Isolation and Developmental Expression of Growth Hormone-Releasing Hormone (GRF), Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) and their Receptors in the Zebrafish, *Danio rerio*

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We accept this dissertation as conforming to the required standard

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ABSTRACT

The growth and development of an organism requires the coordinated actions of many factors. During development individual cells undergo proliferation, migration and differentiation to form the adult organism. Two structurally related members of the glucagon superfamily, growth hormone-releasing hormone (GRF) and pituitary adenylate cyclase-activating polypeptide (PACAP), are thought to modulate vertebrate development. In mammals, GRF modulates the development of pituitary somatotrophs and the release of fetal growth hormone. In contrast, PACAP appears to have a more general role during development. PACAP may be involved in the patterning of the embryonic axis and in the development of the neural tube. The objectives of my study were to isolate GRF, PACAP and their receptors from the zebrafish, characterize their expression in the developing embryo and adult embryo and examine the role of PACAP during brain development.

To study the role of GRF and PACAP, I isolated a genomic clone encoding the GRF and PACAP peptides from the zebrafish genomic library and characterized its gene copy number and adult tissue expression pattern. The GRF-PACAP gene isolated from the zebrafish was comprised of five exons with the GRF peptide encoded on the fourth exon and the PACAP peptide encoded on the fifth exon. This gene structure is similar to that found in other non-mammalian vertebrates and supports the hypothesis that the gene duplication leading to the encoding of the GRF and PACAP peptides on separate genes occurred later in evolution. In addition, the zebrafish genome was found to contain only one copy of

the GRF-PACAP gene. The GRF-PACAP gene was widely expressed in the adult zebrafish in tissues developmentally derived from all three germ layers, suggesting that the gene may be widely expressed in the embryo as well.

To examine the functional significance of the co-expression of GRF and PACAP in zebrafish, I isolated the GRF and PACAP receptors and characterized their expression pattern. I isolated three distinct cDNAs from zebrafish encoding the GRF receptor, the PACAP specific PAC_1 receptor and the shared vasoactive intestinal peptide/PACAP receptor $VPAC_1$. In addition, four isoforms of the PAC_1 receptor were isolated from zebrafish including a novel isoform found in the gill. All three receptors were widely expressed in adult zebrafish and receptors for both GRF and PACAP were found in most tissues. This indicates that GRF and PACAP may modulate each other's function.

To determine the developmental role of GRF and PACAP, I characterized the expression pattern of the GRF-PACAP gene and the GRF, PAC_1 and $VPAC_1$ receptors in the zebrafish embryo. The GRF and PAC_1 receptors are the earliest to be expressed in development starting at the cleavage stage. Later, the GRF-PACAP gene and the $VPAC_1$ receptor are first expressed at the late blastula/early gastrula stage in the zebrafish and are expressed throughout the developmental period. Strong expression of the GRF, PACAP and their receptors during mid gastrulation indicates that these peptides may be involved in modulating the formation of the embryonic axis. During the segmentation period the GRF-PACAP gene is widely expressed in the zebrafish embryo and the PAC_1 receptor short and hop isoforms are differentially expressed. Therefore, PACAP may regulate cell cycle exit or cell

proliferation through activation of different PAC_1 receptor isoforms during the segmentation stage. In the subsequent pharyngula period, the GRF-PACAP transcript is localized mainly to the hatching gland. However, expression is seen also in tissues that undergo differentiation during this stage. Therefore, the timing of the expression of the GRF-PACAP gene indicates that it may be involved in early patterning events and promoting cell cycle exit prior to differentiation. To investigate the role of GRF and PACAP in the developing brain, I localized the expression of GRF, PACAP and the PAC_1 receptor in neuroblasts derived from an embryonic day 3.5 chick. PACAP was found to stimulate the cAMP pathway in these cells, indicating that PACAP may modulate brain development. This work indicates that GRF and PACAP play an important role in vertebrate development.

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LIST OF ABREVIATIONS

bp: base pairs

cAMP: cyclic adenosine monophosphate

cDNA: complementary deoxyribonucleic acid

Da: Daltons

DTT: dithiotreitol

E: embryonic day

GI: gastrointestinal tract

GIP: glucose dependent insulinotropic peptide

GLP: glucagon like peptide

GRF: growth hormone-releasing hormone

mRNA: messenger ribonucleic acid

PAC_1: pituitary adenylate cyclase-activating polypeptide type 1 receptor

PACAP: pituitary adenylate cyclase-activating polypeptide

PHI: peptide histidine isoleucine

PHM: peptide histidine methionine

PCR: polymerase chain reaction

RT: reverse transcriptase

SDS: sodium dodecyl sulfate

SP: signal peptide

SSC: saline sodium citrate

TBST: Tris buffered saline with 0.1% tween-20

VIP: vasoactive intestinal peptide

VPAC: pituitary adenylate cyclase-activating polypeptide type 2 receptor

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I would like to dedicate this thesis

with love to my brother Robert

who has always believed in my abilities.

CHAPTER 1

GENERAL INTRODUCTION

Growth and development are complex processes that require the coordinated activity of many factors throughout the life of an organism. Early studies by developmental biologists focused on embryonic induction showing that neighboring cells and tissues were important for stimulating the development of a tissue. Evidence that genes influenced embryonic induction came from studies by Levi-Montalcini and Hamburger. They were the first to show that proteins called growth factors were involved in embryonic development. These researchers found that nerve growth factor promoted neurite outgrowth from spinal and sympathetic ganglia in the chick (Levi-Montalcini et al. 1954). Further evidence for the role of genes during development came from mutant studies in the *Drosophila*. Therefore, understanding gene expression is key to elucidating the developmental mechanism.

All vertebrates undergo a similar developmental mechanism and in particular, early developmental processes are highly conserved. During embryonic development, cells undergo a period of rapid proliferation (cleavage) followed by a period of cell migration that results in the formation of the three germ layers, the endoderm, the ectoderm and the mesoderm (gastrulation). Once the germ layers are formed, further proliferation, migration and differentiation results in the formation of specific tissues and organs during the segmentation and pharyngula periods. The ectoderm layer envelops the embryo and gives rise to the epidermis, nerve and glial cells of the central and peripheral nervous systems, and some connective tissues. The mesoderm gives rise to the muscle, bone, connective tissue, dermis, urogenital system, heart and lung. The endoderm layer forms the gut and associate glands, including the esophagus, stomach, intestines, liver, pancreas and gallbladder. Postembryonic development involves further

stages of proliferation and differentiation to form the adult organism. After birth an organism must significantly increase in size through skeletal-muscular growth and metabolic changes; gonad tissues must differentiate to become reproductively mature; and the central and peripheral nervous systems continue to develop.

How an individual cell acquires its phenotype during development is influenced by internal and external cues. Internal cues include the particular gene expression pattern in an individual cell, whereas a cell's environment is influenced by the expression of genes in neighboring cells encoding specific structural or chemical cues, such as growth factors or hormones. Therefore, important questions in developmental biology are what genes are expressed, where are they being expressed and at what time are they being expressed during development. Recently, it has been found that hormones that function in the adult organism are also being expressed in the embryo. In particular, researchers have shown that members of the glucagon superfamily are expressed in the developing embryo where their functions are poorly understood (Sherwood et al. 2000).

The Glucagon Superfamily

The glucagon superfamily is comprised of hormones that are involved in the regulation of development, growth and metabolism. In humans there are nine members of the superfamily: pituitary adenylate cyclase-activating polypeptide (PACAP), growth hormone-releasing factor (GRF), vasoactive intestinal peptide (VIP), peptide histidine methionine (PHM), glucagon, glucagon-like peptide-1 (GLP-1), glucagon-like peptide–2 (GLP-2), secretin and glucose-dependent insulinotropic polypeptide (GIP). In vertebrates other than humans, peptide histidine isoleucine (PHI) is found in place of PHM

(Campbell and Scanes 1992). In mammals six structurally related genes encode the nine members of the superfamily (Fig. 1.1). All of the genes encode a precursor with a signal peptide, cryptic peptide and one to three bioactive peptides. The genes for PACAP, GRF, GIP and secretin encode a single bioactive peptide, whereas the genes for vasoactive intestinal peptide and glucagon encode two or three bioactive peptides respectively (Fig. 1.1).

The glucagon superfamily is thought to have arisen by exon and gene duplication events. A hypothetical model for the evolution of the glucagon superfamily is presented in Figure 1.2. In an ancestral invertebrate prior to the protochordate lineage, an exon duplication is hypothesized to have taken place followed by a gene duplication to form the ancestral PACAP and glucagon genes. The glucagon gene is hypothesized to have undergone two separate duplication events leading to the secretin and glucose-dependent insulinotropic peptide genes. Although the timing of these duplication events is not known, we can speculate that the duplication leading to the secretin gene occurred prior to the divergence of the fish, because secretin-like immunoreactivity has been noted in bony and cartilaginous fish (Sherwood et al. 2000). GIP has only been isolated in mammals, but it shares moderate sequence identity with glucagon. Therefore the duplication leading to this gene likely occurred in an ancestral tetrapod, if not earlier in evolution. In the PACAP gene lineage, it appears that a second duplication in an ancestral tunicate led to the presence of two PACAP genes (McRory and Sherwood 1997). The second gene is hypothesized to be the precursor to the VIP gene found in all vertebrates. A more recent duplication of the PACAP gene prior to the divergence of the mammals led to the presence of distinct GRF and PACAP genes.

Figure 1.1 Schematic representation of the genes for the nine members of the glucagon superfamily in humans. Exons are shown as boxes and introns as lines. Shaded boxes represent exons encoding a bioactive peptide. Abbreviations are SP, signal peptide; PRP, PACAP-related peptide (Modified from Sherwood et al. 2000).

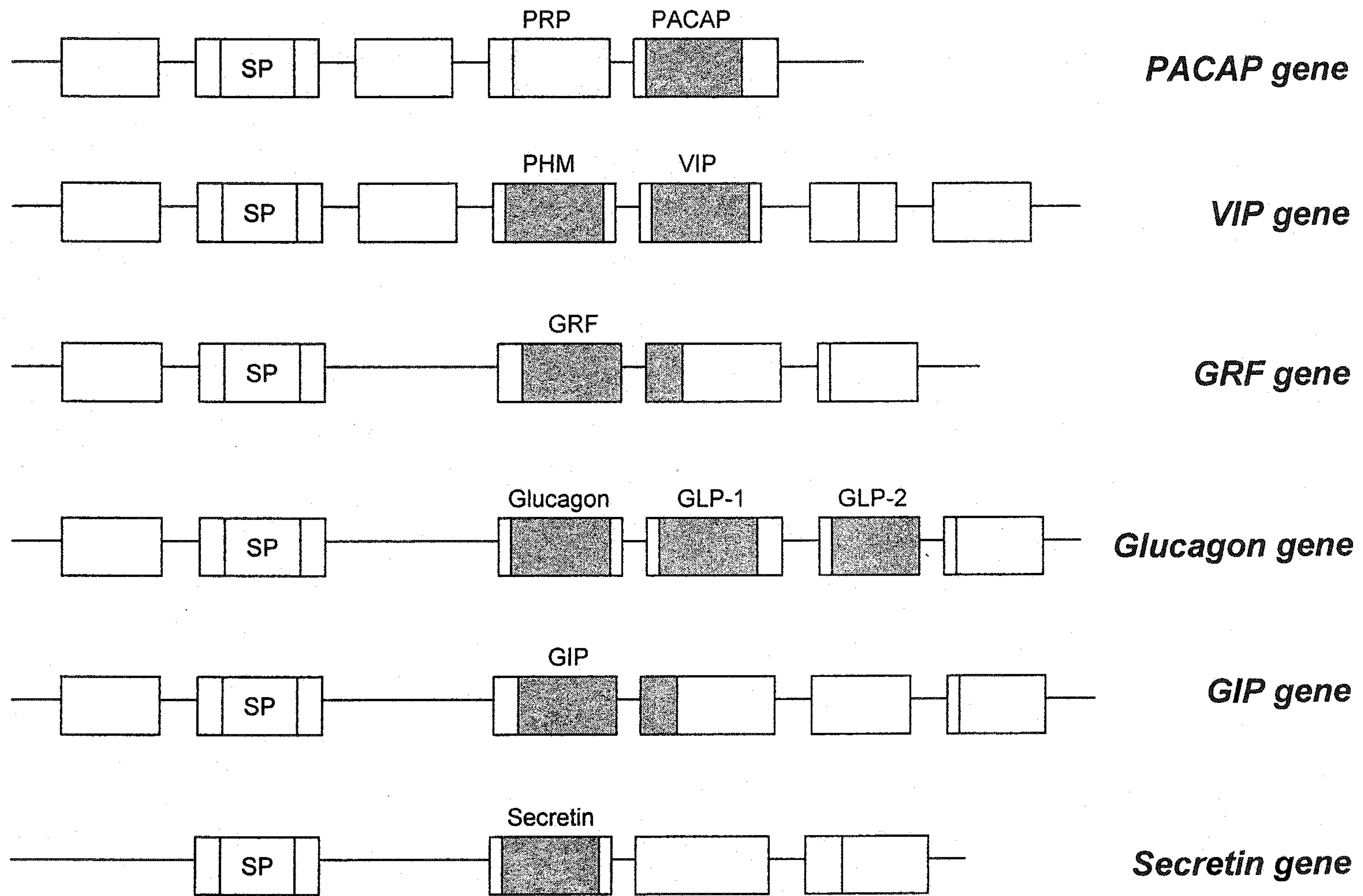
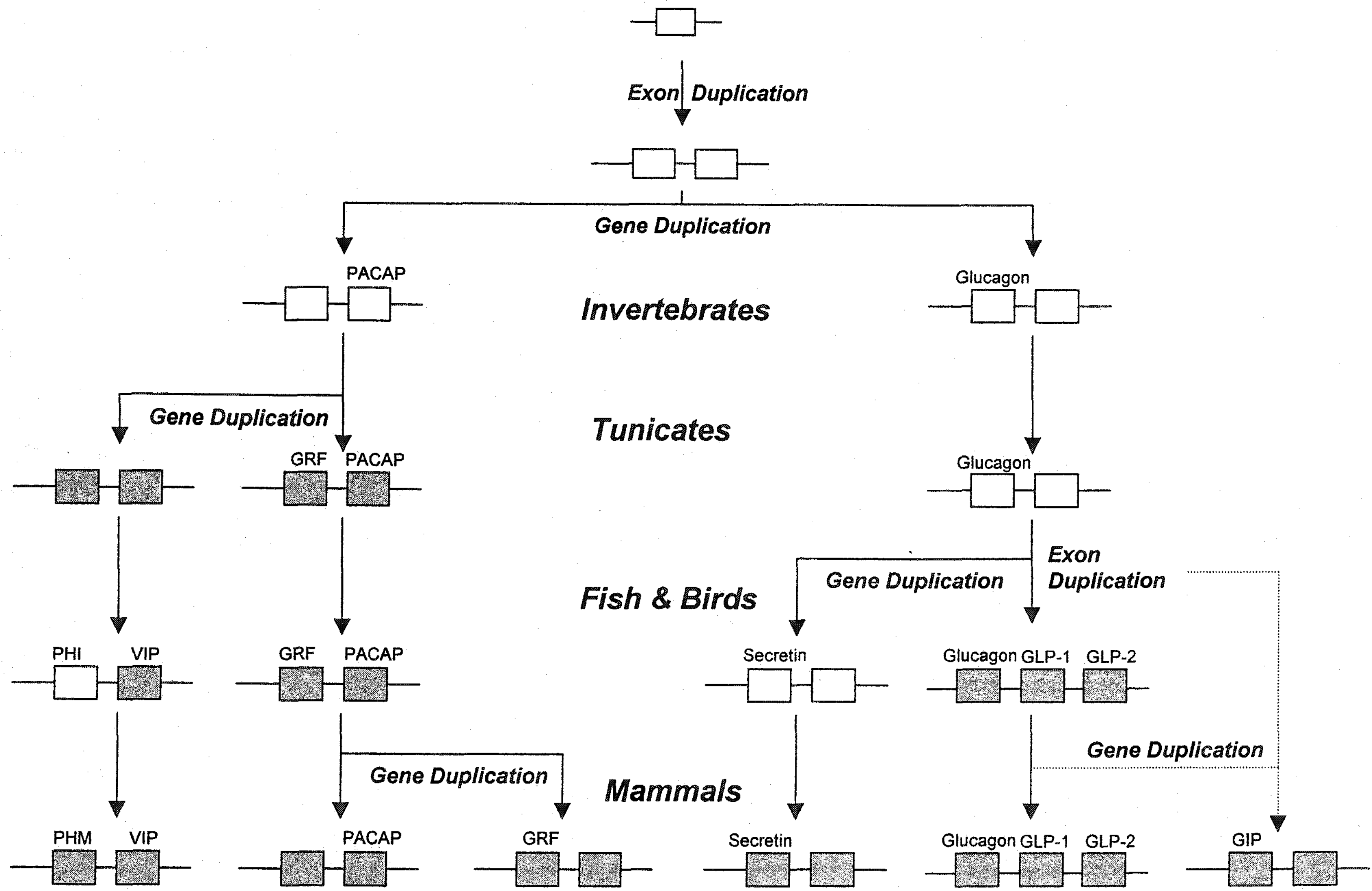

PRP
PACAP
SP
PACAP gene
PHM
VIP
SP
VIP gene
GRF
SP
GRF gene
Glucagon
GLP-1
GLP-2
SP
Glucagon gene
GIP
SP
GIP gene
Secretin
SP
Secretin gene

Figure 1.2 Hypothetical model for the evolution of the glucagon superfamily. The model is based on available data for the presence of members of the superfamily in representatives of each class of animal and on sequence identities between peptide members. Dark gray boxes represent genes where the cDNA or gene is known for a representative of that class and light gray boxes indicate where immunoreactivity for the peptide is found (Modified from Sherwood et al. 2000).

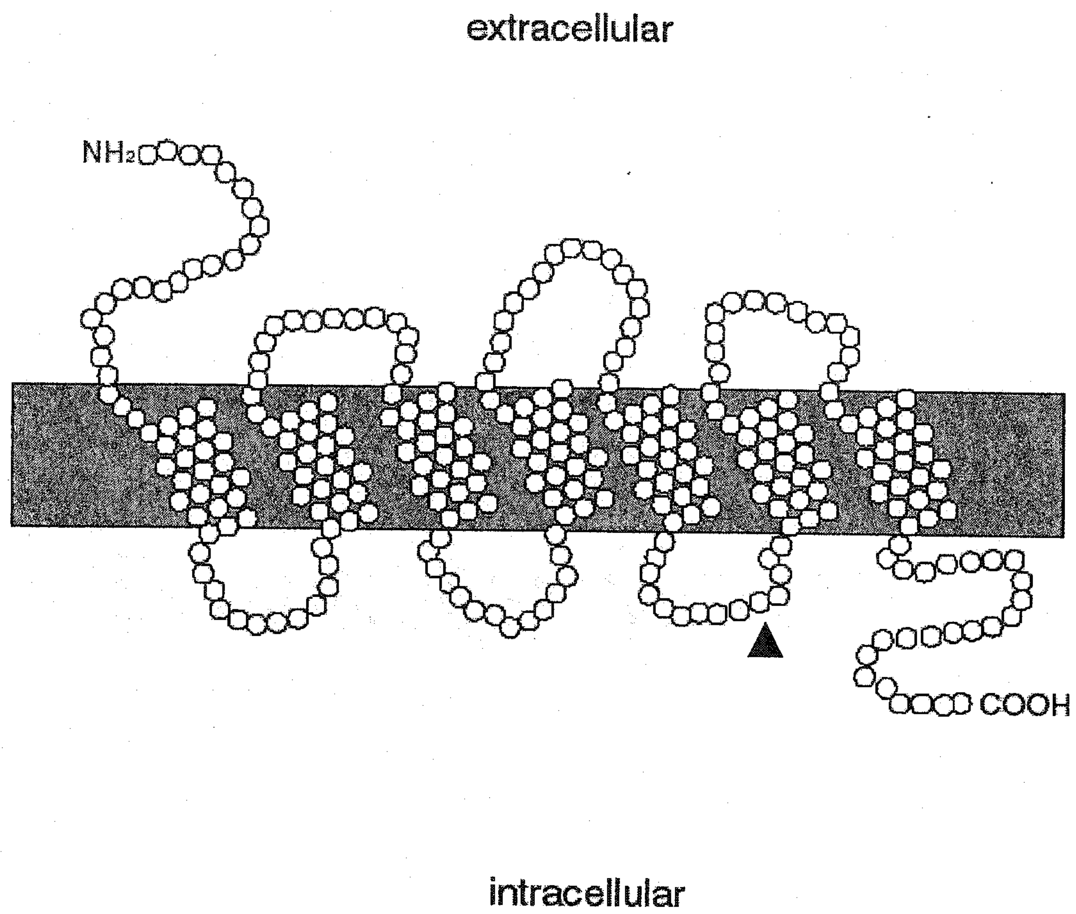

Exon Duplication
Gene Duplication
PACAP
Glucagon
Invertebrates
Gene Duplication
Tunicates
GRF
PACAP
Glucagon
Exon
Duplication
Gene Duplication
Fish & Birds
PHI
VIP
GRF
PACAP
Secretin
Glucagon
GLP-1
GLP-2
Gene Duplication
Gene Duplication
Mammals
PHM
VIP
PACAP
GRF
Secretin
Glucagon
GLP-1
GLP-2
GIP

Our laboratory hypothesizes that PACAP is the ancestral molecule from which the remainder of the family arose. This is based on the strong conservation of the PACAP peptide, 96% between tunicate and human, and that it is the only member of the superfamily to be isolated from a non-vertebrate (McRory and Sherwood 1997). The strong conservation of the PACAP peptide indicates that it may have an important function throughout evolution. Additionally, the co-localization of GRF and PACAP on the same gene in non-mammalian vertebrates indicates that these two peptides must be considered together. The physiological actions of GRF and PACAP are mediated by activation of four related 7 transmembrane G protein-coupled receptors (Fig. 1.3). The receptors for all members of the glucagon superfamily belong to Family B-III of the G protein-coupled receptor superfamily family. Family B-III is characterized by long N-terminal domains and the presence of six conserved cysteine residues putatively involved in ligand binding. In addition, receptors in this family do not exhibit the fingerprint residues of the rhodopsin/β-adrenergic receptor family (Ulloa-Aguirre and Conn 2000).

Growth hormone-releasing hormone (GRF)

GRF was first isolated from a human pancreatic tumour that caused acromegaly based on its ability to stimulate growth hormone release (Guillemin et al. 1982; Rivier et al. 1982). This peptide was later found to be identical to the human hypothalamic form of GRF (Spiess et al. 1983). The GRF peptide is 40-46 amino acids in length depending on the species. In humans two forms of GRF were secreted from the hypothalamus, $GRF_{1-44}NH_2$ and $GRF_{1-40}OH$. The variability in peptide length is possible since only the

Figure 1.3 Schematic representation of a 7 transmembrane G-protein coupled receptor. Arrowhead indicates the region in the third intracellular loop that is involved in G protein interaction and where alternative splicing can result in the addition of a cassette to generate different receptor isoforms (Modified from Ulloa-Aguirre and Conn 2000).


extracellular
NH2
COOH
intracellular

first 29 amino acids are required for biological activity (Frohman et al. 1989). Although the GRF peptide is not highly conserved, the first 29 amino acids display a higher degree of conservation across species than the full-length molecule, possibly due to functional constraints.

GRF is mainly synthesized in the neuronal perikarya of the hypothalamic arcuate nucleus in mammals (Bloch et al. 1983; Ishikawa et al. 1986; Sasaki et al. 1994). In the primate GRF immunoreactive nerve fibers extend from the hypothalamus to the portal vessels, which, along with other evidence, indicates that GRF is a hypophysiotropic factor (Bloch et al. 1983). GRF-like immunoreactivity has also been seen in the hypothalamo-hypophyseal system of amphibians (Marivoet et al. 1998) and fish (Olivereau et al. 1990). In addition to the brain, GRF is produced in a number of peripheral tissues including the testis (Berry and Pescovitz 1988), ovary (Moretti et al. 1990), gastrointestinal tract (Bruhn et al. 1985) and pancreas (Bosman et al. 1984) where it may act as a paracrine factor.

The mRNA transcripts for GRF in the testis and placenta have distinct 5'untranslated regions. In the placenta, the GRF transcript is generated by an alternative promoter approximately 10.7 kilobase pairs upstream from the hypothalamus promoter and the transcript contains an alternative placental exon 1 (Gonzalez-Crespo and Boronat 1991). In the testis, three different transcripts were observed. The different transcripts were generated by use of an alternative promoter approximately 700 basepairs upstream of the placental promoter and by alternative splicing of three possible first exons (Srivastava et al. 1995).

The GRF receptor

The physiological actions of GRF are mediated through a GRF-specific receptor. The cDNA for the GRF receptor has been isolated from human (Lin et al. 1992), rat (Mayo 1992), mouse (Gaylinn et al. 1993), pig (Hsiung et al. 1993), sheep (Horikawa et al. 2001) and goldfish (Wong et al. 1999). The cDNA encodes a protein with seven transmembrane spanning motifs and a G protein recognition site. The GRF receptor is structurally related to the receptors of other members of the glucagon superfamily and belongs to family B-III of the G protein-coupled receptor superfamily (Mayo et al. 2000). In addition to the cDNA, the gene for the GRF receptor was characterized from the rat. The gene is comprised of 14 exons spanning approximately 15 kilobases of genomic DNA (Miller et al. 1999). The GRF receptor has only been isolated from one non-mammalian species, the goldfish. In this fish, the mRNA transcript encodes a protein that shares 43% amino acid sequence identity with the human GRF receptor (Chan et al. 1998).

Several variants of the GRF receptor have been described in mammals. It was identified that exon 11 could be alternatively spliced to generate a variant with a 123 nucleotide insertion in the third intracellular loop of the receptor (Lin et al. 1992; Mayo et al. 1992). However, this long form of the receptor does not appear to stimulate the cAMP or phospholipase C pathways (Mayo et al. 2000). In another variant found in humans, alternative splicing of the GRF receptor gene results in a truncated receptor (Hashimoto et al. 1995) that appears to inhibit the signaling of the normal receptor (Motomura et al. 1998). Additionally, the sheep and goat GRF receptors have a 16 amino acid deletion at the C-terminal end in comparison to other mammalian GRF receptors.

The truncation of the GRF receptor appears to enhance cAMP signaling relative to non-truncated receptors (Horikawa et al. 2001).

In mammals, the GRF receptor is predominantly expressed in the pituitary, placenta and kidney (Mayo et al. 2000). However, low levels of expression have been noted in other tissues including the brain, gonads and gastrointestinal tract. In the goldfish, the GRF receptor was detected in the brain, pituitary, gill, heart, gonad, gastrointestinal tract and spleen (Chan et al. 1998). The wide distribution of the GRF receptor indicates that this peptide may have other functions in addition to the regulation of growth hormone release.

