Cloning and expression of guanylin and guanylate cyclase-C in the European eel, *Anguilla anguilla.*

Muriel M. Comrie

Thesis submitted for the degree of Doctor of Philosophy
University of St Andrews.

September 2000

(i) I, Muriel M. Comrie, hereby certify that this thesis, which is approximately 50,000 words in length, has been written by me, that it is a record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

Date: 26/9/00 Signature of candidate:

(ii) I was admitted as a research student in October 1996 and as a candidate for the degree of Ph.D. in October 1997; the higher study for which this is a record was carried out in the University of St Andrews between 1996 and 2000.

Date: 26/9/00 Signature of candidate:

(iii) I hereby certify that the candidate has fulfilled the conditions of the Resolutions and Regulations appropriate for the degree of Ph.D. in the University of St Andrews and that the candidate is qualified to submit this thesis in application for that degree.

Date: 26/9/00 Signature of supervisor:

In submitting this thesis to the University of St Andrews I understand that I am giving permission for it to be made available for use in accordance with the regulations of the University Library for the time being in force, subject to any copyright vested in the work not being affected thereby. I also understand that the title and abstract will be published, and that a copy of the work may be made and supplied to any *bona fide* library or research worker.

Date: 26/9/00 Signature of candidate:

Acknowledgements

I would like to express my gratitude to several people for their support during this Ph.D. project. Firstly, sincere thanks are due to my supervisor, Dr. Gordon Cramb, for his excellent guidance, advice and good nature- not all students are so lucky! I would also like to thank Dr. Chris Cutler for his technical help, advice on problem-solving and explanations in general. Thanks are also due to everybody on "D" floor for their help throughout, sense of humour and friendship during the last four years; Mary Wilson, Iain Laurie, Joyce Horsburgh and Dr Simon Guild, with a special mention for Dr Lynne Birrell, Dr Stephane Brezillon and Berengere Cocteau (Berengere, your cartoons were a welcome diversion!). Life would also have been a great deal duller without the daily "meetings" of the postgrad. coffee "club" and the strange edible items collected on their travels- I wish them all the very best.

I would also like to express my gratitude to my parents, Douglas and Margaret Boag, and my mother-in-law, Gladys Comrie, for their continuous support and sterling work in the child-minding department; I couldn't have managed without them. Last, but by no means least, I would like to thank my husband and children, Alan, Sarah and Sean for their support, patience and tremendous help around the house (writing up a Ph.D. thesis is a great way of getting out of the ironing!). My sincere thanks to all of you.

Abstract

Full length complementary DNA (cDNA) of the guanylin peptide, which displayed high amino acid homology to mammalian guanylin within the active peptide region, was cloned and sequenced from SW-acclimated yellow eel intestine. Reverse transcription PCR (RT-PCR) amplified guanylin cDNA in the gill, intestine and kidney of fresh water (FW) and seawater (SW)-acclimated yellow and silver eels and Northern blots detected transcripts of 1.0 kb in the kidney and intestine of all four groups. Sequence data demonstrated that long and short forms of eel guanylin are expressed. Transfer of FW-acclimated yellow and silver eels to SW resulted in a significant increase in guanylin mRNA expression levels in the intestine but not in the kidney. In addition, cDNA fragments of two isoforms of eel guanylate cyclase-C (GC-C), termed GC-C(1) and GC-C(2), were cloned and sequenced from SW-acclimated yellow eel intestine. RT-PCR amplified GC-C(1) and GC-C(2) in the intestine and kidney of all four experimental groups but no amplification was demonstrated in the gill. RT-PCR carried out on gut sections also demonstrated that GC-C(2) is not expressed in the posterior gut section of yellow FW-acclimated eels. Northern blotting demonstrated that probes of the GC-C(2) isoform hybridised to transcripts of 4.8 kb in the intestine and kidney of all four groups. Transfer of yellow FW-acclimated eels to SW also resulted in a significant increase in GC-C(2) expression in the intestine. Comparison of FW-acclimated silver and yellow eels also revealed a substantial rise in the intestinal expression levels of GC-C(2) in silver eels compared to their yellow counterparts.

Contents | Page No.

List of Figures

Chapter 5

List of Abbreviations

a.a.	Amino acid
ACE	Angiotensin converting enzyme
Ang I	Angiotensin I
Ang II	Angiotensin II
ANOVA	Analysis of Variance
ANP	Atrial natriuretic peptide
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
AVT	Arginine vasotocin
BNP	Brain natriuretic peptide
cAMP	Cyclic adenosine monophosphate
cDNA	complementary DNA
CFTR	Cystic fibrosis transmembrane conductance regulator
cGMP	Cyclic guanosine monophosphate
CNP	C-type natriuretic peptide
cpm	counts per minute
EDTA	Ethylenediamine tetraacetic acid
DD-PCR	Differential display PCR
DNA	Deoxyribose nucleic acid
dNTP	deoxynucleotide triphosphate
DTT	Dithiothreitol
FW	Fresh water
GC-A	Guanylate cyclase A
GC-B	Guanylate cyclase B
GC-C	Guanylate cyclase C
GFR	Glomerular filtration rate
GH	Growth hormone
mRNA	messenger RNA
NPR-A	Natriuretic peptide receptor A

NPR-B	Natriuretic peptide receptor B
NPR-C	Natriuretic peptide clearance receptor
PCR	Polymerase chain reaction
RAS	Renin angiotensin system
RNA	Ribosomal nucleic acid
RT-PCR	Reverse transcription PCR
SDEV	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SFW	Silver fresh water
SW	Seawater
SSW	silver seawater
St_a	Heat-stable enterotoxin
YFW	Yellow fresh water
YSW	Yellow seawater

Chapter 1

Introduction

1. INTRODUCTION

Guanylin is a member of a family of small bioactive peptides which regulate ion and water homeostasis in several mammalian epithelial tissues such as the intestine. These peptides act by binding to cell-surface receptors that possess guanylate cyclase activity, thus stimulating the production of cyclic GMP and leading to the secretion of ions. The actions of guanylin in stimulating ion secretion in the intestine and other tissues of mammals suggests a possible role for this peptide signalling system in teleost osmoregulation.

Prior to discussing the background of the guanylin peptide signalling system which was the main focus of this research project, a brief description of the experimental organism, the European eel, will be given in addition to an overview of the main aspects of teleost osmoregulation.

An additional aim of the research project was to identify any genes which may be differentially expressed according to environmental conditions or developmental stages of the eel. This second aspect of the project will be discussed in Chapter 5.

1.1 The European Eel

The European eel, *Anguilla anguilla*, is a teleost of the Anguilliforme family, belonging to the super-order Elopomorpha. The Elopomorphs evolved in the recent Cretaceous period and they represent some of the most primitive extant fish of the present day (Young, 1992 ; Harvey-Pough *et.al* , 1989).

Eels are euryhaline fish that can withstand a wide range of salinities from twice seawater (SW) to freshwater (FW). This is an adaptation to their migratory lifestyle and allows the exploitation of two different habitats; a marine environment for the breeding of young and an inland freshwater habitat for the growth and maturation of

the juvenile and adult stages. Unlike the salmonids which display an anadromous migration upstream to FW spawning grounds, the eel displays a catadromous migration downstream to marine spawning grounds.

The life-cycle of the eel can be summarised briefly as follows:

A) Eggs hatch out in the marine environment to produce pelagic leptocephalii larvae.

B) Leptocephalii drift eastwards on the ocean currents until reaching the coastal waters of Europe where they develop into "Glass eels".

C) Glass eels begin the ascent up estuaries attracted by freshwater and develop into pigmented elvers.

D) Elvers continue upstream to lakes and rivers where they develop into yellow eels.

E) Yellow eels grow and mature here for several years before developing into fully mature and sexually differentiated silver eels.

F) Silver eels journey downstream and begin the migration towards the spawning grounds in the Sargasso Sea (Bertin, 1956 ; Tesch , 1977).

Yellow and silver eels display different morphology and physiology; silver eels have larger eyes, thicker skin, an increased blood supply to the swim bladder and they adapt more quickly to seawater. At a molecular level, there are differences in gene expression even when both are acclimated to FW or SW.

1.2 Osmoregulation

Animals which are not osmo-conformers have to maintain a stable physiological state within their bodies despite the prevailing conditions of their environment. This regulation of the internal environment is termed homeostasis and refers to all

forms of regulation including body temperature, blood pressure and osmotic concentration of extracellular and intracellular body fluids.

Maintenance of osmotic concentration is termed **osmoregulation.** The osmotic concentration refers to total concentration of solutes in body fluids.

The osmotic concentrations of intracellular and extracellular fluids are normally identical but of different composition i.e. high K^+ (120-150mM), low Na^+ (20mM) and Cl^- (4-10 mM) for intracellular fluids but high Na^+ (140-180mM) and Cl^- (100-110 mM), low K^+ (4-5 mM) for extracellular fluids. However, both may have very different concentrations to the external environment which the animal inhabits. It is clear therefore that aquatic animals must osmoregulate whether they inhabit FW or SW. A euryhaline fish , such as the eel , is one which can succesfully osmoregulate across a wide range of external salinities from FW to full SW allowing migration between these two extremes of environment.The yellow eel is the sexually undifferentiated FW stage of the European eel which inhabits inland water systems (although it is also capable of adaptation to brackish water and SW) . The yellow eel therefore, has to osmoregulate against the influx of water from the hypo-osmotic external medium and the efflux of ions from the body. To counteract the influx of water, FW teleosts such as the yellow eel will produce large quantities of dilute urine as a consequence of the reabsorption of ions in the bladder and kidney. There will also be active uptake of ions across the gill membranes (Evans, 1975). The teleost gill, gut and kidneys are the three main tissues which co-ordinate ion transport and water absorption between the body and the external environment. In contrast to the yellow eel, the silver eel, which embarks on the the migration to the marine breeding

grounds, has rather different osmoregulatory problems; the hyper-osmotic nature of the external medium means the eel must drink large amounts of SW to counteract water loss through the gills and skin, reduce urine production to avoid further water loss and excrete, via the gills, any excess ions derived from the ingested seawater. The gill, gut and renal system will each be dealt with separately.

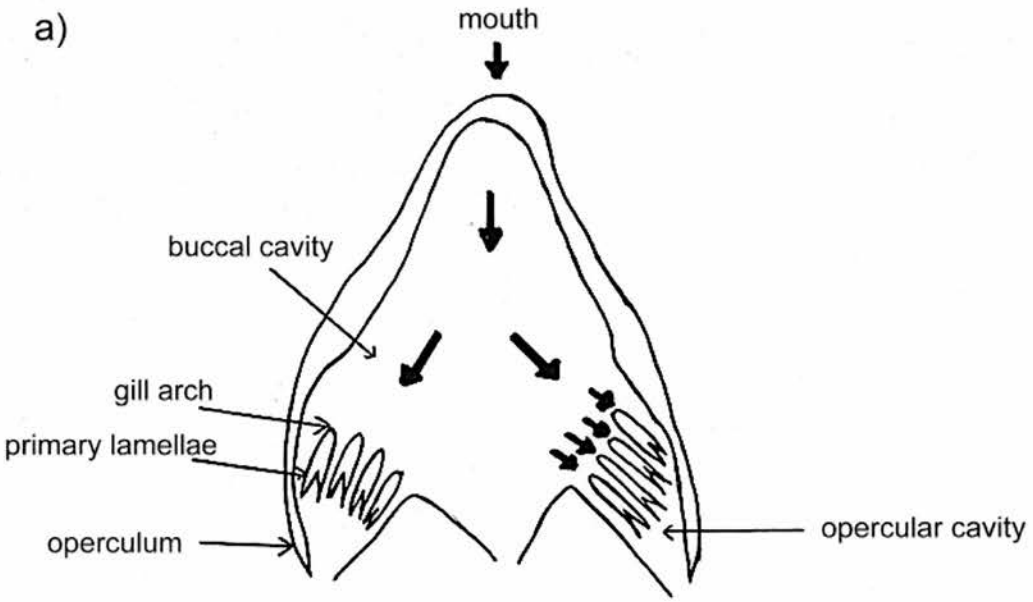
1.2.1 The Gill

In addition to their respiratory function, teleost gills have long been recognised as a major site of ion transport and acid base balance, with an important role in osmoregulation.

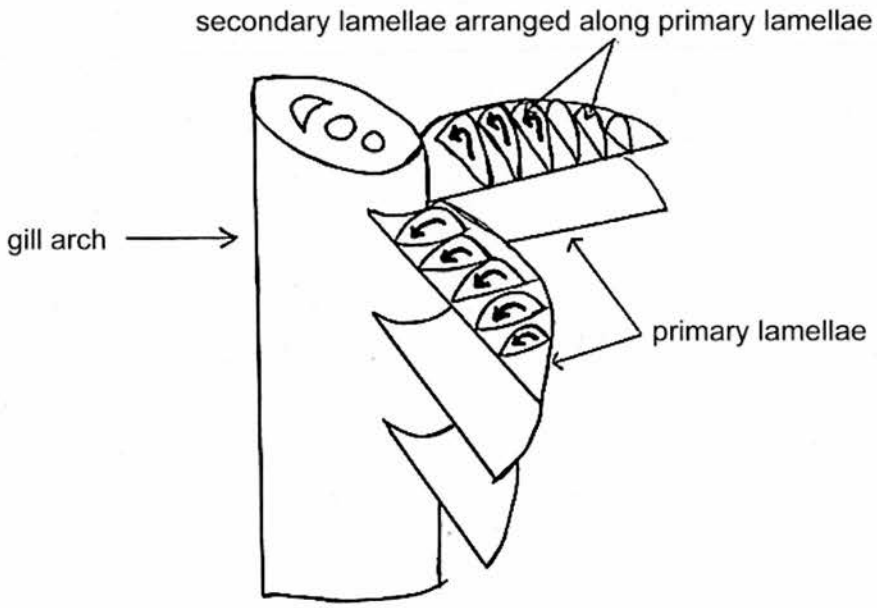
The basic morphological features of the teleost gill are shown in Fig. 1.1 (a) and (b). The gill is covered by an operculum which overlays several branchial arches, each of which bears a paired row of filaments (primary lamellae) and every filament carries a series of secondary lamellae. The primary and secondary lamellae have different epithelial surfaces - the primary and secondary epithelia respectively - which are composed of four cell types; chloride cells (or mitochondria-rich cells), mucous-secretory cells, neuro-epithelial cells and pavement/respiratory cells (Laurent, 1984). It is the gill chloride cells (also found in the opercular epithelium), discovered by Keys and Willmer (1932), that have been shown to be the main site of ion secretion in the SW-acclimated teleost gill. Evidence for this was demonstrated by various research groups; the number of chloride cells were shown to increase upon transfer of euryhaline fish from FW to SW (Utida *et al.*, 1971; Thomson and Sargent, 1977; Foskett *et al.*, 1981), high levels of the sodium transporting enzyme Na^+,K^+-ATPase were detected in chloride cells of SW-acclimated fish (Kamiya,1972; Sargent *et al.*

Fig1.1 Diagrammatic representation of teleost gill showing a) horizontal section through head with arrangement of gill arches and gill filaments and b) arrangement of secondary lamellae, lying perpendicular to primary lamellae (gill filaments), ensuring a large surface area is exposed to the water flow. Short bold arrows indicate direction of water flow.

a)


mouth
buccal cavity
gill arch
primary lamellae
operculum
opercular cavity

b)

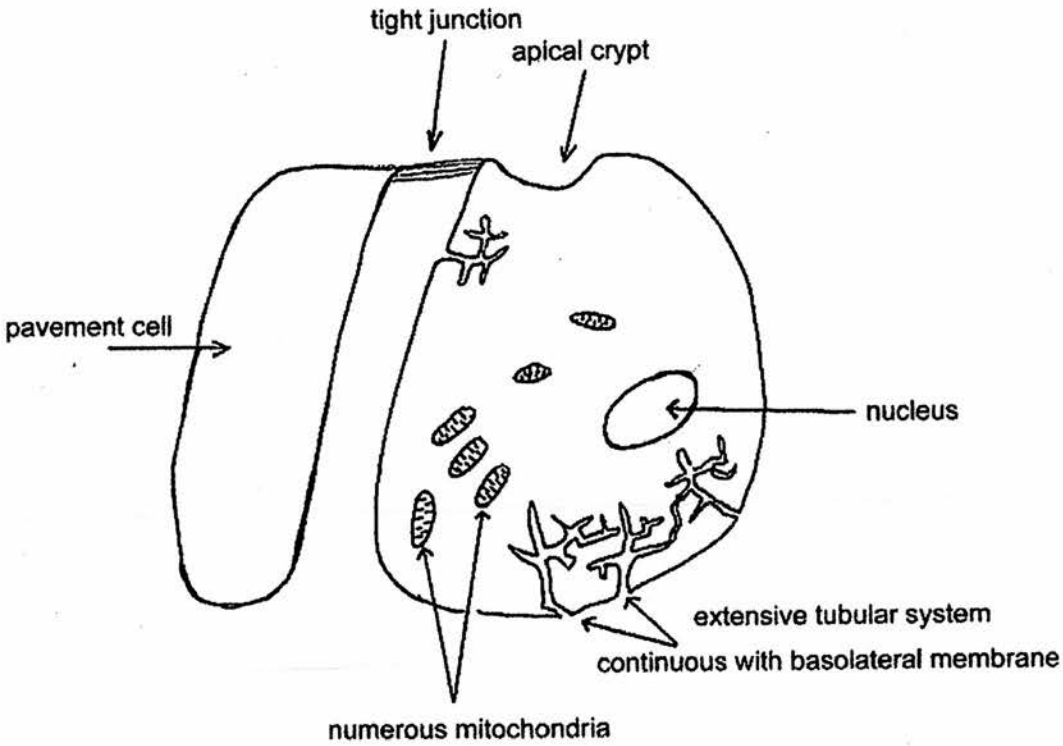

secondary lamellae arranged along primary lamellae
gill arch
primary lamellae

1975; Karnaky *et al.*, 1976; Hootman and Philpott, 1979; Karnaky, 1986; McCormick, 1990 b) and conclusive proof that the chloride cell was the site of active Cl^- release was provided by Foskett and Scheffey (1982) using the vibrating probe technique. The isolated opercular epithelium, rich in chloride cells, has proved to be a useful model for ion transport studies in SW-acclimated fish such as *Fundulus heteroclitus* and *Oreochromis mossambicus* (Karnaky *et al.*, 1977; Degnan *et al.*, 1977, Karnaky and Kinter, 1977; Foskett and Scheffey, 1982).

The basic morphology of the chloride cell (see Fig. 1.2) is that of a large, mitochondria-rich, mononucleate cell containing an extensive tubular system which is continuous with the basolateral membrane (Laurent and Dunel,1980; Karnaky, 1986). The apical surface of the cell is in contact with the external environment and the basolateral surface is exposed to the capillary bloodstream. The chloride cells of FW and SW-acclimated fish display different morphologies; two types of chloride cell are suggested to exist in FW (designated α and β), reported by Pisam *et al.*, (1987), but in SW there is only one type of chloride cell (α) often found in association with another cell type, the accessory cell (Sardet *et al.*, 1979; Hootman and Philpott, 1980; Laurent and Dunel, 1980; Lacy, 1983), which may or may not represent a different developmental stage of the chloride cell. Between the chloride and pavement cells in FW there exists a deep tight junction, but in SW the chloride and accessory cells are joined by a shallower, more "leaky" junction (Sardet *et al.*, 1979) and deeper apical crypts are present in chloride cells of SW-acclimated fish (Foskett *et al.*, 1981; Pisam *et al.*, 1987; King *et al.*, 1989; Lee *et al.*, 1996).

As has been described, the role of the chloride cell in ion excretion has been clearly demonstrated in the SW environment and a current model of the mechanisms of ion

Fig. 1.2 Representation of a chloride cell and adjacent pavement cell.


tight junction
apical crypt
pavement cell
nucleus
extensive tubular system
continuous with basolateral membrane
numerous mitochondria

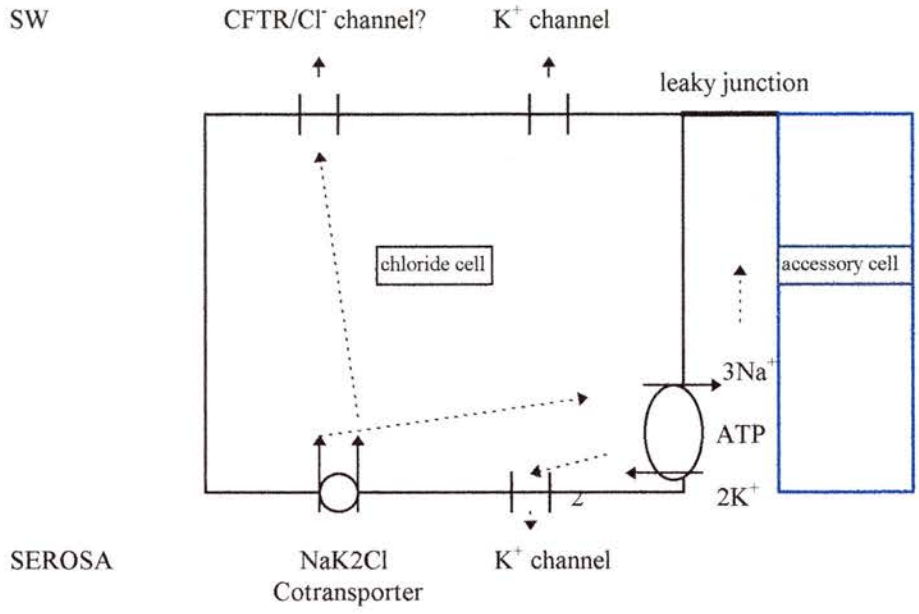
transport in the chloride cell (as proposed by Silva *et al.*, 1977) will be given - see Fig. 1.3 (a) and (b) for models of ion transport in chloride cells of FW and SW-acclimated fish. Excess Na^+ and Cl^-, ingested by the fish in SW , are carried in the bloodstream to the gill chloride cells where they are transported into the cell by means of a basolaterally located furosemide/bumetanide sensitive Na/K/2Cl cotransporter (Degnan *et al,* 1977; Karnaky *et al.*, 1977; Zadunaisky, 1984; Zadunaisky *et al.*, 1995; Zadunaisky, 1997). The increase in plasma osmolality is thought to be the rapid signal for secretion of Cl^- by the chloride cells (Zadunaisky *et al.*, 1995). An elasmobranch homologue of the Na/K/2Cl cotransporter has also been demonstrated in the rectal gland tubule of the dogfish (Greger and Schlatter, 1984a,b), which is the major site of sodium and chloride excretion in elasmobranch fish, and located to the basolateral surface of the chloride cell (Lyttle *et al.*, 1992). The driving force for the uptake of ions into the chloride cell is thought to be the basolaterally located (Karnaky *et al.*, 1976; Hootman and Philpott, 1979) Na^+,K^+-ATPase which concentrates Na^+ ions in the tubular system before they exit to the extracellular environment via a paracellular route (Silva *et al.*, 1977; Karnaky, 1986). This creates a sodium gradient that facilitates the accumulation of chloride ions inside the cell due to the activity of the Na/K/2Cl cotransporter. Consequent studies with ouabain (an inhibitor of Na^+, K^+ATPase) have demonstrated a marked reduction in chloride efflux from the cell and hence a reduction in short-circuit current (Karnaky *et al.*, 1977; Marshall, 1977; Silva *et al.*, 1977). The movement of Na^+ ions down the paracellular pathway to the external environment is driven by the transepithelial potential created by the efflux of chloride ions. The chloride ions themselves exit the cell via apically located anion channels. The nature of the apical

chloride channel is uncertain but one candidate is a teleost equivalent of the cystic fibrosis transmembrane conductance Regulator (CFTR); a chloride channel that is defective in the disease cystic fibrosis (Riordan *et al.*, 1989). An elasmobranch homologue of CFTR has been identified in dogfish rectal gland (Marshall *et al.*, 1991). Marshall *et al.*, (1995) also demonstrated the presence of an apically located 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid(DIDS)-insensitive anion channel in primary cultures of cells from *F.heteroclitus* opercular epithelium which displayed similarities to CFTR. Subsequently, Singer *et al.*, (1998) reported a CFTR homologue that had been isolated and sequenced from a SW-acclimated killifish gill cDNA library. In addition, a homologue of CFTR has been isolated from *Anguilla anguilla* (Cutler *et al.*, 1996). It must be noted, however, that some debate exists as to whether the CFTR could be the sole chloride channel. The uptake of chloride ions into the cell is driven not only bythe Na^+ gradient induced by the Na^+, K^+ATPase, but also by recycling of intracellular K^+ ions via a barium sensitive K^+ channel (Degnan, 1985). Zadunaisky *et al.*, (1995) provided evidence for a basolaterally located DIDS-sensitive Cl^-/HCO_3^- exchanger in isolated opercular epithelium of *F. heteroclitus* and demonstrated that the presence of bicarbonate was required for Cl^- secretion.

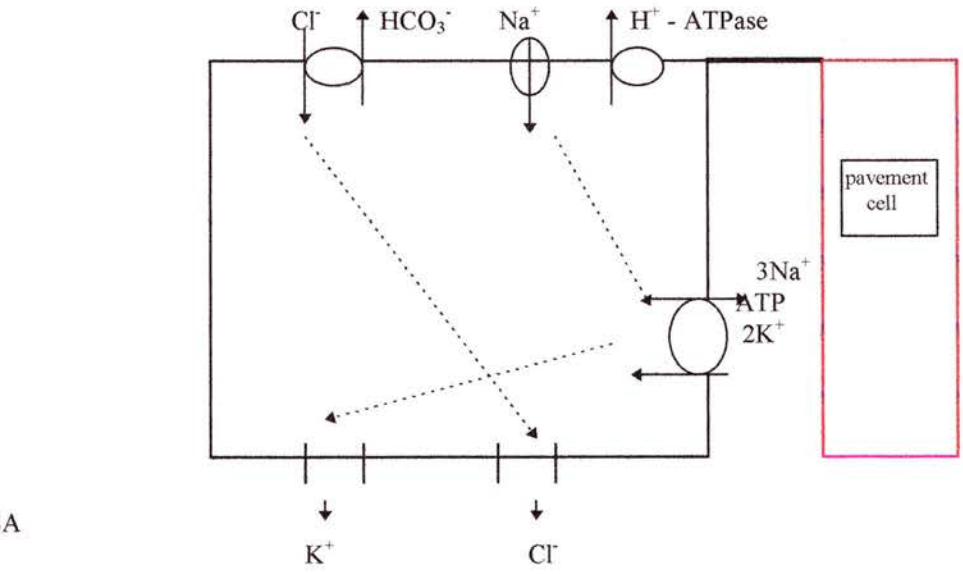
The role of the chloride cell in ion uptake in FW is far less clear although the gills themselves are known to be involved in the uptake of Na^+ and Cl^- in FW (Evans, 1975; Girard and Payan, 1977; Girard and Payan, 1980). Uptake of these ions is counterbalanced by excretion of H^+/NH_4^+ and HCO_3^- respectively, thereby serving a dual role in osmoregulation and acid-base balance. Current opinion suggests an apical Cl^-/HCO_3^- exchange mechanism in the chloride cell for Cl^- uptake (Perry *et al.*, 1981; Perry and Randall, 1981; Marshall, 1995; Sullivan *et al.*, 1996; Perry,1997) while

Fig. 1.3 Current proposed models of ion transport in a) SW chloride cell and b) FW chloride cell.

a)


SW
CFTR/Cl⁻ channel?
K⁺ channel
leaky junction
chloride cell
accessory cell
3Na⁺
ATP
2K⁺
SEROSA
NaK2Cl
Cotransporter
K⁺ channel

b)


FW
Cl⁻
HCO₃⁻
Na⁺
H⁺ - ATPase
pavement cell
3Na⁺
ATP
2K⁺
SEROSA
K⁺
Cl⁻

Na^+ uptake is driven by a negative intracellular potential created during excretion of H^+ ions by an apically located H^+-ATPase (Avella and Bornancin, 1989; Lin and Randall, 1991; Lin and Randall, 1993; Lin *et al.*, 1994; Marshall, 1995; Sullivan *et al.* 1995; Sullivan *et al.*, 1996; Perry, 1997). Studies by Lee *et al.*, (1996) demonstrated several distinct types of chloride cell in *O.mossambicus* that had varying surface morphologies depending on the hypotonicity of the water. These morphologies observed under hard FW (local FW containing 212.3-244.3 ppm $CaCO_3$), local FW (containing 24.7-41.2 ppm $CaCO_3$) and 5% SW were termed wavy convex, shallow basin and deep hole respectively. The morphological effects on the cells of the external medium were reversible suggesting that cell morphology may be involved in adaptation to salinity. Although it is generally accepted that chloride cells play a major role in Cl^- uptake in FW, debate exists as to the site of Na^+ uptake (see Perry, 1997 for discussion).

1.2.2 The Gastro-Intestinal Tract

The teleost intestine, in addition to its role in nutrient absorption, is a major osmoregulatory organ in the SW environment. When a euryhaline fish such as the eel is transferred from FW to SW, an immediate increased drinking response is observed (Oide and Utida, 1967; Kirsch and Mayer-Gostan, 1973; Hirano, 1974). The drinking response is necessary to replace water lost by the fish to the hyperosmotic environment. The ingested SW, on entering the the oesophagous, is subjected to a desalination process where Na^+ and Cl^- are actively and passively removed (Hirano and Mayer-Gostan, 1976; Sleet and Weber, 1982; Parmalee and Renfro, 1983; Simmoneaux *et al.*, 1987a; Nagashimo and Ando, 1994). This process of desalination

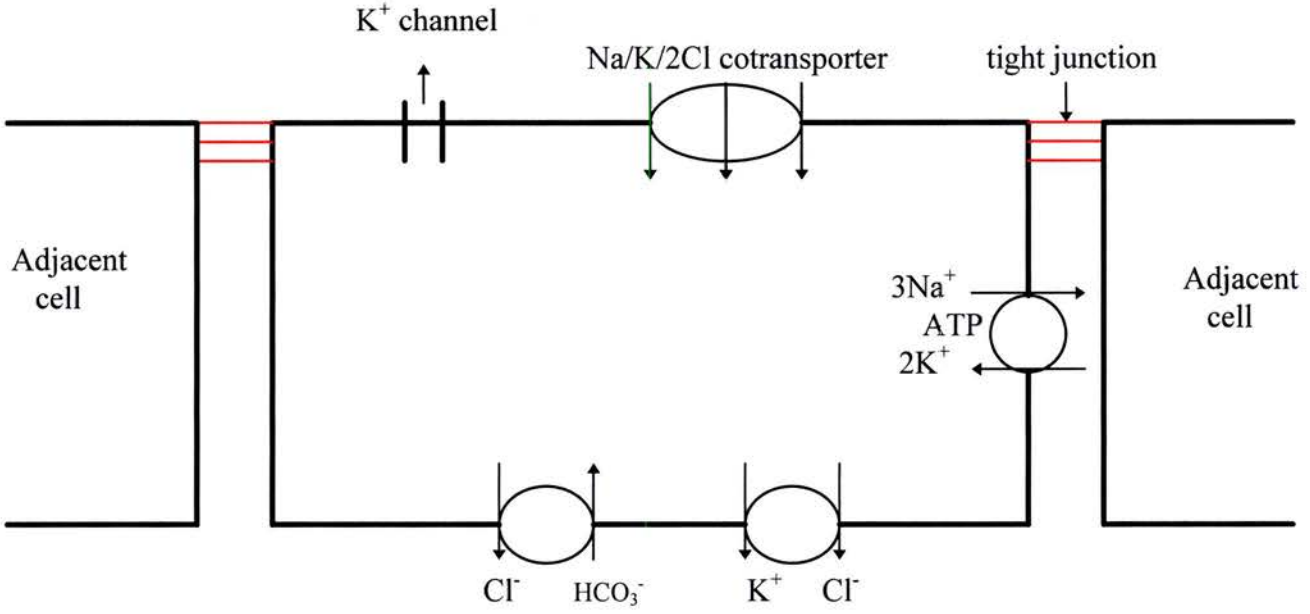
is facilitated by the morphological changes that occur in the SW oesophageal epithelium as noted in both *A.japonica* and *A.anguilla* (Yamamoto and Hirano, 1978; Laurent and Kirsch, 1985), namely the epithelium becomes more columnar and vascularised and there is increased folding of the surface. Yamamoto and Hirano (1978) demonstrated changes to the epithelium occurring 3-4 days after transfer of the eels to SW. It is also thought that the mucous layer of the SW oesophagous forms a diffusion barrier for ion absorption that prevents direct contact between the epithelium and the SW (Simmoneaux *et al.*, 1987a). The absorbed ions are then removed to the gills where they are excreted, as previously described, by the chloride cells.

On leaving the oesophagous, the ingested SW enters the stomach and by the time it reaches the anterior intestine is iso-osmotic to blood plasma (Shedadeh and Gordon, 1969; Parmalee and Renfro, 1983). Skadhauge (1969) demonstrated that SW eel intestine displayed increased permeability to water, and absorption of Na^+ and Cl^- also increased (Oide and Utida, 1967; Skadhauge, 1969). Within the intestine there is active absorption of Na^+ and Cl^- ions which then increases the uptake of water due to the osmotic gradient (Skadhauge, 1969; Utida *et al.*, 1972; Ando, 1974; Skadhauge, 1974; Ando *et al.*, 1975; Ando, 1980, 1983, 1985). The absorbed ions are then removed via the bloodstream to be excreted by the chloride cells. As with the chloride cells in the gill, transepithelial ion movement across the intestinal enterocytes occurs by means of ion transport proteins present in the cell membranes but the direction of ion movement is from the luminal (apical) to serosal surface so the positioning of the transport proteins is essentially opposite to that in the chloride cells of SW-acclimated fish. Although the exact pathways of ion transport in the SW-

acclimated teleost intestine are still uncertain, Fig.1.4 shows the current model of ion transport which will be discussed in the text. The involvement of a basolaterally located Na^+,K^+-ATPase in Na^+ absorption has been demonstrated by ouabain binding studies (Ando and Kobayashi, 1978; Field *et al.*, 1978; Mackay and Janicki, 1979; Parmalee and Renfro, 1983; Ando, 1985; Simmoneaux *et al.*, 1987b; Baldisserotto and Mimura, 1994; Nagashima and Ando, 1994) and levels of Na^+, K^+-ATPase activity have been shown to increase upon transfer of fish to SW (Jampol and Epstein, 1970). In a similar manner to the pathway in the chloride cell, the Na^+, K^+-ATPase transports Na^+ into the intercellular space where it diffuses to the basolateral side of the cell and back diffusion of K^+ creates a membrane potential for Cl^- extrusion from the cell into the bloodstream. The Na^+ and Cl^- ions are taken up from the lumen by an apically located Na/K/2Cl cotransporter; evidence for this cotransporter has been provided by studies using loop diuretics (which inhibit Na/K/2Cl cotransporters) such as bumetanide (Musch *et al.*, 1992; Halm *et al.*, 1985b; Aguenaou *et al.*, 1989; Trischitta *et al.*, 1992) and furosemide (Eveloff, *et al*, 1990; Musch *et al.*, 1982; Baldisserotto and Mimura, 1994). The uptake of ions by the Na/K/2Cl cotransporter is facilitated by the gradient created by the extrusion of Na^+ by the Na^+,K^+-ATPase. The Cl^- ions are transported to the serosa possibly via a basolateral, DIDS-sensitive, Cl^-/HCO_3^- exchanger (Ando and Subramanyam, 1990; Baldisserotto and Mimura, 1994) or a basolateral K/Cl cotransporter (Ando, 1983; Ando, 1985; Halm *et al.*, 1985a). Whilst some studies have put forward evidence for an apical Cl^-/HCO_3^- exchanger (Howard and Ahearn, 1988; Ando, 1990; Ando and Subramanyam, 1990) others have found no evidence

Fig. 1.4 Current model of ion transport pathways in the intestine of SW-adapted fish.

Mucosa



Serosa

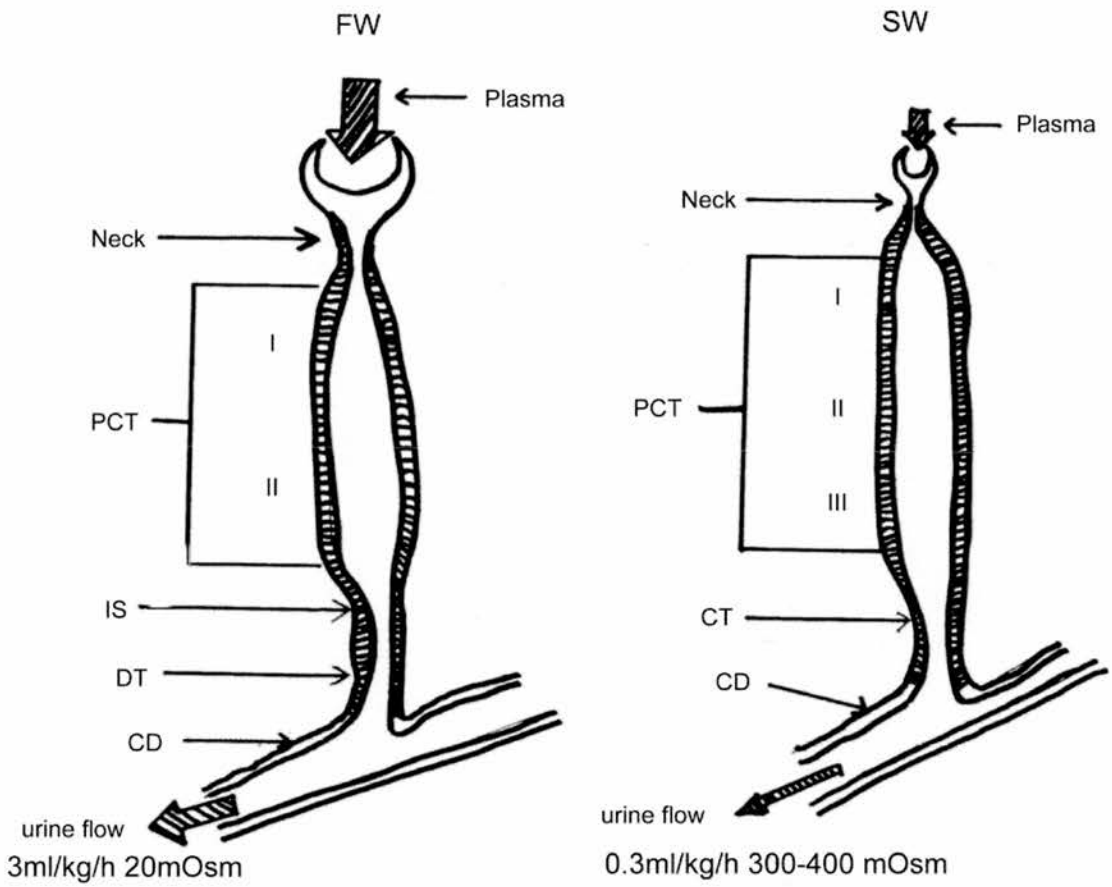
for this (Trischitta *et al.*, 1992; Baldisserotto and Mimura, 1994). The recycling of K^+ ions is thought to occur via barium-sensitive K^+ channels on the apical surface (Simmoneaux *et al.*, 1987b; Ando, 1990; Baldisserotto and Mimura, 1994). The uptake of water in the intestine is linked to the uptake of ions and the reduction in osmolality of the intestinal fluid. Recent research has demonstrated the presence of water channels in mammals which allow the passive diffusion of water across cell membranes, these channels are termed aquaporins and for recent reviews see Agre *et al.*, (1998) and Deen and van Os, (1998). Evidence now exists for teleost homologues of aquaporins (Cutler, C.P. pers. comm.) which may play an important role in water transport across teleost osmoregulatory tissues.

1.2.3 The Teleost Renal System

In teleosts both the kidney and urinary bladder contribute to osmoregulation; FW-acclimated fish must excrete large quantities of excess fluid as urine whilst retaining ions, whereas in SW, urine production is reduced to conserve water. Excess ions cannot be excreted via the urine in SW because the kidneys of teleosts lack a loop of Henle so no counter-current system exists to further concentrate the urine. The urine therefore, is never more concentrated than blood plasma, and excess ions such as Na^+ and Cl^- are excreted via the chloride cells of the gill, although the kidney is the major site of excretion of divalent ions such as Mg^{2+} and SO_4^{2-}.

The teleost kidney is composed of nephrons which contain a glomerulus and a proximal tubule leading from the Bowman's capsule, a distal tubule and a collecting duct (Withers, 1992). Some structural differences do exist between species, see Fig. 1.5 for model of the teleost kidney. As blood enters the glomerulus, fluid, referred to as glomerular filtrate, is forced out into the Bowman's capsule and enters the

Fig. 1.5 Representation of teleost nephron in A) FW and B) SW, giving values for urine flow rates in both environments. PS is proximal segment, IS is intermediate segment, DS is distal segment, CT is collecting tubule and CD is collecting duct.


FW
Plasma
Neck
I
PCT
II
IS
DT
CD
urine flow
3ml/kg/h 20mOsm
SW
Plasma
Neck
I
PCT
II
III
CT
CD
urine flow
0.3ml/kg/h 300-400 mOsm

proximal tubule where in FW-acclimated fish valuable ions such as Na^+ and Cl^- are reabsorbed together with glucose and amino acids. In FW teleosts, nephrons have a high glomerular filtration rate (GFR) and urine flow rate. In SW, the glomeruli may be very small and GFR is greatly reduced with decreased urine flow (Brown *et al.*, 1980). Some teleosts have aglomerular, secretory kidneys which further reduces water loss. Studies on teleost kidney function have investigated GFR (Oide and Utida, 1968; Sokabe, 1973), urine production rates (Brown *et al.*, 1980) and perfusion techniques have also been employed (see Beyenbach, 1995 for a review) but further investigation of ion transport pathways is necessary.

The urinary bladder itself acts to modify the urine in teleosts; in SW it is more permeable to water which can be linked to uptake of ions (Utida, 1972). Utida *et al.*, (1974) demonstrated that urinary bladder Na^+,K^+-ATPase activity was higher after transfer of *Platichthys stellatus* to SW. In FW the urinary bladder reabsorbs sodium chloride but because of low permeability of the epithelium, uptake of water is minimal, this is in contrast to SW acclimated fish where increased permeability of the urinary bladder allows uptake of water (see Marshall, 1995 for a review).

1.2.4 Molecular Investigation of Ion Transport Proteins

In recent years, modern molecular techniques such as gene cloning have been applied to the study of ion transport processes and hormonal function in teleosts and elasmobranchs; such techniques can provide valuable data on tissue expression of genes, changes in levels of expression under different environmental conditions, protein structure and isoform expression, potential binding sites for regulatory and transcriptional factors and the degree of evolutionary conservation of gene and protein structure between teleost species and other vertebrate groups.

To date, the best characterised ion transport protein in teleosts is the Na^+, K^+-ATPase. Studies in mammals and other vertebrates has generated a considerable amount of data and it is known that the enzyme consists of an α and β subunit, both of which occur in a number of isoforms (see Lingrel and Kuntzweiler, 1994 for a review). A γ subunit also occurs in some tissues. In teleost fish, two α subunit isoforms (α1 and α3) have been identified in *A.anguilla* (Cutler *et al.*, 1996); the full length α1 subunit isoform has been cloned and sequenced (Cutler *et al.*, 1995a). Other α subunit cDNAs have been identified in white sucker (Schonrock *et al.*, 1991) and rainbow trout (Kisen *et al.*, 1994). Several isoforms of the β subunit have been detected in eel (Cutler *et al.*, 1996), namely β1, β3, β233, β179, β185 and β185b. Of these, the full length cDNA of β1 has been cloned and sequenced (Cutler *et al.*, 1995b) as has β233 (Cutler *et al.*, 2000) and a partial β3 cDNA has been cloned and sequenced from brain (Cutler *et al.*, 1997). Expression studies of the eel α and β subunit isoforms have demonstrated that the α1 isoform is expressed in most tissues except skeletal muscle whereas the α3 isoform is expressed primarily in brain, gill, intestine and kidney (Cutler *et al.*, 1995a; Cutler *et al.*, 1996; Cutler, C.P. pers.comm). The isoforms of the β subunits are expressed as follows; β1 in most tissues except muscle, β3 in brain, β233 in brain, gill, intestine and kidney and β179 β185 and β185a in brain. Several of the β subunit isoforms are unique to teleosts (Cutler *et al.*, 1996). Northern blot analysis on branchial α1 and β1 showed increased mRNA levels of both subunits upon transfer of eels to SW (Cutler *et al.*, 1995a,b). The mRNA expression levels of α3 and β233 were also found to increase substantially in mid-gut sections of SW-acclimated eels (Cutler *et al.*, 1996).

A study carried out by Sullivan *et.al*, (1996), using *in-situ* hybridisation, demonstrated the presence of both an H^+-ATPase and a Cl^-/HCO_3^- exchanger in gill, however the probes used were designed from bovine and rat sequences respectively and in order to ensure, as far as possible, that there is no binding of the heterologous probes to non-target sequences, an acceptable negative control is needed. It is generally suggested that a sense probe (identical in sequence to the target mRNA and therefore incapable of binding) is used. Recently three isoforms of a Cl^-/HCO_3^- exchanger from eel gill have been cloned (C.P. Cutler, pers.comm) one of which bears homology to the rainbow trout erythrocyte Cl^-/HCO_3^- exchanger described by Hubner *et al.*, (1992). The sequence information derived from these isoforms should allow the design of eel-specific probes for *in-situ* hybridisation. Three eel isoforms of this exchanger have been shown to be expressed in gill, intestine and kidney (Cutler, C.P. pers.comm).

A Na/K/2Cl cotransporter has been cloned from dogfish rectal gland (Xu *et al.*, 1994) and several isoforms of an eel homologue of this cotransporter have been isolated and cloned and named cot1, 2, 3, 4, 5 and 6 (see Cutler *et al*, 1996). Tissue expression studies of these isoforms have shown that cot1 is present in gill with some expression in kidney and intestine. In addition, there is an increase in gill expression when yellow eels are transferred from FW to SW but the expression levels decrease with the same treatment in silver eels (Cutler *et al.*, 1996). The cot2 isoform is expressed at high levels in the intestine of both FW and SW-acclimated silver eels but the expression levels in yellow eels are higher in SW (Cutler, C.P. pers.comm.). It is clear from these data that the different isoforms of these ion-transporting genes may

play different roles in adaptation to FW and SW and in osmoregulation in general. Homologues of CFTR have also been cloned and sequenced from shark rectal gland (Marshall *et al.*, 1991), killifish gill (Singer *et al.*, 1998) and eel gill (Cutler *et al*, 1996). Molecular techniques are also being applied to the study of hormones and hormone receptors in elasmobranchs and teleosts and this will be discussed below within the context of hormonal regulation of ion transfer.

1.3 The Role of Hormones in Osmoregulation

The endocrine system of any animal is the means by which normal physiological functions and responses to physiological challenge are integrated and controlled. Hormones (and neurotransmitters of the nervous and neuroendocrine system) are essentially chemical messengers which may be delivered to the target tissues or cells by different routes such as; a) systemically as an endocrine factor, b) by local paracrine or autocrine routes and c) by neuronal delivery to specific target cells in the case of neurotransmitters. The study of endocrinology in fish has many complicating features, not least because of the vast diversity of species; hormone and receptor structure may display inter-specific differences reflecting variation in activity, potency and possibly even function. Added to this, the fact that many studies have employed mammalian or non-specific (heterologous) hormones in assays means that interpretation of results may prove difficult. That said, current understanding of the main aspects of hormonal actions in teleosts will be discussed.

1.3.1 Prolactin

Prolactin (PRL) is one of the prolactin family of hormones produced in the adenohypophysis of both elasmobranchs and teleosts. Prolactin has many functions in

vertebrate groups e.g. in mammals PRL stimulates milk production, hair growth, sebaceous gland growth and has gonadotropic effects (Withers, 1992), but it has, in addition, an important role in water and electrolyte balance in all vertebrates and an important role in the survival of euryhaline fish in FW.

