilise the peptide and make it more resilient to chymotrypsin-like proteases in the kidney. Another explanation is that renoguanylin mRNA is not expressed in the proximal tubule and is only expressed in kidney tubules which do not possess chymotrypsin-like proteases or renoguanylin may be secreted into tubules distal to the chymotrypsin-like proteases. Therefore renoguanylin may only act in distal and collecting tubule segments where the chymotrypsin-like proteases are potentially not present. Alternatively renoguanylin expressed by the kidney may have no biological

function in this tissue. The renal renoguanylin may be secreted into the bloodstream and transported to another tissue where it may bind to and activate GC-C. Although GC-C has only been found to be in the intestine and the kidney in the European eel this receptor may be present in other tissues at levels where Northern blot analysis is not sensitive enough to detect expression (Comrie *et al.*, 2001[b]). Recent studies in mice support the hypothesis that guanylin-like peptides may also bind to another, as yet unknown, receptor (Forte, 2003). When receptor GC-C$^{-/-}$ mice were given an intravenous salt load the blood pressure and renal sodium excretion remained the same as that in mice with the GC-C receptor present (Carrithers *et al.*, 2004). From these findings it was concluded that urinary sodium excretion was not mediated by GC-C but by another receptor activated by uroguanylin (Forte, 2003). Further research is required to establish a clearer picture of renoguanylin function and its exact location in the kidney.

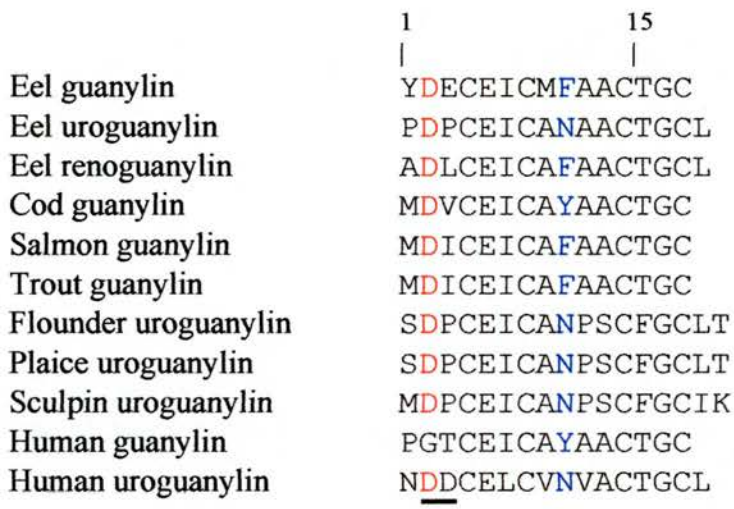


Fig. 4.1. The active peptide region of guanylin, renoguanylin and uroguanylin. The amino acid at position 9 is highlighted in blue. An aspartic acid residue which has been conserved in all of the teleosts is highlighted in red, this may the cleavage site of the prohormone to yield the active peptide. Underlined are the acidic residues of human uroguanylin.

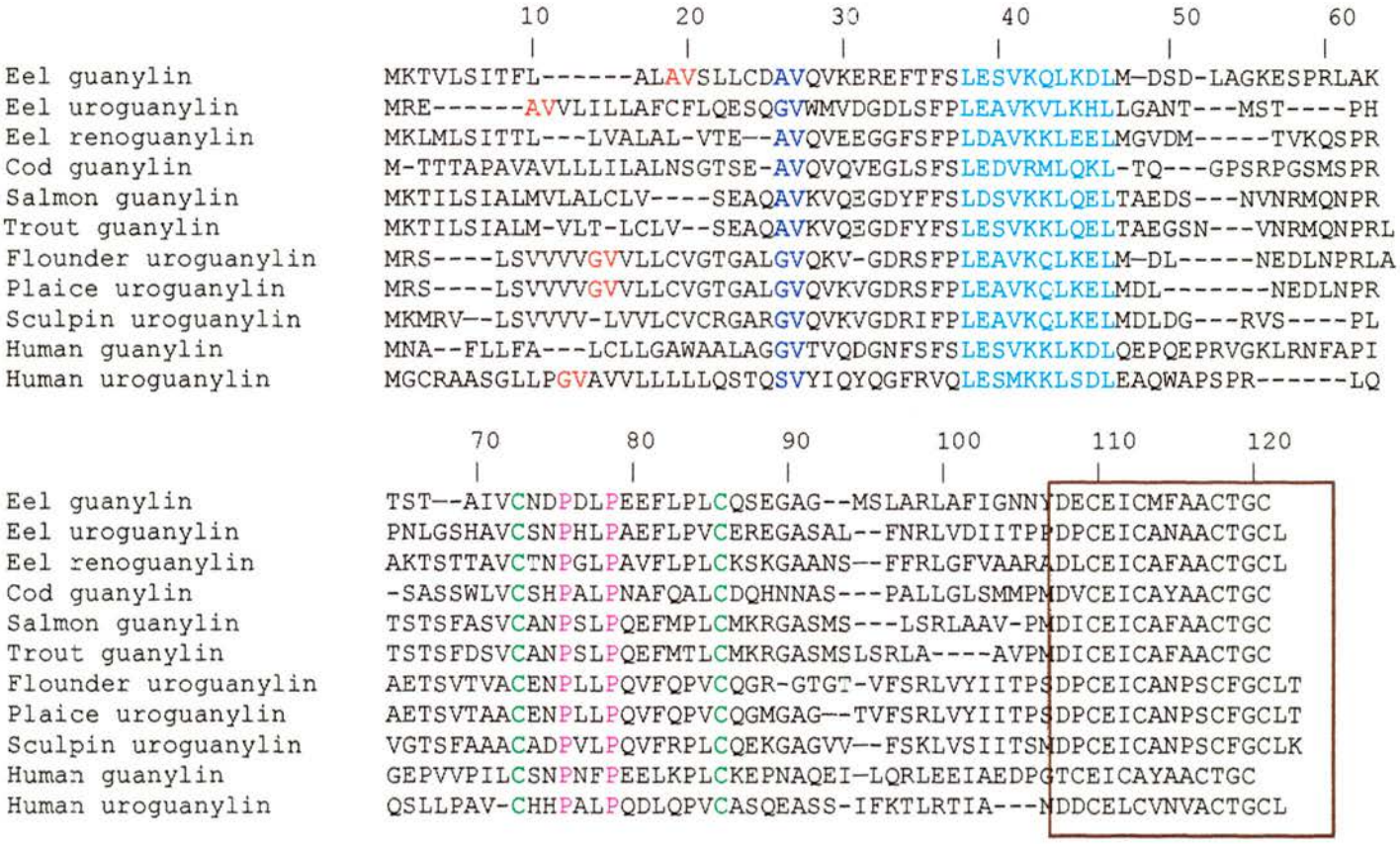


Figure 4.2 The amino acid alignment of the N terminal region of the preprohomone of guanylin, renoguanylin and uroguanylin. The amino acids highlighted in blue (amino acids 26 and 27) are the proposed cleavage site to yield a mature prohormone. The amino acids highlighted in red are other potential cleavage sites. The amino acids highlighted in aqua blue are a structural motif which may be important for folding and processing of the prohormone. Highlighted in pink are two proline residues and in green two cysteine residues which may also be important for folding of the prohormone. The boxed region highlights the active peptide.

Chapter 5 Results

The effect of SW-acclimation on expression of guanylin-like peptide mRNAs.

Northern Hybridisation

This technique was used to investigate the tissue distribution and levels of mRNA expression for guanylin, renoguanylin and uroguanylin in the European eel. Northern blots were conducted to see how expression varied in individual tissues following acclimation to SW. The same Northern blot membranes were used for each of the three guanylin-like peptides. A summary of these results can be found in Appendix 4.

5.1 Effect of SW-acclimation on guanylin mRNA expression

A series of time courses were carried out to investigate whether there were any changes in expression of guanylin mRNA during the process of acclimation of both non-migratory yellow and migratory silver eels to SW.

5.1.1 Yellow and Silver eel intestine

Figure 5.1 (a) and 5.2 (a) show the autoradiographs of the Northern blots, from two separate experiments, which represent guanylin mRNA expression in the intestine of yellow and silver eels respectively. The data were quantified as explained in section 2.14.3 and the values at each time point were compared using a two-way ANOVA followed by Fisher's PLSD post analysis of significance. The bar charts in figures 5.1 (b) and 5.2 (b) represent the quantitative expression of guanylin mRNA in yellow eels and silver eels over the first 7 or 140 days of acclimation respectively. There were no significant changes in the expression of guanylin mRNA at any of the time points in yellow eels. In silver eels however there was a significant increase, of approximately 3 fold, in the expression of guanylin mRNA in silver eels transferred to SW for 2 days and 140 days.

5.1.2 Intestine sections

The intestines from yellow FW (YFW) and 3 week-acclimated SW eels (YSW) and silver FW (SFW) and 3 week-acclimated SW eels (SSW) were divided into three equal length segments labelled, anterior, mid and posterior and run on the same RNA denaturing agarose gel. The relative levels of guanylin mRNA expression in each section was determined as explained in section 2.14.3. The

autoradiograph of the blot is shown in figure 5.3 (a). In the intestine a higher molecular weight mRNA was more prominent in the anterior and mid regions. It is assumed that this is an immature mRNA precursor of guanylin. Figure 5.3 (b) shows the quantified levels of expression of guanylin mRNA in all test groups. Only the 1 kb band was used for quantification. Significant increases in expression were only found in the anterior and posterior intestine sections of the silver eels when acclimated to SW. The increase found in the mid intestine section just failed to be significant. There were no changes in expression in the yellow eel intestine.

5.2 Effect of SW-acclimation on renoguanylin mRNA expression

5.2.1 Yellow and Silver eel intestine

Figure 5.4 (a) and figure 5.5 (a) show the autoradiographs of Northern blots of renoguanylin mRNA expression in the intestine of FW/FW or FW/SW transferred yellow eels and silver eels respectively. The quantitative expression of renoguanylin mRNA in the intestine of yellow and silver eels at selected time points after SW transfer are shown in figures 5.4 (b) and figure 5.5 (b) respectively. The data (at each time point) were compared using two-way ANOVA and Fisher's PLSD post analysis of significance. In yellow eels there was no significant increases in the expression of renoguanylin mRNA but in silver eels there was a marked and significant 2 fold increase in expression ($p < 0.001$) of renoguanylin mRNA in fish transferred to SW for 2 days. This increase in mRNA expression was not sustained as there was no significant difference found 5 days later at the 7 day time point.

5.2.2 Silver eel kidney

Figure 5.6 (a) shows the relative expression of renoguanylin mRNA in the renal kidney of silver eels at selected times following acute transfer of fish from FW to FW or from FW to SW. The data were quantified as explained in section 2.14.3 and the values at each time point were compared using two-way ANOVA followed by Fisher's PLSD post analysis of significance. The bar chart in figure 5.6 (b) represents the quantitative expression of renoguanylin mRNA in the kidney of silver eels. There was an approximate 50 % decrease in the FW/SW

transfer group after 7 days compared to the FW/FW controls. This decrease may be due to an increase in the mRNA expression of the eels in FW rather than a change in expression in the mRNA levels of eels acclimated to SW.

5.2.3 Intestine sections

The autoradiograph of the blot showing the relative expression of renoguanylin mRNA in the intestine which had been divided into three equal lengths labelled, anterior, mid and posterior sections in the four test groups, yellow FW, yellow SW (acclimated for 3 weeks), silver FW and silver SW (acclimated for 3 weeks) is shown in figure 5.7 (a). In the mid intestine sections of both FW and SW silver eels there were higher molecular weight mRNAs present which may be immature precursors of renoguanylin. Only the 1kb band was used for quantification (explained in section 2.14.3) and the quantified data is shown in figure 5.7 (b). The data were compared using two-way ANOVA followed by Fisher's PLSD post analysis of significance. Significant changes in renoguanylin mRNA expression (~ 1.5 fold increase) were only observed in the mid intestine sections of silver eels transferred to SW.

5.3 Effects of SW acclimation on uroguanylin expression

In all of the Northern blots used to study the effects of SW acclimation on uroguanylin mRNA expression three different sized transcripts were observed. The major uroguanylin transcript was a 1kb fragment with much lower levels of expression of two larger fragments which were approximately 2 and 6 kb. In these studies all three bands were used when calculating the levels of expression of uroguanylin mRNA because unlike the mRNA expression of other guanylin-like peptides such as renoguanylin the higher molecular weight fragments were present in all of the samples.

5.3.1 Yellow and Silver eel intestine

In two separate experiments yellow and silver eels were transferred from FW to FW or from FW to SW for up to 7 or 140 days. Northern blots showing the relative expression of uroguanylin mRNA in the intestine of yellow and silver eels are shown in figures 5.8 (a) and 5.9 (a) respectively. The data were

quantified as explained in section 2.14.3 and the values at each time point were compared using two-way ANOVA followed by Fisher's PLSD post analysis of significance. The quantitative expression of uroguanylin mRNA in yellow and silver eels is shown in figures 5.8 (b) and figure 5.9 (b) respectively. In yellow eels a significant 2.5 fold increase in the expression of intestinal uroguanylin mRNA was found after 7 days in SW. In silver eels uroguanylin mRNA expression in the intestine of SW-acclimated fish increased significantly ($p< 0.05$) after 2 days and levels of uroguanylin mRNA remained elevated after 140 days. This agrees with previous studies from our laboratory (Comrie *et al.*, 2001).

5.3.2 Silver eel kidney

The autoradiograph showing the relative expression of uroguanylin mRNA in the renal kidney of silver eels at selected time points after acute transfer of fish from FW to FW or FW to SW is shown in figure 5.10 (a). The data were quantified (figure 5.10 (b)) and analysed using two-way ANOVA and Fisher's PLSD post analysis of significance and there were no significant changes in the expression of uroguanylin mRNA. There was high variability in mRNA expression from the different fish and these differences may occlude finding any significant changes in uroguanylin mRNA expression in the kidney.

5.3.3 Intestine sections

Figure 5.11 (a) shows the autoradiograph of the Northern blot illustrating the relative expression of uroguanylin mRNA along the length of the intestine which had been divided into three equal lengths labelled, posterior, mid and anterior. Four test groups were analysed, yellow FW, yellow SW, silver FW and silver SW eels all which had been acclimated for three weeks. Three different sized mRNA transcripts were present in the majority of the samples, the predominant 1kb band and the usual two higher molecular weight fragments. All three of these bands were used for quantification as they were present in most samples and in SSW mid samples the bands were very intense and therefore it would have been too difficult to separate them into three distinct bands. The quantified data of the expression of uroguanylin mRNA along the length of the intestine is

shown in figure 5.11 (b). There were significant increases in mRNA expression of uroguanylin in all sections of the intestine in silver eels transferred to SW compared to mRNA expression in FW fish. In yellow eels there was similar but smaller increases in expression in all sections of the intestine although these were only significant in the mid and posterior sections.

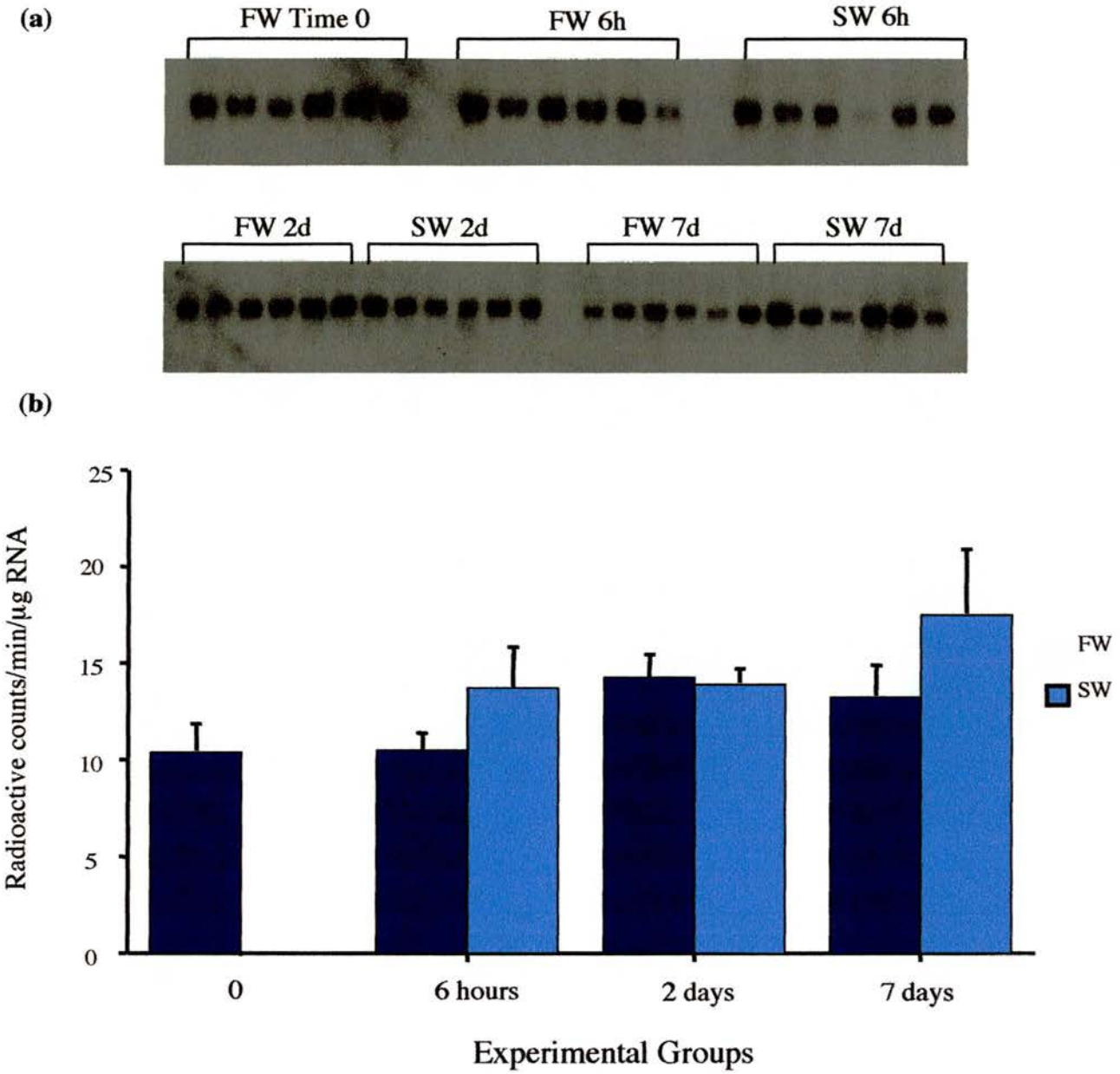


Fig. 5.1 (a) A Northern blot time course showing the relative expression of guanylin mRNA in the intestine of yellow eels following acute FW to FW or FW to SW transfer. There was 10 µg total RNA in each sample and fish were sampled at the time points indicated.
(b) A bar chart showing expression levels of the data from fig. 5.1 (a) (mean ± s.e.). n = 6 fish in all groups. Fisher's PLSD indicated no significant difference in the expression of guanylin mRNA in any of the groups.

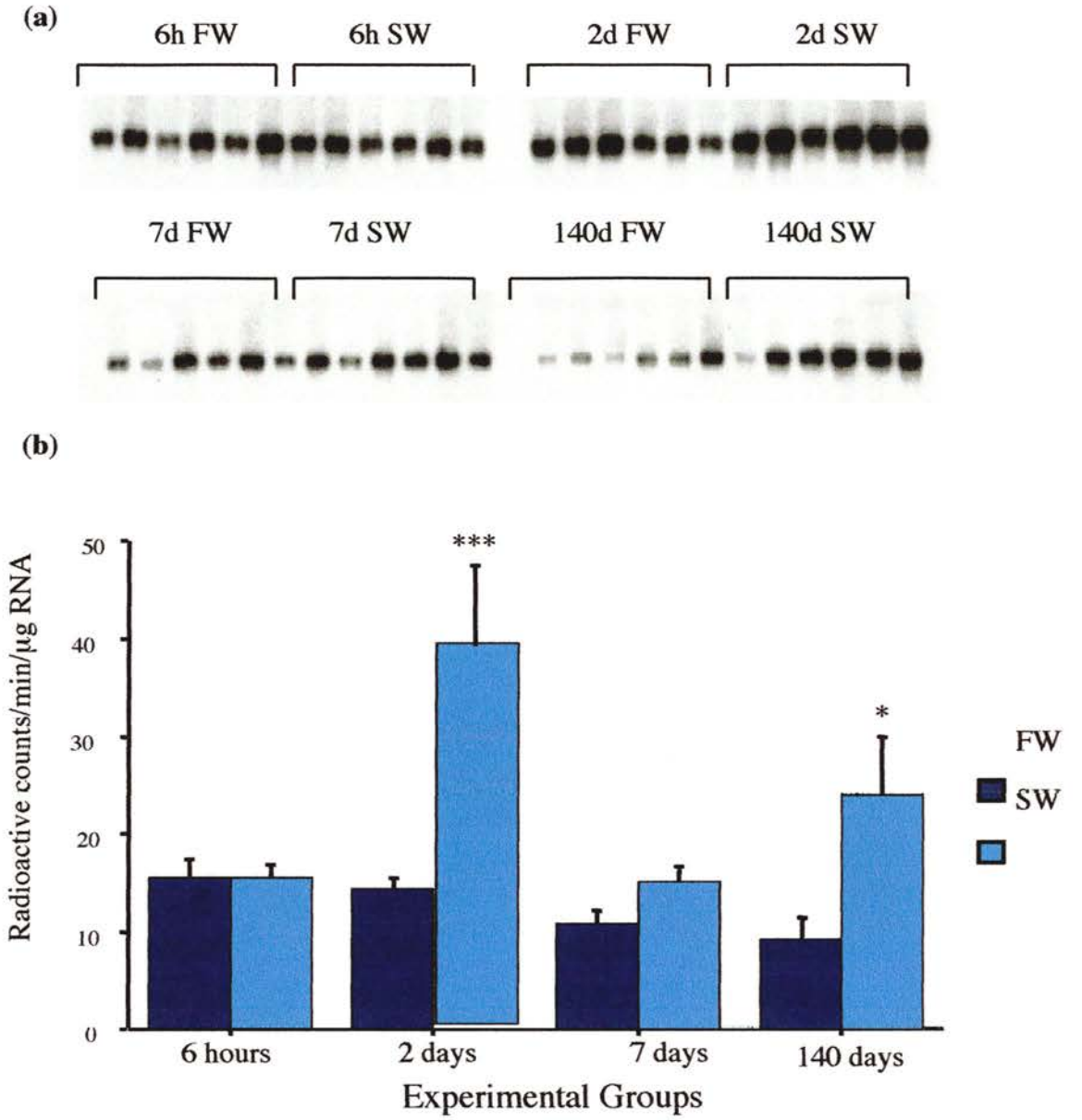


Fig. 5.2 (a) A Northern blot time course showing the relative expression of guanylin mRNA in the intestine of silver eels following acute FW to FW or FW to SW transfer. There was 10 µg total RNA in each sample and fish were sampled at the time points indicated after acute transfer to FW or SW.
(b) A bar chart showing expression levels of the data from fig. 5.2 (a) (mean ± s.e.) and n = 6 fish in all groups. Analysis using Fisher's PLSD showed that SW values were significantly different to the FW group at the same time point as indicated; * $p < 0.05$, *** $p < 0.001$.