The physiological action of GRF

The primary function of GRF is to regulate linear growth. GRF is released from the hypothalamus and acts on the pituitary to stimulate the release of growth hormone from somatotroph cells (Mayo et al. 1995). Mice lacking a functional GRF receptor display severe pituitary hypoplasia indicating that GRF promotes the proliferation of somatotroph cells during postnatal development. In addition these mice exhibit severe dwarfism and have substantially reduced plasma growth hormone levels. Mice that over express GRF have pituitary hyperplasia and exhibit increased growth rates as a result of increased growth hormone levels (Mayo et al. 2000).

However, GRF is also produced in a number of peripheral tissues. In the gastrointestinal tract, plasma GRF is elevated after eating and may modulate the release of insulin from pancreatic islets (Sherwood et al. 2000). In the gonads GRF may act as a paracrine factor to modulate germ cell maturation. In the testis GRF is produced in

Leydig cells and immature sperm cells and acts on Sertoli cells to enhance follicle stimulating hormone-stimulated cAMP accumulation (Fabbri et al. 1995). A larger GRF mRNA transcript is found in the testis; it is thought to arise from alternative splicing and is controlled by a different promoter (Srivastava et al. 1995). In the ovary, GRF may influence granulosa cell differentiation by enhancing follicle stimulating hormone-stimulated cAMP accumulation (Moretti et al. 1990; Bagnato et al. 1991). In addition, GRF is found to activate granulosa cell proliferation during follicular maturation (Karakji and Tsang 1995). In all tissues studied to date, the physiological actions of GRF are primarily elicited through the activation of the cAMP pathway.

GRF and development

Developmental studies involving GRF and its receptor have focused on fetal growth. The GRF peptide and its receptor are first expressed at embryonic day 16 of a 21-day gestational period in the rat (Lin et al. 1992). This corresponds to when the fetal pituitary somatotrophs differentiate and start producing growth hormone. In the chick, GRF appears to be important in regulating somatotroph differentiation and growth hormone secretion. GRF was found to enhance corticosterone stimulated somatotroph differentiation in embryonic day 12 chicks (Dean et al. 1999; Dean and Porter 1999). Additionally, in the chick GRF was found to stimulate the release of growth hormone from differentiated somatotroph cells with increasing sensitivity from embryonic days 16 to 20 (Porter et al. 1995; Dean et al. 1997). It was found that GRF receptor mRNA expression is age dependent in the rat with highest levels of expression during late fetal

development and at the onset of sexual maturation (Koryko et al. 1996). These studies indicate that GRF may be an important regulator of fetal growth hormone secretion.

In mammals the placenta is an important source of hypophysiotropic hormones and may play a role in fetal development (Baird et al. 1985). Immunoreactive GRF was found in the porcine placenta (Farmer and Gaudreau 1997), rat placenta (Baird et al. 1985; Goh et al. 1988; Margioris et al. 1990) and in the mouse placenta, maternal blood and amniotic fluid (Mizobuchi et al. 1995). In addition, the GRF gene was found to be actively transcribed in the human (Berry et al. 1992), rat (Margioris et al. 1990), mouse (Suhr et al. 1989) and sheep (Lacroix et al. 1996) placenta and was localized to the cytotrophoblasts. Placental GRF has been isolated at embryonic day 7 in the rat and reaches a maximal level at day 17 of gestation; it is thought to regulate fetal production of insulin like growth factor-II (Spatola et al. 1991). Also, placental GRF peaks at the same time as somatotrophs become responsive to GRF. In non-placental mammals, GRF mRNA is expressed at a much earlier stage of development. In fish, GRF-PACAP mRNA is expressed during the blastula stage (Krueckl et al. 2001) and in the chick it is expressed during early organogenesis (Erhardt et al. 2001). Therefore, GRF may be important for early development and in mammals the placenta is the source of GRF during early development.

Studies by Kentroli and Vernadakis examined the role of human GRF on differentiation of neuroblasts in the chick. GRF was found to influence catecholaminergic, cholinergic and GABAergic development of undifferentiated neuroblasts from embryonic days 1 to 3. GRF treatment during this period resulted in an increase in tyrosine hydroxylase activity, a sensitive marker for catecholaminergic

neurons, and increased choline acetyltransferase activity, a biochemical marker for cholinergic neurons (Kentroli and Vernadakis 1989; 1990). In contrast, administration of GRF caused a decrease in glutamate decarboxylase activity, a marker for GABAergic neurons (Kentroli and Vernadakis 1991). Therefore, these studies suggest that GRF promotes the differentiation of neuroblasts into catecholaminergic and cholinergic neurons and inhibits the differentiation of neuroblasts into GABAergic neurons. It is unclear whether these studies indicate an *in vivo* physiological role for GRF in neuroblast differentiation, since the GRF transcript is only detected in neuroblasts from embryonic day 3.5 chicks. In addition, GRF did not activate the cAMP pathway in neuroblasts derived from embryonic day 3.5 chicks (Erhardt et al. 2001). Further work is required to characterize the developmental expression of the GRF receptor in non-mammalian vertebrates in order to elucidate the developmental role of GRF.

Pituitary adenylate cyclase-activating polypeptide (PACAP)

PACAP was first isolated from ovine hypothalamic extracts based on its ability to stimulate adenylyl cyclase in rat pituitary cell cultures (Miyata et al. 1989). PACAP is known to exist in two amidated forms: a 38 amino acid form (PACAP-38) and a form comprised of the 27 N-terminal amino acids of PACAP-38 (PACAP-27) (Miyata et al. 1990). Evidence indicates that both forms of PACAP are derived from the same gene and mRNA precursor (Arimura and Shioda 1995). PACAP is widely expressed in the central and peripheral nervous systems and in a number of peripheral tissues including the adrenal gland, pancreas and gonads. PACAP is a pleiotropic hormone that functions in mammals as a neuromodulator, neurotrophic factor and vasodilator. It acts as a

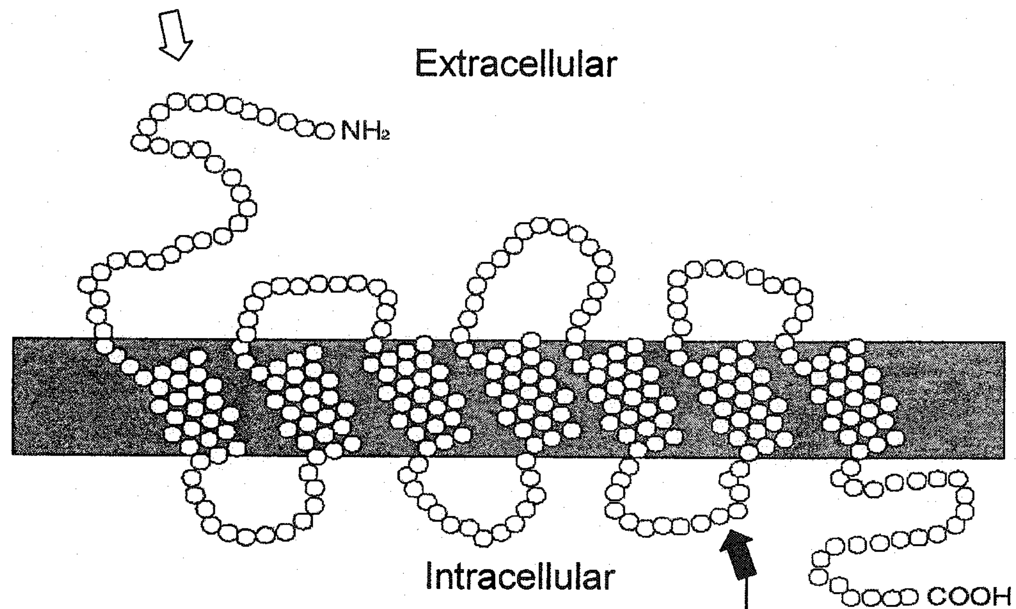
releasing factor in the pituitary and stimulates the release of catecholamines from the adrenal medulla and the release of insulin from pancreatic β-cells. Also, PACAP may have a regulatory role in the maturation of germ cells and the development of the brain (Sherwood et al. 2000).

The PACAP receptors

The diverse physiological actions of PACAP are mediated by three receptors encoded by distinct genes in mammals. PACAP shares its binding sites with its closely related glucagon superfamily member vasoactive intestinal peptide (VIP) and the three receptors are named based on their binding affinities for the two peptides. Two of the receptors called $VPAC_1$ and $VPAC_2$ bind VIP and PACAP with equal affinity, and the third receptor PAC_1 binds PACAP with more than 100-fold greater affinity than it binds VIP. All three receptors belong to Family B-III of the 7-transmembrane G protein-coupled receptor superfamily (Ulloa-Aguirre and Conn 2000).

The functional diversity of PACAP is also mediated through nine PAC_1 receptor isoforms. Six of the isoforms are generated by alternative splicing of the PAC_1 gene at the C-terminal end of the third intracellular loop. These receptor subtypes involve the inclusion or exclusion of three cassettes, termed hop1, hop2 and hip, alone or in combination. The six isoforms are termed PAC_1 short (for the receptor without a cassette), PAC_1 hop1, PAC_1 hop2, PAC_1 hip, PAC_1 hiphop1 and PAC_1 hiphop2 (Fig. 1.4). The hop cassette contains a motif to phosphorylate protein kinase C and the hip cassette appears to impede both adenylyl cyclase and phospholipase C activation, indicating that the generation of different splice variants affects signal transduction pathways (Spengler

Figure 1.4 Schematic representation of the PAC_1 receptor. The clear arrow indicates the region of the receptor where alternative splicing can be used to generate the very short receptor isoforms. The black arrow indicates the region in the third intracellular loop where alternative splicing can result in the inclusion or exclusion of the hip and hop cassettes. Amino acid sequences of the hip, hop1, hop2 and hiphop1 cassettes are indicated (Modified from Spengler et al. 1993).




```
Short  IleTyrLeu-.......................................................................................
hip    IleTyrLeu-ThrAsnLeuArgLeuArgValProLysLysThrArgGluAspProLeuProValProSerAspGlnHisSerProProPheLeu
hop1   IleTyrPhe-.......................................................................................
hop2   IleTyrPhe-.......................................................................................
hiphop IleTyrLeu-ThrAsnLeuArgLeuArgValProLysLysThrArgGluAspProLeuProValProSerAspGlnHisSerProProPheLeu

  .....................................................................................-ArgLeuAla
  .....................................................................................-ArgLeuAla
  SerCysValGlnLysCysTyrCysLysProGlnArgAlaGlnGlnHisSerCysLysMetSerGluLeuSerThrIleThrLeu-ArgLeuAla
  ...CysValGlnLysCysTyrCysLysProGlnArgAlaGlnGlnHisSerCysLysMetSerGluLeuSerThrIleThrLeu-ArgLeuAla
  SerCysValGlnLysCysTyrCysLysProGlnArgAlaGlnGlnHisSerCysLysMetSerGluLeuSerThrIleThrLeu-ArgLeuAla
```

et al. 1993). Two additional isoforms PAC_1 very short 21 (vs-21) and PAC_1 very short 57 (vs-57), are generated by alternative splicing of the N-terminal end of the receptor. The PAC_1 vs-21 isoform is characterized by a 21 amino acid deletion in the N-terminal extracelluar domain that modulates receptor selectivity to PACAP-38 and –27 causing PACAP-27 to stimulate the phospholipase C pathway with equal potency as PACAP-38 (Pantaloni et al. 1996). The PAC_1 vs-57 isoform is characterized by a 57 amino acid deletion in the N-terminal extracellular domain and exhibits reduced affinity for PACAP (Dautzenberg et al. 1999). The ninth PAC_1 receptor isoform, PAC_1 TM4, is characterized by alterations in transmembrane regions 2 and 4. In contrast to the other PAC_1 isoforms, the PAC_1 TM4 isoform is coupled to the Ca^{2+} intracellular signaling pathway through activation of L-type Ca^{2+} channels and not to the adenylyl cyclase and phospholipase C pathways (Chatterjee et al. 1996).

In non-mammalian vertebrates the PAC_1 receptor has been isolated from the goldfish (Wong et al. 1998), zebrafish (Wei et al. 1998), frog (Hu et al. 2000) and chicken (Peeters et al. 1999). However, the sequence for the zebrafish PAC_1 receptor has not been published. Only three of the nine PAC_1 receptor isoforms have been found in non-mammalian vertebrates. In the zebrafish, the PAC_1 short and hop2 isoforms were reported (Wei et al. 1998) and in the chick the PAC_1 short and hop1 isoforms were isolated (Peeters et al. 1999). A cDNA for the VPAC receptor has been isolated from the goldfish (Chow et al. 1997) and frog (Alexandre et al. 1999). In addition, a partial cDNA spanning transmembrane domains 2 to 6 of the VPAC receptor has been isolated from the pigeon, chicken, lizard and salmon (Chow et al. 1997). The isolated VPAC receptors from non-mammalian vertebrates exhibit higher sequence identity with the mammalian

$VPAC_1$ receptor than with the $VPAC_2$ receptor. It is hypothesized that an early duplication event led to the existence of the PAC_1 and $VPAC_1$ receptors and a second duplication of the PAC_1 receptor led to the $VPAC_2$ receptor (Vaudry et al. 2000b).

In mammals the PAC_1 receptor is distributed in the central nervous system, peripheral nervous system, eye, pituitary, adrenal gland, pancreas, liver, ovary, lung and stomach (Vaudry et al. 2000b). The wide spread expression pattern of the PAC_1 receptor appears to be conserved throughout evolution. In the chicken, the PAC_1 receptor is expressed predominantly in the brain, ovary and testis. However, expression is also noted in the pituitary, adrenal gland, pancreas, kidney, lung, heart and intestine (Peeters et al. 1999). In goldfish expression of the PAC_1 receptor is predominantly found in the brain, pituitary and heart. In addition, low levels of expression are seen in the liver, gonads, gills, gall bladder, intestine, kidney, skeletal muscle and spleen (Wong et al. 1998).

In mammals the $VPAC_1$ receptor and $VPAC_2$ receptor have different expression patterns, although some overlap is seen. The $VPAC_1$ receptor is found in the central nervous system, lung, adrenal gland, small intestine and thymus and the $VPAC_2$ receptor is found in the hypothalamus, pituitary, adrenal gland, pancreas, stomach, ovary and testes (Usdin et al. 1994). Only one VPAC receptor has been isolated from non-mammalian species and sequence identity indicates that this receptor is the $VPAC_1$ receptor (Chow et al. 1997). The cloned frog VPAC receptor exhibited highest sequence identity with the mammalian $VPAC_1$ receptor. However, the expression pattern of the frog VPAC receptor overlaps with that of both the mammalian $VPAC_1$ and $VPAC_2$ receptors and the frog VPAC receptor showed pharmacological characteristics of the

$VPAC_2$ receptor (Alexandre et al. 1999). Therefore, it appears that the frog VPAC receptor is a hybrid between the $VPAC_1$ and $VPAC_2$ receptors. Therefore, it is hypothesized that the $VPAC_2$ receptor arose from a duplication of the PAC_1 receptor gene after the divergence of the amphibian lineage. The chromosomal localization of the three receptors in the rat and human supports this hypothesis. In the rat and human the PAC_1 and $VPAC_2$ receptors are localized to the same chromosome, whereas the $VPAC_1$ receptor is localized to a separate chromosome (Vaudry et al. 2000b).

The physiological actions of PACAP

In the pituitary PACAP acts as a hypophysiotropic factor on a variety of cells types to stimulate the release of a variety of hormones through either the cAMP pathway or the inositol (1,4,5) triphosphate pathway with Ca^{2+} mobilization. In gonadotroph cells, PACAP is thought to modulate luteinizing hormone and follicle stimulating hormone expression by activation of the cAMP pathway and of Ca^{2+} mobilization. In somatotroph cells, PACAP modulates the expression and release of growth hormone by activation of the cAMP pathway and Ca^{2+} mobilization. In lactotroph cells, PACAP stimulates Ca^{2+} mobilization causing an increase in prolactin secretion. PACAP stimulates adrenocorticotropic hormone release in corticotroph cells and thyroid stimulating hormone release in thyrotroph cells by Ca^{2+} mobilization, and α-melanocyte stimulating hormone release from melanotroph cells by activation of the cAMP pathway (Rawlings and Hezareh 1996).

PACAP and its receptor have been localized to adrenal chromaffin cells in mammals, frogs and fish where it is found to stimulate catecholamine secretion

(Sherwood et al. 2000). It appears that PACAP modulates catecholamine synthesis and secretion through activation of the PAC_1 receptor (Shioda et al. 2000). Catecholamine is synthesized in the adrenal chromaffin cells by a cascade of enzymes in which tyrosine hydroxylase is the rate limiting enzyme. PACAP appears to stimulate catecholamine synthesis by activation of tyrosine hydroxylase through the protein kinase A pathway (Marley et al. 1996). In contrast, PACAP stimulates the release of catecholamines through activation of the L-type Ca^{2+} channel (Lamouche and Yamaguchi 2001).

In the cardiovascular system PACAP causes the relaxation of smooth muscle resulting in dilation of airways in the lung and vasodilation of blood vessels. However, administration of PACAP first causes hypotension followed by prolonged hypertension or high blood pressure. The initial hypotension is due to the vasodilation and the hypertension is caused by the release of catecholamines from the adrenal gland (Sherwood et al. 2000; Vaudry et al. 2000b). A more recent study indicates that PACAP may directly cause hypertension through activation of L-type Ca^{2+} channels, possibly via a protein kinase C and not a protein kinase A dependent pathway (Li et al. 2001). Therefore, the opposing actions of PACAP on vascular smooth muscle may be due to activation of different signaling pathways. In the heart, PACAP causes an increase in heart rate and contractile force (Sherwood et al. 2000; Vaudry et al. 2000b).

In the gastrointestinal tract PACAP has been shown to have diverse effects including relaxation of smooth muscle contraction, and modulation of exocrine and endocrine secretions from the stomach and pancreas (Sherwood et al. 2000). In the stomach PACAP was shown to stimulate histamine release from enterochromaffin-like cells causing the release of gastric acid. To cause histamine release, PACAP acts through

a Ca^{2+} dependent pathway via activation of the PAC_1 receptor. In D cells of the gastrointestinal tract, PACAP stimulates the release of somatostatin via the $VPAC_1$ receptor causing a decrease in gastric acid secretion (Zeng et al. 1999; Pisgna et al. 2000). Therefore, it appears that PACAP may act as a positive and negative regulator of gastric acid secretion. In the pancreatic β-cells that express all three PACAP receptors, PACAP has a direct and indirect effect on insulin secretion. PACAP was found to up-regulate the glucose stimulated expression of GLUT 1 and hexokinase 1 that, in turn, stimulate insulin synthesis and to directly stimulate insulin synthesis through activation of the cAMP pathway (Borboni et al. 1999). In addition, PACAP has been shown to stimulate insulin release from pancreatic β-cells by increasing the activity of L-type Ca^{2+} channels (Yada et al. 1994). It is likely that this effect is mediated through the activation of the PAC_1 TM4 receptor that is predominantly expressed in β-cells and is known to activate this type of calcium channel (Chatterjee et al. 1996). In the exocrine pancreas, PACAP stimulates amylase secretion by activation of the phospholipase C pathway (Barnhardt et al. 1997).

PACAP and its receptors have been found in the male and female reproductive system and may be involved in the regulation of germ cell maturation. The testes are a major site of PACAP production. The mRNA transcript and mature PACAP peptide have been localized to spermatogonia, primary spermatocytes and immature spermatids near the perimeter of the seminiferous tubules (Hannibal and Fahrenkrug 1995; Shioda et al. 1994). The expression of PACAP in the testis appears to be controlled by an alternative promoter located 13.5 kilobase pairs upstream from the translational start site (Daniel and Habener 2000). In addition, the testis transcript contains a novel 5’

untranslated region corresponding to a testis-specific exon 1 (Hurley et al. 1995; Daniel and Habener 2000). Therefore, a testis-specific promoter regulates the expression of PACAP and expression appears to be under the control of pituitary gonadotropins (Shuto et al. 1995). PACAP is expressed in a stage-specific manner in early developing germ cells and may play an important role in the stage-specific cAMP fluctuations in the rat seminiferous tubules (Kononen et al. 1994). This indicates that PACAP may play an important role in spermatogenesis.

In the female reproductive system, mRNA transcripts for PACAP and the PAC_1 receptor have been isolated from the rat ovary. Analysis of the PAC_1 receptor splice variants revealed that the short, hip/hop and either the hip or hop isoforms are found in the ovary (Scaldaferri et al. 1996). In the rat ovary, expression of PACAP and the PAC_1 receptor was localized to granulosa cells of preovulatory follicles (Koh et al. 2000; Park et al. 2000). Only minor expression of PACAP was seen in other ovarian tissues including the thecal cells and corpus luteum (Koh et al. 2000). Treatment of preovulatory follicles with either human chorionic gonadotropin or luteinizing hormone caused a dose dependent increase in the expression of the PAC_1 receptor (Park et al. 2000). *In vitro* studies showed that PACAP stimulated an increase in cAMP in granulosa cells that was coupled to the stimulation of estradiol and progesterone secretion (Heindel et al. 1996). In addition, PACAP was shown to stimulate meiotic maturation of rat oocytes (Apa et al. 1997). A surge of luteinizing hormone is responsible for stimulating final maturation of oocytes and the production of progesterone that induces ovulation. This surge also stimulates the production of PACAP that appears to be involved in the

stimulation of both oocyte maturation and progesterone secretion. Therefore, it appears that PACAP may play an important role in ovulation.

Some functions of PACAP have been studied in non-mammalian vertebrates. In the frog, PACAP has been localized in the brain and adrenal gland (Chartrel et al. 1991; Yon et al. 1992, 1993). PACAP was found to produce a dose-dependent increase in cAMP from anterior pituitary fragments in the frog (Chartrel et al. 1991). In fish PACAP was found to stimulate growth hormone release in the goldfish (Wong et al. 1998), eel (Montero et al. 1998) and salmon (Parker et al. 1997), and to modulate gonatotropin-II release in the goldfish (Chang et al. 2001). In the frog adrenal, PACAP was found to stimulate corticosteroid-producing cells and to increase intracellular calcium in chromaffin cells (Yon et al. 1994). In the crested newt, PACAP immunoreactivity is localized in the brain and ovary and is thought to modulate steroid synthesis in the ovary (Gobbetti et al. 1997).

PACAP and development

Developmental studies regarding PACAP have focused on the central and peripheral nervous systems. In the mouse, PACAP and PAC_1 receptor mRNA transcripts are first expressed at embryonic day 9.5 corresponding to the early organogenesis developmental stage (Shuto et al. 1996). The PAC_1 receptor was localized to the floor and roof plates of the neural tube of embryonic day 9.5 mouse embryos and later in the rhombencephalon of embryonic day 10.5 to 11.5 mouse embryos (Sheward et al. 1996; 1998). In contrast, PACAP was distributed in the ventromedial and dorsolateral cells of the neural tube (Sheward et al. 1998). In addition, PACAP and the PAC_1 receptor were

expressed in the developing sympathetic chain in developmental precursor sites of the dorsal root and trigeminal ganglia where the two genes are expressed in the adult (Sheward et al. 1998). Similarly, in the rat PACAP and the PAC_1 receptor transcripts were detected at embryonic day 10 (Basille et al. 2000; Zhou et al. 1999) and the PACAP peptide and its binding sites were detected at embryonic day 14 (Tatsuno et al. 1994). In addition, the $VPAC_1$ receptor was detected at embryonic day 11 (Pei 1997) and the $VPAC_2$ receptor was detected at embryonic day 10 at lower levels than the PAC_1 receptor (Bassille et al. 2000). Three of the PAC_1 receptor isoforms are expressed during development. The PAC_1 short and hop isoforms are expressed from embryonic day 14 onwards and the PAC_1 hiphop isoform is expressed from embryonic day 17 in the rat (Bassille et al. 2000). The mRNA transcripts for PACAP, PAC_1 receptor and $VPAC_1$ receptor showed similar distribution patterns with expression in all germinative neuroepithelia throughout development. In contrast the $VPAC_2$ receptor was expressed only in nongerminative areas such as the suprachiasmatic and thalamic nuclei (Basille et al. 2000). PACAP mRNA was widely distributed throughout the rat brain during development and expression diminished towards adulthood (Skoglosa et al. 1999). In contrast, levels of the PACAP peptide and its binding sites increased throughout the developmental period until reaching adult levels (Arimura et al. 1994). The presence of PACAP and its receptors in germinative areas of the developing nervous system indicate that PACAP may have a trophic role during development.

Evidence from the mouse and frog indicate that PACAP may play a role in the early patterning of the neural tube. At embryonic day 10.5 in the mouse PACAP inhibits DNA synthesis in neural precursors and down regulates the expression of *sonic hedgehog*

and *gli*-1. *Gli*-1 and *sonic hedgehog* are known to control the dorsal/ventral patterning of the vertebrate neural tube by promoting the ventral phenotype (Waschek et al. 1998). In addition, studies in the *Xenopus* indicate that PACAP may act as a dorsalizing factor in the neural tube. Ventral administration of PACAP led to down-regulation of ventral marker genes *sizzled, wnt 8 and vent* 1, and upregulation of dorsal maker genes *chordin, goosecoid* and *otx* 2. Also, overexpression of PACAP led to strong anteriorization of the embryo (Otto et al. 2000).

After formation of the neural tube, the anterior nervous system divides into three vesicles, the forebrain, midbrain and hindbrain. Further development causes the forebrain to divide into the telencephalon and diencephalon. The cerebral cortex is composed of six cell layers in mammals and arises from the paired telencephalic vesicles. Cortical precursors proliferate in the neuroepithelium of the ventricular zone and their cell fate is determined by the time at which a precursor exits the cell cycle. In cortical precursors, PACAP and the PAC_1 receptor are expressed and appear to be involved in cell cycle regulation. Administration of PACAP inhibited mitosis and stimulated differentiation of cortical precursors (Lu and DiCicco-Bloom 1997; DiCicco-Bloom et al. 1998; Suh et al. 2001). However, PACAP can also stimulate neuroblast proliferation and its regulation of neuroblast mitotic activity is attributed to the activation of different PAC_1 receptor isoforms. PACAP inhibited proliferation of neuroblasts through activation of the PAC_1 short isoform and promoted proliferation through activation of the PAC_1 hop isoform (Nicot and Di-Cicoo-Bloom 2001). These results support the anti-mitotic role of PACAP in the developing cortex since the cortical neuroblasts express the short isoform (DiCicco-Bloom et al. 1998). Therefore, PACAP may play an important

role in the development of the cerebral cortex by controlling the timing of cell cycle exit, which regulates the fate of cortical precursors.

Also, PACAP may act as a neurotrophic factor in neural crest derived sympathetic neuroblasts. In the chick embryo *in ovo,* PACAP administration increased the survival of sensory and motor neurons of the dorsal root ganglion (Arimura et al. 1994). PACAP activates both the adenylyl cyclase and phospholipase C signaling pathways in sympathetic neuroblasts through activation of the hop PAC_1 receptor isoform promoting precursor proliferation (DiCicco-Bloom et al. 2000; Lu et al. 1998). Therefore, PACAP appears to play an important role in regulating the proliferation of neuroblasts. The differential expression of the PAC1 receptor short and hop isoforms seems to determine the inhibitory or stimulatory effect of PACAP on neuroblast proliferation.