Pickford and Phillips (1959) first established a role for PRL in the osmoregulation of FW-acclimated teleosts when they found that following hypophysectomy, *F.heteroclitus* could survive in FW only if injected with ovine PRL. Since then other studies have shown PRL acts by reducing ion and water permeability in the intestine of both SW *Anguilla japonica* (Utida *et al.*, 1972) and SW-acclimated *Oncorynchus mykiss* (Morley *et al.*, 1981). However , Mainoya (1982) found that in SW-acclimated *O.mossambicus*, where there is **decreased** uptake of NaCl in the anterior intestine (in contrast to other SW-acclimated teleosts), PRL produced the opposite effect and increased permeability of the epithelium to ions and water. Research has also shown that PRL exerts effects on the gill; branchial Na^+, K^+-ATPase activity was reduced when hypophysectomised FW-acclimated *F.heteroclitus* were treated with PRL (Pickford *et al.*, 1970a). Foskett *et al.*, (1982) demonstrated that injection of SW acclimated tilapia with PRL resulted in a dose-dependent reduction in net chloride secretion (short circuit current) and transepithelial potential in the opercular epithelium. Furthermore, when tilapia acclimated to 20% SW were injected with homologous PRL, the chloride cell population in the gill changed; β-type chloride cells (normally a characteristic feature of FW gill) appeared and α-type chloride cell sizes were reduced (Pisam *et al.*, 1993). During the smoltification process, plasma PRL levels in *Salmo salar* were also shown to decrease (Prunet *et al.*, 1989). Two forms of PRL have been identified in tilapia, $tPRL_{188}$ and $tPRL_{177}$, where the numbers

188 and 177 refer to the number of amino acid residues in each species, (Specker *et al.*, 1985) which share 73% homology and have different potencies; $tPRL_{188}$ is thought to induce increases in plasma Na^+ and Cl^- to a greater degree than $tPRL_{177}$ *in vivo* in brackish-water tilapia (Auperin *et al.*, 1994). Sakamoto *et al.*, (1997) demonstrated that $tPRL_{188}$ significantly reduced Na^+, K^+-ATPase activity levels in hypophysectomised tilapia at low doses (0.02μg/g) whereas $tPRL_{177}$ only reduced enzyme activity at higher doses (0.2-0.5 μg/g). To date only one PRL receptor has been identified (Prunet *et al.*, 1996)

1.3.2 Growth Hormone

As the name suggests, Growth Hormone (GH) has growth-promoting actions in fish as in all verterbrates and is another member of the PRL family of hormones produced by the adenohypophysis. In salmonids and other teleosts it has also been shown to contribute to osmoregulatory function (Sakamoto *et al.*, 1993; Sakamoto *et al.*, 1997; Mancera and McCormick, 1998).

Richmann and Zaugg (1987) demonstrated that in *Oncorynchus kisutch*, bovine GH increased gill Na^+, K^+-ATPase activity in both pre-smolts and smolts. Further to this Richmann *et al.*, (1987) reported that, in hypophysectomised *O.kisutch*, GH could partially restore SW tolerance although gill Na^+, K^+-ATPase activity was unchanged. In contrast, Bjornsson *et al.*, (1987) demonstrated that GH did restore gill Na^+, K^+-ATPase activity in hypophysectomised *O.kisutch.* More recent research has demonstrated that immature *O.mykiss*, exposed to SW and pretreated with ovine GH or ovine GH plus cortisol, had enhanced Na^+,K^+-ATPase activity and increased inter-lamellar chloride cell density, with the greatest effects being seen in those fish that

had cortisol plus GH (Madsen, 1990b). Similar effects were observed in *Salmo salar* parr and pre-smolts treated with GH (Prunet *et al.* 1994) and in tilapia (Sakamoto *et al.*, 1997) where homologous GH caused stimulation of gill Na^+,K^+-ATPase activity and improved the ability of the fish to decrease plasma osmolality on transfer to SW. GH stimulates production of insulin-like growth factor 1 (IGF-1) and the GH/IGF-1 axis is thought to contribute to osmoregulatory function in salmonids and other teleosts although the pathways have not been elucidated (McCormick, 1995; Sakamoto *et al.*, 1993; see Mancera and McCormick, 1998 for a review).

1.3.3 Cortisol

The major corticosteroid released by teleost interrenal tissue, cortisol, is involved in the stress response, energy metabolism and ion regulation. Cortisol in fish has both mineralocorticoid and glucocorticoid properties which are normally the function of aldosterone and cortisol respectively in terrestial vertebrates. Aldosterone has only been demonstrated to be present in small amounts in fish and appears to have no known physiological significance (Wendelaar Bonga, 1997). Release of cortisol is controlled, in part, by adrenocorticotropic hormone (ACTH) which is a component of the brain-pituitary-interrenal axis. Mayer *et al.*, (1967) found that cortisol, administered to adrenalectomised eels, reduced the levels of plasma Na^+ and Cl^- ions which had risen after the transfer of the eels to SW. Cortisol also increases Na^+, K^+-ATPase activity in; gill, kidney and intestine of *F.heteroclitus* (Pickford *et al.*, 1970), the gill and intestine of *A.rostrata* (Butler and Carmichael, 1971; Epstein *et al.*, 1971; Forrest *et al.*, 1973a) and the gill and intestine of *O.mykiss* (Madsen,

1990a). Morgan and Iwama (1996), however, did not detect any changes in branchial Na^+, K^+-ATPase activity in FW-acclimated *Oncorhynchus clarki clarki* after treatment with cortisol, but fish with intraperitoneal cortisol implants exposed to SW for 24hr did have increased Na^+K^+ATPase activity compared to sham-operated control fish and demonstrated improved ion regulatory ability compared to the controls. The size and number of chloride cells are also increased in response to cortisol (Foskett *et al*, 1981; Madsen, 1990c; Bindon *et al.*, 1994). In a study using the euryhaline thick-lipped mullet, *Chelon labrosus*, which have **greater** Na^+K^+ATPase activity in FW than SW, cortisol induced a marked decrease in branchial and renal Na^+, K^+-ATPase activity in short term FW-acclimated fish and a slight increase in branchial Na^+, K^+-ATPase activity in SW-acclimated fish (Gallis *et al.*, 1979). When *Anguilla rostrata* were transferred from FW to SW there was a rapid but transient increase in plasma cortisol levels which returned to FW levels after full SW adaptation (Forrest *et al.*, 1973b). McLeese *et al.*, (1994) found a seasonal increase in plasma cortisol and gill cortisol receptors during smoltification of *O.mykiss* which suggested a preparation for the migration to the SW environment. Although cortisol has traditionally been regarded as the so-called SW hormone, some evidence in recent years has pointed to a role for cortisol in FW adaptation. Laurent and Perry (1990) demonstrated that there was an increased branchial influx of Na^+ and Cl^- ions when FW *O.mykiss* were treated with cortisol and cortisol has been shown to contribute to hydromineral balance in both FW and SW-acclimated fish (Wendelaar Bonga, 1997).

1.3.4 The Renin Angiotensin System (RAS)

Present in all vertebrate groups, the RAS is involved in fluid and electrolyte balance and maintenance of blood volume and pressure. In teleost fish the RAS is thought to be important for controlling the drinking response in euryhaline species transferred to SW (Takei *et al.*, 1979; Fuentes and Eddy, 1997).

The RAS is a cascade which operates in the following way; renin, produced in kidney and other tissues, and acting locally or systematically, cleaves a protein substrate (angiotensinogen) to produce a 10 amino acid peptide, angiotensin I (Ang I). Angiotensin converting enzyme (ACE) then cleaves two amino acids from the carboxy-terminal of Ang I to produce the physiologically active angiotensin II (Ang II), which is subsequently cleaved by aminopeptidases between the first and second residues and between the second and third residues to produce angiotensin III (Ang III) and angiotensin IV respectively (Brown and Balment, 1997; Fuentes and Eddy, 1997). Activation of the RAS, and hence stimulation in drinking, is thought to occur as a result of a drop in blood pressure, demonstrated by studies using the muscle relaxant papavarine which induces hypotension (Balment and Carrick, 1983, Perrot *et al.*,1992; Tierney *et al.*, 1995a,b). Ang II has been shown to elicit a drinking response in several fish species; *F.heteroclitus* (Malvin *et al.*, 1980), *Carassius auratus* (Kobayashi *et al.*, 1983), *A.japonica* (Hirano and Hasegawa, 1984), *A.anguilla* (Perrot *et al.*, 1992) and *O.mykiss* (Fuentes and Eddy, 1996). Ang I is believed to be biologically inactive and Ang III, although non-dipsogenic in eels (Hirano and Hasegawa, 1984), was found to depress urine production in a perfused *in-situ* trout kidney (Brown and Balment, 1997).

1.3.5 Natriuretic Peptides

A recent development in the study of teleost endocrinology has followed the discovery of the natriuretic peptides. De Bold *et al.*, (1981) first described a factor in an extract from rat atrium which had both natriuretic and diuretic properties. The unknown agent was named atrial natriuretic factor and then subsequently atrial natriuretic peptide (ANP) once the structure of the peptide hormone was elucidated. Following on from these earlier studies it was apparent that ANP was a member of a small family of related peptides which have been identified in many vertebrate groups including teleosts and elasmobranchs. These peptides are released into the circulation but may also act locally in a paracrine manner. The four main types of natriuretic peptide described to date are; ANP which has been identified in several mammals, amphibians and in *A.japonica* (Takei *et al*, 1989; Takei, 1994), brain natriuretic peptide (BNP) which has been identified in mammals and birds but not in teleosts to date (Takei, 1994; Hazon and Balment, 1998), C-type natriuretic peptide (CNP) identified in mammals, teleosts and elasmobranchs (Takei, 1994; Hazon and Balment, 1998) and ventricular natriuretic peptide (VNP) which has been isolated only from teleosts (Takei, 1994; Hazon and Balment, 1998). Both ANP and VNP have also been reported to be synthesised in the intestine of *A.japonica* (Loretz *et al.*, 1997). The natriuretic peptides act by binding to guanylate cyclase-linked receptors on the cell membrane, generating the production of the intracellular second messenger, cyclic GMP, which will be discussed later in the text.

1.3.5. (i) ANP

ANP was first isolated in teleosts from eel atria (Takei *et al.*, 1989) where it was found to be in the highest concentration although detectable amounts were present in ventricle and brain. The major circulating form of ANP in plasma is a 3kDa, 27 amino acid (a.a.) form but it is synthesised as 14kDa preproANP (140 a.a.) before being processed to the mature form (Takei *et al.*, 1992). Early studies of the actions of ANP in teleosts employed heterologous peptides of mammalian origin which presented a conflicting picture of the possible actions of ANP; for example the biological activity of homologous ANP was found to be 100 times greater in eels than mammalian ANP. In mammals, ANP is known to promote both natriuresis and diuresis in the kidney and cause relaxation of vascular smooth muscle. With the use of homologous peptides and antibodies raised against eel ANP, the physiological functions of this peptide are beginning to be elucidated. When *A.japonica* is transferred from FW to SW there is a brief increase in plasma levels of ANP which then gradually decreases to FW levels (Kaiya and Takei, 1996b). A previous study on eels acclimated to SW for two weeks had not detected this transient rise in plasma ANP levels (Kaiya and Takei, 1996a). A further study indicated that the increase in plasma ANP was the result of cellular dehydration of osmoreceptive cells in the heart and, to a lesser extent, by increased blood volume (Kaiya and Takei, 1996c) both of which stimulated the release of ANP . Eel ANP was shown to decrease both NaCl (Ando *et al.*, 1992; Loretz and Takei, 1997) and water (Ando *et al.*, 1992) absorption across SW eel intestine and had a potency 100-fold greater

than human or rat ANP (Ando *et al.*, 1992). Rat ANP also inhibits ion absorption in the intestine of the winter flounder (O'Grady *et al.*, 1995). Loretz *et al*, (1997) reported that ANP is synthesised locally in eel intestine. Other reported effects of ANP in teleosts include; reduction in dorsal aortic blood pressure in FW eel (Oudit and Butler, 1995), a biphasic pressor/depressor response and diuresis in trout and eel (Olson and Duff, 1992; McKendry *et al.*, 1999) and stimulation of cortisol release in carp (Kloas *et al.*, 1994) and flounder (Arnold-Reed *et al.*, 1991). ANP has also been shown to inhibit the drinking response and decrease plasma Ang II levels in FW and SW-acclimated eels (Tsuchida and Takei, 1998) suggesting that ANP may inhibit part of the RAS. Conflicting reports exist as to the natriuretic and diuretic properties of ANP in teleosts; whilst some reports showed natriuresis and diuresis in trout administered with heterologous ANP (Duff and Olson, 1986), more recent work employing homologous ANP demonstrated that the peptide was antidiuretic in FW eels (Takei and Balment, 1993; Takei, 1994). Such contradictions may be due to differences between teleost species or the use of heterologous peptides, but nevertheless serve to highlight the importance of using specific homologous peptides to elucidate the physiological actions of hormones in teleosts actions.

1.3.5.(ii) VNP

VNP has been isolated from the ventricles of eel and rainbow trout (Takei *et al.*, 1991; Takei, 1994) but has not been found in mammals to date. VNP is a major

circulatory peptide in eels and plasma levels are three times higher than ANP (Takei, 1994). As with ANP, secretion of VNP appears to be regulated by both osmotic challenge (cellular dehydration of osmoreceptive cells in the heart) and blood volume but the increase in VNP levels after transfer of fish to SW was more marked than than the increase of ANP (Kaiya and Takei, 1996c). The biological activity of VNP mirrors that of ANP, causing antidiuresis and the inhibition of the drinking response (Takei and Balment, 1993).

1.3.5 (iii) CNP

The third type of natriuretic peptide found in teleosts is CNP, which has been isolated from the brain of *A.japonica* (Takei *et al.*, 1990) and *F.heteroclitus* (Price *et al.*, 1990). CNP has also been isolated from the hearts of four species of elasmobranch; *Scyliorhinus canicula* (Suzuki *et al.*, 1991), *Triakis scyllia* (Suzuki *et al.*, 1992), *Squalus acanthias* (Schofield *et al.*, 1991) and *Lamna ditropis* (Takano *et al*, 1994). To date CNP is the sole cardiac peptide isolated from elasmobranchs and has been shown to stimulate chloride secretion in the shark rectal gland (Solomon *et al.*, 1992). CNP is therefore thought to play a significant role in elasmobranch osmoregulation.

Current knowledge of natriuretic peptide activity, with particular reference to ANP, can be summarised as follows; when fish are transferred to SW, levels of ANP rise immediately in response to increased plasma osmolality and cardiac cell dehydration, causing an inhibition of the drinking response and decreased NaCl absorption in the

intestine, possibly to prevent salt overload. Urine volume decreases and hence urine Na^+ concentration rises and there is stimulation of cortisol secretion (Takei.Y. pers.comm.). The release of ANP is therefore an immediate short-term response to osmotic challenge; Kaiya and Takei, (1996c) reported that plasma ANP and VNP levels rose thirty minutes after *A.japonica* was injected with hypertonic NaCl , ANP then fell to pre-injection levels after three hours although plasma VNP remained elevated for twelve hours post-injection.

1.3.5 (iv) Natriuretic Peptide Receptors

The natriuretic peptide receptors were initially characterised in mammals and fall into two categories; those that contain an intracellular guanylate-cyclase catalytic domain and those that do not, the latter category being called "clearance receptors". Only two types of guanylate cyclase receptors, having different binding affinities for natriuretic peptides, have been reported in teleosts and are termed natriuretic peptide receptor A (NPR-A) which binds ANP and BNP, and natriuretic peptide receptor B (NPR-B) which binds CNP. The single isoform of the "clearance receptor" subclass is termed NPR-C. Both NPR-A and NPR-B have been cloned from eel (Katafuchi *et al.*, 1994; Kashiwagi *et al.*, 1999). NPR-A is present in gill, kidney, intestine and urinary bladder (Mishina and Takei, 1997; Kashiwagi *et al*, 1999) and NPR-B in gill, liver, heart, intestine and kidney of eels (Katafuchi *et al.*, 1994; Mishina and Takei, 1997). Levels of NPR-B mRNA were shown to decrease substantially in liver, heart and gill of two week SW-acclimated eels (Katafuchi *et al.*, 1994).

1.3.6 Arginine Vasotocin (AVT)

AVT is produced in the neurohypophysis of teleosts and belongs to the vasopressin-like peptide family (Hazon and Balment, 1998). AVT is the dominant vasopressin in non-mammalian groups and possesses antidiuretic and vasopressor properties (Sawyer, 1987). The role of AVT in teleost osmoregulation is unclear but there is evidence for a vasoconstrictive response in eel and flounder (Oudit and Butler, 1995; Warne and Balment, 1997) and an increase in cardiac output (Oudit and Butler, 1995). AVT in physiological doses produces antidiuresis in eel (Balment *et al.*, 1993) and trout (Brown and Balment, 1997). In addition, there is a transient increase in plasma AVT levels following transfer of eels to SW (Balment *et al.*, 1993).

1.3.7 Catecholamines and other Hormones

The catecholamines, epinephrine and norepinephrine, are synthesised mainly by the chromaffin tissue in the teleost head kidney. Plasma levels of catecholamines rise in response to stress and their actions are mediated by two categories of receptor (α and β) which themselves fall into sub-groups; $\alpha 1$, $\alpha 2$, $\beta 1$ and $\beta 2$ (Randall and Perry, 1992). Both catecholamines stimulate ion and water uptake in the intestine of SW eels (Ando and Omura, 1993) and chloride secretion has been shown to be inhibited by epinephrine in the opercular epithelium of *F.heteroclitus* (Degnan *et al.*, 1977) and in the skin of *Gillicthys mirabilis* (Marshall and Bern, 1980). This inhibition is thought to be mediated by $\alpha 2$ receptors (Foskett *et al.*, 1982). Other hormones with suggested roles in fish osmoregulation include the urotensins I and II, the thyroid hormones T_3 and T_4 but little work has been carried out on their functions (see Hazon and Balment, 1998 for discussion).

1.4 Cyclic Nucleotides

Both cyclic AMP (cAMP) and cyclic GMP (cGMP) have long been recognised as intracellular second messengers which regulate many intracellular reactions in response to a variety of stimuli. Levels of both cyclic nucleotides are raised in response to the binding of a variety of extracellular ligands (e.g. hormones) to cell surface receptors. In the case of cAMP, the extracellular ligand binds to a hetero-trimeric G-protein-linked receptor which in turn binds the G-protein that activates or inactivates the plasma membrane adenylate cyclase. The adenylate cyclase then catalyses the synthesis of cAMP from ATP (Alberts *et al.*, 1989; Rang *et al.*, 1995). The cellular responses mediated by cAMP as a result of hormonal stimuli are numerous and are brought about principally by the the activation of cAMP-dependent protein kinases which phosphorylate proteins by transferring the terminal phosphate on ATP to serine and/or threonine residues. The phosphorylation of these proteins acts to regulate their function e.g. the transport of ions (Aidley and Stanfield, 1996). The cGMP second messenger system is rather different to that of cAMP; the membrane associated and soluble guanylate cyclases are receptor molecules which contain both the extracellular ligand-binding domain and guanylate cyclase catalytic domain on the same protein and are directly activated to raise cGMP levels (for recent reviews on guanylate cyclases see Wedel and Garbers, 1997, 1998). Increases in cGMP can act directly to alter the function of proteins (i.e. ion channels) or indirectly via phosphorylation resulting from the activation of cGMP- dependent protein kinases. Both cAMP and cGMP have been shown to inhibit ion absorption in teleost intestine (Field *et al.*, 1980; Rao *et al*, 1984; Trischitta *et al.*, 1996) but cGMP is more potent (Trischitta *et al.*, 1996). In addition, stimulation of chloride

secretion associated with a rise in cAMP has been demonstrated in opercular epithelium of *F.heteroclitus* (Degnan, 1986) and *O.mossambicus* (May and Degnan, 1984).

1.5 The Guanylin peptide signal transduction system

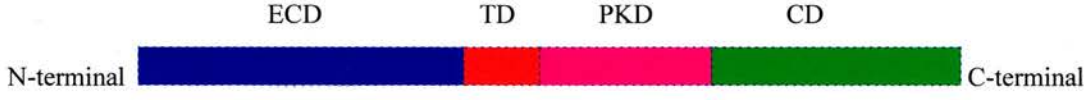
As previously described, cGMP is an important intracellular second messenger produced by receptor guanylate cyclase enzymes. The guanylate cyclases exist either as cytosolic soluble or membrane-associated forms; the soluble forms are activated by nitric oxide or carbon monoxide and the membrane forms are single pass integral membrane proteins which are activated by various endogenous peptides. However, several orphan receptors have been identified in recent years and research has indicated that the nematode *C.elegans*, has genes that may encode as many as 26 guanylate cyclases (Wedel and Garbers, 1997; Wedel and Garbers 1998). The endogenous peptide ligands that have been identified so far are the natriuretic peptide family, which have already been discussed, and the guanylin peptide family, a novel group of peptides which to date consists of three members; guanylin, uroguanylin and the most recently discovered lymphoguanylin. The guanylin peptide family and their common receptor are part of a regulatory signalling system, operating in a variety of tissues, which contribute to ion and water homeostasis in mammals (see Krause *et al.*, 1997 , Forte, 1999, Forte *et al.*, 2000 for comprehensive reviews) and these peptides will be discussed in section 1.5.2 below. Since the discovery of the receptor for the guanylin peptides preceded the discovery of the peptides themselves, the receptor will be discussed first.

In the majority of vertebrate literature the natriuretic peptide receptors are termed guanylate cyclase A (GC-A) and guanylate cyclase B (GC-B); whereas in teleost literature they are termed NPR-A and NPR-B respectively. The guanylin peptide receptor is named guanylate cyclase C (GC-C) and this terminology will be used throughout; in teleosts NPR-C refers to the natriuretic peptide "clearance receptor".

1.5.1 The Guanylin Receptor

The guanylin receptor (GC-C), first identified by Field *et al.* (1978) as an intestinal guanylate cyclase which was activated by *E.coli* heat stable enterotoxin (ST_a), was initially cloned and sequenced from rat intestinal mucosa (Schulz *et al.*, 1990). In common with other members of the membrane guanylate cyclase family, it has an N-terminal extracellular ligand-binding domain, a single-pass transmembrane domain, an intracellular protein kinase-like domain and a C-terminal cyclase catalytic domain (Schulz *et al.*, 1990; Wedel and Garbers, 1997). Fig. 1.6 shows a diagrammatic representation of membrane or particulate guanylate cyclase structure. Mann *et al.*, (1996) located the GC-C gene to mouse chromosome 6 and human chromosome 12. Initial binding studies with ST_a also revealed the presence of GC-C-like receptors in various epithelia of the North American opossum including the kidney, trachea, and gall bladder (Forte *et al.*, 1988; Forte *et al.*, 1989; Krause *et al.*, 1990), with the strongest signal occurring in the intestinal tract. Since then, studies have demonstrated that GC-C is expressed in the intestine of all mammals examined to date including man (Krause *et al.*, 1994) and in the intestine of birds and reptiles (Krause *et al.*, 1995; Krause *et al.*, 1997c). Preliminary binding studies have also indicated the

Fig. 1.6 Diagrammatic model of membrane guanylate cyclase structure showing domain sizes and positioning.



ECD = extracellular domain (~ 460 - 500 aa)

TD = transmembrane domain (~ 20 aa)

PKD = protein kinase-like domain (~300 aa)

CD = catalytic domain (~ 270 aa)

presence of a GC-C homologue in the intestine of fish (Forte *et al*, 1997a, meeting abstract), and a full length cDNA homologue of GC-C has been sequenced very recently from medaka, *Oryzias latipes*, embryos (Mantoku *et al.*, 1999). Schulz *et al.* (1992) found that GC-C was expressed in several rat tissues in addition to intestine, including airway epithelium, adrenal gland, brain and olfactory and tracheal mucosa. Studies using *in-situ* hybridisation techniques, binding studies with ^{125}I-labelled ST_a, and the polymerase chain reaction have shown that GC-C is present in highest density in cells at the basal portion of the intestinal villi and in the crypts of Lieberkuhn of the rat (Cohen *et al.*, 1992; Li and Goy, 1993) and other mammals (Krause *et al.*, 1994). Nandi *et al.*(1997), using a highly specific antibody, found a similar cellular distribution of GC-C in rat, pig and bonnet monkey and Swenson *et al.* (1996) found expression of GC-C in crypts and apical epithelium of mouse intestine using *in-situ* hybridisation.

Comparison of primary structures of GC-C between species such as pig (Wada *et al.*, 1994), human (De Sauvage *et al.*, 1991) and rat (Schulz *et al.*, 1990) show high homology between the catalytic and protein kinase-like domain with structural diversity between species ocurring mainly in the extracellular ligand-binding domain. GC-C appears to exist as a homotrimeric complex (Vandraager *et al.*, 1994) that may be activated by a conformational change upon binding of the ligand and a report by Scheving *et al.*, (1996) demonstrated that the aggregative properties of intestinal GC-C in rats could be modulated by fasting and that GC-C was redistributed to intracellular sites in fasted rats. Hasegawa *et al.*, (1999), using site-directed mutagenesis, protease digestion and the use of a labelled analogue of ST_a determined

that the ST_a binding site of GC-C was associated with residues 387-393, close to the transmembrane region.

The role of the protein kinase-like domain (PKLD) remains uncertain but in the case of GC-A the PKLD contains an ATP-binding site and ATP binding has been shown to be necessary for catalytic activity (Kurose *et al.*, 1987). If the PKLD is deleted then GC-A is constitutively active suggesting a negative regulatory role for PKLD via the binding, but not hydrolysis of, ATP (Chinkers and Garbers, 1989; Wedel and Garbers, 1998). In GC-C however, the PKLD has no ATP-binding site (Schulz *et al.*, 1990) and ST_a activation of GC-C occurs in the absence of ATP, although ATP has been shown to potentiate ST_a activation of GC-C (Vandraager *et al.*, 1993; Bhandari *et al.*, 1999).

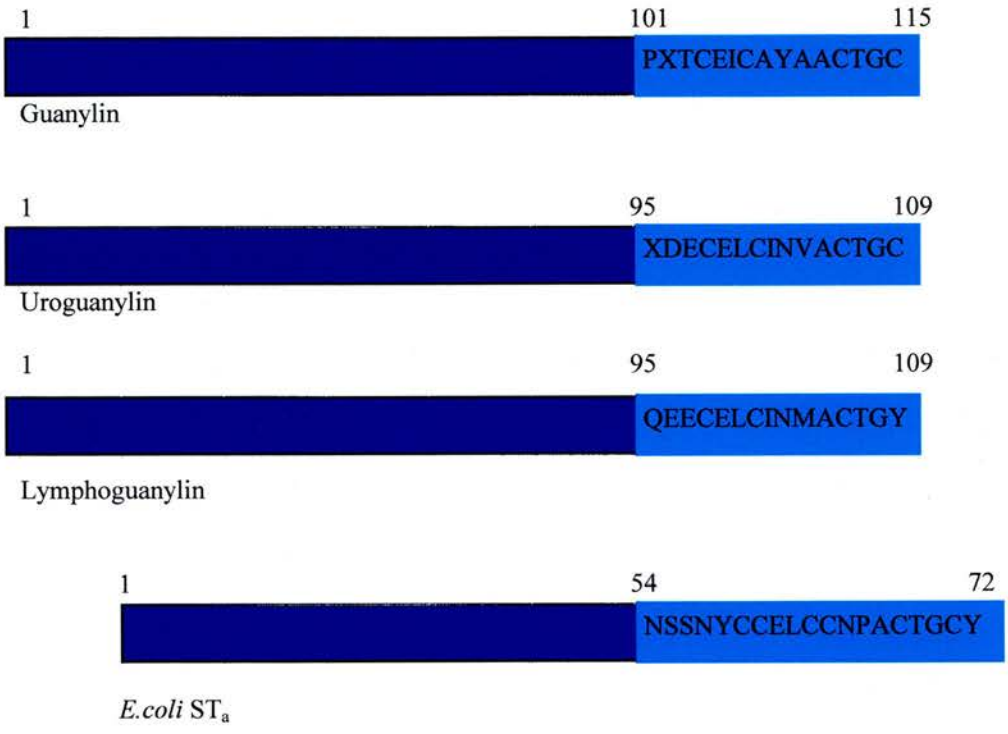
Whilst GC-C has been shown to bind ST_a and the guanylin family of peptides, some data have pointed to the presence of other additional receptors which also bind these ligands; Mann *et al.*, (1993) reported an ST_a receptor in IEC-6 rat intestinal cells that exhibited no guanylate cyclase activity and intestine of GC-C knockout mice contained binding sites for ST_a (Mann *et al.*, 1997), although binding was not associated with any stimulation of fluid secretion. Scheving and Chong (1997) however, demonstrated that proteolytic processing of GC-C occurs along the villus to crypt axis in the intestine, which could in part explain data that suggest the presence of other receptors; proteolysis may cleave off the guanylate cyclase catalytic domain from the rest of the protein thereby abolishing the activity of the receptor but not its ability to bind the ligand. Recently, an opossum kidney guanylate cyclase has been identified (London *et al.*, 1999) by molecular cloning which is activated by ST_a and the guanylin peptides. This receptor has 90% a.a. homology to the GC-C molecule at

the cyclase catalytic domain but only 50% a.a. homology within the ligand binding domain and may represent a new member of the guanylate cyclase receptor family (London *et al.*, 1999).

1.5.2 The Guanylin Family of Peptides

The discovery of the guanylin peptide (Currie *et al.*, 1992) followed on from previous research which demonstrated that ST_a produced by *E.coli* (a major cause of "Traveller's diarrhea" and infant mortality in developing countries) stimulated cGMP by selectively targetting an intestinal guanylate cyclase (Field *et al.*, 1978). The resulting rise in cGMP stimulates the secretion of fluid and electrolytes. Subsequently, the discovery that receptors for St_a (i.e. GC-C) were present in several other opossum tissues (see above) led researchers to hypothesise that there must be an endogenous activator of this guanylate cyclase since these other tissues would not be exposed to ST_a under normal circumstances. Currie *et al.*, (1992) employed the T84 cell line from human colon, which responds sensitively to ST_a but not to the natriuretic peptides or nitric oxide, for use in an assay with purified extracts from rat jejunum. These extracts elicited a cGMP response in the T84 cells and a 15 amino acid peptide was then isolated and purified which they termed guanylin. The second member of the guanylin peptide family, uroguanylin, also an activator of GC-C, was isolated from opossum urine (Hamra *et al.*, 1993) and has a different primary structure to guanylin. Within the last year a third member of the guanylin family, lymphoguanylin, has been identified in lymphoid tissues of the opossum (Forte *et al.*, 1999) - see Fig. 1.7 for sequences of the active peptides and structures of the precursors of the guanylin peptides and St_a.

Fig. 1.7 General structures of guanylin peptides and ST_a showing active peptide at C-terminal where X represents a variable amino acid.


1
101
115
PXTCEICAYAACTGC
Guanylin
1
95
109
XDECELCINVACTGC
Uroguanylin
1
95
109
QEECELCINMACTGY
Lymphoguanylin
1
54
72
NSSNYCCELCCNPACTGCY
E.coli ST a
+
= preprohormone
= active peptide sequence

1.5.2.(i) Structure and Expression of Guanylin Peptides

1.5.2. (i). (a) Guanylin

After the initial identification of guanylin by Currie *et al.*, (1992), a precursor molecule, preproguanylin, was identified from a rat jejunum cDNA library (Wiegand *et al.*, 1992). This and subsequent studies in human and mouse (De Sauvage *et al.*, 1992) demonstrated that the preproguanylin molecule comprises 115-116 a.a., containing the active 15 a.a. guanylin molecule at the C-terminal end. The active guanylin molecule has four cysteine residues at positions 4, 7, 12 and 15 which are capable of forming disulphide bridges; cGMP studies and binding assays have shown that only guanylin with the 4-12 and 7-15 conformation of disulphide bonds is biologically active (Nokihara *et al.*, 1997). Comparison of the active peptide sequences from all known species reveals a high degree of homology although the remainder of the preproguanylin sequence is highly diverse across the range of species. Expression studies have shown the presence of guanylin mRNA in rat intestine, adrenal gland, kidney and uterus (Schulz *et al*, 1992), in human intestine (De Sauvage *et al.*, 1992) and in opossum intestine, kidney, spleen and testis (Fan *et al.*, 1997). The presence of guanylin-like peptides has also been demonstrated in reptilian intestine (Krause *et al.*, 1997c). The expression of guanylin is optimal in the intestine with highest mRNA levels found in ileum and colon of both humans and rats (Wiegand *et al*, 1992a,b).

Some controversy exists over the exact cellular site of guanylin expression in the intestine; Cetin *et al*, (1994) reported it to be in the enterochromaffin cells of guinea pig intestine but other workers found it to be mainly in the goblet cells of rat intestine

(Cohen *et al.*, 1995; Li *et al.*, 1995) whereas De Sauvage *et al.*, (1992) located expression in the Paneth cells of human intestine.

The human guanylin gene, located on chromosome 1, and named GCAP-I has been characterised and shown to consist of 3 exons and 2 introns with a glucocorticoid response element present in the first intron (Hill *et al.*, 1995a). The presence of the glucocorticoid response element suggests a possible role for glucocorticoids in regulating guanylin expression.

1.5.2. (i). (b) Uroguanylin

After Hamra *et al.*, (1993) isolated uroguanylin from opossum urine as a 15 a.a. peptide, subsequent investigation showed that like guanylin, it is produced as a precursor molecule, preprouroguanylin. The preprohormone comprises 112 a.a. in humans (Miyazato *et al.*, 1996) and 109 a.a. in opossum (Fan *et al.*, 1996), with the active peptide being located at the C-terminal (see Fig. 1.7).The human active uroguanylin peptide is 16a.a. whilst in other species it is 15 a.a. In common with guanylin the C-terminal active peptide sequence displays the highest homology between species. Uroguanylin also contains four cysteine residues which form disulphide bonds in the same pattern as guanylin (Hamra *et al.*, 1993; Hidaka *et al*, 1998).

Expression studies have located uroguanylin mRNA in human colon, stomach, gall bladder, lung and pancreas but not kidney (Hill *et al*, 1995b; Magert *et al*, 1998), whereas in the rat, although expression is found in proximal intestine and in rat lung, pancreas and kidney (Miyazato *et al.*, 1996) it is virtually absent from colon (Li *et al.*, 1997). The intestinal cellular location of uroguanylin has been reported to be

enterochromaffin cells (Perkins *et al.*, 1997; Magert *et al.*, 1998; Nakazato *et al*, 1998), which could explain the observation of Cetin *et al*, (1994) who found this to be the site of guanylin expression; it is possible that the anti-guanylin antibodies used may have cross-reacted with uroguanylin in these cells (Forte, 1999). Magert *et al.*, (1998) also demonstrated storage of uroguanylin in gastrointestinal D-cells. In patients with chronic renal failure, the major plasma and urine form of uroguanylin is the prohormone form (Nakazoto *et al.*, 1996) raising the suggestion that under normal circumstances the kidney would process the pro-uroguanylin to the mature uroguanylin but that this process is impaired with the onset of kidney disease.
The human uroguanylin gene (GCAP-II) is localised on the same chromosome as guanylin, chromosome 1, and has 3 exons and 2 introns (Magert *et al.*, 1998).

1.5.2.(i).(c) Lymphoguanylin

The most recent member of the guanylin family to be identified is lymphoguanylin which was isolated from lymphoid tissues of the opossum (Forte *et al.*, 1999). Whilst both uroguanylin and guanylin have four cysteine residues in the active peptide, lymphoguanylin has only three and hence is only capable of forming one disulphide bond (Forte *et al.*, 1999) although no information is available on the conformation of the bonds to date. Synthetic lymphoguanylin was found to elicit a cGMP response in T84 cells and opossum kidney cells (Forte *et al.*, 1999) but was less potent than either guanylin or uroguanylin.

1.5.2.(ii) Activity of Guanylin and Uroguanylin

As previously described, the guanylin peptides are produced as preprohormones before being released as prohormones which are biologically inactive (Schulz *et al*, 1992; Hamra *et al*, 1996;), but after treatment with extracellular proteases they bind to their specific receptors and stimulate a cGMP response (Hamra *et al.*, 1996). The guanylin peptides have similar actions to ST_a in the intestine, i.e. they stimulate secretion of Cl^- and HCO_3^- ions which in turn drives the secretion of Na^+ and water (Currie *et al.*, 1992; Forte *et al.*, 1993; Kita *et al.*, 1994; Guba *et al.*, 1996; Hamra *et al.*, 1997). Whilst the secretion of these ions is known to be mediated by cGMP, the exact cellular mechanisms are unclear. The consensus view is that activation is associated with the activation of a cGMP-dependent protein kinase II (PKG-II) which is localised mainly in brain, lung and intestinal mucosa and/or cAMP-dependent protein kinase (PKA) (Markert *et al.*, 1995; Pfeifer *et al.*, 1996; Lohmann *et al.*, 1997; Forte, 1999). Protein kinase I (PKG-I) is a cGMP-dependent protein kinase which is expressed in the smooth muscle cells. Activation of PKG-II then leads to phosphorylation of the apically-located CFTR, the cystic fibrosis transmembrane conductance regulator, leading to the secretion of Cl^- and HCO_3^- (Cuthbert *et al.*, 1994; Guba *et al.*, 1996). Fig.1.8 shows a model for the actions of the guanylin peptides and the cellular pathways involved. However, it has been found that the small intestine of CFTR knockout mice still retain a substantial, but reduced, response to uroguanylin (Joo *et al.*, 1998) but not guanylin (Cuthbert *et al.*, 1994). These mice were found to express a functional chloride channel (CLC-2) which could provide an alternative pathway for uroguanylin stimulation of ion secretion (Joo *et al.*, 1999; Forte,1999).

Studies have shown that the effects of guanylin and uroguanylin are influenced by extracellular pH; in assays employing T84 cells as a model epithelium guanylin was more effective in stimulating accumulation of cGMP than uroguanylin at pH 8.0 but uroguanylin was more effective than guanylin in the acidic range pH 5.0-6.0 (Hamra *et al.*, 1997). Interestingly, ST was more effective than either across the range of pH values so is clearly not affected by this aspect of extracellular environment (Hamra *et al.*, 1997). Extracellular acidic pH was shown to decrease the binding capacity of guanylin to the receptors and, conversely, increase the binding capacity of uroguanylin (Hamra *et al.*, 1997). Hamra *et al.*, (1997) suggest that the two acidic residues at the N-terminus of uroguanylin are a requirement for binding of the peptide to the receptor under acidic conditions as these conditions may influence the conformational state of uroguanylin. This difference of binding capacities in response to pH confers a degree of flexibility and implies that there is an advantage in having two peptides that bind under different conditions and would increase the range of activity of the guanylin family in salt and water regulation (Forte, 1999). The profile of expression of the two peptides, as discussed earlier, may also reflect the pH range present along the length of the GI tract i.e.in the acidic environment of the proximal small intestine, uroguanylin will have a greater binding capacity for the GC-C receptor and can act to regulate bicarbonate secretion which will contribute to acid/base balance. In contrast, guanylin will be effective in regions where the environment is more alkali. Similarly, the pH microclimate varies along the villus to crypt axis and this will inevitably affect the activities of guanylin and uroguanylin.

Both prohormones of guanylin and uroguanylin are found in the circulation (Kuhn *et al.*, 1993; Hess *et*

al., 1995; Fan *et al.*, 1996), but whilst the major circulating form of guanylin is the inactive

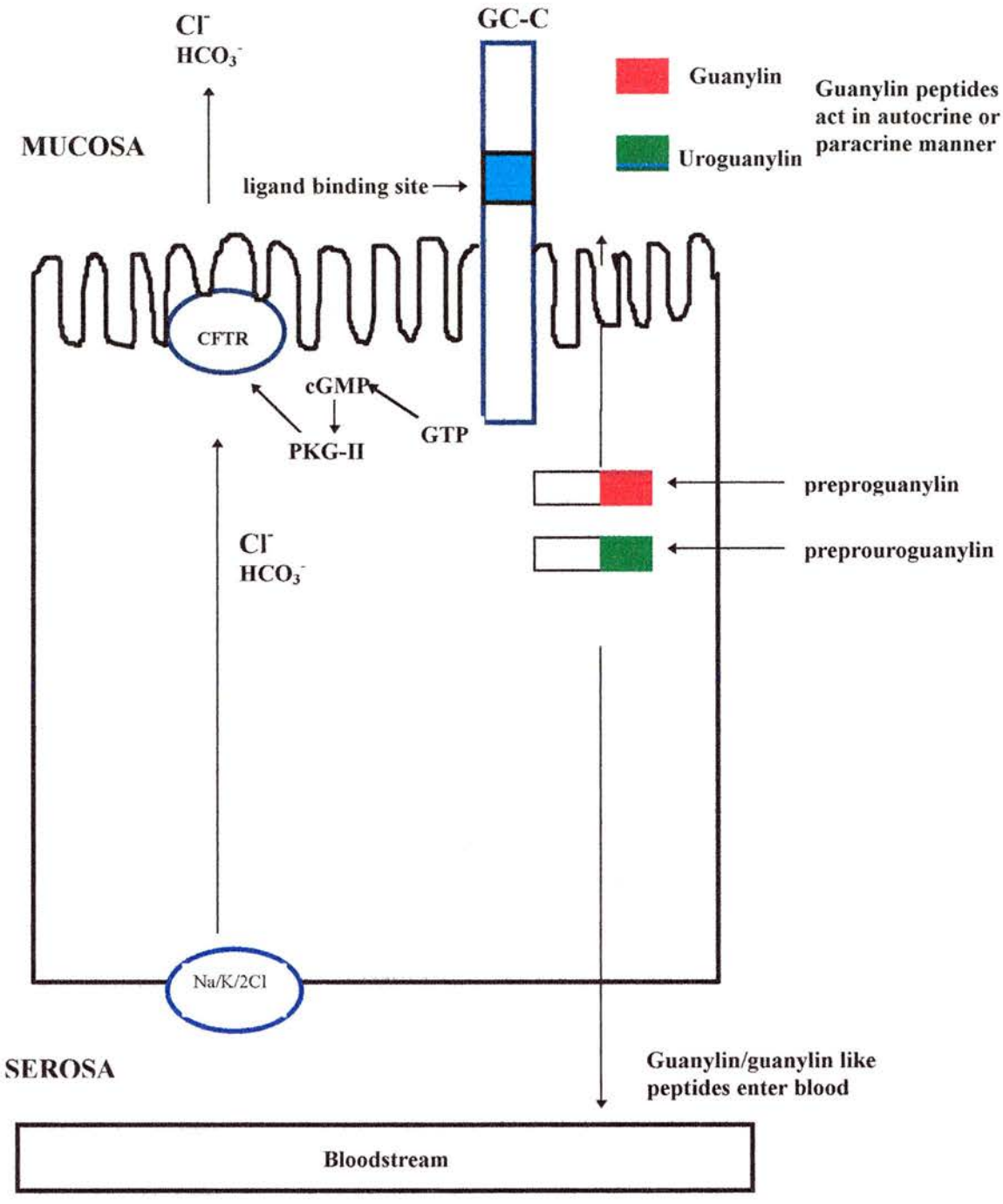
prohormone, both prouroguanylin and active uroguanylin are

Studies have shown that the effects of guanylin and uroguanylin are influenced by extracellular pH; in assays employing T84 cells as a model epithelium, guanylin was more effective in stimulating accumulation of cGMP than uroguanylin at pH 8.0 but uroguanylin was more effective than guanylin in the acidic range pH 5.0-6.0 (Hamra *et al.*, 1997). Interestingly, ST_a was more effective than either in the pH 5.0 - 8.0 range so is clearly not affected by this aspect of extracellular environment (Hamra *et al.*, 1997). Extracellular acidic pH was shown to decrease the binding capacity of guanylin to the receptors and, conversely, increase the binding capacity of uroguanylin (Hamra *et al.*, 1997). Hamra *et al.*, (1997) suggest that the two acidic residues at the N-terminus of uroguanylin are a requirement for binding of the peptide to the receptor under acidic conditions as these conditions may influence the conformational state of uroguanylin. This difference of binding capacities in response to pH confers a degree of flexibility and implies that there is an advantage in having two peptides that bind under different conditions and would increase the range of activity of the guanylin family in salt and water regulation (Forte, 1999). The profile of expression of the two peptides, as discussed earlier, may also reflect the pH range present along the length of the GI tract i.e.in the acidic environment of the proximal small intestine, uroguanylin will have a greater binding capacity for the GC-C receptor and can act to regulate bicarbonate secretion which will contribute to acid/base balance. In contrast, guanylin will be effective in regions where the environment is more alkali. Similarly, the pH microclimate varies along the villus to crypt axis and this will inevitably affect the activities of guanylin and uroguanylin. Both prohormones of guanylin and uroguanylin are found in the circulation (Kuhn *et al.*, 1993; Hess *et al.*, 1995; Fan *et al.*, 1996), but whilst the major circulating form of

guanylin is the inactive prohormone, both prouroguanylin and active uroguanylin are present in plasma (Hess *et al.*, 1995; Fan *et al.*, 1996). This, together with reports that uroguanylin stimulated Na^+, K^+ and Cl^- secretion in perfused rat kidney (Fonteles *et al.*, 1998), and stimulated Na^+, and K^+ secretion and water loss when administered intravenously to mice (Greenberg *et al.*, 1997), support the hypothesis that uroguanylin is part of an endocrine pathway that links the intestine and kidney to regulate ion and water homeostasis (Forte, 1999), whereas guanylin was either far less potent or exhibited no effect. Kinoshita *et al.*, (1997a) reported elevated urine levels of uroguanylin in patients on high salt diet and that levels of uroguanylin in the urine correlated proportionately with excretion of Na^+, K^+ and Cl^-. A recent study by Furuya *et al.* (1998) showed that intravenous injection of guanylin caused a rapid induction of mucous secretion in rat crypt goblet cells and transient oedema in the intestinal mucosa suggesting that there may be an endocrine role for circulating guanylin.

Expression of guanylin has also been detected in bronchiolar non-ciliated secretory cells - termed CLARA cells - (Cetin *et al.*, 1995) suggesting a paracrine mechanism for guanylin regulation of electrolyte and water transport in the lung. This is supported by Zhang *et al.* (1998) who demonstrated a guanylin-induced outwardly rectifying chloride current in human bronchial epithelium.

Fig. 1.8 Model of suggested cellular mechanisms of guanylin peptide activity in the intestine. Adapted from Krause *et al.*, (1997 d).


Cl⁻
HCO3⁻
GC-C
Guanylin
Uroguanylin
Guanylin peptides act in autocrine or paracrine manner
MUCOSA
ligand binding site
CFTR
cGMP
PKG-II
GTP
preproguanylin
preprouroguanylin
Cl⁻
HCO3⁻
Na/K/2Cl
SEROSA
Guanylin/guanylin like peptides enter blood
Bloodstream

Many aspects of guanylin peptide regulation and function have yet to be clarified using modern molecular techniques. Together with the study of disease pathology and the growing use of transgenic animals these investigations will all contribute to the understanding of this regulatory signalling system.

1.6 Research Project Aims

The primary aim of this research project was to investigate the presence of a guanylin/guanylate cyclase C signalling system in the euryhaline European eel. Since this system contributes to ion and water homeostasis in mammals it was postulated that should such a system exist in teleost fish, it may have an important role in osmoregulation. A euryhaline species such as the eel, which can survive a range of salinities, is a valuable model organism to study any changes in gene expression which may occur in response to the environment, particularly with regard to proteins involved in osmoregulation. The objectives of this project were; a) to use molecular methods based on the polymerase chain reaction (PCR) to detect the presence of guanylin and GC-C in RNA extracted from the osmoregulatory tissues of both yellow and silver, FW and SW-acclimated eels, b) to clone and sequence PCR products obtained from complementary DNA (cDNA) synthesised from these RNA samples c) to investigate possible changes in expression levels of these genes between the four experimental groups using Northern Hybridisation d) to raise antibodies to the receptor molecule for subsequent use in immunological studies such as Western blotting to assess the levels of expression of this protein in various tissues under different developmental/salinity acclimated states.