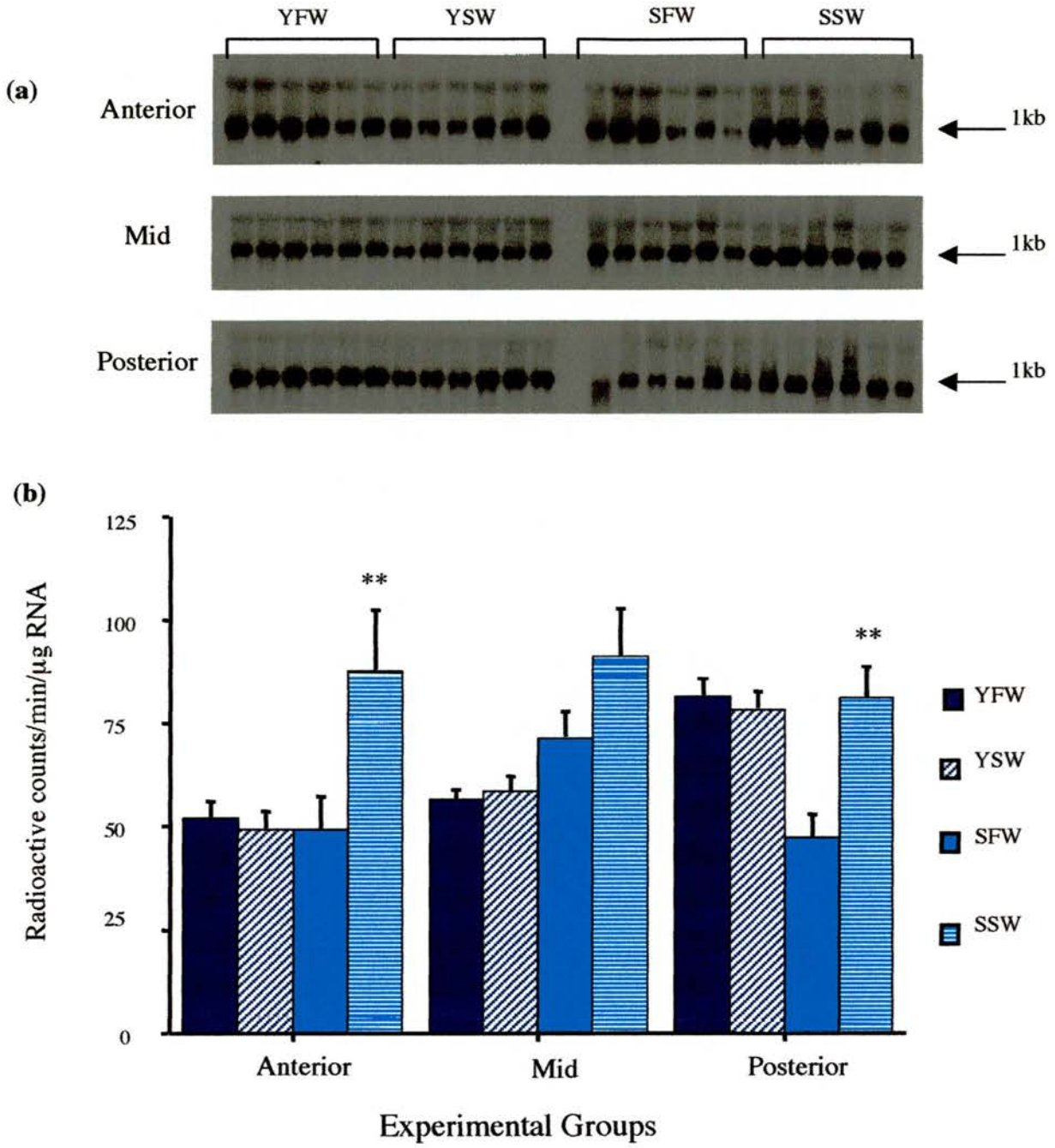
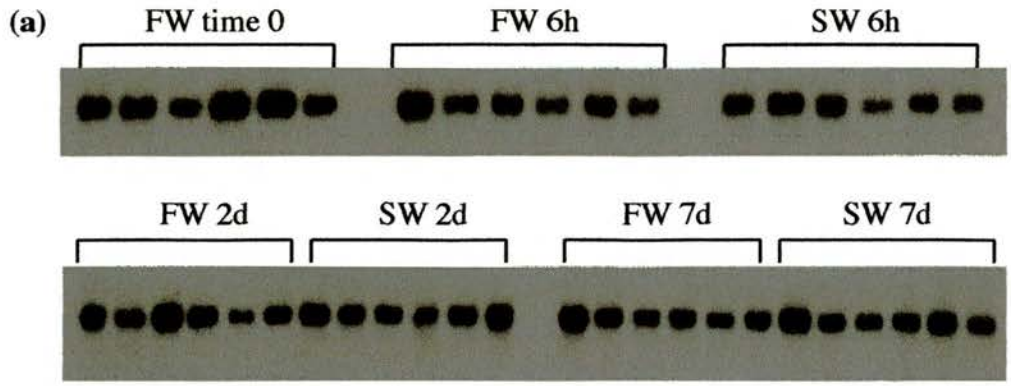


Fig 5.3 (a) A Northern blot showing the relative levels of expression of guanylin mRNA in three regions (anterior, mid and posterior) of the eel. There were four experimental groups of eels, yellow freshwater (YFW), yellow seawater (YSW), silver freshwater (SFW) and silver seawater (SSW). Eels were acclimated to SW for three weeks.
(b) A bar chart showing expression levels (mean ± s.e.) of the data from fig 5.3 (a). There was 10 µg of RNA in each sample and n = 6 fish in all groups. Two-way ANOVA and Fisher's PLSD test was used to analyse the data. ** indicated that levels of expression of guanylin between anterior SFW and anterior SSW and between posterior SFW and posterior SSW were significant at the 1% level ($p < 0.01$).



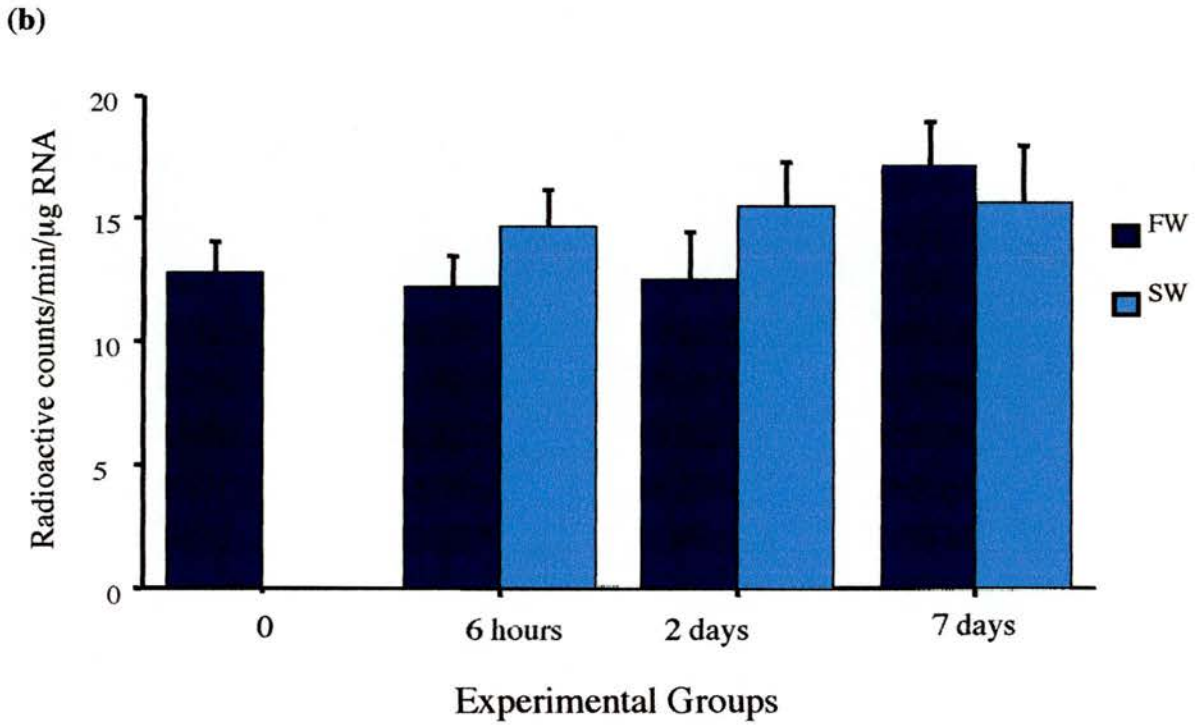


Fig. 5.4 (a) A Northern blot showing the relative expression of renoguanylin in the intestine of yellow eels at selected times following acute FW to FW and FW to SW transfer.
(b) A bar chart showing the expression levels (mean ± s.e.) of the data from fig. 5.4 (a). There was 10 µg RNA in each sample and n = 6 fish in all experimental groups. Data were quantified and analysed using two-way ANOVA and Fisher's PLSD test. There were no significant changes in expression of renoguanylin between any of the groups in the intestine of yellow eels.

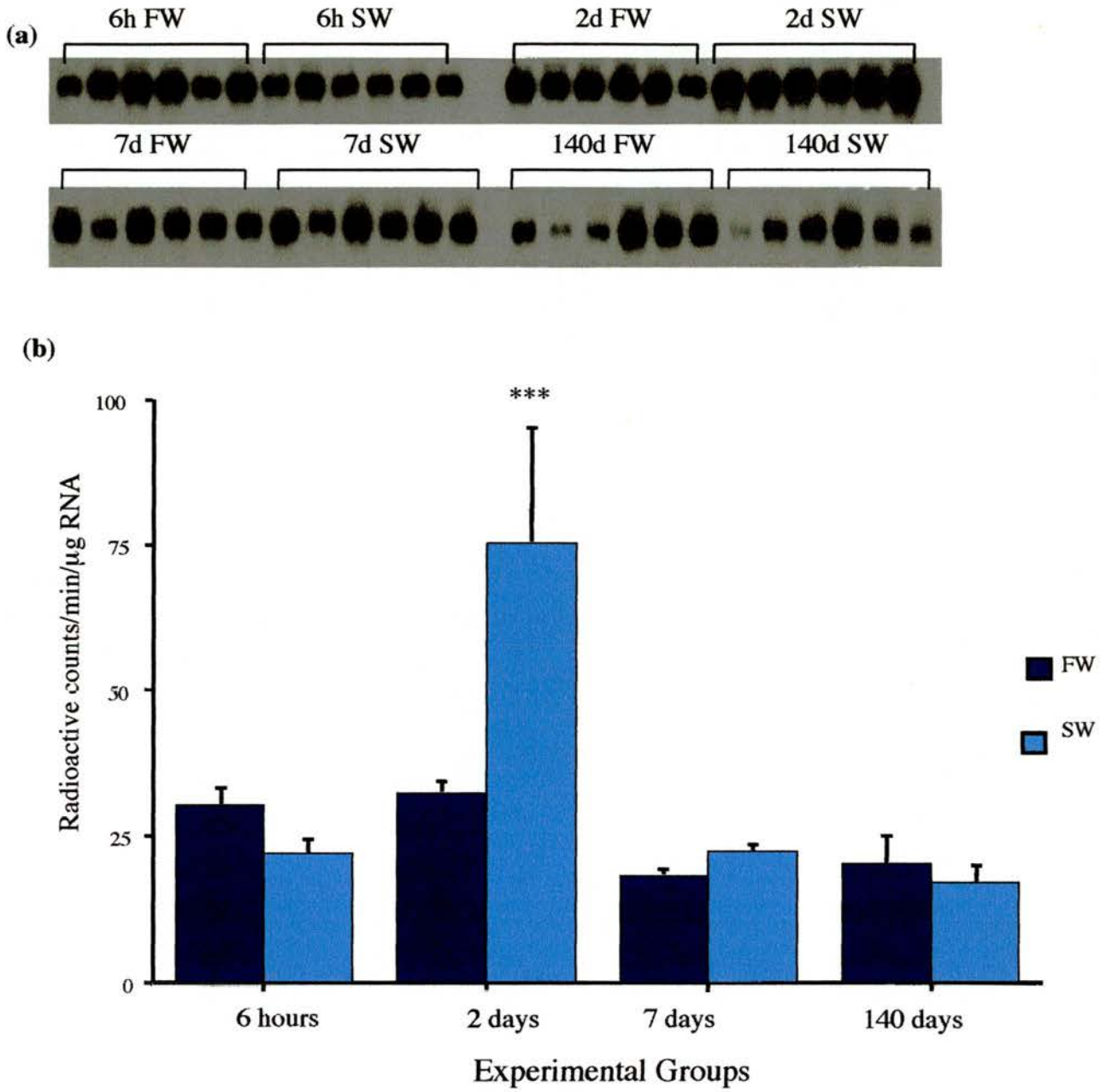


Fig. 5.5 (a) A Northern blot showing the relative expression of renoguanylin mRNA in the intestine of silver eels at selected times following acute FW to FW or FW to SW transfer.
(b) A bar chart showing the expression levels (mean ± s.e.) of the data from fig 5.5 (a). There was 10 µg total RNA in each sample and n = 6 fish in all experimental groups. Statistical analysis was by two-way ANOVA and Fisher's PLSD post hoc test. *** indicates a significant difference in the level of expression of renoguanylin at $P < 0.001$ between 2 day-acclimated FW and SW groups.

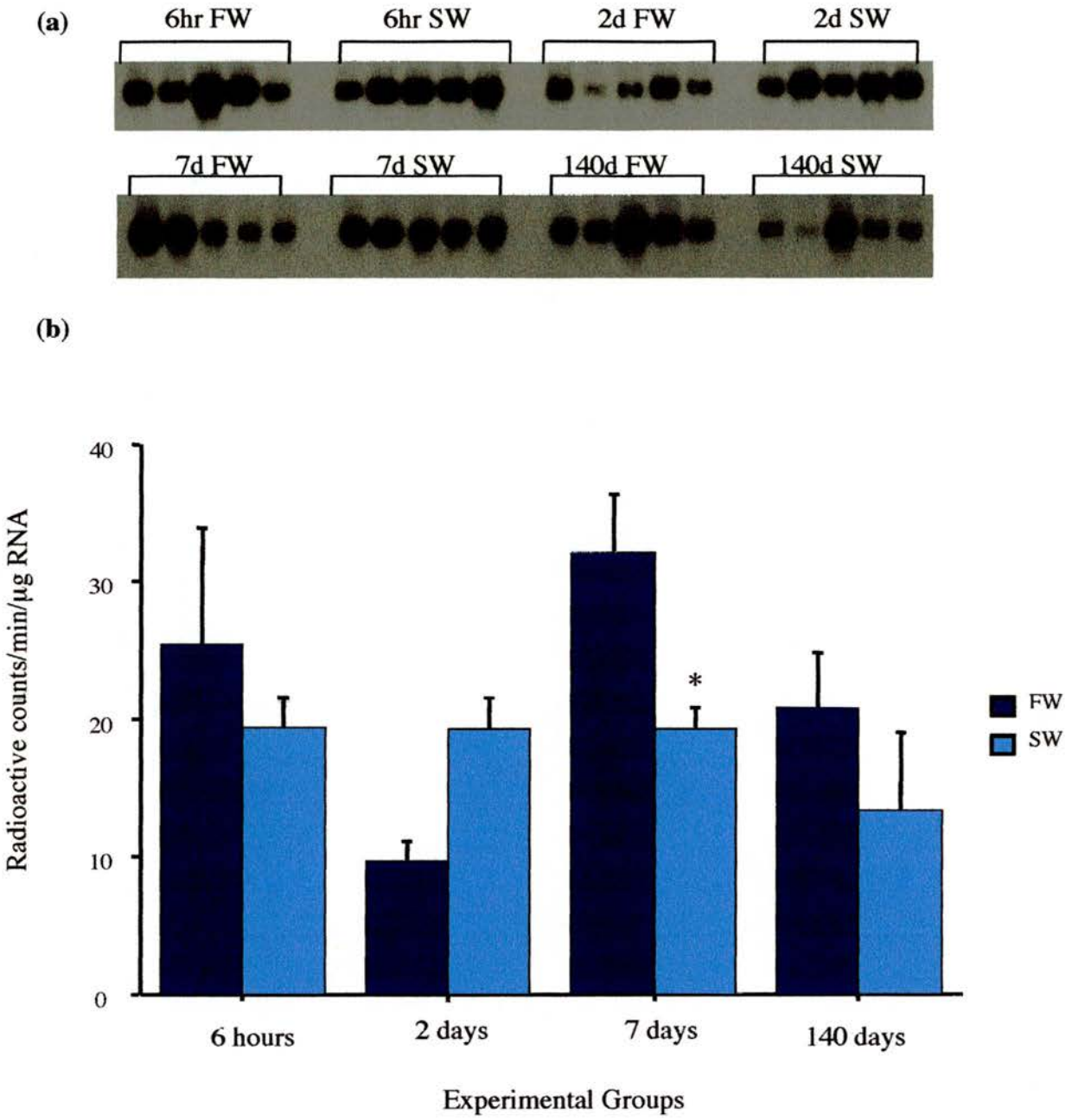


Fig. 5.6 (a) A Northern blot showing the relative expression of renoguanylin mRNA in the renal kidney of silver eels at selected time points following acute FW to FW or FW to SW transfer.
(b) A bar chart showing the expression levels (mean ± s.e.) of the data from fig 5.6 (a). There was 10 µg RNA in each sample and n = 5 fish in all experimental groups. Two-way ANOVA and Fisher's PLSD analysis found * significance at the 5% level ($p < 0.05$) between 7 day transferred FW and SW groups. This is probably due to changes in the FW group rather than changes in the level of mRNA in the eels acclimated to SW for 7 days.

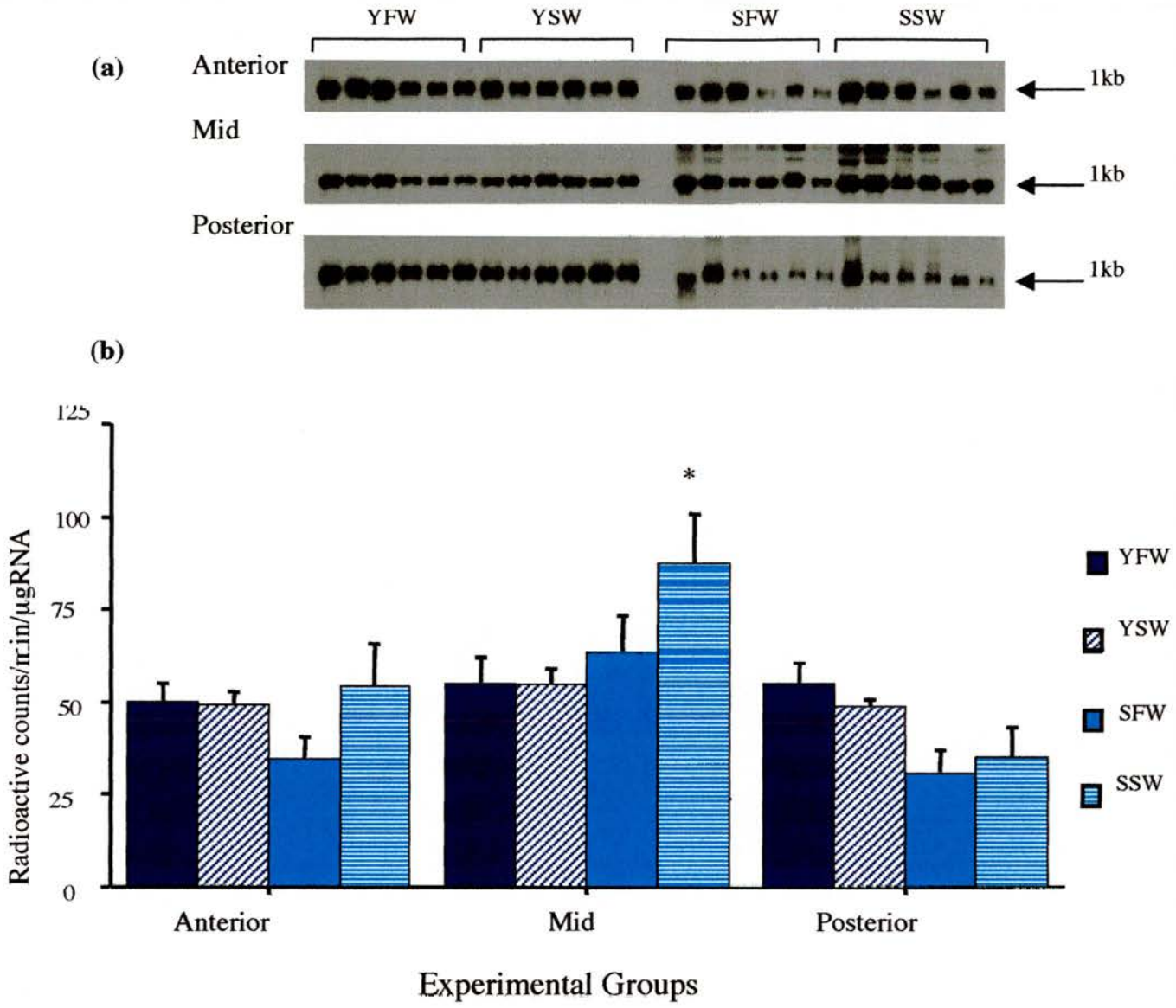


Fig. 5.7 (a) A Northern blot showing the relative expression of renoguanylin mRNA in the anterior, mid and posterior regions of the eel intestine. There were four experimental groups of eels, yellow freshwater (YFW), yellow seawater (YSW), silver freshwater (SFW) and silver seawater (SSW). Eels were acclimated to SW for three weeks.
(b) A bar chart showing expression levels (mean ± s.e.) from the data shown in fig. 5.7 (a). There was 10 µg RNA in each sample and n = 6 fish in all experimental groups. Fisher's PLSD test showed that was a significant difference of * $p < 0.05$ between mid intestine SFW and mid intestine SSW groups.