During postnatal development the cerebellar cortex undergoes a period of neurogenesis from postnatal day 4 to 20 in the rat. During this period, PACAP and its binding sites are present in the cerebellum. Expression peaks at postnatal day 8 and then declines to adult levels (Nielsen et al. 1998; Basille et al. 1994). In particular it was found that PACAP was localized to Purkinje cells and its binding sites were present on immature granule cells of the external granule layer that generates the majority of cerebellar neurons (Basille et al. 1993; 1994). In cerebellar neuroblast culture from 8-day-old rats, PACAP was found to stimulate the adenylyl cyclase and phospholipase C signaling pathways (Basille et al. 1995). This is possibly mediated through activation of the PAC_1 hop receptor isoform that is expressed at high levels in cerebellar neuroblasts (D'Agata et al. 1996; Campard et al. 1997). In cultured cerebellar neuroblasts PACAP administration was found to promote cell survival (Cavallaro et al. 1996; Campard et al.

1997; Gonzalez et al. 1997; Villalba et al. 1997). The neuroprotective actions of PACAP appear to be mediated by cAMP dependent activation of the mitogen-activated protein kinase pathway (Campard et al. 1997; Villalba et al. 1997). Similarly, *in vivo* studies indicate that PACAP administration caused an increase in the proliferation of cerebellar granule cells and stimulated migration of external granule cells toward the internal cell layers (Vaudry et al. 2000a). Therefore, these studies provide substantial evidence that PACAP is involved in the neurogenesis of the cerebellum.

PACAP has also been implicated in the development of other tissues including the adrenal, liver and pancreas (Sherwood et al. 2000). PACAP and its binding sites have been localized in the human fetal adrenal gland at a time when chromaffin cells are migrating (Yon et al. 1998). In addition, administration of PACAP to cultured sympathoadrenal cells resulted in the promotion of neurite outgrowth (Deutsch and Sun 1992; Hernandez et al. 1995). PACAP has been localized to cells innervating chromaffin cells and apprears to stimulate the development of the neuronal phenotype in chromaffin cells (Wolf and Krieglstein 1995). Therefore, it appears that PACAP may play a role in the development of tissues in which it is expressed in the adult.

OBJECTIVES

Previous work in the rat and mouse indicates that both GRF and PACAP are involved in vertebrate development. In particular, both GRF and PACAP are thought to modulate brain development in vertebrates. However, the wide expression pattern of both GRF and PACAP in the adult suggests that these peptides may be involved in the development of other systems. In addition, the evolutionary relationship between GRF

and PACAP may have a functional significance. My objectives were to examine the role of GRF and PACAP during vertebrate development using the zebrafish as a model system. The zebrafish is an excellent developmental model because it has a short generation time, rapid embryogenesis and transparent embryos. In comparison with other developmental models, such as the chick or mouse, the developing zebrafish embryo is more accessible and its optical transparency makes it ideal for examining gene expression. In addition, the use of the zebrafish allowed me to examine whether the developmental role of these two peptides is conserved throughout evolution and to address the functional implications of the co-expression of GRF and PACAP in fish. I hypothesized that GRF and PACAP modulate the development of the vertebrate embryo and that due to the strong conservation of the PACAP peptide this role is conserved in all vertebrates.

To study the gene expression of GRF and PACAP in the zebrafish I isolated and characterized the GRF-PACAP gene in the zebrafish. I isolated a clone from a zebrafish genomic library containing a gene that encoded the GRF and PACAP peptides. The gene isolated from the zebrafish had a similar arrangement as was found in the salmon (Parker et al. 1993) and chicken (McRory et al. 1997). It is hypothesized that a genome duplication event occurred in an ancestral teleost indicating that the zebrafish genome may have more than one copy of the GRF-PACAP gene. Therefore, I examined the GRF-PACAP gene copy number by Southern analysis and results indicated that only one copy of the gene was present in the zebrafish genome. In addition, I examined the tissue expression pattern in the adult zebrafish. This study confirmed that the isolated gene was transcribed and that it is widely expressed in the adult zebrafish. The GRF-PACAP gene

was expressed in tissues that were developmentally derived from all three germ layers, endoderm, ectoderm and mesoderm. This suggests that the GRF-PACAP gene is widely expressed in the developing embryo.

To examine whether GRF and PACAP function in a paracrine or endocrine manner in these tissues and to determine the functional significance of the co-expression of GRF and PACAP, I isolated and characterized their receptors in the adult zebrafish. I amplified three cDNAs from a zebrafish brain cDNA library corresponding to the GRF receptor, PAC_1 receptor and $VPAC_1$ receptor. All three receptors were widely expressed in the adult zebrafish, indicating that GRF and PACAP have a variety of functions in fish as well as mammals. In addition, three isoforms of the PAC_1 receptor were isolated from the adult zebrafish including a novel isoform from the gill.

Previously in our laboratory it was found that the GRF-PACAP transcript is expressed at an earlier developmental stage in rainbow trout than in birds or mammals. Therefore, to examine the role of GRF and PACAP during early development, I characterized the expression of transcripts for GRF-PACAP, GRF receptor, PAC_1 receptor and $VPAC_1$ receptor in the developing zebrafish embryo. This study confirmed the early expression of the GRF-PACAP transcript in fish. In addition, all three receptors were expressed during the late blastula, gastrula and segmentation periods. This suggests that both GRF and PACAP modulate early development when the embryonic body is being established in the zebrafish.

In mammals, studies indicate that GRF and PACAP are involved in brain development. To examine the possible role of GRF and PACAP during brain development in the fish, in collaboration with Sandra Krueckl, I localized the expression

of the GRF-PACAP transcript by *in situ* hybridization during the segmentation and pharyngula periods in the zebrafish. Widespread expression of the GRF-PACAP transcript was found in the zebrafish embryo throughout the segmentation period. The transcript was highly expressed in the neural tube indicating that GRF and PACAP may be involved in brain development. In addition, strong expression was found in the prechordal plate and later in the hatching gland that is derived from the prechordal plate. The timing and widespread expression of the GRF-PACAP transcript in the developing embryo indicates that these hormones may modulate the cell cycle during development.

CHAPTER 2

Isolation and characterization of the zebrafish *(Danio rerio)* GRF-PACAP gene

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INTRODUCTION

GRF and PACAP are structurally related members of the glucagon superfamily that are implicated in growth and development. PACAP is the most highly conserved member of the glucagon superfamily. Mammalian and tunicate PACAP-27 share 96% amino acid sequence identity (McRory and Sherwood 1997). Additionally, PACAP shares high amino acid sequence identity with VIP, 68% in humans, indicating that these two members of the glucagon superfamily are highly related (Campbell and Scanes 1992). In contrast, GRF is only moderately conserved. Mammalian and salmon GRF share only 41% amino acid sequence identity (Parker et al. 1997). The strong conservation of the PACAP peptide may indicate an important physiological role.

PACAP was isolated from ovine hypothalamic extracts based on its ability to stimulate adenylyl cyclase in rat pituitary cultures (Miyata et al. 1989). PACAP is widely expressed in the central nervous system and peripheral nervous system innervating the pituitary, eye, digestive system, urogenital system and respiratory tract. In addition, PACAP is produced in non-neural tissues including the adrenal and pancreas (Sherwood et al. 2000). Full characterization of PACAP's physiological role is incomplete. However, it is known to function as a neuromodulator, as a neurotrophic factor, as a vasodilator and as a releasing factor in the pituitary, adrenal gland and pancreas (Sherwood et al. 2000).

Similarly, GRF also exhibits a wide tissue distribution and diverse physiological actions. GRF is primarily produced in the hypothalamus and acts on the pituitary to cause the release of growth hormone from somatotroph cells (Mayo et al. 1995). In addition GRF is produced in a number of peripheral tissues including the pituitary, lung, thyroid,

adrenal, kidney (Shibasaki et al. 1984), gastrointestinal tract (Bosman et al. 1984), placenta (Margioris et al. 1990), ovary (Bagnato et al. 1992) and testis (Berry and Pescovitz 1988; Pescovitz et al. 1990). Both of these peptides are expressed in the developing embryo, but their function during development is not fully characterized.

In fish, a cDNA for both GRF and PACAP has been isolated from the salmon and catfish indicating that the two peptides are encoded by a single gene (Parker et al.1993; McRory et al.1995). The GRF-PACAP gene has only been isolated in one fish species, the salmon (Parker et al. 1997). In contrast to fish, GRF and PACAP are encoded on distinct genes in mammals. It is hypothesized that a gene duplication after the divergence of the reptilian and mammalian lineages led to the existence of two separate genes in mammals (Fig. 2.1). Therefore, a key question relates to the functional significance of the gene arrangement found in fish.

To investigate the structural and functional evolution of GRF and PACAP, and to lay the foundation for developmental studies, I have isolated the GRF-PACAP gene from the zebrafish genomic library. Here I describe the full nucleotide sequence for the GRF-PACAP gene and its copy number for the zebrafish. Additionally, the tissue expression of the GRF-PACAP mRNA transcript in the adult zebrafish is described.

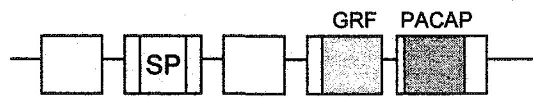
Materials and Methods

Synthesis of a zebrafish PACAP specific probe

A region of the GRF-PACAP gene was amplified by PCR from zebrafish genomic DNA with primers zebra 1 and 3'zebra2 (Table 2.1). Zebra 1 was designed to hybridize with the beginning of the first exon and 3'zebra 2 with the end of the second

Figure 2.1 Schematic representation of the proposed mechanism of evolution of the GRF and PACAP gene from fish to mammals. It is hypothesized that a gene duplication with chromosomal rearrangement occurred after the divergence of the mammalian lineage. Exons are shown as boxes, introns are shown as lines and shaded boxes indicate important peptides.

FISH/BIRDS



Gene Duplication and Rearrangement

MAMMALS

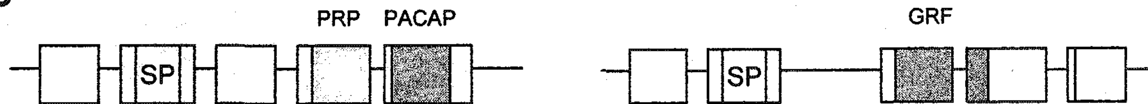


Table 2.1 Primer sequences for zebrafish PACAP gene and tubulin gene reported in the 5' to 3' direction of the sense strand for forward primers and antisense strand for reverse primers. Direction of primer is represented by the arrows, forward primers are (←) and reverse primers are (→).

Primer Name	Direction	Sequence 5'to 3'
zebra 1	→	ATATCTCGCCTCAGATCCGT
3'zebra 2	←	CACATTGCATTGAACTAGGAGC
PA-1	→	CATTCGGATGGGATCTTCACGGATAG
3'PA	←	TACATGTTTAAAGAACACAAGAGCG
3'zebra 5	←	GCTTGCTCCACATAGCATCTGTCTATT
zebra 2	→	CGACTCTTGCTTTCCTCATC
3'UTR-R	←	GCATTGTCAGGTGCGTCAGTA
zebra 3	→	GAGACTGCAGGATTTGATGAGG
3'zebra 4	←	TCGCATCAGTGTATGCAGGTACTTC
Tubulin 10	→	CAGGTGTCCACGGCTGTGGTG
Tubulin 11	←	AGGGCTCCATCGAAACGCAG

exon of the GRF-PACAP gene (Fig. 2.2). The reaction was carried out in a 50 μl volume containing: 200 μM dNTPs, 2 mM MgCl, 0.4 μM of each primer and 2.5 units of *Taq* DNA polymerase (Promega, Mississauga, ON). The reaction was heated to 94 °C for 1 minute, then cycled 30 times (94°C for 1 min, 48°C for 1 min, 72°C for 1 min). An aliqout of the PCR product was cloned into pGEM-T vector (Promega, Mississauga, ON) as outlined by the manufacturer. A recombinant clone was sequenced manually using [$^{\alpha\text{-}35}$S]-dATP with the Sequenase 2.0 kit (US Biochemical, Cleveland OH) as specified by the manufacturer and run on a 6 % polyacrylamide/7 M urea wedge gel that was dried under vacuum and exposed to Kodak Max film (Kodak, Rochester, NY). The sequence matched the 5' untranslated region and signal peptide of the zebrafish GRF-PACAP cDNA (Fig. 2.2). The probe used to screen the zebrafish genomic library was amplified by PCR using primers zebra 1 and 3'zebra 2 as described above from the sequenced clone. The amplified product was labeled with [α-^{32}P]-dCTP (NEN Life Science Products, Guelph, ON) using the Random Primers DNA Labeling System (Gibco BRL, Burlington, ON) as specified by the manufacturer. Before use unincorporated dNTPs were removed by eluting the reaction from a NAP 5 column (Pharmacia, Baie d'Urfe, QB).

Screening of the zebrafish genomic library

A zebrafish genomic library in λ fix II (a gift from Jonathan Alexander, University of California, Berkeley) was used to isolate the GRF-PACAP gene. A total of $5x10^5$ plaque forming units were screened using a 267 bp [α-^{32}P]-dCTP labeled PACAP specific probe. Phage were diluted in SM buffer and incubated with 600 μl of XL1 Blue MRA cells (O.D. 260 = 0.5) for 15 minutes at 37 °C. Then 10 ml of NZY top agarose

Figure 2.2 PCR amplified probe used to screen a zebrafish genomic library. (A) Sequence of amplified product corresponding to the zebrafish GRF-PACAP gene. Capital letters represent coding regions of exons 1 and 2 and lowercase letters represent intron sequence. Primer sequences are underlined. (B) Schematic of GRF-PACAP gene indicating primer locations and probe used for screening.