Chapter 2

Methods

2. GENERAL METHODS

Yellow and migrating silver eels (250-750 g,) from the River Tay catchment area (Lunan Burn and Butterstone Loch) were obtained from private suppliers in Blairgowrie, Perthshire. The eels were maintained without feeding in a 12 hour light/dark cycle at ambient temperature, in tanks at the Gatty Marine Laboratory , St Andrews. After transport to the Gatty Marine Lab the eels were placed in freshwater tanks, to recover from any stress, for at least 4 days before being transferred to either fresh water (FW) or seawater (SW) for three weeks. Previous experiments showed that three weeks was a sufficient time for both yellow and silver eels to become fully acclimated to SW. The FW to FW transfers were controls to ensure that any changes were not brought about by handling stress. Yellow eels are sexually undifferentiated but male and female silver eels were used in the experiments. Addresses for suppliers of equipment and materials are listed in Appendix 1 and sequences / sites of all primers - including those supplied with commercial kits - are listed in Appendix 2. Unless otherwise stated all chemicals were Analar® grade supplied by BDH Laboratory supplies, Poole, Dorset or Sigma Aldrich Ltd. Poole, Dorset

2.1 Reverse transcription polymerase chain reaction (RT-PCR) - overview

The method of RT-PCR was employed to detect expression of genes in various tissues (gill, intestine and kidney) of yellow and silver eels adapted to either FW or SW . Total RNA was extracted from tissues of interest and complementary DNA (cDNA) synthesised from the endogenous messenger RNA (mRNA) template using an oligo dT primer, which hybridises to the poly A tail of mRNA, and the addition of reverse transcriptase enzyme and deoxy nucleotide triphosphates (dNTPs).The resulting cDNA therefore represented genes which were expressed in the tissues of interest. To

determine whether a particular gene was expressed and therefore present in this representative cDNA, degenerate PCR primers were designed. Known amino acid sequences determined from the genes across a variety of species (deposited in the GenBank database) were compared to elucidate the most highly conserved regions - this indicated that amino acid sequences within these same genes from the eel may also be conserved. Degenerate sense and anti-sense primers , i.e. those containing nucleotide wobbles or inosine residues to accommodate the degeneracy of the genetic code, were then synthesised and used in PCR with cDNA from various eel tissues. Inosine is an analogue of guanine and may be used on its own, or in conjunction with cytosine, at positions where the codon sequences are unknown. Products of these PCRs were loaded onto agarose gels containing ethidium bromide and, after electrophoresis, viewed under UV light to detect whether amplified fragments of the expected size were present.

2.1.1 Isolation of total RNA

Total RNA was isolated by a modification of the high salt precipitation method of Chomzynski and MacKay (1997). At all times surgical gloves were worn and autoclaved or disposable equipment used to prevent contamination by RNases which would destroy the RNA. Tissues to be investigated were dissected as rapidly as possible from eels and homogenised, using a Polytron PT.10 probe 9 (Kinematica Ltd.) set at speed 4-5 for 20-30sec., in Solution 'D'- 4M guanidinium isothiocyanate, 25mM sodium citrate, 0.5% sarkosyl (v/v) and 0.1M beta-mercaptoethanol in diethylpyrocarbonate-treated H_2O (DEPC) in the proportion of 5ml solution per 1g of tissue. The addition of DEPC to the H_2O destroys any endogenous RNases that may

be present and so ensures that RNA will not be degraded. In the case of gill and intestine, epithelial scrapings were taken. Homogenates could either be processed immediately or stored at - 20^0C. Processing of the denatured extract was continued by the addition of the following solutions :-2M sodium acetate (pH 4) (0.5ml per 1g of tissue) , water-saturated phenol (2.5ml per 1g of tissue) , chloroform:isoamyl alchohol (24:1 v/v) (1ml per 1g of tissue).

Tubes were vortexed briefly between the addition of each solution. After the three reagents were added the tubes were centrifuged at 3,954g for 30 min. at 4°C in the Beckman J6-MC centrifuge, rotor 4.2 (Beckman Instruments Inc.). After centrifugation, the aqueous phase was carefully transferred to a fresh tube, and then 2.5 vol. of 2-propanol and 0.2 vol. of high salt buffer (1.2M NaCl , 0.8M sodium citrate pH 7) was added sequentially with vortexing. The resulting solution was incubated at room temperature for 10 min. before centrifugation at 3,954g for 20 min. The supernatant was poured off and the pellet washed twice in 80% ethanol before drying under vacuum at room temperature for 5 min. After resuspension of the pellet in DEPC water, diluted samples (1:100) were prepared and the absorbance was measured at 260nm and 280nm using a spectrophotometer (PU 8620 UV/VIS/NIR, Philips) to determine both the concentration and purity of the samples. RNA samples from each extract were also run on a denaturing formaldehyde gels and stained with ethidium bromide (see below) to ensure that no degradation of the RNA had occurred.

2.1.2 RNA Denaturing Agarose Gel

RNA denaturing gels - 1.2% agarose w/v (Biogene Ltd.), 1X MOPS (20 mM 3-[N-Morpholino]-propanesulphonic acid, 8mM sodium acetate, 1mM EDTA) and 6.7% formaldehyde (v/v) - were prepared then poured into a 11cm X 14.9cm gel rig (Scotlab Ltd.) containing the most appropriately sized comb and allowed to set. Samples were diluted in sample denaturing buffer; 5µg RNA in 50 % formamide (v/v), 6.7% formaldehyde (v/v), 1X MOPS (final volume 40µl). The samples were denatured at 65^0C for 15 min. and snap cooled on ice before adding 0.1 vol of 5% "Loading Dyes" (0.025% bromophenol blue, 0.025% xylene cyanol and 50% glycerol; all w/v) and loaded onto the gel. The samples were electrophoresed at 135 volts (5 volts/cm) (Pharmacia EPS 500/400 powerpack, Pharmacia Ltd.) for 1.5 - 2 hours in 1X MOPS. After electrophoresis, gels were washed in 0.1M sodium acetate in ethidium bromide solution (0.1M ammonium acetate plus 1µg/ml ethidium bromide) for 45 min. then destained in 0.1M ammonium acetate for 1-2 hours before viewing on the UV transilluminator (UVT-20M Trans-luminator, Herolab).

2.1.3 Synthesis of single-stranded complementary DNA (cDNA)

Synthesis of cDNA was carried out by reverse transcription using total RNA extracted from tissues of yellow and silver FW and SW eels.

Synthesis of cDNA was carried out by adding 5µg of the appropriate total RNA and 2µl of 0.2 mM oligo $dT_{12\text{-}18}$ primer (Pharmacia Ltd.) to a 0.2ml microfuge tube (the volume was made up to 5µl with DEPC H_2O if necessary) and then incubating at 70°C for10 min. in the thermal cycler (Techne Progene, Techne Ltd.) before snap-cooling on ice and centrifuging briefly at room temperature at 11,336g in a bench top

microfuge (Biofuge A, Heraus Christ). Following this, 15µl of a reaction mix comprising First Strand Buffer (75mM KCl, 3mM $MgCl_2$, 50mM Tris-HCl, pH 8.3 Gibco BRL Life Technologies Ltd. UK) , 10mM DTT (Dithiothreitol) , 1mM dNTPs (Promega Corporation UK) and 200 units of SuperScript II™ Reverse Transcriptase (GIBCO BRL Life Technologies Ltd.) was added, with further mixing, before incubating at 45°C in the thermal cycler for 4-5 hours. The resulting cDNA was then stored at -20°C for future use.

2.1.4 Polymerase Chain Reaction (PCR)

The PCR technique is a process whereby denatured template DNA is amplified many times by the binding, at homologous regions on the template strands, of sense and antisense primers and the subsequent synthesis of complementary strands in the presence of thermostable DNA polymerase and deoxynucleotide triphosphates (dNTPs). Since amplification of the original template occurs in an exponential manner, large numbers of identical DNA molecules can be generated. Complementary DNA (cDNA), synthesized as described, was used as the template in standard PCR in conjunction with degenerate primers (Severn Biotech Ltd.or MWG Biotech Ltd.) designed to homologous regions within the mRNA of interest (see Appendix 2 for primer sites and sequences). In the initial stages of the project only mammalian sequences of GC-C and guanylin were available in the database so highly conserved regions were chosen from these sequences, the rationale being that these sequences would also be conserved in teleosts if a guanylin / GC-C signal transduction system was present. Towards the end of the project, a zebrafish guanylin sequence derived from a cDNA library was deposited in the GenBank database and a

medaka fish homologue of GC-C was published (Mantoku *et al.*, 1999) which enabled the design of additional primers. Figs. 2.1 and 2.2 show the primer sites selected from guanylin and GC-C sequences respectively of several vertebrate species.

All reagents were defrosted on ice and a 20μl reaction mixture was set up in a microfuge tube; 0.5μl of cDNA and 1μl each of appropriate sense and antisense primer at 100μM (final concentration 5μM) were pipetted into a microfuge tube before setting up a "master mix" of the other reagents (if more than one cDNA and/or primer set were being tested). It should be noted that in cases where specific, homologous primers were used, the stock primers were at a concentration of 4μM (final concentration 0.2 μM). A master mix was prepared by multiplying the appropriate quantities of each reagent by the number of reaction tubes plus one; final concentrations were 200μM dNTP mix,1X PCR Buffer (20mMTris-HCl pH 8.4, 50mM KCl, 1.5mM $MgCl_2$; Amersham Pharmacia Biotech Ltd.) and 0.625 units/20μl of *Taq* DNA polymerase (Amersham Pharmacia Biotech Ltd or BiogeneLtd). Preparation of a master mix reduced the risk of pipetting errors. Samples were then placed in the thermal cycler (Techne PHC-3 or Techne Progene, Techne Ltd.) to complete the PCR using the following typical parameters; an initial incubation at 92°C for 2 min., followed by 40 cycles of 94°C for 4sec., 52-55°C for 30sec. and 72°C for 0.5 - 2 min (depending on the expected fragment size; 1 min. extension time for every 1.0 kb of the fragment) before a final extension time of 10 min. at 72°C. PCR annealing temperatures varied depending on the primers; some degenerate primer pairs required lower temperatures to accomodate nucleotide mismatches.

Fig. 2.1 a) Sites chosen for the design of degenerate primers to amplify guanylin cDNA. The red box represents the degenerate sense primer, Guanylin sense, and the blue represents the degenerate Guanylin anti primer (see Appendix 2). **Fig. 2.1 b)** demonstrates the sites chosen from eel sequence data for the design of specific primers used in 5′ and 3′ RACE PCR, where 1 is the 5′ RACE 1 primer and 2 and 3 are 3′ RACE 1 and 3′ RACE 2 respectively.

a)


1
Zebrafish part guanylin AA seq --AFLVVALCLV------CDAVEVKEGDFSFTLESVRILQQLAEQQKT
Guinea Pig guanylin AA seq MNTFLLSALCL-GAWAALVGAVTVQDGDFSFSLESVKKLKDLQEAPES
Human guanylin AA seq MNAFLLFALCLLGAWAALAGGVTVQDGNFSFSLESVKKLKDLQEPQEP
Mouse guanylin AA seq MNACVLSVLCLLGALAVLVEGVTVQDGDLSFPLESVKKLKGLREVQEP
Pig guanylin AA seq MNTFLFPTLCLLGVWAALAGGVTVKDGEFSFSLESVKKLKDLQELQKP
Zebrafish part guanylin AA seq Q-------NPRLAKTSYYSVCS-NPSLPQEFVPLCMQRGATMSFARLA
Guinea Pig guanylin AA seq KVQGRRKF-------VAPPLCSF-SGFPEELRPVCKEPNSQDILNRLA
Human guanylin AA seq RVGKLRNFAPIPGEPVVPILCSN-PNFPEELKPLCKEPNAQEILQRLE
Mouse guanylin AA seq RLVSHKKFAPRLLQPVAPQLCSSHSALPEALRPVCEKPNAEEILQRLE
Pig guanylin AA seq R--NPRNL----DGPIIPVLCNS-PKFPEELKPICQKPNAEEILERLE
1
Zebrafish part guanylin AA seq SVPVD--VCEICAFAACT----------GC
Guinea Pig guanylin AA seq VIAQDPSTCEICAYAACA----------GC
Human guanylin AA seq EIAEDPGTCEICAYAACT----------GC
Mouse guanylin AA seq AIAQDPNTCEICAYAACT----------GC
Pig guanylin AA seq TIAQDPSTCEICAYAACA----------GC

b)

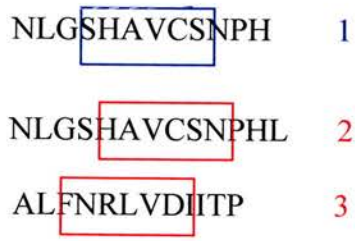
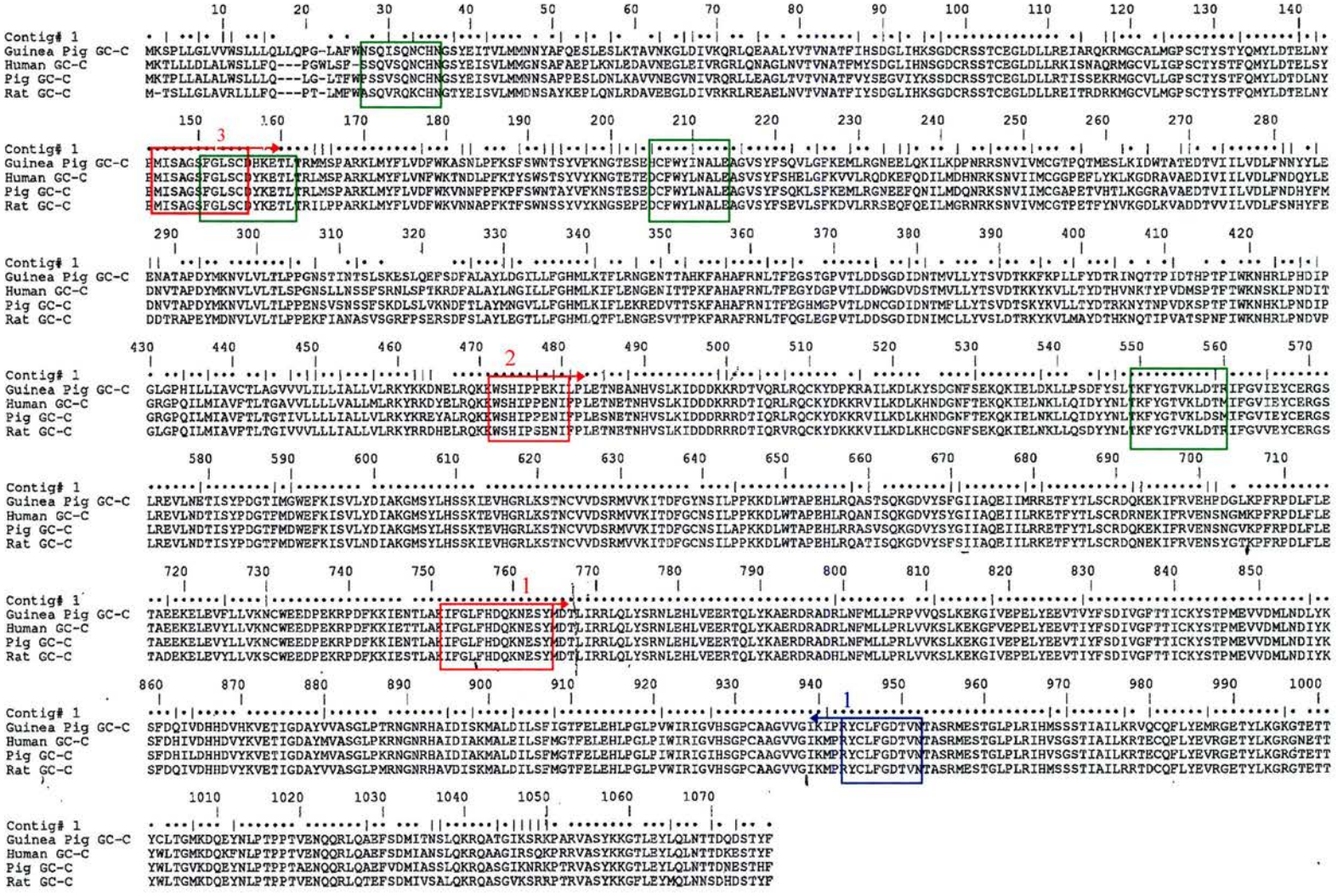
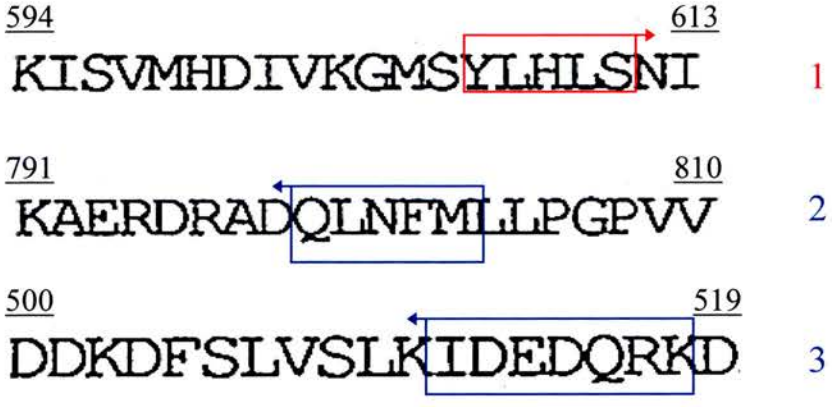

NLGSHAVCSNPH 1
NLGSHAVCSNPHL 2
ALFNRLVDIITP 3

Fig. 2.2 a) Sites chosen for the design of degenerate GC-C primers from conserved regions of mammalian species. Red boxes numbered 1, 2 and 3 represent GC-C sense, GC-C4 sense and GC-C6 sense respectively, the blue box 1 represents GC-C anti (see Appendix 2). Green boxes represent sites of unsuccessful primers. **Fig. 2.2 b)** shows the sites chosen from eel GC-C sequence data for the design of specific primers where 1 is GC-C4 seq sense, 2 is GC-C4 seq anti and 3 is GC-C(2) specific anti (see Appendix 2).


Contig# 1
Guinea Pig GC-C
Human GC-C
Pig GC-C
Rat GC-C
MKSPLLGLVVWSLLLQLLQPG-LAFWNSQISQNCHNGSYEITVLMMNNYAFQESLESLKTAVNKGLDIVKQRLQEAALYVTVNATFIHSDGLIHKSGDCRSSTCEGLDLLREIARQKRMGCALMGPSCTYSTYQMYLDTELNY
MKTLLLDLALWSLLFQ---PGWLSF-SSQVSQNCHNGSYEISVLMMGNSAFAEPLKNLEDAVNEGLEIVRGRLQNAGLNVTVNATPMYSDGLIHNSGDCRSSTCEGLDLLRKISNAQRMGCVLIGPSCTYSTFQMYLDTELSY
MKTPLLALALWSLLLQ---LG-LTFWPSSVSQNCHNGSYEISVLMMNNSAPPESLDNLKAVVNEGVNIVRQRLLEAGLTVTVNATFVYSEGVIYKSSDCRSSTCEGLDLLRTISSEKRMGCVLLGPSCTYSTFQMYLDTDLNY
M-TSLLGLAVRLLLFQ---PT-LMFWASQVRQKCHNGTYEISVLMMDNSAYKEPLQNLRDAVEEGLDIVRKRLREAELNVTVNATFIYSDGLIHKSGDCRSSTCEGLDLLREITRDRKMGCVLMGPSCTYSTFQMYLDTELNY
MISAGSFGLSCDYKETLTRMMSPARKLMYFLVDFWKASNLPFKSFSWNTSYVFKNGTESEHCFWYINALEAGVSYFSQVLGFKEMLRGNEELQKILKDPNRRSNVIVMCGTPQTMESLKIDWTATEDTVIILVDLFNNYYLE
MISAGSFGLSCDYKETLTRLMSPARKLMYFLVNFWKTNDLPFKTYSWSTSYVYKNGTETEDCFWYLNALEASVSYFSHELGFKVVLRQDKEFQDILMDHNRKSNVIIMCGGPEFLYKLKGDRAVAEDIVIILVDLFNDQYLE
MISAGSFGLSCDYKETLTRLMSPARKLMYFLVDFWKVNNPPFKPFSWNTAYVFKNSTESEDCFWYLNALEAGVSYFSQKLSPKEMLRGNEEFQNILMDQNRKSNVIIMCGAPETVHTLKGGRAVAEDTVIILVDLFNDHYFM
MISAGSFGLSCDYKETLTRILPPARKLMYFLVDFWKVNNAPFKTFSWNSSYVYKNGSEPEDCFWYLNALEAGVSYFSEVLSFKDVLRRSEQFQEILMGRNRKSNVIVMCGTPETFYNVKGDLKVADDTVVILVDLFSNHYFE
ENATAPDYMKNVLVLTLPPGNSTINTSLSKESLQEFSDFALAYLDGILLFGHMLKTFLRNGENTTAHKFAHAFRNLTFEGSTGPVTLDDSGDIDNTMVLLYTSVDTKKFKPLLFYDTRINQTTPIDTHPTFIWKNHRLPHDIP
DNVTAPDYMKNVLVLTLSPGNSLLNSSFSRNLSPTKRDFALAYLNGILLFGHMLKIFLENGENITTPKFAHAFRNLTFEGYDGPVTLDDWGDVDSTMVLLYTSVDTKKYKVLLTYDTHVNKTYPVDMSPTFTWKNSKLPNDIT
DNVTAPDYMKNVLVLTLPPENSVSNSSFSKDLSLVKNDFTLAYMNGVLLFGHMLKIFLEKREDVTTSKFAHAFRNITFEGHMGPVTLDNCGDIDNTMFLLYTSVDTSKYKVLLTYDTRKNYTNPVDKSPTFIWKNHKLPNDIP
DDTRAPEYMDNVLVLTLPPEKFIANASVSGRFPSERSDFSLAYLEGTLLFGHMLQTFLENGESVTTPKFARAFRNLTFQGLEGPVTLDDSGDIDNIMCLLYVSLDTRKYKVLMAYDTHKNQTIPVATSPNFIWKNHRLPNDVP
GLGPHILLIAVCTLAGVVVLILLIALLVLRKYKKDNELRQKKWSHIPPEKILPLETNBANHVSLKIDDDKKRDTVQRLRQCKYDPKRAILKDLKYSDGNFSEKQKIELDKLLPSDFYSLTKFYGTVKLDTRIFGVIEYCERGS
GRGPQILMIAVFTLTGAVVLLLLVALLMLRKYRKDYEELRQKKWSHIPPENIPPLETNETNHVSLKIDDDKRRDTIQRLRQCKYDKKRVILKDLKHNDGNFTEKQKIELNKLLQIDYYNLTKFYGTVKLDTMIFGVIEYCERGS
GRGPQILMIAVFTLTGTIVVLLLIALLVLRKYKREYALRQKKWSHIPPENIPPLESNETNHVSLKIDDDRRRDTIQRLRQCKYDKKRVILKDLKHNDGNFTEKQKIELNKLLQIDYYNLTKFYGTVKLDSMIFGVIEYCERGS
GLGPQILMIAVFTLTGIVVVLLLIALLVLRKYRRDHELRQKKWSHIPSENIPPLETNETNHVSLKIDDDRRRDTIQRVRQCKYDKKKVILKDLKHCDGNFSEKQKIELNKLLQSDYYNLTKFYGTVKLDTRIFGVVEYCERGS
LREVLNETISYPDGTIMGWEFKISVLYDIAKGMSYLHSSKIEVHGRLKSTNCVVDSRMVVKITDFGYNSILPPKKDLWTAPEHLRQASTSQKGDVYSFGIIAQEIIMRRETFYTLSCRDQKEKIFRVEHPDGLKPFRPDLFLE
LREVLNDTISYPDGTFMDWEFKISVLYDIAKGMSYLHSSKTEVHGRLKSTNCVVDSRMVVKITDFGCNSILPPKKDLWTAPEHLRQANISQKGDVYSYGIIAQEIILRKETFYTLSCRDRNEKIFRVENSNGMKPFRPDLFLE
LREVLNDTISYPDGTFMDWEFKISVLYDIAKGMSYLHSSKTEVHGRLKSTNCVVDSRMVVKITDFGCNSILAPKKDLWTAPEHLRRASVSQKGDVYSYGIIAQEIILRRETFYTLSCRDQKEKIFRVENSNGVKPFRPDLFLE
LREVLNDTISYPDGTFMDWEFKISVLNDIAKGMSYLHSSKIEVHGRLKSTNCVVDSRMVVKITDFGCNSILPPKKDLWTAPEHLRQATISQKGDVYSFSIIAQEIILRKETFYTLSCRDQNEKIFRVENSYGTKPFRPDLFLE
TAEEKELEVFLLVKNCWEEDPEKRPDFKKIENTLAKIFGLFHDQKNESYMDTLIRRLQLYSRNLEHLVEERTQLYKAERDRADRLNFMLLPRPVVQSLKEKGIVEPELYEEVTVYFSDIVGFTTICKYSTPMEVVDMLNDLYK
TAEEKELEVYLLVKNCWEEDPEKRPDFKKIETTLAKIFGLFHDQKNESYMDTLIRRLQLYSRNLEHLVEERTQLYKAERDRADRLNFMLLPRLVVKSLKEKGFVEPELYEEVTIYFSDIVGFTTICKYSTPMEVVDMLNDIYK
TAEEKELEVYLLVKNCWEEDPEKRPDFKKIENTLAKIFGLFHDQKNESYMDTLIRRLQLYSRNLEHLVEERTQLYKAERDRADRLNFMLLPRLVVKSLKEKGIVEPELYEEVTIYFSDIVGFTTICKYSTPMEVVDMLNDIYK
TADEKELEVYLLVKSCWEEDPEKRPDFKKIESTLAKIFGLFHDQKNESYMDTLIRRLQLYSRNLEHLVEERTQLYKAERDRADHLNFMLLPRLVVKSLKEKGIVEPELYEEVTIYFSDIVGFTTICKYSTPMEVVDMLNDIYK
SFDQIVDHHDVHKVETIGDAYVVASGLPTRNGNRHAIDISKMALDILSFIGTFELEHLPGLPVWIRIGVHSGPCAAGVVGIKIPRYCLFGDTVNTASRMESTGLPLRIHMSSSTIAILKRVQCQFLYEMRGETYLKGKGTETT
SFDHIVDHHDVYKVETIGDAYMVASGLPKRNGNRHAIDIAKMALEILSFMGTFELEHLPGLPIWIRIGVHSGPCAAGVVGIKMPRYCLFGDTVNTASRMESTGLPLRIHVSGSTIAILKRTECQFLYEVRGETYLKGRGNETT
SFDHILDHHDVYKVETIGDAYMVASGLPKRNGNRHAIDIAKMALDILSFMGTFELEHLPGLPIWIRIGIHSGPCAAGVVGIKMPRYCLFGDTVNTASRMESTGLPLRIHVSGSTIAILKRTECQFLYEVRGETYLKGRGTETT
SFDQIVDHHDVYKVETIGDAYVVASGLPMRNGNRHAVDISKMALDILSFMGTFELEHLPGLPVWIRIGVHSGPCAAGVVGIKMPRYCLFGDTVNTASRMESTGLPLRIHMSSSTIAILRRTDCQFLYEVRGETYLKGRGTETT
YCLTGMKDQEYNLPTPPTVENQQRLQAEFSDMITNSLQKRQATGIKSRKPARVASYKKGTLEYLQLNTTDQDSTYF
YWLTGMKDQKFNLPTPPTVENQQRLQAEFSDMIANSLQKRQAAGIRSQKPRRVASYKKGTLEYLQLNTTDKESTYF
YWLTGVKDQEYNLPTPPTAENQQRLQAEFVDMIASSLQKRQASGIKNRKPTRVASYKKGTLEYLQLNTTDNESTHF
YWLTGMKDQEYNLPTPPTVENQQRLQTEFSDMIVSALQKRQASGVKSRRPTRVASYKKGFLEYMQLNNSDHDSTYF

b)


594
613
KISVMHDIVKGMSYLHLSNI
1
791
810
KAERDRADQLNFMLLPGPVV
2
500
519
DDKDFSLVSLKIDEDQRKD
3

After PCR was complete the samples were electrophoresed on ethidium bromide agarose gel, along with a size marker such as a 100bp Step Ladder (Promega Corporation UK Ltd.) to detect the presence of amplified fragments. During PCR, positive controls were run using a primer set designed for the amplification of the highly expressed gene, α-subunit of Na^+K^+ ATPase, to test both cDNA quality and PCR efficiency, whilst negative controls were carried out using each primer only to eliminate the possibility of non-specific priming.

2.1.5 Standard Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to visualise the products obtained from PCR. Agarose gels were made up at a concentration of 1.5% w / v in 1X TAE (40mM Tris base, 0.35% glacial acetic acid (v/v), 10mM EDTA, pH 8) buffer to a volume of either 30ml or 100ml depending on the number of samples to be run. The agarose was dissolved in the buffer by boiling until vigorous foaming had subsided. The agarose solution was cooled to about 55°C before adding ethidium bromide (1 μg/ml). The gel was then poured into a sealed gel rig with comb and allowed to set on a flat surface. 5% loading dyes were added to the samples 1:10 v/v before loading on the gel. Electrophoresis was carried out at 125 volts or 70 volts depending on gel size (5 volts/cm) for approximately 1 - 1.5 hours. Finally, once electrophoresis was complete, the gel was viewed on the UV transilluminator to detect the presence of PCR products.

2.2 Cloning of PCR products of interest

Amplified fragments of interest were cloned by the procedure detailed below. In some cases where low yields of fragments were found, several identical PCRs were carried out and the products "pooled" together before agarose gel electrophoresis.

2.2.1 Purification of Fragments

A Geneclean® kit (Bio 101 Inc.) was used to extract and purify the DNA from agarose gels. Briefly, the gel slices were weighed and 3 vols (w/v) of 3M Sodium Iodide added before melting the gel in a dri-block at 55°C. Glassmilk resin (5µl) was added to the melted gel and the solution was incubated on ice for 5-10 min. before centrifugation at 11,336g for 30 sec in a bench top microfuge. The supernatant was carefully aspirated and the pellet washed three times with 500µl of New Wash solution - supplied with the kit . The supernatant was discarded after each centrifugation at 8,700g for 5 seconds. After the final wash 70µl of MilliQ® water, pre-heated to 55^{0}C, was added to the pellet which was then incubated for 2 min. at 55^{0}C, before being re-centrifuged at 11,336g for 30 sec.The supernatant was carefully removed and placed in a spin-column (Bio 101 inc.). The column was centrifuged at 8,700g for 30 sec. and the eluate collected. The pelleted resin was then pre-incubated with another aliquot of 70µl (pre-heated) water at 55^{0}C and re-processed as above giving a final volume of 140µl. The DNA was then precipitated at - 20^{0}C for 2 hours by addition of 2.5 vol. of absolute ethanol and 0.1 vol. of 3M sodium acetate (pH 4.6) and collected by centrifugation at 36,249g for 30 min. at 4^{0}C (Beckman J2-MC, JA18.1 rotor). The supernatant was removed, the pellet washed twice with 200µl 70% ethanol then resuspended in 8.6µl of distilled water before carrying out a

blunt-end reaction (see below) in preparation for cloning using the Invitrogen Zero Blunt® TOPO® cloning kit - version B or C (Invitrogen).

In the case of an amplification which was known to contain only one product an alternative purification was carried out using a Quick Step PCR Purification Kit (Edge Biosystems Ltd.). In this procedure, the PCR product was incubated with SOPE™ resin (5µl per 20µl reaction) for 2-5 minutes at room temperature, followed by filtration of the solution in a spin column, containing size separation gel matrix, by centrifugation in a bench top microfuge for 2 min. at 750g. The resulting eluate was then used in the blunt end reaction (see below), in the same way as Geneclean® purified products.

2.2.2 Blunt End cloning reaction

The vector supplied with the Invitrogen kit accepts blunt-ended fragments, therefore PCR products amplified with *Taq* DNA polymerase (which adds A "overhangs" to the ends of fragments) had to undergo a blunt-end reaction (using Pfu polymerase which does not create A overhangs) before cloning could be carried out. The reactions were carried out in 10µl volumes (8.6µl purified PCR product, 1µl of 10mM dNTPs, 1µl (1unit/µl) Turbo Pfu (Stratagene Ltd.). The resulting reaction mix was then incubated at 72^{O}C for 10 min. before carrying out the TOPO™ cloning reaction.

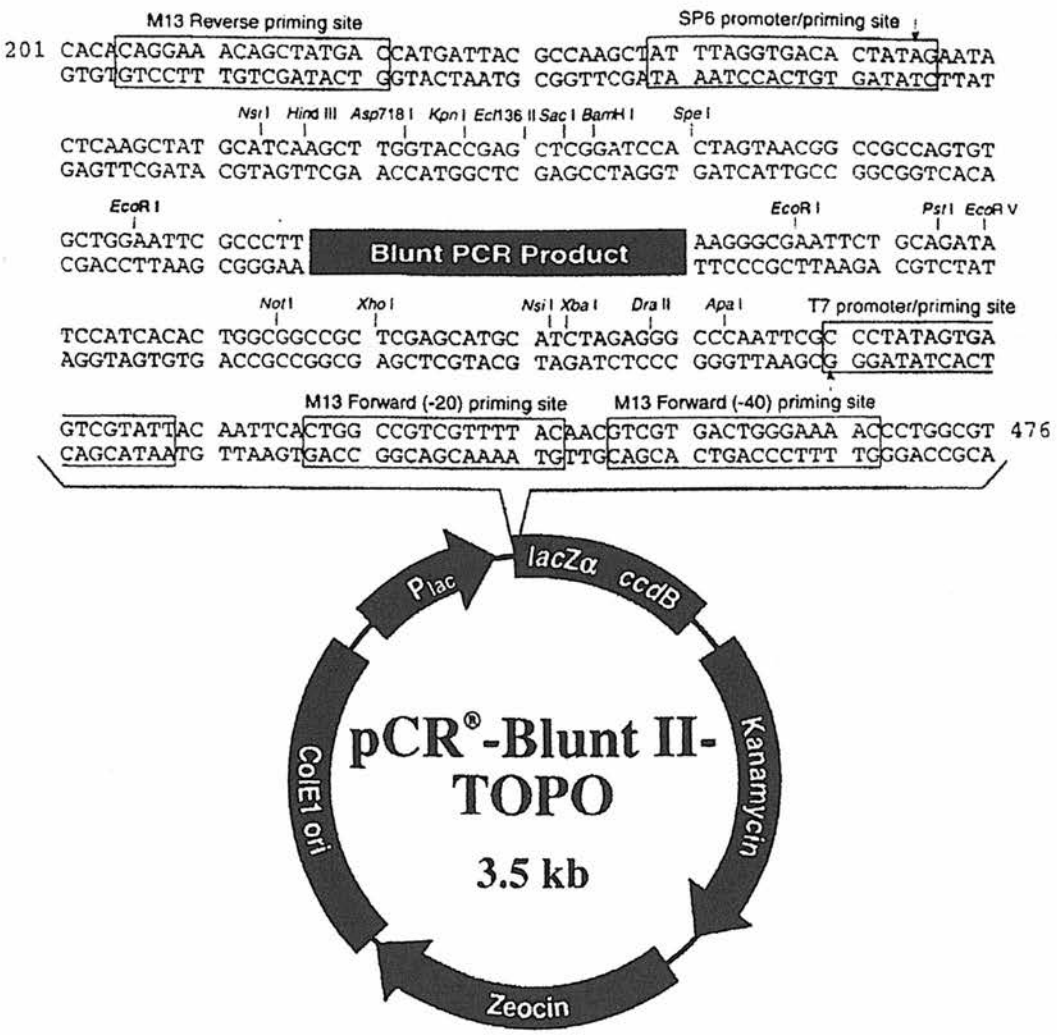
In the case of the Invitrogen Zero Blunt® TOPO® Cloning Kit a blunt end PCR product is inserted into the pCR®4 Blunt-TOPO plasmid vector which is linearised and has topoisomerase 1 covalently bound to the 3′ ends. Topoisomerase 1 displays ligation activity and when vector and insert are incubated together the insert is ligated

into the vector causing disruption of the lethal *E.coli* gene , *ccd*B. This ensures that only those transformed cells which contain a vector plus insert will survive.

2.2.3 Cloning Reaction

Blunt-ended PCR products were ligated into the cloning vector as described in the Invitrogen Zero Blunt® TOPO® Cloning Kit manual. Briefly, 2μl of the blunt - end reaction was placed in a 0.2ml microfuge tube with 0.5μl of pCR4®-Blunt-TOPO vector (see Fig. 2.3) and incubated at room temperature for 5min. After the incubation, Stop Buffer (1μl) was added to terminate the reaction and 2μl of the mix was added to one vial of TOP10™ competent cells. The cells were incubated on ice for 30 min. before heat shock treatment at 42^0C for 30 seconds. Following heat shock, 250μl of SOC medium (supplied with kit) was added to the transformed cells which were then incubated at 37^0C for 1 hour on a rotating wheel. After the 1 hour incubation, 50 - 70μl aliquots of the transformed cells were dispensed onto separate pre-warmed sterile autoclaved LB agarose plates containing 50μg/ml kanamycin. The addition of kanamycin selected for transformed cells containing the pCR4®-Blunt-TOPO vector, which has a kanamycin resistance gene, and reduced the risk of contamination of the plates by non-transformed cells or airborne bacteria. These plates were then incubated overnight at 37^0C and examined for colonies the following day. Theoretically, all colonies which were present should be positive for vector and insert. For each successful colony reaction, several colonies were selected and cultured overnight at 37^0C in 1ml of Terrific Broth containing kanamycin and glycerol. Stock Terrific Broth was made up by adding the following reagents to 1L of

Fig. 2.3 - Diagrammatic representation of cloning vector pCR®4 Blunt-TOPO with cloning site and T7 and M13 primer sites. Taken from Invitrogen Zero Blunt TOPO Cloning manual (version C).


M13 Reverse priming site
SP6 promoter/priming site
201 CACACAGGAA ACAGCTATGA CCATGATTAC GCCAAGCTAT TTAGGTGACA CTATAGAATA
GTGTGTCCTT TGTCGATACT GGTACTAATG CGGTTCGATA AATCCACTGT GATATCTTAT
NsiI HindIII Asp718I KpnI Ecl136II SacI BamHI SpeI
CTCAAGCTAT GCATCAAGCT TGGTACCGAG CTCGGATCCA CTAGTAACGG CCGCCAGTGT
GAGTTCGATA CGTAGTTCGA ACCATGGCTC GAGCCTAGGT GATCATTGCC GGCGGTCACA
EcoRI
EcoRI PstI EcoRV
GCTGGAATTC GCCCTT Blunt PCR Product AAGGGCGAATTCT GCAGATA
CGACCTTAAG CGGGAA TTCCCGCTTAAGA CGTCTAT
NotI XhoI NsiI XbaI DraII ApaI T7 promoter/priming site
TCCATCACAC TGGCGGCCGC TCGAGCATGC ATCTAGAGGG CCCAATTCGC CCTATAGTGA
AGGTAGTGTG ACCGCCGGCG AGCTCGTACG TAGATCTCCC GGGTTAAGCG GGATATCACT
M13 Forward (-20) priming site
M13 Forward (-40) priming site
GTCGTATTAC AATTCACTGG CCGTCGTTTT ACAACGTCGT GACTGGGAAA ACCCTGGCGT 476
CAGCATAATG TTAAGTGACC GGCAGCAAAA TGTTGCAGCA CTGACCCTTT TGGGACCGCA
Plac
lacZα ccdB
Kanamycin
Zeocin
ColE1 ori
pCR®-Blunt II-TOPO
3.5 kb

MilliQ® water; 12g of enzymatic casein digest, 24g of yeast extract, 9.4g of di-potassium hydrogen phosphate and 2.2g of potassium dihydrogen phosphate. This solution was autoclaved, stored at 4^0C and used in 100ml aliquots that contained 0.8 ml of glycerol and 0.5 ml of kanamycin (10mg/ml solution).

2.3 Colony PCR of cloned fragments

To determine if cloning of the fragments of interest had been successful, colony PCR was carried out using the vector primers T7 sense and M13 anti-sense, which are present within the multiple cloning site (see Fig. 2.3). These primers, when used in PCR, would amplify across the region of the insert producing a product of the same size as the original fragment **plus** the flanking regions of the vector included downstream from both primer binding sites. After the overnight incubation in Terrific Broth, 50µl of each sample was diluted 1 in 4 with MilliQ® water before centrifugation at 11,336g for 30 seconds in a benchtop centrifuge at room temperature. Aliquots of 0.5µl of these samples could then be used as templates in colony PCR, carried out in the standard manner described, using the T7 and M13 primers (stock concentration of 4µM) and visualised on ethidium bromide agarose gel to detect which samples contained the insert.

2.4 Sequencing of cloned fragments

Three positive colony PCR samples were chosen for sequencing purposes; if any errors in sequencing occurred within a single clone, the other two copies would provide a consensus sequence as they would be unlikely to contain nucleotide errors in the same position. Fragments from three colonies were sequenced in both

directions (sense and anti-sense strands) to produce six sequence reactions for each fragment. When the sequencing reactions did not continue to the end of a large fragment, or at least overlap with the sequence for the opposite strand, then further internal primers were designed to obtain a sequence for the internal section of the fragment.

For sequencing, fragments from the selected clones were amplified by colony PCR and purified by Quick Step PCR Purification Kit as previously described.

Sequencing reactions were carried out using an ABI PRISM™Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Perkin Elmer Ltd.). The sequencing reactions (described below) were carried out in the laboratory before sending the samples to commercial companies (Oswel DNA sequencing, Southampton University or PNACL, Leicester University) for electrophoresis and analysis.

The sequencing method is a modern adaptation of the dideoxy chain termination technique (Sanger *et.al.*, 1977) but in this system each dideoxy nucleotide (ddNTP) incorporates a fluorescein donor linked to a rhodamine acceptor dye. The reaction mix also contains standard dNTPs but when a ddNTP (present in minor proportions) is incorporated into the chain of DNA it blocks addition of the next nucleotide. The reaction therefore generates a ladder of DNA fragments, all ending with one of the four fluorescent ddNTPs, at any of the numerous possible sites for each ddNTP along the length of the DNA strand. The sample is electrophoresed on a polyacrylamide gel, and each of the terminated fragments are detected by a flourescent analyser (ABI Prism 310 Genetic Analyser, Perkin Elmer Corporation) as coloured bands on the

gel. The resulting chromatogram provides a read-out of the nucleotide sequence by means of the emission spectra.

Before carrying out sequencing reactions the concentration of the purified colony PCR fragments were calculated to determine the correct volume of sample for use in the reactions.

2.4.1 Calculation of sample concentration

After purification of the insert obtained by colony PCR, the DNA fragment concentration was determined in the following way; a 1µl aliquot of the PCR sample was run on an agarose gel alongside 1µl of HaeIII digest/ØX174 marker. Following staining with ethidium bromide, the intensity of the band of interest could be compared to the intensity of the marker bands and the approximate DNA concentration of the PCR product determined. The size marker which exhibited the closest flourescence intensity to the unknown PCR product was used to estimate the concentration of DNA in the unknown sample as shown below;

$$\frac{\text{size (bp) of marker band with equivalent intensity to unknown}}{5386} \times 850$$

where 5386 is the total size (bp) of ØX174 and 850 is the concentration (ng/µl) of the marker. The calculated value gives the estimated concentration of the sample in ng/µl.

2.4.2 Sequencing Reaction

When the concentration of the fragment has been determined, a 10µl sequencing reaction was set up in a 0.2ml microfuge tube; the appropriate volume of sample containing 25 ng of DNA, 0.4µl of primer (i.e. T7, M13 or specific sequencing primer at 4µM), 2µl ABI PRISM™Big Dye™ Terminator Reaction Mix (containing dNTPs, Dye terminators, AmpliTaq® DNA Polymerase, $MgCl_2$ and Tris-HCl) and H_2O as required to make volume up to 10µl.

The resulting reaction mix was then placed in the thermal cycler to undergo cycle sequencing, with the following parameters, for 25 cycles; 96^0C for 10 sec. 55^0C for 5 sec., 60^0C for 4min.The sample was then held at 4^0C until ready for precipitation. To precipitate the reaction, 0.1 vol. of 3M sodium acetate (pH 4.6) and 2.5 vol. of absolute ethanol were added before placing the tube on ice for 10 min. The tube was then centrifuged at 11,336g for 20 min. in a bench top microfuge and the supernatant carefully removed by pipetting before washing with 200µl of 70% ethanol. After the wash step the ethanol was removed and the sample allowed to air dry for 15-20 min.before being sent for analysis.

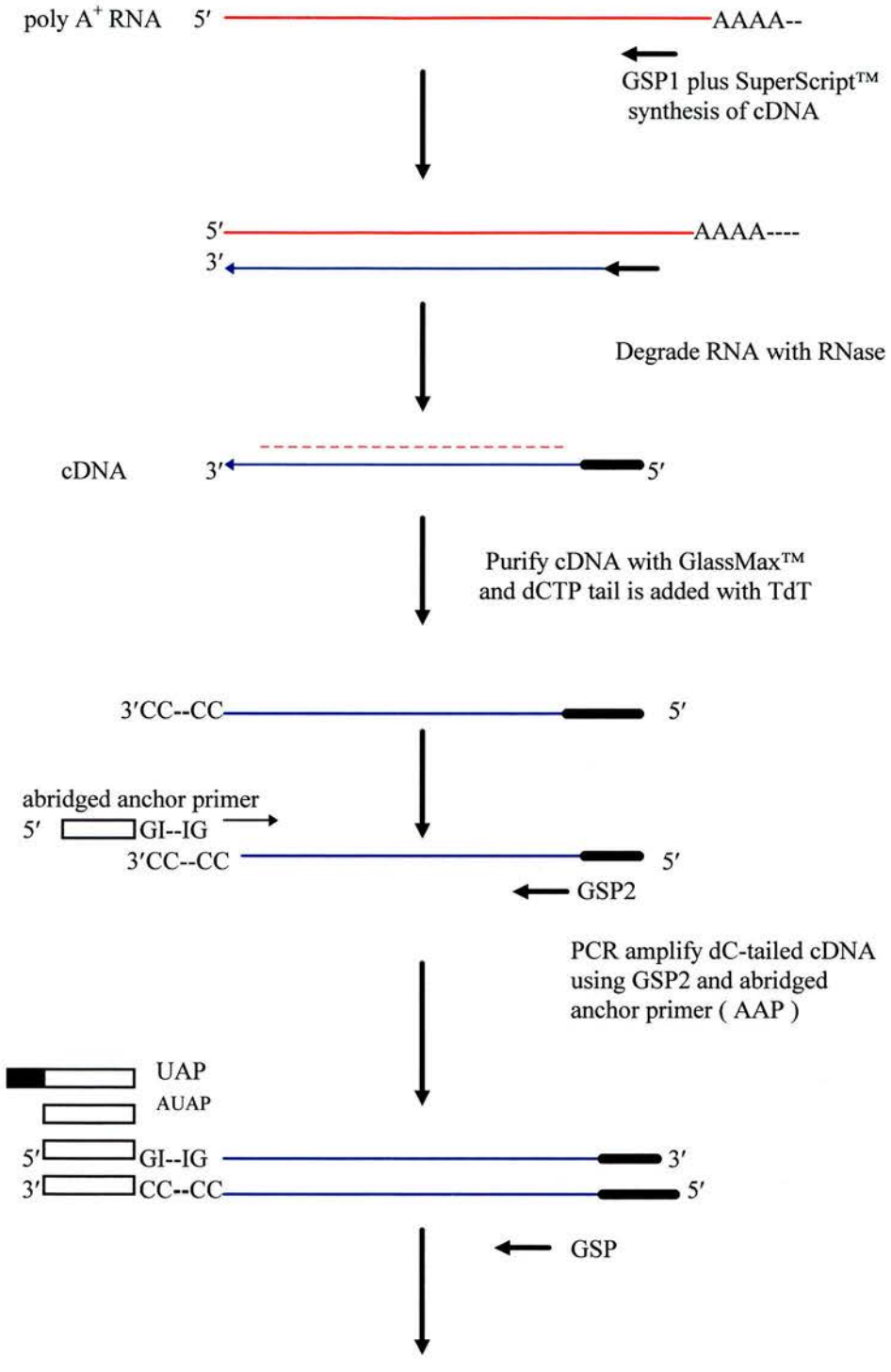
2.5 Rapid amplification of cDNA ends (5′ and 3′- race)-overview

In an effort to amplify the 5′ and 3′ unknown regions, outwith the coding sequences of the mRNA, the RACE technique was employed which will be briefly explained before fully describing the methods.