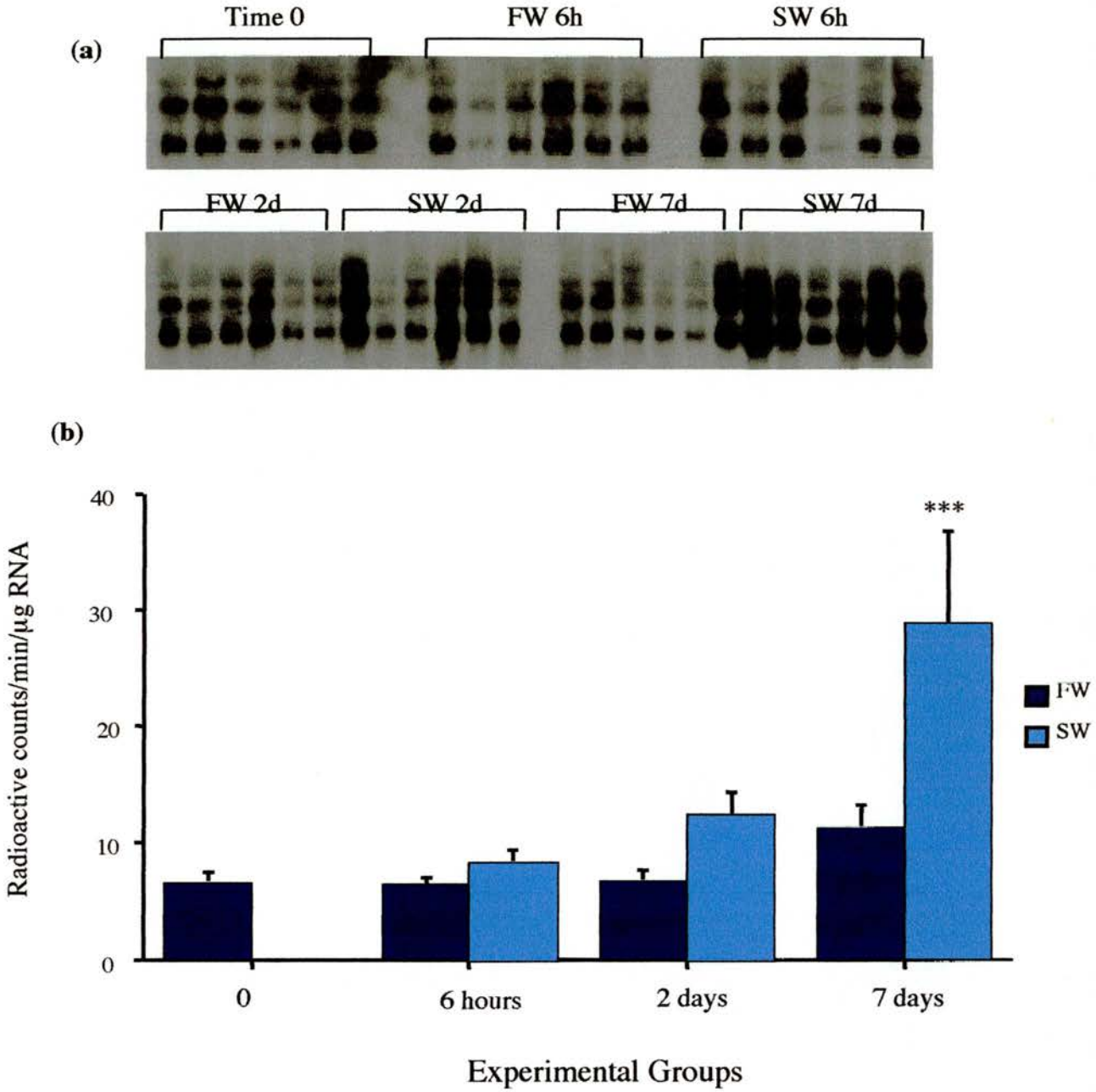


Fig. 5.8 (a) A Northern blot showing the time course effects of FW/FW or FW/SW transfer on the relative expression of uroguanylin mRNA in the intestine of yellow eels. (b) A bar chart showing the quantified expression levels (mean ± s.e.) of the data from the fig. 5.8 (a) and n = 6 fish in all experimental groups and there was 10 µg total RNA in each sample. Data was analysed using two-way ANOVA and Fisher's PLSD test. *** indicates a significant difference in the level of expression of uroguanylin at $p < 0.001$ between 7 day acclimated FW and SW fish.

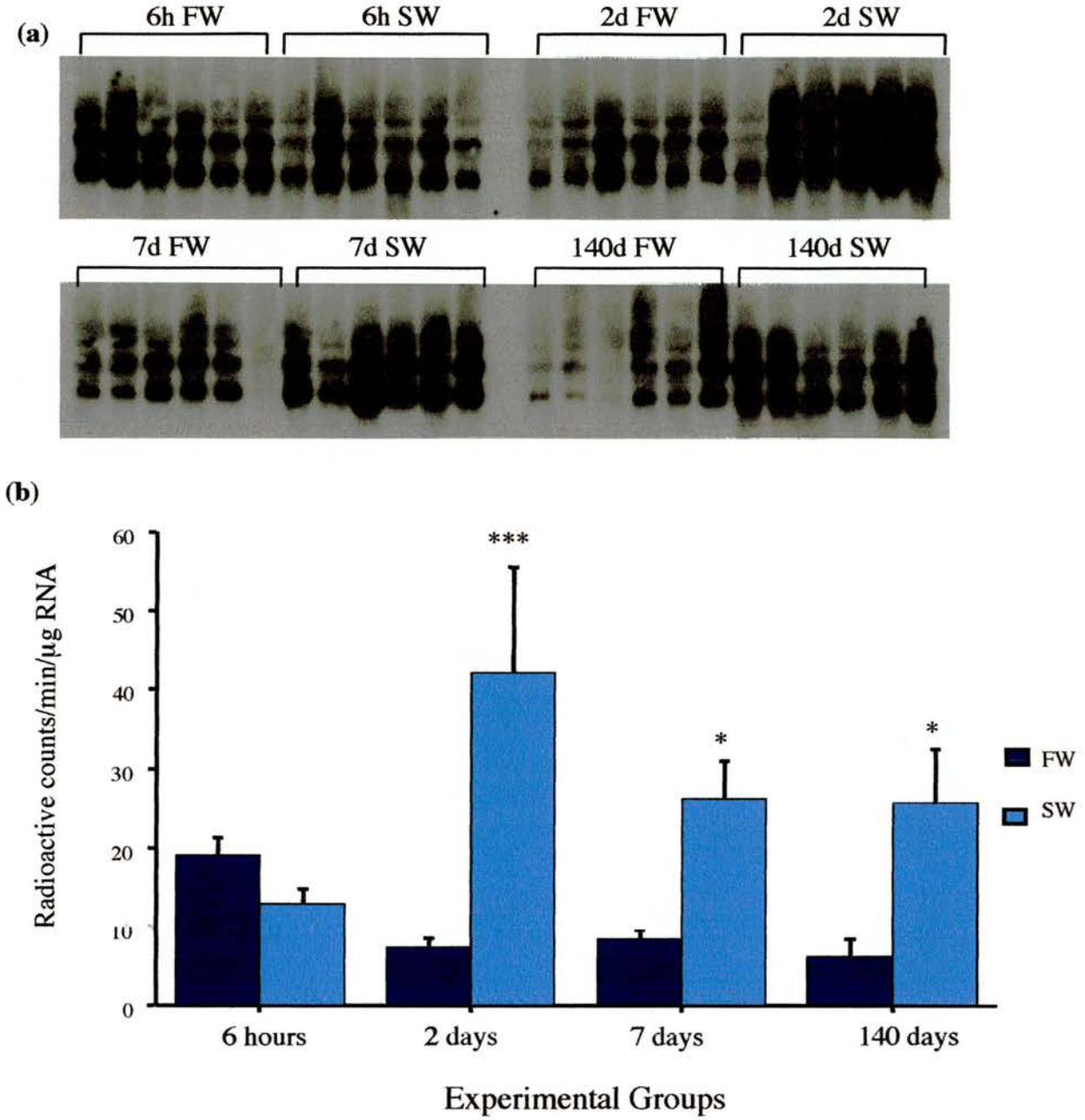


Fig.5.9 (a) A Northern blot showing the time course of effects of FW/FW or FW/SW transfer on the relative expression of uroguanylin mRNA in the intestine of silver eels following acute FW to FW or FW to SW transfer.
(b) A bar chart showing the quantified expression levels (mean ± s.e.) of the data from fig. 5.9 (a). There was 10 µg RNA in each sample and n = 6 fish in all experimental groups. The data was analysed using two-way ANOVA and Fisher's PLSD test. *** indicates a significant difference in the level of expression of uroguanylin at $p < 0.001$ between 2 day acclimated FW and SW fish and * indicates a significant difference in the level of expression of $p < 0.05$ between 7 day acclimated FW and SW fish and between long term acclimated FW and SW fish.

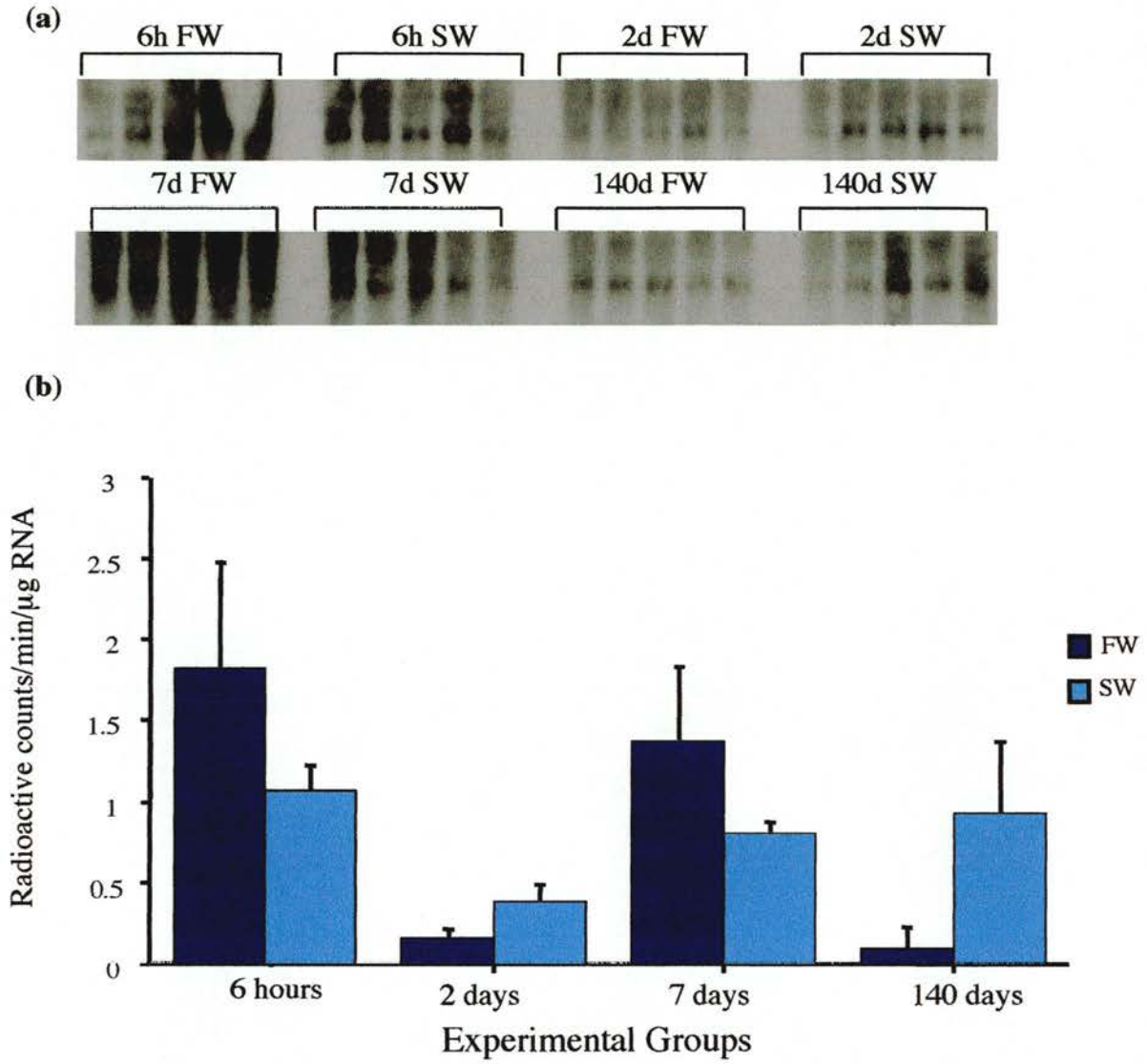


Fig. 5.10 (a) A Northern blot showing the time course of expression of uroguanylin mRNA in the kidney of silver eels following acute FW to FW or FW to SW transfer. Fish were sampled at the time points indicated.
(b) A bar chart showing the expression levels (mean ± s.e.) of the data from fig. 5.10 (a). There was 10 µg total RNA in each sample and n = 5 in all groups. The data was analysed using a two-way ANOVA and Fisher's PLSD test. There were no significant differences in the level of expression of uroguanylin between fish transferred to FW or SW at any of the time points.

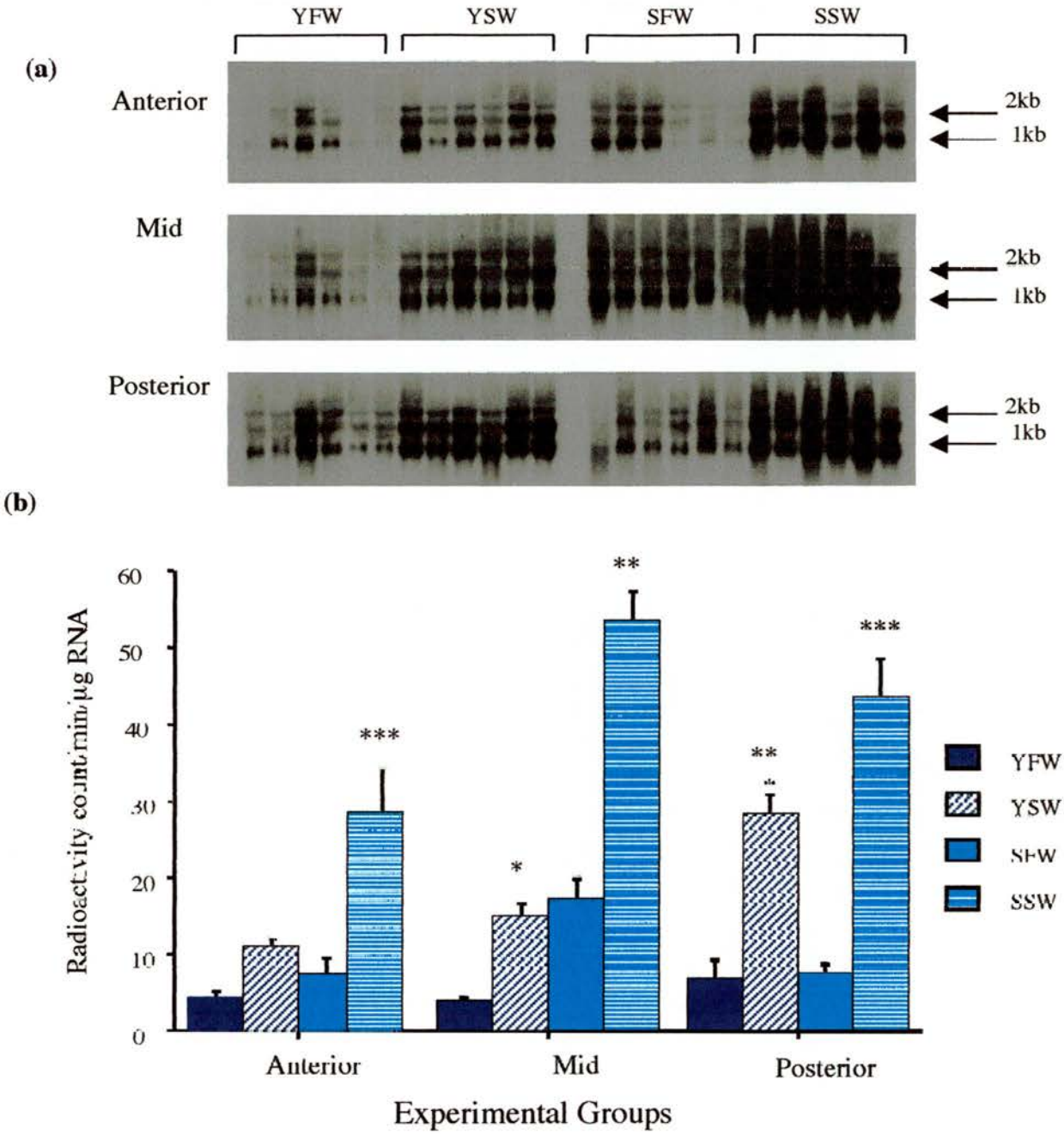


Fig. 5.11 (a) A Northern blot showing the relative expression levels of uroguanylin mRNA in the anterior, mid and posterior regions of the eel intestine. There were four experimental groups of eels, yellow freshwater (YFW), yellow seawater (YSW), silver freshwater (SFW), silver seawater (SSW). Eels were acclimated for three weeks.
(b) A bar chart showing expression levels (mean ± s.e.) of the data from fig 5.11(a). There was 10 µg total RNA in each sample and n = 6 fish in all experimental groups. Fisher's PLSD test indicates a * significance level of $p < 0.05$ between YFW and YSW eels in the mid section, ** a significance level at $p < 0.01$ between SFW and SSW in the mid section and between YFW and YSW in the posterior section and *** a significance level of $p < 0.001$ between SFW and SSW in the anterior section and between SFW and SSW in the posterior regions of the intestine.

Chapter 6 Discussion

The effect of SW-acclimation on the expression of guanylin-like peptide mRNAs.

6.1 Expression of Guanylin-like mRNA in the intestine of *A. Anguilla.*

In mammals it is believed that expression of uroguanylin mRNA is under the influence of NaCl in the diet (Forte, 2003) and a study by Kita *et al.*, (1999) showed that the concentration of uroguanylin present in the urine was increased in mammals on a high salt diet. Further support for guanylin peptides participating in the control of body sodium balance came from a study by Potthast *et al.*, (2001) who found that low salt consumption significantly decreased the levels of uroguanylin and guanylin mRNA expression in the small intestine. Mammalian studies have found that administration of an oral salt load causes a much larger natriuresis than the intravenous administration of saline (Forte *et al.*, 2000[b]) and oral administration of salt to rats augmented uroguanylin mRNA levels in the intestine and the kidneys (Potthast *et al.*, 2001). When eels are transferred to a SW environment they drink their external medium (~ 0.5 ml/kg/h) and this should elicit a similar effect on the mRNA expression of guanylin-like peptides as administration of an oral salt load to mammals. Northern blots using total RNA from the intestine of both yellow and silver eels acclimated to SW for varying periods of time were used to study changes in the expression of the three guanylin-like peptides, guanylin, renoguanylin and uroguanylin identified in *A. anguilla*.

In the intestine of yellow eels there were a number of differences between the levels of mRNA expression of the three guanylin-like peptides. Uroguanylin mRNA was expressed at much lower levels (approximately 2 - 3 fold less) than either guanylin or renoguanylin in both FW- or SW-acclimated yellow eels. Although there was no significant change in the levels of expression of guanylin or renoguanylin mRNA following transfer of yellow eels to SW, expression of uroguanylin mRNA was significantly upregulated in yellow eels transferred to SW for 7 days compared to yellow eels transferred from FW to FW for the same period of time. Therefore in yellow eels intestinal uroguanylin may be involved in osmoregulatory processes associated with FW/SW transfer.