A

```
ATATCTCGCCTCAGATCCGTCCGACTACGAAGACCTGAGAGAGAGAGAGGGAGGA  55
AAGATACAGACGCTGTGGGTAACAAAGTGACGCGTTGAAAAGTTTAAAGAGCAAG  110
ACTGGGAGAGAAAGGAGAGAGAGAGAGAGAGCTGGAGAATTTCATCTCATTCTGG  165
ACGCAGCCTCCATTGGACAGCATCCGTCCGCTGCCGCAGgtaaatgcaaaacttc  220
tgccagatattttaattcttgattttgggaatcgtacttcatttatagcaattag  275
aaacagttaaaccacaactatactagattattacacaggaatatcgaagattgaa  330
agtgctttttagacagaaatatggtactaatggatgttttattctacagAATGA  385
TTACGAGCAGCAAAACGACTCTTGCTTTCCTCATCTATGGGCTCCTAGTTCAATG  440
CAATGTG                                                  447
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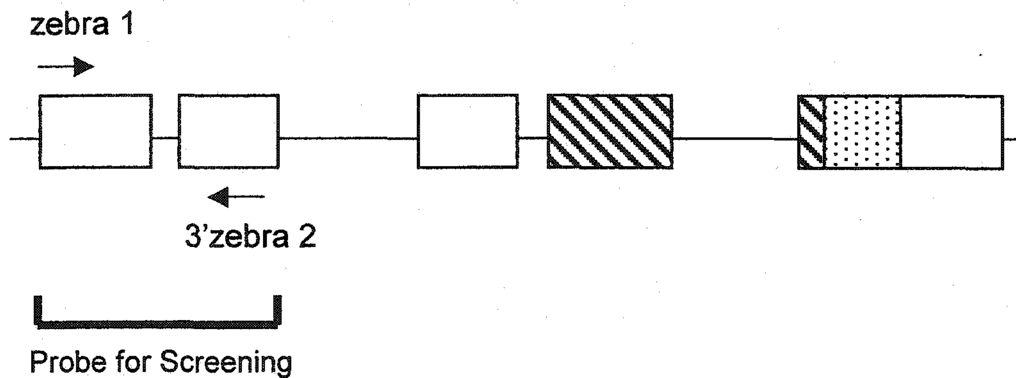
B



was added to the phage/cell suspension and the mixture was plated on 150 mm NZY agar plates. The plates were incubated at 37 °C for 10-12 hours, then placed at 4 °C for 1 hour. Duplicate nylon membrane (NEN Life Science Products, Guelph, ON) lifts were carried out. The first filter was placed on the cool plate for 1 minute and the second filter was placed on the plate for 3 minutes. Filters were alkaline denatured for 10 minutes in 0.5 M NaOH/ 1.5 M NaCl, neutralized for 2 x 10 minutes in 1.5 M NaCl/0.5 M Tris-HCl pH 8.0 and then placed in 2 x SSC for 2 x 5 minutes. The filters were air dried at room temperature for 1 hour.

Filters were soaked in 6x SSC for 5 minutes then, prehybridized at 50°C in a solution containing: 6x SSC, 5x Denhardt's solution, 0.5% SDS and 300 μg of blocking DNA for 4 hours. For hybridization, the above solution and the [α-^{32}P]-dCTP random labeled probe (7.5×10^6 cpm/ml) were added to the membranes and incubated at 55°C for 14 hours. The membranes were washed twice with 2x SSC and 0.1% SDS at 55°C for 30 minutes, twice with 1x SSC and 0.1% SDS at 55°C for 30 minutes, and twice with 0.5x SSC and 0.1% SDS at 55°C for 30 minutes. The membranes were air dried and then exposed to BioMax film (Kodak, Rochester, NY) for five days at -80°C. The autoradiographs of the duplicate membranes were compared to identify putative positive plaques. Regions of the plate corresponding to positive plaques were cored using a 1ml sterile pipette tip. Agar plugs were placed in 1.5 ml microfuge tubes with 1 ml of SM buffer and 10 μl of chloroform, and stored at 4 °C. Putative positives were confirmed by PCR using primers zebra 1 and 3'zebra 2 at an annealing temperature of 52°C. Positive plugs were re-screened at 1000 pfu and then at 100 pfu until single isolated plaques were obtained. Three rounds of screening were required to isolate five single positive clones:

λA2, λL2 λM4, λN1 and λO1. The presence of the GRF-PACAP gene in all five clones was confirmed by PCR amplification with two different primer sets specific for the GRF-PACAP gene, primers zebra 1 and 3'zebra 2, and primers zebra 3 and 3'zebra 4 (Table 2.1). Clones λN1 and λO1 yielded inconsistent results and were excluded from further analysis. Further, the orientation and location of the GRF-PACAP gene within the insert of each of the three remaining clones was investigated by PCR with a gene specific and a vector specific primer. Clones λA2 and λL2 were found to contain an incomplete exon 5 and were excluded from further analysis. Only the λM4 clone yielded positive results with both the gene specific primer sets and the insert contained the full length gene. Therefore, the λM4 clone was selected for further characterization.

Lambda DNA isolation

Plate lysates of the λM4 clone were done by plating 50 000 pfu/plate on 10 x 150 mm NZY agarose plates as previously described and grown at 37 °C until confluent lysis occurred. Plates were cooled at 4 °C for 6 hours. The plates were covered with 10 ml of SM buffer and swirled gently for 12 to 14 hours. The lysate solution was removed and pooled. The plates were then washed with an additional 5 ml of SM buffer and swirled gently for 3 hours. The wash was added to the lysate solution and 3 ml of choloroform was added. The lysate solution was stored at 4 °C overnight. Bacteriophage DNA was prepared from 50 ml of the lysate using the λ midi DNA preparation kit (Qiagen, Mississauga, ON) as specified by the manufacturer.

Characterization of the λM4 clone

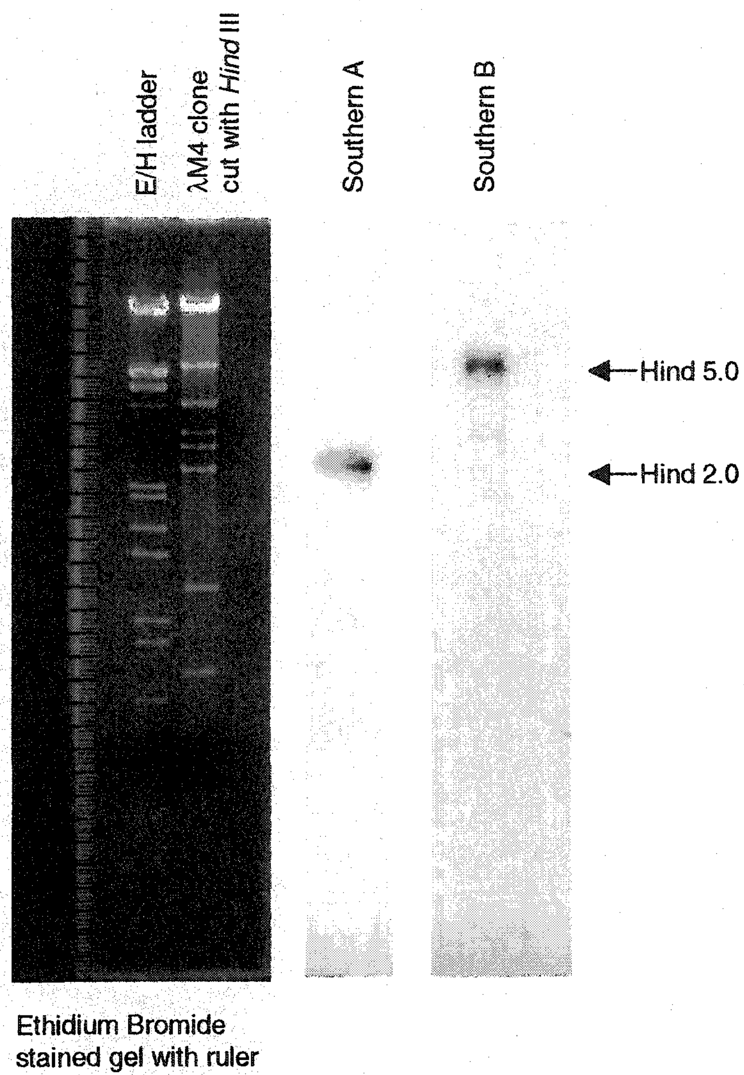
The purified λM4 clone was cut with a variety of enzymes and *Hind* III was found to cut the insert out of the vector arms and into 6 fragments from 5 kb to 0.6 kb in

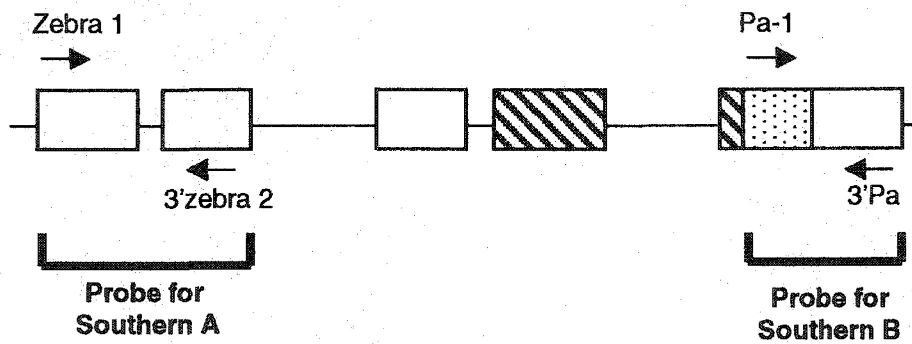
length. Southern analysis using probes to the beginning and end of the gene was performed to determine which fragments contained the gene. The λM4 clone was digested with *Hind* III, (NEB, Mississauga, ON) and electrophoresed on a 1% agarose gel. DNA was capillary transferred according to the manufacturer's specifications to a nylon membrane (NEN Life Science Products, Guelph, ON). The membrane was prehybridized at 55°C in 6x SSC, 5x Denhardt's solution, 0.5% SDS and 300 μg of blocking DNA for 4 hours. Using primers zebra 1 and 3'zebra 2 a probe spanning from the first exon to the second exon (Fig. 2.3) was amplified by PCR from zebrafish genomic DNA as described above at an annealing temperature of 52°C. The probe was radiolabeled with [α-^{32}P]-dCTP as described previously. The radiolabeled probe (3.9×10^7 cpm/ml) was hybridized at 55°C in 6x SSC, 5x Denhardt's solution, 0.5% SDS and 300 μg of blocking DNA for 12 hours. The membrane was washed twice in 2x SSC and 0.1% SDS at 50°C for 30 minutes, and twice in 0.5x SSC and 0.1% SDS at 50°C for 30 minutes, and twice in 0.1% SSC and 0.1% SDS at 50°C for 30 minutes. The membrane was allowed an air dry then exposed to BioMax film (Kodak, Rochester, NY) for 5 days at -80°C. This procedure was repeated with a probe amplified using primers PA-1 and 3'PA (Table 2.1) from zebrafish genomic DNA, corresponding to exon 5 of the zebrafish gene (Fig. 2.3). Southern A shows that the 2 kb band (Hind 2.0) contains the 5' end of the GRF-PACAP gene and Southern B shows that the 5 kb band (Hind 5.0) contains the 3' end of the GRF-PACAP gene (Fig. 2.3)

Cloning and sequencing of the GRF-PACAP gene

The λM4 clone and the pBluescript II KS+ vector were cut with *Hind* III. The λM4 5 kb and 2 kb bands that were determined to contain the GRF-PACAP gene were

Figure 2.3 Southern analysis of λM4 clone cut with *Hind* III. Shown are: ethidium bromide gel of digested clone with size marker and ruler, Southern A results with probe to 5' end of gene, Southern B results with probe to 3' end of gene and gene schematic showing primer and probe locations. The Hind 5.0 band containing the 3' end of the GRF-PACAP gene and the Hind 2.0 band containing the 5' end of the GRF-PACAP gene are indicated.


E/H ladder
λM4 clone cut with Hind III
Southern A
Southern B
Hind 5.0
Hind 2.0
Ethidium Bromide stained gel with ruler


Zebra 1
Pa-1
3'zebra 2
3'Pa
Probe for Southern A
Probe for Southern B

gel purified using the ultrafree-MC centrifugal filter units (Millipore, Bedford, MA) as outlined by the manufacturer. The insert and vector DNA were heated to 70 °C for 5 minutes, then placed on ice. The insert DNA was ligated into pBluescript II at 16 °C overnight (in 1 mM ATP, 10 mM DTT, 1x ligase buffer) using 400 units of T4 DNA ligase (Gibco BRL, Burlington, ON). Recombinant plasmids were cloned in XL 2 Blue MRF' host cells (Stratagene, La Jolla, CA) and identified by blue-white selection. Plasmid DNA was isoated using the QIAprep miniprep kit (Qiagen, Mississauga, ON) as outlined by the manufacturer. Both strands from the recombinant plasmids were sequenced using an Applied Biosystems Incorporated Prism 377 DNA Sequencer, first with forward and reverse primers, then with internal primers designed against the zebrafish sequences.

Southern analysis of genomic DNA

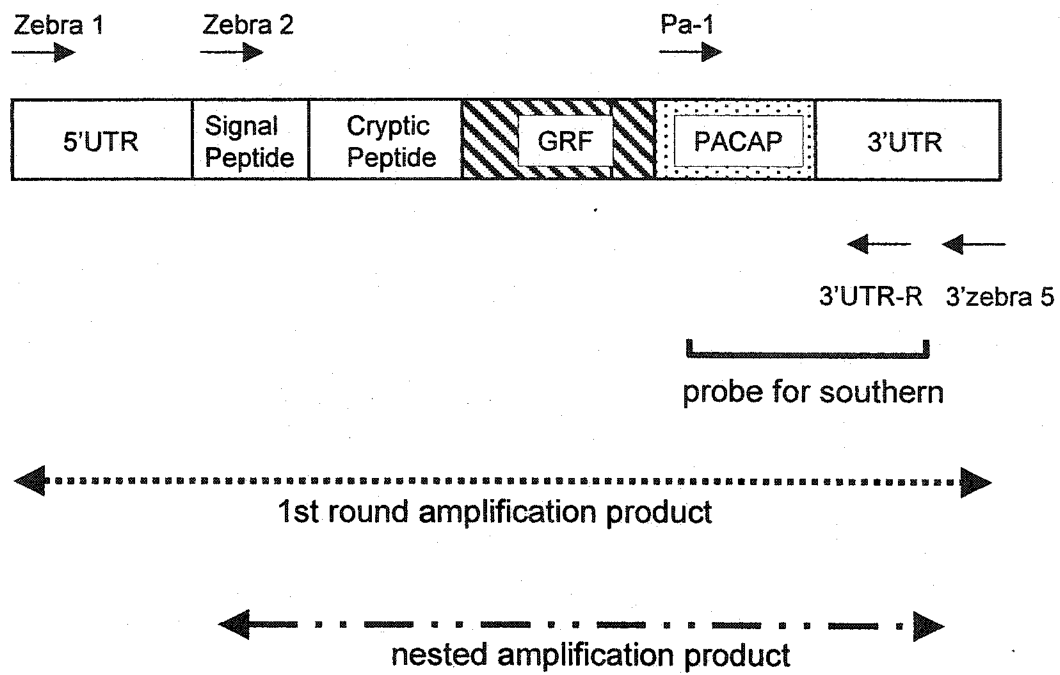
An adult zebrafish was ground in liquid nitrogen and digested with 10 mg of Proteinase K (Sigma, Oakville, ON) in 10 mM Tris-HCl pH 8.0, 100 mM EDTA and 0.5% SDS at 55°C overnight. The DNA was purified with one phenol extraction and two phenol/chloroform/isoamyl alcohol (24:24:1) extractions. A total of 13 μg of zebrafish genomic DNA per reaction was digested with *Hind* III, *EcoR* I, *Nco* I, *Not* I, *Sal* I, or *Sph* I (NEB, Mississauga, ON) and electrophoresed on a 1% agarose gel. DNA was capillary transferred according to the manufacturer's specifications to a nylon membrane (NEN Life Science Products, Guelph, ON). The membrane was prehybridized at 42°C in 6x SSC, 5x Denhardt's solution, 0.5% SDS and 300 μg of blocking DNA for 12 hours. Using primers PA-1 and 3'zebra 5 (Table 2.1) a 433 bp probe corresponding to exon 5 of the GRF-PACAP gene was amplified from zebrafish genomic DNA, as described above

at an annealing temperature of 52°C. The probe was radiolabeled with [α-^{32}P]-dCTP as described previously. The radiolabeled probe (3.6×10^7 cpm/ml) was hybridized at 54°C in 6x SSC, 5x Denhardt's solution, 0.5% SDS and 300 µg of blocking DNA for 12 hours. The membrane was washed twice in 2x SSC and 0.1% SDS at 54°C for 45 and 30 minutes, respectively, and twice in 1x SSC and 0.1% SDS at 50°C for 30 and 10 minutes, respectively. An additional wash in 0.5% SSC and 0.1% SDS was carried out for the high stringency condition. The membrane was then exposed to BioMax film (Kodak, Rochester, NY) for 5 days at -80°C.

mRNA isolation and cDNA synthesis

Both male and female adult zebrafish were over anaesthetized using MS222. Brain, heart, gill, gastrointestinal (GI) tract, gonad and muscle tissues were isolated and immediately frozen in liquid nitrogen. The tissues were individually ground to a fine powder using a micropestle (Diamed, Mississauga, ON) in 1.5 ml tubes that were chilled with liquid nitrogen. mRNA was isolated using the Poly (A) Pure Kit (Ambion, Austin, TX) as outlined by the manufacturer. Single stranded cDNA was synthesized using 2 mM of oligo(dT)$_{20}$ in 1x First Strand Buffer, 1.6 mM dNTPs, 8 mM DTT, 16 units of RNAguard (Pharmacia) and 200 units of Superscript II (GibcoBRL, Burlington, ON) to a final volume of 25 µl. The reaction was incubated at 42°C for 90 minutes and at 95°C for 10 minutes. The GRF-PACAP transcript was amplified by two successive rounds of PCR using nested primer sets (Fig. 2.4). Primers zebra 1 and 3'zebra 5 were used with 1 µl of cDNA as a template for the first PCR amplification, at an annealing temperature of 52°C. The GRF-PACAP transcript was amplified from 1 µl of the previous reaction using nested primers zebra 2 and 3'UTR-R (Table 2.1). PCR products (10 µl) from the

Figure 2.4 Schematic showing the primers used to amplify the GRF-PACAP mRNA transcript. First round and nested round PCR products are depicted by dashed arrows and the probe used for Southern analysis is shown.


Zebra 1
Zebra 2
Pa-1
5'UTR
Signal Peptide
Cryptic Peptide
GRF
PACAP
3'UTR
3'UTR-R
3'zebra 5
probe for southern
1st round amplification product
nested amplification product

second round of amplification were separated on a 1.5% agarose gel. Southern analysis to confirm the amplified products was performed as described by Lueders and Fewell (1994). The gel was dried on a vacuum gel drier for 30 minutes at room temperature and 30 minutes at 60°C. The dried gel was then hybridized to an [α-^{32}P]-dCTP labelled probe amplified with primers Pa-1 and 3'UTR-R (Fig. 2.4). An aliquot (2 μl) of the PCR products from the second round of amplification of the brain, eye, GI tract, ovary and testis was ligated into pGEM-T vector (Promega, Madison, WI) as recommended by the manufacturer and cloned. Recombinant plasmids were sequenced as previously described using forward and reverse primers. A control reaction using 1 μl of cDNA amplified with tubulin primers Tubulin 10 and Tubulin 11 (Table 2.1) was performed.

RESULTS

Isolation of zebrafish GRF-PACAP genomic clone

Approximately 500 000 plaque-forming units of recombinant phage from a zebrafish genomic library were screened with a 267 bp fragment amplified from zebrafish genomic DNA using gene specific primers. Five clones isolated after three successive rounds of screening were shown by PCR and sequencing to contain the zebrafish PACAP gene. Based on PCR results confirming that the full length GRF-PACAP gene was present, the λM4 clone was selected for further analysis and was digested with a number of restriction enzymes. Hind III was found to cut the λM4 insert into six fragments and Southern analysis revealed that the GRF-PACAP gene was localized in the 2.0 kb and the 5.0 kb fragments (Fig. 2.3). These fragments were subcloned and both strands were sequenced using gene specific primers.

Structure of the GRF-PACAP gene

The GRF-PACAP gene spans approximately 6.2 kb of genomic DNA and consists of five exons (Fig. 2.5 and Fig. 2.6). The intron exon junctions were confirmed by comparison with the cDNA sequence and all translated exons begin with the third nucleotide of a codon. The first exon encodes the 5'UTR of the GRF-PACAP mRNA. The second exon encodes the signal peptide and the amino terminal of the cryptic peptide. The third exon encodes more of the cryptic peptide. The fourth exon encodes the remainder of the cryptic peptide and the first 31 amino acids of the GRF-like peptide. The fifth exon encodes the remainder of the GRF-like peptide, the PACAP peptide and the 3'UTR (Fig. 2.5 and Fig. 2.6). The gene encodes an open reading frame of 173 amino acids (Fig. 2.6). The deduced amino acid sequence for the zebrafish GRF peptide shows high sequence identity (60-95%) with other fish species and only moderate sequence identity (35-41%) with mammalian species (Table 2.2). The deduced amino acid sequence for the zebrafish PACAP peptide shows high sequence identity with other vertebrates (84-92%) (Table 2.2).

Gene copy number of the GRF-PACAP gene

To determine the copy number of the zebrafish GRF-PACAP gene, total genomic DNA isolated from an adult zebrafish was digested and hybridized to a 422 bp PACAP specific probe. Single bands were found in all lanes except lane 2 in which two bands were seen (Fig. 2.7). Genomic DNA in this lane was digested with the enzyme *EcoR* I which cuts within the probe (Fig. 2.7). DNA digested with *Hind* III yielded a band at approximately 5 kb corresponding to know cut sites within the gene (Fig. 2.7).

Figure 2.5 Structural organization of the zebrafish GRF-PACAP genomic clone. *Hind* III subclones used to sequence the gene are shown above and the mRNA precursor structure is shown below.

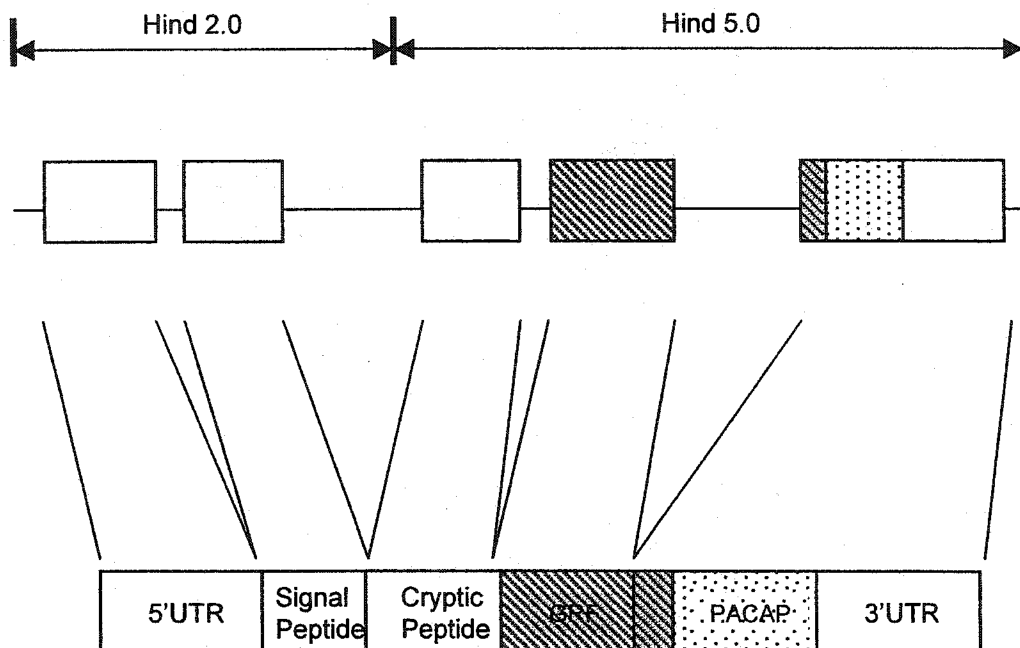

Hind 2.0
Hind 5.0
5'UTR
Signal Peptide
Cryptic Peptide
GRF
PACAP
3'UTR

Figure 2.6 Nucleotide sequence of the zebrafish GRF-PACAP gene. Exons are in bold letters with the translated exons in capitals and the introns and flanking regions in normal lowercase letters. The amino acid sequence for the precursor peptide is shown and numbered beginning with the methionine in the signal peptide. The sequence encoding the GRF peptide is double underlined and the sequence encoding the PACAP peptide is single underlined. Consensus sites for the TATA and CAAT boxes are underlined in the 5'flanking regions and four possible polyadenylation sites are underlined in the 3' region. The transcription start site and polyadenylation site are not known. Therefore, these exon boundaries are putative.

ttaatatttgcatttttccttttacaaatgggatttttttataatatctgaaaaataaatgact 64

gaaccgtcaaaaatgcccag**tgaactgtccatcagtcaatctgtttatctttatgtgacgattt** 128

aaaaaaatcaatctaagcacagaaagtacagtcggctcctaattagacttatttgggtctatat 192

ttgtatatggaagaagggaaattgcggtcgtgtctccatagtaaaggaggatgacgtggatcga 256

cgcgatctccaacgcggggagtgaaacggtgcataagtaagagctggaagaataaaactcgcga 320

tactgtaatgagagatgaagacaaacgatatctcgcctcaaatccgtccgactacgaagacctg 384

agagagagagagggaggaaagatacagacgctgtgggtaacaaagtgacgcgttgaaaagttta 448

aagagcaagactgggagagaaaggagagagagagagagagctggagaatttcatctcattctgg 512

acgcagcctccattggacagcatccgtccgctgccgcag gtaaatgcaaaacttctgccagatatt 576

ttaattcttgattttgggaatcgtacttcatttatagcaattagaaacagttaaaccacaactatactaga 640

ttattacacaggaatatcgaagattgaaagtgcttttttagacagaaatatggtactaatggatgtttttat 704

tctacag **a ATG ATT ACG AGC AGC AAA ACG ACT CTT GCT TTC CTC ATC TAT** 754

M I T S S K T T L A F L I Y **14**

GGG CTC CTA GTT CAA TGC AAT GTG TGT TCG CCT CTG AGT TAC CCG AAA 800

G L L V H C N V C S P L S Y P K **30**

ATC AG gtacgcatacggagcttcattgatatcaacgcacgggcgaggatttggagtttagatcacgag 868

I R **32**

aaacgttatttaagcggtgaa ← INTRON 2 → taaagtaacttttacaaatga 2587

aatgccttgatgtcttgttctag **A ATG GAG ACT GCA GGA TTT GAT GAG GAG GGA** 2641

M E T A G F D E E G **42**

AAC TCA TTA ACG GAT GTA ACA TTT GAC AGT GAC CAG ATC ACT ATA CGA 2689

N S L T D V T F D S D Q I T I R **58**

AGC TCT CCT ACA GTC ACT GAA GAC GCA TAC ACG TTA TTT AGT CCT CCA 2737

S S P T V T E D A Y T L F S P P **74**

TCA AAA AG gtgatcttcatactttacctccaaactcccattgcaggcatgtttatcattgtaatcgg 2804

S K R **77**

tcaggtctgtttttctgcgttaaggcatttggtgcacctgctgatttatgcaaagacatgaactggagaga 2875

ctgaaaagtcatgacaactgtgatgttctattagatgacatgaaattaatttgcatctctacgtgtttgt 2946

acaatctgccgttttgtcgcgcag **A CTG GAA AGG CAC GCT GAC GGG ATG TTT AAT** 3001

L E R H A D G M F N **87**

AAA GCC TAC AGG AAA GCG CTC GGC CAG TTA TCC GCG AGG AAG TAC CTG 3049

K A Y R K A L G Q L S A R K Y L **103**

CAT ACA CTG ATG GCA AAA CGT GTT GG gtaagatagactatatcttcatcttaatctct 3107

H T L M A K R V G **112**

ctctcttacttatccattatatggc ← INTRON 4 → tcttgaatgctttgtcctatttctc 5177

taacacag **A GGA GGG AGC ACA ACA GAA GAT GAC AAT GAA CCA CTC TCA**$_{45}$ 5225

G G S T T E D D N E P L S **125**

AAA CGT CAC TCG GAT GGG GTT TTC ACG GAC AGC TAC AGT CGC TAC CGG 5273

K R H S D G V F T D S Y S R Y R **141**

AAG CAA ATG GCC GTG AAG AAG TAT CTG GCC ACG GTC CTT$_{27}$**GGC AAA AGG** 5321

K Q M A V K K Y L A T V L G K R **157**

TAT AGA CAG AGA TAT AGA AGC AAA$_{38}$**GGA CGG CGG CTC GCT TAT TTG tag** 5369

Y R Q R Y R S K G R R L A Y L **stop** **173**

aatttttcaaatgcgtctctctctaaccatcaatgtacagctcctgtagcaagtcactattaag 5433

gctatggacctgtgtcctttctaaacgtgtatttatgtattaagtaaagatattacaatgaata 5497

ttttgataataatattgcttttcttttgtactgacgcacctgacaatgcaacaatctgccttgt 5561

ggatcagtcgtatagtctgaactttgaacaaatctaactatactacttatttatttatttattc 5625

agttgtttgtttatcatgcttgctccaatacactgtctattaaatacatcagtctgaatatgag 5689

taaataaatagtctcaatgactactttgaatgaaaccgtccttctttcactcctgacattaaga 5753

aagaagtgtttttatttacaacagccctgaagacttagatttgcaccaatttctacaagacgta 5817

gactttggtgtgctcccggatgaggatgagattgaggctttttttgttggattggctgagacagt 5881

tttcttctgaattcccggtgagtttttataaggctcagagggctgtctctcgctctgttcatac 5945

gcttttactcacattaaattcttcacttcccatctttggcttaaatccaagtcattcatctctt 6009

gcacaaacctttcaactattagtaatgctctacctttgattctgttctgaaatatttagcctgt 6073

aactgatcaaataacgctgaatctaaatggcatcttcagcattcttaattgattaaacactttc 6137

cacttcctcctgactttactgaatgtgcctttggtgccttttgaatcttcaccaaagaattaa 6201

agtctttgtctgtgaatttgtgtcttgtgtattacaatttattagtcagtggtttgtgattctt 6265

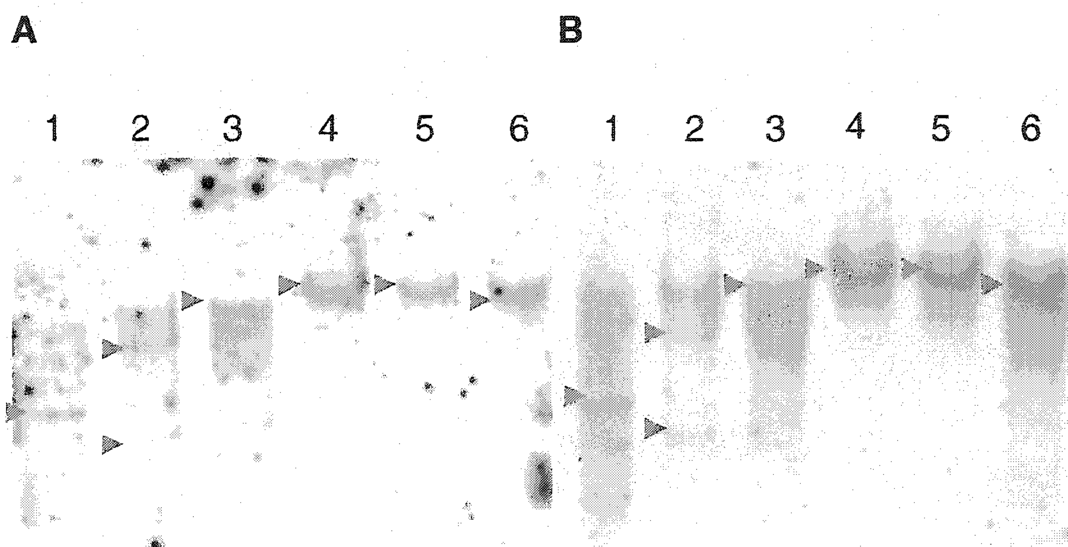
Table 2.2 The amino acid sequence identity of other forms of the GRF and PACAP peptides in comparison to the zebrafish. Dots indicate identical amino acids and the alternative amino acid is indicated when the sequences differ.

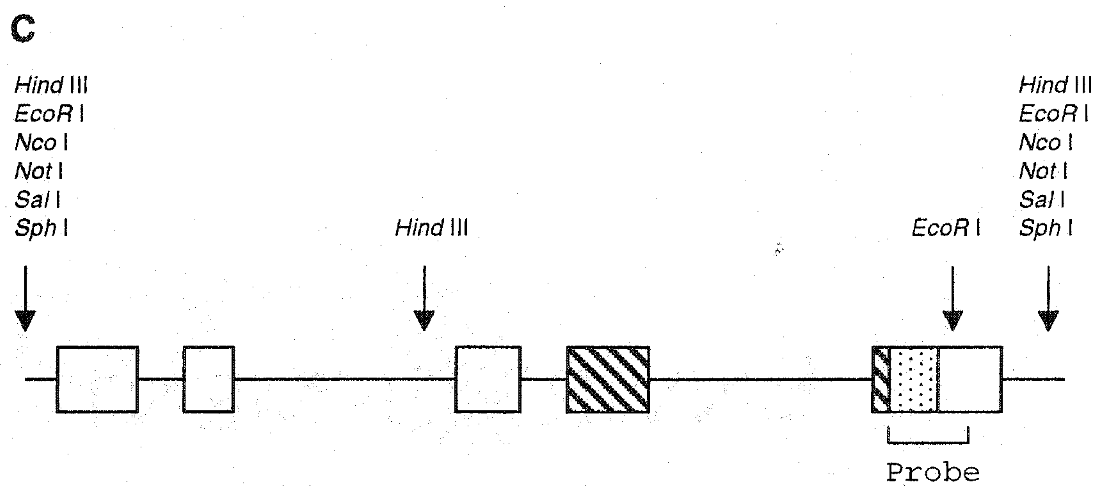
GRF		% IDENTITY
Zebrafish	$HADGMFNKAYRKALGQLSARKYLHTLMAKRVGGGSTTEDDNEPLS_{45}$	100
Carp[a]	$.................................MI........_{45}$	95
Salmon[b,c]	$.......................S...........M...S...._{45}$	93
Catfish[d]	$....LLDR.L.DI.V.........S.T.V...EEEED.E.S...._{45}$	60
Chicken[e]	$....I.S.....L.......N...S........A.SGLG.EAEPLS_{46}$	66
Rat[f]	$...AI.TSS..RI....Y...L..EI.NRQQ.ERNQEQRSRFN_{43}$	39
Mouse[g]	$.V.AI.TTN...L.S..Y...VIQDI.NKQ-.ERIQEQRARLS_{42}$	35
Human[h]	$Y..AI.TNS...V........L.QDI.SRQQ.ESNQERGARAR._{44}$	41

PACAP		% IDENTITY
Zebrafish	$HSDGVFTDSYSRYRKQMAVKKYLATVL_{27}GKRYRQRYRSK_{38}$	100
Salmon[b,c]	$....I...................A.._{27}..........N._{38}$	92
Catfish[d]	$....I...................A.._{27}.R.....F.N._{38}$	87
Stargazer[i]	$....I..............Q....A.._{27}.R.....V.N._{38}$	84
Stingray[j]	$....I...................A.._{27}....KPKVKNS_{38}$	76
Frog[k]	$....I...................A.._{27}....K..IKN._{38}$	84
Chicken[e]	$.I..I...................A.._{27}....K..VKN._{38}$	82
Mammalian[l]	$....I...................A.._{27}....K..VKN._{38}$	84

[a]Vaughan et al. 1992; [b]Parker et al. 1995; [c]Parker et al. 1997; [d]McRory et al. 1995; [e]McRory et al., 1997; [f] Spiess et al. 1983; [g]Frohman et al. 1989; [h]Ling et al. 1984a; [i]Matsuda et al. 1997; [j] Matsuda et al. 1998; [k]Chartrel et al. 1991; [l] Miyata et al. 1989

Figure 2.7 Southern blot analysis of zebrafish genomic DNA using a 422 bp zebrafish PACAP specific probe. (A) low stringency washes of 1x SSC and 0.1% SDS and (B) high stringency washes of 0.5x SSC and 0.1% SDS. Lane 1, *Hind* III; lane 2 *Eco R*I; lane 3, *Nco* I; lane 4, *Not* I; lane 5, *Sal* I; lane 6, *Sph* I. (C) Schematic of the zebrafish GRF-PACAP gene indicating restriction enzyme cut sites and the probe.


A
B
1
2
3
4
5
6
1
2
3
4
5
6


C
Hind III
EcoR I
Nco I
Not I
Sal I
Sph I
Hind III
EcoR I
Hind III
EcoR I
Nco I
Not I
Sal I
Sph I
Probe

Additionally, PCR products from the 5 clones isolated from the genomic library were identical.

Tissue expression of GRF-PACAP in the zebrafish

To determine the tissue distribution of the GRF-PACAP transcript in adult zebrafish, RT-PCR and subsequent Southern analysis were performed for both male and female fish. A nested primer set was used to amplify a transcript of approximately 650 bp from the brain, eye, GI tract, ovary and testis, but not from gill, heart and skeletal muscle (Fig. 2.8A). The tissue expression pattern and product size was identical in male and female fish (data for male not shown). The observed fragment was confirmed to represent the GRF-PACAP transcript by Southern analysis using a PACAP specific probe (Fig. 2.8B). The transcript from each tissue was sequenced and they were found to be identical to each other and to the coding region of the isolated genomic clone. Additionally, a 250 bp product was amplified from each tissue using the tubulin primers (Fig. 2.9). This indicated that the mRNA isolation and cDNA synthesis were successful in each tissue.

Discussion

Structure of the GRF-PACAP gene

A clone that encodes the GRF-PACAP gene was isolated from a zebrafish genomic library and its genomic sequence was determined. The genomic sequence for the GRF-PACAP gene has only been isolated in one other fish species, the salmon (Parker et al. 1997). The zebrafish GRF-PACAP gene encodes a signal peptide, cryptic peptide, and mature GRF and PACAP peptides. This gene arrangement has also been found in the

Figure 2.8 (A) RT-PCR amplification of the GRF-PACAP transcript using the nested primer set zebra 2 and 3'UTR-R from mRNA isolated from adult zebrafish tissues. (B) Southern blot analysis of the RT-PCR products using a 233 bp zebrafish PACAP specific probe. The amplified GRF-PACAP transcript is approximately 650 bp.

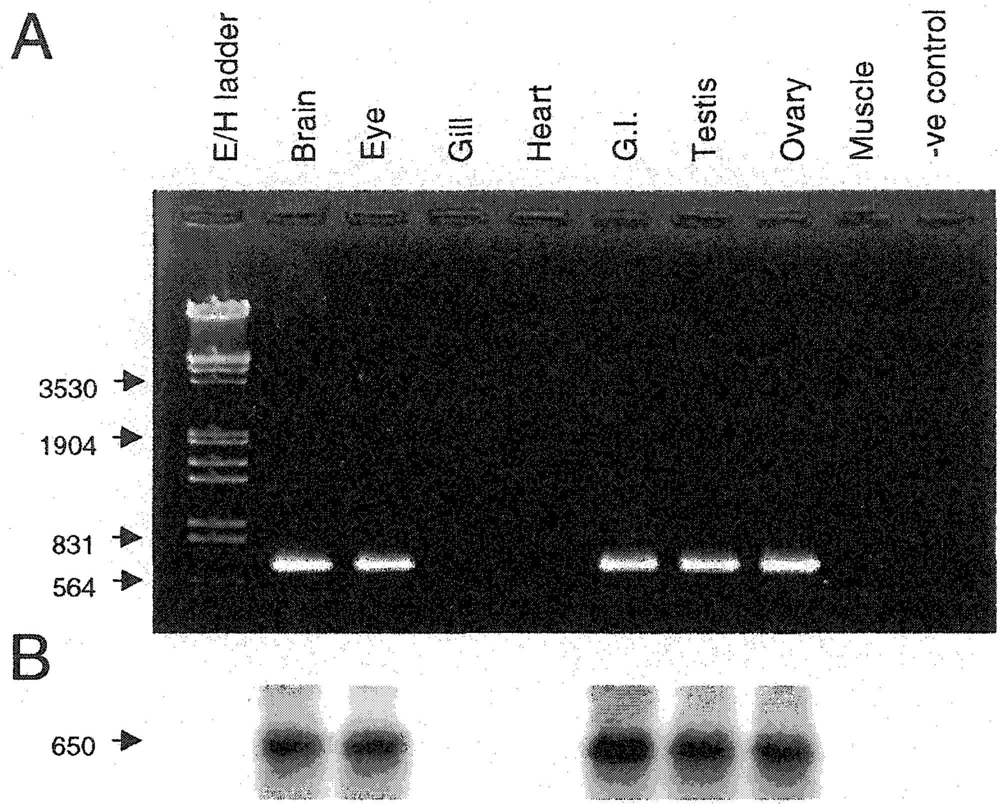
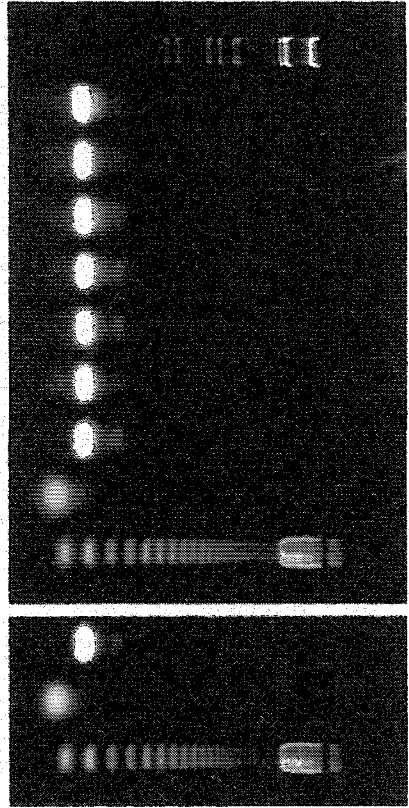

A
E/H ladder
Brain
Eye
Gill
Heart
G.I.
Testis
Ovary
Muscle
-ve control
3530
1904
831
564
B
650

Figure 2.9 RT-PCR amplification of the tubulin transcript from mRNA isolated from adult zebrafish tissues to confirm mRNA quality. The tubulin transcript is approximately 250 bp.


E/H ladder
Brain
Eye
Gill
Heart
G.I. tract
Ovary
Muscle
-ve control
123 bp ladder
Testis
-ve control
123 bp ladder
246 bp

salmon, chicken and frog (Parker et al. 1997; McRory et al. 1997; Alexandre et al. 2000). In contrast, the GRF and PACAP peptides are encoded on separate genes in mammals. It is hypothesized that a gene duplication event led to the mammalian organization (Sherwood et al. 1994).

GRF and PACAP are classified as members of the glucagon superfamily based on sequence identity, gene organization and precursor structure. Some members are encoded together on a single gene (PACAP/GRF, glucagon/GLP-1/GLP-2, VIP/PHM) and others are encoded singly (GIP, secretin, and in mammals GRF and PACAP). In humans, PACAP-28 and VIP share 68% sequence identity, whereas PACAP-38 and GRF share 24% sequence identity. Zebrafish GRF is 45 amino acids in length and is only moderately conserved; it shares 93% sequence identity with salmon GRF and only 41% identity with human GRF. Additionally, the length of the GRF peptide is not conserved; it varies between 46 amino acids in the chicken to 42 amino acids in the mouse. However, the variability in the length of the GRF molecule does not affect its ability to stimulate the release of GH from the pituitary somatotroph cells. It has been shown that only the first 29 amino acids are required for full biological activity (Ling et al. 1984b). Increased sequence identity, 55% between zebrafish and human GRF, is observed when only the first 29 amino acids are compared. In contrast to the GRF peptide, zebrafish PACAP-38 is highly conserved; it shares 92% sequence identity with salmon PACAP and 84% sequence identity with mammalian PACAP. Zebrafish PACAP is unusual in that there are two amino acid changes in the first 27 amino acids at positions 5 and 25 compared to human PACAP. This is in contrast to other PACAP-27 forms that are identical to human PACAP (Table 2.2).

The relationship of the GRF-PACAP gene to the PHI-VIP gene

The zebrafish GRF-PACAP gene has a clear structural relationship with the VIP gene. The organization of the two genes is similar in that the VIP gene encodes two peptides (PHI and VIP), as does the GRF-PACAP gene. Also, the sequence identities between PACAP and VIP, and GRF and PHI are higher than usual suggesting a duplication event in evolution. Zebrafish PACAP shares 78% sequence identity with VIP from either cod or dogfish (Thwaites et al. 1989; Dimaline et al. 1989). This is considerably higher than the 68% identity shared by PACAP and VIP in mammals. Additionally, zebrafish GRF shares 66% sequence identity with human PHM (Itoh et al. 1983), and 62% sequence identity with mouse PHI (Lamperti et al. 1991) but the identity to fish PHI can not be compared as the latter sequence is not known. The high amino acid sequence identity of zebrafish GRF and PACAP with PHM/I and VIP from other species supports the hypothesis that the VIP gene arose from a duplication of the PACAP gene. Also, the higher identity of fish VIP and PACAP compared to that of mammalian VIP and PACAP indicates that the duplication event may have occurred at some point close to the divergence of the piscine lineages.

Gene copy number

Our results to date indicate that the GRF-PACAP gene has only one copy in the zebrafish genome. However, analysis of the HOX clusters in the zebrafish indicates that a full or partial genome duplication took place in an ancestral fish. Phylogenetic analysis revealed that this was a fish-specific duplication event that occurred before the divergence of the zebrafish lineage (Amores et al. 1998). This duplication event would have occurred approximately 300 million years ago (Vogel, 1998). Therefore, the

presence of a single copy of the GRF-PACAP gene in the zebrafish may be due to a duplication event with only minor nucleotide substitutions or to chromosome loss after the duplication event. The first alternative is found in the salmon, but the two copies are distinguishable as distinct bands in a Southern blot (Parker et al. 1993; Krueckl and Sherwood 2001). The second alternative of chromosomal loss or partial duplication might explain the single copy of the GRF-PACAP gene in the zebrafish. Evidence from comparative genomics studies supports the second alternative. In zebrafish it appears that 20% of duplicated genes are retained after the duplication event (Postlethwait et al. 2000). However, further work is required to confirm the copy number of the GRF-PACAP gene in the zebrafish and map its chromosomal location.

Tissue expression pattern

In the zebrafish mRNA expression was noted in the brain, eye, GI tract, ovary and testis. These results confirm and extend previous studies in the catfish, salmon and goldfish. In the catfish mRNA expression was noted in the brain, stomach, testis and ovary (McRory et al. 1995). In the salmon expression was noted in the brain, pyloric cecum and intestine (Parker et al. 1997). In the goldfish expression was noted in the brain, pituitary, gill, gastrointestinal tract, kidney, ovary and testis (Wong et al. 1998). However, this is the first report of GRF-PACAP mRNA expression in the eye of a fish species.

The transcript isolated from each of these tissues encoded the full precursor including the signal peptide, cryptic peptide, GRF and PACAP. Comparison of the transcript sequence with the genomic clone revealed that it was identical to the coding region of the isolated GRF-PACAP gene. This indicates that a functional gene was

isolated from the zebrafish genomic library. In the salmon two transcripts are observed in the brain, pyloric cecum and intestine (Parker et al. 1997) and in the channel catfish two transcripts are observed in the brain, pituitary, fat GI, ovary, testis and skeletal muscle (Small and Nonneman 2001). In the chicken two transcripts are observed in the brain as well (McRory et al. 1997). In both catfish, salmon and chicken, the longer transcript contains the full precursor observed in the zebrafish, whereas the shorter transcript corresponds to an alternatively spliced mRNA lacking exon 4, which encodes the majority of the GRF domain (Small and Nonneman 2001; Parker et al. 1997; McRory et al. 1997). These alternative transcripts were not seen in either the zebrafish or the Thai catfish (McRory et al. 1995). This could indicate that the shorter transcript is not produced in these species or that it is produced at levels that were not detectable.

The endocrine significance of the presence of the GRF and PACAP peptides in the same gene is interesting. It means that in the zebrafish the expression of both peptides is controlled by the same regulatory mechanisms. Physiologically, one possibility is that both peptides have the same function, such as the release of GH. Another possibility is that one or both peptides modulate other factors in a tissue. In mammals the two peptides are encoded on separate genes, but they are still localized in some of the same tissues. Both GRF and PACAP are expressed in the brain, pancreas, intestine, ovary and testis (Arimura and Shioda, 1995; Mayo et al. 1995). This may indicate that the coordinated expression of GRF and PACAP is still conserved to some extent in mammals.

The expression pattern of zebrafish GRF-PACAP is similar to that found in other non-mammalian vertebrates. In the brain, PACAP has been shown to stimulate GH

release from salmon, eel and goldfish pituitary cells in a dose dependent manner (Parker et al. 1997; Montero et al. 1998; Wong et al. 1998) and to influence cAMP accumulation and calcium mobilization in the frog pituitary (Chartrel et al. 1991; Gracia-Navarro et al. 1992). In the ovary of the crested newt, PACAP may modulate steroid synthesis through the mediation of prostaglandins (Gobbetti et al. 1997). In the Atlantic cod PACAP immunoreactivity is found in nerve fibers throughout the GI tract and it has been shown to inhibit spontaneous contractions of smooth muscle of the proximal intestine (Olsson and Holmgren, 1994, 1999).

In conclusion, the zebrafish GRF-PACAP gene is similar in structure to that found in other non-mammalian vertebrates. The PACAP peptide is highly conserved in all species studied to date. In addition, zebrafish PACAP shows strong sequence identity with VIP providing further evidence for the evolution of the glucagon superfamily by gene duplication. The strong conservation of the PACAP peptide and its tissue distribution in a variety of vertebrates indicates that its function may be conserved. To further investigate the functional significance of the gene arrangement in fish and birds, characterization of the GRF and PACAP receptors is required. In the adult zebrafish, GRF-PACAP mRNA expression is found in tissues that are derived from all three embryonic germ layers. Therefore, two key questions are whether the GRF-PACAP gene is widely expressed in the embryo and whether the gene products influence the development of organs in which expression is found in the adult.

CHAPTER 3

Isolation and characterization of the receptors for GRF and PACAP in the zebrafish

INTRODUCTION

The receptors for GRF and PACAP are members of the G-protein-coupled receptor superfamily. All G-protein-coupled receptors share a common architecture comprised of seven transmembrane helices linking intracellular and extracellular loops. The extracellular domains of the receptors are involved in ligand binding and the intracellular domains are involved in G-protein activation (Wess 1997). In particular, the second intracellular loop, third intracellular loop and the intracellular carboxyl tail of the receptor appear to be important for G-protein activation (Spiegel et al. 1995).

The GRF receptor has been isolated from six mammalian species and one fish species (Chan et al. 1998). Two transcripts for the GRF receptor were isolated from the rat, one encoding a 423 amino acid product, and the other encoding a 464 amino acid product with a 41 amino acid insert in the third intracellular loop (Lin et al. 1992). Although both isoforms bind GRF with equal affinity the long isoform does not activate an intracellular signaling pathway (Mayo et al. 2000). Alternative processing of the GRF receptor transcript was found in humans, as well, resulting in the retention of an intron that leads to truncation of the receptor after the fifth transmembrane domain (Hashimoto et al. 1995). Additionally, the sheep and goat GRF receptors have a 16 amino acid deletion at their C-terminal end (Horikawa et al. 2001).

In mammals, the GRF receptor is expressed in the hypothalamus, pituitary, kidney, placenta, testis and ovary (Mayo et al. 2000). The distribution of the GRF receptor in the goldfish was similar to the distribution found in mammals. The transcript was detected in the brain, pituitary, gill, heart, gastrointestinal tract, spleen, testis and ovary (Chan et al. 1998). The presence of the GRF receptor in these tissues indicates that

GRF has a functional role in these tissues. Although the function of GRF in the hypothalamus, pituitary and gonads has been investigated, little is know about its function in other tissues.

The physiological actions of PACAP are mediated through the activation of two types of receptors. In one type of receptor, PACAP shares its binding sites with vasoactive intestinal peptide (VIP), which is a related family member. The second type of receptor binds specifically with PACAP. Thus, the PACAP receptors are classified based on their binding affinities for the two peptides. The PACAP-specific (PAC_1) receptor exhibits 100 to 1000 fold greater affinity for PACAP over VIP, whereas the receptors shared by VIP and PACAP (VPAC) exhibit equal binding affinities for both peptides (Rawlings and Hezareh 1996). In mammals three receptors have been isolated, the PAC_1 receptor, $VPAC_1$ receptor and $VPAC_2$ receptor. In addition, nine isoforms of the PAC_1 receptor have been isolated from mammals. Six of these isoforms involve the inclusion or exclusion of three cassettes alone or in combination, termed hip, hop1 and hop2, in the third intracellular loop, which is involved in G-protein activation (Spengler et al. 1993). Two other isoforms involve amino acid deletion of 21 (PAC_1vs-21) or 57 (PAC_1vs-57) residues at the N-terminal end of the receptor, which is involved in ligand binding (Pantaloni et al. 1996; Dautzenberg et al. 1999). The ninth isoform PAC_1TM4 involves alterations in transmembrane domains 2 and 4 (Chatterjee et al. 1996). In non-mammalian vertebrates the PAC_1 receptor has been identified in the goldfish (Wong et al. 1998), zebrafish (Wei et al. 1998), frog (Hu et al. 2000), and chicken (Peeters et al. 1999). However, the sequence for the PAC_1 receptor in zebrafish has not been published. Of the nine PAC_1 receptor isoforms found in mammals, only the short and hop1 isoforms

from chicken and short and hop2 isoforms from zebrafish have been isolated in non-mammalian vertebrates (Peeters et al. 1999; Wei et al. 1998). The $VPAC_1$ receptor has been identified in the goldfish, salmon, frog, lizard, pigeon and chicken (Chow et al. 1997; Alexandre et al. 1999). Attempts to clone the $VPAC_2$ receptor from a non-mammalian vertebrate have been unsuccessful.

In mammals the PAC_1 receptor is distributed in the central nervous system, peripheral nervous system, eye, pituitary, adrenal gland, pancreas, liver, ovary, lung and stomach (Vaudry et al. 2000b). The wide spread expression pattern of the PAC_1 receptor appears to be conserved throughout evolution. In the chicken and the goldfish, the PAC_1 receptor is expressed in the brain and a variety of peripheral tissues (Peeters et al. 1999; Wong et al. 1998). In mammals the $VPAC_1$ receptor and $VPAC_2$ receptor have different expression patterns, although some overlap is seen. The $VPAC_1$ receptor is found in the central nervous system, lung, adrenal gland, small intestine and thymus and the $VPAC_2$ receptor is found in the hypothalamus, pituitary, adrenal gland, pancreas, stomach, ovary and testis (Usdin et al. 1994). Similarly to the PAC_1 receptor, expression of the VPAC receptor appears to be conserved in non-mammalian vertebrates. The frog VPAC receptor is widely expressed and the expression overlaps with that of both the $VPAC_1$and $VPAC_2$ receptors found in mammals (Alexandre et al. 1999).

To understand the physiological actions of GRF and PACAP in the zebrafish, their receptors must be isolated and their distribution characterized. Therefore, the objectives of this study were to isolate, sequence and characterize the expression of the GRF, PAC_1 and VPAC receptors in the adult zebrafish. In this chapter, I describe the

partial nucleotide sequences for the GRF, PAC_1 and VPAC receptor transcripts and the tissue expression of the GRF, PAC_1 and VPAC receptor transcripts in the adult zebrafish.

MATERIALS AND METHODS

Amplification of partial cDNAs for the GRF, PAC_1 and VPAC receptors

PCR amplification of the GRF receptor was performed using primers GRF 5 and GRF 7 (Table 3.1) that were designed against transmembrane domains 5 and 7 of the goldfish GRF receptor (Wong et al. 1998). PCR amplification of the PAC_1 and $VPAC_1$ receptors was performed using primers GF-PAC1-FW and GF-PAC1-RV (Table 3.1). I designed these primers against conserved regions in transmembrane domain 2 and 7 of the goldfish PAC_1 receptor (Wong et al. 1998). A 1μl aliquot of the zebrafish cDNA library (a gift from Christiane Goblet, Institut Pasteur, France) was added to a 50 μl volume containing: 200 μM dNTPs, 2 mM $MgCl_2$, 0.4 μM of each primer and 2.5 units of *Taq* DNA polymerase (Gibco BRL, Burlington, ON). The reaction was heated to 94 °C for 2 minutes, then cycled 30 times at 94°C for 30 sec., 52°C for 45 sec., 72°C for 1 min. Aliquots of 10 μl from the PCR products were separated on a 1.5% agarose gel. The PCR products were ligated into pGEM-T vector as specified by the manufacturer (Promega, Madison, WI) and cloned. Recombinant plasmids were sequenced using an Applied Biosystems Incorporated Prism 377 DNA Sequencer.

mRNA isolation and cDNA synthesis

For the tissue expression study, both male and female adult zebrafish were over anaesthetised using MS222. Brain, eye, heart, gill, gastrointestinal (GI) tract, gonad, skeletal muscle and swim bladder tissues were isolated and immediately frozen in liquid

Table 3.1 Primer sequences for the zebrafish GRF receptor, PAC_1 receptor and VPAC receptor reported in the 5' to 3' direction of the sense strand for forward primers and 5' to 3' direction of the antisense strand for reverse primers. Direction of the primer is represented by the arrows, forward primers are represented by (→) and reverse primers are represented by (←).

Primer Name	Direction	Location	Gene Target	Sequence 5' to 3'
GRF 5	→	TMD 5	GRF receptor	TGGTGGATCATTAAAGGAC
GRF 7	←	TMD7	GRF receptor	AAGCCCTGGAAAGAGCCCAG
gf-PAC1-FW	→	TMD2	PACAP receptor	ATCCACATGAACCTGTTTGT
gf-PAC1-RV	←	TMD 7	PACAP receptor	TCCAACTCCTCCATTTCCTCTT
PAC1R5'	→	ECL 1	PAC_1 receptor	TGCTTCGTTCACACTGTGGGCT
PAC1R3'1	←	ICL 3	PAC_1 receptor	AATGCTGGACTCGTTTCCGCCG
PAC1R3'2	←	ECL 3	PAC_1 receptor	TGCGCTCCCGCTTGCTGAAATC
VPAC FW	→	ECL 1	$VPAC_1$ receptor	TGCTCCACTGGTTCTGTTGGCT
VPAC3'	←	ICL 3	$VPAC_1$ receptor	TTGGTTTGATTCATTTCTCCCG
VPAC RV	←	ECL 3	$VPAC_1$ receptor	GTACAGCGACCACAAAACCCTG

Note: Abbreviations are: ECL, extracellular loop; ICL, intracellular loop;TMD, transmembrane domain

nitrogen. The tissues were individually ground to a fine powder using a micropestle (Diamed, Mississauga, ON) in 1.5 ml microfuge tubes that were chilled with liquid nitrogen. The mRNA was isolated using the Poly (A) Pure Kit (Ambion, Austin, TX) as outlined by the manufacturer. Single stranded cDNA was synthesized using 2 mM of oligo$(dT)_{20}$ in 1x First Strand Buffer, 1.6 mM dNTPs, 8 mM DTT, 16 units of RNAguard (GibcoBRL, Burlington, ON) and 200 units of Superscript II (GibcoBRL, Burlington, ON) to a final volume of 50 µl. The oligo dT primer was annealed to the mRNA at 70°C for 10 minutes, then the remaining reagents were added and the reaction was incubated at 42°C for 90 minutes and at 95°C for 10 minutes.

PCR amplification of the receptors

PCR amplification of the GRF receptor was performed using the primers GRF 5 and GRF 7 (Table 3.1). Amplification of the PAC_1 receptor was performed using the primers PAC1R5' and PAC1R3'2 and amplification of the VPAC receptor was performed using primers VPAC FW and VPAC RV (Table 3.1). The primers were designed against extracellular loops 2 and 4 of the partial cDNA sequences for the appropriate receptors that I had previously isolated from the zebrafish cDNA library (Fig. 3.1). Receptor transcripts were amplified using 3 µl of the cDNA as a template as described above at an annealing temperature of 55°C. PCR products (15 µl) were separated on a 2% agarose gel. A control reaction using 2 µl of cDNA amplified with tubulin primers Tubulin 10 and Tubulin 11 (see Table 2.1) was performed.

For tissues in which two bands were amplified, the eye and testis for the GRF receptor, and the brain, gill, testis and ovary for the PAC_1 receptor, a 3 µl aliquot of the PCR products was ligated into pGEM-T vector (Promega, madison WI) as recommended

by the manufacturer and cloned. In addition, a 3 μl aliquot of the PCR products of the VPAC receptor from the testis and ovary were ligated into pGEM-T vector (Promega, Madison, WI) as recommended by the manufacturer and cloned. Recombinant plasmids were isolated and sequenced as previously described to determine which isoforms of the receptors were found in each tissue.

Southern analysis of RT-PCR results

The PCR products (15 μl) were separated on a 1% agarose gel and the DNA was capillary transferred in 0.4 M NaOH according to the manufacturer's specifications to a GeneScreen nylon membrane (NEN Life Science Products, Guelph, ON). The membrane was washed in 6x SSC for 2 minutes and then allowed to air dry. It was then prehybridized at 56°C in 6x SSC, 5x Denhardt's solution, 0.5% SDS and 15 μg of blocking DNA for 3 hours. Zebrafish specific probes for each receptor of approximately 250 to 400 nucleotides in length were amplified. A 266 bp probe for the GRF receptor was amplified from the previously isolated GRF receptor clone using primers GRF 5 and GRF 7 (Table 3.1). A 387 bp probe was PCR amplified from the previously isolated PAC_1 receptor clone using primers PAC1R5' and PAC1R3'1 (Table 3.1). Similarly, a 390 bp probe was PCR amplified from the VPAC receptor clone using primers VPAC FW and VPAC3' (Table 3.1). PCR amplification was carried out as previously described at an annealing temperature of 55°C. The GRF, PAC_1 and VPAC receptor probes were labeled with [α-^{32}P]-dCTP (NEN Life Science Products, Guelph ON) using the Random Primers DNA Labelling System (Gibco BRL, Burlington, ON) as specified by the manufacturer. The membranes were hybridized separately in a solution containing 6x SSC, 5x Denhardt's solution, 0.5% SDS, 15 μg of blocking DNA and either $8.3x10^7$

cpm/ml of the GRF probe, $5.8x10^7$ cpm/ml of the PAC_1 probe or $4.7x10^7$ cpm/ml of the VPAC probe at 55°C overnight. The membranes were washed once with 1x SSC and 0.1% SDS, twice with 0.5x SSC and 0.1% SDS, and once with 0.1x SSC and 0.1% SDS at 55°C for 30 minutes each. The membranes were then exposed to a phosphor screen (Molecular Dynamics) for 30 minutes at room temperature. The exposed screen was scanned with a Storm 860 scanner (Molecular Dynamics) and analysed using ImageQuant software.

RESULTS

Isolation of the GRF receptor

To isolate the GRF receptor from the zebrafish, the goldfish cDNA was compared to the human cDNA and primers were designed against conserved regions in transmembrane domains 5 and 7. A partial cDNA clone that shared 91% sequence identity with the goldfish GRF receptor was isolated from the adult zebrafish brain. The transcript was 266 nucleotides in length spanning from transmembrane domain 5 to the beginning of transmembrane domain 7 (Fig. 3.1).

Isolation of the PAC_1 and VPAC receptors

To isolate the PAC_1 receptor from the zebrafish, regions of the goldfish cDNA were compared with the human, rat and mouse PAC_1 receptor, since the sequence for the zebrafish receptor is not published. Primers were designed against conserved regions of the goldfish receptor in transmembrane domain 2 and the C-terminal cytoplasmic tail. In my study a partial cDNA clone with high sequence identity to the PAC_1 receptor was isolated from the adult zebrafish brain. The transcript was 706 nucleotides in length and

Figure 3.1 Partial nucleotide sequence from cDNA isolated from an adult zebrafish brain encoding the GRF receptor. Transmembrane domains are singly underlined and primer sequences are underlined with dashes.