2.5.1 5′-RACE method

In 5′RACE (carried out using the GIBCO BRL Life Technologies 5′RACE System Version 2.0; see Fig.2.4) a gene-specific anti-sense primer (GSP1), designed to a known sequence near the 5′ end of the previously cloned fragment, was used to synthesise single-stranded cDNA from mRNA using reverse transcriptase. Following degradation of the original mRNA by RNase, a homopolymeric tail was added to the cDNA at the 3′ end (i.e. a poly C tail) using the terminal deoxynucleotide transferase enzyme (TdT). A first PCR carried out with a second (nested) gene-specific anti-sense primer (GSP2) for a known sequence at the 5′end of the cDNA and an abridged anchor sense primer (AAP which binds to the poly C tail) 5′ of the extending product (see Fig. 2.4). The product could then be re-amplified with a universal amplification primer (UAP see Fig. 2.4, supplied with the kit) and a further nested gene-specific primer (GSP) designed to the known sequence; if the first and second PCR product bands differ from each other by the same number of base pairs as the two gene specific primers this would indicate a positive result. Gene specific primers must fulfil the criteria for all PCR primers; the G/C content must not be too high, the annealing temperature (T_m) must be high enough to prevent non-specific binding during PCR and pallindromic sequences must be avoided, so care was taken during primer design to select regions of the known 5′ sequence that met these requirements. All primer sequences are given in Appendix 2, including those supplied in the RACE kits.

Fig. 2.4 - Diagrammatic overview of 5' RACE technique adapted from Gibco BRL Life Technologies 5' RACE system manual.



Further PCR amplifications may be carried out with a nested GSP and the universal amplification primer (UAP) or abridged universal amplification primer (AUAP).

2.5.1 (i) First Strand cDNA Synthesis

A 24µl reaction was set up in a 0.2ml microcentrifuge tube; 0.625 µl of 4µM Gene Specific Primer (GSP1), 1µl of total RNA (1µg/µl), 2.5µl of 10X PCR buffer (Amersham Pharmacia Biotech Ltd.), 1µl of 10mM dNTPs, 2.5 µl of 0.1M DTT and sterile water to bring the final volume to 24µl .The GSP1 and total RNA were incubated at 70^0C for 10 min., then chilled on ice for 1 min., before pulse centrifugation at 11,336g and addition of the remaining reagents. The contents were gently mixed and centrifuged as above, then incubated for 1 min. at 42^0C before the addition of 200 units of SuperScript II™ Reverse Transcriptase (Gibco BRL Ltd.). The contents were again mixed gently and incubated for 50 min. at 42^0C. The reaction was terminated by incubation at 70^0C for 15 min. before brief centrifugation and the addition of 1µl of RNase mix (supplied with kit) and incubation at 37^0C for 30 min. to degrade the RNA. The reaction was placed on ice before proceeding to the purification step.

2.5.1 (ii) GlassMax™ DNA isolation and purification of single stranded cDNA

The 6M sodium iodide solution (supplied with the kit) was equilibrated to room temperature and MilliQ® water to be used in the reaction was pre-heated to 65^0C. After equilibration, 120µl of the 6M sodium iodide was added to the volume of cDNA from the first strand synthesis reaction and the whole mix transferred to a GlassMax™ spin cartridge (supplied with kit) which was then centrifuged at 11,336g for 20 sec at room temperature and the eluate discarded. The cartridge insert was washed with 0.4ml of cold 1X Wash Buffer (supplied with kit) before centrifugation at 11,336g for 30 sec. The eluate was discarded and this step repeated three times

before washing the cartridge twice with cold 70% ethanol. After the final ethanol wash the tube was centrifuged at 11,336g for 1 min. The cartridge insert was then placed in a fresh tube and 50µl of the pre-heated MilliQ® water added before centrifugation in the bench top microfuge at 11,336g for 20 sec. to elute the cDNA.

2.5.1 (iii) Terminal deoxynucleotidyl transferase (TdT) tailing reaction

The following components were added to 10µl of the purified cDNA eluted from the glassmilk reagent above ; 6.5µl DEPC H_2O, 5.0µl of Tailing Buffer and 2.5µl of 2mM dCTP (both supplied with kit) before incubation at 94^0C for 2-3 min. The reaction mix was rapidly chilled on ice for 1 min., pulse centrifuged and placed on ice again before the addition of 1µl of TdT and incubation at 37^0C for 10 min. The enzyme was inactivated at 65^0C for 10 min. then the solution was pulse-centrifuged and placed on ice.

2.5.1 (iv) PCR of dC-Tailed cDNA

The initial PCR is carried out to determine that the cDNA synthesised by the binding of the gene specific primer (GSP1) to the template mRNA does represent the gene of interest and this is achieved by using a further, nested, gene specific primer (GSP2) in conjunction with the abridged anchor primer (AAP) as represented in Fig. 2.4

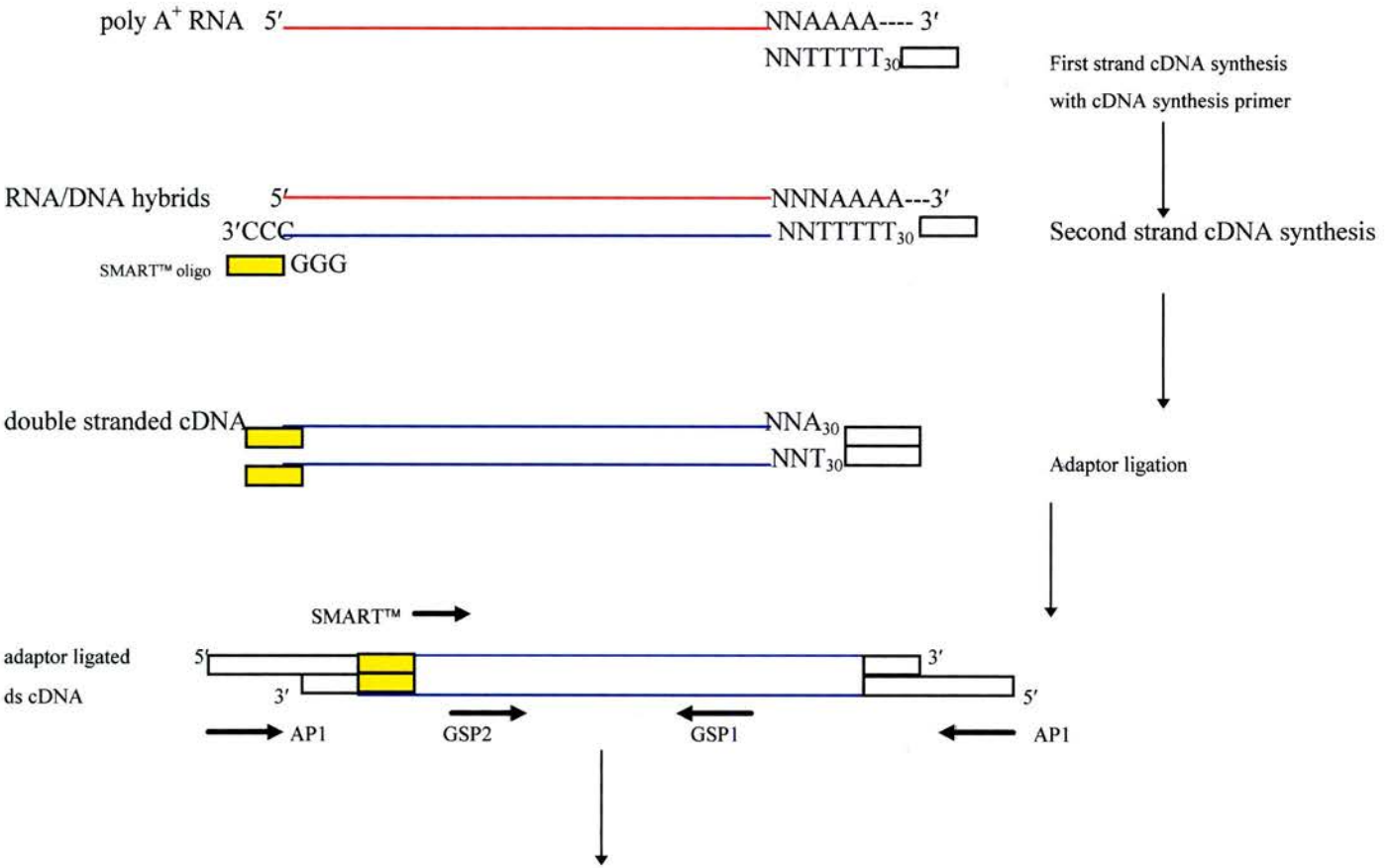
The dC-tailed cDNA prepared from the previous step was used in PCR, set up as a 50µl reaction in a 0.2ml microfuge tube comprising 5µl of 10X PCR buffer (Amersham Pharmacia Biotech Ltd), 2µl of 10µM GSP2, 2µl of 10µM AAP, 1µl of 10mM dNTPs and containing 5µl of dC-tailed cDNA product. The contents were

gently mixed before addition of 0.5μl of *Taq* DNA Polymerase (5units/μl, Amersham Pharmacia Biotech Ltd.). The reaction was then placed immediately into the thermal cycler for the following protocol: 94^0C for 2 min, then 40 cycles as follows: 94^0C for 30 sec., 55^0C for 30 sec., 72^0C for 2 min followed by a final extension at 72^0C for 10 min. The results of the 5′ RACE could then be analysed by agarose gel electrophoresis. Further PCR could be run, using the first 5′ RACE PCR products as a template, with another nested gene specific antisense primer (GSP) and either the Universal Amplification Primer (UAP) or Abridged Universal Amplification Primer (AUAP) supplied with the kit. Bands of interest were cloned and sequenced to confirm the result.

2.5.2 Clontech Marathon™ cDNA amplification kit

In the 3′ RACE method using the Clontech Marathon™ RACE kit , double-stranded cDNA was synthesised using an oligo dT primer and in an adaptation of the protocol described in the manual, a Clontech SMART™ oligonucleotide was included in the synthesis reaction. This oligonucleotide binds to the dC tail added to the 3′ end of the cDNA by the MMLV enzyme and serves as an extended template for reverse transcription. Since MMLV only adds a dC tail when the end of the mRNA template has been reached, the SMART™ oligo only binds when full length cDNA has been synthesised and the SMART™ oligo could also be used as a primer in subsequent 5′ RACE PCR. A blunt-ended adaptor primer site was ligated onto both ends of the cDNA (this cDNA can be used for both 5′ and 3′ RACE) and PCR carried out using the adaptor primer in conjunction with a gene specific primer (GSP) designed from known sequence near the 3′ end of the coding region. Further nested specific primers

Fig. 2. 5 - Diagrammatic overview of 3' RACE technique adapted from Clontech Marathon™ cDNA manual.



5′ RACE using GSP1 and AP1 or 3′ RACE using GSP2 and API primers

could be used in subsequent PCR to confirm that any bands obtained were good candidates for cloning and sequencing - as with 5′ RACE, nested reactions should produce a band that is smaller than the first by the same number of base pairs as that between the two specific primers. See Fig.2.5 for a diagrammatic overview of the 3′ RACE method and Appendix 3 for primer sequences.

The Marathon™ cDNA, produced using the method described below, synthesised from yellow eel SW gut, was provided by Dr C. Cutler for use in the RACE-PCR

2.5.2 (i) First Strand cDNA Synthesis

A reaction was set up for first-strand cDNA synthesis containing 1μg of total RNA in a reaction mix comprising; 10μM SMART™ oligo, 1μM cDNA synthesis primer (see Appendix 3), 1X First Strand Buffer (50mM Tris pH 8.3, 6mM $MgCl_2$), 1mM dNTP mix and 200 units of MMLV reverse transcriptase in a final vol. of 10μl. The total RNA and cDNA synthesis primer (total volume made up to 5μl with H_2O if necessary) were incubated together at 70^0C for 2 min. before cooling on ice and adding the remaining components. The tube was again briefly centrifuged before incubating at 42^0C in the thermal cycler for 2 hours. The tube was placed on ice to terminate the reaction before proceeding to second strand cDNA synthesis.

2.5.2 (ii) Second strand cDNA synthesis

The following components were added to the first strand reaction to give a final volume of 80 μl and concentration of reagents at; 1X second strand buffer (100mM KCl, 10mM ammonium sulphate, 5mM $MgCl_2$, 0.15mM β-NAD, 20mM Tris

(pH 7.5) and 50μg/ml bovine serum albumin), 200μM dNTP mix and 1X second strand enzyme cocktail (*E.coli* DNA polymerase (0.3 units/μl), *E.coli* DNA ligase (0.06 units/μl) and *E.coli* RNase H (0.0125 units/μl), these enzymes degrade the RNA and synthesise the second cDNA strand . The contents of the tube were mixed and centrifuged briefly before incubating at 16^0C for 1.5 hour. After incubation, 2 units of T4 DNA Polymerase was added to create blunt ends on the ds cDNA and a further 1.5 hour incubation at 16^0C was carried out before termination of the second strand reaction by the addition of 4μl of a solution comprising 0.2M EDTA / 2mg/ml glycogen. The next step was to purify and extract the cDNA in the following way; 100μl of phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v) was added to the ds cDNA and the contents mixed by vigorous vortexing before centrifugation at 11,336g for 20 min. at room temperature in a bench top centifuge. The top aqueous layer was gently removed and transferred to a clean tube and 100μl of chloroform:isoamyl alcohol (24:1 v/v) added. The contents were vigorously mixed and centrifuged as above to separate the phases; again the upper aqueous layer was removed and transferred to a clean tube. To precipitate the cDNA, 0.5 vol of 4M ammonium acetate and 2.5 vol of absolute ethanol were added, the tube was centrifuged as above and the supernatant removed. The pellet was washed with 300μl of 80% ethanol and centrifuged at 11,336g for 20 min. before removing the supernatant, air - drying the pellet at room temperature for 2-5 min. and finally resuspending in 10μl of MilliQ® H_2O.

The next step of the procedure was adaptor ligation.

2.5.2 (iii) Adaptor Ligation

The Marathon™ cDNA adaptor is partially double stranded with an extended 5′ region (see Fig. 2.5) that has no binding site for the AP1 adaptor primer unless one is generated by the gene specific primer. The 3′ end of the adaptor is blocked by an amine group to prevent extension and hence non-specific amplification by the generation of an AP1 binding site.

To ligate the adaptor to both ends of the double stranded cDNA, a 10μl reaction volume was set up comprising the following; 2 μM Marathon™ cDNA adaptor, 1X Ligation buffer (250mM Tris-HCl pH 7.8, 50mM $MgCl_2$, 5mM DTT, 5mM ATP, and 5% (w/v) polyethylene glycol),1unit of T4 DNA ligase, and containing 5μl of ds cDNA from previous reaction. The reaction was either incubated at 16^0C overnight or at room temperature for 3-4 hours, then the ligase inactivated by heating at 70^0C for 5 min.

2.5.2 (iv) 3′ RACE PCR

Using the adaptor-ligated ds cDNA synthesised as described and supplied by Dr C.Cutler, 3′ RACE PCR could be carried out in the manner previously described ; PCR was set up in the standard way but using the adaptor primer 1 (AP1) which would bind to the adaptor region of the cDNA and a gene specific primer (GSP2) designed to a known sequence near the 3′ end of the existing cDNA fragment. Since the size of the 3′ untranslated region is usually unknown, nested reactions can be carried out (as in 5′ RACE) using a second gene specific primer and analysis conducted by agarose gel electrophoresis. 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. The bands could then be excised, purified and cloned in preparation for sequencing to confirm identity.

2.6 Northern blotting

Northern blotting was carried out to detect expression of genes of interest in various eel tissues.

In this technique radiolabelled PCR products, representing fragments of the gene of interest, are incubated under stringent conditions with total or poly A^+ RNA which had been size-separated by denaturing agarose gel electrophoresis and subsequently electro-blotted onto a nylon membrane. Under suitably stringent conditions, identified PCR-amplified cDNA fragments would only bind to highly homologous regions of mRNA, thereby indicating the presence of the gene of interest in that tissue. Two Northern blots were carried out, either tissue blots - where total RNA from a range of different tissues was hybridised with the probe - or quantitative blots in which known amounts of total RNA from tissues of interest were compared by Northern hybridisation and autoradiography to detect differences in expression levels of genes of interest. Both blots were conducted in the same manner but in the case of quantitative blots the object was to compare any changes in gene expression between groups of fish e.g. FW or SW, silver or yellow eels. In theory, a comparison of gene expression levels should be achievable by simply running the same amount of RNA from each sample, then determining the relative amount of radio-labelled probe that had hybridised by means of electronic autoradiography (Packard Instant Imager) - which detects radioactivity as counts per minute (cpm) for each band of interest on the blot. As an alternative to electronic autoradiography, autoradiographs could also

be assessed by a scanning densitometer (Shimadzu CS9000, Shimadzu corporation Ltd.) which assigned values to each sample, corresponding to the relative optical density at 550nm wavelength of the band on the autoradiograph. In practice, loading the correct amount of RNA from each individual sample can be highly variable; RNA samples can be difficult to dissolve completely and may contain various contaminants inducing errors in A_{260} readings and consequently concentrations can be difficult to assess by absorption. A combination of 28s and/or 18S RNA ethidium bromide staining intensity, and quantification (in counts per minute) of bound radiolabelled probe, or scanning densitometry, was therefore used to determine relative changes in gene expression. A gel documentation system (GeneSnap, Syngene Ltd.,) was used to assign values for intensity of ethidium bromide staining to the 18S and 28s RNA for each sample. The values obtained from the gel documentation system were then compared to that of one sample, which had been chosen as the standard, to give a figure to which each sample could be directly related. In this way, the radioactive or scanning densitometry values determined for all samples could be adjusted by a correction factor which corresponded to the amount of RNA blotted for each sample. The final adjusted values therefore gave a more accurate reflection of any changes in gene expression between groups of fish. A full description of Northern hybridisation is given below.

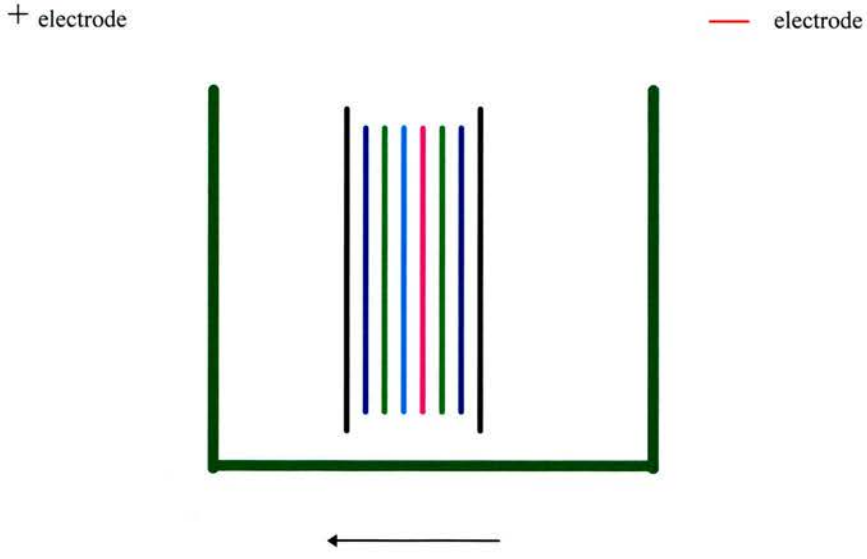
A denaturing RNA gel was run, as described in section 2.1.2, using total RNA isolated from several eel tissues. After electrophoresis was completed, the Millenium™ size markers (Sigma Ltd.) were carefully excised and following a 20 min. rinse in distilled water, were placed in ethidium bromide stain solution

(0.1M ammonium acetate, 1μg/ml ethidium bromide) for one hour and then transferred to de-stain solution (0.1M ammonium acetate) for 1- 1.5 hours before measuring the distance migrated by the separated band (for a quantitative blot the whole gel including the tissue RNA samples was stained for analysis). During staining of the markers, the remainder of the gel was electro-blotted in the manner described below:-

2.6.1 Electro-Blotting

A plastic cassette was set up containing a "sandwich" of Scotchbrite™ pad , Whatman® 3MM filter paper , ZetaProbe™ membrane (BioRad Laboratories Ltd.) , RNA gel , Whatman® 3MM filter paper (Whatman International Ltd.), Scotchbrite™ pad in that order (see Fig. 2.6). The cassette was then closed and placed in a pre-cooled rig (BioRad Laboratories Ltd.) containing 1X TAE buffer and the blot run at 25 volts or 0.8 milliAmps for 3 hours or 10 volts overnight (power supply, BioRad 250/2.5. Biorad Laboratories Ltd.). A magnetic stirrer was placed in the tank so that the buffer was continuously circulated and cooled by a water pump. After electrophoresis was complete the cassette was removed and the positions of the wells on the agarose gel were marked on the ZetaProbe™ membrane by pencil, before removing the gel. The membrane was carefully removed and placed between two sheets of filter paper. When the membrane was nearly dry, the RNA samples were covalently fixed to the nylon by UV cross-linking (XL 1500 UV Crosslinker, Spectronics Corporation) and placed at -20^0 C until needed.

Fig. 2.6 Diagrammatic representation of Northern blot electrophoresis. The arrow indicates the movement of the RNA from gel onto membrane.



= plastic cassette casing

= Scotchbrite™ pads

= Whatman® 3MM filter paper

= ZetaProbe™ membrane

= denaturing RNA gel

= electrophoresis tank filled with 1X TAE buffer

2.6.2 Labelling Of Probe

The next step in the procedure was to label the cloned PCR product of interest with γ-^{32}P dCTP using a Megaprime labelling kit (Amersham Pharmacia Biotech Ltd.). In this procedure, random nonamer primers bind to the PCR product template and, in the presence of the Klenow fragment of DNA Polymerase 1, γ-^{32} P dCTP and unlabelled dNTPs (N= A, G and T), generate γ-^{32}P labelled probes for use in hybridisation reactions.

For the labelling reaction, 25ng of cloned PCR product was added to a 0.5ml microfuge tube , made up to 5µl with MilliQ® water and 5µl of the Primers solution supplied with the Megaprime™ kit was added. This solution was boiled for 5min. to denature the DNA then cooled to room temperature. After a pulse centrifugation,10µl of labelling buffer (containing all dNTPs except dCTP), 23µl of H_2O, 5µl of [γ-^{32}P] dCTP - 6000Ci/µmol - (Amersham Pharmacia Biotech Ltd.) and 2µl of Klenow DNA Polymerase 1 enzyme, 1unit/µl (New England Biolabs Ltd.) were added and the resulting reaction mixture incubated at 37^{0}C for 30 min. before a further centrifugation. Gel filtration chromatography was used to separate radiolabelled probe from the non-incorporated nucleotides. This was conducted using a column comprising a 2ml siliconised glass pipette containing Sephadex G50™. The pipette was plugged with siliconised glass wool and glass beads, then the pre-swollen Sephadex G50™ in water was poured up to a level of 2ml. This step eliminated non-incorporated radiolabelled nucleotides and helped reduce background. The column was continuously monitored with a Geiger counter to determine the point at which the labelled probe was eluted, and fractions of four drops each were collected and aliquots (1µl) analysed on the scintillation counter (Packard TRI-CARB 1600 TR

Liquid Scintillation Analyser, Packard Ltd.). The fractions representing the radiolabelled probe were those which contained the peak counts per minute. The fractions containing non-incorporated [γ^{32}P]-dCTP were discarded. The incorporation of labelled nucleotide was calculated as a percentage in the following way:

$$\% \text{ incorporation} = \frac{\text{incorporated cpm}}{\text{total cpm}} \times 100$$

2.6.3 Hybridisation

The hybridisation flasks were thoroughly rinsed out with DEPC water and the hybridisation oven (Techne Ltd.) set at 47^0 C. A pre-hybridisation solution of 10ml was made up comprising 50% of de-ionised formamide, 5X Denhardts (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, all w/v), 1M NaCl, 50mM phosphate buffered saline (PBS), 5mM EDTA, 1% SDS (v/v), 0.5 mg/ml yeast tRNA and 0.5 mg/ml sonicated calf thymus DNA (both denatured at 95^{0}C for 15 min then snap cooled on ice before adding to the pre-hybridisation solution. The resulting pre-hybridisation solution was then heated to 47^{0}C in the oven. In later hybridisation reactions a proprietary hybridisation buffer (Ambion UltraHyb™, Ambion Inc.) was also employed which displayed increased hybridisation efficiency thus allowing detection of lower levels of mRNA.

The cross-linked filter was placed in a clean flask, rinsed briefly in DEPC H_2O and warmed to 47^{0}C before adding the pre-hybridisation solution. The flask was then placed in the oven and the filter membrane allowed to pre-hybridise at 47^{0}C for at least 4 hours. This ensured blocking of non-specific binding sites before addition of

the radio-labelled probe. Before adding to the flask, the radiolabelled cDNA probe was denatured by boiling for 5 min before rapidly cooling on ice. The hybridisation was then allowed to continue overnight before the filter was given two 30 min. washes in Solution 1 (44.5mM NaCl, 0.5% SDS v/v) and one 20 min. wash in Solution 2 (44.5mM NaCl, 0.1% SDS v/v). After washing was completed the filter was sealed in polythene and analysed in the Instant Imager (Packard Ltd.). After this an autoradiograph could be obtained by exposing the filter to X-Ray film (Kodak Biomax™ film and Kodak X-Omatic cassette, Eastman Kodak Company). Once the film had been developed, the marked wells on the filter could be aligned with the film and any visible bands measured to determine the distance travelled from the wells. Using the ethidium bromide-stained RNA standards a graph was drawn of the log of the marker sizes against the distance travelled; using this standard graph, the distance travelled by any radioactive bands of interest present on the autoradiograph could be assessed in terms of mRNA size. Northern Blots thus provided information about the levels of expression of mRNA in various tissues and also estimates of the lengths of these mRNAs.

2.7 Western blotting

In addition to the PCR-based molecular techniques described, attempts were made to evaluate protein expression by the immunological method of Western Blotting. This method is similar to other hybridisation techniques but instead of nucleic acid hybridisations, Western blots involve protein:protein interactions. Membrane protein samples (prepared as described below) from gill and intestinal epithelia were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-

PAGE), electro-blotted onto polyvinylidene fluoride (PVDF) membrane then hybridised with polyclonal antibodies raised to epitopes on the guanylin receptor, GC-C. Bands, produced by binding of antibody to antigen, were visualised by an enzyme -substrate colour reaction, in which a secondary antibody, conjugated to alkaline phosphatase, (or horseradish peroxidase as used in the ELISA method described below) binds to the primary antibody. In the presence of substrate solution the enzyme- substrate reaction produces a colour change which can be visualised as bands on the Western blot membrane.

2.7.1.(i) Membrane Protein Preparations

Membrane preparations, isolated from eel gill and intestine were prepared by Dr G. Cramb. Tissues of interest, gill and intestine, were removed from eels and cooled on ice before washing in ice cold PBS, blotting and weighing. A 1g portion of tissue (epithelial scrapings from either gill or intestine) was then homogenised (Polytron® with PT 104 probe, Kinematica) in 5 volumes of homogenisation buffer comprising, 25mM Hepes, 0.25M sucrose pH 7.4, 5mM $MgCl_2$, 1mM $CaCl_2$ containing 0.18mg/ml of phenylmethylsulphonylfluoride (PMSF) and 0.1mM dithiothreitol (DTT). The resulting homogenate was filtered through 4 layers of muslin guaze then made up to to 10ml with the same homogenisation buffer containing an inhibitor cocktail with final concentrations of, 0.1μM pepstatin, 1μM leupeptin and 0.08 TIU of aprotinin. Two volumes of 25mM Hepes, 70% w/v sucrose, 0.1mM DTT pH 7.4 was then added, mixed, and the solution placed in the bottom of a 38ml Ultra Clear™ centrifuge tube (Beckman Inc.). This was then overlaid carefully with 5ml of 25mM Hepes, 43% (w/v) sucrose, 0.1mM of DTT pH 7.4, and

overlaid finally with 2ml of 25mM Hepes, 8% (w/v) sucrose 0.1mM DTT pH 7.4. The tube was then centrifuged at 100,000g in a Beckman L7 Ultracentrifuge, SW 28 rotor (Beckman Instruments Inc.) for 2 hours at 4^0C. The band of material at the 8-43% sucrose interface was removed with a large bore needle and syringe then diluted to 40ml with 25mM Hepes, 0.25M sucrose, 0.1mM DTT pH 7.4.

A further centrifugation was then carried out at 31,360g (Beckman J2 MC, JA-20 rotor) for 40 min at 4^0C. The resulting pellets were resuspended in 1-2ml of 25mM Hepes, 0.25M sucrose, 0.1mM DTT pH 7.4, aliquoted and stored at -90^0C. The protein concentration of the samples were determined by the Bradford assay.

2.7.1.(ii) Bradford Assay

Protein standards, using Bovine Serum Albumin (BSA) were made up in MilliQ® water, in doubling dilutions, ranging from 0.125 up to 1mg/ml. For each sample and standard, duplicate aliquots of 100µl were removed and thoroughly mixed with 2ml of Bradford's Reagent. Bradford's reagent (which must be stored away from light) is made by dissolving 0.1g Coomassie brilliant blue G250 in 50ml absolute ethanol before adding 100ml of 85% orthophosphoric acid, making up to 1L with MilliQ® water and filtering through a Whatman No.1 filter paper. The tubes were allowed to stand at room temperature for 5 min. to allow the colour reaction to develop before reading absorbance on the spectrophotometer at 600nm. Blanks of water and resuspension buffer were also used. The absorbance readings obtained from the standards were plotted on a graph and the test samples read off the curve produced to determine the protein concentration.

2.7.2 Antibody Production

A sequence of 15 amino acids (LYKAERDRADQLNFM) was chosen from within the catalytic domain of the eel homologue of GC-C, after comparison with the same region of eel GC-B, to ensure no cross-reactivity would occur. This sequence was synthesised as an 8-branched Multiple Antigen Peptide, or MAP (Genosys Biotechnologies Inc.) which was done by attaching first alanine, then lysine residues to a solid resin core thus creating branched "arms" to bind peptide molecules. This results in an amplification of antigen surface area to present to B-cells and therefore, theoretically, an increased immune response in the sheep/rabbit. A 1mg amount of this peptide was sent to the Scottish Antibody Production Unit (SAPU, Common Services Agency) where it was made up as 1mg/ml in PBS. Four injections of 250µl of this solution, made up in Freund's Adjuvant, were used to immunise one sheep; following the first immunisation, three injections were administered to the sheep at 4-weekly intervals and bleeds were taken seven days after each of the injections. In addition the peptide was also used to immunise a rabbit to provide another possible source of anti-sera. To immunise the rabbit, the peptide was diluted to 0.5mg/ml with PBS then 0.5ml of the peptide was blasted, by syringe, into a well mixed emulsion of the oil Drakeoyl 6UR and the emulsifier ARACEL A (10:1 v/v). The resulting solution was mixed by syringing before adding 1ml of 2% Tween® 80 in PBS and mixing again. This solution was then injected subcutaneously into the rabbit at 3-4 sites with subsequent injections every two weeks prepared in the same way.

Prior to any immunisation of the sheep or rabbit, a pre-immune bleed was taken to be used as a control in Western blots and ELISAs. Post-immunisation bleeds and pre-

immune sera were tested by enzyme linked immunosorbent assay (ELISA) to determine the antibody titre of the serum.

2.7.2 ELISA -measurement of antibody titre

To determine the antibody titre present in both the sheep and rabbit immune antisera, it was necessary to perform an ELISA, in which the peptide (antigen) is bound to micro-titre plates before addition of anti-serum at varying dilutions. A secondary antibody conjugated to horseradish peroxidase was used for colorimetric detection of any primary antibody present in the anti-sera. The stronger the colour reaction, the higher the level of antibody in the serum and the appropriate dilution factor for use in subsequent techniques such as Western Blotting can be determined. The ELISA test was performed on antisera from the third bleeds and the titres compared with those present in the pre-immune serum. In addition, a positive control for the ELISA technique was carried out using a previously characterised antibody, β233 (an anti-serum to an isoform of the β subunit of the eel Na^+, K^+- ATPase), which served to confirm the ELISA was working correctly.

To begin the protocol, ninety-six well micro-titre plates (Dynatech M29A, sterile,flat-bottomed, Dynex Technologies) were treated with 100μl of 1% (w/v) glutaraldehyde:PBS solution and incubated in the refrigerator overnight. The following day the gluteraldehyde:PBS solution was removed by aspiration and 50μl of 10μg/ml peptide solution (i.e. the peptide antigen) added to the wells for another overnight incubation in the refrigerator. The peptide solution was removed by aspiration and the plates allowed to dry at 37^0C for 15 min. The wells were then blocked (from non-specific binding) by the addition of 150 μl 10% skimmed milk

protein (Boots Company PLC.):PBS (w/v) and incubated at 37^0C for 3 hours. The blocking solution was then removed by aspiration and the plates washed three times in 1% milk / PBS (w/v) following which, 100µl of the relevant anti-serum (doubling dilutions ranging from 1:5 up to 1:5,120 in 1% milk / PBS) was added to the wells in sets of 4 replicates per dilution. The plates were then left at room temperature for 1 hour. The three washes in 1% milk / PBS were repeated, then 50µl of horseradish peroxidase(HRP)-conjugated donkey anti-sheep (1:1000 in 1% milk / PBS) or HRP-conjugated donkey anti-rabbit (1:500 in 1% milk / PBS) - both supplied by SAPU - was added to each of the wells for another 1 hour incubation at room temp. The three washes of 1% milk:PBS were repeated followed by a further two washes in phosphate / citrate Buffer (0.025 M citric Acid, 0.05M disodium hydrogen orthophosphate). The final step was the addition of 100µl of the substrate solution (0.08% w/v of Orthophenylenediamine- OPDA- and 0.03% v/v hydrogen peroxide in phosphate / citrate buffer) to each well, followed by the incubation of the plates at room temperature, in darkness, for 30-60 min. The optical density of the wells at 450nm was assessed using a plate reader (Dynatech MRX, Dynex Technologies). Results were plotted on a graph and the titre evaluated.

2.7.3 Western Blotting

Once the titre of the antibody had been assessed by ELISA an appropriate dilution around the 1/2 max titre was chosen for use in Western blotting which was carried out as described below.

2.7.3 (i) Protein separation by electrophoresis

Thawed membrane preparations from gill and intestine were centrifuged at 8,700g on the bench top microfuge for 15 sec. and a volume equivalent to 100μg of protein from each membrane preparation was aliquoted into fresh microfuge tubes. Each sample was made up to 35 - 40μl with 1X solubilisation buffer (5mM Tris, 0.5mM EDTA, 1.25% SDS w/v) before addition of an equal volume of 2X loading buffer (0.75M Tris, 30mM DTT, 40% glycerol v/v, 0.006% bromophenol blue w/v, pH 6.8). Samples were then denatured at 95^0C for 10 min. then cooled on ice in readiness for loading onto SDS-PAGE gels alongside a sample of molecular weight markers (Amersham Pharmacia Biotech Ltd.). Sufficient replicates of each membrane sample (20μg per lane) were loaded for separate incubations with either immune antiserum or control pre-immune serum. As with the ELISA, β233 immune serum was included as a positive control to identify the β subunit of eel Na^+, K^+-ATPase. The SDS-PAGE gels were prepared by the following method; 8% running gel (8% 37:19:1 acrylamide mix v/v (Sigma Aldrich Ltd.), 0.375M Tris pH 8.8, 0.1% SDS v/v, 0.1% ammonium persulphate w/v, 0.1% N,N,N,N-tetramethylethylenediamine-TEMED v/v) was prepared and, before polymerisation , poured into the gel rig (18 cm by 16 cm with 1mm spacers, Biorad Laboratories Ltd.) and overlaid with butanol. After polymerisation the top of the gel was washed with running buffer (see below) then a 4% stacking gel (4% 37.1 acrylamide v/v, 0.19M Tris pH8.8, 0.1% SDS v/v, 0.1% ammonium persulphate w/v, 0.1% TEMED v/v) was poured on top and a gel comb inserted. Once the stacking gel had set and the comb was removed, the samples were loaded into the wells through the buffer reservoir and the whole apparatus set into the electrophoresis tank which contained running buffer (200mM glycine, 25mM Tris

base and 0.1% SDS) and electrophoresed at 7 volts/cm for 4-5 hours. After electrophoretic separation, the gel was removed and placed in a cassette for the electro-blotting procedure.

2.7.3 (ii) Electro-blotting

As previously described for Northern blotting (see Fig. 2.6), a "sandwich" of Scotchbrite pads, filter paper, Electran® PVDF membrane (BDH) and the gel was set up. The membrane had been pre-prepared by soaking consecutively in methanol for 30 sec., in water for10 min. and finally in blot buffer for15 min. The gel was set in plastic casing, placed in a blotting rig (Hoefer Scientific Instruments) and run in transfer buffer (48mM Tris, 1M glycine, 20% methanol v/v and 3.75% SDS v/v pH 8.6) overnight at 30 volts. After the blotting procedure was complete the PVDF membrane was ready for incubation with the antiserum.

2.7.3 (iii) Western blotting

The membrane was cut into strips to separate the replicate samples destined for incubations with different anti-sera or pre-immune sera . The first incubation was a blocking step in which the membranes were washed in a blocking solution comprising 20% milk; 0.2% Tween 80 in PBS (w/v/v) for 1 hour at room temperature followed by three 15 min. washes in 0.1% milk; 0.2% Tween 80 in PBS. The membranes were then incubated for 1 hour at room temperature in the appropriate anti-serum diluted in 1% milk; 0.2% Tween 80 in PBS. The sheep anti-serum for GC-C was used at 1:20 dilution, the rabbit anti-serum for GC-C was used at 1:10 and 1:20 dilution and the β233 anti-serum at 1:1000 dilution. Incubations in pre-immune serum were carried

out at the same dilutions as the corresponding anti-serum. A further three 15 min. washes in 0.1% milk; 0.2% Tween 80 in PBS were performed before incubation for one hour in a 1:10,000 dilution of alkaline phosphatase-conjugated donkey anti-Sheep/Rabbit serum (Sigma Aldrich Company Ltd.) in 1% milk; 0.2% Tween 80 in PBS. Following the incubation with secondary antibody, the blots were processed with three 15 min. washes in 0.1% milk; 0.2% Tween 80 in PBS, two 15 min. washes in PBS and one 5 min wash in distilled water before incubating the membranes in Western Blue® Stabilised Substrate for alkaline phosphatase (Promega UK Ltd.). Once the colour had developed - after approx. 5 min.- the membranes were washed in distilled water and allowed to dry. Any bands of interest were compared with the protein molecular weight standards (Pharmacia Biotech Ltd.) to determine size.

Statistical analysis

StatView 4.01 software (Abacus Concepts) was used to perform ANOVA, followed by Fisher's PLSD post analysis of significance, on the raw data obtained from the quantitative Northern blots. ANOVA can be used to compare the effects of more than two treatments (i.e. in this case FW or SW and the two life stages, yellow and silver eels) when samples fall within a normal distribution range and the variability between each treatment is similar.

Chapter 3

Results

3. RESULTS

Degenerate primer sites as selected from guanylin and GC-C a.a. sequences of several vertebrate species were shown previously in Chapter 2, Figs. 2.1 a) and 2.2 a) respectively. Where specific primers were designed from eel sequences, these sites are indicated separately in Figs. 2.1 b) and 2.2 b) respectively.

3.1 Guanylin

3.1.1 RT-PCR

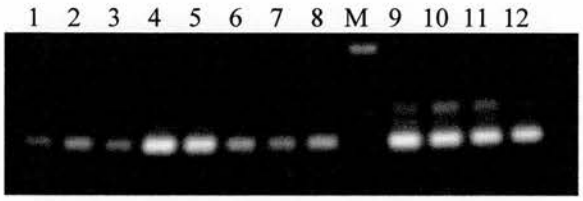
The degenerate primers, guanylin sense and guanylin antisense (see Appendix 2) were designed to amplify a 234 bp region of guanylin cDNA. Template cDNAs, synthesised from gill, intestine and kidney, of yellow and silver FW and SW-acclimated eels were used in RT-PCR with these primers. Fig. 3.1 shows the products of these reactions visualised by gel electrophoresis as described in section 2.1.5. A band of the expected size of ~234 bp was amplified in all three tissues from yellow and silver eels acclimated to both FW and SW conditions. A number of primer pairs were tried before successfully amplifying the 234 bp fragment using a primer pair designed with the aid of additional sequence data, recently deposited in the GenBank database, from a zebrafish cDNA library. A fragment, purified from agarose gel and re-amplified, from yellow SW eel intestine was selected for cloning and sequencing.

3.1.2 RACE-PCR

3.1.2 (ii) 3′ RACE-PCR

The guanylin 3′ RACE PCR was first carried out using a template Marathon™ cDNA synthesised from YSW eel gill, which was available at the time, but this proved to be

Fig 3.1 Results of RT-PCR using degenerate guanylin primers (see Appendix 2) with template cDNA synthesised from RNA extracts of the intestine, gill and kidney of the four experimental groups. Lanes 1, 2, 3 and 4 are amplifications using intestinal cDNA templates from yellow fresh water (YFW), yellow seawater (YSW), silver fresh water (SFW) and silver seawater (SSW) acclimated eels respectively, lanes 5, 6, 7 and 8 are amplifications using gill cDNA templates from the same groups in the same order and lanes 9, 10, 11 and 12 are amplifications using kidney cDNA templates from the same groups in the same order. The letter M indicates the 100 bp step ladder (Promega Ltd.)


1 2 3 4 5 6 7 8 M 9 10 11 12
← 234 bp

unsuccessful. However, a 3′ RACE PCR product of 800 bp, was amplified independently by Dr G.Cramb, using template Marathon™ cDNA (see section 2.5.2) synthesised from YSW eel intestine, in a first PCR using the GSP1 sense primer (3′ RACE 1 sense, Appendix 2) and the AP1 antisense primer (see Appendix 2). Two separate nested PCR were then carried out, using the first PCR as template, with GSP2 sense primer (3′ RACE 2 sense, see Appendix 2) and either AP1 or AP2. Two fragments were amplified in the nested PCR, as shown in Fig 3.2, of ~ 750 and 700 bp. The 700 bp fragment was cloned and sequenced.

3.1.2 (i) 5′ RACE-PCR

Initial efforts were made to amplify the 5′ untranslated region of guanylin cDNA using cDNA synthesised from intestine of yellow SW-acclimated eels by the GIBCO BRL 5′ RACE kit but these attempts were unsuccessful (data not shown). Consequently, as with 3′ RACE, cDNA synthesised from yellow SW eel intestine by the Clontech Marathon™ technique was used as template for the 5′RACE PCR. The synthesis of this cDNA involved a step whereby a SMART™ oligonucleotide (supplied with the Clontech SMART™ cDNA synthesis kit, see Appendix 2) was ligated to the 5′ end of the cDNA before adaptor ligation. The SMART™ oligonucleotide binds to the "C" overhang, which reverse transcriptase adds to the end of full length cDNA, providing a primer site which will only be present if the cDNA is full length. The SMART™ oligonucleotide could then, if necessary, be used as a sense primer in nested PCR. The first 5′ RACE PCR was carried out using the degenerate Guanylin antisense primer (Guanylin anti, see Appendix 2) and the AP1 sense primer

Fig. 3.2 Results of 3 ′ RACE-PCR using Marathon™ cDNA synthesised from the intestine of yellow SW-acclimated eels. Lane 3 is a strong band of ~700 bp amplified using template PCR product (amplified with 3′RACE 1 plus AP1) of lane 1 where the band is not visible. Lane 2, where the amplified fragment was also very faint was an amplification using template PCR product from the lane 1 reaction and 3′ RACE 2 sense and AP1 primers. The strong band of 700 bp shown in lane 3 was purified and cloned for sequencing.

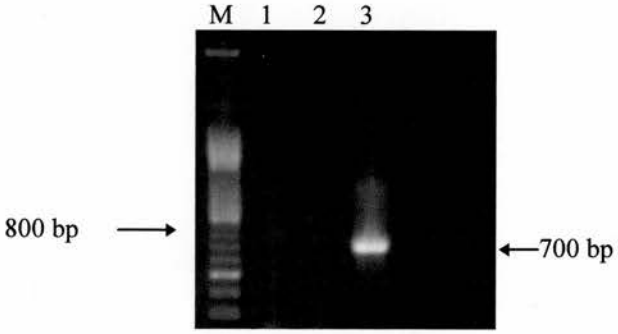
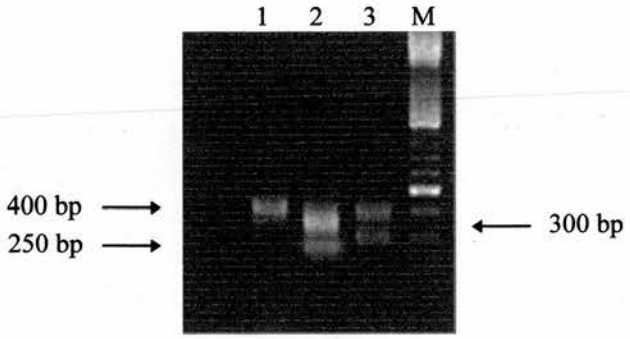

M 1 2 3
800 bp
700 bp

Fig. 3.3 Results of 5 ′ RACE-PCR using Marathon™ cDNA synthesised from the intestine of yellow SW-acclimated eels. Lane 1 is amplification of the template cDNA using the degenerate guanylin sense primer and AP1 primer (see Appendix 2), and a band of 400 bp is present. This first PCR was used as template in two further nested PCR; lane 2 shows fragments of 320 and 250 bp amplified with the 5′ RACE antisense and AP2 primers (see Appendix 2) and lane 3 shows fragments of 380 bp and 280 bp amplified with the SMART™ oligonucleotide and 5′ RACE 1 antisense primers (see Appendix 2). All four fragments were cloned and sequenced.


1
2
3
M
400 bp
250 bp
300 bp

supplied with the Marathon™ kit. A band of 400bp (see Fig. 3.3) was amplified by this reaction. The product of this first PCR was then used as template in two separate nested PCR; one PCR was carried out with the Marathon™ AP2 sense primer and a GSP antisense primer (5′ RACE 1 anti, see Appendix 2), designed from sequence data obtained from the 234 bp fragment amplified by RT-PCR (see section 3.1.1), while the other nested PCR was carried out with the SMART™ oligonucleotide as sense primer and the GSP antisense primer (see Appendix 2). The two nested PCR amplified three separate fragments, as shown in Fig. 3.3, which were all purified and cloned for sequencing.

3.1.3 Cloning and sequencing

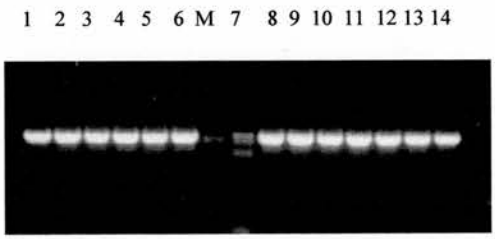
3.1.3 (i) Cloning

All the fragments amplified by RT-PCR, 5′ and 3′ RACE PCR were successfully cloned as described (see section 2.2 and 2.4). After selection of clones, colony PCR, as described in section 2.3, was carried out to confirm the presence of an insert. Fig.3.4 demonstrates an example of colony PCR carried out on clones containing the 380bp guanylin fragment, one of the fragments amplified by 5′ RACE-PCR, (see section 3.1.1), as visualised on an ethidium bromide-stained agarose gel. The fragment is larger than 380 bp because it contains regions of the vector spanned by the T7 and M13 vector primers.

3.1.3 (ii) Sequencing

Sequencing reactions were carried out in both sense and antisense directions using three separate clones per fragment but since four separate fragments were generated

Fig. 3.4 Example of colony PCR confirming the presence of inserts in cloned fragments. The example shown is a set of clones, lanes 1-14, containing the 380 bp fragment that was amplified in the nested 5′ RACE PCR. The fragment is larger because the amplification also contains region of the vector spanned by the T7 sense and M13 antisense primers.


1 2 3 4 5 6 M 7 8 9 10 11 12 13 14
← 480 bp (vector plus insert)

by 5′ RACE, two clones of each fragment were selected for sequencing. The sequencing results indicated that the 250bp and 280 bp fragments were truncated versions of the 320 bp and 380 bp fragments and represented different 5′ spliceoforms, hence the different sizes of the 5′ fragments. The sequence data was compared and combined to produce the full length eel guanylin nucleotide and a.a. sequence represented in Fig. 3.5. A comparison of the eel guanylin a.a. sequence with those of other species is shown in Fig. 3.6. In mammals the cleavage site between the precursor and the active peptide is situated between the aspartic acid (D) and the proline (P). The possible cleavage site of the eel active guanylin peptide is indicated on Fig. 3.6.