In contrast, in silver eels transferred to SW for two days mRNA expression of all three guanylin-like peptides was upregulated in the intestine. The expression of renoguanylin mRNA decreased again over the remainder of the time course and expression of guanylin mRNA fell in the 7 day SW eels but increased again in

silver eels transferred to SW for 140 days. The expression of uroguanylin mRNA in SW eels was lower in silver eels transferred to SW for 7 days and 140 days compared to the silver eels transferred to SW for 2 days but the level of uroguanylin mRNA expression remained significantly higher compared to the expression of uroguanylin mRNA in the FW/FW transferred fish at these time points. The upregulation of uroguanylin mRNA took approximately 5 days longer to occur in the intestine of yellow eels compared to silver eels acclimated to SW. Silver eels may have mechanisms already activated which initiate the upregulation of uroguanylin mRNA as silver eels normally migrate from FW to SW unlike yellow eels whose natural habitat is FW. This could explain why it took just 2 days for significant changes in mRNA expression to occur in silver eels compared to 7 days in yellow eels. This data suggests that uroguanylin and guanylin, and to a lesser extent renoguanylin, are involved in osmoregulation in silver eels during FW/SW transfer. Of the three peptides, uroguanylin may be the most important for long-term adaptation to SW. These change sin mRNA expression may not necessarily translate into changes in peptide secretion and thus activity of the system.

The levels of expression of mammalian guanylin mRNA are known to vary along the longitudinal axis of the digestive tract. Uroguanylin is predominately found in the proximal small intestine whereas guanylin mRNA levels peak in the distal small intestine and the large intestine (Forte, 1999[b]). To establish if the mRNAs for teleost guanylin-like peptides were differentially expressed throughout different regions of the intestine RNA was isolated from the intestines from both yellow and silver FW- and SW-acclimated eels and the intestines were divided into three equal length segments (termed anterior, mid and posterior). The results suggest that mRNAs for all three of the guanylin-like peptides are expressed along the length of the eel intestine, which differs from the differential levels of mRNA expression of guanylin and uroguanylin along the mammalian digestive tract (Forte, 1999[b]). The mRNA expression of guanylin was significantly increased in the anterior and posterior regions of the intestine of silver eels acclimated to SW compared to FW/FW transferred silver eels however, in yellow eels there were no significant changes in the mRNA expression of guanylin. The mRNA expression of renoguanylin was only

significantly upregulated by 25 – 30% in the mid section of silver eels acclimated to SW. Again there was no change in renoguanylin mRNA expression in yellow eels. The results of the mRNA expression of guanylin and renoguanylin in yellow eels differ from the mRNA expression of these peptides in *Anguilla japonica* (Yuge *et al.*, 2003). Yuge *et al* found that expression levels of guanylin mRNA were significantly elevated in the anterior and posterior regions and renoguanylin mRNA expression was significantly increased in the anterior region of the intestine of yellow *A. japonica* following acclimation to SW. There are a number of possible reasons for the differences in these two seta of data from *A. anguilla* and *A. japonica*. One reason may be that the *A. japonica* yellow eels were small (weighing < 200g) and were farmed whereas the *A. anguilla* eels were caught in the river Tay and were larger in size (weighing > 200g). This difference in size and therefore maturity of the two sets of eels may be one reason why there was a difference in mRNA expression. A second explanation may be the difference in the way the data was quantified. For this study variations in the amount of RNA in each sample were determined by quantification of the ethidium bromide stained 18S and 28S ribosomal RNA bands on the gels and these values were used to adjust the final radioactive signals obtained per normalised microgram of total RNA. In contrast Yuge *et al* (2003) used RT-PCR to measure the levels of RNA and values were expressed relative to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene. This method may be flawed as the mRNA levels of GAPDH also increased (but perhaps not to the same extent) following SW acclimation and this would affect the relative expression levels of the guanylin-like peptide.

In yellow SW-acclimated eels mRNA expression of uroguanylin was significantly increased in the mid and posterior sections of the intestine compared to FW/FW transferred eels and in silver SW-acclimated eels, mRNA expression (compared to FW/FW transferred eels) was significantly elevated in all three regions of the intestine. From these studies on the intestine of yellow and silver eels it can be concluded that SW-acclimation predominately causes an upregulation in mRNA expression of uroguanylin in both yellow and silver eels and to a lesser extent guanylin and renoguanylin exclusively in silver eels, and

that the guanylin-like peptides are therefore possibly involved in osmoregulation. The effects of SW acclimation on the mRNA expression levels of uroguanylin in *A. anguilla* are similar to the effect of salt ingestion on the upregulation of this gene in mammals. Mammalian studies have found that there is an increase in the levels of uroguanylin mRNA expression in the intestine following an oral salt load (Fonteles *et al.*, 1998). In SW, eels lose a large amount of water across body surfaces, predominately the gills. To compensate for this loss and to maintain internal plasma levels at approximately 350 $mOsmkg^{-1}$ eels drink large volumes of the SW and absorb the water in the intestine whilst reducing their urinary water output. It may therefore seem unusual that in a situation where water has to be conserved there is upregulation of the guanylin/GC-C signalling system which results in secretion of water and ions into the lumen of the intestine. It is thought that the guanylin signalling system may antagonise the rate of salt and water uptake in the intestine and this would prevent any sudden increases in blood volume and plasma osmolality which would follow drinking (Comrie *et al.*, 2001[b]). Alternatively when the eels are in SW the guanylin-like peptides may be synthesised in the intestine then transported via the bloodstream to the gills where they may activate as yet unknown receptors to help excrete salt.

6.2 Expression of guanylin-like mRNAs in the kidney of *A. anguilla*.

Northern blots using total RNA from the kidney of FW- and SW- acclimated silver eels were used to study the mRNA expression of uroguanylin and renoguanylin in this tissue. Northern blot analysis indicates that guanylin was not expressed in this tissue at any measurable level.

There was no significant upregulation or downregulation of renoguanylin mRNA in the kidney of silver eels at any of the time points following acclimation to SW. These findings are in agreement with the study by Yuge *et al.*, (2003). Yuge *et al* found no significant changes in the expression of renoguanylin in the kidney of *Anguilla japonica* following transfer to SW for 2 weeks. From this data Yuge *et al.*, (2003) proposed that in the kidney, renoguanylin is not affected by environmental salinity and may have a different function in the kidney other than osmoregulation. Further research is required to determine the exact function of renoguanylin in the kidney.

There were no significant changes in the mRNA expression of uroguanylin in the kidney of silver eels acclimated to SW over the time course. This data suggests that uroguanylin does not play a role in osmoregulation in the kidney following transfer to SW, but these results differ from those in mammals and *A. japonica*. In mammals Potthast *et al.*, (2001) found that the mRNA expression of uroguanylin was upregulated in the kidney in response to a high salt diet. In *A. japonica* Yuge *et al.*, (2003) found a significant 2-fold increase in uroguanylin mRNA expression following transfer to SW for 2 weeks. The different results from *A. anguilla* and *A. japonica* cannot be compared because Yuge *et al.*, (2003) used immature yellow eels but for this study, mature silver *A. anguilla* were used. The experiments need to be repeated using mRNA from the kidney of yellow eels (unfortunately these tissues were not available for this research project) to enable a comparison between the different eel species.

This current analysis of data obtained from our experiments should not rule out the possibility that uroguanylin may play a role in osmoregulation in the kidney of *A. anguilla*. Mammalian studies have found that uroguanylin expressed in the intestine is secreted into the bloodstream then transported to the kidney (Forte *et al.*, 2000[b]). In the kidney, uroguanylin transported from the intestine and uroguanylin that has been expressed and released in the kidney act together to activate GC-C receptors located in the kidney to cause natriuresis and diuresis (Fonteles *et al.*, 1998). A similar uroguanylin intestine-kidney endocrine axis may be present in teleost fish. This could mean that in *A. anguilla,* uroguanylin, which is expressed at much higher levels in the intestine than the kidney may be transported via the bloodstream to have physiological actions within the kidney. This would result in higher levels of uroguanylin within the tubules of the kidney and may mean that uroguanylin would have a role to play in osmoregulation in the kidney but further research is required to confirm this theory.

Chapter 7 Results

Bacterial expression and immunohistochemistry of guanylin-like peptides.

For the following Western blots and immunohistochemistry data the proguanylin and prouroguanylin antibodies used were obtained from the fourth bleed and purified as described in section 2.19.1.

7.1 Testing of antibody specificity and optimal dilution

The specificity and optimal concentrations of the antibodies raised to proguanylin and prouroguanylin were determined using the Western blot technique. The blots were prepared by running a series of SDS gels, using proguanylin and prouroguanylin samples amplified as described in section 2.15, and the gels transferred to PVDF membrane (section 2.18).

Figure 7.1 (a) shows a membrane with 1 μg and 10μg prouroguanylin samples. A 10 kDa band was present which corresponds to prouroguanylin. The samples were run on a second membrane, shown in figure 7.1 (b). The primary antibody used was guanylin diluted 1 in 10 and no band was present. These results demonstrate that the guanylin antibodies do not cross react with uroguanylin.

Figures 7.2 (a) is a membrane of serial dilutions of proguanylin and the primary antibody used was guanylin diluted 1 in 10. Figure 7.2 (b) shows serial dilutions of prouroguanylin and the primary antibody used was uroguanylin diluted 1 in 10. On both membranes a dominant band of 28 kDa was present which corresponds to the prohormone fused to thyrodoxone. The lowest protein concentration, which was detected by the antibodies, was approximately 1 μg. Figures 7.3 (a) and (b) show membranes incubated with serial dilutions of guanylin and uroguanylin antibodies to determine which dilutions of the primary antibody could detect 1 μg of protein. The optimal dilutions were 1 in 500 for uroguanylin and 1 in 1000 for guanylin.

7.2 Bacterial expression of guanylin-like proteins

Using the techniques described in section 2.15 proguanylin, prorenoguanylin and prouroguanylin were successfully expressed. Two 12% SDS gels were run which followed the expression of run of proguanylin, one gel was transferred to PVDF membrane for Western blotting and the other was stained with Coomassie blue. Figures 7.4 (a) and (b), 7.6 and 7.7 show the expression and isolation from the

bacterial cell line (as described in section 2.15.5) of proguanylin, prorenoguanylin and prouroguanylin following the addition of IPTG to the bacterial culture. A protein of 28 kDa was expressed which corresponds to the prohormone fused to a larger thyrodoxone protein. The membranes also followed the purification of the prohormone using a Ni^{2+} chelation technique described in section 2.15.6 (i). The 28 kDa protein was displaced from the Ni^{2+} column using 250 mM imidazole and bands of this size were detected on the Coomassie blue stained gels and the Western blots. Prior to digestion of the prohormone from the fusion protein the protein samples had to be dialysed against 50 mM Tris, 150 mM NaCl and 1 mM EDTA, pH 7 to lower the concentration of imidazole as the imidazole would have inhibited the enzyme activity. The enzyme used for the digest was PreScission™ protease. A sequence of amino acids, Leu-Phe-Gln-Gly-Pro had been incorporated at the 5' end of the prohormone, as described in section 2. 15, and these amino acids are recognised by PreScission™ protease which cleaves between the glutamine and glycine residues. Figure 7.5 shows the Western blot treated with proguanylin specific antiserum looking at the optimal time for the PreScission™ protease digest (as described in section 2.15.6(ii)). Two bands were detected, the 17 kDA fragment is the thyrodoxone protein and the 10 kDa band is the proguanylin which has been digested away from the fusion protein. This indicates that although there was a 10 fold difference in the concentration of PreScission™ protease used in each digest, 0.2 units and 2 units, both digests were able to cleave proguanylin from the thyrodoxone fusion protein. The digest using 0.2 units of enzyme produced levels of product equivalent to the digest using 2 units of enzyme after 8 h. Irrespective of the concentration of PreScission™ protease enzyme used the 28 kDa proguanylin fusion protein sample was never completely digested. Either more enzyme is required or a digest longer than 24 h is required but due to time constraints this was not investigated.

7.3 Identification of endogenous guanylin-like peptides.

Three techniques were attempted to isolate endogenous guanylin-like peptides from the intestine of the European eel. The first technique used to isolate endogenous peptides from the eel intestine was a discontinuous sucrose gradient (explained in section 2.16.1). Two 12 % SDS gels using the protein samples collected from the interface between each layer of sucrose were run and transferred to PVDF membrane and as a control 1 μg and 10 μg samples of bacterially expressed prouroguanylin were included. Figure 7.8 (a) shows the results of a membrane hybridised with guanylin antibodies diluted 1 in 10 and figure 7.8 (b) shows the results of a membrane hybridised with uroguanylin antibodies diluted 1 in 10. Neither endogenous guanylin or uroguanylin were detectable using the current approaches but the uroguanylin antibodies did bind to the two control samples which indicated that the Western blot technique had worked.

In mammalian studies two techniques have been used to isolate endogenous guanylin-like peptides. Cetin *et al* (1994) boiled the samples in 1 M acetic acid for 10 min and Li *et al* (1997) homogenised the tissue samples in the presence of a cocktail of protease inhibitors. These methods described in section 2.16.2 were used to isolate endogenous guanylin-like peptides from the European eel. The Western blots using the proguanylin specific antiserum following treatment of eel intestine tissue with 1 M acetic acid are shown in figure 7.9 (a) and results following homogenisation of the intestinal tissue are shown in figure 7.9 (b). Unfortunately to date neither of these techniques have proved successful in isolating endogenous peptides from the European eel although attempts spiking with recombinant peptide did allow detection of peptide these methods were not effective in recovering native peptide from tissues.

7.4 Immunhistochemistry

Fixation in 4 % PFA and paraffin embedding procedures yielded good structural preservation shown in the phase contrast images, figures 7.10 a, c and e and 7.11 a, c and e. These tissues were used to determine the cellular location of guanylin-like peptides in the intestine of the European eel. In figures 7.10 and 7.11 the phase

contrast images are shown on the left and the corresponding immunofluoresence image is shown on the right. The control for the guanylin and uroguanylin antibodies using the pre-immune serum showed no positive immunoreactivity with only non-specific auto fluoresence being restricted to red blood cells (figures 7.10 (b) and 7.11 (b)). Using guanylin antibodies diluted 1:5 and 1:2 specific staining was observed in cells in the intestine of yellow eels adapted to FW which are probably goblet cells as they had the same structure as other goblet cells (figures 7.10 c and d and e and f). The staining was predominantly found at in the mucus of the goblet cells. These findings differ slightly from a study by Yuge *et al* (2003). Yuge *et al* (2003) found that guanylin was localised exclusively in some goblet cells but the signal was present in the cytoplasm of the goblet cells but absent in the mucus of these cells. Further research is required to completely comprehend the cellular location of guanylin in the intestine of the European eel.

Shown in figures 7.11 a – f are the results using uroguanylin antibodies on longitudinal sections from yellow eels adapted to FW. Unfortunately no specific staining was observed, only non-specific autofluoresence of the red blood cells. An antigen retrieval technique using 10 mM citrate buffer was attempted by my colleague Dr A-S Martinez but this method did not yield any specific fluoresence and further experiments are required to try and obtain specific fluoresence.

Due to delays in synthesising the renoguanylin antiserum it was not possible to carry out immunohistochemistry experiments for this peptide during this project.

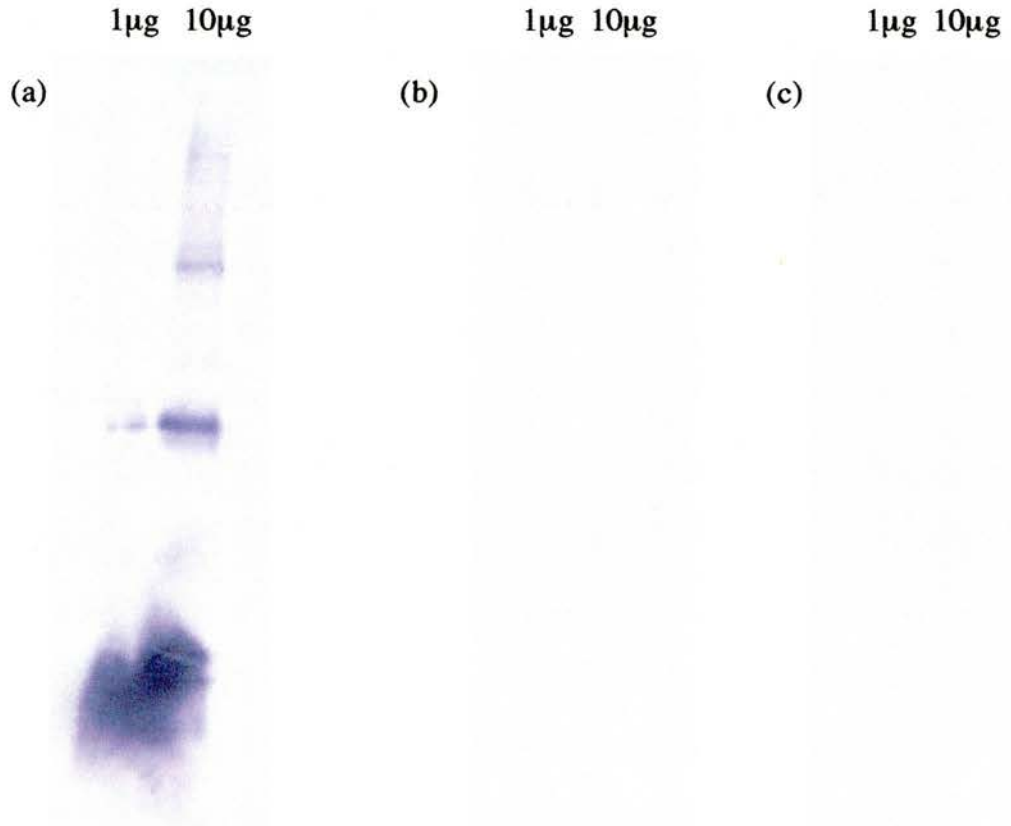


Fig. 7.1 Western blots where recombinant prouroguanylin (prepared as described in section 2.15) samples of 1 and 10 µg were probed with either (a) affinity purified prouroguanylin antisera (diluted 1:10) or (b) affinity purified proguanylin antisera (diluted 1:10). (c) recombinant proguanylin-Trx fusion protein 1 and 10 µg samples were probed with purified prouroguanylin antisera.

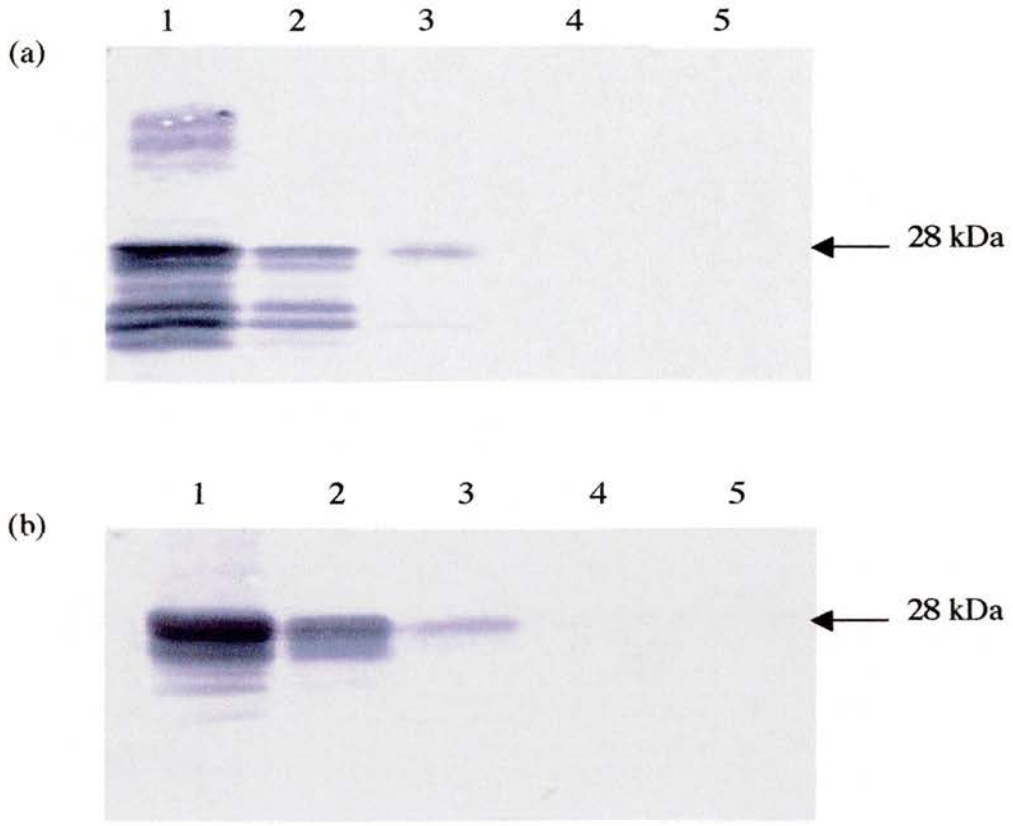


Fig. 7.2 A Western blot to determine the minimum protein concentration required for detection by the proguanylin and prouroguanylin antibodies.
(a) The membrane was incubated with proguanylin antibody diluted 1 in 100. The protein samples were serial dilutions of the proguanylin Trx fusion protein, lane 1 – 72.5 μg, 2 – 7.25 μg, 3 – 0.725 μg, 4 - 0.0725 μg and 5 – 7.25 ng.
(b) The membrane was hybridised with prouroguanylin antibody diluted 1 in 100. The protein samples were serial dilutions of the prouroguanylin Trx fusion protein, lane 1 – 40 μg, 2 – 4.0 μg, 3 – 0.4 μg, 4 - 0.04 μg and 5 – 4 ng.