```
TGG TGG ATC ATT AAA GGA CCG ATC ACC GCT TCA CTG TTT     39
                        TMD-5
GCT AAT ATC ATC ATC TTC CTG AAT GTG ATT CGC ATA TTG     78

GTT CAG AAA TTG AAA AGT CCT GGA GTC GGG GGC AAT GAT    117

ACG GGT CAC TTC ATG AGA CTG GCC AAG TCC ACA CTG TTT    156

CTG ATT CCT CTG TTC GGG ATG CAC TAC ACT CTG TTC GCC    195
                        TMD-6
TTC CTG CCA GAA AAC ACT GGA GAA ATA ATC CGC TTT TAC    234

ATC GAG CTC GGC CTG GGC TCT TTC CAG GGC TT             266
                        TMD-7
```

spanned from transmembrane domain 2 to the beginning of the C-terminal cytoplasmic tail involved in G protein binding (Fig. 3.2). The isolated zebrafish PAC_1 receptor shared high sequence similarity with other PAC_1 receptors, 87% with the goldfish PAC_1 receptor and 75% the with human PAC_1 receptor (Table 3.2). In addition, the hop cassette isolated from the zebrafish shared 71% amino acid and nucleotide sequence identity with the rat hop cassette.

The same primers that were used to isolate the PAC_1 receptor amplified a second clone. This second partial cDNA showed high sequence identity with the VPAC receptor. The second transcript was 699 nucleotides in length and spanned the same region as seen in the PAC_1 receptor from transmembrane domain 2 to the beginning of the C-terminal cytoplasmic tail (Fig. 3.3). The zebrafish VPAC clone shared high (90%) sequence similarity with the goldfish VPAC receptor and moderate (58%) sequence identity with the human $VPAC_1$ receptor (Table 3.3). In addition, the zebrafish PAC_1 and VPAC receptors shared only moderate (57%) sequence similarity. This level of sequence similarity between the PAC_1 and $VPAC_1$ receptors is found in other species including the goldfish (56%) and human (54%). Therefore, both receptors have been conserved throughout evolution, with the PAC_1 receptor being more tightly conserved.

Tissue expression of the GRF receptor in the adult zebrafish

To determine where GRF may elicit a physiological action, the tissue distribution of its receptor was determined. The GRF receptor transcript is expressed in the brain, eye, gill, GI tract, testis and swim bladder (Fig. 3.4). In both the eye and testis Southern analysis revealed the presence of a second longer transcript (Fig. 3.4). It appears that this

Figure 3.2 Partial nucleotide sequence from cDNA isolated from an adult zebrafish brain encoding the PAC_1 receptor. Transmembrane domains are singly underlined and primer sequences are underlined with dashes. The arrow at nucleotide position 485 indicates the site of insertion of the 3rd intracellular loop cassettes.