3.1.4 Northern hybridisation

3.1.4 (i) Northern tissue blot

Total RNA (5μg) from a variety of yellow eel tissues was electrophoresed and hybridised, as described in section 2.6, with purified, [γ^{32}P]-dCTP-labelled 234bp guanylin fragment. Fig. 3.7 shows the autoradiograph with distinct bands of ~ 1 kb present in FW and SW intestine and a faint band present in FW kidney. A 2kb band is also present in the intestine samples but sequence results showed the long and short form of guanylin to be 1029 bp and 933 bp respectively and the nature of the 2kb band is unknown. In contrast to the RT-PCR, no transcripts of either size were detected in the gill.

3.1.4 (ii) Quantitative Northern hybridisation

Quantitative Northern blots were carried out to determine any changes in guanylin expression levels between yellow and silver, FW and SW-acclimated eels. Total RNA

Fig. 3.5 a) Interleaved nucleotide and derived a.a.. sequence of eel guanylin showing the 5′ untranslated region of the long form.

Fig. 3.5 b) The 5′ untranslated region of the short form of eel guanylin up to the methionine start codon.

a)

```
   1            GAGATGGCCACTTCCACCGAGCGCGGAGCTTCGGGAGGGACCCACGTGGAGCGGTG
  57 AGGGAGGAAGGGGACACCCGCCTAGCCAGATCAGCCGAATCAACCCTAGCGATCAATGGGGTAACAG
     Met Ser Gln Arg Asp Leu Cys Glu Met Arg Glu Ala Val Val Leu Ile Leu    17
 124 ATG TCG CAG CGT GAT CTG TGT GAG ATG AGA GAG GCA GTG GTT CTG ATA CTT
     Leu Ala Phe Cys Phe Leu Gln Glu Ser Gln Gly Val Trp Val Met Asp Gly    34
 175 CTG GCT TTC TGC TTC CTT CAG GAA TCG CAG GGC GTG TGG GTG ATG GAT GGA
     Asp Leu Ser Phe Pro Leu Glu Ala Val Lys Val Leu Lys His Leu Leu Gly    51
 226 GAC CTT AGC TTC CCC CTG GAG GCC GTG AAA GTC CTG AAG CAC CTT CTG GGG
     Ala Asn Thr Met Ser Thr Pro His Pro Pro Asn Leu Gly Ser His Ala Val    68
 277 GCC AAC ACT ATG TCC ACC CCC CAC CCT CCA AAC CTT GGC TCA CAT GCC GTT
     Cys Ser Asn Pro His Leu Pro Ala Glu Phe Leu Pro Val Cys Glu Arg Glu    85
 328 TGT TCC AAC CCC CAC CTT CCA GCT GAA TTC TTA CCT GTA TGT GAG AGA GAG
     Gly Ala Ser Ala Leu Phe Asn Arg Leu Val Asp Ile Ile Thr Pro Pro Asp   102
 379 GGG GCC AGT GCA CTA TTC AAC AGG CTG GTG GAC ATA ATC ACT CCA CCA GAT
     Pro Cys Glu Ile Cys Ala Asn Ala Ala Cys Thr Gly Cys Leu Stop          116
 430 CCA TGT GAA ATC TGC GCT AAT GCT GCC TGC ACT GGA TGC CTG TAG ATACTCG
 482 ATCAAGAAGCTTGACTGAGGGATTAGAACATCTTCAGCTGCATTCTTCAGACTGCAAAGGACAAAGC
 549 TAAACAAGAAGCATTCAGTTATTCAGAGAGGTCTGGTTTATCTGTGATGAGCTCCAGGCCTGAACAC
 616 ACTGAGCATGTGGGTAGACATGATGTACAATCTGGAAAAAGGACCTCTGGAGGAAGACAAATTACTG
 683 AAAATATGCTGTAAAAGCTGTAAAACACTTCTTGTTAGTTTTAAAACTTTTCAATATATATTTTAAA
 750 TGGTTTAAATGTTAACAATAGCTTACAACAGTAGGTTCCATGACTGCATTTGTAGAAAGACAATGTC
 817 CTCTAGTGAAGTACATCAGCGAAAGTTGGGTCCATATACAGTACATTCATTTTACATTGGTAAATGC
 884 AATTGGACATAGATATACAGTTTCTTAAACCACAATGTATCTTTCAATTGCCAATATTTTANAAAGC
 951 ACTGCAGTTGCATGTATAAGCTCTGTCTAAAAAATTTTGAATGTAGGTTTGAAGCTCAAGACAGATG
1018 AGTTTTCCTCTGAAAAAAAA
```

b)

5′-TTTTTTAACCATTTTGGACCCAGAGGCAGGACGACCCGTGATCTGTGA**ATG**

Fig 3.6 Alignment of eel guanylin a.a. sequence with those of other vertebrate species. Black dots indicate conserved amino acids and dashes indicate spaces inserted for alignment purposes. The long and short form of eel guanylin differ by 8 amino acids at the 5′ amino terminal. The red boxed area indicates the probable cleavage site of the active guanylin peptide in mammals and the green boxed area represents a putative cleavage site for the eel guanylin peptide.

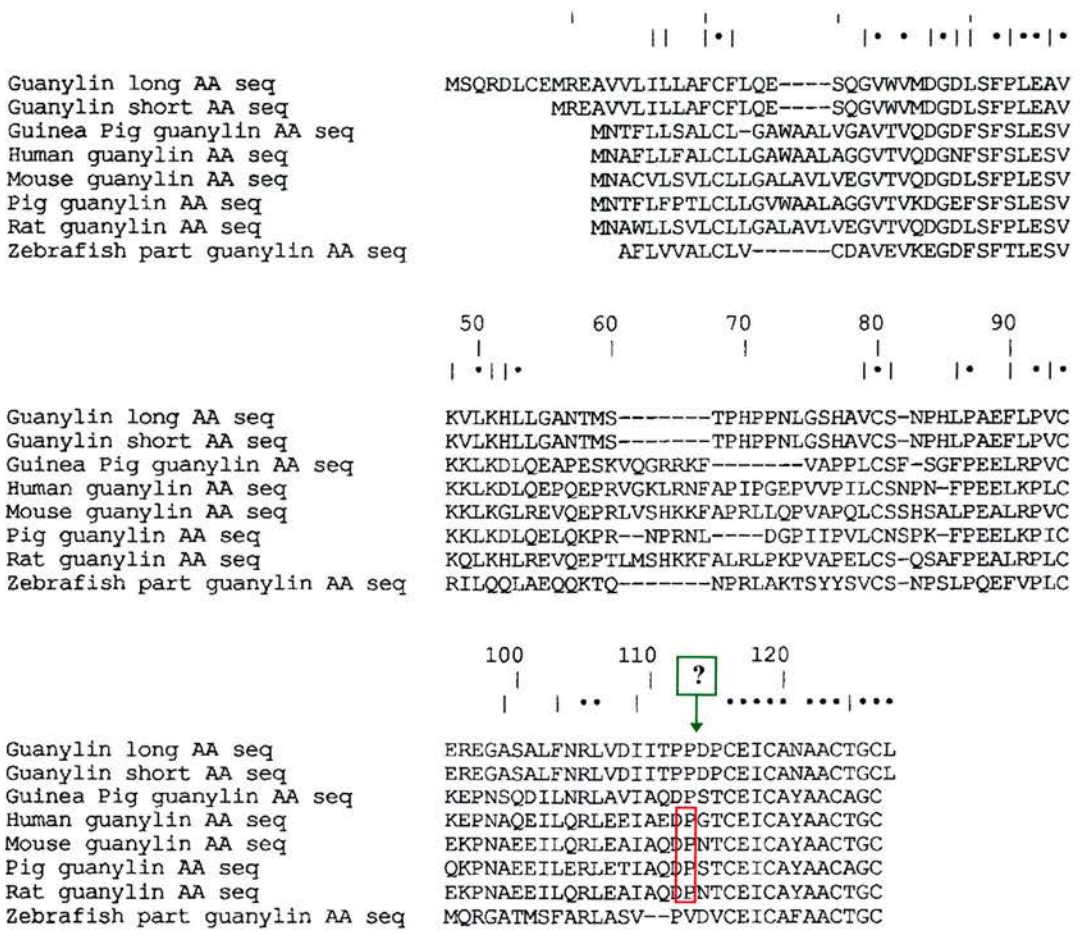
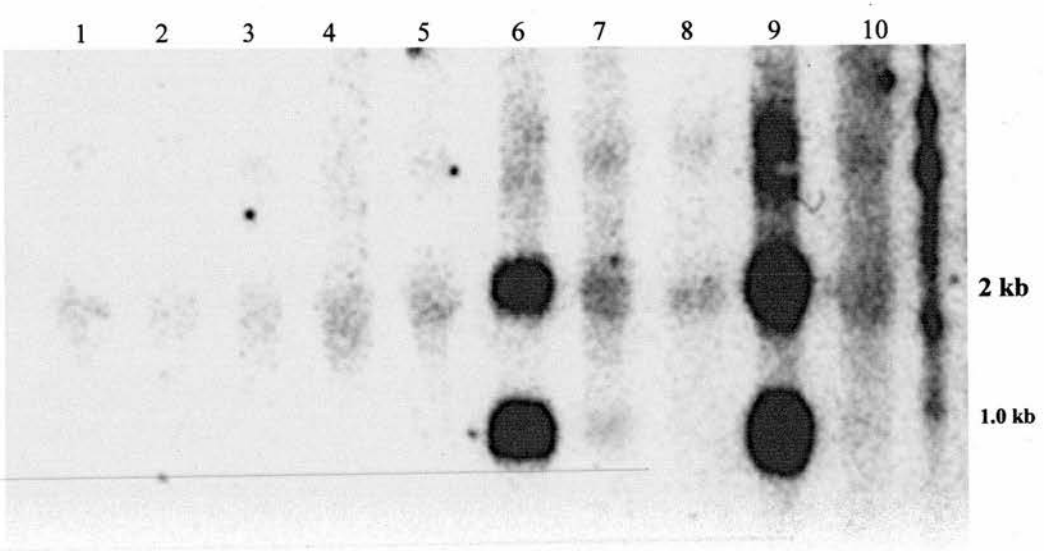

Guanylin long AA seq MSQRDLCEMREAVVLILLAFCFLQE----SQGVWVMDGDLSFPLEAV
Guanylin short AA seq MREAVVLILLAFCFLQE----SQGVWVMDGDLSFPLEAV
Guinea Pig guanylin AA seq MNTFLLSALCL-GAWAALVGAVTVQDGDFSFSLESV
Human guanylin AA seq MNAFLLFALCLLGAWAALAGGVTVQDGNFSFSLESV
Mouse guanylin AA seq MNACVLSVLCLLGALAVLVEGVTVQDGDLSFPLESV
Pig guanylin AA seq MNTFLFPTLCLLGVWAALAGGVTVKDGEFSFSLESV
Rat guanylin AA seq MNAWLLSVLCLLGALAVLVEGVTVQDGDLSFPLESV
Zebrafish part guanylin AA seq AFLVVALCLV------CDAVEVKEGDFSFTLESV
50
60
70
80
90
Guanylin long AA seq KVLKHLLGANTMS-------TPHPPNLGSHAVCS-NPHLPAEFLPVC
Guanylin short AA seq KVLKHLLGANTMS-------TPHPPNLGSHAVCS-NPHLPAEFLPVC
Guinea Pig guanylin AA seq KKLKDLQEAPESKVQGRRKF-------VAPPLCSF-SGFPEELRPVC
Human guanylin AA seq KKLKDLQEPQEPRVGKLRNFAPIPGEPVVPILCSNPN-FPEELKPLC
Mouse guanylin AA seq KKLKGLREVQEPRLVSHKKFAPRLLQPVAPQLCSSHSALPEALRPVC
Pig guanylin AA seq KKLKDLQELQKPR--NPRNL----DGPIIPVLCNSPK-FPEELKPIC
Rat guanylin AA seq KQLKHLREVQEPTLMSHKKFALRLPKPVAPELCS-QSAFPEALRPLC
Zebrafish part guanylin AA seq RILQQLAEQQKTQ-------NPRLAKTSYYSVCS-NPSLPQEFVPLC
100
110
120
?
Guanylin long AA seq EREGASALFNRLVDIITPPDPCEICANAACTGCL
Guanylin short AA seq EREGASALFNRLVDIITPPDPCEICANAACTGCL
Guinea Pig guanylin AA seq KEPNSQDILNRLAVIAQDPSTCEICAYAACAGC
Human guanylin AA seq KEPNAQEILQRLEEIAEDPGTCEICAYAACTGC
Mouse guanylin AA seq EKPNAEEILQRLEAIAQDPNTCEICAYAACTGC
Pig guanylin AA seq QKPNAEEILERLETIAQDPSTCEICAYAACAGC
Rat guanylin AA seq EKPNAEEILQRLEAIAQDPNTCEICAYAACTGC
Zebrafish part guanylin AA seq MQRGATMSFARLASV--PVDVCEICAFAACTGC

Fig. 3.7 Northern blot showing tissue expression of guanylin mRNA in yellow eels. Lanes 1-10 from left to right are; 1- white skeletal muscle, 2- liver, 3- stomach, 4- oesophagus (all SW-acclimated eels), 5- FW gill, 6- FW intestine, 7- FW kidney, 8- SW gill, 9-SW intestine and 10- SW kidney.


1
2
3
4
5
6
7
8
9
10
2 kb
1.0 kb

(5μg) from intestine, kidney and gill (not shown) of six individual eels from each experimental group was hybridised with [γ^{32}P]-dCTP labelled 234bp guanylin fragment as above (see Fig. 3.8 for autoradiograph). Expression was found in the intestine and kidney but not in gill.The 1kb band was quantified because sequencing results indicated that this was the expected size of the mRNA. Appendix 3 lists the raw volume of RNA applied to the gel for each sample as determined from the intensity of ethidium bromide staining by the GeneSnap gel documentation system. The RNA sample with the highest value for raw volume was chosen, from both intestine and kidney samples, as a reference sample. All other samples were then related to the reference to provide a correction factor for the amount of RNA loaded on the gel for each sample which was used to adjust the values determined from densitometry for RNA loading. Appendix 5 shows the statistical analysis of the data, demonstrating a significant rise in intestinal expression levels of guanylin in SW-acclimated yellow and silver eels compared to FW-acclimated eels. The bar chart in Fig. 3.9 represents the averaged expression levels of guanylin in each tissue from the four experimental groups.

3.2 Guanylate cyclase C

3.2.1 RT-PCR

A partial fragment of 2.4 kb of eel GC-C was amplified in a series of RT-PCRs. Each of the resulting amplified fragments were sequenced and the data used to design specific primers for subsequent PCR in an effort to amplify the full length GC-C cDNA. The first fragment to be amplified with the degenerate GC-C sense and GC-C antisense primer pair (see Appendix 2) was a 598 bp fragment spanning the protein kinase-like and cyclase catalytic domains (see primer sites in Fig. 2.2). The primers were used in RT-PCR with template cDNA from the intestine, gill and kidney of

Fig. 3.8 Quantitative Northern blot showing expression levels of guanylin mRNA in the intestine and kidney of six individual eels from each test group; yellow fresh water (YFW), yellow seawater (YSW), silver fresh water (SFW) and silver seawater (SSW) acclimated eels.

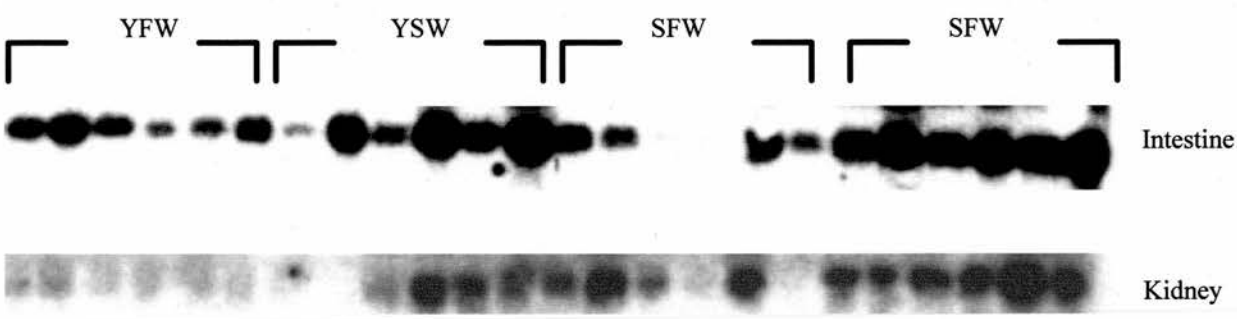
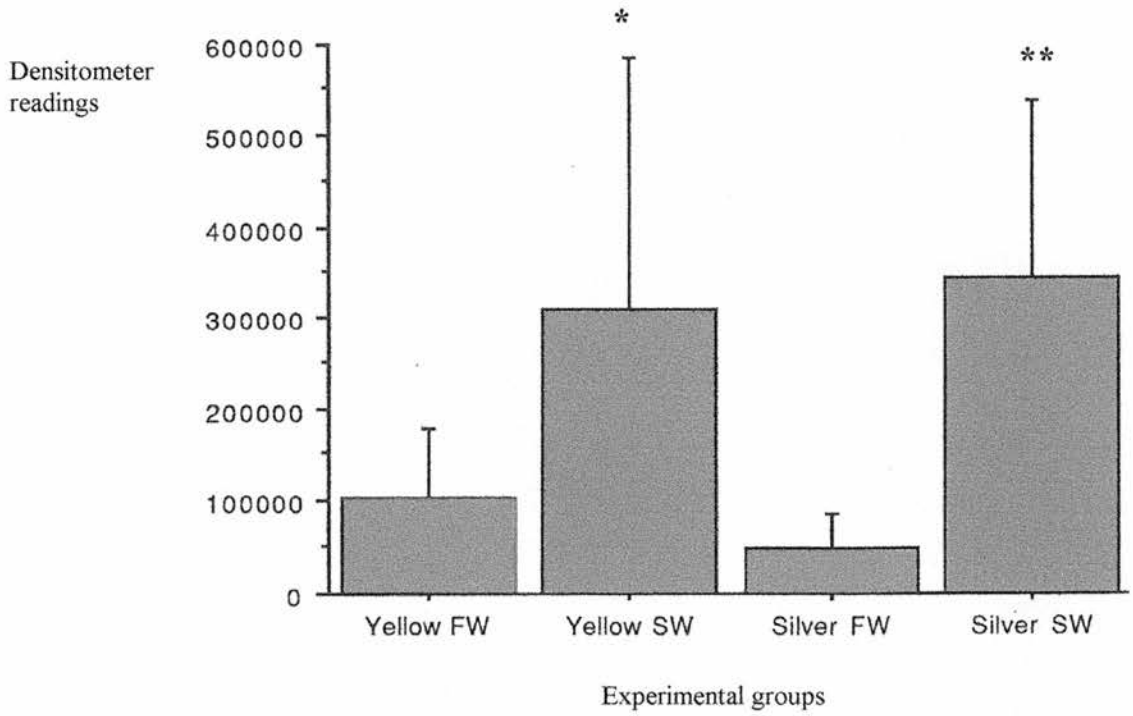

YFW
YSW
SFW
SFW
Intestine
Kidney

Fig. 3.9 Bar chart showing expression levels (mean, ± S.E.) of guanylin mRNA in a) the intestine, and b) the kidney, of eels from each test group (n=6 in all groups) where * indicates a significant difference in expression levels of guanylin at $P<0.05$ (Fisher's) between yellow FW and Yellow SW eel intestine and ** indicates a significant difference in expression levels at $P<0.01$ (Fisher's) between silver FW and silver SW eel intestine. No significant differences were observed in kidney guanylin expression levels between test groups.

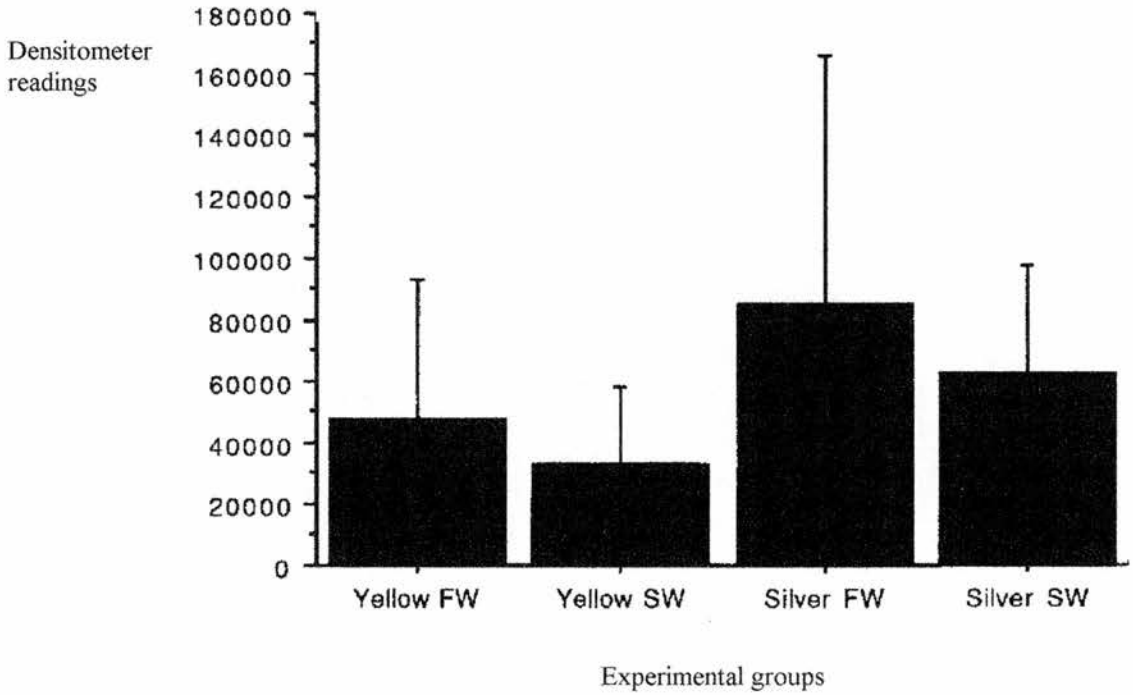
Intestine

a)



b)

Kidney



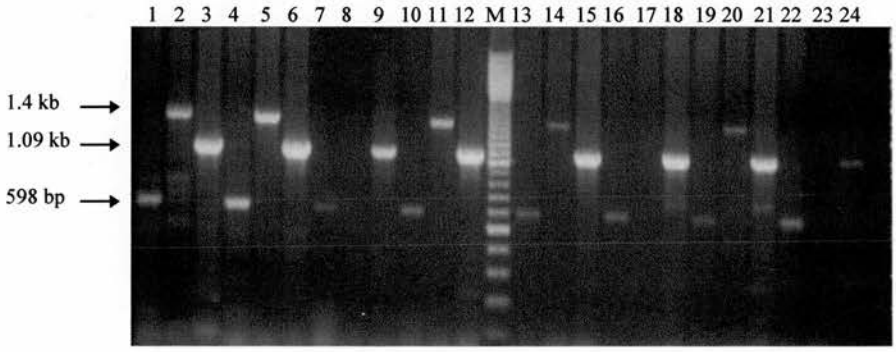
yellow and silver FW and SW eels. A 598 bp fragment was amplified in the intestine and kidney cDNA of all four experimental groups as shown in Fig. 3.10 a). A second fragment of 1.4 kb (incorporating the original 598 bp region and extending into the extracellular domain) was amplified with the degenerate GC-C4 sense primer (see Appendix 2) and the degenerate GC-C antisense primer (see Fig. 2.2). An attempt was made to design a specific antisense primer, designed from the sequence data obtained from the 598 bp fragment, but without success. The 1.4 kb fragment was amplified in cDNA from intestine and kidney of all four experimental groups and although Fig. 3.10 a) does not indicate amplification of the 1.4 kb fragment in YSW eel kidney subsequent PCR did confirm it to be present in this tissue (see Fig. 3.10 b). The final fragment amplified by RT-PCR was a 1.09 kb region towards the 5′ end of the molecule which was amplified with the degenerate GC-C6 sense primer (designed following the incorporation of the medaka fish sequences in the GenBank and the known mammalian sequences to a region near the 5′ end of the GC-C gene) and GC-C (2) specific antisense primer (see Fig. 2.2) designed to a region near the 5′ end of the known sequence of the 1.4 kb fragment. The final fragment was amplified in the cDNA of intestine and kidney from all four experimental groups- see Fig 3.10 a). No amplification was seen in any gill cDNA with any primer set (data not shown).

3.2.2 5′ and 3′ RACE PCR

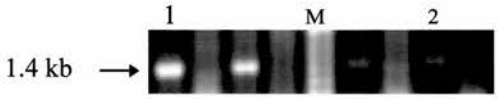
Attempts to amplify both the remaining 5′ and 3′ regions of the eel GC-C were unsuccessful; time constraints towards the end of the project did not permit further investigation.

Fig. 3.10 a) Results of RT-PCR using degenerate GC-C primers in template cDNA synthesised from RNA extracted from the intestine and kidney of yellow fresh water (YFW), yellow seawater (YSW), silver fresh water (SFW) and silver seawater (SSW) acclimated eels. Lanes 1-12 a) are amplifications of intestinal cDNA of YFW (lanes 1-3), YSW (lanes 4-6), SFW (lanes 7-9) and SSW (lanes 10-12) respectively. Lanes 13-24 a) are amplifications of kidney cDNA in the same test groups in the same order. The 1.4 kb band was amplified using the GC-C4 sense/GC-C antisense primer set, the 1.09 kb band was amplified using the GC-C6 sense/ GC-C(2) antisense primer set and the 598 bp band was amplified using the GC-C sense/GC-C antisense primer set (see Appendix 2 for primer sequences).
Fig. 3.10 a) does not demonstrate amplification of the 1.4 kb fragment from SFW intestine cDNA, YSW kidney cDNA or SSW kidney cDNA as the bands were very faint but **Fig. 3.10 b)**, lanes 1 and 2, shows amplification of the 1.4 kb band in SFW intestine and YSW kidney respectively and **Fig. 3.10 c)** lane 1 shows amplification of the 1.4 kb band in SSW kidney. The 100 bp DNA step ladder is represented by the letter M.

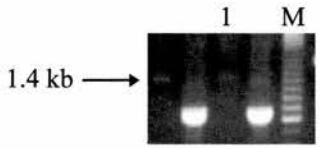
a)


1 2 3 4 5 6 7 8 9 10 11 12 M 13 14 15 16 17 18 19 20 21 22 23 24
1.4 kb
1.09 kb
598 bp

b)


1
M
2
1.4 kb

c)


1
M
1.4 kb

3.2.3 Cloning and sequencing of RT-PCR fragments

3.2.3 (i) Cloning

All three fragments of GC-C, amplified from intestine cDNA isolated from yellow SW-acclimated fish, were purified and cloned as described (see section 2.2 and 2.4). Positive clones were selected and colony PCR (see section 2.3) carried out to confirm the presence of inserts.

3.2.3 (ii) Sequencing

The nucleotide and derived amino acid sequence data of the 598 bp fragment is demonstrated in Fig. 3.11 and represents consensus sequences derived from three separate clones in both sense and antisense directions.The primer sites were excluded from the reported sequence in case of mismatching during annealing of the primers to the cDNA, resulting in a sequence of 529 bp. The short length of the fragment allowed complete sequencing of the fragment using the T7 sense and M13 antisense vector primers. Fig 3.12 demonstrates the nucleotide sequence and derived amino acid sequence of the combined 1.4 kb and 1.09 kb fragments, excluding the primer sites at the 5′ and 3′ ends of the combined sequence. Three clones containing the 1.4 kb fragment were sequenced in two steps; T7 and M13 vector primers provided sequence data of approximately 600 bp from each end of the sequence. The remaining unsequenced central region of the 1.4 kb fragment was determined by sequencing with specific nested primers (GC-C sense seq and GC-C antisense seq, see Appendix 2 and Fig. 2.2) designed from the data provided by the T7 and M13 sequencing reactions. Comparison of the 529 bp fragment with the same region of the 1.4 kb fragment indicated differences at the nucleotide and amino acid level, suggesting the

Fig. 3.11 Interleaved nucleotide and derived a.a.. sequence of the 529 bp fragment of eel GC-C(1). Primer sites at the 5′ and 3′ ends of the fragment are not included in the sequence.

```
    Leu Ile Arg Arg Leu Gln Ile Tyr Ser Arg Asn Leu Glu Asn Leu Val Glu     17
  2 CTG ATT CGC CGC CTG CAG ATC TAC TCC CGC AAC CTG GAG AAC CTG GTG GAG
    Gln Arg Thr Ala Leu Tyr Lys Ala Glu Arg Asp Arg Ala Asp Gln Leu Asn     34
 53 CAG CGG ACC GCC CTG TAC AAG GCG GAG AGG GAC AGG GCC GAC CAG CTC AAC
    Phe Met Leu Leu Pro Gly Pro Val Val Arg Ser Leu Lys Glu Thr Gly Ser     51
104 TTC ATG CTG CTG CCT GGG CCA GTG GTA CGC TCC CTG AAG GAG ACG GGG AGC
    Val Glu Pro Glu Leu Phe Asp Glu Val Thr Ile Tyr Phe Ser Asp Ile Val     68
155 GTG GAG CCT GAG CTT TTC GAC GAG GTG ACC ATC TAC TTC AGC GAC ATC GTG
    Gly Phe Thr Thr Val Cys Gln Tyr Ser Thr Pro Met Glu Val Val Asp Met     85
206 GGC TTC ACC ACC GTC TGC CAG TAC AGC ACG CCT ATG GAG GTG GTG GAC ATG
    Leu Asn Asp Ile Tyr Lys Asn Phe Asp Arg Ile Leu Asp Asn His Asp Val    102
257 CTG AAT GAC ATC TAC AAG AAC TTC GAC AGG ATC CTC GAC AAT CAC GAT GTT
    Tyr Lys Val Glu Thr Ile Gly Asp Ala Tyr Met Val Val Ser Gly Leu Pro    119
308 TAC AAG GTG GAG ACG ATT GGC GAC GCC TAC ATG GTG GTG TCG GGG CTC CCG
    Arg Arg Asn Gly Asn Arg His Ala Val Asp Ile Cys Arg Met Ala Leu Asp    136
359 CGC CGG AAC GGG AAC AGG CAC GCG GTG GAC ATC TGC CGC ATG GCG CTG GAC
    Ile Val Ala Phe Met Gly Thr Phe Glu Leu Gln His Leu Pro Gly Leu Pro    153
410 ATC GTG GCC TTC ATG GGT ACC TTC GAG CTG CAG CAC CTG CCG GGG CTG CCC
    Val Trp Ile Arg Ile Gly Val His Ser Gly Pro Cys Ala Ala Gly Val Val    170
461 GTG TGG ATC CGC ATC GGA GTG CAC TCT GGT CCC TGT GCA GCG GGA GTG GTG
    Gly Ser Lys Met Pro Arg                                                176
512 GGG AGC AAG ATG CCT CGG
```

Fig. 3.12 Interleaved and combined nucleotide and derived a.a. sequence of the 1.4 kb and 1.09 kb fragments of eel GC-C(2).

```
     Arg Ala Tyr Leu Thr Arg Leu Leu Pro Pro Ala Arg Lys Ile Ser Asn Leu  17
   1 AGG GCC TAC CTA ACG CGG CTG CTT CCC CCG GCG CGA AAG ATC TCC AAC CTC
     Phe Ile Glu Phe Leu Arg Phe Glu Glu Ser Ser Leu Lys Pro Arg Trp Glu  34
  52 TTC ATC GAG TTC CTG CGC TTT GAA GAG AGC AGC CTG AAG CCG CGT TGG GAA
     Ala Val Tyr Val Tyr Lys Lys Pro Glu Asn Ser Glu Asp Cys Phe Trp Tyr  51
 103 GCG GTC TAC GTC TAC AAG AAA CCG GAG AAT TCG GAG GAC TGC TTC TGG TAC
     Ile Asn Ala Leu Asp Ala Pro Ser Ala Ala Phe Asn Ser Ala Ile Thr Arg  68
 154 ATC AAC GCC CTT GAT GCC CCG TCA GCT GCA TTT AAC TCA GCA ATA ACG AGG
     Lys Val Leu Arg Lys Pro Glu Glu Leu His Ser Ala Phe Glu Arg Lys Asn  85
 205 AAG GTC CTG CGC AAA CCG GAG GAA CTG CAC TCA GCC TTC GAG AGG AAG AAC
     Arg Thr Ser Asn Leu Phe Ile Ile Cys Gly Thr Pro Glu Asp Val Ala Asn 102
 256 AGG ACG AGC AAC TTG TTT ATT ATC TGT GGG ACC CCC GAG GAC GTT GCC AAT
     Leu Thr Asp Asn Gly Arg Arg Leu Glu Pro Asp Ile Val Ile Leu Leu Ile 119
 307 TTG ACA GAC AAT GGA CGC CGT CTT GAG CCG GAC ATT GTC ATC CTT CTG ATT
     Asp Leu Tyr Asn His Glu Tyr His Ser Ala Ala Gly Ser Pro Ala Met Ser 136
 358 GAT CTT TAC AAC CAC GAG TAC CAC AGT GCT GCA GGC AGT CCG GCT ATG AGC
     Asn Val Leu Val Ile Thr Met Pro Asn Ile Arg Asn Tyr Thr Glu Gly Trp 153
 409 AAC GTG CTG GTC ATA ACC ATG CCC AAC ATA AGG AAC TAC ACA GAG GGC TGG
     Thr Asp Asn Gly Thr Leu Pro Glu Met Asn Asp Tyr Val Ala Gly Tyr His 170
 460 ACC GAC AAT GGC ACG CTA CCG GAG ATG AAT GAC TAT GTT GCG GGG TAC CAT
     Asp Gly Val His Leu Phe Gly Leu Val Leu Arg Gln Lys Met Leu Tyr Gly 187
 511 GAC GGG GTG CAC CTG TTT GGT TTG GTG CTG AGG CAG AAG ATG CTC TAT GGT
     Glu Gly Ser Val Glu Glu Asn Ala Ser Val Glu Asn Pro Phe Lys Asn Ile 204
 562 GAA GGC AGT GTT GAA GAG AAC GCC TCA GTT GAA AAC CCC TTC AAG AAC ATC
     Ser Phe Ser Gly Ile Gly Gly Gln Tyr Val Leu Asp Glu His Gly Asp Arg 221
 613 TCC TTC AGT GGT ATT GGT GGG CAA TAT GTG CTA GAC GAA CAT GGA GAC CGA
     Asp Val Asn Phe Ser Val Met Tyr Met Ser Thr Thr Asp Ser Gln Tyr Lys 238
 664 GAT GTA AAT TTT TCG GTG ATG TAC ATG TCG ACC ACT GAC AGC CAG TAT AAA
     Val Leu Phe Glu Phe Asp Thr Ser Thr Asn Asn Thr Ala Val Val Asp Ala 255
 715 GTT CTG TTT GAG TTT GAC ACG TCC ACC AAC AAC ACG GCA GTC GTA GAC GCT
     Asn Pro Thr Trp His Trp Lys Ser Ser Arg Leu Pro Asp Asp Arg Pro Ala 272
 766 AAC CCG ACC TGG CAC TGG AAG AGC TCC CGG CTG CCG GAC GAC AGG CCC GCC
     Gln Glu Gln Val Leu Leu Ala Thr Gln Asp Ile Ile Val Ile Val Leu Gly 289
 817 CAG GAG CAG GTG CTG CTG GCC ACT CAG GAC ATC ATC GTC ATC GTC CTG GGC
     Ile Ser Val Val Val Val Thr Val Phe Ala Leu Ile Phe Tyr Arg Arg Asn 306
 868 ATC TCT GTG GTG GTG GTG ACG GTC TTC GCC CTC ATC TTT TAC AGG CGA AAT
     Ile Lys Asp Arg Gln Ala Gln Lys Gln Trp Ser His Ile Pro Pro Asp Arg 323
 919 ATT AAA GAC CGG CAG GCT CAA AAG CAG TGG TCC CAC ATC CCT CCG GAC CGG
     Ile Thr Pro Val Asp Asp Lys Asp Phe Ser Leu Val Ser Leu Lys Ile Asp 340
 970 ATC ACC CCT GTG GAC GAC AAA GAC TTC AGC CTG GTC TCC CTC AAG ATT GAT
     Glu Asp Gln Arg Lys Asp Ser Ile Asp Arg Ile Arg Arg Gly Arg Tyr Asp 357
1021 GAG GAC CAG AGG AAG GAC AGC ATT GAC AGA ATT CGC CGG GGA CGC TAC GAT
     Gln Lys Pro Ile Ile Leu Lys Glu Leu Lys Asn Thr Glu Gly Tyr Phe Ser 374
1072 CAA AAG CCC ATC ATT CTG AAG GAG CTG AAG AAC ACG GAG GGG TAC TTC AGC
     Glu Glu Lys Arg Ile Glu Leu Asn Thr Leu Leu Arg Ile Asp Tyr Tyr Asn 391
1123 GAG GAG AAG AGG ATC GAG CTT AAC ACG CTC CTG CGC ATC GAT TAC TAC AAC
     Leu Thr Lys Phe Tyr Gly Thr Val Lys Phe Glu Tyr Gly Val Phe Gly Val 408
1174 CTG ACC AAG TTC TAC GGC ACG GTC AAG TTT GAG TAC GGC GTG TTT GGA GTG
     Tyr Glu Phe Cys Glu Arg Gly Ser Leu Arg Tyr Val Leu Asn Asp Lys Ile 425
1225 TAT GAA TTC TGC GAG AGA GGA TCG CTG CGG TAC GTG CTG AAT GAC AAG ATC
     Ser Tyr Pro Glu Glu Thr Phe Met Asp Leu Glu Phe Lys Ile Ser Val Met 442
1276 TCT TAT CCA GAG GAG ACC TTC ATG GAC CTG GAG TTT AAA ATC TCT GTC ATG
     His Asp Ile Val Lys Gly Met Ser Tyr Leu His Leu Ser Asn Ile Ala Val 459
1327 CAT GAC ATT GTT AAG GGC ATG TCC TAC CTC CAC CTG AGC AAC ATC GCA GTC
     His Gly Arg Leu Lys Ser Thr Asn Cys Val Val Asp Ala Arg Met Val Val 476
1378 CAC GGC CGC CTA AAA TCC ACA AAC TGC GTG GTG GAC GCC CGC ATG GTG GTC
     Lys Ile Thr Asp Phe Gly Cys Asn Asn Ile Leu Arg Pro Ser Arg Asp Leu 493
1429 AAA ATC ACG GAT TTC GGC TGC AAC AAC ATC CTT CGT CCC AGC AGA GAC CTG
     Trp Thr Ala Pro Glu His Leu Arg Asn Pro Gly Thr Ser Gln Lys Gly Asp 510
1480 TGG ACG GCC CCG GAG CAC CTG CGT AAC CCC GGG ACC TCC CAG AAG GGA GAC
     Val Tyr Ser Phe Gly Ile Ile Ser Gln Glu Ile Leu Leu Arg Lys Cys Thr 527
1531 GTG TAC AGC TTC GGC ATC ATC AGC CAG GAG ATC CTG CTG AGG AAG TGC ACC
     Phe Tyr Thr Ala Ala Cys Ser Asp Arg Ala Glu Lys Thr Tyr Arg Val Gln 544
1582 TTC TAC ACC GCC GCG TGT TCC GAT CGA GCG GAG AAG ACA TAC AGA GTG CAG
     Phe Pro Asp Ser Cys Ser Phe Phe Arg Pro Asp Leu Asn Leu Glu Ser Ala 561
1633 TTC CCG GAC TCC TGC TCC TTC TTC AGG CCC GAC CTG AAT TTG GAG TCG GCA
     Gly Glu Arg Glu Arg Glu Leu Cys Gly Leu Ile Lys Asn Cys Trp Asp Glu 578
1684 GGG GAG AGA GAG CGG GAG CTA TGT GGG CTC ATT AAG AAC TGC TGG GAT GAG
     Asp Pro Glu Lys Arg Pro Asp Phe Lys Lys Ile Glu Ser Cys Leu Val Lys 595
```

```
1735 GAC CCA GAG AAG AGG CCG GAT TTC AAG AAG ATA GAA AGC TGT CTG GTC AAG
     Ile Ser Ser Leu His Ser Gln Gly Asn Glu Ser Tyr Met Asp Ser Met Ile 612
1786 ATC AGC AGC CTG CAC AGC CAG GGC AAT GAG AGC TAC ATG GAC AGC ATG ATC
     Arg Arg Leu Gln Met Tyr Ser Arg Asn Leu Glu His Leu Val Glu Glu Arg 629
1837 CGG CGG CTG CAG ATG TAC TCA CGC AAC CTG GAG CAC CTG GTG GAG GAG AGG
     Thr Leu Tyr Lys Arg Ala Glu Arg Asp Arg Ala Asp Gln Leu Asn Phe Met 646
1888 ACG TTG TAC AAG AGG GCC GAG AGG GAC CGA GCC GAC CAG CTC AAC TTC ATG
     Leu Leu Pro Gly Pro Val Val Gln Ser Leu Lys Glu Arg Gly Tyr Val Glu 663
1939 CTG CTG CCT GGG CCT GTG GTA CAG TCC CTG AAG GAG CGT GGC TAT GTG GAG
     Pro Glu Leu Phe Asp Glu Val Ser Ile Tyr Phe Ser Asp Ile Val Gly Phe 680
1990 CCA GAG CTG TTT GAC GAG GTG AGC ATC TAC TTC AGC GAC ATC GTG GGC TTC
     Thr Thr Leu Cys Gln Tyr Ser Thr Pro Met Glu Val Val Asn Met Leu Asn 697
2041 ACC ACC CTG TGC CAG TAC AGC ACG CCC ATG GAG GTG GTG AAC ATG CTG AAT
     Glu Ile Tyr Lys Asn Phe Asp Ser Ile Leu Asp Asn His Asp Val Tyr Lys 714
2092 GAA ATC TAC AAG AAC TTC GAC AGC ATC CTC GAC AAC CAC GAC GTC TAC AAG
     Val Glu Thr Ile Gly Asp Ala Tyr Met Val Ala Ser Gly Leu Pro Asn Arg 731
2143 GTG GAG ACG ATC GGT GAT GCG TAC ATG GTG GCA TCG GGG CTG CCC AAC AGG
     Asn Gly Asn Arg His Ala Val Asp Ile Ser Arg Met Ala Leu Asp Ile Leu 748
2194 AAT GGG AAC AGG CAT GCG GTG GAC ATC TCC CGC ATG GCG CTG GAC ATC CTG
     Ser Phe Met Gly Thr Phe Arg Leu Gln His Leu Gln Glu Leu Pro Val Trp 765
2245 TCC TTC ATG GGC ACC TTT CGG CTG CAA CAC CTG CAG GAG CTG CCC GTG TGG
     Ile Arg Ile Gly Val His Ser Gly Pro Cys Ala Ala Gly Val Val Gly Ile 782
2296 ATC CGC ATC GGG GTG CAC TCA GGT CCC TGT GCA GCA GGG GTG GTG GGA ATT
     Lys Met Pro Arg                                                     786
2347 AAG ATG CCC CGA
```

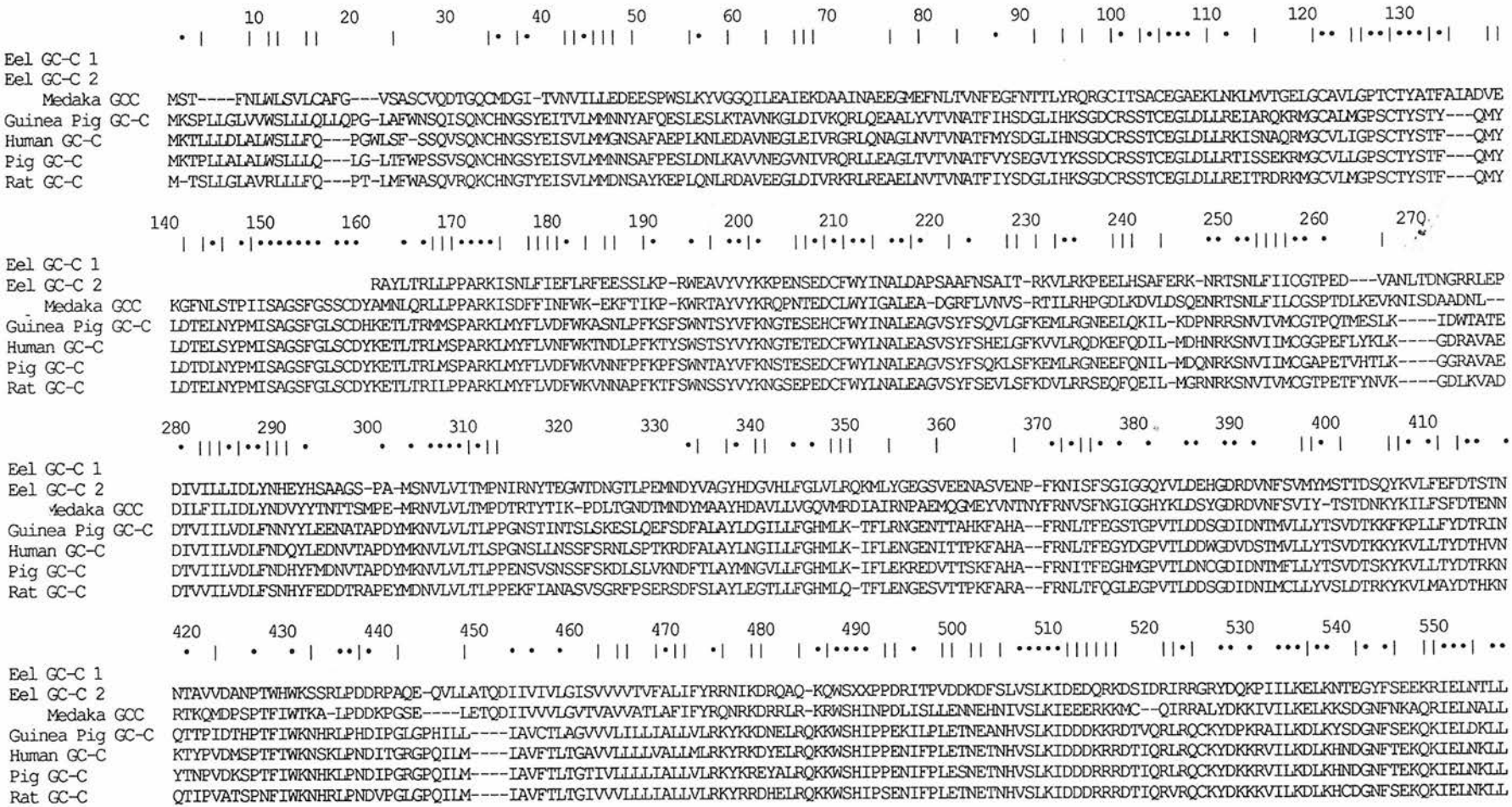
presence of two isoforms of eel GC-C. The two isoforms were termed GC-C (1) and GC-C (2) respectively but attempts to amplify further regions of the GC-C (1) isoform, represented by the 529 bp fragment, proved unsuccessful. Two specific anti sense primers - GC-C (1) specific antisense and GC-C (2) specific antisense, see Appendix 2 - designed from the 529 bp and 1.4 kb fragment sequence data respectively, were used in RT-PCR with separate degenerate sense primers. The 1.09 kb fragment which was amplified by RT-PCR using GC-C6 degenerate sense and GC-C (2) specific antisense primers (see section 3.2.1) was sequenced from three separate clones using the T7 sense and M13 antisense vector primers. The sequence data provided by each primer overlapped in the central region of the fragment but each primer did not reach the opposite end of the fragment. The sequence data corresponding to the 1.09 kb fragment, as shown in Fig. 3.12, therefore represents a consensus of the available information from three clones with each primer. Time constraints did not permit further nested sequencing reactions on each strand of the 1.09 kb fragment. Alignment of the derived a.a. sequences for the 529 bp and 2.4 kb eel GC-C fragments with those of other species is shown in Fig. 3.13 and homology between the GC-C(1), GC-C(2) and GC-C from other species is 84-87% in the cyclase catalytic and protein kinase-like region.