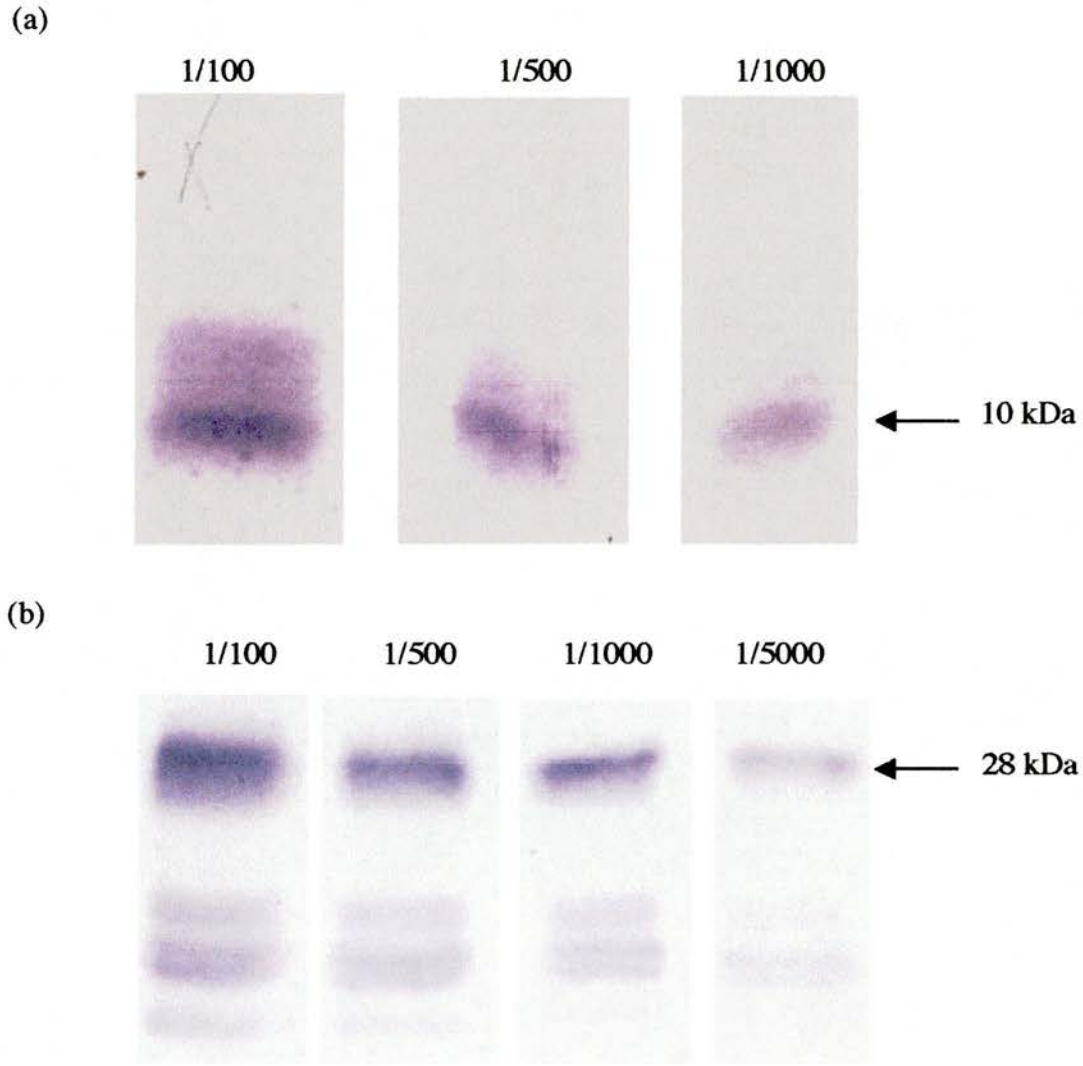


Fig. 7.3 (a) Western blots where 1 μg samples of recombinant prouroguanylin were probed with purified prouroguanylin antibody with final dilutions of 1/100, 1/500 and 1/1000.
(b) Western blots of 1 μg samples of $(His)_6$-tagged proguanylin-Trx fusion protein incubated with the proguanylin purified antibody with final dilutions of 1/100, 1/500, 1/1000 and 1/5000.

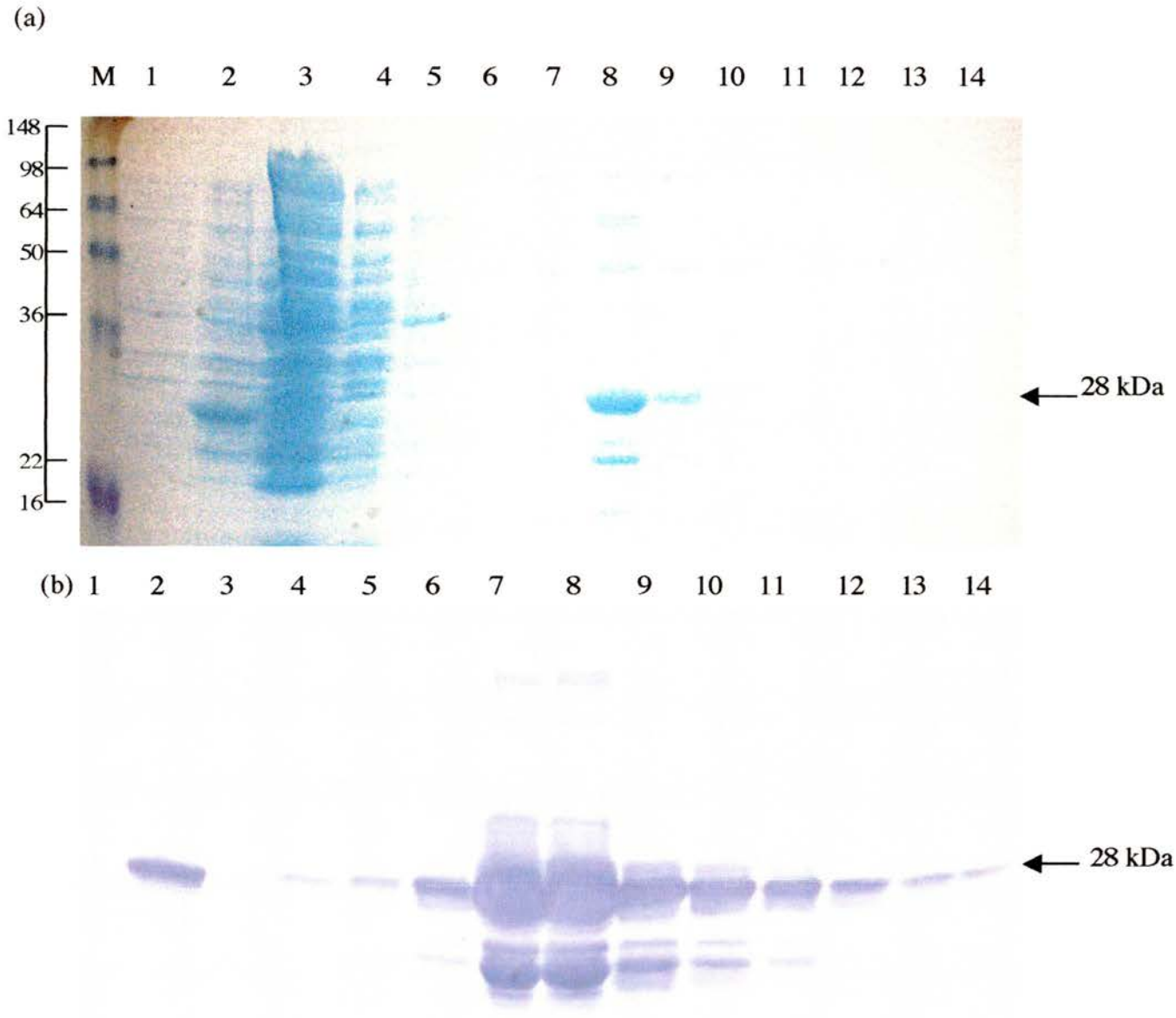


Fig. 7.4 (a) Coomassie Blue stained 12% SDS PAGE gel of proguanylin-Trx $(His)_6$ fusion protein isolated from Origami B cells. Lanes are: - M – Invitrogen See Blue® Plus2 Pre-stained standard, 1 – cells before protein induction with IPTG, 2 – cells after 2.5 h incubation with 1 mM IPTG, 3 – after total protein extraction from the bacteria (described in section 2.15.5), Lanes 4 – 14 are the fractions collected as the sample was added to a Ni^{2+} column, as described in section 2.15.6 (i). Lane 4 – flow through from the Ni^{2+} column, lane 5 – 10 mM imidazole wash of the Ni^{2+} column, lane 6 – 20 mM imidazole wash of the Ni^{2+} column, lanes 7 – 14 fractions 1 – 8 of the elution of the $(His)_6$-tagged proguanylin-Trx fusion protein from the Ni^{2+} column with 250 mM imidazole.

(b) Western blot of the proguanylin-Trx fusion protein isolated from Origami B cells probed with purified proguanylin antibody diluted 1 in 100. Lanes are: - 1 – cells before protein induction with IPTG, 2 – cells after 2.5 h incubation with 1 mM IPTG, Lanes 3 – 14 are the fractions collected as the sample was added to a Ni^{2+} column, as described in section 2.15.6 (i). Lane 3 – flow through from the Ni^{2+} column, lane 4 – 10 mM imidazole wash of the Ni^{2+} column, lane 5 – 20 mM imidazole wash of the Ni^{2+} column, lanes 6 – 14 fractions 1 – 9 of the elution of the $(His)_6$-tagged proguanylin-Trx fusion protein from the Ni^{2+} column with 250 mM imidazole. In the Western blot the 28 kDa proguanylin-Trx fusion protein was observed at higher levels because the antibodies were more sensitive than Coomassie blue stain.

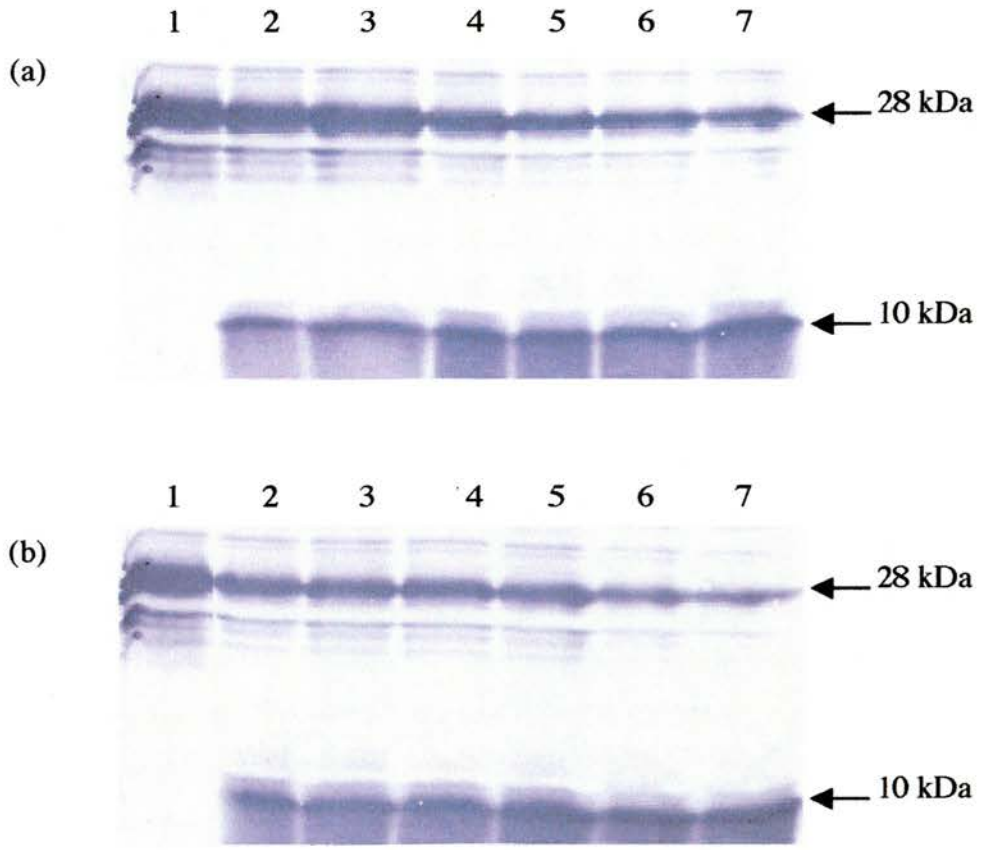


Fig 7.5 Western blot analysis using proguanylin antisera diluted 1:1000 to detect the 10 kDa proguanylin peptide after digestion of 50 _g of the proguanylin Trx fusion protein using (a) 0.2 units PreScission™ protease enzyme and (b) 2 units PreScission™ protease enzyme.
Lanes: - 1 – no enzyme, 2 – 1 h, 3 – 2 h, 4 – 4 h, 5 – 8 h, 6 – 12 h and 7 – 24 h incubation with the PreScission™ protease enzyme at 4 °C.

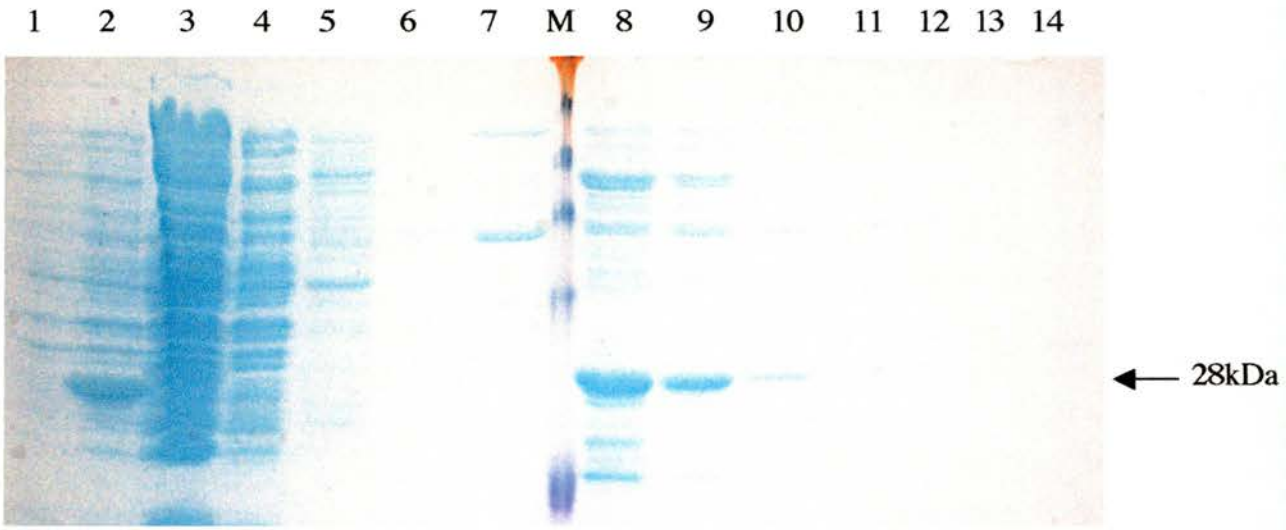


Fig. 7.6 A Coomassie Blue stained 12 % SDS PAGE gel of $(His)_6$-tagged prorenoguanylin-Trx fusion protein isolated from Origami B cells. M – Invitrogen See Blue® Plus2 Pre-stained standard. Lanes left to right are: - lane 1 – cells before protein induction with IPTG, lane 2 – cells after a 2.5 h incubation with 1 mM IPTG, lane 3 – after protein extraction from the bacteria (described in section 2.15.5), lanes 4 – 14 are the fractions collected as the sample was added to a Ni^{2+} column, as described in section 2.15.6 (i). Lane 4 – flow through from the Ni^{2+} column, lane 5 – 10 mM imidazole wash of the Ni^{2+} column, lane 6 – 20 mM imidazole wash of the Ni^{2+} column, lanes 7 – 14 fractions 1 – 8 of the elution of the $(His)_6$-tagged prorenoguanylin-Trx fusion protein from the Ni^{2+} column with 250 mM .

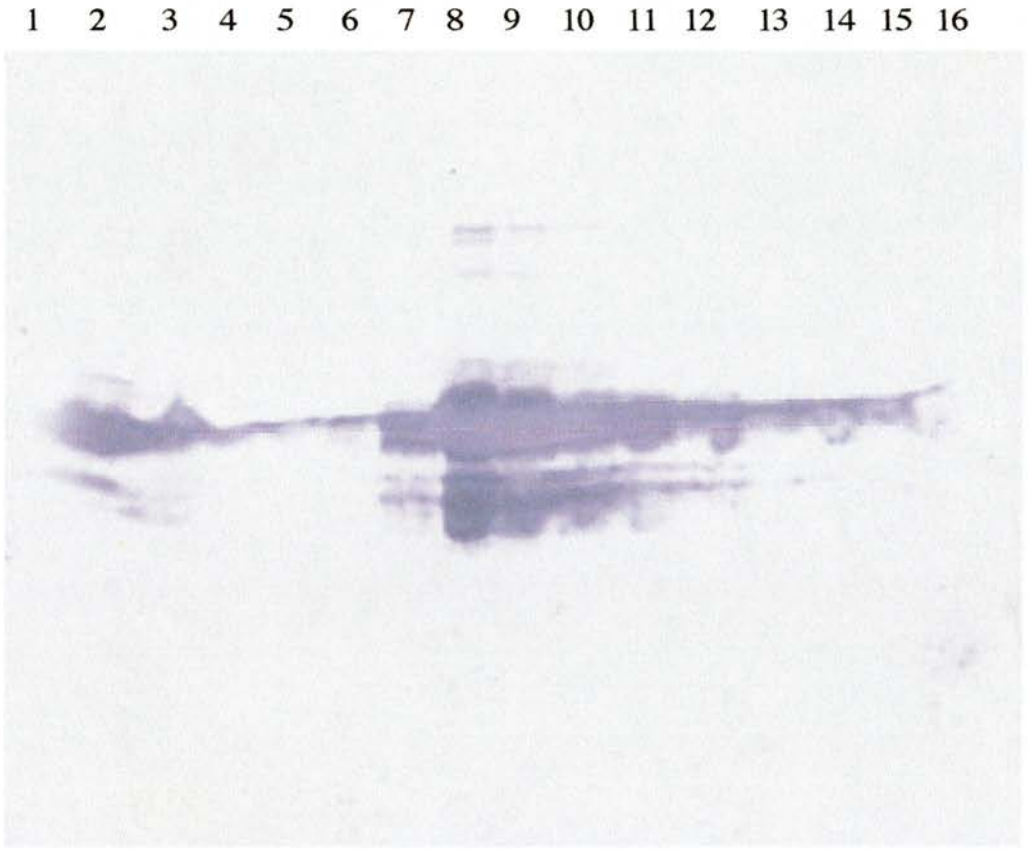


Fig. 7.7 Western blot of $(His)_6$-tagged prouroguanylin-Trx fusion protein isolated from Origami B cells incubated with a purified prouroguanylin antibody diluted 1 in 100. Lanes left to right are :- lane 1 – cells before protein induction with IPTG, lane 2 – cells after 2.5 h incubation with 1 mM IPTG, lane 3 – after protein extraction from bacteria (described in section 2.15.5), Lanes 4 – 14 are the fractions collected as the sample was added to a Ni^{2+} column, as described in section 2.15.6 (i). Lane 4 – flow through from the Ni^{2+} column, lane 5 – 10 mM imidazole wash of the Ni^{2+} column, 6 – 20 mM imidazole wash of the Ni^{2+} column, lanes 7 – 14 fractions 1 – 8 of the elution of the $(His)_6$-tagged prouroguanylin-Trx fusion protein from the Ni^{2+} column with 250 mM imidazole.