```
ATC CAC ATG AAC CTG TTT GTG TCC TTC ATC CTG AGG GCC    39
                         TMD-2
ATT TCT GTC TTT ATT AAA GAT GGA GTT CTG TAT GCA GAG    78

GAG GAC AGT GAC CAC TGC TTC GTT CAC ACT GTG GGC TGT   117

AAA GCG GTG ATG GTG TTT TTC CAC TAC TGC GTG ATG TCA   156
                         TMD-3
AAC TAC TTC TGG CTC TTC ATC GAA GGT CTT TAT CTC TTT   195

ACT CTT CTG GTC GAA ACC TTC TTT CCA GAG AGA CGC TAC   234

TTT TAC TGG TAC ACC ATC ATC GGC TGG GGA ACG CCC ACC   273
                         TMD-4
ATT TGT GTG ACC ATA TGG GCC GTT CTG CGC CTT CAT TTT   312

GAT GAT TCT GGC TGC TGG GAC ATG AAT GAT AAC ACT GCC   351

CTC TGG TGG GTG ATC AAG GGC CCT GTG GTG GCA TCA ATC   390
                         TMD-5
ATG ATT AAC TTT GTG CTG TTC ATT GGG ATC ATC ATA ATC   429

CTG GTG CAG AAG CTT CAG TCT CCA GAC ATC GGC GGA AAC   468
                     ⇩
GAG TCC AGC ATT TAT CTA CGT CTG GCG CGC TCC ACC CTT   507

CTC CTC ATT CCT CTG TTT GGG ATC CAC TAC ACT GTG TTT   546
                         TMD-6
GCC TTT TCC CCG GAG GAT TTC AGC AAG CGG GAG CGA CTG   585

GTC TTT GAG CTC GGC CTG GGC TCC TTC CAA GGG TTT GTT   624
                         TMD-7
GTG GCT GTG CTG TAC TGC TTT CTG AAT GGA GAG TTT CTC   663

CCT AAA GGT GCA GTC GGA GAT AAA GAG GAA ATG GAG GAG   702

TTG G                                                 706
```

Table 3.2 Nucleotide sequence pair distances for the partial cDNA of the PAC_1 receptor, using Clustral method.

Percent Similarity

	1	2	3	4	5	6		
1		87.0	74.6	75.4	73.5	74.6	**1**	*zebrafish*
2	10.0		74.6	79.0	70.9	79.0	**2**	*goldfish*
3	28.1	27.2		75.8	66.5	75.3	**3**	*frog*
4	24.1	21.3	27.5		86.0	90.0	**4**	*rat*
5	38.8	44.8	53.1	27.7		79.3	**5**	*mouse*
6	24.3	20.8	28.0	10.5	34.4		**6**	*human*

Percent Divergence

Figure 3.3 Partial nucleotide sequence from the cDNA isolated from an adult zebrafish brain encoding the VPAC receptor. Transmembrane domains are singly underlined and primer sequences are underlined with dashes.

```
ATC CAC ATG AAC CTG TTT GTG TCC TTC ATT CTG AAA GCC    39
                        TMD-2
ATC GCG GTT TTC GTC AAA GAT GCA GTG CTG TAC GAT GTT    78

GTC CAG GAG TCT GAC AAC TGC TCC ACT GGT TCT GTT GGC   117

TGC AAA GCT GTG ATT GTG TTT TTC CAG TAC TGC ATT ATG   156
                        TMD-3
GCC AGT TTC TTC TGG CTG CTG GTG GAA GGT CTG TAC ATT   195

CAT GCT CTT CTC GCC GTC TCC TTC TTT TCT GAG AGG AAG   234

TAC TTC TGG TGC TAC ATC CTC ATC GGT TGG GGA GGC CCA   273
                        TMD-4
ACC GTT TTC ATC ATG GCC TGG AGC TTT GCC AAG GCT TAC   312

TTC AAT GAT GTT GGA TGC TGG GAT ATA ATT GAA AAT TCC   351

GAT CCT TTT TGG TGG ATT ATC AAA ACC CCG ATA TTA GCT   390
                        TMD-5
TCC ATT CTG ATG AAC TTT ATT CTG TTC ATT TGT ATC ATC   429

CGG ATA CTG AGG CAG AAG ATC AAC TGT CCT GAC ATC GGG   468

AGA AAT GAA TCA AAC CAA TAT TCG AGG CTG GCA AAA TCA   507

ACA CTT CTG TTG ATT CCA CTC TTT GGA ATA AAC TTT ATC   546
                        TMD-6
ATA TTC GCC TTC ATT CCG GAG AAC ATC AAA ACC GAG CTA   585

CGG CTT GTG TTT GAC CTC ATT CTC GGT TCA TTT CAG GGT   624
                        TMD-7
TTT GTG GTC GCT GTA CTC TAC TGT TTC CTA AAT GGA GAG   663