3.2.4 Northern hybridisation

3.2.4 (i) Tissue blot

Total RNA extracted from a range of yellow eel tissues was hybridised with [γ^{32}P-dCTP]-labelled GC-C probe. The chosen probe was purified 1.09 kb fragment, amplified with GC-C6 sense and GC-C (2) specific antisense primers (nucleotides 1 - 988, excluding the primer sites, see Fig. 3.12) , which represented the GC-C(2) isoform. Fig. 3.14 demonstrates that GC-C(2) is expressed in the intestine and kidney

Fig. 3.13 Alignment of a.a. sequences of eel GC-C(1) and eel GC-C(2) isoform fragments with GC-C a.a. sequences from other vertebrate species. Black dots indicate conserved a.a. sequences across the species range.


Eel GC-C 1
Eel GC-C 2
Medaka GCC
Guinea Pig GC-C
Human GC-C
Pig GC-C
Rat GC-C

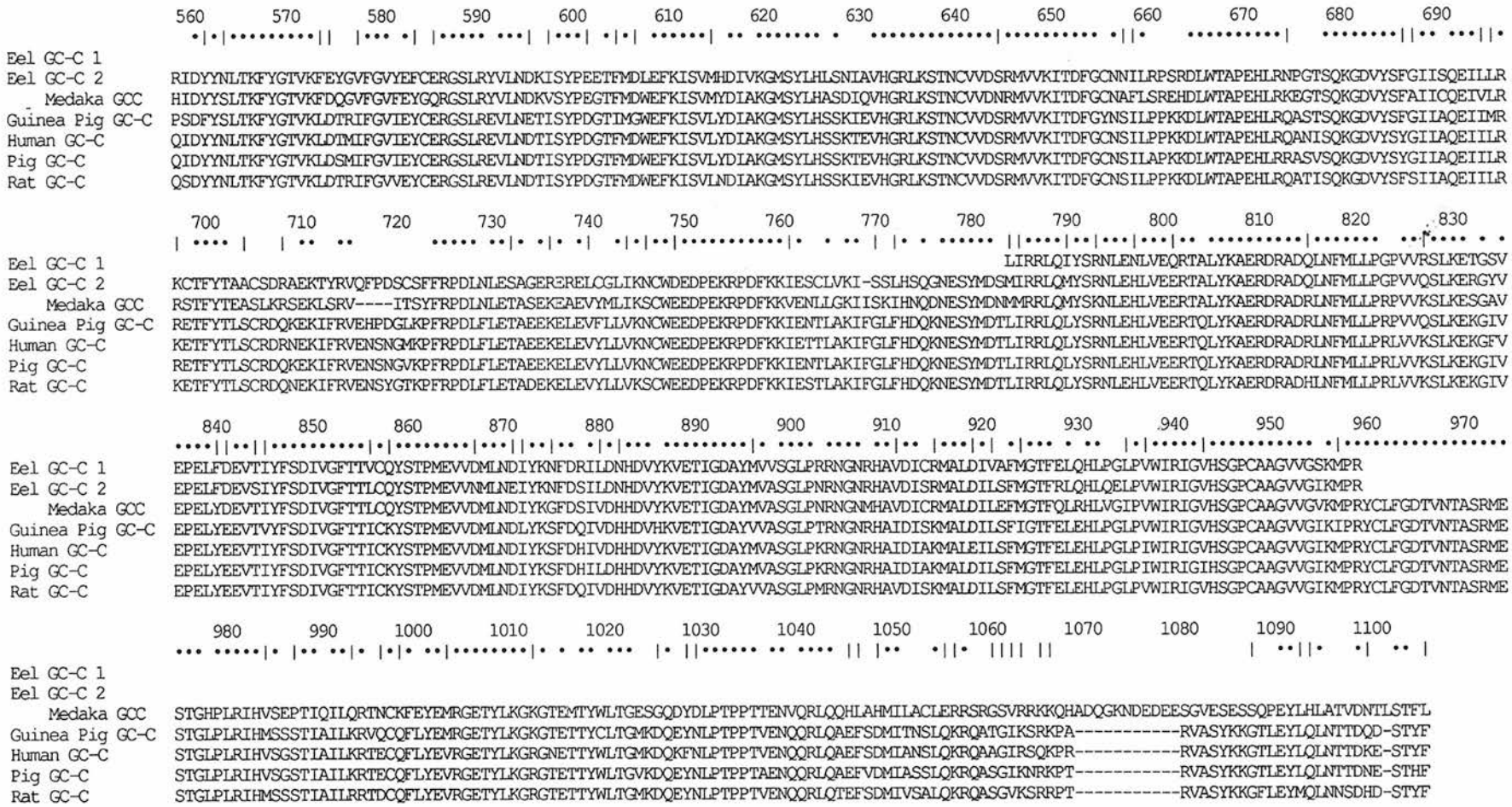
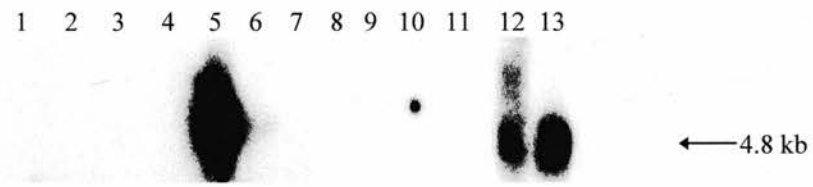

560 570 580 590 600 610 620 630 640 650 660 670 680 690
Eel GC-C 1
Eel GC-C 2 RIDYYNLTKFYGTVKFEYGVFGVYEFCERGSLRYVLNDKISYPEETFMDLEFKISVMHDIVKGMSYLHLSNIAVHGRLKSTNCVVDSRMVVKITDFGCNNILRPSRDLWTAPEHLRNPGTSQKGDVYSFGIISQEILLR
Medaka GCC HIDYYSLTKFYGTVKFDQGVFGVFEYGQRGSLRYVLNDKVSYPEGTFMDWEFKISVMYDIAKGMSYLHASDIQVHGRLKSTNCVVDNRMVVKITDFGCNAFLSREHDLWTAPEHLRKEGTSQKGDVYSFAIICQEIVLR
Guinea Pig GC-C PSDFYSLTKFYGTVKLDTRIFGVIEYCERGSLREVLNETISYPDGTIMGWEFKISVLYDIAKGMSYLHSSKIEVHGRLKSTNCVVDSRMVVKITDFGYNSILPPKKDLWTAPEHLRQASTSQKGDVYSFGIIAQEIIMR
Human GC-C QIDYYNLTKFYGTVKLDTMIFGVIEYCERGSLREVLNDTISYPDGTFMDWEFKISVLYDIAKGMSYLHSSKTEVHGRLKSTNCVVDSRMVVKITDFGCNSILPPKKDLWTAPEHLRQANISQKGDVYSYGIIAQEIILR
Pig GC-C QIDYYNLTKFYGTVKLDSMIFGVIEYCERGSLREVLNDTISYPDGTFMDWEFKISVLYDIAKGMSYLHSSKTEVHGRLKSTNCVVDSRMVVKITDFGCNSILAPKKDLWTAPEHLRRASVSQKGDVYSYGIIAQEIILR
Rat GC-C QSDYYNLTKFYGTVKLDTRIFGVVEYCERGSLREVLNDTISYPDGTFMDWEFKISVLNDIAKGMSYLHSSKIEVHGRLKSTNCVVDSRMVVKITDFGCNSILPPKKDLWTAPEHLRQATISQKGDVYSFSIIAQEIILR
700 710 720 730 740 750 760 770 780 790 800 810 820 830
Eel GC-C 1 LIRRLQIYSRNLENLVEQRTALYKAERDRADQLNFMLLPGPVVRSLKETGSV
Eel GC-C 2 KCTFYTAACSDRAEKTYRVQFPDSCSFFRPDLNLESAGERERELCGLIKNCWDEDPEKRPDFKKIESCLVKI-SSLHSQGNESYMDSMIRRLQMYSRNLEHLVEERTALYKAERDRADQLNFMLLPGPVVQSLKERGYV
Medaka GCC RSTFYTEASLKRSEKLSRV----ITSYFRPDLNLETASEKEAEVYMLIKSCWEEDPEKRPDFKKVENLLGKIISKIHNQDNESYMDNMRRLQMYSKNLEHLVEERTALYKAERDRADRLNFMLLPRPVVKSLKESGAV
Guinea Pig GC-C RETFYTLSCRDQKEKIFRVEHPDGLKPFRPDLFLETAEEKELEVFLLVKNCWEEDPEKRPDFKKIENTLAKIFGLFHDQKNESYMDTLIRRLQLYSRNLEHLVEERTQLYKAERDRADRLNFMLLPRPVVQSLKEKGIV
Human GC-C KETFYTLSCRDRNEKIFRVENSNGMKPFRPDLFLETAEEKELEVYLLVKNCWEEDPEKRPDFKKIETTLAKIFGLFHDQKNESYMDTLIRRLQLYSRNLEHLVEERTQLYKAERDRADRLNFMLLPRLVVKSLKEKGFV
Pig GC-C RETFYTLSCRDQKEKIFRVENSNGVKPFRPDLFLETAEEKELEVYLLVKNCWEEDPEKRPDFKKIENTLAKIFGLFHDQKNESYMDTLIRRLQLYSRNLEHLVEERTQLYKAERDRADRLNFMLLPRLVVKSLKEKGIV
Rat GC-C KETFYTLSCRDQNEKIFRVENSYGTKPFRPDLFLETADEKELEVYLLVKSCWEEDPEKRPDFKKIESTLAKIFGLFHDQKNESYMDTLIRRLQLYSRNLEHLVEERTQLYKAERDRADHLNFMLLPRLVVKSLKEKGIV
840 850 860 870 880 890 900 910 920 930 940 950 960 970
Eel GC-C 1 EPELFDEVTIYFSDIVGFTTVCQYSTPMEVVDMLNDIYKNFDRILDNHDVYKVETIGDAYMVVSGLPRRNGNRHAVDICRMALDIVAFMGTFELQHLPGLPVWIRIGVHSGPCAAGVVGSKMPR
Eel GC-C 2 EPELFDEVSIYFSDIVGFTTLCQYSTPMEVVNMLNEIYKNFDSILDNHDVYKVETIGDAYMVASGLPNRNGNRHAVDISRMALDILSFMGTFRLQHLQELPVWIRIGVHSGPCAAGVVGIKMPR
Medaka GCC EPELYDEVTIYFSDIVGFTTLCQYSTPMEVVDMLNDIYKGFDSIVDHHDVYKVETIGDAYMVASGLPNRNGNMHAVDICRMALDILEFMGTFQLRHLVGIPVWIRIGVHSGPCAAGVVGVKMPRYCLFGDTVNTASRME
Guinea Pig GC-C EPELYEEVTVYFSDIVGFTTICKYSTPMEVVDMLNDLYKSFDQIVDHHDVHKVETIGDAYVVASGLPTRNGNRHAIDISKMALDILSFIGTFELEHLPGLPVWIRIGVHSGPCAAGVVGIKIPRYCLFGDTVNTASRME
Human GC-C EPELYEEVTIYFSDIVGFTTICKYSTPMEVVDMLNDIYKSFDHIVDHHDVYKVETIGDAYMVASGLPKRNGNRHAIDIAKMALEILSFMGTFELEHLPGLPIWIRIGVHSGPCAAGVVGIKMPRYCLFGDTVNTASRME
Pig GC-C EPELYEEVTIYFSDIVGFTTICKYSTPMEVVDMLNDIYKSFDHILDHHDVYKVETIGDAYMVASGLPKRNGNRHAIDIAKMALDILSFMGTFELEHLPGLPIWIRIGIHSGPCAAGVVGIKMPRYCLFGDTVNTASRME
Rat GC-C EPELYEEVTIYFSDIVGFTTICKYSTPMEVVDMLNDIYKSFDQIVDHHDVYKVETIGDAYVVASGLPMRNGNRHAVDISKMALDILSFMGTFELEHLPGLPVWIRIGVHSGPCAAGVVGIKMPRYCLFGDTVNTASRME
980 990 1000 1010 1020 1030 1040 1050 1060 1070 1080 1090 1100
Eel GC-C 1
Eel GC-C 2
Medaka GCC STGHPLRIHVSEPTIQILQRTNCKFEYEMRGETYLKGKGTEMTYWLTGESGQDYDLPTPPTTENVQRLQQHLAHMILACLERRSRGSVRRKKQHADQGKNDEDEESGVESESSQPEYLHLATVDNTLSTFL
Guinea Pig GC-C STGLPLRIHMSSSTIAILKRVQCQFLYEMRGETYLKGKGTETTYCLTGMKDQEYNLPTPPTVENQQRLQAEFSDMITNSLQKRQATGIKSRKPA----------RVASYKKGTLEYLQLNTTDQD-STYF
Human GC-C STGLPLRIHVSGSTIAILKRTECQFLYEVRGETYLKGRGNETTYWLTGMKDQKFNLPTPPTVENQQRLQAEFSDMIANSLQKRQAAGIRSQKPR----------RVASYKKGTLEYLQLNTTDKE-STYF
Pig GC-C STGLPLRIHVSGSTIAILKRTECQFLYEVRGETYLKGRGTETTYWLTGVKDQEYNLPTPPTAENQQRLQAEFVDMIASSLQKRQASGIKNRKPT----------RVASYKKGTLEYLQLNTTDNE-STHF
Rat GC-C STGLPLRIHMSSSTIAILRRTDCQFLYEVRGETYLKGRGTETTYWLTGMKDQEYNLPTPPTVENQQRLQTEFSDMIVSALQKRQASGVKSRRPT----------RVASYKKGFLEYMQLNNSDHD-STYF

Fig 3.14 Northern blot showing tissue expression of GC-C(2) mRNA in yellow eels. Lanes 1-13 from left to right are; 1- brain, 2- heart, 3- white skeletal muscle, 4- gill, 5- intestine, 6- kidney, 7- liver, 8- stomach, 9- oesophagous and 10- eye, all SW-acclimated eels, and 11- gill, 12- intestine and 13- kidney, from FW-acclimated eels.


1 2 3 4 5 6 7 8 9 10 11 12 13
4.8 kb

of FW and SW-acclimated fish. The transcript size was estimated to be 4.8 kb. Since no sequence data is available for the GC-C(1) isoform in the extracellular domain, it is not known whether sufficient homology exists between GC-C(1) and GC-C(2) for the probe also to detect the GC-C(1) isoform.

3.2.4 (ii) Quantitative blot

As with guanylin, expression levels were compared between the intestine, kidney and gill (not shown) of yellow and silver, FW and SW-acclimated eels. The same membrane as that used in the guanylin quantitative blot (see section 3.2.4 (i)) was stripped and re-probed with [γ^{32}P]-dCTP-labelled 1.09 kb GC-C(2) fragment (see Fig. 3.15).Transcripts were not detected in gill samples. Appendix 4 gives the raw data for GC-C(2) expression levels in kidney and intestine. Radioactive counts for the probe on the Northern blot were determined by the Instant Imager (see Appendix 4) and adjusted for differences in RNA loading as previously described (see section 3.1.4 (ii)). This demonstrated that expression levels of GC-C(2) in the intestine of yellow SW-acclimated eels were significantly higher than in yellow FW-acclimated eels. Expression levels of GC-C(2) were also significantly increased in silver SW-acclimated eel intestine compared to yellow FW-acclimated eels. Expression levels of GC-C(2) were considerably higher in the intestine of silver FW-acclimated eels compared to yellow FW-acclimated eels although not statistically significant. Appendix 6 shows the statistical analysis of the data. Fig. 3.16 shows a graph of comparative, averaged GC-C(2) expression levels represented in radioactive counts of the probe on the Northern blot. As a result of the extremely low and highly variable expression levels of GC-C(2) in kidney no analysis was conducted.

Fig. 3.15 Quantitative Northern blot demonstrating expression levels of eel GC-C(2) in the intestine and kidney of six individual eels from each experimental group, yellow fresh water (YFW), yellow seawater (YSW), silver fresh water (SFW) and silver seawater (SSW) eels.

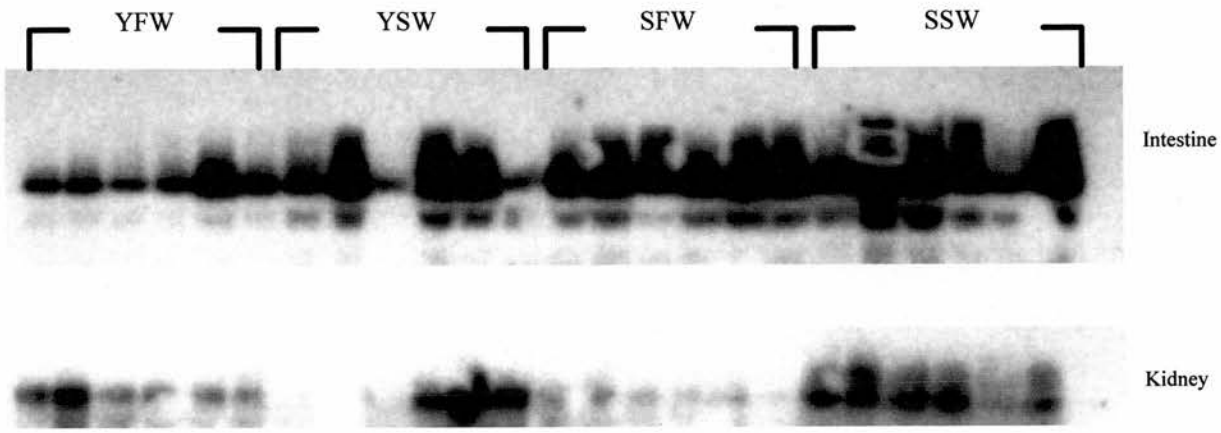
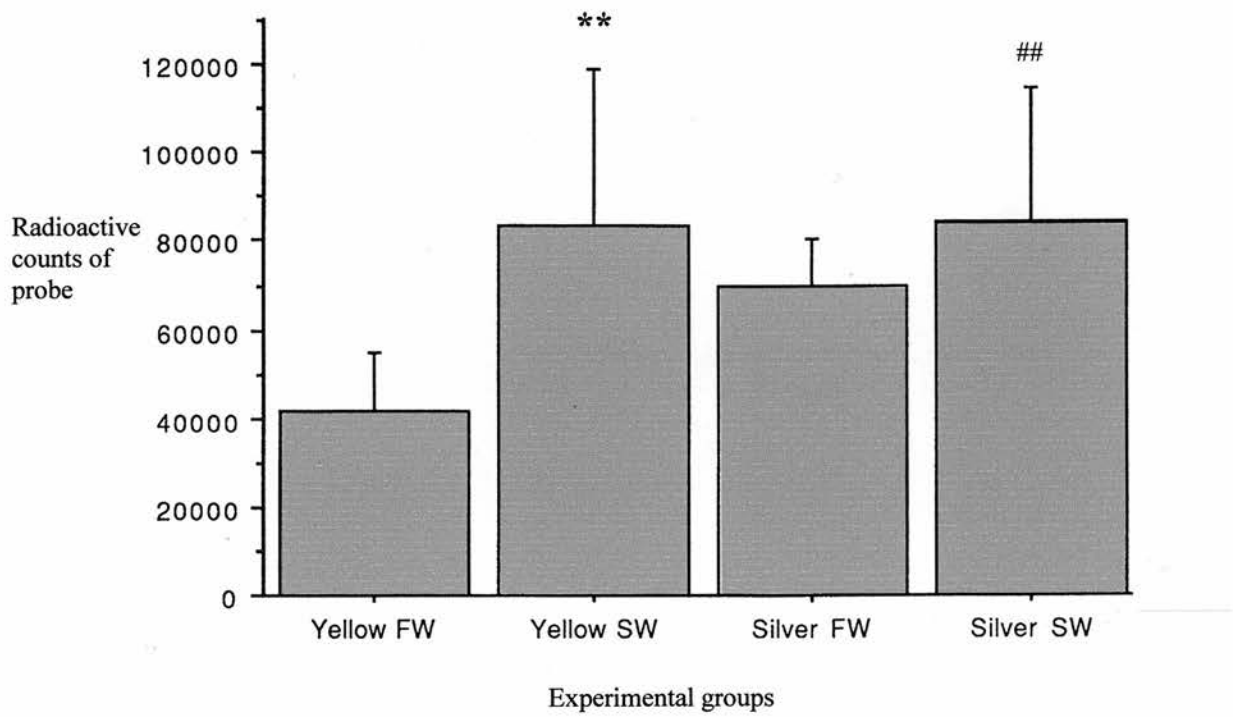

YFW
YSW
SFW
SSW
Intestine
Kidney

Fig. 3.16 Bar chart demonstrating comparative mRNA expression (mean ± S.E.) of eel GC-C(2) in the intestine of each experimental group (n=6 in all groups) where ** indicates a significant difference at P<0.01 (Fisher's) between yellow FW-acclimated and Yellow SW-acclimated fish, and ## indicates a significant difference between silver SW-acclimated and yellow FW-acclimated fish at P<0.01 (Fisher's).


**
##
120000
100000
80000
60000
40000
20000
0
Radioactive counts of probe
Yellow FW
Yellow SW
Silver FW
Silver SW
Experimental groups

3.2.5 Western blotting

3.2.5 (i) ELISA determination of antibody titres

The titres of both GC-C antibodies, raised in sheep and rabbit, were very low. The positive control, carried out using a previously characterised antibody to an isoform of the Na^+, K^+-ATPase β-subunit (β233), gave the expected high titre in the ELISA (see Fig. 3.17 for graph of ELISA results). The sheep and rabbit antisera had very low cross-reactivity at all dilutions used with the highest optical density of 0.29 O.D. units found with the 1/10 dilution for the rabbit antiserum and 0.15 O.D. units at 1/5 dilution for the sheep antiserum.

3.2.5 (ii) Western blot

Despite the low titre of each antibody, a western blot was carried out in an attempt to detect the GC-C protein in membrane extracts prepared from the intestine of yellow and silver FW and SW eels. A band of 45 kDA was detected as expected by the β233 positive control at 1/1000 dilution but no bands of the expected molecular size of ~145-160 kDa were detected by the sheep or rabbit GC-C antisera which were both used at two dilutions, 1/10 and 1/20 (see section 2.7.3 (iii)). Fig 3.18 demonstrates the results of the Western blots. Data from mammalian GC-C sequences indicate a protein size of ~145-160 kDa and no bands of a comparable size were detected on the Western blot.

Fig. 3.17 Graph demonstrating the results of the ELISA, showing titres for the eel GC-C antibodies raised in sheep (GC-C S) and rabbit (GC-C R) and the β233 positive control.

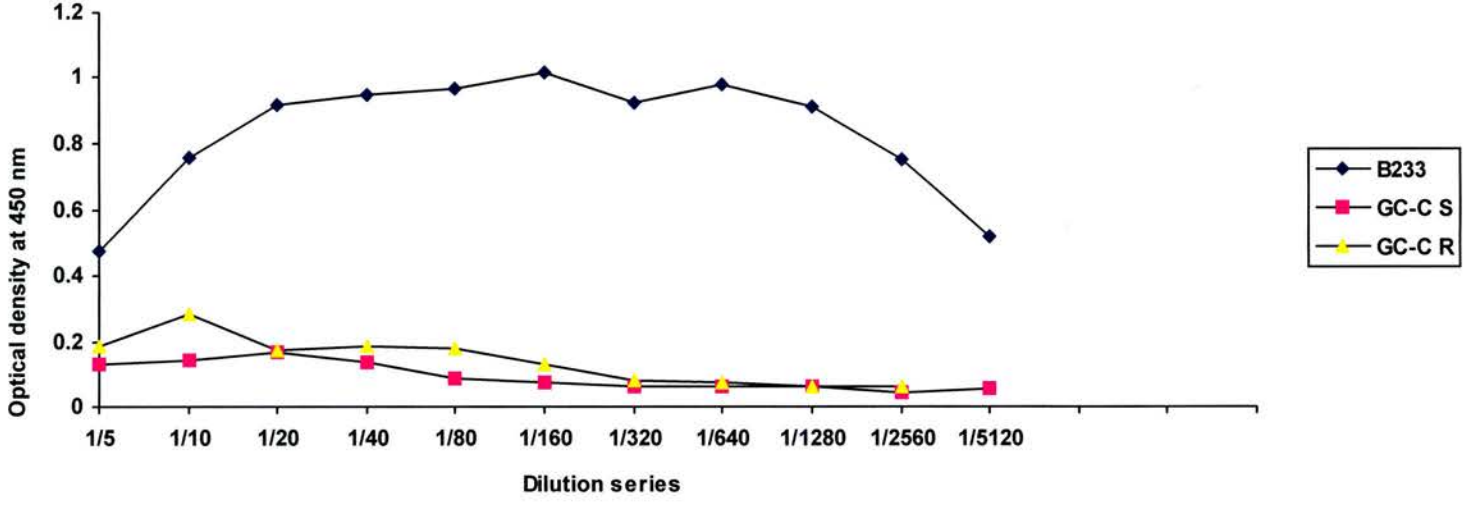
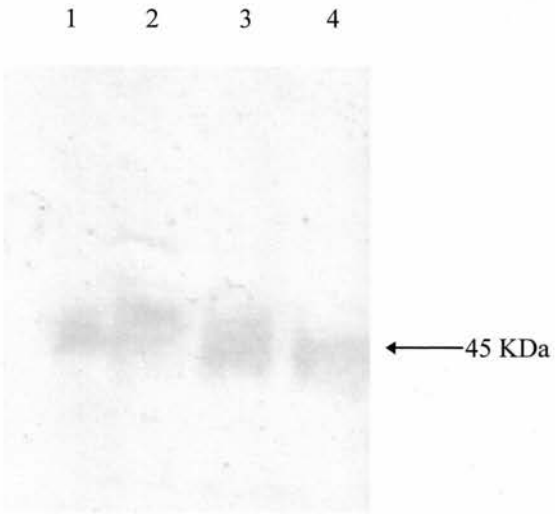

1.2
1
0.8
0.6
0.4
0.2
0
Optical density at 450 nm
1/5
1/10
1/20
1/40
1/80
1/160
1/320
1/640
1/1280
1/2560
1/5120
Dilution series
B233
GC-C S
GC-C R

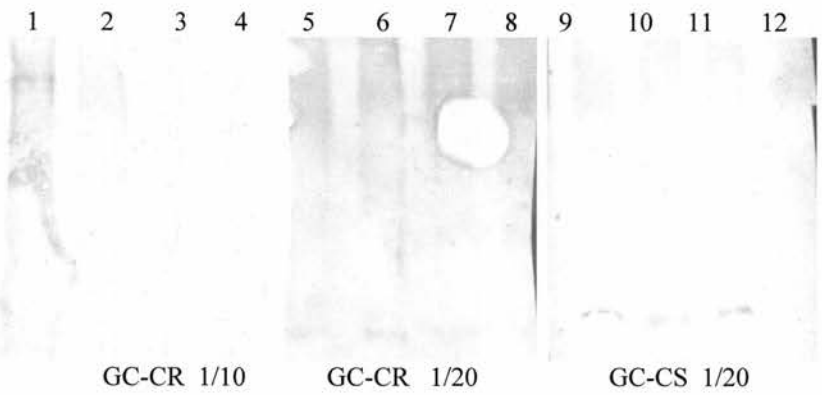
Fig. 3.18a) Western blot showing hybridisation of β233 antibodies (positive control) to gill and intestinal membrane samples from silver FW and SW-acclimated eels. Lanes 1and 2 are gill samples from silver FW-acclimated and silver SW-acclimated fish respectively, and lanes 3 and 4 are intestine samples from silver FW-acclimated and silver SW-acclimated fish respectively.The dilution of β233 was 1/1000 and bands of the expected molecular weight of 45 kDa were detected in all membrane samples.

Fig. 3.18 b) Results of Western blot using GC-C antibodies raised in rabbit (GC-CR) and sheep (GC-CS). The membrane samples are from the same test groups as described in Fig. 3.18 a) in the same order; Lanes 1-4 are results using GC-CR at 1/10 dilution, lanes 5-8 are results using GC-CR at 1/20 dilution and lanes 9-12 are the results using GC-CS at 1/20 dilution. No bands of the expected molecular weight of ~145-160 kDa were detected.

a)



b)



3.2.6 Distribution of GC-C within eel intestine

RT-PCR was carried out on template cDNA, synthesised in the standard way, from the anterior, mid and posterior intestinal segments of eels from all four experimental groups using the GC-C6 sense/GC-C(2) specific antisense primer set. The GC-C6/GC-C(2) primer set amplified a 1.09 kb fragment in all segments except the posterior of yellow FW-acclimated eels (see Fig.3.19). Further RT-PCR using the GC-C4 sense/GC-C anti primer set also failed to amplify GC-C(2) in cDNA from the posterior gut segment of yellow FW-acclimated eels.

3.2.7 RT-PCR in additional fish species

RT-PCR was carried out on template cDNA, synthesised from the gill, intestine and kidney of *Myxocephalus scorpius* (sculpin), *Pleuronectus platessa* (plaice), *Merlangus merlangus* (whiting) and *Gadus morhua* (cod), and the gill, intestine, kidney and rectal gland of the elasmobranch *Scyliorhinus canicula* (dogfish).
The primer set used was GC-C sense/ GC-C antisense (see Appendix 2), which amplified the sequence shown in Fig. 3.11, and resulted in amplification of a fragment of the expected size of just under 600bp in the intestine of all the teleosts and the kidney of sculpin, plaice and cod (see Fig.3.20). A slightly larger fragment of ~610 bp was also amplified from the plaice gill cDNA. No fragment was amplified from any dogfish cDNAs. Subsequent PCR using the other primer sets (i.e. GC-C4 sense/GC-C anti and GC-C6 sense/GC-C(2) specific anti) were unsuccessful.
The lack of success may have been because the primer sets were not suitable for other fish species. Attempts were made to clone and sequence the 600 bp fragment from

Fig. 3.19 Results of RT-PCR in separate intestinal regions from eels of all four experimental groups. PCR was carried out using the GC-C6 sense/ GC-C(2) anti primer set; lanes 1-3 are the results with anterior, mid and posterior segments, in that order, from yellow FW-acclimated eels, lanes 4-6 are the same gut segments from yellow SW-acclimated eels, lanes 7-9 are the same the same gut segments from silver FW-acclimated eels and lanes 10-12 are the same gut segments from silver SW-acclimated eels. A band of the expected size of 1.09 kb was amplified in all cDNA samples except the posterior segment of yellow FW-acclimated fish.

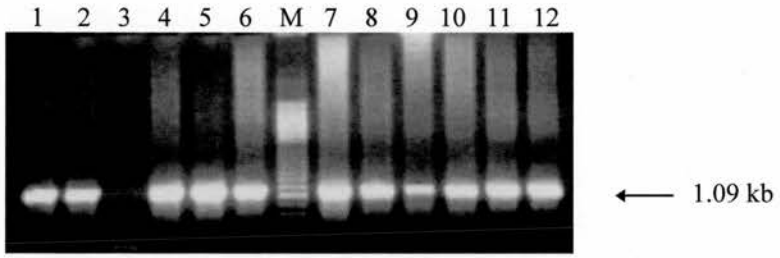
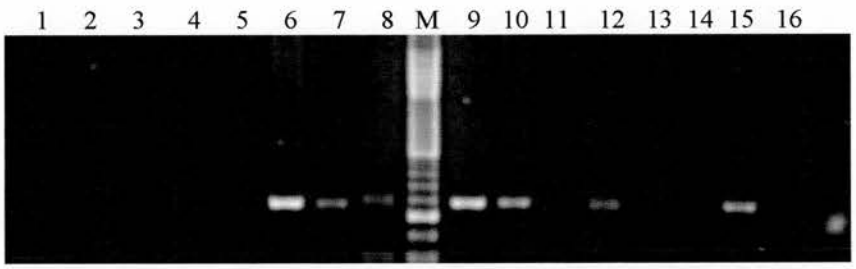

1 2 3 4 5 6 M 7 8 9 10 11 12
1.09 kb

Fig. 3.20 Results of RT-PCR with the GC-C sense/ GC-C anti primers using template cDNA from the gill, intestine, kidney and rectal gland of dogfish (lanes 1-4) and the gill, intestine and kidney of sculpin (lanes 5-7), plaice (lanes 8-10), whiting (lanes11-13) and cod (lanes 14-16).


1 2 3 4 5 6 7 8 M 9 10 11 12 13 14 15 16
598 bp

and the resulting sequences were poor, indicating that perhaps the cloning itself was not successful.

Chapter 4

Discussion

4. DISCUSSION

4.1 Tissue expression of eel guanylin and GC-C

The European eel, *Anguilla anguilla*, has been shown to express homologues of both guanylin and its receptor, GC-C. Two isoforms of eel GC-C were detected, termed GC-C(1) and GC-C(2).

The results of RT-PCR and Northern blotting indicate that eel guanylin and eel GC-C(2) are both expressed in the intestine and kidney of all four experimental groups of fish studied namely, FW or SW-acclimated yellow and silver eels. Guanylin was also detected in the gill by RT-PCR but this was not confirmed by Northern blotting (see Figs. 3.3, 3.9, 3.12, and 3.16). RT-PCR was not carried out to detect expression of either guanylin or GC-C in tissues other than gill, intestine or kidney but Mantoku *et al.*, (1999) reported RT-PCR amplification of GC-C in many organs of adult medaka including spleen, liver, pancreas, gallbladder, ovary, testis brain and eye. The presence of these transcripts were not confirmed by Northern blotting but the results of the RT-PCR suggest a diverse tissue distribution of GC-C in this teleost fish.

Transcript sizes for guanylin and GC-C(2) were determined to be ~1.0 kb and 4.8 kb respectively from Northern blots although sequence data for guanylin cDNA gave sizes of 1.03 kb and 0.94 kb for the long and short forms of guanylin and Northern blots can only provide an estimate of size. In mammals, transcript sizes of ~0.6-0.8 kb for guanylin and ~ 3.8 kb for GC-C have been reported.

The technique of RT-PCR is more sensitive than Northern blotting and can detect mRNA transcripts present at low levels. Whilst the possibility remains that the product amplified by the guanylin primers in the gill samples may be due to the presence of contaminating genomic DNA, examination of the human (Hill *et al.*,

1995a) and mouse (Sciaky *et al.*, 1994) genomic sequence of the guanylin gene demonstrates two introns in the region spanned by the eel guanylin primers. If the guanylin gene sequence is conserved between teleosts and mammals then these introns should also be present in the eel guanylin gene and amplification of genomic DNA would result in a fragment that was ~ 200 bp larger than the expected size. A band of around 2 kb, slightly larger than the 18s rRNA, was also observed in the guanylin blot (see Fig. 3.9). Forte *et al.*, (1999) has reported that lymphoguanylin has a considerably larger transcript (~ 1.6 kb) than either guanylin or uroguanylin and it is possible that eels express another higher molecular weight guanylin-like peptide.

The results of RT-PCR experiments on anterior, mid and posterior intestinal cDNA indicate that GC-C(2) is expressed along the entire length of the eel intestine, except in yellow FW-acclimated posterior intestine, but do not indicate if expression levels differ between these regions (see Fig. 3.19). In mammals, GC-C is expressed along the full length of the gastrointestinal tract from duodenum to colon but expression is reduced along the length of the small intestine and colon (Krause *et al.*, 1997 d). Receptor density also varies along the vertical axis of the intestine, i.e. the villus to crypt axis with the highest receptor density occuring in epithelial cells at the base of the villi (Cohen *et al*, 1992; Krause *et al.*, 1994; Krause *et al.*, 1997 d). Teleost and reptilian intestine, however do not possess true crypts or villi, and the distribution of the GC-C receptor is uniform along the vertical axis (Forte *et al.*, 1997 a, Krause *et al.* 1997 c, Krause *et al.*, 1997 d).

The failure of two primer sets to amplify the 1.4 kb and 1.09 kb G-C(2) fragments in the intestine or kidney of other teleosts may be due to sequence diversity of teleost

GC-C between species. The GC-C sense/ GC-C antisense primer set, which did amplify the GC-C(1) 598 bp fragment in most samples, spans a region between the catalytic cyclase and protein kinase-like domains which is highly conserved across the species range. The nucleotide sequence of the 5′ end of the gene, such as those spanned by the GC-C6 sense, GC-C(2) specific anti and GC-C4 sense primers in the extracellular domain are less conserved, and these extracellular domains may be highly diverse between fish species.

4. 2 Sequences of eel guanylin and GC-C

4.2.1 Guanylin

Comparison of the deduced amino acid sequence of eel guanylin with those of zebrafish and mammals (see Fig. 3.8) indicates high homology (around 80%) between species in the sequence of the active peptide region but the tyrosine residue in the active mammalian sequences is replaced by asparagine and phenylalanine in eel and zebrafish respectively. The finding of a long and short form of eel guanylin, with different 5′ ends, was interesting and may indicate that two spliceoforms, with different 5′ splice sites have arisen from the same gene. The differential processing of pre-mRNA to produce alternatively spliced transcripts is a phenomenon which leads to the expression of different proteins from one gene, enabling the production of tissue-specific or cell-specific proteins (Gilbert, 1994). The design of labelled oligonucleotide probes that were specific for the 5′ end of each mRNA would enable an investigation of cellular expression sites both forms in a variety of tissues.A second possibility is that a duplication of the eel guanylin gene has occurred relatively recently during the evolutionary history of the eel. An early gene duplication event

would have resulted in higher diversity between the long and short forms of eel guanylin.

The cleavage site of the eel active peptide from the prohormone has yet to be determined but the mammalian cleavage site is thought to occur between the aspartic acid and the proline at the positions shown on Fig 3.8. The eel guanylin also has aspartic acid and proline residues (see Fig. 3.8) which may be a potential site of cleavage.The amino terminal sequence of the precursor molecule is highly diverse across the species range.

4.2.2 GC-C

The two isoforms of the eel homologue of GC-C, GC-C(1) and GC-C(2), (see sequence comparisons in Fig. 3.15) which were identified may represent a gene duplication event during teleost evolution. To date, no other reports exist of other isoforms of GC-C in mammals. The high homology (87%) between the 529 bp GC-C(1) fragment and the corresponding region of the GC-C(2) sequence could have resulted in cross-hybridisation during Northern blotting to determine GC-C(1) expression levels. For this reason, a probe that is highly specific for GC-C(1) would be desirable for future experiments to determine if there are differences between expression levels of GC-C(1) and GC-C(2) in the four experimental groups. As stated earlier, several unsuccessful attempts were made to amplify other regions of GC-C(1) and it later became clear that the quality of primers supplied by one company was highly variable, possibly contributing to this lack of success.

Comparison of the 2.4 kb eel GC-C(2) fragment with medaka fish and mammalian sequences demonstrates an overall a.a. homology of around 50% with the highest homology of over 80% in the protein kinase-like and cyclase catalytic domains (see Fig. 3.15). The reasons for the failure to amplify the 5′ and 3′ regions of GC-C(2) are not known but are the subject of continuing investigations in the laboratory.

4.3 Expression levels of eel guanylin and GC-C

The quantitative Northern blots indicated significantly increased expression of guanylin and GC-C(2) in the intestine of yellow SW-acclimated and silver SW-acclimated eels compared to FW-acclimated eels. Interestingly, levels of expression of GC-C(2) in silver FW-acclimated eel intestine were also ~70% higher than yellow FW-acclimated eels (see Tables 3.1, 3.2 and Fig. 3. 11, 3.20). This finding may suggest a degree of pre-adaptation in FW-acclimated silver eels in preparation for their migration to the SW environment. The observed increase in expression levels of eel guanylin and eel GC-C(2) in the intestine of SW-acclimated eels would seem to indicate a role for this regulatory signalling system in adaptation of the fish to the SW environment.

Levels of guanylin expression in the kidney were not significantly different between the four experimental groups (see Fig. 3.9 b) and Appendix 5) but were much lower than those observed in the intestine (see Fig. 3.9 a)). These data, together with the highly variable levels of expression of GC-C(2) in the kidney (see Appendix 4) may indicate that, in contrast to the teleost intestine, the guanylin/GC-C signal transduction system in the teleost kidney does not play a significant role in SW-

acclimation. A further investigation of both guanylin and GC-C expression in the kidney could be carried out using *in-situ* hybridisation to determine those regions of the teleost kidney which display the highest densities of receptor and/or guanylin expression. Krause *et al.*, (1997 d) reports that straight proximal tubules have the highest density of guanylin/ST_a receptor expression compared to other regions of opossum kidney.

Fonteles *et al.*, (1998) reported that uroguanylin was more potent than guanylin in stimulating natriuresis, kaliuresis and diuresis in the perfused rat kidney, indicating that uroguanylin may be the major guanylin peptide regulating GC-C activity in the kidney. Levels of circulating proguanylin are considerably higher than those of prouroguanylin (Forte *et al.*, 2000) and there is a pronounced increase in both in patients suffering from renal disease (Kinoshita *et al.*, 1997 a, b). These data together with the finding that guanylin (but not uroguanylin or St_a) is sensitive to the actions of proteases at aromatic residues in the COOH terminal (Hamra *et al.*, 1996, Forte *et al.*, 2000) suggest that the kidney is the site where guanylin is cleared from the circulation and inactivated. Prouroguanylin in contrast, is converted to active uroguanylin by proteases in the lumen of the kidney tubules to stimulate natriuresis, kaliuresis and diuresis (Greenberg *et* al., 1996, Fonteles *et al.*, 1998). It is not known if teleost fish express a homologue of mammalian uroguanylin but, interestingly, a comparison of the eel guanylin active peptide with that of uroguanylin demonstrates an asparagine residue in the same position, instead of the tyrosine common to mammalian sequences (see below). This asparagine may confer on the eel guanylin a resistance to protease digestion as it does in mammalian uroguanylin.

PPDPCEICANAACTGCL eel guanylin
QEDCELCINVACTGC uroguanylin

Eel guanylin therefore may carry out the functions of both guanylin and uroguanylin.

4.4 Regulation of guanylin and GC-C expression

The mechanisms which regulate expression of guanylin and GC-C in teleosts have yet to be determined but hepatocyte nuclear factor-α (HNF-α) has been shown to stimulate guanylin mRNA transcription in the mouse guanylin gene *in vitro* (Hochman *et al.*, 1997) and possible sites for the transcription factors known as activator protein-1(AP-1) and activator protein-2 (AP-2) have been demonstrated in the mouse guanylin gene (Sciaky *et al.*, 1994). Li *et al.*, (1996) reported a down-regulation in expression of both guanylin and GC-C in the distal colon of rats fed on a low salt diet, and this is probably an adaptive response to maintain sodium levels, continued expression of guanylin and GC-C would lead to depletion of cellular sodium, as a result of continued Cl^- secretion. No changes in expression levels were detected in rats fed on a high salt diet but the authors state that high salt intake may increase expression of guanylin and GC-C in tissues other than intestine, such as kidney where recent research has demonstrated elevated levels of plasma and urine uroguanylin in humans on high-salt diets (Kinoshita *et al.*, 1997). These findings support the hypothesis that uroguanylin is an endocrine factor which acts to influence urinary salt excretion.

A glucocorticoid response element has also been identified in the mammalian guanylin gene promoter (Sciaky *et al.*, 1994; Hill *et al.*, 1995) indicating that, in

mammals at least, aldosterone may contribute to the regulation of the guanylin gene. In teleosts the major corticosteroid released by the interrenal tissues is cortisol, which has both mineralocorticoid and glucocorticoid properties, and the increase in plasma cortisol levels that accompany the transfer of fish to SW (Forrest *et al.*, 1973 b) may provide the signal for the observed increases in expression of guanylin in the intestine. However, many factors may be involved in the regulation of teleost guanylin and GC-C expression.

The genomic sequence of medaka GC-C (Mantoku *et al.*, 1999) consists of 27 exons and the 5′ flanking region of the gene contains consensus binding sites for transcription factors expressed in mammalian intestine, such as Cdx-2 and the GATA family of transcription factors, indicating that these transcription factors may contribute to the regulation of expression of GC-C in mammals and may themselves be expressed in response to dietary stimuli.

An investigation by Swenson *et al.*, (1999) demonstrated that hepatocyte nuclear factor-4 (HNF-4) was a key regulator of GC-C expression in the intestine. These workers identified a critical HNF-4 binding site which, when mutated, abolished activation of the GC-C gene. Scheving *et al.*, (1996) reported the redistribution of GC-C from the cell surface to intracellular sites of enterocytes in fasted rats which suggests an adaptive response to salt restriction such as that reported by Li *et al.*, (1996) for the down regulation of guanylin and GC-C expression in the intestine of rats on a low salt diet.

4.5 Activity of the eel guanylin/GC-C signal transduction system

The presence of eel guanylin and eel GC-C in the intestine and kidney suggests that there is a role for the guanylin/GC-C signal transduction system in ion and fluid homeostasis that has yet to be fully characterised in teleosts. Forte *et al.*, (1997a) found that guanylin and ST_a stimulated increases in cGMP in the intestinal mucosa of several fish species. Guanylin is known to stimulate secretion of both Cl^- and HCO_3^- in mammals via the CFTR and possibly by other functional chloride channels (Currie *et al.*, 1992; Forte *et al.*, Hamra *et al.*, 1993; Kita *et al.*, 1994; Kuhn *et al.*, 1994; Guba *et al.*, 1996; Joo *et al.*, 1998). Guanylin may carry out a similar function in teleosts, but the upregulation of guanylin and GC-C in the SW-acclimated intestine, which is known to be involved in the absorption of Cl^- and Na^+ ions, is an interesting finding. Wilson *et al.*, (1996) reported a substantial secretion of endogenous base (HCO_3^- and CO_3^{2-}) into the intestine of SW-acclimated *O.mykiss* and noted that the rectal fluids of these fish had a pH of 8.9. Shedadeh and Gordon (1969) also reported a high pH of rectal fluids in SW-acclimated *O.mykiss* and had observed carbonate deposits within the intestinal mucous. Deposits were also noted in the intestine of SW-acclimated eels during tissue removal for this research project. These findings suggest that the intestine of SW-acclimated teleosts may also have a physiological function in acid-base balance and that the guanylin/GC-C signalling pathway may play an important role. The teleost homologue of the CFTR, isolated from *F.heteroclitus* (Singer *et al.*, 1998), was reported to be expressed at high levels in the intestine of SW-acclimated fish, inviting speculation that the teleost guanylin/ GC-C signal transduction system may regulate the secretion of HCO_3^- and/or Cl^-, via the CFTR, in the intestine of SW-acclimated fish.

If the guanylin/GC-C signalling stimulates secretion of Cl^- and/or prevents absorption of Cl^- and Na^+ in the intestine of SW-acclimated fish, this may aid the prevention of salt "overload" in the fish, acting in a similar manner to teleost natriuretic peptides which inhibit ion and water absorption in the intestine of SW-acclimated eels. The second messenger cGMP has been shown to be a potent inhibitor of ion absorption in teleost intestine (Trischitta *et al.*, 1996) and the rise in intracellular cGMP caused by the binding of guanylin to GC-C may contribute to the inhibition of ion absorption. In addition, 8-bromo-cGMP has been reported to stimulate secretion of proguanylin both apically and basolaterally in rat colonic mucosa (Martin *et al.*, 1999) with a concomitant release of bioactive guanylin at the apical surface only, leading to the suggestion that natriuretic peptides may be involved in the control of guanylin secretion and synthesis. The observed increase in plasma ANP and VNP levels in eels transferred to SW may therefore provide a stimulus for guanylin secretion in the teleost intestine. Guanylin itself may be part of a positive feedback mechanism regulating its own release. If guanylin receptors are present on the same cell types as those that synthesise guanylin then a positive feedback mechanism may operate. Future functional studies will be required to examine the role of this signalling system in the intestine of teleost fish and to determine whether guanylin acts in an endocrine manner by circulating in the plasma. Studies in mammals have shown that uroguanylin and prouroguanylin are present plasma (Kuhn *et al.*, 1993; Hess *et al.*, 1995; Fan *et al.*, 1996) indicating that an endocrine axis may exist between the intestine and kidney that regulates natriuresis and contributes to Na^+ balance within the body.