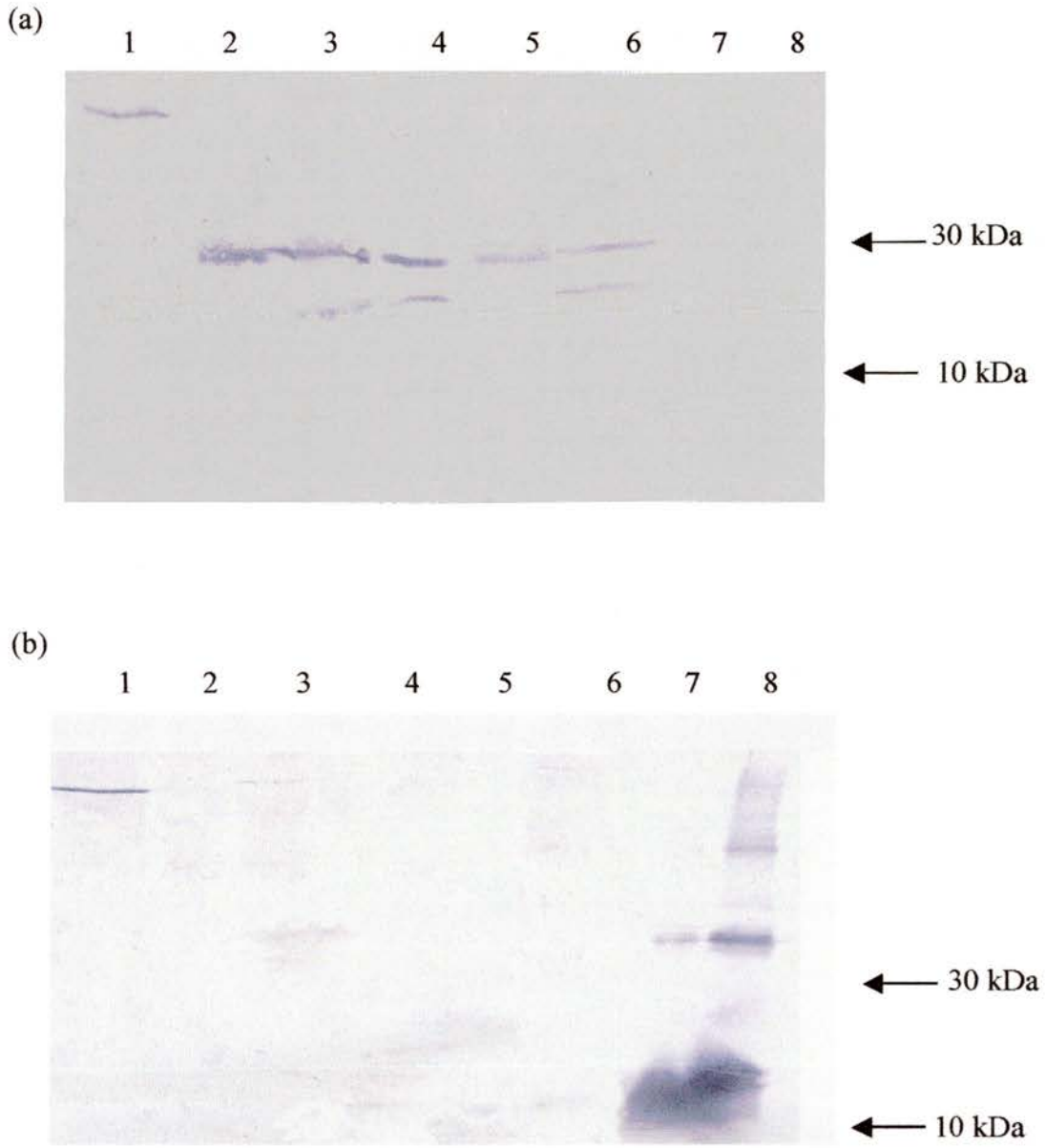


Fig. 7.8 (a) Western blots of extracted eel intestinal proteins probed with proguanylin antibodies and (b) with prouroguanylin antibodies. The samples in lanes 1 – 5 were 300 μg wet weight of the fractions obtained from each layer of a sucrose gradient used in the membrane preparation of the intestine from a silver SW eel. Lane 1 – soluble fraction, lane 2, 8 % - 40% interface fraction, lane 3, 40 % - 46% interface fraction, lane 4, 46 % - 50% interface fraction and lane 5, 50 % fraction. Lane 6 was 300 μg protein of the 40 % fraction boiled in 1M acetic acid. Lanes 7 and 8 are 1 μg and 10 μg of recombinant uroguanylin peptide, which acted as a control. The dilution of proguanylin and prouroguanylin antibodies was 1/10 and no bands of the expected molecular weight of 10 kDa were detected in any of the samples incubated with proguanylin antibodies. A 10 kDa fragment was only detected in the recombinant uroguanylin peptide samples incubated with prouroguanylin antibodies.

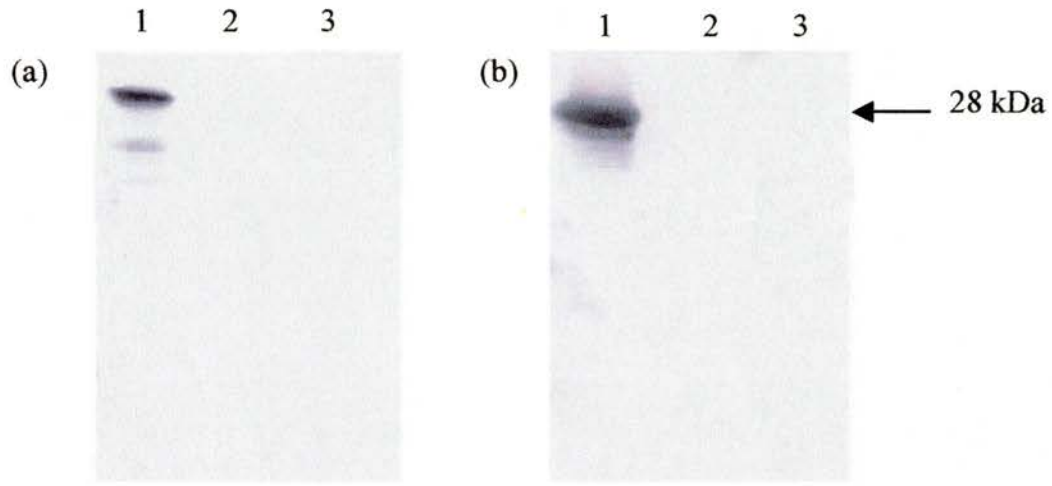


Fig 7.9 Western blots following the extraction of endogenous guanylin-like prohormones. The intestine from two silver SW eels was boiled and divided into two, one half was treated with acetic acid and the other half was homogenised using non-denaturing buffers (as described in section 2.16.2). (a) Lane 1 – 30 μg bacterial recombinant $(His)_6$-tagged proguanylin-Trx fusion protein, lane 2 – intestinal tissue extracted with 1 M acetic acid, lane 3 –intestinal tissue homogenised using non-denaturing buffers . The membrane was probed with proguanylin antibody diluted 1 in 1000. (b) Lane 1 – 30 μg bacterial recombinant $(His)_6$-tagged prouroguanylin-Trx fusion protein, lane 2 – intestinal tissue extracted with 1 M acetic acid, lane 3 – intestinal tissue homogenised in non-denaturing buffers. The membrane was probed with prouroguanylin antibody diluted 1 in 500.

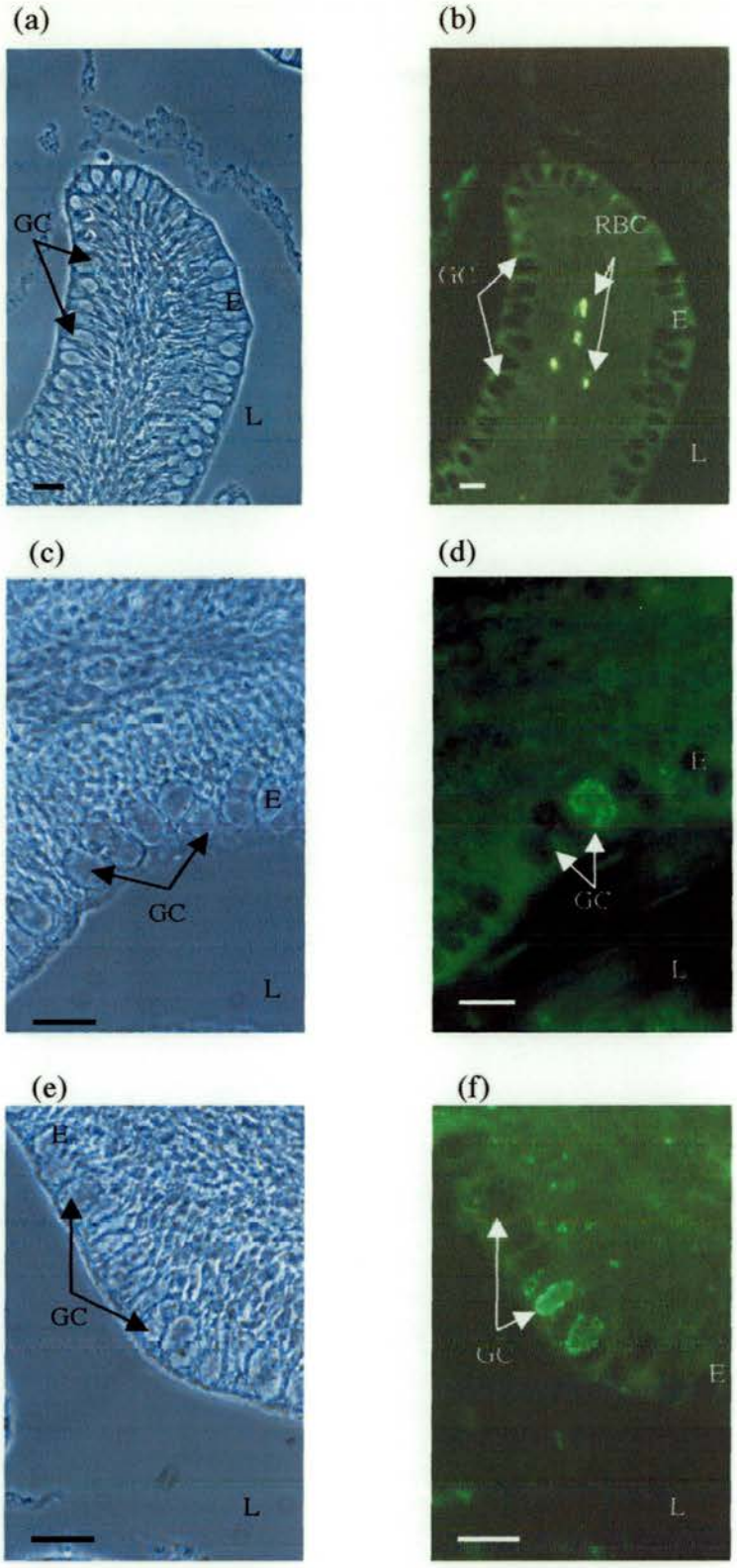


Fig. 7.10 Longitudinal intestinal sections from yellow eels adapted to FW. (a) and (b) incubated with guanylin pre-immune serum. (c) and (d) incubated with guanylin antiserum diluted 1:5, (e) and (f) incubated with guanylin antibodies diluted 1:2. E, epithelium; L, lumen; GC, goblet cell; RBC, red blood cell. Bars, 25μm.

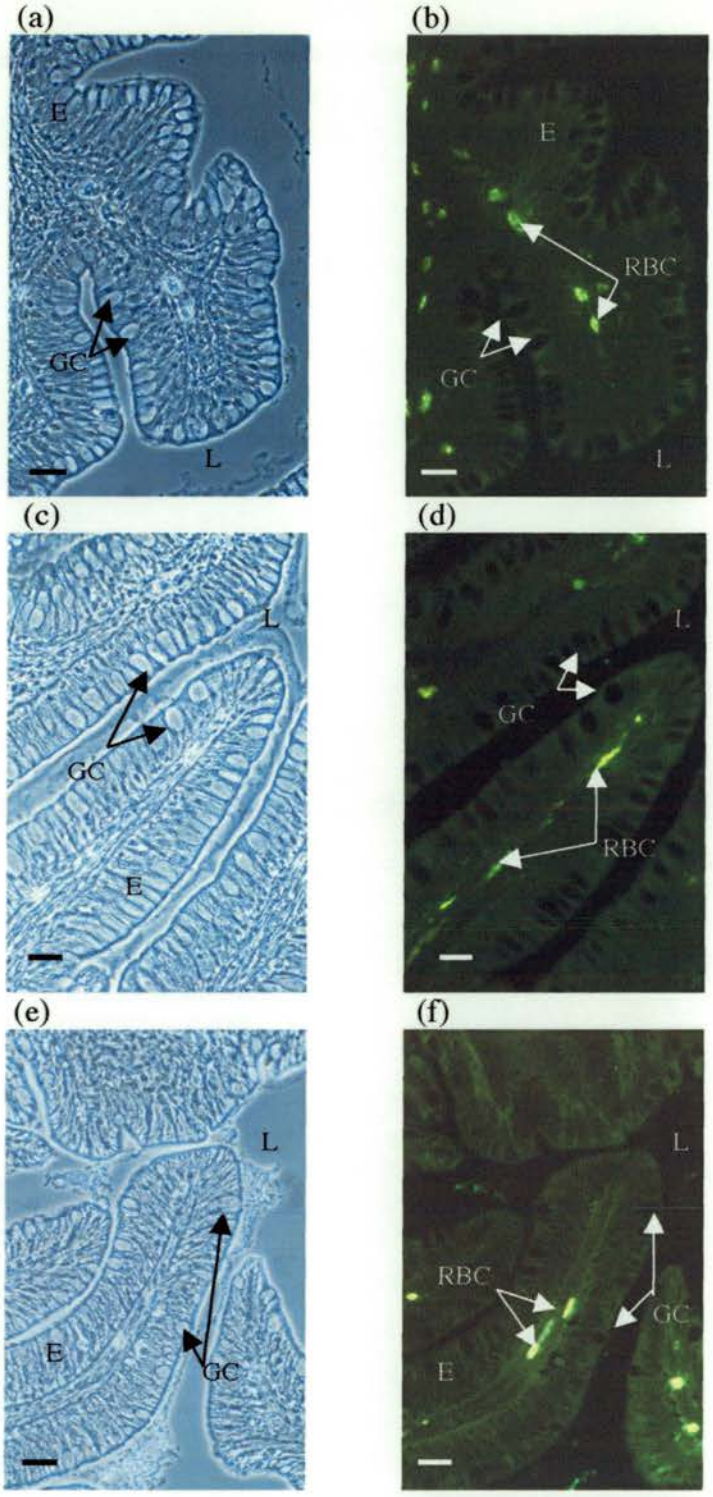


Fig. 7.11 Longitudinal intestinal sections from yellow eels adapted to FW. (a) and (b) incubated with uroguanylin pre-immune serum. (c) and (d) incubated with uroguanylin antiserum diluted 1:25 , (e) and (f) antigen retrieval technique using 10 mM citrate buffer pH 6.0 and incubated with uroguanylin antibodies diluted 1:25. E, epithelium; L, lumen; GC, goblet cell; RBC, red blood cell. Bars, 25μm.

Chapter 8 Discussion

Bacterial expression and immunohistochemistry of guanylin-like peptides.

8.1 Antibody preparation

Previous studies using antibodies raised to guanylin-like peptides (Nakazato *et al.*, 1998; Yuge *et al.*, 2003) have used the active peptide region as the antigen. Due to the high homology between guanylin, renoguanylin and uroguanylin within the active peptide region sequences within the prohormone were selected instead as this would decrease or even eliminate any cross reactivity between the antibodies. The amino acid sequences selected for antibody production were guanylin (ESVKQLKDLMDSDLC), renoguanylin (KLEELMGVDMTVKQC) and uroguanylin (MSTPHPPNLGSHAVC). The antibodies were used for Western blots and for immunohistochemistry in an attempt to determine the protein abundance and cellular location of guanylin-like peptides.

8.2 Preparation of recombinant proguanylin-like peptides

Lauber *et al.*, (2002) have successfully used the pET expression system to amplify human proguanylin and they have successfully purified this proguanylin. Using similar procedures (described in section 2.15) teleost proguanylin, prorenoguanylin and prouroguanylin were amplified and then purified. These recombinant peptides have been used to determine if the antibodies raised to proguanylin, prorenoguanylin and prouroguanylin were working and to ensure that these antibodies did not cross react with the other guanylin-like peptides. The three recombinant peptides can be used for future studies looking at the structure of guanylin-like peptides and these recombinant peptides can be used to investigate the physiological actions of the guanylin-like peptides. Studies looking at the structure of guanylin-like peptides may reveal where the cleavage sites are located within the prohormone to produce the active peptide and the enzymes present in the intestinal lumen which hydrolyse the prohormone may be identified.

8.3 Isolation of endogenous guanylin-like peptides from the eel intestine

Unfortunately attempts at isolating endogenous guanylin-like peptides have been unsuccessful. Three different techniques were attempted but no peptide corresponding to the size of proguanylin (10 kDa) was detected by SDS PAGE/ Western blotting. One of the techniques in which the intestinal tissue was boiled

in 1 M acetic acid was successfully used by Cetin *et al.* (1994) to isolate proguanylin from the guinea pig intestine. This study found that guanylin antisera revealed an immunoreactive band of 10 – 12 kDa in tissue from the large intestine. In a study by Li *et al.*, (1997) the mucosal layer was stripped free from the muscle layer of each tissue of rat intestine and homogenised in a buffer containing a cocktail of protease inhibitors such as aprotinin and leupeptin. Like the study by Cetin *et al.*, Li *et al.* detected a 10 kDa band in tissue taken from the colon using an antibody which had been raised against an amino terminal domain of the guanylin prohormone. This method was also used to extract intestinal tissue from eels and like the acetic acid treated samples no immunoreactive bands were detected in Western blots. This data does not correlate with the results of the Northern blots which indicate that substantial amounts of guanylin, renoguanylin and uroguanylin mRNA are expressed throughout the intestine of yellow and silver eels. It is possible that insufficient intestinal tissue was used for the extraction procedure. The mammalian studies do not specify how much tissue they had to use to enable immunodetection of the 10 kDa proguanylin band. Therefore the tissue from a number of yellow or silver eels may need to be pooled to enable isolation of endogenous proguanylin-like peptides from the European eel. Another possible explanation for the lack of endogenous guanylin-like peptides is that they are being lost during the extraction procedure.

8.4 Cellular location of guanylin-like peptides

In situ hybridisation and immunohistochemical studies in mammals (Nakazato *et al.*, 1998; Forte *et al.*, 2000[b]) indicate that uroguanylin is expressed in enterochromaffin cells, which are tall, flask-shaped cells shaped cells (Nakazato *et al.*, 1998). Enterochromaffin cells are the most abundant type of entero-endocrine cell and these cells are widely distributed throughout the intestine and are known to secrete serotonin and other peptides into the lumen of the intestine Forte *et al.*, 2000[b]). In rats the enterochromaffin cells, which were immunoreactive for uroguanylin, were predominately found in the midvilli of the small intestine along the crypt villus axis (Nakazato *et al.*, 1998). Unfortunately in the European eel the cellular location of uroguanylin has not been elucidated. Different dilutions of the specific eel uroguanylin antibodies have been tried and

a number of antigen retrieval techniques included in the procedures but only non-specific fluorescence of the red blood cells was observed. There may be a number of reasons why these antibodies which have been shown to specifically identify the recombinant prouroguanylin protein have failed to detect the specific antigen. This study on the European eel used antibodies which had been raised to a N terminal region of prouroguanylin (fig. 2.7) whereas the study by Nakazato *et al.*, (1998) on the rat intestine used three different uroguanylin antibodies with only one being successful. Two of the antibodies that had been produced, had been raised to N terminal regions of prouroguanylin and the third antibody had been raised to the 15 amino acid active peptide region. Specific immunofluoresence was only found using the antibody raised to the active peptide region, whereas the other two antibodies displayed indistinguishable staining patterns. Nakazato *et al.*, (1998) suggested that the two antibodies may not have worked due to the tertiary structure of prouroguanylin which may have prevented the antibodies from binding to the antigenic peptide epitope. This may also be the reason why the uroguanylin antibody has been unsuccessful in immunohistochemistry with tissues taken from the eel. As yet there is no information regarding the structure and folding of prouroguanylin. Now that prouroguanylin has been produced via bacterial expression a clearer picture of the structure of uroguanylin may emerge and this would enable antibodies to be raised which would bind to prouroguanylin and therefore the cellular location of uroguanylin in the European eel may be revealed.

Using the eel specific guanylin antibodies, fluorescence was observed in the apical region of some cells which appear to be goblet cells, a cell type important for secreting mucus in the intestine. Like this study, a study on *Anguilla japonica* (Yuge *et al.*, 2003) also found that guanylin was expressed in some but not all goblet cells. Mammalian studies have also found immunostaining of guanylin in goblet cells (Cohen *et al.*, 1995; Forte *et al.*, 2000[b]) and Cohen *et al.*, (1995) have suggested that because guanylin is localised to mucin-secreting cells then the secretion of mucus and the release of guanylin may be co-regulated. Cohen *et al.*, (1995) found that in the rat, like *Anguilla anguilla* and *Anguilla japonica,* only some of the goblet cells were immunoreactive to guanylin. This raises the question, why do some goblet cells and not others secrete guanylin? In general

there are biochemically distinct populations of goblet cells and each population of cells produces a different type of mucin and other secretory products (Yuge *et al.*, 2003). Therefore it is possible that guanylin is only secreted by certain types of goblet cells which would explain why in these mammalian and teleost studies only some goblet cells are immunoreactive to guanylin. There is still a discrepancy about where the exact location of guanylin is within the goblet cells. This study on *Anguilla anguilla* found fluorescence of the guanylin antibodies within asymmetrical vesicles found mainly at the apical region of the goblet cells. This would seem a logical place to find proguanylin as it is known that proguanylin is secreted into the intestinal lumen where it is hydrolysed by as yet unknown protease or proteases to form the biologically active guanylin peptide. In the study by Yuge *et al.*, (2003) on *Anguilla japonica,* the fluorescence of guanylin was found within the cytoplasm at the centre of the goblet cells, not in the apical region of the cell. It is plausible that proguanylin is stored within the cytoplasm of the goblet cells and then moves towards the apical side of the goblet cell prior to secretion. If this was correct then why do you not see fluorescence in all regions of the goblet cell? Further research is required to establish exactly where guanylin is located within the goblet cell and future work may also determine which type of goblet cells secrete guanylin.

Chapter 9 Results

The cloning and expression of Antisecretory Factor in *A. anguilla.*

9.1 RT-PCR amplification of AF from eel brain cDNA

The reverse-transcriptase polymerase chain reaction was used to amplify an antisecretory factor (AF) DNA homologue from mRNA isolated from the brain of yellow SW eels (section 2.5). The degenerate primers, AF sense and antisense (Appendix 2) were designed using the amino acid sequence information published in the gene bank for human and rat AF. The amplified products were electrophoresed on a 1% agarose gel (section 2.9) and a band of the expected size of 1 kb was observed (figure 9.1). The fragment was purified, cloned and sequenced as described in sections 2.10, 2.11 and 2.13 respectively.