GTC CAA GGT GAA ATC AAG AGG AAA TGG AGG AGT TGG       699
```

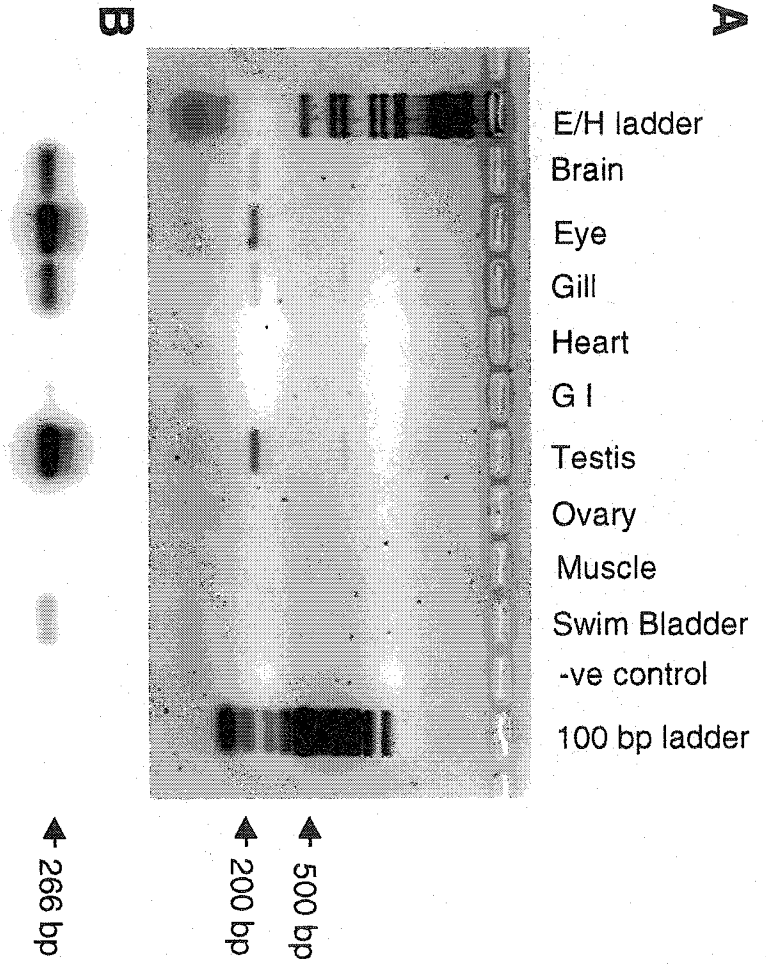
Table 3.3 Nucleotide sequence pair distances for the partial cDNA of the $VPAC_1$ receptor, using Clustral method.

Percent Similarity

	1	2	3	4	5	6		
1		89.5	64.3	61.7	60.2	58.4	**1**	*zebrafish*
2	9.0		66.0	66.4	62.6	60.9	**2**	*goldfish*
3	35.2	35.6		73.5	68.4	67.3	**3**	*frog*
4	37.8	33.6	28.0		67.6	66.3	**4**	*chicken*
5	41.3	40.5	33.6	33.8		85.3	**5**	*mouse*
6	42.7	41.1	35.5	34.1	13.9		**6**	*human*

Percent Divergence

Figure 3.4 (A) RT-PCR amplification of the GRF receptor transcript from mRNA isolated from adult zebrafish tissue. (B) Southern blot analysis of the RT-PCR products using a 266 bp zebrafish GRF receptor specific probe. The amplified transcript is 266 bp in length.


A
B
E/H ladder
Brain
Eye
Gill
Heart
G I
Testis
Ovary
Muscle
Swim Bladder
-ve control
100 bp ladder
266 bp
200 bp
500 bp

transcript is expressed at lower levels than the normal transcript, which may explain why attempts to clone and sequence the longer transcript were unsuccessful.

Tissue expression of the VPAC and PAC_1 receptors in the adult zebrafish

To determine the tissues in which PACAP may elicit a physiological action, the tissue distribution of both the PAC_1 and VPAC receptors was determined. The PAC_1 receptor is widely expressed in the adult zebrafish and the mRNA transcript is found in the brain, eye, gill, gastrointestinal tract, testis, ovary and skeletal muscle. Two transcripts are evident in the brain, gill, testis and ovary (Fig. 3.5). The short transcript encodes the PAC_1 short receptor isoform. In the brain and testis, the long transcript encodes a PAC_1 receptor isoform with the hop1 cassette in the third intracellular loop, whereas in the ovary the long transcript encodes the hop2 cassette (Fig. 3.6 and Table 3.4). In the gill, the long transcript encodes a novel PAC_1 receptor isoform. This transcript encodes a novel 106 nucleotide cassette in the third intracellular loop that causes a frameshift (Fig. 3.7). A possible transmembrane domain 6 that has a high (65%) concentration of hydrophobic amino acid residues is identified after the frameshift (Fig. 3.7).

In the adult zebrafish the VPAC receptor is more widely expressed than the PAC_1 receptor. The mRNA transcript for the VPAC receptor is found in all tissues studied, including the brain, eye, gill, heart, gastrointestinal tract, testis, ovary, skeletal muscle and swim bladder (Fig. 3.8). The tissues in which the GRF-PACAP, GRF receptor, PAC_1 receptor and VPAC receptor transcripts are expressed are summarized in Table 3.4. The quality of the cDNA was confirmed by amplification of the tubulin transcript from

Figure 3.5 (A) RT-PCR amplification of the PAC_1 receptor transcript from mRNA isolated from adult zebrafish tissues. (B) Southern blot analysis of the RT-PCR products using a 387 bp zebrafish PAC_1 receptor specific probe. The amplified transcripts were approximately 490 and 575 nucleotide in length.

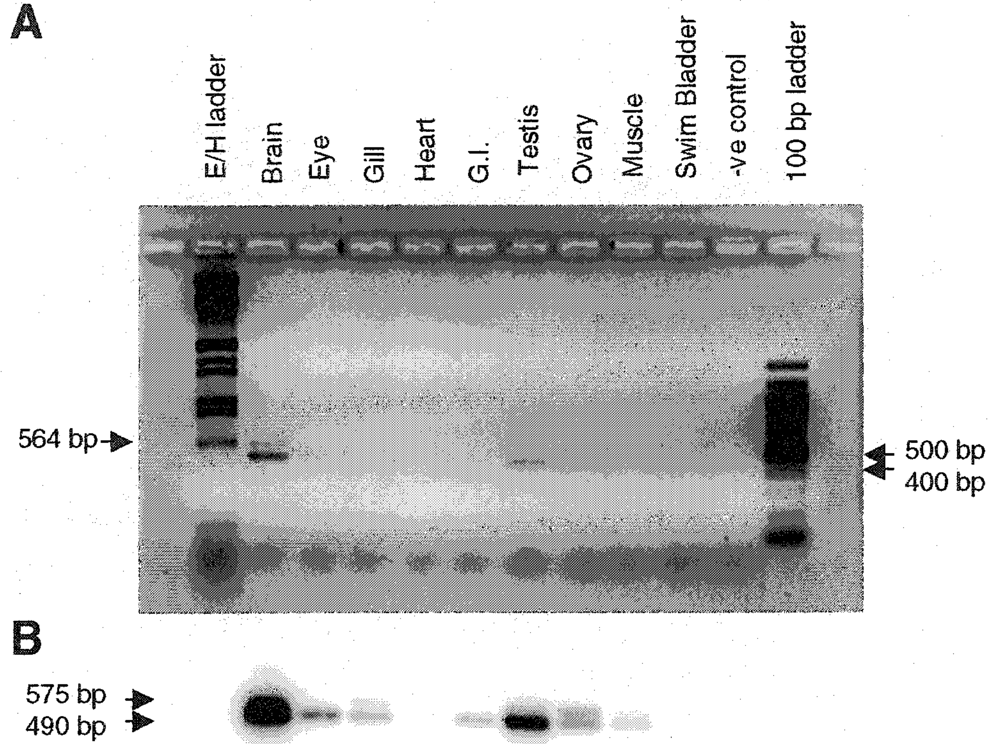
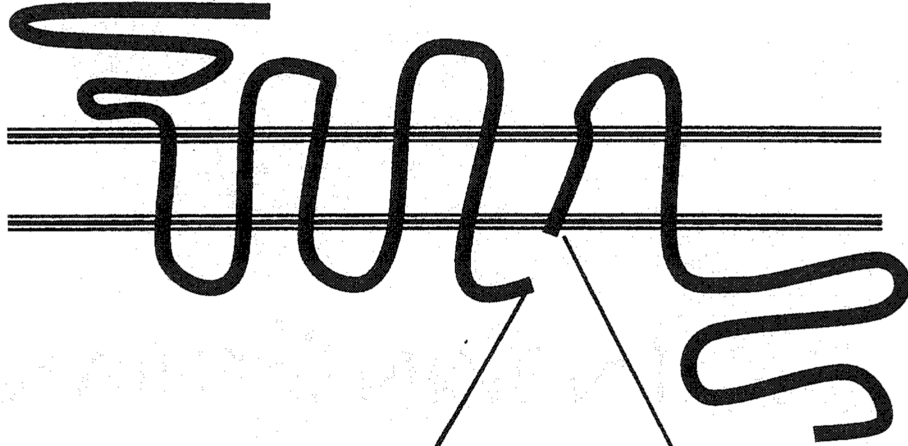

A
E/H ladder
Brain
Eye
Gill
Heart
G.I.
Testis
Ovary
Muscle
Swim Bladder
-ve control
100 bp ladder
564 bp
500 bp
400 bp
B
575 bp
490 bp

Figure 3.6 Schematic representation of a seven transmembrane spanning G-protein-coupled receptor showing the site of insertion and nucleotide sequences of the hop cassettes in the 3rd intracellular loop. The nucleotide sequences correspond to the hop 1 and hop 2 cassettes isolated from the adult zebrafish.



Hop1- gcatttatct-CAGCTGTGCACAGAAATGCTTCAGTGAGCCCACACAGGCT
Hop2- gcatttatct-   CTGTGCACAGAAATGCTTCAGTGAGCCCACACAGGCT

GTCCAGCATTCGTGCAGGATGTCAGAGCTTTCTACCATCACGCT-acgtctggc -Hop1
GTCCAGCATTCGTGCAGGATGTCAGAGCTTTCTACCATCACGCT-acgtctggc -Hop2

Table 3.4 Tissue distribution of the mRNA transcripts for the GRF-PACAP gene and its receptors in the adult zebrafish.

TISSUE	GRF-PACAP	GRF R		VPAC R	PAC_1-R			
		(S)	(L)		short	Hop 1	Hop 2	Novel
Brain	*	*		*	*	*		
Eye	*	*	*	*	*			
Gill		*		*	*			*
Heart				*				
G.I.	*	*		*	*			
Testis	*	*	*	*	*	*		
Ovary	*			*	*		*	
Sk. Muscle				*	*			
Swim Bladder		*		*				

Note: Sk. represents skeletal

Figure 3.7 Partial nucleotide sequence from cDNA isolated from the gill of an adult zebrafish encoding a novel isoform of the PAC_1 receptor. Transmembrane domains are singly underlined and the novel 106 nucleotide cassette in the third intracellular loop is in bold italics. Primer sequences are underlined with dashes.

TGC TTC GTT CAC ACT GTG GGC TGT AAA GCG GTG ATG GTG 39

TTT TTC CAC TAC TGC GTG ATG TCA AAC TAC TTC TGG CTC 78

TMD-3

TTC ATC GAA GGT CTT TAT CTT TTT ACT CTT CTG GTC GAA 117

ACC TTC TTT CCA GAG AGA CGC TAC TTC TAC TGG TAC ACC 156

ATC ATC GGC TGG GGA ACG CCC ACC ATT TGT GTG ACC ATA 195

TMD-4

TGG GCC GTT CTG CGC CTT CAT TTT GAT GAT TCT GGC TGC 234

TGG GAC ATG AAT GAT AAC ACT GCC CTC TGG TGG GTG ATC 273

AAG GGC CCT GTG GTG GCA TCA ATC ATG ATT AAC TTT GTG 312

TMD-5

CTG TTC ATT GGG ATC ATC ATA ATC CTG GTG CAG AAG CTT 351

CAG TCT CCA GAC ATC GGC GGA AAC GAG TCC AGC ATT TAT 390

CT***C TTG CTT GCT TCA CCG GAC TTA AAT CCG AGG GCT TTG*** 429

TAC ACC GTA CAA TGT AAG CTA CTG AGC AAA CTG ACC ACA 468

CCA GAA AAG TTG ACC ATG TGG ACA TAG ATA GAC GTC TGG 507

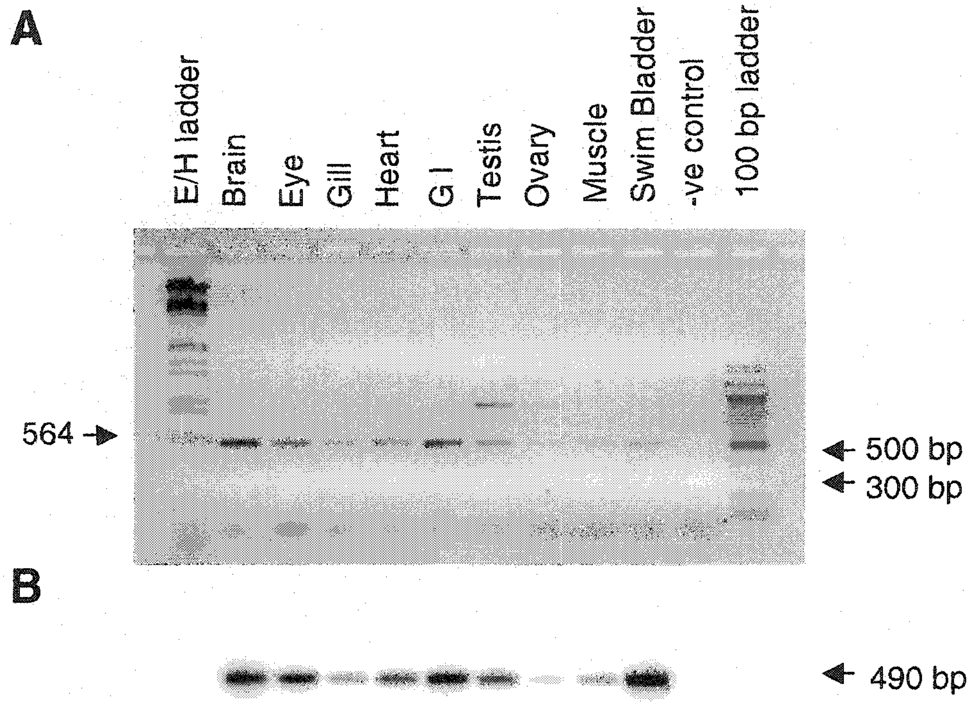
CGC GCT CCA CCC TTC TCC TCA TTC CTC TGT TTG GGA TCC 546

possible TMD-6

ACT ACA CTG TGT TTG CCT TTT CCC CGG AGG ATT TCA GCA 585

AGC GGG AGC GAC 597

Figure 3.8 (A) RT-PCR amplification of the $VPAC_1$ receptor transcript from mRNA isolated from adult zebrafish tissues. (B) Southern blot analysis of the RT-PCR products using a 390 bp zebrafish $VPAC_1$ receptor specific probe. The amplified transcript was approximately 490 bp in length.


A
E/H ladder
Brain
Eye
Gill
Heart
G I
Testis
Ovary
Muscle
Swim Bladder
-ve control
100 bp ladder
564
500 bp
300 bp
B
490 bp

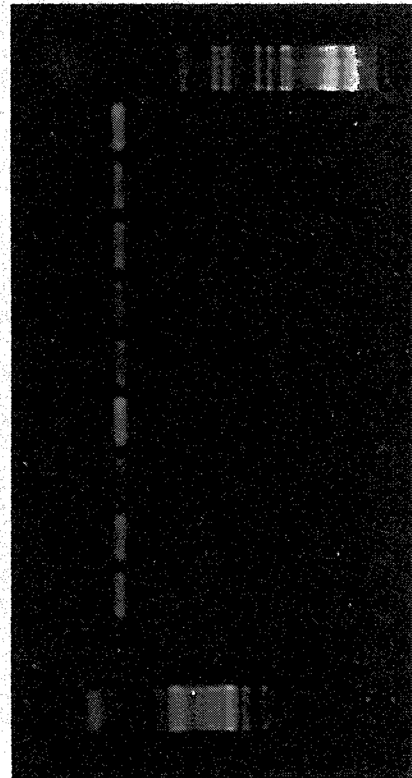
each tissue. A 250 nucleotide band corresponding to the tubulin transcript was amplified from all of the tissues (Fig. 3.9).

DISCUSSION

Isolation of the GRF receptor

The GRF receptor has been isolated from six mammalian species including the human, rat (Mayo 1992), mouse (Lin et al. 1992), pig (Hsiung et al. 1993), sheep and cow (Horikawa et al. 2001). However, it has only been isolated in one non-mammalian species, the goldfish (Chan et a. 1998). The identification of the GRF receptor in fish is difficult due to the low degree of conservation of the receptor amoung vertebrates. The goldfish GRF receptor shares only 43% amino acid sequence identity with mammalian GRF receptors (Chan et al. 1998). This corresponds to the low conservation of the GRF peptide amoung vertebrates (Fradinger and Sherwood 2000). The isolation of the GRF receptor in the goldfish was critical to the isolation of the receptor in the zebrafish, because it allowed for sequence comparison with the mammalian receptors. In this study primers were designed against regions in transmembrane domains 5 and 7 of the goldfish receptor (Chan et al. 1998) that were conserved with the human receptor (Lin et al. 1992). The isolated partial cDNA for the zebrafish GRF receptor shares high nucleotide sequence identity (91%) with the goldfish GRF receptor. The high degree of conservation between the zebrafish and goldfish receptors is similar to the degree of conservation of the GRF peptides (~90%) in these two species. However, isolation of the full-length cDNA will be required to further characterize the zebrafish GRF receptor.

Figure 3.9 RT-PCR amplification of the tubulin transcript from mRNA isolated from adult zebrafish tissues. This control PCR was done at the same time as those to amplify the GRF-PACAP and receptor transcripts to confirm cDNA quality. The tubulin transcript is approximately 250 bp in length.



E/H ladder
Brain
Eye
Gill
Heart
GI
Testis
Ovary
Muscle
Swim Bladder
-ve control
100 bp ladder

← 200 bp
← 500 bp

Isolation of the PACAP receptors

To isolate the PACAP receptor from the zebrafish, primers were designed against conserved regions between the human PAC_1 and goldfish PAC_1 receptors in transmembrane domains 2 and 7. Using these primers I isolated two partial cDNAs encoding PACAP receptors from the adult zebrafish brain (Fig. 3.2 and 3.3). Based on sequence similarity one partial cDNA corresponds to the zebrafish PAC_1 receptor and the other corresponds to the zebrafish $VPAC_1$ receptor (Table 3.2 and 3.3). The zebrafish PAC_1 receptor shares 87% nucleotide sequence similarity with the goldfish PAC_1 receptor. The goldfish receptor shared high sequence identity with the PAC_1 receptor and displayed pharmacological characteristics of PAC_1 receptors (Wong et al. 1998), indicating that the zebrafish receptor is another fish PAC_1 receptor. The zebrafish VPAC receptor shares 89.5% nucleotide sequence similarity with the goldfish $VPAC_1$ receptor. Phylogenetic analysis grouped the goldfish receptor with the mammalian $VPAC_1$ receptors (Chow et al. 1997), indicating that the zebrafish receptor is another fish $VPAC_1$ receptor. In addition, for both zebrafish receptors, the two cysteine residues found in the first extracellular loop of the PAC_1 and $VPAC_1$ receptors are conserved.

In mammals, the physiological actions of PACAP are mediated by the PAC_1, $VPAC_1$ and $VPAC_2$ receptors. However, only the PAC_1 and $VPAC_1$ receptors have been isolated from non-mammalian vertebrates including the chicken, frog, goldfish (Peeters et al. 1999; Alexandre et al. 1999; Wong et al. 1998; Chow et al. 1997) and now zebrafish. The isolation of the PACAP receptors from the zebrafish provides further information with which to analyze the evolution of the PACAP receptors, since the tetraploid nature of the goldfish genome makes it difficult to use this fish for analysis of gene duplications.

It is hypothesized that the mammalian $VPAC_2$ receptor arose from a gene duplication event of the PAC_1 receptor (Vaudry et al. 2000b). Current evidence indicates that this duplication may have taken place in an ancestral mammal, but further study is required to confirm this hypothesis.

Additionally, the PAC_1 receptor is more highly conserved than the $VPAC_1$ receptor throughout evolution. This may indicate that the PAC_1 receptor was the ancestral receptor and that the VPAC receptors arose from two separate gene duplications of this receptor. This would support the hypothesis that PACAP is the ancestral peptide from which the VIP peptide arose (Sherwood et al. 2000). Alternatively, the conservation of the two receptors may be due to the conservation of the PACAP and VIP peptides. The PACAP peptide is highly conserved throughout evolution, whereas the VIP peptide is only moderately conserved (Sherwood et al. 2000).

Tissue distribution of the receptors

In the adult zebrafish, the GRF and PACAP receptors were expressed in the brain and a variety of peripheral tissues (Table 3.4). Transcripts encoding the GRF and PACAP peptides, in addition to transcripts for their receptors, are expressed in the brain, eye, gastrointestinal tract and testis indicating that the two hormones may have an autocrine/paracrine action in these tissues. In addition, transcripts encoding the GRF and PACAP peptides but only the PACAP receptors are expressed in the ovary; other peripheral tissues that do not express the GRF-PACAP transcript, nonetheless express one or more of the GRF, PAC_1 or VPAC receptors indicating that these hormones have an endocrine effect in these tissues. Receptors for both GRF and PACAP are found in the gill and swim bladder, whereas only PACAP receptors are found in the heart and

skeletal muscle. In addition, of the two PACAP receptors, only the VPAC receptor is expressed in the heart and swim bladder. This could indicate that VIP has a more important physiological role in these two tissues. Although GRF and PACAP are encoded by the same gene in fish, the differential expression of the GRF and PACAP receptors provides a mechanism for functional regulation in each tissue.

In the adult zebrafish the GRF receptor was expressed in the brain, eye, gill, gastrointestinal tract, testis and swim bladder. This distribution is similar to that found in the goldfish (Chan et al. 1998). However, in the goldfish the GRF receptor transcript is more widely expressed. The transcript is found in the heart, ovary and skeletal muscle in addition to the above tissues, although expression was not examined for the eye and swim bladder in the goldfish (Chan et al. 1998). In the rat, the GRF receptor transcript is expressed predominantly in the pituitary, placenta and kidney, but low levels of expression are noted in a variety of peripheral tissues (Matsubara et al. 1995). Therefore, it appears that the expression of the GRF receptor is conserved throughout evolution.

The tissue expression pattern of the PAC_1 receptor in the zebrafish is similar to that found in the goldfish (Wong et al. 1998). In both fish, expression was found in the brain, gill, gastrointestinal tract, gonads and skeletal muscle. However, strong expression of the PAC_1 receptor was found in the heart of the goldfish (Wong et al. 1998), but expression was not detected in this tissue for the zebrafish. The tubulin control for heart indicated that the lack of PAC_1 receptor transcript in this tissue was not due to the quality of the cDNA. In addition, expression of the PAC_1 receptor was found in the eye of the zebrafish, but this tissue was not studied in the goldfish. The expression of the PAC_1 receptor in fish is consistent to that found in the rat (Vaudry et al. 2000b). This may

indicate that the functional role of PACAP in these tissues has been conserved throughout evolution.

In the zebrafish, the $VPAC_1$ receptor was expressed in all tissues studied including the brain, eye, gill, heart, gastrointestinal tract, gonads, skeletal muscle and swim bladder. In contrast, the goldfish does not show expression of the $VPAC_1$ receptor in the gonads; the eye and swim bladder were not studied (Chow 1997). The wide distribution of the $VPAC_1$ receptor is also seen in the frog (Alexandre et al. 1999). Therefore, similarly to the frog and goldfish, the zebrafish $VPAC_1$ receptor shows a distribution pattern that overlaps with both the $VPAC_1$ and $VPAC_2$ receptors in mammals. In mammals the $VPAC_2$ receptor, and not the $VPAC_1$ receptor, is expressed in the gonads and skeletal muscle (Vaudry et al. 2000b). Therefore, data from the zebrafish support the hypothesis that a gene duplication event leading to the $VPAC_2$ receptor occurred some time after the divergence of the amphibian lineage (Alexandre et al. 1999). Alternatively, the $VPAC_2$ receptor may exist in non-mammalian vertebrates, but has not been found.

The functional significance of the receptor distribution in fish is still unknown. It has been shown that both GRF and PACAP stimulate the release of GH in the goldfish, eel and salmon (Wong et al. 2000; Montero et al. 1998; Parker et al. 1997). In addition, PACAP has been shown to inhibit gut motility in the Atlantic cod (Olsson and Holmgren 1998). However, little is know about the functions of GRF and PACAP in other tissues. In mammalian peripheral tissues, GRF and PACAP have been shown to affect the maturation of germ cells. In addition, PACAP influences contraction of cardiac muscle and smooth muscle in the respiratory and gastrointestinal tract. PACAP also modulates

endocrine or exocrine secretions from the heart, adrenal, pancreas and gastrointestinal tract (Sherwood et al. 2000). The conservation of expression of GRF, PACAP and their receptors between fish and mammals may indicate that the physiological function of GRF and PACAP in these tissues is conserved as well.

Functional implications of the PAC_1 receptor isoforms

The diverse physiological functions of PACAP can be explained by the activation of both different receptor types and different receptor isoforms generated by alternative splicing. In mammals, it has been shown that the different PAC_1 receptor splice variants of the third intracellular loop have different signaling properties. In particular, the short isoform, that does not contain a cassette in the third intracellular loop, is widely expressed and stimulates the cAMP and phospholipase C signaling pathways (Spengler et al. 1993). In contrast, the hip cassette appears to impair stimulation of the cAMP pathway and inhibits stimulation the phospholipase C signaling (Spengler et al. 1993; Journot et al. 1994). The presence of the hiphop cassette impairs both the cAMP and phospholipase C pathways (Spengler et al. 1993). In the adult zebrafish the hop 1 isoform has been isolated from the brain and testis and the hop 2 isoform has been isolated from the ovary. In the rat, the hop1 isoform is the most abundant variant of the PAC_1 receptor and it is predominantly expressed in the brain and testis (Spengler et al. 1993). The presence of the hop cassette does not affect cAMP or phospholipase C signaling in comparison to the short isoform. The PAC_1 short, hop 1 and hop 2 isoforms display similar activation of the cAMP and phospholipase C signaling pathways. However, in addition to activating the cAMP pathway, PACAP has been shown to affect the mitogen-activated protein-kinase signaling pathway through stimulation of adenylyl

cyclase and to stimulate calcium mobilization (Vaudry et al. 2000b). Therefore, further biochemical characterization is required to determine the functional significance of the hop cassette. In addition, a novel isoform was isolated from the gill of the adult zebrafish. Further work will be required to characterize this isoform and determine it's functional significance.

In conclusion, the isolation of the GRF and PACAP receptors provides further information with which to evaluate the evolution of this family of G-protein-coupled receptors. Results from the zebrafish confirm the hypothesis that the $VPAC_2$ receptor arose later in evolution. In addition, the rates of nucleotide change in the receptors mirror those of the GRF, PACAP and VIP peptides, indicating that the peptides may exert a selective pressure on their receptors or vice versa. The tissue distribution of the GRF receptor, PAC_1 receptor short and hop isoforms, and $VPAC_1$ receptor appears to be conserved between fish and mammals, possibly indicating conservation of function. In all tissues studied, with the exception of the ovary, receptors for both GRF and PACAP are present in tissue expressing the GRF-PACAP gene. Differential expression of the receptors is only seen in tissues where the peptides have an endocrine function. The functional significance of this expression pattern must still be investigated to determine whether the two hormones modulate each other's function or if they have separate functions in tissues where their receptors are co-localized.

CHAPTER 4

Early developmental expression of GRF, PACAP and their receptors in the zebrafish embryo

INTRODUCTION

The development of the vertebrate embryo is characterized by periods of cell proliferation, migration and differentiation. To understand the developmental program, it is important to characterize the factors involved in these processes. In mammals GRF, PACAP and their receptors are expressed in the developing embryo during organogenesis (Sherwood et al. 2000). Therefore, it appears that both hormones may have a physiological role during development.

GRF and its receptor are first expressed on embryonic day 16.5 in the mouse and GRF is thought to be involved in maturation of pituitary somatotroph and release of fetal growth hormone (Lin et al. 1992). PACAP and its receptors are expressed at an earlier stage of development in the mouse and rat. The PAC_1 receptor and its ligand are first expressed at embryonic day 9.5 in the mouse (Shuto et al. 1996; Sheward et al. 1996) and at embryonic day 10 in the rat (Basille et al. 2000). The $VPAC_1$ receptor is expressed at embryonic day 11 in the rat (Pei 1997). At this developmental stage, PACAP is thought to influence neural tube patterning (Washeck et al. 1998). In contrast, the $VPAC_2$ receptor is expressed at embryonic day 14 in the mouse, which is a slightly later stage of development (Waschek et al. 1996). The expression of PACAP has been investigated in non-mammalian vertebrates as well. In the chick embryo, PACAP and the PAC_1 receptor are expressed in neuroblasts at embryonic day 3.5 (Erhardt et al. 2001). This corresponds to the same developmental stage as is found in mammals. However, in fish the GRF-PACAP transcript is expressed at a much earlier developmental stage. In the rainbow trout, expression of the GRF-PACAP transcript is first expressed during the late blastula stage and expression continues throughout the developmental period (Krueckl and

Sherwood 2001). However, the developmental expression of the GRF and PACAP receptors has not been examined in fish.

The early expression of GRF, PACAP and their receptors in the developing vertebrate embryo provides strong evidence that these two hormones may have an important role during development. In addition, the evolutionary significance of the early expression of GRF and PACAP in the fish is yet to be elucidated. The objective of this study was to confirm the early expression of the GRF-PACAP gene and to examine the expression of the GRF, PAC_1 and $VPAC_1$ receptors in the developing zebrafish embryo during the cleavage, blastula, gastrula and early segmentation periods. Comparison of the expression of the GRF and PACAP receptors will allow us to determine if one or both hormones have a physiological role during early development.

Materials and Methods

Fish breeding and egg collection

Adult zebrafish were housed at 28.5°C with a 14 hour light and 10 hour dark photoperiod. Male and female fish were kept separately for one week prior to spawning. On the day prior to spawning, one male and five female fish were transferred to the spawning tank in the evening, 2 hours after their afternoon feeding. The bottom of the spawning tank was lined with plastic mesh to keep adult fish away from the eggs. Spawning occurs at the onset of light (9:00 am) and adults were removed from the tank at 10 am. Developing embryos can be easily distinguished from unfertilized eggs based on transparency. Unfertilized eggs are white in colour and completely opaque. Therefore, developing embryos were collected at 7 time intervals (Table 4.1). As much water as

Table 4.1 Zebrafish embryo collection schedule. Adults were bred under 14 hours of daylight beginning at 9:00 am.

HOURS POST-FERTILIZATION	TIME	STAGE	DEVELOPMENTAL PERIOD
1-2	11:00 am	4-64 cells	Cleavage
3-4	1:00 pm	1000 cells	Blastula
5-6	3:00 pm	shield	Gastrulation
7-8	5:00pm	75% epiboly	Gastrulation
9-10	7:00 pm	bud	Segmentation
13-14	11:00 pm	8-10 somites	Segmentation
17-18	3:00 am	18 somites	Segmentation

possible was removed from the collected embryos and they were flash frozen in liquid nitrogen and stored at –80°C.

mRNA isolation and cDNA synthesis

For the developmental expression study, five embryos from each developmental stage were ground to a fine powder using a micropestle (Diamed, Mississauga, ON) in 1.5 ml microfuge tubes that were chilled with liquid nitrogen. The mRNA was isolated using the Poly (A) Pure Kit (Ambion, Austin, TX) as outlined by the manufacturer. Single stranded cDNA was synthesized using 2 mM of oligo$(dT)_{20}$ in 1x First Strand Buffer, 1.6 mM dNTPs, 8 mM DTT, 16 units of RNAguard (Gibco BRL, Burlington, ON) and 200 units of Superscript II (GibcoBRL, Burlington, ON) to a final volume of 50 μl. The oligo dT primer was annealed to the mRNA at 70°C for 10 minutes. Then, the other reagents were added and the reaction was incubated at 42°C for 90 minutes and at 95°C for 10 minutes.

PCR amplification of GRF-PACAP and the GRF, PAC_1 and $VPAC_1$ receptors

PCR amplification of the GRF-PACAP transcript was performed using the primers zebra1 and 3'UTR-R, designed to hybridize to exon 1 and the 3'UTR of the zebrafish cDNA sequence (Table 4.2). Amplification of the GRF receptor was performed using primers GRF5 and GRF7, designed to hybridize to transmembrane domains 5 and 7 of the goldfish GRF receptor (Table 4.2). PCR amplification of the PAC_1 receptor was performed using the primers PAC1R5' and PAC1R3'2 and amplification of the $VPAC_1$ receptor was performed using primers VPAC FW and VPAC RV (Table 4.2). The primers for both the PAC_1 and $VPAC_1$ receptors were designed against extracellular

Table 4.2 Primer sequences for the zebrafish GRF-PACAP gene, GRF receptor, PAC_1 receptor and VPAC receptor reported in the 5' to 3' direction of the sense strand for the forward primers and antisense strand for the reverse primers. Direction of the primer is represented by the arrows, forward primers are represented by (◄——) and reverse primers are represented by (——►).

Primer Name	Direction	Location	Gene Target	Sequence 5' to 3'
zebra1	——►	Exon 1	GRF-PACAP	ATATCTCGCCTCAGATCCGT
zebra2	——►	Exon 2	GRF-PACAP	CGACTCTTGCTTTCCTCATC
3'UTR-R	◄——	3'UTR	GRF-PACAP	GCATTGTCAGGTGCGTCAGTA
GRF 5	——►	TMD 5	GRF receptor	TGGTGGATCATTAAAGGAC
GRF 7	◄——	TMD 7	GRF receptor	AAGCCCTGGAAAGAGCCCAG
PAC1R5'	——►	ECL 1	PAC_1 receptor	TGCTTCGTTCACACTGTGGGCT
PAC1R3'1	◄——	ICL 3	PAC_1 receptor	AATGCTGGACTCGTTTCCGCCG
PAC1R3'2	◄——	ECL 3	PAC_1 receptor	TGCGCTCCCGCTTGCTGAAATC
VPAC FW	——►	ECL 1	$VPAC_1$ receptor	TGCTCCACTGGTTCTGTTGGCT
VPAC3'1	◄——	ICL 3	$VPAC_1$ receptor	TTGGTTTGATTCATTTCTCCCG
VPAC RV	◄——	ECL 3	$VPAC_1$ receptor	GTACAGCGACCACAAAACCCTG

Note: Abbreviations are: ECL, extracellular loop; ICL, intracellular loop; TMD, transmembrane domain; UTR, untranslated region

loops 1 and 3 of the partial cDNA sequences for the appropriate receptors that were previously isolated from the zebrafish cDNA library (Chapter 3). I had to design the primers to hybridize against the extracellular domains to specifically amplify either the PAC_1 receptor or $VPAC_1$ receptor. Since, the extracellular domains of the receptors are less conserved than the intracellular domains, this eliminated the possibility of the primers annealing to both receptor transcripts. A 3μl aliquot of the isolated cDNA was added to a 50 μl volume containing: 200 μM dNTPs, 2 mM $MgCl_2$, 0.4 μM of each primer and 2.5 units of *Taq* DNA polymerase (Gibco BRL, Burlington, ON). The reaction was heated to 94 °C for 2 minutes, then cycled 30 times at 94°C for 30 sec., 55°C for 45 sec. and 72°C for 1 min. A control reaction using 2 μl of cDNA amplified with tubulin primers Tubulin 10 and Tubulin 11 (see Table 2.1) was performed. The tubulin reactions (10 μl) were separated on a 2% agarose gel, stained with eithidium bromide and photographed.

Southern analysis of RT-PCR results

The PCR products (10 μl) from each primer set were separated on 2% agarose gels. The DNA was capillary transferred in 0.4 M NaOH according to the manufacturer's specifications to four GeneScreen nylon membranes (NEN Life Science Products, Guelph, ON). The membranes were washed in 6x SSC for 2 minutes and then allowed to air dry. Then, it was prehybridized at 56°C in 6x SSC, 5x Denhardt's solution, 0.5% SDS and 15 μg of blocking DNA for 3 hours. A 680 bp probe for the GRF-PACAP cDNA was amplified from the previously isolated clone using primers zebra2 and 3'UTR-R (Table 4.2). A 266 bp probe for the GRF receptor was amplified from the previously isolated GRF receptor clone using primers GRF 5 and GRF 7 (Table 4.2). A

387 bp probe was PCR amplified from the previously isolated PAC_1 receptor clone using primers PAC1R5' and PAC1R3'1 (Table 4.2). Similarly, a 390 bp probe was PCR amplified from the VPAC receptor clone using primers VPAC FW and VPAC3'1 (Table 4.2). PCR amplification was carried out as described in the above section at an annealing temperature of 56°C. The GRF, PAC_1 and $VPAC_1$ receptor probes were labelled with [α-32P]-dCTP (NEN Life Science Products, Guelph ON) using the Random Primers DNA Labelling System (Gibco BRL, Burlington, ON) as specified by the manufacturer. The membranes were hybridized separately in a solution containing 6x SSC, 5x Denhardt's solution, 0.5% SDS, 15 µg of blocking DNA and 1 ml of the appropriate probe (7.5×10^7 cpm/ml of the GRF-PACAP probe, 6.3×10^7 cpm/ml of the GRF probe, 7.05×10^7 cpm/ml of the PAC_1 probe or 6.1×10^7 cpm/ml of the VPAC probe) at 56°C overnight. The membranes were washed once with 1x SSC and 0.5% SDS, twice with 0.5x SSC and 0.5% SDS, and once with 0.1x SSC and 0.5% SDS at 55°C for 30 minutes each. Then, the membranes were exposed to a phosphor screen (Molecular Dynamics) for 30 minutes at room temperature. The exposed screen was scanned with a Storm 860 scanner (Molecular Dynamics) and analyzed using ImageQuant software.

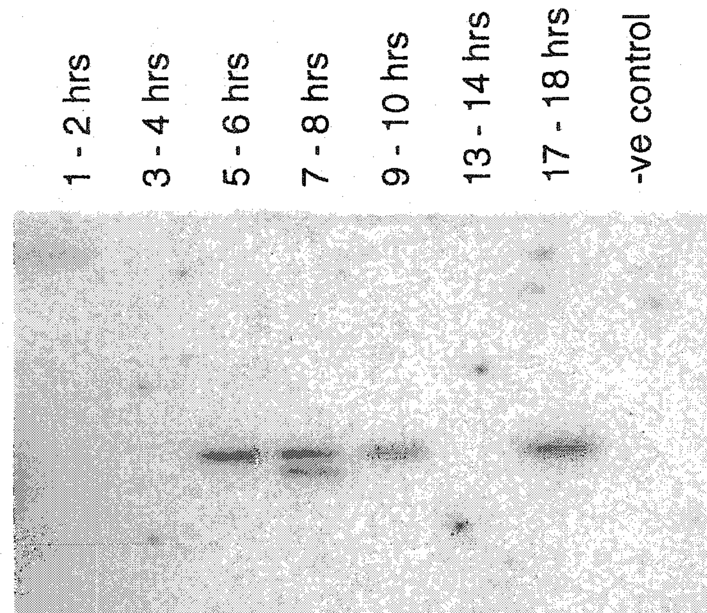
RESULTS

Developmental expression of the GRF-PACAP gene

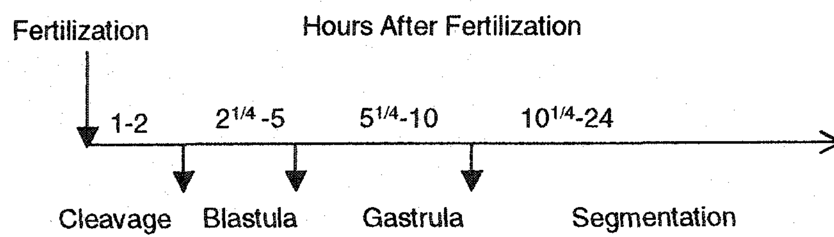
The GRF-PACAP transcript is first expressed at 5-6 hours after fertilization, which corresponds to the late blastula early gastrula stage of development in the zebrafish (Fig 4.1). During the mid-gastrula period when the primary germ layers and embryonic axis are forming, two GRF-PACAP transcripts are observed at 7-8 hours after