Martin *et al.*, (1999) reported that proguanylin is secreted to the luminal side of isolated rat colonic mucosa with no secretion to the basolateral side. Incubation with carbachol and 8- bromo-cGMP however, stimulated secretion of proguanylin across both basolateral and luminal surfaces, suggesting that in mammals the secretion of guanylin may be under the control of cholinergic and cGMP dependent mechanisms. The release of proguanylin across the basolateral surface may provide a circulating source of the peptide which acts in an endocrine manner, after proteolytic processing at or near the target tissue. In the mammalian intestine, proguanylin and prouroguanylin, released at the apical surface of enterocytes are proteolytically processed to produce the bioactive peptides (Hamra *et al.*, 1996) which then act in an autocrine and/or paracrine manner on other enterocytes.

Preliminary attempts to amplify an eel homologue of uroguanylin by RT-PCR were unsuccessful (data not shown), so it is not known whether guanylin is the only peptide of this type expressed in the eel. Since uroguanylin and guanylin mRNAs have been reported to display different expression patterns in the gastrointestinal tract of several mammalian species(Krause *et al.*, 1997 d ; Forte, 1999), the expression pattern of the long and short forms of eel guanylin along the teleost intestine may be an interesting comparative area of study, together with further attempts to amplify an eel homologue of uroguanylin and possibly lymphoguanylin, which is structurally quite different and may have diverged early in vertebrate evolution.

The increase in expression levels of GC-C(2) observed in the intestine of SW-acclimated yellow eels should be examined further to determine whether this is either due to a general increase in GC-C(2) expression levels along the entire length of the intestine or to the expression of GC-C(2) in the posterior segment of the intestine.

Expression of GC-C(2) was detected in the posterior intestine of silver FW-acclimated eels which may indicate some pre-adaptation for the migration to the marine environment. The increase in expression levels of intestinal GC-C(2) between silver SW-acclimated eels and silver FW-acclimated eels was not statistically significant as was that between yellow SW-acclimated and yellow FW-acclimated eels. This may indicate that the increase in expression levels of GC-C(2) in the intestine of yellow SW-acclimated eels was due to the expression of GC-C(2) in the posterior intestine.

The detection of guanylin in the gill samples by RT-PCR, which was not confirmed by Northern blotting, requires further investigation. The gill chloride cells of SW-acclimated fish are the major site of excess ion excretion so the expression of guanylin in the gill is not unexpected but RT-PCR and Northern blotting failed to detect GC-C(1) or GC-C(2) in the gill. Some evidence does exists for other, as yet unidentified, guanylin peptide/ST_a receptors; Mann *et al.*, (1997) reported binding sites for ^{125}I-ST_a in the intestine of GC-C knockout mice and the possibility may exist that teleosts express another receptor for guanylin. However, the expression of guanylin in the gill should also be confirmed by sequencing the fragment.

If expression of guanylin in the gill is confirmed,it is possible that guanylin is expressed in the gill before entering the circulation to act in an endocrine manner on another tissue in a similar way to guanylin and uroguanylin in mammals. Furuya *et al.*, (1998) demonstrated an endocrine role for guanylin in mammals when they showed that intravenous injection of guanylin induced mucous secretion in rat duodenum goblet cells. These findings indicate a possible receptor for guanylin on the serosal surface of the intestinal epithelium. Mucous acts as a diffusion barrier in the

intestine and oesophagous of SW-acclimated teleosts, and circulating guanylin may induce the secretion of intestinal mucous in teleost fish as it does in rats.

The discovery that guanylin, uroguanylin, lymphoguanylin and GC-C are expressed in several mammalian tissues including intestine, kidney, lung, pancreas and heart suggests that this signal transduction system may play diverse physiological roles which have yet to be fully characterised. Guanylin is also reported to stimulate cGMP production and secretion of chromogranin A and γ-aminobutyric acid in neuroendocrine pancreatic cells (John *et al.*, 1998) and may act to regulate secretion from other neuroendocrine tissues. The second messenger cGMP is now increasingly regarded as an important regulator of cell function and the guanylin peptides may prove to have equally important functions.

Further investigations into teleost guanylin and GC-C at both the molecular and physiological levels should provide valuable new insights into the role(s) of this signalling system with respect to teleost osmoregulatory mechanisms.

4.6 Western blotting

The failure of the Western blotting technique to detect eel GC-C in membrane preparations was in all likelihood due to the poor antibody titre of the sheep and rabbit anti-sera (see Fig. 3.17). The chosen peptide sequence may have had a low capacity to produce an immune response in the animals and financial constraints did not allow further attempts to raise antibodies using peptides designed from other regions of GC-C.

4.7 Conclusions

European eels have been shown to express homologues of both guanylin and GC-C. Full length eel guanylin cDNA has been amplified and sequenced and a 2.4 kb partial eel GC-C(2) and a partial 529 bp eel GC-C(1) have also been amplified and sequenced. The expression of two isoforms of eel GC-C is interesting because this has not been reported in any mammalian studies. The expression of a long and short form of eel guanylin also requires further investigation to determine cellular sites of expression and possible differences in distribution in a variety of tissues.

Expression levels of guanylin and GC-C were shown to increase in the intestine when eels were transferred to SW indicating a role for the intestinal guanylin/GC-C signal transduction system in SW acclimation. The results for the kidney were less clear and may indicate that in the kidney, the guanylin/GC-C signalling system does not play a direct role in SW acclimation.

4.8 Future Work

Further work on the eel guanylin/GC-C signal transduction system would include the complete sequencing of both the GC-C(1) and GC-C(2) receptor cDNAs and expression studies to determine the tissue distribution of each isoform. In addition, the sequencing of eel guanylin and eel GC-C should allow the design of homologous probes for *in-situ* hybridisation to determine cellular sites of guanylin and GC-C expression in tissues of interest. Further investigations should also be carried out to determine the significance of the two spliceoforms of eel guanylin.

Purification of eel guanylin from intestinal mucosa and/or synthesis of eel guanylin peptide would permit functional studies to be carried out using a homologous peptide.

This would require the determination of the correct cleavage site of the bioactive eel guanylin peptide.

Expression levels of both guanylin and GC-C along the length of the intestine should be investigated by Northern blotting, *in situ* hybridisation and immunohistochemistry. Time course studies would also demonstrate expression levels of both guanylin and GC-C in eels that had been adapted to SW for different periods of time.

Further sequence data on the eel GC-C(1) and GC-C(2) receptor isoforms should permit the design of other peptides for the production of antibodies. Successful antibody production would enable Western blotting and immunohistochemical studies to be carried out.

It is important that further studies should be carried out to elucidate the endocrine and physiological roles of the guanylin/guanylate cyclase C signalling system in teleost fish. The role of this signalling system in teleosts may be very different to that in mammals and awaits further investigation.

Chapter 5

Differential gene expression in yellow and silver eels

5. DIFFERENTIAL GENE EXPRESSION IN YELLOW AND SILVER EELS

5.1 Introduction

In addition to the investigation of the guanylin/GC-C signal transduction system in the European eel, a second aspect of the research project involved attempts to identify genes which may be differentially expressed in yellow and silver FW and SW-acclimated eels. During the development of the sexually immature yellow eel into the sexually mature, migratory silver eel and the migration from FW to SW, several adaptive changes occur which must be regulated by gene expression. The aim of this aspect of the project was, therefore, to use PCR-based techniques which would allow the identification of genes which may be switched on or off following these developmental and environmental changes.

One technique chosen to investigate gene expression was differential display PCR (DD-PCR), a method developed by Liang and Pardee (1992), which allows the identification and analysis of altered gene expression at the mRNA level in the following way; populations of total RNA from two tissue or cell samples, for example FW and SW intestine, are extracted and reverse transcription is carried out with three one-base-anchored oligo-dT primers in separate reactions, resulting in three separate cDNA preparations for each tissue. Several independent PCR amplifications are then carried out in the presence of one of a number of short primers, with arbitary sequences, and the one-base-anchored oligo-dT primer used to synthesise the cDNA. The PCR-amplified products, obtained from both RNA populations, with the same primer sets, can then be directly compared after polyacrylamide gel electrophoresis and silver staining. Direct comparison of the banding patterns then allows the identification of any differentially expressed genes. Fig. 5.1 shows a flow diagram of the steps in the DD-PCR technique.

Fig. 5.1 Diagrammatic overview of the DD-PCR technique.

mRNA population

1. Reverse transcription

5′-GTTTGCGGCCGCTTTTTTTTTTTN-3′ (N=G,C or A)

dNTPs

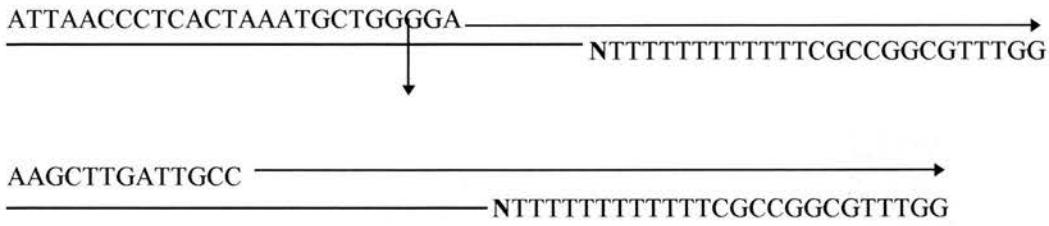
MMLV reverse transcriptase

2. PCR amplification

5′-ATTAACCCTCACTAAATGCTGGGGA-3′ (random primer)

dNTPs

Taq DNA polymerase



3. Denaturing polyacrylamide gel

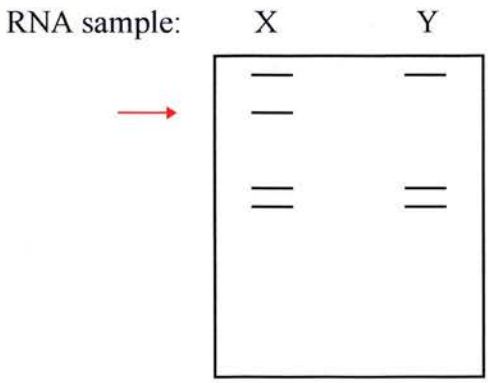


Fig. 5.2 Flow diagram of the steps carried out in suppression subtractive cDNA analysis.

cDNA synthesis

Tester and Driver ds cDNA are prepared from the two mRNA samples under comparison

↓

***Rsa* I digestion**

Tester and Driver cDNA are separately digested to obtain shorter, blunt-ended molecules

↓

Adaptor ligation

Two Tester populations are created with different adaptors, but Driver cDNA has no adaptors

↓

First hybridisation

Hybridisation kinetics lead to equalisation and enrichment of differentially expressed sequences

↓

Second hybridisation

Templates for PCR amplification are generated from differentially expressed sequences

↓

First PCR amplification

Using suppression PCR only differentially expressed sequences are amplified exponentially

↓

Second PCR amplification

Background is reduced and differentially expressed sequences further enriched

A second technique chosen to analyse differential gene expression was suppression subtractive cDNA analysis. The basic principle underlying this technique involves the hybridisation of two populations of ds cDNAs, synthesised, for example, from FW and SW-acclimated eel intestine. One population is the "Tester"cDNA, subdivided into two samples, each of which has a different adaptor ligated to the 5′ end of each strand. The other is the "Driver" cDNA which has no adaptors ligated. After hybridisation of the Tester and Driver populations and the removal of the hybrid sequences, the remaining unhybridised fraction represents those genes that are expressed in the Tester population but not the Driver population. Fig. 5.2 is a flow diagram showing the steps of suppression subtractive hybridisation.

5.2 Method

5.2.1 DD-PCR

The DD-PCR method is based on the reverse transcription of mRNA and therefore the first step in the protocol was the extraction of total RNA from the tissues to be compared; in this case total RNA was extracted from the brain, gill and intestine of FW and SW-acclimated silver eels in order to detect differential gene expression in each tissue under two salinity conditions. The total RNA was extracted and samples were run on denaturing formaldehyde gels stained with ethidium bromide, as previously described in sections 2.1.1 and 2.1.2 respectively to ensure no degradation of the RNA had occurred. The synthesis of cDNA and the subsequent PCR amplifications only differed from the methods described in sections 2.1.3 and 2.1.4 by the use of the three one-base-anchored oligo-dT cDNA synthesis primers and the arbitrary PCR primers (see Appendix 7). The cycling parameters for the PCR were

the same as described in section 2.1.4 but the annealing temperature used was 40^0C for two minutes.

Following PCR , the samples were electrophoresed on a 6% polyacrylamide sequencing gel of the following composition, 42% urea w/v, 1X TBE buffer, 6% acrylamide v/v (19:1, Scotlab), 0.05% ammonium persulphate w/v and 0.005% of TEMED v/v. The gel solution was thoroughly mixed and syringed between two glass electrophoresis plates with 0.4mm spacers, which had been clamped together and laid flat, and an appropriately sized comb placed in the gel between the two plates. PCR products were visualised by silver staining of the gel. To carry out silver staining of thin gels the glass plates were treated to ensure that the gel adhered to one plate. This was achieved by treating one plate with SigmaCote® (Sigma Aldrich) and the other with Bind Silane solution (Fluka Chemicals). The gel was allowed to polymerise overnight before being placed in the electrophoresis rig containing 2X TBE buffer. A 3.5µl aliquot of each PCR reaction was mixed with 2µl of loading buffer (95% formamide, 10mM EDTA pH 8.0, 0.09% xylene cyanol w/v and 0.09% bromophenol blue w/v) before denaturing at 80^0C for 2 min. then immediately loading onto the gel. Electrophoresis was carried out at 60 watts for 4 hours. When electrophoresis was complete the plate treated with SigmaCote® was carefully removed ensuring the gel was not damaged. The remaining plate with the affixed gel was placed in fix solution (10% glacial acetic acid) and agitated for 20 min. before three washes in MilliQ® water. The gel was then placed in stain solution (6mM silver nitrate, 0.06% formaldehyde), and agitated for 30 min. at room temperature before rinsing very briefly (5-10 seconds) in MilliQ® water and transferring immediately to chilled developing solution (28 mM sodium carbonate, 8µM sodium thiosulphate and 0.06%

formaldehyde v/v). The gel was agitated in developing solution until bands appeared, before the direct addition of fix solution to terminate the development reaction and two further brief rinses in MilliQ® water. The gel could then be photographed to provide a permanent record and any bands of interest could be excised for reamplification, cloning and sequencing. Any sequences obtained could then be compared to existing sequences in the GenBank database to identify possible functions of the proteins.

5.2.2 Suppression subtractive cDNA analysis

The technique of suppression subtractive cDNA analysis was carried out using the commercially available procedure used in the Clontech PCR-Select™ cDNA Subtraction Kit. The protocol requires that ds cDNA is synthesised from Poly A^+ RNA. After extraction of total RNA in the manner described in section 2.1.1, Poly A^+ RNA was prepared as described below.

A 2ml sterile syringe was clamped upright on a stand and a small piece of boiled cotton wool pushed down to the neck of the syringe. Approximately 250g or less of Oligo dT cellulose was weighed out and suspended in DEPC H_2O. The solution was allowed to settle, and the supernatant containing fine cellulose particles carefully aspirated off. This process was repeated, allowing the fine cellulose particles to be eliminated. A final small volume of DEPC water was added to the cellulose which was then decanted into the column . Silicon tubing was attached to the neck of the syringe barrel. The solids settled at the bottom of the syringe and the whole column was connected by the tubing to a micro-flow cuvette placed in the spectrophotometer. The flow rate through the column was controlled by a peristaltic pump and the flow of RNA through the column could be monitored by the spectrophotometer to detect the peaks of absorbance represented by ribosomal and polyA^+ RNA. After washing and equilibrating the column with DEPC H_2O 1X Binding Buffer was added (10mM

Tris pH 7.5, 0.5M NaCl, 1mM EDTA, 0.5% SDS) then an equal volume of 2X Binding Buffer. The RNA samples (which were 3-5 mg of pooled total RNA extracted from the gill, intestine and brain of six FW and SW-acclimated silver eels) were heated to 65°C for 10 min. An equal volume of 2X Binding Buffer was added to each sample and then the sample carefully added to the column ensuring that the cellulose never dried out (DEPC H_2O was added to the column between buffers and samples). The UV absorbance increased sharply as non-poly A^+ mRNA came through the spectrophotometer but poly A^+ mRNA was retained by the Oligo dT in the cellulose. Once the sample and buffer had entered the column, 1X Binding Buffer was added. When all non-polyA^+ RNA had passed through the cuvette,as determined by the resulting absorbance peak on the spectrophotometer, a 3-4 ml volume of Wash Buffer (10mM Tris pH 7.5, 0.1M NaCl, 1mM EDTA) was added to the column before the addition of Elution Buffer (10mM Tris pH 7.5, 1mM EDTA) which elutes the poly A^+mRNA from the cellulose. The eluant was collected after travelling through the cuvette then precipitated by the addition of 0.1 vol 3M NaOAc and 2.5 vol of absolute ethanol. The column was regenerated by flushing with Regeneration Solution (0.1M NaOH) and stored at 4^0C under DEPC H_2O in the short term. The samples of poly A^+ mRNA from each tissue of SW and FW-acclimated silver eels were stored at -80°C until needed. Synthesis of first and second strand cDNA was carried out using the Clontech Marathon™ protocol as described in sections 2.5.2 (i) and (ii) respectively.

5.2.3 Digestion of cDNA with *Rsa* 1

This procedure was carried out with each of the samples to obtain blunt-ended molecules for adaptor ligation. *Rsa* 1 is four-base cutter that will recognise a $GT^{\downarrow}AC$ restriction site and will hence generate many blunt-ended fragments from substrate cDNA. In a clean 0.5ml tube a 50µl reaction volume was set up comprising the following components; 43.5µl of double-stranded (ds) cDNA synthesised in steps (i) and (ii) , 5µl 10X *Rsa* 1 restriction buffer (100mM Bis Tris propane-HCl pH 7.0,

100mM $MgCl_2$ and 1mM DTT) and 1.5µl of *Rsa* 1 (10 units/µl). The tubes were vortexed and pulse-centrifuged before incubation at 37°C for 90 min. A 5µl aliquot was removed to carry out agarose gel electrophoresis for the purpose of analysing restriction efficiency. Comparison of uncut ds cDNA with the digested cDNA should demonstrate a size range of 0.5-10 kb for uncut cDNA and 0.1-2 kb for digested cDNA. To terminate the restriction digest 2.5µl of 0.2M EDTA was added and phenol extraction carried out as previously described. The resultant pellet of digested ds cDNA was resuspended in 5.5µl of H_2O and stored at -20°C . At the end of these steps preparation of Driver cDNA for both SSW and SFW gill $PolyA^+$mRNA was complete. Control human skeletal muscle $PolyA^+$ mRNA , provided with the kit, was also used for synthesis of cDNA and processed in the same way as all other samples to ensure the success of the technique.

The next step was to prepare two populations of Tester cDNA by the ligation of two different adaptors as described below in section 5.2.4.

5.2.4

To produce Tester cDNA for each sample 1.5µl of Driver DNA was diluted up to 9µl with sterile water. Two 2µl aliquots of this dilution were then combined with 2µl of H_2O , 4µl of Master Mix (comprising 1µl of H_2O, 2µl of 5X ligation buffer (250mM Tris-HCl pH 7.8, 50mM $MgCl_2$ and 10mM DTT),1µl of T4 DNA ligase (400 units/µl) and 2µl of either Adaptor 1 or Adaptor 2 (supplied with the kit) to produce two Tester populations of the same cDNA with different adaptors ligated to each population. In a separate tube, 2µl of each population was mixed to produce an unsubtracted Tester control. All tubes were then centrifuged and incubated overnight at 16°C following which the reaction was terminated by the addition of 1µl of EDTA/Glycogen (0.2M EDTA, 0.1% glycogen w/v) then the samples heated at 72°C for 5min. At the end of this process, in addition to the unsubtracted Tester control samples, there were two complete cDNA Tester populations sub-divided into two; Adaptor 1- ligated and Adaptor 2 - ligated. A 1µl aliquot was removed from both sets

of unsubtracted Tester control and diluted up to 1ml with H_2O to be used in PCR analysis.

PCR was carried out using primers supplied with the kit and primers designed to the eel α-subunit of the Na^+, K^+-ATPase (supplied by Dr C.Cutler) but analysis suggested that either second strand cDNA synthesis or adaptor ligation was possibly of poor efficiency despite successful control reactions using the human skeletal muscle Poly A^+ RNA supplied with the kit. Subsequent steps in the protocol were therefore not carried out

5.3 Results and discussion

Despite several attempts, over a considerable period of time, to achieve results from both DD-PCR and suppression subtractive cDNA analysis, these efforts were unsuccessful. Very few fragments were detected on any of the DD-PCR gels despite various modifications to PCR parameters and re-synthesis of the cDNA samples. Successful control reactions to test the cDNA were carried out using primers designed to amplify the α-subunit of the Na^+, K^+-ATPase. This may indicate that the arbitrary short primers were unsuitable since successful amplification was demonstrated in control reactions. The silver staining technique was also tested by running DNA size markers, which were successfully detected, ruling out any problem with the staining method.

The problems encountered with the suppression subtractive cDNA technique were possibly due to the adaptor ligation stage; discussions with technical services at Clontech revealed that other kit users had experienced similar difficulties. As with the DD-PCR method, cDNA was re-synthesised and successful control PCR carried out to test the cDNA. Due to both financial and time constraints no further work was carried

out on this aspect of the project but if future investigations overcame the difficulties, valuable data could be obtained with respect to the genes which regulate the adaptive changes occurring in yellow and silver eels.

References

REFERENCES

Agre, P., Bonhivers, M. and Borgnia, M.J. (1998). The aquaporins, blueprints for cellular plumbing systems. *The Journal of Biological Chemistry*, 273, 24, 14659-14662.

Aguenaou, H., Hubsch, A. and Colin, D.A. (1989). Is there a Cl^--OH^- exchange (Cl^--OH^- cotransport) mechanism in the brush-border membrane of the intestine of the fresh water trout (*Salmo gairdneri*, R.)?. *The Journal of Membrane Biology*, 108, 13-20.

Aidley, D.J. and Stanfield, P.R. (1996). Ion channels (First edition). Cambridge University Press.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J.D. (1992). Molecular Biology of the Cell (second edition). Garland Publishing, London.

Ando, M. (1974). Effects of cortisol on water transport across the eel intestine. *Endocrinology Japan*, 21, 539-546.

Ando, M. (1980). Chloride-dependent sodium and water transport in the seawater eel intestine. *Journal of Comparative Physiology*, 138, 87-91.

Ando, M. (1983). Potassium-dependent chloride and water transport across the seawater eel intestine. *Journal of membrane biology*, 73, 125-130.

Ando, M. (1985). Relationship between coupled Na^+-K^+-Cl^- transport and water absorption across the seawater eel intestine. *Journal of Comparative Physiology B*, 155, 311-317.

Ando, M. (1990). Effects of bicarbonate on salt and water transport across the intestine of the seawater eel. *Journal of Experimental Biology*, 150, 367-379.

Ando, M. and Kobayashi, M. (1978). Effects of stripping of the outer layers of the eel intestine on salt and water transport. *Comparative Biochemistry and Physiology*, 61A, 497-501.

Ando, M. and Subramanyam, M.V.V. (1990). Bicarbonate transport systems in the intestine of the seawater eel. *Journal of Experimental Biology*, 150, 381-394.

Ando, M. and Omura, E. (1993). Catecholamine receptor in the seawater eel intestine. *Journal of Comparative Physiology*, 163, 64-69.

Ando, M., Utida, S. and Nagahama, H. (1975).Active transport of chloride in eel intestine with special reference to seawater adaptation. *Comparative Biochemistry and Physiology*, 51A, 27-32.

Ando, M., Kondo, K. and Takei, Y. (1992). Effects of eel atrial natriuretic peptide on NaCl and water transport across the intestine of the seawater eel. *Journal of Comparative Physiology B*, 162, 436-439.

Arnold-Reed, D., Hazon, N. and Balment, R.J. (1991). Biological actions of atrial natriuretic factor in flatfish. *Fish Physiology and Biochemistry*, 9, 3, 271-277.

Auperin, B., Reutier-Delrue, F., Mortial, J.A. and Prunet, P. (1994). Evidence that two tilapia (*Oreochromis niloticus*) prolactins have different osmoregulatory functions during adaptation to a hyperosmotic environment. *Journal of Molecular Endocrinology*, 12, 13-24.

Baldisserotto, B. and Mimura, O.M. (1994). Ion transport across the isolated intestinal mucosa of *Anguilla anguilla* (Pisces). *Comparative Biochemistry and Physiology*, 108A, 2/3, 297-302.

Balment, R.J., Warne, J.M., Tierney, M. and Hazon, N. (1993). Arginine vasotocin and fish osmoregulation. *Fish Physiology and Biochemistry*, 11, 189-194.

Bertin, L. (1956). Eels. Clever-Hulme Press, London.

Beyenbach, K.W. (1995). Secretory electrolyte transport in renal proximal tubules of fish. In: Wood, C.M. and Shuttleworth, T.J. (eds) Cellular and molecular approaches to fish ionic regulation. *Fish Physiology*, 14, 85-105.

Bhandari, R., Suguna, K. and Visweswariah, S.S. (1999). Guanylate cyclase C receptor: regulation of catalytic activity by ATP. *Bioscience Reports*, 19, 3, 179-188.

Bindon, S.D., Gilmour, K.M., Fenwick, J.C. and Perry, S.F. (1994). The effects of branchial chloride cell proliferation on respiratory function in the rainbow trout *Oncorhynchus mykiss*. *Journal of Experimental Biology*, 197, 47-63.

Bjornsson, B.TH., Yamauchi, K., Nishioka, R.S., Deftos, L.J. and Bern, H.A. (1987). Effects of hypophysectomy and subsequent hormonal replacement therapy on hormonal and osmoregulatory status of coho salmon *Oncorhynchus kisutch*. *General and Comparative Endocrinology*, 68, 421-430.

Brown, J.A. and Balment, R.J.(1997). Teleost renal function: regulation by arginine vasotocin and by angiotensins. In: Ionic regulation in animals: a tribute to Professor W.T.W. Potts, (eds, Hazon, N., Eddy, F.B. and Flik, G.), Springer Verlag.

Brown, J.A., Oliver, J.A., Henderson, I.W. and Jackson, B.A. (1980). Angiotensin and single nephron function in the trout, *Salmo gairdneri*. *American Journal of Physiology*, 239, R509-R514.

Butler, D.G. and Carmichael, F.J. (1972). (Na^+-K^+)-ATPase activity in the eel (*Angilla rostrata*) in relation to changes in environmental salinity: role of adrenocortical steroids. *General and Comparative Endocrinology*, 19, 421-427.

Cetin, Y., Kuhn, M., Kulaksiz, H., Adermann, K., Bargsten, G., Grube, D. and Forssmann, W-G. (1994). Enterochromaffin cells of the digestive system: cellular source of guanylin, a guanylate cyclase-activating peptide. *Proceedings of the National Academy of Sciences USA*, 91, 2935-2939.

Cetin, Y., Kulaksiz, H., Redecker, P., Bargsten, G., Adermann, K., Grube, D. and Forssmann, W-G. (1995). Bronchiolar nonciliated secretory cells: source of guanylin in the mammalian lung. *Proceedings of the National Academy of Sciences USA*, 92, 5925-5929.

Chinkers, M. and Garbers, D.L. (1989). The protein kinase domain of the ANP receptor is required for signaling. *Science*, 245, 1392-1394.

Chomzynski, P. and MacKay, K. (1995). Modification of the TRI reagent procedure for the isolation of RNA from polysaccharide- and proteoglycans-rich sources. *Biotechniques*, 19, 942-945.

Cohen, M.B., Mann, E.M., Lau, C., Henning, S.J. and Giannella, R.A. (1992). A gradient in expression of the *Escherichia* coli heat-stable enterotoxin receptor exists along the villusto-crypt axis of rat small intestine. *Biochemical and Biochemical Research Communications*, 186, 483-490.

Cohen, M.B., Witte, D.P., Hawkins, J.A. and Currie, M.G. (1995). Immunohistochemical localisation of guanylin in the rat small intestine and colon. *Biochemical and Biophysical Research communications*, 209, 3, 803-808.

Currie, M.G., Fok, K.F., Kato, J., Moore, R.J., Hamra, F.K., Duffin, K.L. and Smith, C.E. (1992). Guanylin: an endogenous activator of intestinal guanylate cyclase. *Proceedings of the National Academy of Sciences USA*, 89, 947-951.

Cuthbert, A.W., Hickman, M.E., MacVinish, L.J., Evans, M.J., Colledge, W.H., Ratcliffe, R., Seale, P.W. and Humphrey, P.P.A. (1994). Chloride secretion in response to guanylin in colonic epithelia from normal and transgenic cystic fibrosis mice. *British Journal of Pharmacology*, 112, 31-36.

Cutler, C.P., Sanders, I.L., Hazon, N. and Cramb, G. (1995a). Primary sequence, tissue specificity and expression of the Na^+, K^+-ATPase α1 subunit in the European eel (*Anguilla anguilla*). *Comparative Biochemistry and Physiology*, 111B, 567-573.

Cutler, C.P., Sanders, I.L., Hazon, N. and Cramb, G. (1995b). Primary sequence, tissue specificity and mRNA expression of the Na^+, K^+-ATPase β1 subunit in the European eel (*Anguilla anguilla*). *Fish Physiology and Biochemistry*, 14, 5, 423-429.

Cutler, C.P., Sanders, I.L., Luke, G., Hazon, N. and Cramb, G. (1996). Ion transport in teleosts: identification and expression of ion transporting proteins in branchial and intestinal epithelia of the European eel. In: Gene Expression and Manipulation in Aquatic Organisms, (eds, Ennion, S.J. and Goldspink, G.),Cambridge University Press.

De Bold, A.J., Borenstein, H.B., Veress, AT. and Sonnemberg, H. (1981). A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats. *Life Sciences*, 28, 89-94.

Deen, P.M.T. and Van Os, C.H. (1998). Epithelial aquaporins. *Current Opinions in Cell Biology*, 10, 435-442.

Degnan, K.J. (1985). The role of K^+ and Cl^- conductances in chloride secretion by the opercular epithelium. *Journal of Experimental Zoology*, 236, 19-25.

Degnan, K.J. (1986). Cyclic AMP stimulation of Cl^- secretion by the opercular epithelium: the apical membrane chloride conductance. *The Journal of Experimental Zoology*, 238, 141-146.

Degnan, K.J., Karnaky, K.J. and Zadunaisky, J.A. (1977). Active chloride transport in the *in-vitro* opercular skin of a teleost (*Fundulus heteroclitus*), a gill-like epithelium rich in chloride cells. *Journal of Physiology*, 271, 155-191.

De Sauvage, F.J., Camerato, T.R. and Goeddel, D.V. (1991). Primary structure and functional expression of the human receptor for *Escherichia coli* heat-stable enterotoxin.

De Sauvage, F.J., Keshav, S., Kuang, W-J., Gillett, N., Henzel, W. and Goeddel, D.V. (1992). Precursor structure, expression and tissue distribution of human guanylin. *Proceedings of the National Academy of Sciences USA*, 89, 9089-9093.

Duff, D.W. and Olson, K.R. (1986). Trout vascular and renal responses to atrial natriuretic factor and heart extracts. *American Journal of Physiology*, 251, R639-R642.

Epstein, F.H., Cynamon, M. and McKay, W. (1971). Endocrine control of Na^+, K^+-ATPase and seawater adaptation in *Anguilla rostrata*. *General and Comparative Endocrinology*, 16, 323-328.

Evans, D.H. (1975). Ionic exchange mechanisms in fish gills. *Comparitive Biochemistry and Physiology*, 51A, 491-495.

Eveloff, J., Field, M., Kinne, R. and Murer, H. (1980). Sodium-cotransport systems in intestine and kidney of winter flounder. *Journal of Comparative Physiology*, 135, 175-182.

Fan, X., Hamra, F.K., Freeman, R.H., Eber, S.L., Krause, W.J., Lim, R.W., Pace, V.M., Currie, M.G. and Forte, L.R. (1996). Uroguanylin: cloning of a preprouroguanylin cDNA, mRNA expression in the intestine and heart and isolation of uroguanylin and prouroguanylin from plasma. *Biochemical and Biophysical Research Communications*, 219, 457-462.

Fan, X., Wang, Y., London, R.M., Eber, S.L., Krause, W.J., Freeman, R.H. and Forte, L.R. (1997). Signaling pathways for guanylin and uroguanylin in the digestive, renal, central nervous, reproductive and lymphoid systems. *Endocrinology*, 138, 4636-4648

Field, M., Graf, L.H., Laird, W.J. and Smith, P.L. (1978). Heat-stable enterotoxin of *Escherichia coli*: In vitro effects of guanylate cyclase activity, cGMP concentration and ion transport in small intestine. *Proceedings of the National Academy of Sciences USA*, 75, 2800-2804.

Field, M.; Karnaky, K.J., Smith, P.L, Bolton, J.E. and Kinter, W.B. (1978). Ion transport across the intestinal mucosa of the winter flounder, *Pseudopleuronectus americanus* I. *Journal of Membrane Biology*, 41, 265-293.

Field, M., Smith, P.L. and Bolton, J.E. (1980). Ion transport across the isolated intestinal mucosa of the winter flounder, *Pseudopleuronectus americanus* II. *Journal of Membrane Biology*, 55, 157-163.

Fonteles, M.C., Greenberg, R.N., Monteiro, H.S.A., Currie, M.G. and Forte, L.R. (1998). Natriuretic and kaliuretic properties of guanylin and uroguanylin in the perfused rat kidney. *American Journal of Physiology*, 275, F191-F197.

Forrest, J.N., Cohen, A.D., Schon, D.A. and Epstein, F.H. (1973 a). Na transport and Na^+, K^+-ATPase in gills during adaptation to seawater: effects of cortisol. *American Journal of Physiology*, 224, 3, 709-713

Forrest, J.N., MacKay, W.C., Gallagher, B. and Epstein, F.H. (1973 b). Plasma cortisol response to saltwater adaptation in the American eel *Anguilla rostrata. American Journal of Physiology*, 224, 3, 714-717.

Forte, L.R. (1999). Guanylin regulatory peptides: structures, biological activities mediated by cGMP and pathobiology. *Regulatory Peptides*, 81, 25-39.

Forte, L.R. and Hamra, F.K. (1996). Guanylin and uroguanylin: intestinal peptide hormones that regulate epithelial transport. *News in Physiological Sciences*, 11, 17-24.

Forte, L.R., Krause, W.J., and Freeman, R.H. (1988). Receptors and cGMP signalling mechanism for *E.coli* enterotoxin in opossum kidney. *American Journal of Physiology*, 255, F1040-F1046.

Forte, L.R., Krause, W.J., and Freeman, R.H. (1989). Escherichia coli enterotoxin receptors: localisation in opossum kidney, intestine and testis. *American Journal of Physiology*, 257, F874-F881.

Forte, L.R., Eber, S.L., Turner, J.T., Freeman, R.H., Fok, K.F. and Currie, M.G. (1993). Guanylin stimulation of Cl^- secretion in human intestinal T_{84} cells via cyclic guanosine monophosphate. *The Journal of Clinical Investigation*, 91, 2423-2428.

Forte, L.R., London, R.M., Eber, S.L., Freeman, R.H. and Krause, W.J. (1997a). Guanylin-like petides and receptors in fish intestine. *FASEB Journal*, 11, A297.

Forte, L.R., Eber, S.L., Fan, X., London, R.M., Wang, Y., Rowland, L.M., Chin, D.T., Freeman, R.H. and Krause, W.J. (1999). Lymphoguanylin: cloning and characterization of a unique member of the guanylin peptide family. *Endocrinology*, 140, 4, 1800-1806.

Forte, L.R., London, R.M., Freeman, R.H. and Krause, W.J.(2000). Guanylin peptides: renal actions mediated by cyclic GMP. *American Journal of Physiology*, 278, 2, F180-F191.

Foskett, J.K. and Scheffey, C. (1982). The chloride cell: definitive identification as the salt-secretory cell in teleosts. *Science*, 215, 164-166.

Foskett, J.K., Logsdon, G.D., Turner, T., Machen, T.E. and Bern, H.A. (1981). Differentiation of the chloride extrusion method during seawater adaptation of a teleost fish, the cichlid *Sarotherodon mossambicus*. *Journal of Experimental Biology*, 93, 209-224.

Foskett, J.K., Hubbard, G.M., Machen, T.E. and Bern, H.A. (1982). Effects of epinephrine, glucagon and vasoactive intestinal peptide on chloride secretion by teleost opercular epithelium. *Journal of Comparative Physiology*, 146, 27-34.

Foskett, J.K., Machen, T.E. and Bern, H.A. (1982). Chloride secretion and conductance of teleost opercular membrane: effects of prolactin. *American Journal of* Physiology, 242, R380-R389.

Fuentes, J. and Eddy, F.B.(1997). Drinking in marine, euryhaline and freshwater teleost fish. In: Ionic regulation in animals: a tribute to Professor W.T.W. Potts, (eds, Hazon, N., Eddy, F.B. and Flik, G.)

Furuya, S., Naruse, S. and Hayakawa, T. (1998). Intravenous injection of guanylin induces mucus secretion from goblet cells in rat duodenal crypts. *Anatomy and Embryology*, 197, 359-367.

Gallis, J-L., Lasserre, P. and Belloc, F. (1979). Freshwater adaptation in the euryhaline teleost, *Chelon labrosus*. *General and Comparative Endocrinology*, 39, 1-10.

Girard, J.P. and Payan, P. (1980). Ion exchanges through respiratory and chloride cells in freshwater- and seawater-adapted teleosteans. *American Journal of Physiology*, 238, R260-R268.

Greenberg, R.N., Hill, M., Crytzer, J., Krause, W.J., Eber, S.L. Hamra, F.K. and Forte, L.R. (1997). Comparison of effects of uroguanylin, guanylin and *Escherichia coli* heat-stable enterotoxin St_a in mouse intestine and kidney. Evidence that uroguanylin is an intestinal natriuretic hormone. *Journal of Investigative Medicine*, 45, 276-283.

Gilbert, S.F. (1994). Developmental Biology (4th Edition). Sinauer Associates Inc. USA.

Greger, R. and Schlatter, E. (1984a). Mechanism of NaCl secretion in the rectal gland of spiny dogfish (*Squalus acanthias*). I : Experiments in isolated in vitro perfused rectal gland tubules. *Pflugers Archives*, 402, 63-75.

Greger, R. and Schlatter, E. (1984b). Mechanism of NaCl secretion in the rectal gland of spiny dogfish (*Squalus acanthias*). II: Effects of inhibitors. *Pflugers Archives*, 402, 364-375.

Guba, M., Kuhn, M., Forssmann, W-G., Classen, M., Gregor, M. and Seidler, U. (1996). Guanylin strongly stimulates rat duodenal HCO_3^- secretion: proposed mechanism and comparison with other secretagogues. *Gastroenterology*, 111, 1558-1568.

Halm, D.R., Krasny, E.J. and Frizzell, R.A. (1985 a). Electrophysiology of flounder intestinal mucosa I. *Journal of General Physiology*, 85, 843-864.

Halm, D.R., Krasny, E.J. and Frizzell, R.A. (1985 b). Electrophysiology of flounder intestinal mucosa II. *Journal of General Physiology*, 85, 865-883.

Hamra, F.K., Forte, L.R., Eber, S.L., Pidhorodeckyj, N.V., Krause, W.J., Freeman, R.H., Chin, D.T., Tompkins, J.A., Fok, K.F., Smith, C.E., Duffin, K.L., Siegel, N.R. and Currie, M.G. (1993). Uroguanylin: structure and activity of a second endogenous peptide that stimulates intestinal guanylate cyclase. *Proceeding of the National Academy of Sciences USA*, 90, 10464-10468.

Hamra, F.K., Fan, X., Krause, W.J., Freeman, R.H., Chin, D.T., Smith, C.E., Currie, M.G. and Forte, L.R. (1996 a). Prouroguanylin and proguanylin: purification from colon, structure and modulation of bioactivity by proteases. *Endocrinology*, 137, 1, 257-265.

Hamra, F.K., Krause, W.J., Eber, S.L., Freeman, R.H., Smith, C.E., Currie, M.G. and Forte, L.R. (1996 b). Opossum colonic mucosa contains uroguanylin and guanylin peptides. *American Journal of Physiology* (*Gastrointestinal and Liver Physiology* 33) 270, G708-G716.

Hamra, F.K., Eber, S.L., Chin, D.T., Currie, M.G. and Forte, L.R. (1997). Regulation of intestinal uroguanylin/guanylin receptor-mediated responses by mucosal acidity. *Proceedings of the National Academy of Sciences USA*, 94, 2705-2710.

Harvey-Pough, F., Heiser, J.B. and McFarland, W.N.(1989). Vertebrate Life (third edition). Collier MacMillan, London.

Hasegawa, M., Hidaka, Y., Matsumoto, Y., Sanni, T. and Shimonishi, Y. (1999). Determination of the binding site on the extracellular domain of guanylyl cyclase C to heat-stable enterotoxin. *The Journal of Biological Chemistry*, 274, 44, 31713-31718.

Hazon, N. and Balment, R.J. (1998). Endocrinology. In: The Physiology of Fishes, Evans, D.H. (ed.), 2nd. edition, 441-463, (CRC Press, New York.).

Hess, R., Kuhn, M., Schulz-Knappe, P., Raida, M., Fuchs, M., Klodt, J., Adermann, K., Kaever, V., Cetin, Y. and Forssmann, W-G. (1995). GCAP-II: isolation and characterisation of the circulating form of human uroguanylin. *FEBS Letters*, 374, 34-38.

Hill, O., Cetin, Y., Cieslik, A., Magert, H-J. and Forssmann, W-G. (1995a). A new human guanylate cyclase-activating peptide (GCAP-II, uroguanylin): precursor cDNA and colonic expression. *Biochimica et Biophysica Acta*, 1253, 146-149.

Hill, O., Kuhn, M., Zucht, H-D., Cetin, Y., Kulaksiz, H., Adermann, K., Klock, G., Rechkemmer, G., Forssmann, W-G. and Magert, H.J. (1995b). Analysis of the human guanylin gene and the processing and cellular localization of the peptide. *Proceedings of the National Academy of Sciences USA*, 92, 2046-2050.

Hirano, T. and Mayer-Gostan, N. (1976). Eel oesophagous as an osmoregulatory organ. *Proceedings of the National Academy of Sciences USA*, 73, 4, 1348-1350.

Hirano, T. and Hasegawa, S. (1984). Effects of angiotensins and other vasoactive substances on drinking in the eel, *Anguilla japonica*. *Zoological Science*, 1, 106-113.

Hochmann, J.A., Sciaky, D., Whitaker, T.L., Hawkins, J.A. and Cohen, M.B. (1997). Hepatocyte nuclear factor-1α regulates transcription of the guanylin gene. *American Journal of Physiology*, 273, G833-G841.

Hootman, S.R. and Philpott, C.W. (1979). Ultracytochemical localisation of Na^+K^+-ATPase in chloride cells from the gills of a euryhaline teleost. *Anatomical Record*, 193, 99-130.

Howard, J.N. and Ahearn, G.A. (1988). Parallel antiport mechanisms for Na^+ and Cl^- transport in herbivorous teleost intestine. *Journal of Experimental Biology*, 135, 65-76.

Hubner, S., Michel, F., Rudloff, V. and Appelhans, H. (1992). Amino acid sequence of band-3 protein from rainbow trout erythrocytes derived from cDNA. *Biochemical Journal*, 285, 17-23.

Jampol, L.M. and Epstein, F.H. (1970). Sodium- potassium- activated adenosine triphosphatase and osmotic regulation by fishes. *American Journal of Physiology*, 218, 2, 607-611.

John, M., Weidenmann, B., Kruhoffer, M., Adermann, K., Ankorina-Stark, I., Schlatter, E., Ahnert-Hilger, G., Forssmann, W-G and Kuhn, M. (1998). Guanylin stimulates regulated secretion from human neuroendocrine pancreatic cells.*Gastroenterology*, 114, 791-797.

Joo, N.S., London, R.M., Kim, H.D., Forte, L.R. and Clarke, L.L. (1998). Regulation of intestinal Cl^- and HCO_3^- secretion by uroguanylin. *American Journal of Physiology*, 274, G633-G644.

Joo, N.S., Clarke, L.L., Hee, B.H. and Kim, H.D. (1999). Cloning of a CLC-2 chloride channel from murine duodenum and its presence in CFTR knockout mice. *Biochemical and Biophysical Research Communications*,

Kaiya, H. and Takei, Y. (1996a). Atrial and ventricular natriuretic peptide concentrations in plasma of freshwater- and seawater-adapted eels. *General and Comparative Endocrinology*, 102, 2, 183-190.

Kaiya, H. and Takei, Y. (1996b). Changes in plasma atrial and ventricular natriuretic peptide concentrations after transfer of eels from freshwater to seawater or vice versa. *General and Comparative Endocrinology*, 104, 3, 337-345.

Kaiya, H. and Takei, Y. (1996 c). Osmotic and volaemic regulation of atrial and ventricular natriuretic peptide secretion in conscious eels. *Journal of Endocrinology*, 149, 444-1-447.

Kamiya, M. (1972). Sodium-potassium-activated adenosinetriphosphatase in isolated chloride cells from eel gills. *Comparative Biochemistry and Physiology*, 43B, 611-617.

Karnaky, K.J. (1986). Structure and function of the chloride cell of *Fundulus heteroclitus* and other teleosts. *American Zoology*, 26, 209-224.

Karnaky, K.J. and Kinter, W.B. (1977). Killifish opercular skin: a flat epithelium with a high density of chloride cells. *Journal of Experimental Zoology*, 199, 355-364.

Karnaky, K.J., Kinter, L.B., Kinter, W.B. and Stirling, C.E. (1976). Teleost chloride cell. II. Autoradiographic localization of gill Na, K-ATPase in killifish, *Fundulus heteroclitus* adapted to low and high salinity environments. *Journal of Cell Biology*, 70, 157-177.

Karnaky, K.J., Degnan, K.J. and Zadunaisky, J.A. (1977). Chloride transport across isolated opercular epithelium of killifish: a membrane rich in chloride cells. *Science*, 195, 203-205.

Kashiwagi, M., Miyamoto, K., Takei, Y. and Hirose, S. (1999). Cloning, properties and tissue distribution of natriuretic peptide receptor-A of euryhaline eel, *Anguilla japonica*. *European Journal of Biochemistry*, 259, 204-211.

Katafuchi, T., Takashima, A., Kashiwagi, M., Hagiwara, H., Takei, Y. and Hirose, S. (1994). Cloning and expression of eel natriuretic-peptide receptor B and comparison with its mammalian counterparts. *European Journal of Biochemistry*, 222, 835-842.

Keys, A. and Willmer, E.N. (1932). "Chloride secreting cells" in the gills of fishes, with special reference to the common eel. *The Journal of Physiology*, LXXVI, 368-378.

King, J.A.C., Abel, D.C. and DiBona, D.R. (1989). Effects of salinity on chloride cells in the euryhaline cyprinodontid fish *Rivulus marmoratus*. *Cell and Tissue Research*, 257, 367-377.

Kinoshita, H., Fujimoto, S., Nakazato, M., Yokota, N., Date, Y., Yamaguchi, H., Hisanaga, S. and Eto, T. (1997a). Urine and plasma levels of uroguanylin and its molecular forms in renal diseases. *Kidney International*, 52, 1028-1034.

Kinoshita, H., Nakazato, M., Yamaguchi, S., Matsukura, S., Fujimoto, S. and Eto, T. (1997 b). Increased plasma guanylin levels in patients with impaired renal function. *Clinical Nephrology*, 47, 28-32.

Kirsch, R. and Mayer-Gostan, N. (1973). Kinetics of water and chloride exchanges during adaptation of the European eel to sea water. *Journal of Experimental Biology*, 58, 105-121.

Kisen, G., Gallais, C., Auperin, B., Klungland, H., Sandra, O., Prunet, P. and Andersen, O. (1994). Northern blot analysis of the Na^+, K^+-ATPase α-subunit in salmonids. *Comparative Biochemistry and Physiology* B, 107, 255-259.

Kita, T., Smith, C.E., Fok, K.F., Duffin, K.L., Moore, W.M., Karabatsos, P.J., Kachur, J.F., Hamra, F.K., Pidhorodeckyj, N.V., Forte, L.R. and Currie, M.G. (1994). Characterization of human uroguanylin: a member of the guanylin peptide family. *American Journal of Physiology*, 266, F342-F348.