9.2 RACE reactions

Following cloning and sequencing of the initial 1 kb cDNA fragment (figure 9.1) the nucleotide information was used to synthesise specific sense and antisense primers to be used in nested 3' and 5' RACE (rapid amplification of cDNA ends) reactions (Appendix 2). Details of the Clontech Marathon™ 3' and 5' RACE reactions are detailed in section 2.8. Figures 9.2 and 9.3 are examples of 3' RACE fragment and 5' RACE fragments of AF respectively. The 3' RACE product was approximately 800 bp and the fragment amplified from the 5' RACE nested reaction was approximately 300 bp. The 3' and 5' RACE products were purified, cloned and sequenced.

9.3 Tissue distribution of AF

The RT-PCR technique using degenerate eel AF sense and antisense primers (Appendix 2) was used to determine whether AF may be expressed in osmoregulatory tissues as well as the brain. Figure 9.4 shows the results of RT-PCR amplifications from mRNA isolated from brain, intestine, gill and kidney from SW-acclimated eels followed by electrophoresis on an ethidium bromide containing 1% agarose gel. The expected 1 kb fragment was only visible when using brain cDNA as a template. The same results (not shown) were obtained following RT-PCR using mRNA isolated from the brain, gill, kidney and intestine form FW-acclimated eels. The cDNA used from all of the tissues was viable, as it had been tested with degenerate primers designed to amplify the alpha subunits of members of the P-type ATPase family such as the Na^+,K^+

ATPase α subunit. This result suggests that AF is only expressed at significant levels in the brain and not the major osmoregulatory tissues.

Northern blot analysis was attempted to quantitatively determine the mRNA expression levels of AF expression. Unfortunately levels of AF expression were too low for detection and real time PCR experiments are required to ascertain the level of AF expression.

9.4 Sequencing of AF

Sequencing reactions (section 2.13) were carried out in both sense and antisense directions using the vector primers T3 and T7 (Appendix 2). The final collated nucleotide and putative amino acid interleaved sequences of AF from the European eel are shown in figure 9.5. The amino acid sequence of AF from the European eel was compared with two mammalian species; human and rat in figure 9.6. AF is a highly conserved protein; there is 82% homology between the amino acid sequence of AF from human, rat and the European eel. A sequence of amino acids VCHSKTR (amino acids 35 – 42) required for biological activity of AF (Johansson *et al.*, 1997[a]) are conserved in all of the species and this region is highlighted in figure 9.6.

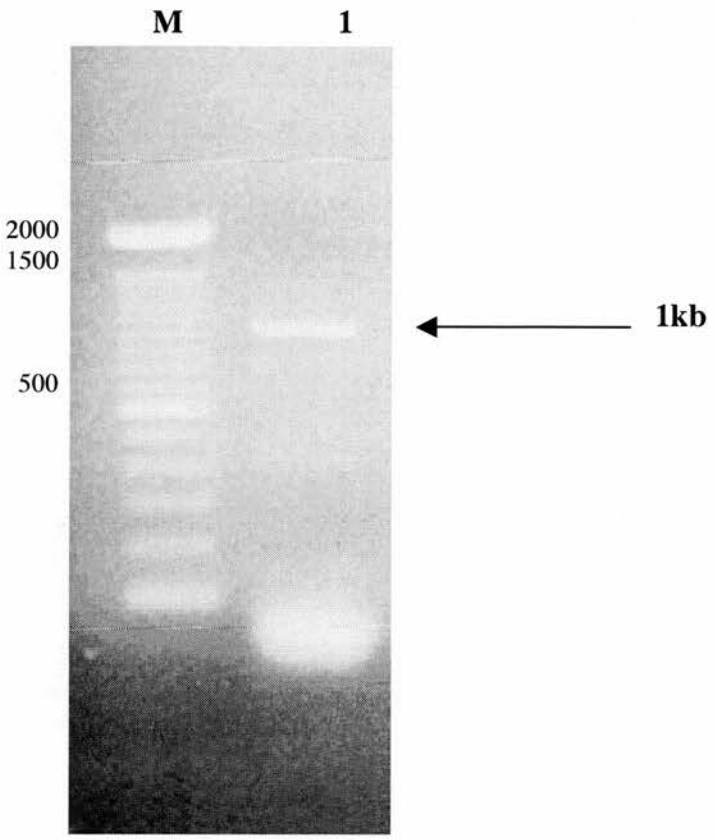


Fig. 9.1 Results of RT-PCR using degenerate antisecretory factor primers (Appendix 2) and cDNA synthesised from mRNA extracted from brain tissue of SW-acclimated eels. M indicates the 100 bp DNA step ladder (NEB) and lane 1 shows the fragment amplified from the PCR reaction.

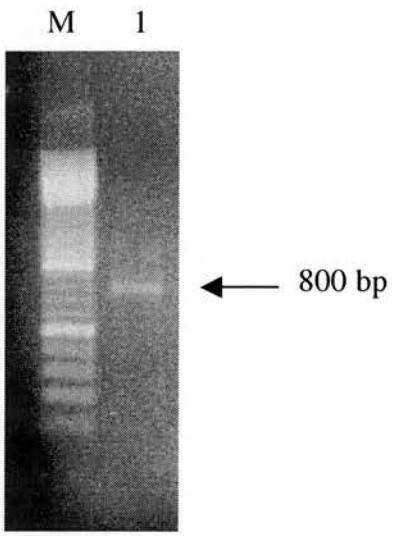


Fig. 9.2. Results of 3' RACE-PCR using Marathon™ cDNA synthesised from the brain of SW-acclimated eels after amplification with eel specific AF 3'RACE primer 1 (Appendix 2) and the kit AP1 primer then eel specific AF 3'RACE primer 2 and the kit AP2 primer (Appendix 2). A fragment of 800 bp was amplified which was cloned and sequenced.

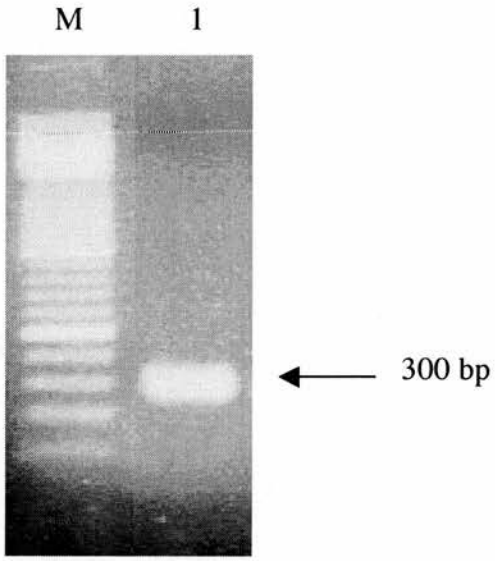


Fig. 9.3 Results of 5' RACE-PCR using Marathon™ cDNA synthesised from the brain of SW-acclimated eels after amplification with eel specific AF 5'RACE primer 1 and the kit AP1 primer (Appendix 2) then eel specific AF 5' RACE primer 2 and the kit AP2 primer (Appendix 2). A fragment of 300 bp was amplified which was cloned and sequenced.

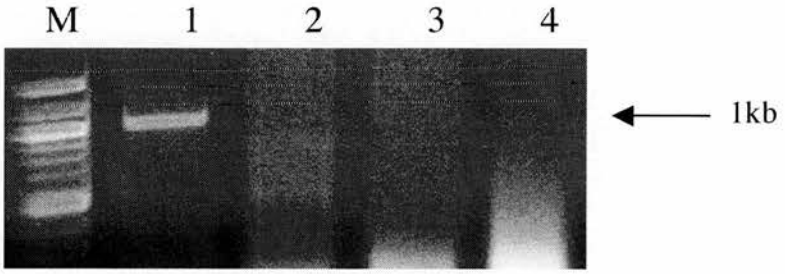


Fig. 9.4. Results of PCR using degenerate AF primers and cDNA from various tissues from SW-acclimated eels; M indicates the 100 bp marker, lane 1 – brain, 2 – gill, 3 – kidney, 4 – intestine.

```
  1                                                                                                                  TGTAGTTACCAATCTCTGTGAGCGGTAGGGGCCTTTGCAAAC
    Met Val Leu Glu Ser Thr Met Val Cys Val Asp Asn Ser Glu Tyr Met Arg Asn Gly Asp Phe Leu Pro Thr Arg Leu Gln Ala Gln Gln     30
 43 ATG GTA CTC GAA AGT ACT ATG GTC TGT GTG GAC AAC AGT GAG TAC ATG CGG AAT GGA GAC TTC TTG CCC ACA AGA TTG CAA GCC CAG CAG
    Asp Ala Val Asn Ile Val Cys His Ser Lys Thr Arg Ser Asn Pro Glu Asn Asn Val Gly Leu Ile Thr Met Ala Asn Asn Cys Glu Val     60
133 GAC GCT GTC AAC ATT GTA TGT CAC TCC AAG ACC CGC AGC AAC CCT GAG AAC AAC GTG GGC CTA ATC ACA ATG GCA AAT AAC TGT GAA GTG
    Leu Thr Thr Leu Thr Pro Asp Thr Gly Arg Ile Leu Ser Lys Leu His Ala Ile Gln Leu Arg Gly Thr Ile Ser Phe Cys Thr Gly Ile     90
223 CTC ACT ACG TTG ACC CCT GAC ACG GGG CGG ATC CTC TCC AAA CTG CAC GCT ATC CAG CTG CGT GGC ACC ATC AGC TTC TGC ACA GGC ATT
    Arg Val Ala His Leu Ala Leu Lys His Arg Gln Gly Lys Asn His Lys Met Arg Ile Ile Ala Phe Val Gly Ser Pro Val Glu Asp Asn    120
313 AGG GTG GCG CAC CTG GCT TTG AAA CAC AGG CAG GGG AAG AAC CAC AAG ATG CGC ATC ATT GCG TTC GTG GGA AGT CCA GTG GAG GAT AAC
    Glu Lys Asp Leu Val Lys Met Ala Lys Arg Leu Lys Lys Glu Lys Val Ser Val Asp Val Ile Asn Phe Gly Glu Glu Glu Arg Asn Thr    150
403 GAG AAG GAT CTG GTT AAA ATG GCC AAA CGG TTG AAG AAA GAG AAA GTC AGT GTG GAC GTA ATC AAC TTC GGT GAA GAG GAG AGG AAC ACA
    Glu Lys Leu Thr Ala Phe Val Asn Ala Leu Asn Gly Lys Glu Gly Gly Gly Ser His Leu Val Thr Val Ser Pro Gly Pro Ser Leu Ala    180
493 GAG AAG CTG ACT GCT TTT GTT AAC GCC CTG AAT GGG AAG GAG GGC GGA GGC TCC CAC CTG GTG ACC GTG TCT CCG GGC CCG AGC CTG GCC
    Asp Ala Leu Leu Ser Ser Pro Val Leu Ala Gly Glu Gly Gly Ser Val Leu Gly Leu Gly Ala Asn Asp Phe Glu Phe Gly Val Asp Pro    210
583 GAC GCC CTG CTG TCC TCT CCT GTC CTG GCT GGG GAG GGA GGC TCT GTG CTG GGC CTG GGG GCC AAC GAC TTT GAG TTT GGA GTC GAC CCC
    Ser Ala Asp Pro Glu Leu Ala Leu Ala Leu Arg Val Ser Met Glu Glu Gln Arg Gln Arg Gln Glu Glu Glu Ala Arg Arg Ala Ala Val    240
673 AGT GCG GAC CCC GAG CTA GCA CTG GCG CTG CGC GTG TCC ATG GAG GAG CAG AGA CAG CGT CAG GAA GAG GAG GCG CGC AGG GCA GCT GTT
    Val Ser Thr Gly Asp Ala Ser Val Pro Thr Pro Thr Gly Asp Glu Ser Asp Glu Ala Leu Leu Gln Met Ser Val Ala Gln Pro Glu Thr    270
763 GTG TCT ACA GGA GAC GCC AGC GTC CCC ACT CCC ACC GGA GAC GAG TCC GAC GAG GCC CTG CTG CAG ATG TCG GTG GCA CAG CCG GAG ACG
    Gly Ser Pro Gly Leu Pro Asp Phe Ser Arg Met Thr Glu Asp Glu Gln Ile Ala Tyr Ala Met Gln Met Ser Leu Gln Gly Ala Gly Gly    300
853 GGC TCC CCA GGC CTG CCG GAC TTC AGC CGG ATG ACA GAG GAC GAG CAG ATT GCC TAT GCC ATG CAG ATG TCC CTG CAG GGG GCT GGT GGT
    Glu Tyr Gly Glu Ala Glu Ser Leu Asp Met Asp Thr Gly Ala Pro Met Glu Thr Ala Glu Ser Val Val Lys Glu Glu Asp Asp Tyr Asp    330
943 GAG TAT GGT GAA GCT GAG TCT TTG GAC ATG GAC ACT GGG GCC CCC ATG GAG ACT GCA GAA TCT GTT GTT AAG GAG GAA GAC GAT TAC GAC
    Met Gln Asp Pro Glu Phe Leu Gln Ser Val Leu Glu Asn Leu Pro Gly Val Asp Pro Asn Asn Glu Ala Ile Arg Asn Ala Met Gly Ser    360
1033 ATG CAG GAT CCG GAG TTC CTG CAA AGC GTC TTG GAA AAC CTG CCA GGC GTG GAT CCG AAC AAC GAA GCC ATT CGC AAC GCC ATG GGC TCC
    Leu Ala Ser Gln Thr Gly Ser Lys Pro Pro Glu Asp Lys Arg Asp Glu Glu Lys Lys Lys Stop                                    380
1123 CTG GCT TCA CAG ACA GGC TCC AAG CCA CCT GAG GAC AAG AGG GAT GAG GAG AAG AAA AAA TGA GACTTGCACTGAGACCTCACTAACTGACAATTGGA
1221 CCAGCACACATTTAAAACCTGAAACAAACATGATCTCATGACGCACACATGCACGTGCACACACACACACACACACACACACACACACATTTTGTTGTAGGTAATTCTGATCATGACTG
1340 ATTGATGACCTCAACGAAAACGTGTAAACACACAAACGCGTTCCCCCTTATGTACTACGTATCTGCCTTGTTTTTTCTTTTCTTTTTTTTGCATTGCCATTAAAAAAATGAACAAAAAA
1459 AAAAAAAAAAAAAAA
```

Fig 9.5 The interleaved nucleotide and derived amino acid sequences of antisecretory factor from *Anguilla anguilla*.

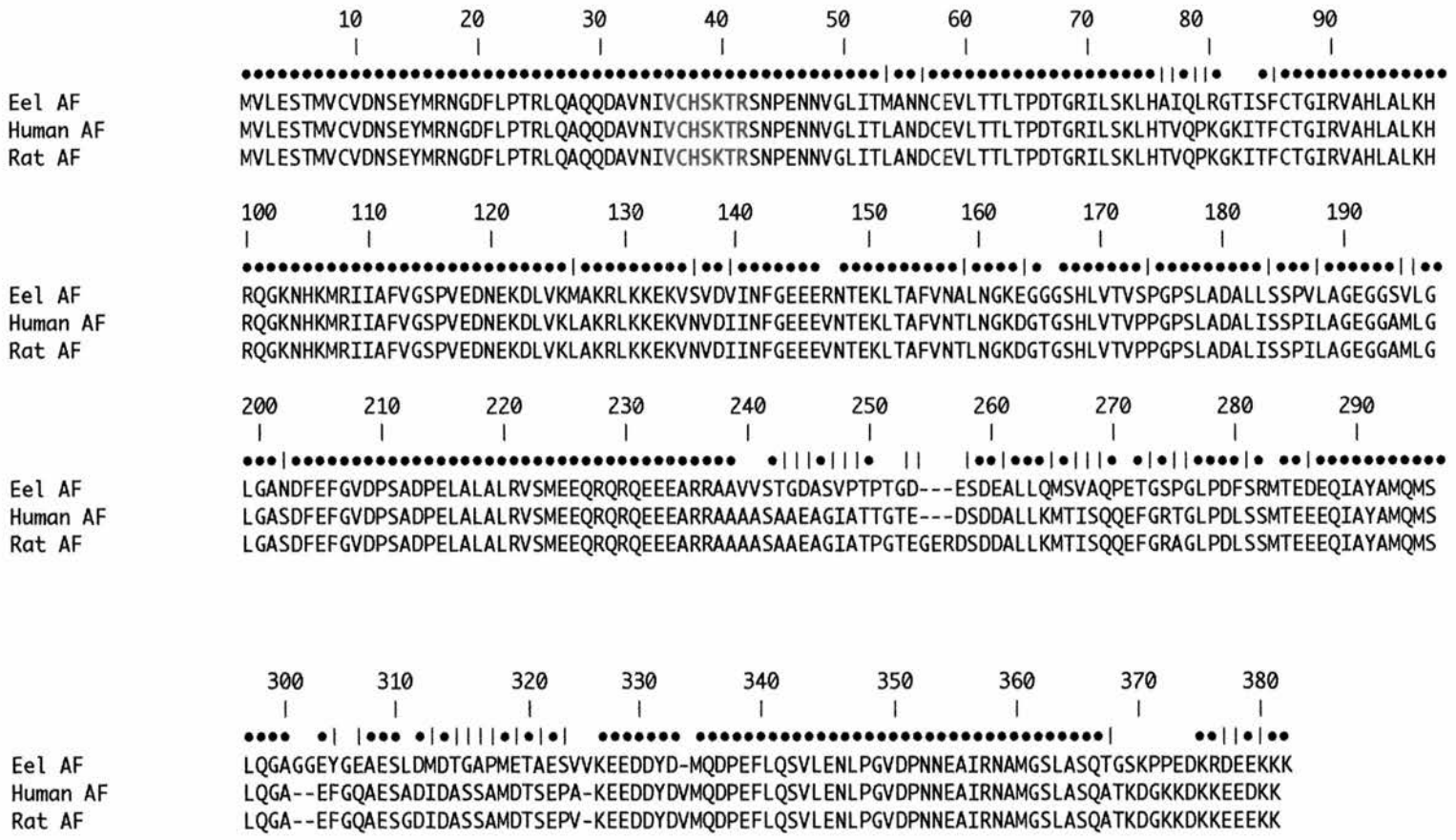


Fig. 9.6 An alignment of the derived amino acid sequence of eel antisecretory factor (AF) protein compared with AF in humans and rats. Symbols in the alignment are as follows: • conserved amino acid, | amino acid with conserved similarity and – indicates spaces introduced to optimise alignment. The amino acids highlighted in blue are essential for the biological activity of AF.

Chapter 10 Discussion

The cloning and expression of Antisecretory Factor in *A. anguilla*.

10.1 Antisecretory Factor

In 1984, Lönnroth and Lange identified a pituitary protein which antagonised the effect of cholera toxin within the intestine and called this protein antisecretory factor (AF). AF is a 41kDa, heat labile protein which is synthesised and stored in the pituitary gland (Björck *et al.*, 2000). Studies have found that AF, like other proteins such as neuropeptide Y, somatostatin and enkephalins, increases the absorption of fluid and electrolytes in the gut (Johansson *et al.*, 1997[a]). In mammals AF has been found to counteract the hypersecretion of electrolytes and water in the intestine induced by noxious bacteria such as *Vibrio cholerae* which produce and secrete cholera toxin (Hansen and Skadhauge, 1995). It is known that AF is secreted from the pituitary gland then transported via the bloodstream to the intestine, but the exact mechanism of action of AF is unknown but a number of possibilities have been suggested. From experiments using isolated cell membranes from Dieters nerve cells it was found that AF blocked ion channels including those channels activated by GABA (Lönnroth and Lange, 1986). Further evidence that the actions of AF may involve neuronal structures comes from a study by Grondahl *et al*, (2002). They carried out *in vitro* Ussing chamber experiments using porcine intestinal tissue stripped of the smooth muscle and they found that the effects of AF lay beyond the surface epithelium and involved neuronal structures in the mucosa. These and other studies in mammals have led to the idea that AF may act on the efferent neuromotor neurones since intestinal fluid secretion is regulated by the enteric nervous system (Johansson *et al.*, 1997[b]) but further research is required to completely understand the mechanism of action of AF.

Unlike mammals, who only experience extreme loss of fluid and ions due to the activity of noxious toxins, teleost fish experience changes in fluid and ion loss on a frequent basis. Euryhaline teleosts such as *A. anguilla* inhabit both FW and SW environments. In SW the fish lose water and gain ions but in FW the fish lose ions and gain water. Irrespective of the external environment *A. anguilla* maintain the plasma osmolality around 350 $mOsmkg^{-1}$ and this is achieved by the actions of an array of hormones. This study looked at the possibility that AF may

have a role in osmoregulation in the known osmoregulatory tissues, the gills, the gut and the kidney.

Initial RT-PCR experiments using cDNA synthesised from RNA extracted from the brain of yellow SW eels confirmed that AF is expressed in teleost fish. In mammals AF is formed in the pituitary gland (Johansson *et al.*, 1995) but it was not possible to determine exactly which region of the brain expressed AF in *A. anguilla* due to the small amount of tissue available from the fish. The fragment amplified from the RT-PCR experiments was cloned and sequenced. The nucleotide sequence contained a large open reading frame and like AF from humans (Johansson *et al.*, 1995) this reading frame coded for a 382 amino acid residue protein. The amino acid sequence of teleost AF was compared with the amino acid sequence of AF from humans and rats and there was an 82% amino acid homology between these species. Johansson *et al.*, (1995) have found that human AF is acidic and concluded that this is due to the high number of acidic residues present in the middle section of the amino acid sequence. Teleost AF is also acidic with a pI of 4.4 and like human AF also has a high number of acidic amino acids in the middle region of the protein.