Kloas, W., Reinecke, M. and Hanke, W. (1994). Role of the atrial natriuretic peptide for adrenal regulation in the teleost fish *Cyprinus carpio*. *American Journal of Physiology*, 267, R1034-R1042.

Kobayashi, H., Umura, H., Takei, Y., Itatsu, N., Ozawa, M. and Ichinohe, K. (1983). Drinking induced by angiotensin II in fishes. *General Comparative Endocrinology*, 49, 295-306.

Krause, W.J., Freemna, R.H. and Forte, L.R. (1990). Autoradiographic demonstration of specific binding sites for *E.coli* enterotoxin in various epithelia of the North American opossum. *Cell and Tissue Research*, 260, 387-394.

Krause, G., Bayerl, A., Heim, J-M., Singh, S. and Gerzer, R. (1994). Distribution of membrane bound guanylyl cyclases in human intestine. *Gut*, 35, 1250-1257.

Krause, W.J., Freeman, R.H., Eber, S.L., Hamra, F.K., Fok, K.F., Currie, M.G. and Forte, L.R. (1995). Distribution of *Escherichia coli* heat-stable enterotoxin/guanylin/uroguanylin receptors in avian intestinal tract. *Acta Anatomica*, 153, 210-219.

Krause, W.J., Freeman, R.H., Eber, S.L., Hamra, F.K., Currie, M.G. and Forte, L.R. (1997 c). Guanylyl cyclase receptors and guanylin-like peptides in reptilian intestine. *General and Comparative Endocrinology*, 107, 229-239.

Krause, W.J., London, R.M., Freeman, R.H. and Forte, L.R. (1997 d). The guanylin and uroguanylin peptide hormones and their receptors. *Acta Anatomica*, 160, 213-231.

Kuhn, M., Raida, M., Adermann, K., Schulz-Knappe, P., Gerzer, R., Heim, J-M. and Forssmann, W-G. (1993). The circulating bioactive form of human guanylin is a high molecular weight peptide (10.3 kDa). *FEBS*, 318, 2, 205-209.

Kuhn, M., Adermann, K., Jahne, J., Forssmann, W-G. and Rechkemmer, G. (1994). Segmental differences in the effects of guanylin and *Escherichia coli* heat-stable enterotoxin on Cl^- secretion in human gut. *Journal of Physiology*, 479, 433-440.

Kurose, H., Inagami, T. and Ui, M. (1987). Participation of adenosine 5'-triphosphate in the activation of membrane bound guanylate cyclase by the atrial natriuretic factor. *FEBS*, 219, 2, 375-379.

Lacy, E.R. (1983). Histochemical and biochemical studies of carbonic anhydrase activity in the opercular epithelium of the euryhaline teleost, *Fundulus heteroclitus*. *The American Journal of Anatomy*, 166, 19-39.

Laurent, P.L. (1984). Gill internal morphology. In *Fish Physiology* Vol Xa (eds, Hoar, W.S and Randall, D.J.). New York Academic press.

Laurent, P. and Dunel, S. (1980). Morphology of gill epithelia in fish. *American Journal of Physiology* (*Regulatory Integrative Comparative Physiology* 7), 238, R147-R159.

Laurent, P. and Perry, S.F. (1990). Effects of cortisol on gill chloride cell morphologyand ionic uptake in the freshwater trout, *Salmo gairdneri*. *Cell and Tissue Research*, 259, 429-442.

Lee, T-H., Hwang, P-P., Lin, H-C. and Huang, F-L. (1996). Mitochondria-rich cells in the branchial epithelium of the teleost, *Orechromis mossambicus*, acclimated to various hypotonic environments. *Fish Physiology and Biochemistry*, 15, 6, 513-523.

Li, Z. and Goy, M.F. (1993). Peptide-regulated guanylate cyclase pathways in rat colon: in situ localisation of GCA, GCC and guanylin mRNA. *American Journal of* Physiology, 265, G394-G402.

Li, Z., Taylor-Blake, B., Light, A.R. and Goy, M.F. (1995). Guanylin, an endogenous ligand for C-type guanylate cyclase, is produced by goblet cells in the rat intestine. *Gastroenterology*, 109, 1863-1875.

Li, Z., Knowles, J.W., Goyeau, D., Prabhakar, S., Short, D.B., Perkins, A.G. and Goy, M.F. (1996). Low salt intake down-regulates the guanylin signalling pathway in rat distal colon. *Gastroenterology*, 111, 1714-1721.

Li, Z., Perkins, A.G., Peters, M., Campa, M.J. and Goy, M.F. (1997). Purification, cDNA sequence, and tissue distribution of rat uroguanylin. *Regulatory Peptides*, 68, 45-56.

Liang, P. and Pardee, A.B. (1992). Differential display of eukaryotic mRNA by means of the polymerase chain reaction. *Science*, 257, 967-970.

Lin, H. and Randall, D. J. (1991). Evidence for the presence of an electrogenic proton pump on the trout gill epithelium. *Journal of Experimental Biology*, 161, 119-134.

Lin, H. and Randall, D.J. (1993). H^+-ATPase activity in crude homogenates of fish gill tissue-inhibitor sensitivity and environmental and hormonal regulation. *Journal of Experimental Biology*, 180, 163-174.

Lin, H., Pfeifer, D.C., Vogl, A.W., Pan, J. and Randall, D.J. (1994). Immunolocalisation of proton-ATPase in the gill epithelia of rainbow trout. *Journal of Experimental Biology*, 195, 169-183.

Lingrel, J.B. and Kuntzweiler, T. (1994). Na^+, K^+-ATPase. *Journal of Biological Chemistry*, 269, 19569-19662.

Lohmann, S.M., Vaandrager, A.B., Smolenski, A., Walter, U. and De Jonge, H.R. (1997). Distinct and specific functions of cGMP-dependent protein kinases. *TIBS*, 22, 307-312.

London, R.M., Eber, S.L., Visweswariah, S.S., Krause, W.J. and Forte, L.R. (1999). Structure and activity of OK-GC: a kidney receptor guanylate cyclase activated by guanylin peptides. *American Journal of Physiology*, 45, F882-F891.

Loretz, C.A., Freel, R.W. and Bern, H.A. (1983). Specificity of response of intestinal ion transport systems to a pair of natural peptide hormone analogs: somatostatin and urotensin II. *General and Comparative Endocrinology*, 52, 198-206.

Loretz, C.A. and Takei, Y. (1997). natriuretic peptide inhibition of intestinal salt absorption in the Japanese eel: physiological significance. *Fish Physiology and Biochemistry*, 17, 1-6, 319-324.

Loretz, C.A., Pollina, C., Kaiya, H., Sakaguchi, H. and Takei, Y. (1997). Local synthesis of natriuretic peptides in the eel intestine. *Biochemical and Biophysical Research Communications*, 238, 3, 817-822.

Madsen, S.S. (1990a). The role of cortisol and growth hormone in seawater adaptation and development of hypoosmoregulatory mechanisms in sea trout parr (*Salmo trutta trutta*). *General and Comparative Endocrinology*, 79, 1-11

Madsen, S.S. (1990b). Cortisol treatment improves the development of hypoosmoregulatory mechanisms in the euryhaline rainbow trout, *Salmo gairdneri*. *Fish Physiology and Biochemistry*, 8, 1, 45-52.

Madsen, S.S. (1990c). Enhanced hypoosmoregulatory ability to growth hormone after cortisol treatment in immature rainbow trout, *Salmo gairdneri*. *Fish Physiology and Biochemistry*, 8, 4, 271-279.

MacKay, W.C. and Janicki, R. (1979). Changes in the eel intestine during seawater adaptation. *Comparative Biochemistry and Physiology*, 62A, 757-761.

Magert, H-J., Reinecke, M., David, I., Raab, H-R., Adermann, K., Zucht, H-D., Hill, O., Hess, R. and Forssmann, W-G. (1998). Uroguanylin: gene structure, expression, processing as a peptide hormone, and co-storage with somatostatin in gastrointestinal D-cells. *Regulatory Peptides*, 73, 165-176.

Mainoya, J.R. (1982). Water and NaCl absorption by the intestine of the tilapia, *Sarotherodon mossambicus*, adapted to fresh water or seawater and the possible role of prolactin and cortisol. *Journal of Comparative Physiology*, 146, 1-7.

Malvin, R.L., Schiff, D. and Eiger, S. (1980). Angiotensin and drinking rates in the euryhaline killifish. *American Journal of Physiology*, 239, R31-R34.

Mancera, J.M. and McCormick, S.D. (1998). Osmoregulatory actions of the GH/IGF axis in non-salmonid teleosts. *Comparative Biochemistry and Physiology B*, 121, 43-48.

Mann, E.A., Cohen, M.B. and Giannella, R.A. (1993). Comparison of receptors for *Escherichia coli* heat-stable enterotoxin: novel receptor present in IEC-6 cells. *American Journal of* Physiology, 264, G172-G178.

Mann, E.A., Jump, M.L. and Giannella, R.A. (1996). Cell line-specific transcriptional activation of the promoter of the human guanylyl cyclase C/heat-stable enterotoxin receptor gene. *Biochimica et Biophysica Acta*, 1305, 7-10.

Mann, E.A., Jump, M.L., Wu, J., Yee, E. and Giannella, R.A. (1997).Mice lacking the guanylyl cyclase C receptor are resistant to St_a-induced intestinal secretion. *Biochemical and Biophysical Research Communications*, 239, 463-466.

Markert, T., Vaandrager, A.B., Gambaryan, S., Pohler, D., Hausler, C., Walter, U., DeJonge, H.R., Jarchau, T. and Lohmann, S.M. (1995). Endogenous expression of type-II cGMP-dependent protein-kinase messenger RNA and protein in rat intestine-implications for cystic fibrosis transmembrane conductance regulator. *Journal of Clinical Investigation*, 96, 2, 822-830.

Marshall, W.S. (1977). Transepithelial potential and short-circuit current across the isolated skin of *Gillichthys mirabilis* (Teleostei: Gobiidae), acclimated to 5% and 100% seawater. *Journal of Comparative Physiology*, 144, 157-165.

Marshall, W.S. (1995). Transport processes in isolated teleost epithelia: opercular epithelium and urinary bladder. In: Cellular and molecular approaches to fish ionic regulation (eds, Wood, C.M. and Shuttleworth, T.J.), Fish Physiology Vol. 14, Academic Press, San Diego.

Marshall, J., Martin, K.A., Picciotto, M., Hockfield, S., Nairn, A.C. and Kaczmarek, L.K. (1991). Identification and localisation of a dogfish homologue of Human Cystic Fibrosis Transmembrane Conductance Regulator. *Journal of Biological Chemistry*, 266, 22749-22754.

Marshall, W.S., Bryson, S.E., Midelfart, A. and Hamilton, F.W. (1995). Low-conductance anion channel activated by cAMP in teleost Cl^--secreting cells. *American Journal of* Physiology, 268.

Mantoku, T., Muramatsu, R., Nakauchi, M., Yamagami, S., Kusakabe, T. and Suzuki, N. (1999). Sequence analysis of cDNA and genomic DNA and mRNA expression of the medaka fish homologue of mammalian GC-C. *Journal of Biochemistry*, 125, 3, 476-486.

Martin, S., Adermann, K., Forrssmann, W-G. and Kuhn, M. (1999). Regulated, side-directed secretion of proguanylin from isolated rat colonic mucosa. *Endocrinology*, 140, 11, 5022-5029.

May, S.A. and Degnan, K.J. (1984). cAMP -mediated regulation of chloride secretion by the opercular epithelium. *American Journal of Physiology*, 246, R741-R746.

Mayer, N., Maetz, J., Chan, D.K.O., Forster, M. and Chester-Jones, I. (1967). Cortisol, a sodium excreting factor in the eel (*Anguilla anguilla* L.) adapted to sea water. *Nature*, 214, 1118-1120.

McCormick, S.D. (1990b). Cortisol directly stimulates differentiation of chloride cells in tilapia opercular membrane. *American Journal of Physiology*, 259, R857-R863.

McCormick, S.D. (1995). Hormonal control of gill Na^+,K^+-ATPase and chloride cell function. In: Cellular and molecular approaches to fish ionic regulation (eds Wood, C.M. and Shuttleworth, T.J.), 285-315. San Diego Academic Press.

McKendry, J.E., Bernier, N.J., Takei, Y., Duff, D.W., Olson, K.R. and Perry, S.F. (1999). Natriuretic peptides and control of catecholamine release in two freshwater teleosts and a marine elasmobranch. *Fish Physiology and Biochemistry*, 20, 1, 61-77.

McLeese, J.M., Johnsson, J., Huntley, F.M., Clarke, W.C. and Weisbart, M. (1994). Seasonal changes in osmoregulation, cortisol and cortisol receptor activity in the gills of parr/smolt of steelhead trout and steelhead-rainbow trout hybrids, *Oncorhynchus mykiss*. *General and Comparative Endocrinology*, 93, 103-113.

Mishina, S. and Takei, Y. (1997). Characterisation of natriuretic peptide receptors in eel gill. *Journal of Endocrinology*, 154, 3, 415-422.

Miyazato, M., Nakazato, M., Yamaguchi, H., Date, Y., Kojima, M., Kangawa, K., Matsuo, H. and Matsukuru, S. (1996a). Cloning and characterisation of a cDNA

encoding a precursor for human uroguanylin. *Biochemical and Biophysical Research Communications*, 219, 644-648.

Miyazato, M., Nakazato, M., Matsukura, S., Kangawa, K. and Matsuo, H. (1996b). Uroguanylin gene expression in the alimentary tract and extra-gastrointestinal tissues. *FEBS Letters*, 398, 170-174.

Morgan, J.D. and Iwama, G.K. (1996). Cortisol-induced changes in oxygen consumption and ionic regulation in coastal cutthroat trout (*Oncorhynchus clarki clarki*) parr. *Fish Physiology and Biochemistry*, 15, 5, 385-394.

Morley, M., Chadwick, A. and El Tounsy, E.M. (1981). The effect of prolactin on water absorption by the intestine of the trout (*Salmo gairdneri*). *General and Comparative Endocrinology*, 44, 64-68.

Musch, M.W., Orellana, S.A., Kimberg, L.S., Field, M., Halm, D.R., Krasny, E.J. and Frizzell, R.A. (1982). Na^+-K^+-Cl^- co-transport in the intestine of a marine teleost. *Nature*, 300, 351-353.

Nagashima, K. and Ando, M. (1994). Characterisation of esophageal desalination in the seawater eel, *Anguilla japonica*, 164, 47-54.

Nakazato, M., Yamaguchi, H., Kinoshita, H., Kangawa, K., Matsuo, H., Chino, N. and Matsukura, S. (1996). Identification of biologically active and inactive human uroguanylins in plasma and urine and their increases in renal insufficiency.

Nakazato, M., Yamaguchi, H., Date, Y., Miyazato, M., Kangawa, K., Goy, M.F., Chino, N. and Matsukura, S. (1998). Tissue distribution, cellular source and structural analysis of rat immunoreactive uroguanylin. *Endocrinology*, 139, 5247-5254.

Nandi, A., Bhandari, R. and Visweswariah, S.S. (1997). Epitope conservation and immunohistochemical localization of the guanylin/heat-stable toxin peptide receptor, guanylyl cyclase C. *Journal of Cell Biochemistry*, 66, 500-511.

Nokihara, K., Wray, V., Ando, E., Naruse, S. and Hayakawa, T. (1997). Synthesis, solution structure, binding activity and cGMP activation of human guanylin and its disulfide isomer. *Regulatory Peptides*, 70, 111-120.

O'Grady, S.M., Field, M., Nash, N.T. and Rao, M.C. (1985). Atrial natriuretic factor inhibits Na-K-Cl cotransport in teleost intestine. *American Journal of Physiology*, 249, C531-C534.

Oide, M. and Utida, S. (1967). Changes in water and ion transport in isolated intestines of the eel during salt adaptation and migration. *Marine Biology*, 1, 102-106.

Oide, M. and Utida, S. (1968). Changes in intestinal absorption and renal excretion of water during adaptation to seawater in the Japaneses eel. *Marine Biology*, 1, 172-177.

Olson, K.R. and Duff, D.W. (1992). Cardiovascular and renal effects of eel and rat atrial natriuretic peptide in rainbow trout, *Salmo gairdneri*. *Journal of Comparative Physiology B*, 162, 408-415.

Oudit, G.Y. and Butler, D.G. (1995). Cardiovascular effects of arginine vasotocin, atrial natriuretic peptide and epinephrine in freshwater eels. *American Journal of Physiology* (*Regulatory Integrative Comparative Physiology* 37), 268, R1273-R1280.

Parmalee, J.T. and Renfro, J.L. (1983). Esophageal desalination of seawater in flounder: role of active sodium transport. *American Journal of Physiology*, 245, R888-R893.

Perkins, A., Goy, M.F. and Li, Z. (1997). Uroguanylin is expressed by enterochromaffin cells in the rat gastrointestinal tract. *Gastroenterology*, 113, 1007-1014.

Perrot, M.N., Grierson, C.E., Hazon, N. and Balment, R.J. (1992). Drinking behaviour in sea water and fresh water teleosts, the role of the renin-angiotensisn system. *Fish Physiology and Biochemistry*, 10, 2, 161-168.

Perry, S.F. (1997). The chloride cell: structure and function in the gills of freshwater fishes. *Annual Review of Physiology*, 59, 325-347.

Perry, S.F and Randall, D.J. (1981). Effects of amiloride and SITS on branchial ion fluxes in rainbow trout, *Salmo gairdneri*. *Journal of Experimental Zoology*, 215, 225-228.

Perry, S.F., Haswell, M.S., Randall, D.J. and Farrell, A.P. (1981). Branchial ionic uptake and acid-base regualtion in the rainbow trout, *Salmo gairdneri*. *Journal of Experimental Biology*, 92, 289-303.

Pfeifer, A., Aszodi, A., Seidler, U., Ruth, P., Hofmann, F. and Fassler, R. (1996). Intestinal secretory defects and dwarfism in mice lacking cGMP-dependent protein kinase II. *Science*, 274, 2082-2085.

Pickford, G.E. and Phillips, J.G. (1959). Prolactin, a factor in promoting survival of hypophysectomised killifish in fresh water. *Science*, 130, 454-455.

Pickford, G.E., Griffith, R.W., Torretti, J., Hendlez, E. and Epstein, F.H. (1970a). Branchial reduction and renal stimulation of Na^+, K^+-ATPase by prolactin in hypophysectomised killifish in freshwater. *Nature*, 228, 378-379.

Pickford, G.E., Pang, P.K., Weinstein, E., Torretti, J., Hendler, E. and Epstein, F.H. (1970b). The response of the hypophysectomised cyprinodont, *Fundulus heteroclitus*, to replacement therapy with cortisol: effects on blood serum and sodium-potassium activated adenosine triphosphatase in the gills kidney and intestinal mucosa. *General and Comparative Endocrinology*, 14, 524-534.

Pisam, M. Caroff, A. and Rambourg, A. (1987). Two types of chloride cells in the gill epithelium of a freshwater-adapted euryhaline fish: *Lebistes reticulatus*, their modifications during adaptation to saltwater. *The American Journal of Anatomy*, 179, 40-50.

Pisam, M., Auperin, B., Prunet, P., Rentier-Delrue, F., Martial, J. and Rambourg, A. (1993). Effects of prolactin on α and β chloride cells in the gill epithelium of saltwater adapted tilapia "*Oreochromis niloticus*". *The Anatomical Record*, 235, 275-284.

Price, D.A., Doble, K.E., Lee, T.D., Gallis, S.M., Dunn, B.M., Parten, B. and Evans, D.H. (1990). The sequencing, synthesis and biological actions of an ANP-like peptide isolated from the brain of the killifish, *Fundulus heteroclitus*. *Biological Bulletin*, 178, 279-285

Prunet, P., Boeuf, G., Bolton, J.P. and Young, G. (1989). Smoltification and seawater adaptation in Atlantic salmon (*Salmo salar*): plasma prolactin, growth hormone and thyroid hormones. *General and Comparative Endocrinology*, 74, 355-364.

Prunet, P., Pisam, M., Claireaux, J.P., Boeuf, G. and Rambourg, A. (1994). Effects of growth hormone on gill chloride cells in juvenile Atlantic salmon (*Salmo salar*). *American Journal of Physiology* (*Regulatory Integrative Comparative Physiology* 35), 266, R850-R857.

Prunet, P., Sandra, O. and Auperin, B. (1996). Molecular characterisation of prolactin receptors in tilapia. In: Gene expression and manipulation in Aquatic organisms (eds. Ennion, S.J and Goldspink, G.), Cambridge University Press.

Randall, D.J. and Perry, S.F. (1992). Catecholamines. In: Fish Physiology vol XII (eds. Hoar, W.S. and Randall, D.J.) Part B, 255-300.

Rang, H.P., Dale, M.M., Ritter, J.M. and Gardner, P. (1995). Pharmacology (third edition). Churchill Livinstone, London.

Rao, M.C., Nash, N.T. and Field, M. (1984). Differing effects of cGMP and cAMP on ion transport across flounder intestine. *American Journal of Physiology*, 246, C167-C171.

Richman III, H.N. and Zaugg, W.S. (1987). Effects of cortisol and growth hormone on osmoregulation in pre- and de-smoltified coho salmon (*Oncorhynchus kisutch*). *General and Comparative Endocrinology*, 65, 189-198.

Richmann, N.H., Nishioka, R.S., Young, G. and Bern, H.A. (1987). Effects of cortisol and growth hormone replacement on osmoregulation in hypophysectomized coho salmon (*Oncorhynchus kisutch*). *General and Comparative Endocrinology*, 67, 194-201.

Riordan, J.R.; Rommens, J.M.; Kerem, B-S.; Alon, N.; Rozmahel, R.; Grzelczak, Z.; Zielinski, J.; Lok, S.; Plavsic, N.; Chou, J-L.; Drumm, M.L.; Iannuzzi, M.C.; Collins, F.S. and Tsui, L-C. (1989). Identification of the cystic fibrosis gene: cloning and characterisation of complementary DNA. *Science*, 245, 1066-1072.

Sakamoto, T., McCormick, S.D. and Hirano, T. (1993). Osmoregulatory actions of growth hormone and its mode of action in salmonids: a review. *Fish Physiology and Biochemistry*, 11, 1-6, 155-164.

Sakamoto, T., Sheperd, B.S., Madsen, S.S., Nishioka, R.S., Siharath, K., Richman, N.H., Bern, H.A. and Grau, E.G. (1997). Osmoregulatory actions of growth hormone and prolactin in an advanced teleost. *General and Comparative Endocrinology*, 106, 95-101.

Sardet, C., Pisam, M. and Maetz, J. (1979). The surface epithelium of teleostean fish gills. Cellular and junctional adaptations of the chloride cell in relation to salt adaptation. *Journal of Cell Biology*, 80, 96-117.

Sargent, J.R., Thomson, A.J. and Bornancin, M. (1975). Activities and localisation of succinic dehydrogenaseand Na^+/K^+-activated adenosine triphosphatase in the gills of fresh water and sea water eels (*Anguilla anguilla*). *Comparative Biochemistry and Physiology*, 51B, 75-79.

Sawyer, W.H. (1987). Neurohypophysial hormones and osmoregulatory adaptations. In: (eds, Kirsch, R. and Lahlou, B.) Comparative Physiology of Environmental Adaptations Vol 1., 8th ESPC conference.

Scheving, L.A. and Russell, W.E. (1996). Guanylyl cyclase C is up-regulated by non-parenchymal cells and hepatocytes in regenerating rat liver. *Cancer Research*, 56, 5186-5191.

Scheving, L.A. and Chong, K-M. (1997). Differential processing of guanylyl cyclase C along villus-crypt axis of rat small intestine. *American Journal of* Physiology, 272, C1995-C2004.

Scheving, L.A., Russell, W.E. and Chong, K-M. (1996). Structure, glycosylation and localisation of rat intestinal guanylyl cyclase C: modulation by fasting. *American Journal of Physiology*, 271, G959-G968.

Schofield, J.P., Jones, D.S.C. and Forrest, J.N Jr. (1991). Identification of C-type natriuretic petide in heart of spiny dogfish shark (*Squalus acanthias*). *American Journal of Physiology*, 261, F734-F739.

Schonrock, C., Morley, S.D., Okawara, Y., Lederis, K. and Richter, D. (1991). Sodium and potassium ATPase of the teleost fish *Catostomus commersoni*. Sequence, protein structure and evolutionary conservation of the α-subunit. *Biological Chemistry Hoppe Seyler*, 372, 279-286.

Schulz, S., Green, C.K., Yeun, P.S.T. and Garbers, D.L. (1990). Guanylyl cyclase is a heat-stable enterotoxin receptor. *Cell*, 63, 941-948.

Schulz, S., Chrisman, T.D. and Garbers, D.L. (1992). Cloning and expression of guanylin. Its existence in various mammalian tissues. *The Journal of Biological Chemistry*, 267, 23, 16019-16021.

Sciaky, D., Kosiba, J.L. and Cohen, M.B. (1994). Genomic sequence of the murine guanylin gene. *Genomics*, 24, 583-587.

Shedadeh, Z.H. and Gordon, M.S. (1969). The role of the intestine in salinity adaptation of the rainbow trout, *Salmo gairdneri*. *Comparative Biochemistry and Physiology*, 30, 397-418.

Silva, P., Solomon, R., Spokes, K. and Epstein, F.H. (1977). Ouabain inhibition of gill Na^+, K^+-ATPase: relationship to active chloride transport. *Journal of Experimental Zoology*, 199, 419-426.

Simmoneaux, V., Barra, J.A., Humbert, W. and Kirsch, R. (1987a). The role of mucus in ion absorption by the oesophagus of the sea-water eel (*Anguilla anguilla* L.). *Journal of Comparative Physiology B*, 157, 187-199.

Simmoneaux, V., Humbert, W. and Kirsch, R. (1987b). Mucus and intestinal ion exchanges in the sea-water adapted eel, *Anguilla anguilla* L. *Journal of Comparative Physiology*, 157, 295-306.

Singer, T.D., Tucker, S.J., Marshall, W.S. and Higgins, C.F. (1998). A divergent CFTR homologue: highly regulated salt transport in the euryhaline teleost *F.heteroclitus*. *American Journal of Physiology* (*Cell Physiology* 43), 274, C715-723.

Skadhauge, E. (1969). The mechanism of salt and water absorption in the intestine of the eel (*Anguilla anguilla*) adapted to waters of various salinities. *Journal of Physiology*, 204, 135-158.

Skadhauge, E. (1974). Coupling of transmural flows of NaCl and water in the intestine of the eel (*Anguilla anguilla*). *Journal of Experimental Biology*, 60, 535-546.

Sleet, R.B. and Webber, L. (1982). The rate and manner of seawater ingestion by a marine teleost and corresponding seawater modification by the gut. *Comparative Biochemistry and Physiology*, 72A, 469-475.

Sokabe, H., Oide, H., Ogawa, M. and Utida, S. (1973). Plasma renin activity in Japanese eels (*Anguilla japonica*) adapted to seawater or in dehydration. *General Comparative Endocrinology*, 21, 160-167.

Solomon, R., Protter, A., McEnroe, G., Porter, J.G. and Silva, P. (1992). C-type natriuretic peptide stimulates chloride secretion in the rectal gland of *Squalus acanthias*. *American Journal of Physiology*, 262, R707-R711.

Specker, J.L., King, D.S., Nishioka, R.S., Shiharata, K., Yamaguchi, K. and Bern, H.A. (1985). Isolation and characterisation of two prolactins released *in vitro* by the pituitary of a cichlid fish. *Proceedings of the National Academy of Sciences USA*, 82, 7490-7494.

Sullivan, G.V., Fryer, J.N. and Perry, S.F. (1995). Immunolocalisation of proton pumps (H^+-ATPase) in pavement cells of rainbow trout gill. *Journal of Experimental Biology*, 198, 2619-2629.

Sullivan, G.V., Fryer, J.N. and Perry, S.F. (1996). Localization of mRNA for the proton pump (H^+-ATPase) and Cl^-/HCO_3^- exchanger in the rainbow trout gill. *Canadian Journal of Zoology*, 74, 2095-2103.

Suzuki, R., Takahashi, A., Hazon, N. and Takei, Y. (1991). Isolation of a high-molecular-weight C-type natriuretic peptide from the heart of a cartilaginous fish (European dogfish, *Scyliorhinus canicula*). *FEBS*, 282, 2, 321-325.

Swenson, E.S., Mann, E.A., Jump, M.L., Witte, D.P. and Giannella, R.A. (1996). The guanylin/ST_a receptor is expressed in crypts and apical epithelium throughout the mouse intestine. *Biochemical and Biophysical Research Communications*, 225, 1009-1014.

Swenson, E.S., Mann, E.A., Jump, M.L. and Giannella, R.A. (1999). Hepatocyte nuclear factor-4 regulates intestinal expression of the guanylin/heat-stable enterotoxin receptor. *American Journal of Physiology (Gastrointestinal and Liver Physiology* 39), 276, G728-G736.

Takano, M., Sasayama, Y. and Takei, Y. (1994). Molecular evolution of shark C-type natriuretic peptides. *Zoological Science*, 11, 451-454.

Takei, Y. (1994). Structure and function of natriuretic peptides in vertebrates. *Perspectives in Comparative Endocrinology*, 149-154.

Takei, Y., Hirano, T. and Kobayashi, H. (1979). Angiotensin and water intake in the Japanese eel, *Anguilla japonica*. *General and Comparative Endocrinology*, 38, 466-475.

Takei, Y., Takahashi, A., Watanabe, T.X., Nakajima, K. and Sakakibara, S. (1989). Amino acid sequence and relative biological activity of eel atrial natriuretic peptide. *Biochemical and Biophysical Research Communications*, 164, 1, 537-543.

Takei, Y., Takahashi, A., Watanabe, T.X., Nakajima, K. and Sakakibara, S., Takao, T. and Shimonishi, Y. (1990).Amino acid sequence and relative biological activity of a natriuretic peptide isolated from eel brain. *Biochemical and Biophysical Research Communications*, 170, 883-891.

Takei, Y., Takahashi, A., Watanabe, T.X., Nakajima, K. and Sakakibara, S. (1991). A novel natriuretic peptide isolated from eel cardiac ventricles. *FEBS*, 282, 2, 317-320.

Takei, Y., Ando, K. and Kawakami, M. (1992). Atrial natriuretic peptide in eel plasma, heart and brain characterized by homologous radioimmunoassay. *Journal of Endocrinology*, 135, 325-331.

Takei, Y. and Balment, R.J. (1993). Biochemistry and physiology of a family of eel natriuretic peptides. *Fish Physiology and Biochemistry*, 11, 183-188.

Tesch, F.W. (1977). The Eel (First edition). Chapman Hall, London.

Thomson, A.J. and Sargent, J.R. (1977). Changes in the levels of chloride cells and ($Na^+ + K^+$)-dependent ATPase in the gills of yellow and silver eels adapting to seawater. *Journal of Experimental Zoology*, 200, 33-40.

Tierney, M.L., Luke, G., Cramb, G. and Hazon, N. (1995a). The role of the renin-angiotensin system in the control of blood pressure and drinking in the European eel, *Anguilla anguilla*. *General and Comparative Endocrinology*, 100, 39-48.

Tierney, M.L., Cramb, G. and Hazon, N. (1995b). Stimulation of the renin-angiotensin system and drinking by papverine in the seawater eel. *Journal of Fish Biology*, 46, 721-724.

Trischitta, F., Denaro, M.G., Faggio, C. and Schettino, T. (1992). Comparison of Cl^- absorption in the intestine of the seawater- and freshwater-adapted eel, *Anguilla anguilla*: evidence for the presence of an Na-K-Cl cotransport system on the luminal membrane of the enterocyte. *The Journal of Experimental Zoology*, 263, 245-253.

Trischitta, F., Denaro, M.G., Faggio, C., Mandolfino, M. and Schettino, T. (1996). Different effects of cGMP and cAMP in the intestine of the European eel, *Anguilla anguilla*. *Journal of Comparative Physiology B*, 166, 30-36.

Tsuchida, T. and Takei, Y. (1998). Effects of homologous atrial natriuretic peptide on drinking and plasma ANG II level in eels. *American Journal of Physiology*, 44, 5, R1605-R1610.

Utida, S., Kamiya, M. and Shirai, N. (1971). Relationship between the activity of Na^+-K^+-activated adenosine triphosphatase and the number of chloride cells in eel gills with special reference to sea-water adaptation. *Comparative Biochemistry and Physiology A*, 38, 443-447.

Utida, S., Hirano, T., Oide, H. and Ando, M. (1972). Hormonal control of the intestine and urinary bladder in teleost osmoregulation. *General and Comparative Endocrinology* supplement 3, 317-327.

Utida, S., Kamiya, M., Johnson, D. and Bern, H.A. (1974). Effects of freshwater adaptation and of prolactin on sodium-potassium-activated adenosine triphosphatase activity in the urinary bladder of two flounder species. *Journal of Endocrinology*, 62, 11-14.

Vaandrager, A.B., van der Weil, E. and De Jonge, H.R. (1993). Heat-stable enterotoxin activation of immunopurified guanylyl cyclase C. *The Journal of Biological Chemistry*, 268, 26, 19598-19603.

Vaandrager, A.B., van der Weil, E., Hom, M.L., Luthjens, L.H. and De Jonge, H.R. (1994). Heat-stable enterotoxin receptor/guanylyl cyclase C is an oligomer consisting of functionally distinct subunits which are non-covalently linked in the intestine.*The Journal of Biological Chemistry*, 269, 23, 16409-16415.

Wada, A., Hirayama, T., Kitao, S., Fujisawa, J-I., Hidaka, Y. and Shimonishi, Y. (1994). Pig intestinal membrane-bound receptor (Guanylyl Cyclase) for heat-stable enterotoxin: cDNA cloning, functional expression and characterization. *Microbiological Immunology*, 38, 7, 535-541.

Warne, J.M. and Balment, R.J. (1997). Vascular actions of neurohypophysial peptides in the flounder. *Fish Physiology and Biochemistry*, 17, 313-318.

Wedel, B.J. and Garbers, D.L. (1997). New insights on the function of the guanylyl cyclase receptors. *FEBS Letters*, 410, 29-33.

Wedel, B.J. and Garbers, D.L. (1998). Guanylyly cyclases: approaching year thirty. *Trends in Endocrinology and Metabolism*, 9, 5, 213-219.

Wendelaar Bonga, S.E. (1997). The stress response in fish. *Physiological Reviews*, 77, 3, 591-623.

Wiegand, R.C., Kato, J. and Currie, M.G. (1992a). Rat guanylin cDNA: characterization of the precursor of an endogenous activator of intestinal guanylate cyclase. *Biochemical and Biophysical Research Communications*, 185, 3, 812-817.

Wiegand, R.C., Kato, J., Huang, M.D., Fok, K.F., Kachur, J.F. and Currie, M.G. (1992b). Human guanylin: cDNA isolation, structure and activity. *FEBS*, 311, 2, 150-154.

Withers, P.C. (1992). Comparative Animal Physiology (International edition). Saunders College Publishing, London.

Wilson, R.W., Gilmour, K.M., Henry, R.P. and Wood, C.M. (1996). Intestinal base excretion in the seawater-adapted rainbow trout: a role in acid-base balance? *The Journal of Experimental Biology*, 199, 2331-2343.

Yamamoto, M. and Hirano, T. (1978). Morphological changes in the esophageal epithelium of the eel, *Anguilla japonica*, during adaptation to seawater. *Cell and Tissue Research*, 192, 25-38.

Young, J.Z. (1992). The Life of Vertebrates (third edition). Clarendon Press, Oxford.

Zadunaisky, J.A. (1984). The chloride cell: the active transport of chloride and the paracellular pathways. In: Gills Part B, *Fish Physiology,* Vol X, (eds, Hoar, W.S. and Randall, D.J.).

Zadunaisky, J.A. (1997). Gill chloride cells activation by plasma osmolarity. In: Ionic regulation in animals: a tribute to Professor W.T.W. Potts (eds, Hazon, N., Eddy, F.B. and Flik, G.

Zadunaisky, J.A., Cardona, S., Au, L., Roberts, D.M., Fisher, E., Lowenstein, B., Cragoe, E.J. and Spring, K.R. (1995). Chloride transport activation by plasma osmolarity during rapid adaptation to high salinity of *Fundulus heteroclitus*. *Journal of Membrane Biology*, 143, 207-217.

Zhang, Z.H., Jow, F., Numann, R. and Hinson, J. (1998). The airway epithelium: a novel site of action by guanylin. *Biochemical and Biophysical Research Communications*, 244, 50-56.

Appendices

Appendix 1-suppliers of chemicals and equipment

BDH Laboratory services, Poole, Dorset, UK.

Sigma Aldririch company Ltd., Poole, Dorset, UK.

Gibco BRL Life Technologies (UK), Paisley, UK.

Promega corporation UK, Southampton, UK.

Amersham Pharmacia Biotech Ltd., Little Chalfont, Buckinghamshire, UK.

Invitrogen, Groningen, The Netherlands.

BIO 101 Inc., Vista, California, USA.

Vh Bio Ltd., Gosforth, Newcastle-upon-Tyne, UK.

Beckman Instruments Inc., Palo Alto, California, USA.

Severn Biotech Ltd., Kidderminster, UK.

Qiagen Ltd. (UK), Crawley, West Sussex, UK.

Fluka Chemicals, Gillingham, Dorset, UK.

Heraus Christ, Osterode, Germany.

Philips, Pye Unicam Ltd., Cambridge, UK.

Dynex Technologies, Billinshurst, West Sussex, UK.

Kinematica, Lucerne, Switzerland.

The Boots Company PLC, Nottingham, UK.

MWG Biotech AG, Ebersberg, Germany.

Biogene Ltd., Kimbolton, Cambridgeshire, UK.

Ambion Inc., suppliers AMS Biotechnology, Abingdon, Oxfordshire, UK.

Techne Ltd., Cambridge, UK.

Herolab GMBH Laboratory, Weisloch, Germany.

IBI, Newhaven, Connecticut, USA.

Eastman Kodak Co., Rochester, New York, USA.

Biorad Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK.

Scotlab, Carluke, UK.

Hoefer Scientific Instruments, San Francisco, California, USA.

Anachem Ltd., Luton, Bedfordshire, UK.

Packard Instruments, Pangbourne, Berkshire, UK.

PE Applied Biosystems, Warrington, UK.

Stratagene,

New England Biolabs Ltd.,

Appendix 2- primer sequences

Degenerate guanylin primers

Guanylin sense 5′-GGI AAI CTI TCI TTC ACI CTI GAA TCI GT-3′
G T T C T G
T

Guanylin anti 5′-GCI GCA AAI GCA CAI ATC TCA CA-3′
G T G T G

Guanylin 3′ RACE primers- gene specific primers (GSP)

3′ RACE 1 (sense) 5′- CAC ATG CCG TTT GTT CCA AC-3′

3′ RACE 2 (sense) 5′-CAA CAG GCT GGT GGA CAT AA-3′

3′ RACE anti (sequencing primer) 5′-GGA AAA CTC ATC TGT CTT GAG-3′
GC T

Guanylin 5′ RACE primer- gene specific primer (GSP)

Guanylin 5′ RACE 1 (anti) 5′-GGA ACA AAC GGC ATG TGA GC-3′

Clontech Marathon™ kit primers

Marathon cDNA synthesis primer 5′-TTC TAG AAT TCA GCG GCC $GC(T)_{30}NN$-3′

Adaptor primer 1 (AP1) 5′-CCA TCC TAA TAC GAC TCA CTA TAG GGC-3′

Adaptor primer 2 (AP2) 5′-ACT CAC TAT AGG GCT CGA GCG GC-3′

SMART™ oligo 5′-AAG CAG TGG TAA CAA CGC AGA GTA CGC GGG-3′

Degenerate GC-C primers

GC-C sense 5′-GTT TGA CCA AAA AAA CGA AAC ITA CAT GGA CAC-3′
T G G T GTG C T T

GC-C anti 5′-GTA TTI ACI GTA TCI CCA AAI AAA CAA TA-3′
G C C G C G C GG G

GC-C4 sense 5′-CTI AGI CAA AAA AAA TGG TCI CAC ATA CC-3′
T C C C G G G C T C
T

GC-C6 sense 5′-GC<u>I</u> GG<u>I</u> AG<u>C</u> TT<u>C</u> GG<u>I</u> <u>CCI</u> TC<u>I</u> TG<u>C</u> GA<u>C</u> <u>C</u>A-3′
C C T T C TTC C T T T

GC-C(2) specific anti 5′-GTC CTT CCT CTG GTC CTC A-3′

GC-C4 sequencing sense 5′-CCT ACC TCC ACC TGA GC-3′

GC-C4 sequencing anti 5′-GCA ACA TGA AGT TGA GCT GG-3′

Guanylin mRNA expression in all tests groups

		RNA raw vol.	CF	Density	Adjusted density		ST DEV
YFW Intestine	1	113850	1.66	55730	92512		
	2	113574	1.7	139498	237147		
	3	100466	1.88	45759	86027		
	4	107901	1.75	22061	38607		
	5	152061	1.24	31312	38827		
	6	115006	1.65	75356	124337	mean= 102910	73639
YSW Intestine	1	116270	1.63	16798	27380		
	2	116692	1.62	232243	376234		
	3	94117	2.01	39482	79359		
	4	108800	1.74	331907	577518		
	5	124582	1.52	107383	163222		
	6	110578	1.71	374258	639981	mean= 310615	260558
SFW Intestine	1	157533	1.2	87282	104738		
	2	168961	1.12	37991	42550		
	3	173955	1.08	14973	16171		
	4	164028	1.15	10182	11709		
	5	*189270	1*	78285	78285		
	6	158848	1.19	32715	38931	mean= 48730	36264
SSW Intestine	1	144297	1.31	97395	127587		
	2	131671	1.44	291134	419233		
	3	137184	1.38	120613	166446		
	4	103914	1.82	274870	500263		
	5	114817	1.65	164969	272199		
	6	110899	1.71	343248	586954	mean= 345414	185800
YFW Kidney	1	77588	3.13	12869	40280		
	2	65258	3.72	33669	125249		
	3	92979	2.61	7762	20259		
	4	102972	2.36	28547	67371		
	5	116292	2.08	8835	18377		
	6	135565	1.8	8349	15028	mean= 47754	42746
YSW Kidney	1	122667	1.98	2977	5894		
	2	109809	2.21	1475	3260		
	3	149322	1.62	26913	43599		
	4	181126	1.34	45290	60689		
	5	212458	1.14	26011	29653		
	6	114291	2.12	25595	54261	mean= 32893	24337
SFW Kidney	1	105636	2.3	21868	50296		
	2	123184	1.97	69026	135981		
	3	105974	2.3	21129	48597		
	4	87439	2.78	11256	31292		
	5	94348	2.57	85612	220023		
	6	91180	2.66	8727	23214	mean= 84900	77539
SSW Kidney	1	*242760	1*	30596	30596		
	2	230102	1.05	30948	32495		
	3	191307	1.27	51495	65399		
	4	192745	1.26	42599	53674		
	5	169825	1.43	83359	119203		
	6	155528	1.56	47738	74471	mean= 62640	32750

GC-C mRNA expression in all tests groups

		RNA raw vol.	CF	Radioactive counts	Adjusted counts		ST DEV
YFW Intestine	1	113850	1.66	18295	30369		
	2	113574	1.7	20083	34141		
	3	100466	1.88	18508	33949		
	4	107901	1.75	21032	36806		
	5	152061	1.24	46860	58106		
	6	115006	1.65	34848	57499	mean=41812	12555
YSW Intestine	1	116270	1.63	41467	67591		
	2	116692	1.62	69068	111890		
	3	94117	2.01	24998	50246		
	4	108800	1.74	72122	125492		
	5	124582	1.52	65683	99838		
	6	110578	1.71	26203	44807	mean= 83327	33740
SFW Intestine	1	157533	1.2	53200	63840		
	2	168961	1.12	60968	67982		
	3	173955	1.1	49233	54156		
	4	164028	1.15	60325	69374		
	5	*189270	1*	78570	78570		
	6	158848	1.19	69527	82737	mean= 69610	102(
SSW Intestine	1	144297	1.31	48213	63159		
	2	131671	1.44	58197	83804		
	3	137184	1.38	56992	78650		
	4	103914	1.82	48757	88738		
	5	114817	1.65	31442	51880		
	6	110899	1.71	79961	136733	mean= 83827	19272
YFW Kidney	1	77588	3.13	1869	5850		
	2	65258	3.72	7133	26535		
	3	92979	2.61	2223	5802		
	4	102972	2.36	3913	9235		
	5	116292	2.08	3021	6284		
	6	135565	1.8	1546	2783		
YSW Kidney	1	122667	1.98	0	0		
	2	109809	2.21	0	0		
	3	149322	1.62	175	284		
	4	181126	1.34	0	0		
	5	212458	1.14	2098	2392		
	6	114291	2.12	5066	10740		
SFW Kidney	1	105636	2.3	102	235		
	2	123184	1.97	155	305		
	3	105974	2.3	976	2245		
	4	87439	2.78	426	1184		
	5	94348	2.57	1267	3256		
	6	91180	2.66	987	2625		
SSW Kidney	1	*242760	1*	2409	2409		
	2	230102	1.05	2921	3067		
	3	191307	1.27	2134	2710		
	4	192745	1.26	1643	2070		
	5	169825	1.43	1373	1963		
	6	155528	1.56	1995	3112		

ANOVA Table	Guanylin mRNA expression levels in the intestine				
	DF	Sum of Squares	Mean Square	F-value	P-value
Intestine	3	394108791048.8	131369597016.2	4.814	0.011
Residual	20	545747330636	27287366531.8		

Fisher's Test for intestinal data	Mean Difference	Critical Difference	P-value
YFW, YSW	-207706.2	198942.183	0.0416
YFW, SFW	54178.9	198942.183	0.5763
YFW, SSW	-242537.5	198942.183	0.0194
YSW, SFW	261885.0	198942.183	0.0125
YSW, SSW	-34831.3	198942.183	0.7188
SFW, SSW	-296716	198942.183	0.005

ANOVA Table	Guanylin mRNA expression levels in kidney				
	DF	Sum of Squares	Mean square	F-value	P-value
Kidney	3	8860579084.1	2953526361.4	1.243	0.325
Residual	20	47522226419.5	2376111320.9		

Statistical analysis of guanylin mRNA expression levels in the kidney and intestine of all test groups. One way analysis of variance (ANOVA) was used, followed by a Fisher test if the ANOVA demonstrated a statistically significant difference between the test groups, and carried out using the StatView computer programme.

ANOVA Table	GC-C(2) Expression levels in the intestine				
	DF	Sum of Squares	Mean square	F-value	P-value
Intestine	3	6975692685.1	2325230895.1	4.114	0.02
Residual	20	11305357542.8	565267877.1		

Fisher's test for intestinal data	Mean difference	Critical difference	P-value
YFW, YSW	-41499.0	28633.4	0.0067
YFW, SFW	-27631.5	28633.4	0.0578
YFW, SSW	-42015.7	28633.4	0.0062
YSW, SFW	13867.5	28633.4	0.3244
YSW, SSW	-516.7	28633.4	0.9703
SFW, SSW	-14384.2	28633.4	0.3072

Statistical analysis of GC-C(2) mRNA expression in the intestine of all test groups. One way analysis of variance (ANOVA) was carried out, followed by a Fisher test, using the StatView computer programme.

Primers for DD-PCR

Primers for cDNA synthesis:

DD1 5′-GTTGCGGCCGCTTTTTTTTTTG-3′

DD2 5′-GTTGCGGCCGCTTTTTTTTTTC-3′

DD3 5′-GTTGCGGCCGCTTTTTTTTTTA-3′

Arbitrary primers for PCR: supplied with Clontech Delta™ Fingerprinting Kit

5′-ATTAACCCTCACTAAATGCTGGGGA-3′

5′-ATTAACCCTCACTAAATCGGTCATAG-3′

5′-ATTAACCCTCACTAAATGCTGGTGG-3′

5′-ATTAACCCTCACTAAATGCTGGTAG-3′

5′-ATTAACCCTCACTAAAGATCTGACTG-3′

5′-ATTAACCCTCACTAAATGCTGGGTG-3′