In mammals AF acts to inhibit excess secretion of fluid and ions in the intestine. Other peptides such as somatostatin also inhibit this excess secretion in the intestine and studies have found that the biological activity of pro-somatostatin resides in a short sequence of amino acids. A study by Johansson *et al.*, (1997[a]) looked at whether AF was stored in the pituitary gland as a larger prohormone then cleaved into a smaller biologically active peptide like pro-somatostatin, which is cleaved into the 14 amino acid (or 22 amino acids in the gut) somatostatin. Johansson *et al.*, (1997[a]) found that when AF was treated with trypsin the ability of AF to inhibit the actions of cholera toxin increased two fold. This indicated that the activity of AF resided in a smaller fragment. After testing a number of truncated AF fragments this study found that only AF fragments containing the sequence VCHSKTR (amino acids 36 – 42) were biologically active and capable of inhibiting the actions of cholera toxin. This sequence of seven amino acids has been conserved in AF from *A. anguilla* and suggests that like mammalian AF this run of amino acids is important for a biologically active

AF protein. Further study is required to conclude if teleost AF is cleaved into a smaller biologically fragment which includes the aforementioned sequence of VCHSKTR.

In mammalian (rat and human) AF there are only four cysteine residues and these amino acids are all situated at the N terminal within the first 90 amino acids of the protein. These four cysteine residues are also present at the same positions in teleost AF. To date, the exact length of the putative biologically active form of the AF peptide has not yet been elucidated but it may incorporate all of these cysteine residues and these cysteine residues may form intramolecular disulphide bonds which may be important for the correct folding of the peptide and for biological activity of AF.

Having confirmed that AF is expressed by *A. anguilla* further RT-PCR experiments were carried out using cDNA derived from yellow SW brain, gill, kidney and intestine to determine if AF was located in any of these important osmoregulatory tissues in addition to the brain. Only a fragment of approximately 1 kb, which corresponded to the expected size of AF, was amplified from the brain cDNA. In the rat, Northern blot analysis suggested that AF mRNA was expressed in the intestinal mucosa as well as in the brain (Tateishi *et al.*, 1999). Attempts to use Northern blot analysis to determine which tissues expressed AF in teleosts were carried out for this study but the results were inconclusive (data not shown). If further experiments confirm that AF is only expressed in the brain then it suggests that AF is transported via the bloodstream to the intestine where it acts to inhibit the secretion of fluid and ions. Due to time constraints this project was unable to determine whether AF plays a role in osmoregulation.

In addition to determining if AF is involved in osmoregulation and trying to understand the mechanism of action of AF, future studies may be able to find answers to the idea that AF may be affecting the activity of other hormones involved in osmoregulation such as guanylin. If, as studies from mammals suggest, AF is acting in the intestine to prevent excessive fluid and ion secretion into the intestinal lumen then the function of AF may antagonise the actions of guanylin-like peptides. The members of the guanylin family are known to bind to

a GC-C receptor, located on the apical membrane of cells in the intestine. Activation of the GC-C receptor initiates a series of steps which results in the secretion of chloride ions and subsequently sodium ions and water into the intestinal lumen. Therefore AF may act to prevent guanylin from causing an excessive loss of water and ions in the intestine.

Chapter 11 Final conclusions

11 Final conclusions

In euryhaline teleosts, mechanisms are in place in the gill, intestine and kidney which help to regulate plasma fluid and ion levels and to maintain plasma osmolality at approximately 350 $mOsmkg^{-1}$ irrespective of the salinity of the external environment. Using RT-PCR, cloning and sequencing techniques, this study has shown that the European eel expresses three members of the guanylin peptide family termed guanylin, renoguanylin and uroguanylin. Similar techniques have shown that a number of other teleost species also express guanylin and/or uroguanylin peptides and it will be interesting to see, from future studies, whether all three guanylin-like peptides are found in both stenohaline and euryhaline fish. From Northern blots it is known that the mRNAs for guanylin, renoguanylin and uroguanylin are expressed in the intestine and renoguanylin and uroguanylin are also expressed in the kidney. No guanylin-like peptides were found to be expressed in the gills.

Experiments were carried out which investigated the effect of SW-acclimation on the expression of the three guanylin-like peptide mRNAs in the intestine of yellow, over a period of 7 days and silver eels over a period of 140 days. In yellow eels only mRNA for uroguanylin increased after 7 days in SW. In silver eels there was a transient increase in mRNA levels of renoguanylin after 2 days, significant increases in guanylin mRNA expression after 2 days and 140 days but of these three peptides it was the mRNA expression of uroguanylin that underwent the most significant increase following acclimation to SW. These findings suggested that uroguanylin and to a lesser extent guanylin was involved in any changes associated with FW/SW acclimation

The mRNA levels of guanylin are relatively high compared to other known transcripts (e.g. Na^+, K^+ ATPase α and β subunits) suggesting that there should be a substantial amount of proguanylin within cells in the intestine (Pers. comm. G Cramb). This data was not supported by the Western blots, possibly because the extraction procedure is not optimised for the eel and the guanylin-like peptides are being lost at some stage or indeed the antisera determinants are degraded. Therefore to date, the abundance of proguanylin protein within the intestine is unknown. From the preliminary immunohistochemistry data immunofluoresence suggests that proguanylin is only found in a few goblet cells

in the intestine. Cetin *et al.*, (1994) have found similar results in rats. They found that high levels of guanylin mRNA expression were found in lower parts of the intestine, but they only found a small number of immunoreactive guanylin cells in this region. Cetin *et al.*, (1994) suggested that the lack of immunofluoresence to guanylin antibodies may be due to high rates of synthesis and secretion of guanylin by these cells and this would result in minimal content or lack of proguanylin within these cells. The goblet cells in *A. anguilla* may also exhibit a high turnover of prohormone with rapid rates of secretion which would explain why there is low fluorescence but high levels of mRNA expression. More work is required to confirm this hypothesis. Perhaps the putative high rates of secretion are also true for renoguanylin and uroguanylin, however high affinity antibodies are required before any conclusions can be made.

From the experiments following the changes in mRNA expression following acclimation to SW over 140 days on the intestine of silver eels it took two days before significant increases in mRNA expression of uroguanylin and guanylin were observed. In terms of physiological function the guanylin peptides induce immediate responses in target tissues by binding to the GC-C receptor, a cell surface receptor found in both intestine and kidney, and inducing rapid and large increases in the levels of the intracellular second messenger, cGMP. This subsequently results in the activation of protein kinase G and the immediate phosphorylation and activation of the CFTR chloride channel. The changes in expression of the guanylin prohormone mRNAs after two days suggests that this system has been upregulated, in some unknown way, during the adaptation of fish to SW. This means that the guanylin-like peptide system, especially that of uroguanylin, has the potential to be more active in the intestine of eels when in a SW environment. The results suggest that this signalling system is more involved in SW-acclimated fish and that uroguanylin and possibly guanylin are part of the osmoregulatory control system helping to regulate the amount of water and ions which are secreted. But why do teleosts express mRNA for three members of the guanylin family in the intestine? It is known that guanylin and uroguanylin bind to the receptor GC-C but does renoguanylin also bind to the GC-C receptor or does it bind to another as yet unidentified receptor? The possibility that another receptor exists other than GC-C comes from studies using GC-C$^{-/-}$ mice (Forte,

2003). This study found that the GC-C$^{-/-}$ mice had normal blood pressure and renal sodium excretion. It was suggested that one explanation for the normal blood pressure and renal sodium excretion was that the actions of uroguanylin were via another unidentified receptor (Forte, 2003). Previous studies from our laboratory have shown that at least two GC-C receptors are present within the intestine and kidney of eels (Comrie *et al.*, 2001[b]). It is indeed possible that guanylin, uroguanylin and renoguanylin bind to different GC-C isoforms. It may also be possible that another receptor which the guanylin-like peptides can bind to exists within the gills and when in a SW environment guanylin and uroguanylin expressed within the intestine are transported to the gill were they activate a receptor resulting in the secretion of excess salt. Future work is also required to understand how renoguanylin works and what its function is in the intestine. Research is also needed to determine whether the three guanylin-like peptides function differently under extremes of physiological conditions. For example in mammals, guanylin has been found to work optimally under basic conditions but uroguanylin works more efficiently in a medium which has an acidic pH (Fan *et al.*, 1997). Therefore in teleosts the three guanylin-like peptides may each have a different pH optimum. If these peptides do function optimally under different physiological conditions then this may explain why three members of the guanylin family have evolved in teleosts.

From mammalian studies in the kidney, uroguanylin does have a role to play in salt and fluid regulation (Potthast *et al.*, 2001). From this study on the kidney of the European eel there was no up-regulation following acclimation of the eels to SW. Renoguanylin and uroguanylin undoubtedly play a role in fluid and ion balance in the kidney however the role of these peptides did not change significantly in SW- compared to FW-acclimated fish. The renal actions of renoguanylin and uroguanylin are likely to differ considerably in FW fish compared to SW fish as there is such a dramatic change in kidney function in these two states. In FW the kidney is producing copious volumes of dilute urine (Hazon and Balment, 1998) but in SW the kidney produces low levels of urine containing divalent ions (Jobling, 1995). Mammalian studies have also suggested that uroguanylin is involved in an endocrine axis connecting the intestine and the kidney (Forte *et al.*, 2000[b]). A similar endocrine axis may also exist in teleosts.

This study also investigated the presence of another protein from *A. anguilla,* termed antisecretory factor (AF), which may be involved in the regulation of intestinal secretion and therefore osmoregulation. As discussed in chapter 10, the actions of AF result in the prevention of excess fluid and ion secretion within the intestine and therefore may act to antagonise the actions of guanylin but further research is required to confirm this hypothesis.

Although this study has answered the original aims the results have led to a number of questions which need to be answered. Future studies can use the recombinant prohormones for guanylin, renoguanylin and uroguanylin, which have been successfully synthesised by bacterial expression, for physiological and structural studies and these studies may determine where the prohormones of the guanylin-like peptides are stored, how and where the prohormones are cleaved and by which proteases. Likewise additional immunohistochemical studies are required to confirm the cellular location of all three prohormones within the intestine and renoguanylin and uroguanylin within the kidney.

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Appendices

Appendix 1 List of suppliers of equipment and chemicals.

Ambion Inc., Abingdon, Oxfordshire, UK

Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK

Anachem, Luton, Bedfordshire, UK

Beckman Instruments Inc. Palo Alto, California

BIO 101 Inc., Anachem Ltd, Luton, UK

Biogene Ltd., Anachem Ltd., Luton, UK

Biosoft, Cambridge, UK

Canberra Packard, Meridan, CA

Clontech, Basingstoke, UK

Eastman Kodak Co., Rochester, New York, USA

Edge Biosystems, Gaithersburg, USA

Gibco Life Technologies Ltd., Paisley, UK

Herolab GMBH laboratory, Weisloch, Germany

Invitrogen, Groningen, The Netherlands

Kinematica, Lucerne, Switzerland

Millipore, Watford, UK

MWG Biotech, Milton Keynes, UK

New England Biolabs, Hitchin, Hertfordshire, UK

Novagen, Merck KgaA, Darmstadt, Germany

Oxoid, Unipath Ltd, UK

Packard Instruments, Pangbourne, Berkshire, UK

PE Applied Biosystems, Perkin Elmer Ltd, Chesire, UK

Pierce, Perbio Science UK Ltd., Chesire, UK

PNACL, Leicester University, Leicester

Promega Corporation UK, Southampton, UK

Scie-Plas, UK

Sigma Aldrich Ltd, Poole, Dorset

Sigma Genosys, Cambridgeshire, UK.

Syngene, Cambridge, UK

Techne Ltd, Cambridge, UK

VWR Laboratory services, Poole, Dorset, UK

Appendix 2 Sequences of Primers

Degenerate Primers

Eel guanylin degenerate 5 sense	5'GGA GA I/C (CT)T I/C (GC)T T(CT)(CT)C(CT)CTGGAG(GT)C I/C AA3'
Eel guanylin degenerate 5 antisense	5'(AG)CA I/C GC I/C GC(AG)(AT)(AT) I/C GC(AG)CA I/C AT(CT)TC(AG)CA 3'
Eel AF sense	5' GAC AGC GTC AGG AAGAGG 3'
Eel AF antisense	5' CCTCTCCTCTTCACCGAA 3'

Clontech Marathon™ Kit Primers

Marathon cDNA synthesis primer	5' GGA ACA AAC GGC ATG TGA GC 3'
SMART™ oligo	5' AAG CAG TGG TAA CAA CGC AGA GTA CGC GGG 3'
Marathon adaptor primer 1 (AP1)	5' CCA TCC TAA T AC GAC TCAC T AT AGG GC 3'
Marathon adaptor primer 2 (AP2)	5' ACT CAC TAT AGG GCT CGA GCG GC 3'

5' RACE Primers

Eel guanylin 5'RACE 1	5' CTG GCA GGC CTG GGT TGG TAC 3'
Eel guanylin 5'RACE 2	5' GAG GGC TCT GTT TCA CTG TCA TGT C 3'
Flounder guanylin A 5'RACE 1	5' GCA GCA GAG GGT TCT CAC AAG C 3'
Flounder guanylin A 5' RACE 2	5' CGG CGA GGC GAG GGT TGA G 3'
Flounder guanylin B 5' RACE 1	5' GCA TCC ACT AGC CTG GAG AAG ACC 3'
Flounder guanylin B 5' RACE 2	5' GTT ACA GAC AGA GAC CAT GTT GGT G 3'
Sculpin guanylin 5' RACE 1	5' CGA AAG ACC TGC GGC AGG AC 3'
Sculpin guanylin 5' RACE 2	5' CCA TAG ACG TGA TGA TTG ACA CCA G 3'
Trout guanylin 5' RACE 1	5' CTA GTC TTG AGA GGG ACA TAC TTG CC 3'
Trout guanylin 5' RACE 2	5' CTC TTC ATG CAA AGG GTC ATG AAC TCC 3'
Whiting guanylin 5' RACE 2	5' CAT GCC GAG TCT GGC GAG AGA G 3'
Eel AF 5' RACE	5' CAG CTG GAT AGC GTG CAG 3'
Japanese Eel guanylin 5' RACE 1	5' GCA ACC GGT GCA GGC AGC AAA CAT GC 3'
Japanese Eel guanylin 5'ß RACE 2	5' TGC CAG CCT GGC GAG CAT GCC 3'

3' RACE Primers

Eel guanylin 3'RACE 1	5' GGCAAAGACCAGTACTACGGCCG 3'
Eel guanylin 3'RACE 2	5' CTCTGCAAGAGCAAAGGAGCGGC 3'
Cod guanylin A 3' RACE 1	5' GCT ACT CCT GAT CCT GGC CCT GAA CTC 3'
Cod guanylin A 3' RACE 2	5' CCT CCG AGG CAG TGC AGG TCC AG 3'
Cod guanylin B 3' RACE 1	5' CAG TGC GCG ATG GTC TGC CAC G 3'
Cod guanylin B 3' RACE 2	5' CCG TGT GTC GGA CAA GGG AGC G 3'
Flounder guanylin A 3' RACE 1	5' CGC CGA GAC AAG TGT CAC GG 3'
Flounder guanylin A 3' RACE 2	5' CTG CTG CCT CAG GTC TTT CAG CC 3'
Flounder guanylin B 3' RACE 1	5' GCT GAT CGA CAC AGA TGC CAT GG 3'
Flounder guanylin B 3' RACE 2	5' CTT GCC ACA GGC CTT CTA TCC AGT G 3'
Sculpin guanylin 3' RACE 1	5' GAT TTA GAC GGC CGC GTC AGC 3'
Sculpin guanylin 3' RACE 2	5' GGA CGA GCT TTG CGG CCG C 3'
Trout guanylin 3'RACE 1	5' GCA GAG GGC AGC AAT GTA AAC AGG 3'
Trout guanylin 3'RACE 2	5' GCA CCA GCT TTG ATT CTG TCT GTG C 3'
Whiting guanylin 3' RACE 1	5' GCA CCG TGA CCC TCT GCG C 3'
Whiting guanylin 3' RACE 2	5' CCA GGC CAG CTG AAC CCT CG 3'
Eel AF 3' RACE	5' GCC TAT GCC ATG CAG ATG TC 3'
Japanese Eel guanylin 3' RACE 1	5' GAC CAG TAC TGC AAT CGT GTG CAA TGA C 3'
Japanese Eel guanylin 3' RACE 2	5' TGT CAG AGC GAA GGA GCT GGC ATG 3'

Cloning and Sequencing Primers

M13 forward	5' GGA ACA AAC GGC ATG TGA GC 3'
M13 reverse	5' AAG CAG TGG TAA CAA CGC AGA GTA CGC GGG3'
T7	5' CCA TCC TAA T AC GAC TCA CT A TAG GGC 3'
T3	5' ACT CAC TAT AGG GCT CGA GCG GC 3'
T7 terminator	5' GTT TTC CCA GTC ACG ACG TTG TAA 3'
Lac(pET) sense	5' TCA CAC AGG AAA CAG CTA TGA CCA 3'

Primers used in Bacterial Protein Expression

Eel uroguanylin pET 32 sense	5' GGT ATT GAG GGT CGC GTG TGG GTG ATG GAT GGA GAC C 3'
Eel uroguanylin pET 32 antisense	5' AGA GGA GAG TTA GAG CCC TAC AGG CAT CCA GTG CAG GC 3'
Eel guanylin pET 32 sense	5' AGC CAT ATG AAG ACC GTG CTC AGC ATC ACT TTC C 3'
Eel guanylin pET 32 antisense	5' ATC CTC GAG TTA GCA ACC GGT GCA GGC AGC 3'
pET 32 Xa/LIC sense	5' CGA ACG CCA GCA CAT GGA CAG CC 3'
pET 32 Xa/LIC antisense	5' CGC AAG CTT GTC GAC GGA GCT CG 3'
Eel guanylin LIC sense	5' GGT ATT GAG GGT CGC GTC CAG GTC AAA GAA AGA GAG TTC AC 3'
Eel guanylin LIC antisense	5' AGA GGA GAG TTA GAG CCT TAG CAA CCG GTG CAG GCA GC 3'
Eel renoguanylin LIC sense	5' GGT ATT GAG GGT CGC GTC CAA GTG GAA GAA GGA GGC 3'
Eel renoguanylin LIC antisense	5' AGA GGA GAG TTA GAG CCT CAG AGG CAC A GTG CAG 3'
Eel guanylin pET 32 with PreScission protease site	5' GGT ATT GAG GGT CGC CTG GAA GTT CTG TTC CAG GGG CC GTC CAG GTC AAA GAA AGA GAGTTC AC 3'
Eel uroguanylin pET 32 with PreScission protease site	5' GGT ATT GAG GGT CGC CTG GAA GTT CTG TTC CAG GGG CC GTG TGG GTG ATG GAT GGA GAC C 3'
Eel renoguanylin pET 32 with PreScission protease site	5' GGT ATT GAG GGT CGC CTG GAA GTT CTG TTC CAG GGG CCC GTC CAA GTG GAA GAA GGA CGC 3'

Appendix 3 Preparation of SDS Acrylamide Gels

Running gel 12%:

	60 ml for 2 gels
37.5:1 acrylamide/bisacrylamide solution v/v (Sigma Aldrich Ltd.)	24
H_2O	19.8
1.5 M Tris pH 8.8	15
10% SDS v/v	0.6
10% NH_4S2O_4 w/v	0.6
Temed (N,N,N,N tetramethylethylenediamine; Sigma Aldrich Ltd.)	0.048

Stacking gel 4%:

	20 ml for 2 gels
37.5:1 acrylamide/bisacrylamide solution v/v (Sigma Aldrich Ltd.)	2.67
H_2O	14.43
1.5 M Tris pH 6.8	2.5
10% SDS v/v	0.2
10% NH_4S2O_4 w/v	0.2
Temed (N,N,N,N tetramethylethylenediamine; Sigma Aldrich Ltd.)	0.02

Preparation of glass plates;

- Clean18 cm by 16 cm glass plates with ethanol
- Cover two edges of a plate with grease; place 1 mm spacers and cover these with grease; add the second glass plate.
- Clamp on the spacers and tighten. Put on the support and screw into place.

Preparation of gels – running and staking gels:

- Make up sufficient gel mixture as listed above.

Running gel: Pour the solution to 3 – 4 cm below the top of the glass with a syringe.

Overlay with water saturated butanol and leave to set for ~ 20 min.

After setting, rinse gel with water before adding the stacking gel.

Stacking gel: Pour the solution with a syringe from one side to the other side. There should be ~ 1 cm of stacking gel between the running gel and the comb.

Put in the comb, remove bubbles and leave to set for ~ 15 min.

Appendix 4

Summary table - The effect of SW- acclimation on the mRNA expression of guanylin, renoguanylin and uroguanylin.

For all peptides at each time point the level of mRNA expression of the peptide in FW fish is compared to the mRNA expression of the peptide in eels acclimated to SW.

		Guanylin	Renoguanylin	Uroguanylin
Intestine yellow eels	6 h	–	–	–
	2 days	–	–	–
	7 days	–	–	–***
Intestine silver eels	6 h	–	–	–
	2 days	–***	–***	–***
	7 days	–	–	–*
	140 days	–**	–	–*
Intestine sections	Anterior Yellow eels	–	–	–
	Anterior silver eels	–**	–	–***
	Mid Yellow eels	–	–	–*
	Mid Silver eels	–	–*	–**
	Posterior Yellow eels	–	–	–**
	Posterior Silver eels	–**	–	–***
Kidney silver eels	6 h		–	–
	2 days		–	–
	7 days		–*	–
	140 days		–	–

The data was analysed using two-way ANOVA and Fisher's PLSD test.

Key: _ = no significant change in the level of mRNA expression in FW eels compared to eels acclimated to SW.

_ = an increase in mRNA expression following acclimation to SW.

_ = a decrease in mRNA expression following acclimation to SW.

* = significant difference in the level of expression of $p < 0.05$

** = significant difference in the level of expression of $p < 0.01$

*** = significant difference in the level of expression of $p < 0.001$