Figure 4.1 (A) Southern blot analysis of the GRF-PACAP transcript from mRNA isolated from zebrafish embryos at different developmental stages. A zebrafish GRF-PACAP-specific probe was used on products amplified by RT-PCR. The amplified GRF-PACAP transcript is approximately 900 bp in length. (B) Schematic showing developmental timeline for the zebrafish embryo.

A



B



fertilization (Fig. 4.1). This corresponds to approximately 70% – 75% epiboly. Epiboly refers to a period of cell movement when the blastoderm spreads over the yolk until the yolk cell is completely encompassed. At approximately 50% epiboly, involution produces the two primary germ layers and then further morphogenesis establishes the embryonic axis. At the end of the gastrula period and the mid to late segmentation period, only the long transcript is observed. The GRF-PACAP transcript was not detected at 13-14 hours after fertilization (Fig. 4.1).

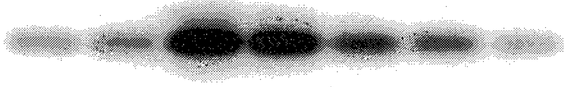
Developmental expression of the GRF and PACAP receptors

Both the GRF and PAC_1 receptors are expressed throughout the early developmental period of the zebrafish (Fig. 4.2 and 4.3). Only one transcript is observed for the GRF receptor and it appears that the highest level of expression occurs between 5 and 8 hours after fertilization (Fig 4.2). Two transcripts are observed for the PAC_1 receptor. The long and short transcripts are both expressed from 1 to 8 hours after fertilization, whereas the only the short transcript is expressed at 9-10 and 17-18 hours after fertilization and only the long transcript is expressed at 13-14 hours after fertilization (Fig. 4.3). In contrast, the VPAC receptor was first expressed at a later stage of development in the zebrafish. A band corresponding to the $VPAC_1$ receptor is first expressed at 5-6 hours after fertilization, peaks at 7-8 hours after fertilization and is present at lower levels throughout the remainder of the early developmental period from 9-18 hours after fertilization. Similar to the GRF receptor, strongest levels of expression of the VPAC receptor are seen at 5-8 hours after fertilization (Fig. 4.4). Amplification of the tubulin transcript from all the developmental stages indicates that the inability to

Figure 4.2 (A) Southern blot analysis of the GRF receptor transcript from mRNA isolated from zebrafish embryos at different developmental stages. A zebrafish GRF receptor-specific probe was used on products amplified by RT-PCR. The amplified GRF receptor transcript is approximately 250 bp in length. (B) Schematic showing developmental timeline for the zebrafish embryo.

A

1 - 2 hrs
3 - 4 hrs
5 - 6 hrs
7 - 8 hrs
9 - 10 hrs
13 - 14 hrs
17 - 18 hrs
-ve control



B

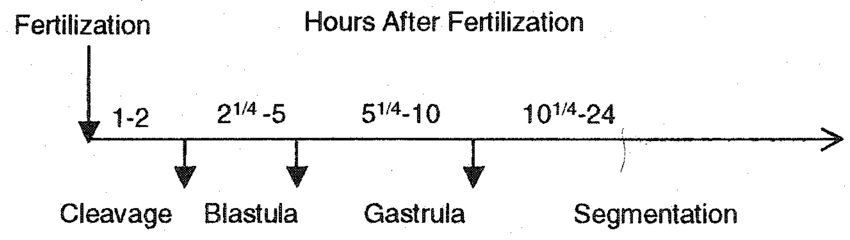
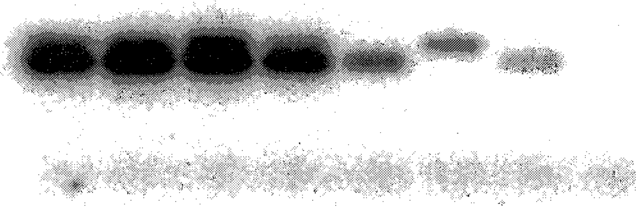


Figure 4.3 (A) Southern blot analysis of the PAC_1 receptor transcript from mRNA isolated from zebrafish embryos at different developmental stages. A zebrafish PAC_1 receptor-specific probe was used on products amplified by RT-PCR. The amplified PAC_1 receptor transcript is approximately 500 bp in length. (B) Schematic showing developmental timeline for the zebrafish embryo.

A

1 - 2 hrs
3 - 4 hrs
5 - 6 hrs
7 - 8 hrs
9 - 10 hrs
13 - 14 hrs
17 - 18 hrs
-ve control



B

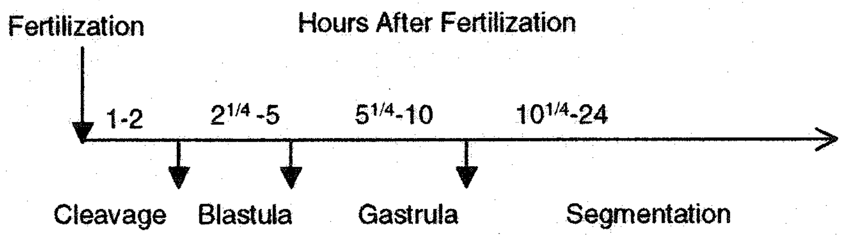
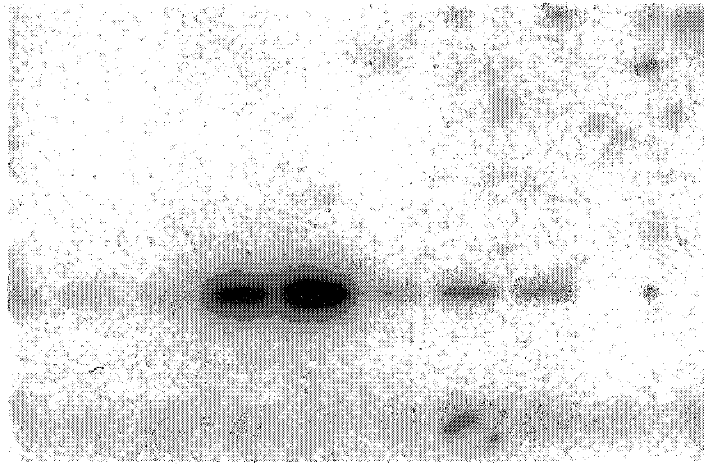
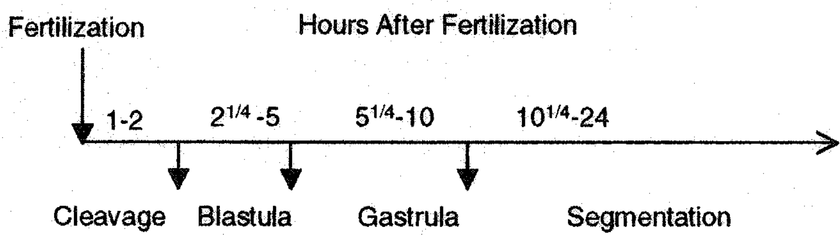


Figure 4.4 (A) Southern blot analysis of the $VPAC_1$ receptor transcript from mRNA isolated from zebrafish embryos at different developmental stages. A zebrafish $VPAC_1$ receptor-specific probe was used on products amplified by RT-PCR. The amplified $VPAC_1$ receptor transcript is approximately 500 bp in length. (B) Schematic showing developmental timeline for the zebrafish embryo.

A



B



amplify a transcript at a developmental stage was not due to cDNA quality. Although, there was lower levels of tubulin amplified from 1 to 4 hours after fertilization (Fig. 4.5). The early developmental expression of GRF-PACAP and their receptors is summarized in Table 4.3.

DISCUSSION

Early developmental expression of the GRF-PACAP transcript

In the zebrafish the GRF-PACAP transcript is first expressed during the late blastula stage. This confirms a study in the rainbow trout in which expression was first noted in the blastula stage as well (Krueckl and Sherwood 2001). In the rainbow trout, the GRF-PACAP gene was expressed throughout the developmental period, at all stages studied. In contrast, no expression was detected in the zebrafish embryo at 13-14 hours after fertilization. This stage corresponds to the early segmentation period (Kimmel et al. 1995). Additionally, alternative splicing of the GRF-PACAP gene to generate a long and short transcript was noted for all developmental stages in the rainbow trout. The long transcript encoded both GRF and PACAP and the short transcript encoded only PACAP. It was found that alternative splicing involving the removal of exon 4 resulted in the production of the short transcript (Krueckl and Sherwood 2001). In contrast, two transcripts were found at only one developmental stage (7-8 hours after fertilization) in the zebrafish. At this time the primary germ layers are forming and the embryonic axis is being established. This may indicate that GRF and PACAP have an important function at this stage of development. It is important to note that this is the fist time that alternative

Figure 4.5 RT-PCR amplification of the tubulin transcript from mRNA isolated from zebrafish embryos at different developmental stages. The amplified tubulin PCR product is approximately 200 bp in length.

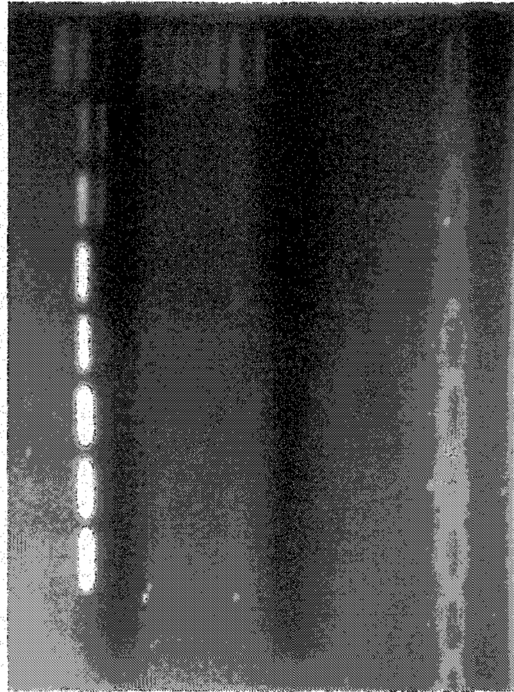

100 bp ladder
1 - 2 hrs
3 - 4 hrs
5 - 6 hrs
7 - 8 hrs
9 - 10 hrs
13 - 14 hrs
17 - 18 hrs
-ve control

Table 4.3 Developmental expression of the mRNA transcripts for the GRF-PACAP gene and its receptors in the zebrafish embryo.

HOURS POST-FERTILIZATION	STAGE	GRF-PACAP		GRF receptor	PAC_1 receptor		$VPAC_1$ receptor
		long	short		long	short	
1-2	4-64 cells			*	*	*	
3-4	1000 cells			*	*	*	
5-6	shield	*		**	*	*	**
7-8	75% epiboly	*	*	**	*	*	**
9-10	bud	*		*		*	*
13-14	8-10 somites			*	*		*
17-18	18 somites	*		*		*	*

Note: ** indicates high level of expression

splicing of the GRF-PACAP gene has been found in the zebrafish. In the adult zebrafish, alternative splicing of the gene was not detected in the brain or peripheral tissues (Fradinger and Sherwood 2000). In contrast, alternative splicing of the GRF-PACAP gene has been found in both the brain and peripheral tissues of the salmon and channel catfish (Parker et al. 1993; Small and Nonneman 2001).

Early developmental expression of the receptors for GRF and PACAP

Receptors for both GRF and PACAP are expressed throughout early development, indicating that both peptides have a physiological role during development. Expression of the GRF receptor and PAC_1 receptor mRNA transcripts is found as early as 1-2 hours after fertilization during the cleavage period. However, the GRF-PACAP transcript is not detected until the late blastula period making it unclear if these peptides function during the cleavage and early blastula period. It is possible that the receptors are not being translated at this stage or that undetectable levels of the GRF-PACAP transcript are being expressed at this time. However, corresponding with the first expression of the GRF-PACAP transcript, higher levels of expression of the GRF receptor are noted at 5-8 hours after fertilization. In addition, the $VPAC_1$ receptor is first expressed at 5-6 hours after fertilization and like the GRF receptor shows highest levels of expression from 5-8 hours after fertilization.

The expression of the GRF-PACAP gene and its receptors is much earlier in fish than in mammals. In the rat the GRF peptide and its receptor are expressed at embryonic day 16 during the segmentation period (Lin et al. 1992). However, the placenta was shown to be an important source of foetal GRF in mammals (Baird et al. 1985) and placental GRF has been detected as early as embryonic day 7 in the rat (Spatola et al.

1991). Therefore, GRF may be required during early development and in mammals the placenta may be the foetal source of GRF before it is expressed in the embryo. However, further investigation into the presence of the GRF receptor during early development is required to confirm this hypothesis. Similarly, the PACAP peptide and the PAC_1 receptor are expressed at embryonic day 9.5 in the mouse during the segmentation stage (Shuto et al 1996) and the $VPAC_1$ receptor is expressed at embryonic day 11 in the rat (Waschek et al. 1996; Pei 1997). Therefore, two key questions are whether 1) maternal or placental sources of the GRF and PACAP peptides account for the later expression of these peptides in mammals and 2) their receptors are expressed during early development.

Functional implications of the early expression of GRF, PACAP and their receptors

In the zebrafish the GRF-PACAP gene is first expressed and the GRF and PACAP receptors are strongly expressed at the beginning of epiboly at the transition between the blastula and gastrula periods. Expression of GRF, PACAP and their receptors continues throughout the gastrula stage when the embryo undergoes substantial morphogenic movements to form the epiblast, hypoblast and embryonic axis. In particular, alternative splicing of the GRF-PACAP gene is seen for the first time in the zebrafish during the gastrulation stage. This indicates that the transcriptional regulation of this gene is under tight control and that the ratio of GRF and PACAP is being altered during epiboly, indicating that PACAP may have an important functional role during gastrulation. In the frog embryo, PACAP appears to promote dorsal and anterior patterning (Otto et al. 2000). In the frog, PACAP upregulated the expression of *goosecoid* an important gene involved in establishment of the embryonic axis (Otto et al. 2000). High levels of *goosecoid* expression are seen during epiboly in the zebrafish

(Schulte-Merker et al. 1994) corresponding to the high levels of expression of GRF-PACAP and their receptors. This provides further evidence that PACAP may be involved in the establishment of the embryonic axis. In addition, PACAP may modulate the early patterning of the neural tube in the mouse by down regulating ventral marker genes (Waschek et al. 1998). This indicates that PACAP may be involved in modulating the formation of the embryonic axis.

The differential expression of the GRF-PACAP transcript and the short and long PAC_1 receptor transcripts during the early segmentation period indicates that PACAP may have an important role at this stage as well. The segmentation stage occurs between 10 and 24 hours after fertilization, and is characterized by periods of proliferation, morphogenic movements and differentiation creating the somites, neuromeres and rudimentary organs (Kimmel et al. 1995). Therefore, in the zebrafish the differential expression of the long and short PAC_1 receptor transcripts during this stage may indicate that PACAP is regulating anti-mitogenic and mitogenic events at the different time periods. The short transcript corresponds to the PAC_1 short isoform that has been associated with anti-mitogenic effects in cortical neuroblast cultures (Nicot and DiCicco-Bloom 2001). The long transcript corresponds to either the PAC_1 hip or hop isoform. Since, the short and hop isoforms have been isolated from the zebrafish (Chapter 3) and they are the predominant forms of the PAC_1 receptor isolated during development (Zhou et al. 2000a,b) it is probable that the long transcript is the hop isoform. The hop isoform appears to stimulate proliferation of cortical neuroblast cultures (Nicot and DiCicco-Bloom 2001). PACAP and the PAC_1 short receptor are expressed in brains from embryonic day 3.5 chick embryos, corresponding to the segmentation stage. Addition of

PACAP to E 3.5 neuroblasts causes an increase in cAMP indicating that PACAP may have a physiological role in the developing chick brain as well (Erhardt et al. 2001). Therefore, at 9-10 and 17-18 hours after fertilization when the PAC_1 short receptor isoform is being expressed, PACAP may be promoting cell cycle exit. At 13-14 hours after fertilization when the PAC_1 hop receptor isoform is being expressed, PACAP may be promoting cell proliferation.

In conclusion, this study confirms the previous study in the rainbow trout indicating that the GRF-PACAP gene is expressed earlier in fish than in mammals. In addition, the expression of GRF and PACAP receptors in the developing embryo indicates that both of these peptides modulate early development. In particular, PACAP appears to have an important role during gastrulation and segmentation. In mammals, PACAP appears to have an important role in brain development. Therefore, to further investigate the role of PACAP during neurulation and brain formation, it will be important to localize the expression of the GRF-PACAP transcript during the segmentation period and the subsequent pharyngula period, which occurs prior to hatching.

CHAPTER 5

Localization of the GRF-PACAP transcript in the developing zebrafish embryo

The whole mount *in situ* hybridization study was done in collaboration with Sandra Krueckl. Embryos for this study were provided by Dr. Cecilia Moens from the Fred Hutchinson Cancer Research Center, Seattle Washingtion.

INTRODUCTION

In the vertebrate embryo, GRF and PACAP have been shown to influence development during the segmentation period. In particular, PACAP is thought to modulate neurulation in the vertebrate embryo and GRF is thought to influence the differentiation of precursor pituitary cells into somatotrophs and embryonic growth hormone release (Sherwood et al. 2000). In the developing nervous system, PACAP has been found to influence patterning of the neural tube in the mouse embryo. It was found to down regulate the expression of two ventral marker genes, *Gli*-1 and *sonic hedgehog* (Waschek et al. 1998). In addition, PACAP has been found to inhibit proliferation in cortical neuroblasts (Lu and DiCicco-Bloom 1997; DiCicco-Bloom et al. 1998; Suh et al. 2001). However, in the cerebellum, PACAP promotes proliferation of granule cells *in vivo* (Vaudry et al. 1999) and promotes cell survival in cultured cerebellar neuroblasts (Cavallaro et al. 1996; Campard et al. 1997; Gonzalez et al. 1997; Villalba et al. 1997). However, the localization of GRF and PACAP expression during the segmentation period has not been fully characterized.

The zebrafish provides an excellent model for studying early development of vertebrates due to its rapid development and clear embryos. The development of the zebrafish embryo can be divided into seven major stages: the zygote period, the cleavage period, the blastula period, the gastrula period, the segmentation period, the pharyngula period and the hatching period (Kimmel et al. 1995). The segmentation stage occurs between 10 and 24 hours after fertilization, and is characterized by morphogenic movements creating the somites, neuromeres and rudimentary organs. In the zebrafish the timing of neurulation and segmentation overlap so that the zebrafish does not have a distinct neurula period. The pharyngula stage, from 24 to 48 hours after fertilization, is

when the embryo starts to straighten, the circulatory system matures and further differentiation of the rudimentary organs occurs. In addition, the embryo starts to acquire its pigmentation and fin buds start to develop. This period is followed by the hatching period during which the final maturation of organs and cartilage development occurs. In the zebrafish, embryos hatch at different times or asynchronously (Kimmel et al. 1995).

Evidence from mammals suggests that GRF and PACAP play an important role in the developing nervous system. The objectives of this study are to characterize the expression of the GRF-PACAP transcript during the segmentation and pharyngula periods; to examine the role of GRF and PACAP during neurulation; to investigate whether these hormones are involved in the development of organs in which they are expressed in the adult. To study the developmental expression of the GRF-PACAP gene during the segmentation and pharyngula periods in the zebrafish, I isolated and cloned a partial cDNA from the zebrafish brain. A probe corresponding to the 5' end of the cDNA to be used for *in situ* hybridization was amplified from the partial cDNA clone. In collaboration with Sandra Krueckl, the expression of GRF-PACAP was localized by whole mount *in situ* hybridization during the segmentation and pharyngula periods in the zebrafish. To further localize the expression of the gene, I embedded and sectioned a second set of whole mount embryos.

MATERIALS AND METHODS

Amplification of partial cDNA for GRF-PACAP from the zebrafish

An adult zebrafish was over anaesthetized using MS222 and the brain was isolated and immediately frozen in liquid nitrogen. The tissue was ground to a fine

powder using a micropestle (Diamed, Mississauga, ON) in a 1.5 ml eppendorf tube that was chilled with liquid nitrogen. The mRNA was isolated using the Poly (A) Pure Kit (Ambion, Austin, TX) as outlined by the manufacturer. Single stranded cDNA was synthesized using 2 mM of oligo$(dT)_{20}$ in 1x First Strand Buffer, 1.6 mM dNTPs, 8 mM DTT, 16 units of RNAguard (GibcoBRL, Burlington, ON) and 200 units of Superscript II (GibcoBRL, Burlington, ON) to a final volume of 25 µl. The reaction was incubated at 42°C for 90 minutes and at 95°C for 10 minutes. The GRF-PACAP transcript was amplified from 2.5 µl of the cDNA using the primers zebra1 and 3'UTR-R, at an annealing temperature of 52°C. An aliquot (2 µl) of the PCR product was ligated into pGEM-T vector (Promega, Madison, WI) as recommended by the manufacturer and cloned. Recombinant plasmids were sequenced as previously described using forward and reverse primers.

Digoxigenin-UTP labeled RNA Probe synthesis

A non-conserved region of the GRF-PACAP transcript was amplified from 1 µl of the cDNA clone using primers to the 5'UTR (zebra1) and the cryptic peptide (3'zebra2) at an annealing temperature of 52°C (Fig. 5.1). The PCR product was ligated into pGEM-T vector (Promega, Madison, WI) and the recombinant plasmids were sequenced to determine their orientation within the vector. A plasmid containing the GRF-PACAP insert in the forward orientation was selected and purified using the Qiagen Miniprep Kit (Qiagen, Mississauga, ON). Then, 20 µg of DNA was linearized by restriction digest with either *Sal* I or *Sac* II (NEB, Mississauga, ON) for the antisense and sense probes respectively (Fig. 5.1). The samples were digested using 5 µg of proteinase K at 37°C for 30 minutes. The DNA was extracted 1x with an equal volume

Figure 5.1 Probe for the GRF-PACAP mRNA transcript used for *in situ* hybridization in the zebrafish embryo. (A) Nucleotide sequence amplified from the isolated partial cDNA for GRF-PACAP encoding 412 bp of the 5' end of the transcript. Primer sequences are underlined and exon barriers are indicated. (B) Schematic of the GRF-PACAP transcript indicating the location of the primer used to amplify the probe. (C) Schematic of the cloned probe indicating the promoters and restriction sites used to generate the antisense (positive) and sense (negative control) RNA probes.

A

[exon 1 ATATCTCGCCTCAGATCCGTCCGACTACGAAGACCTGAGAGAGAGAGA 48

GGGAGGAAAGATACAGACGCTGTGGGTAACAAAGTGACGCGTTGAAAAGTTTAA 102

AGAGCAAGACTGGGAGAGAAAGGAGAGAGAGAGAGAGAGCTGGAGAATTTCATC 156

TCATTCTGGACGCAGCCTCCATTGGACAGCATCCGTCCGCTGCCGCAG] [exon 2 204

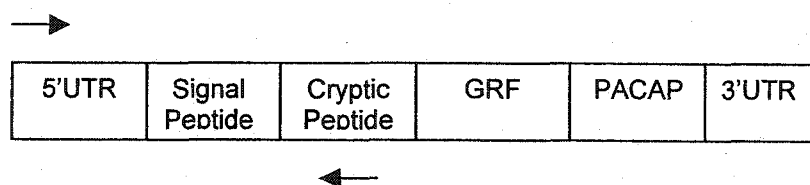
AATGATTACGAGCAGCAAAACGACTCTTGCTTTCCTCATCTATGGGCTCCTAGT 258

TCAATGCAATGTGTGTTCGCCTCTGAGTTACCCGAAAATCAG] [exon 3 AATGG 305

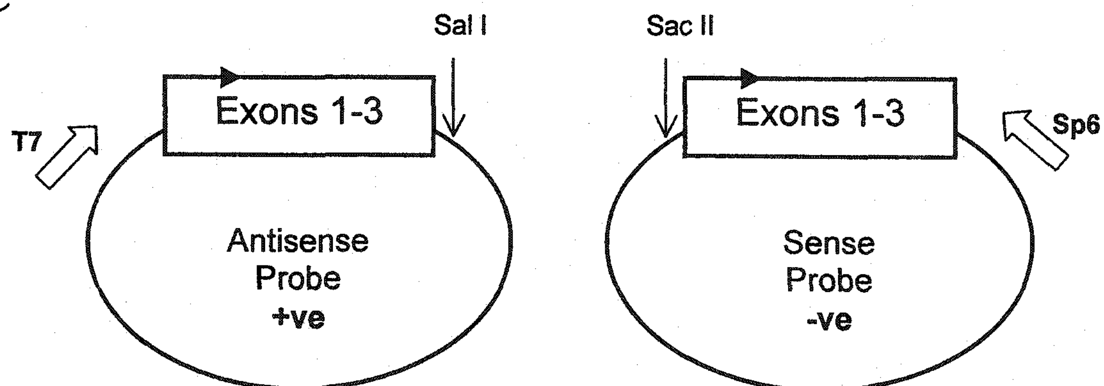
AGACTGCAGGATTTGATGAGGAGGGAAACTCATTAACGGATGTAACATTTGACA 359

GTGACCAGATCACTATACGAAGCTCTCCTACAGTCACTGAAGACGCATACACG] 412

B



C



of phenol and 2x with an equal volume of phenol:chloroform:isoamyl alcohol and the aqueous layer was precipitated with 0.1 volume of 3 M NaOAc pH 5.2 and 2.5 volumes of 100% ethanol overnight at -20°C. The precipitated DNA was spun down and rehydrated in 10 µl of water and the concentration was determined. A 1 µl aliquot was electrophoresed alongside the uncut plasmid on an agarose gel to check the linearization. The single stranded digoxigenin-UTP labeled RNA probe was synthesized from 3.4 µg of linearized plasmid (equivalent to 1 µg of insert) by *in vitro* transcription using the DIG RNA Labeling Kit (Roche, Indianapolis, IN) as outlined by manufacturer. The antisense (positive) probe was synthesized using T7 RNA polymerase and the sense (negative control) probe was synthesized using SP6 RNA polymerase (Fig. 5.1C)

The concentration of the DIG labeled probes was estimated by spot assay. Serial dilutions of the DIG labeled control RNA antisense probe and sense probe were prepared and 1 µl of each dilution was spotted on a nylon membrane. To fix the RNA the membrane was baked at 120°C for 30 minutes. The membrane was washed briefly in TBST, then incubated at room temperature in blocking solution (TBST and 10% rabbit serum) for 30 minutes. Anti-DIG-alkaline phosphatase was diluted 1:5000 in blocking solution and the membrane was incubated in this solution for 30 minutes at room temperature. The membrane was washed 2X in TBST and then briefly in detection solution (100 mM Tris-HCl pH 9.5 and 100 mM NaCl). The colour substrate solution was prepared by adding 45 µl of NBT (75 mg/ml of 70% dimethylformamide) and 35 µl of BCIP (50 mg/ml of 100% dimethyformamide) to 10 ml of detection solution. The colour substrate solution was added to the membrane and the colour was allowed to develop in the dark at room temperature overnight. Intensities of the antisense probe and

sense probe dilutions were compared to the control dilutions and the concentration of the probes was estimated.

Whole mount In situ hybridization

Fixed embryos in 100 % methanol were provided by Dr. Cecilia Moens (Fred Hutchinson Cancer Research Center, Seattle Washington). Embryos were rehydrated in a decreasing methanol series into PBST (75% methanol/25% PBST; 50% methanol/50% PBST; 25% methanol/75% PBST). Embryos were then washed 5x in PBST for 5 minutes at room temperature. Embryos were permeabilized by treatment with 10 μg/ml of proteinase K at room temperature for 1 to 20 minutes, depending on the developmental stage (Table 5.1). The reaction was stopped by rinsing the embryos in PBST; then they were refixed in 4% paraformaldehyde/PBS for 20 minutes at room temperature. Then, the embryos were then washed 5x in PBST for 5 minutes at room temperature. The embryos were prehybridized in hybe buffer (50% formamide, 5x SSC, 50 μg/ml heparin, 500 μg/ml tRNA, 0.1% tween-20 and 0.1 M citric acid) for 2½ hours at 65 °C. The prehybridization solution was removed, pre-warmed hybe buffer with labeled probe (100 ng/200 μl) was added, and the embryos were incubated overnight at 65 °C. Embryos were washed at 65 °C in 1) 66% hybe buffer/33 % 2x SSC, 2) 33% hybe buffer/66% 2x SSC and 3) 2x SSC for 5 minutes each. Then, embryos were washed twice in 0.2x SSC/0.1% tween-20 at 65 °C for 20 minutes and once in 0.1x SSC/0.1% tween-20 at 65 °C for 10 minutes. Finally embryos were washed at room temperature in 66% 0.2x SSC/33% PBST, 33% 0.2x SSC/66% PBST and PBST for 5 minutes each. Embryos were transferred to blocking solution (2% rabbit serum, 2 mg/ml BSA in PBST) and incubated at room temperature for 1 hour. Anti-DIG-alkaline phosphatase antibody was

Table 5.1 Proteinase K digestion times for the permeabilization of zebrafish embryos at different developmental stages.

DEVELOPMENTAL STAGE	PROTEINASE K DIGESTION TIME
Bud	1 minute
18 somites	5 minutes
24 hour	8 minutes
30 hour	15 minutes
48 hour	20 minutes

diluted 1/5000 in blocking solution. Embryos were transferred to the antibody solution and incubated at room temperature for 2 hours. Embryos were washed 5x in PBST for 5 minutes and 4x in colouration buffer (100 mM Tris-HCl ph 9.5, 50 mM $MgCl_2$, 100 mM NaCl and 0.1% tween-20) for 5 minutes. Substrate buffer was prepared by adding 45 µl of nitro-blue tetrazolium (75 mg/ml in 70% dimethylformamide) and 35 µl of 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml in 100% dimethylformamide) to 10 ml of colouration buffer. Embryos were transferred to the substrate buffer and incubated in the dark at room temperature until a purple product was visible. The embryos were washed twice in sterile water to stop the reaction and then stored in PBST at 4 °C.

Embedding and sectioning

Embryos were fixed overnight in 4% paraformaldehyde/PBS at 4°C. After fixation the embryos were washed twice in sterile water and dehydrated in an increasing ethanol series (25%, 50%, 75%, 95% and 100% x3). Embryos were incubated in each solution for 10 minutes. Dehydrated embryos were embedded in Technovit 7100 (Kulzer, Germany) as outline by the manufacturer. The embryos were pre-infiltrated in equal parts of 100% ethanol and Technovit base liquid for 4 hours at room temperature. The embryos were infiltrated overnight at 4°C in three changes of preparation solution and then polymerized for 24 hours as specified by the manufacturer (Kulzer, Germany). Polymerized embryos were serially sectioned at 10 µm using a Sorvall JB-4 microtome.

RESULTS

Isolation of a partial cDNA for GRF-PACAP

A partial cDNA encoding 901 bp of the GRF-PACAP transcript was isolated from

an adult zebrafish brain. The isolated GRF-PACAP cDNA sequence was identical to the coding region of the previously isolated genomic clone (Chapter 2) and encoded part of the 5'UTR, signal peptide, cryptic peptide, GRF peptide, PACAP peptide and part of the 3'UTR (Fig. 5.2). The transcript encodes a 173-amino-acid precursor protein that undergoes post-translational processing to yield the mature GRF and PACAP peptides. The coding region of the GRF peptide shares 66% nucleotide sequence identity with human PHM and the PACAP peptide shares 75% nucleotide identity with human VIP. In contrast, the 5'UTR, signal and cryptic peptide regions of the transcript do not share sequence identity with the PHM-VIP cDNA. Therefore, the 5' region of the transcript was used to localize the developmental expression of the GRF-PACAP gene.

Localization of the GRF-PACAP transcript in the zebrafish embryo

During the segmentation period, from 10 to 24 hours post-fertilization, the GRF-PACAP transcript is widely expressed in the zebrafish embryo. In the bud, 18 somite and 24 hour embryos, a purple stain corresponding to the GRF-PACAP transcript is seen throughout the embryos (Fig. 5.3A,D,G). The expression of the transcript decreases and becomes localized during the pharyngula period. In the 30 hour and 48 hour embryos, the widespread expression of the transcript is no longer evident and appears to be localized to the hatching gland (Fig. 5.3J,M). No purple stain is detected in the negative controls for all five stages (Fig. 5.3B,E,H,K,N).

In the developing nervous system the GRF-PACAP transcript is widely expressed in the neural tube during the segmentation period (bud, 18 somite and 24 hour stages) and little expression is detected during the pharyngula period (30 hour and 48 hour stages) (Fig. 5.3). During the segmentation period, the GRF-PACAP transcript is found in the

Figure 5.2 Nucleotide sequence of the partial cDNA isolated from the zebrafish brain corresponding to the GRF-PACAP transcript. Primers used to amplify the transcript are underlined with dashes and the exon barriers are indicated. In addition, the 5'untranslated region (UTR), signal peptide, cryptic peptide, GRF peptide, PACAP peptide and 3'UTR are indicated. Sequence that is translated is indicated by capital letters and non-translated sequence is indicated by lower case letters. The sequence encoding the signal peptide is underlined with dots and dashes. The sequence encoding the GRF peptide is single underlined and the sequence encoding the PACAP peptide is double underlined.

exon1- [*5'UTR* atatctcgcctcagatccgtccgactacgaagacctgagag 41

agagagggaggaaagatccagacgctgtgggtaacaaagtgacgcgttgaa 92

aagtttaaagagcaagactgggagagaaaggagagagagagagagagctgg 143

agaatttcatctcattctggacgcagcctccattggacagcatccgtccgc 194

tgccgcag -exon2- a] [*Signal Peptide* ATG ATT ACG AGC AGC AAA 221

ACG ACT CTT GCT TTC CTC ATC TAT GGG CTC CTA GTT CAA 260

TGC AAT GTG TGT] [*Cryptic peptide* TCG CCT CTG AGT TAC CCG AAA 293

ATC AG -exon3- A ATG GAG ACT GCA GGA TTT GAT GAG GAG 326

GGA AAC TCA TTA ACG GAT GTA ACA TTT GAC AGT GAC CAG 365

ATC ACT ATA CGA AGC TCT CCT ACA GTC ACT GAA GAC GCA 404

TAC ACG TTA TTT AGT CCT CCA TCA AAA AG -exon4- A CTG 437

GAA] AGG [*GRF* CAC GCT GAC GGG ATG TTT AAT AAA GCC TAC 473

AGG AAA GCG CTC GGC CAG TTA TCC GCG AGG AAG TAC CTG 512

CAT ACA CTG ATG GCA AAA CGT GTT GG -exon5- A GGA GGG 545

AGC ACA ACA GAA GAC GAC AAT GAA CCA CTC TCA] AAA CGT 584

[*PACAP* CAC TCG GAC GGG GTT TTC ACG GAC AGC TAC AGT CGC 620

TAC CGG AAG CAA ATG GCC GTG AAG AAG TAT CTG GCC ACG 659

GTC CTT_{27} GGC AAA AGG TAT AGA CAG AGA TAT AGA AGC 695

AAA_{38}] GGA CGG CGG CTC GCT TAT TTG TAG_{stop} [3'UTR aatttt 728

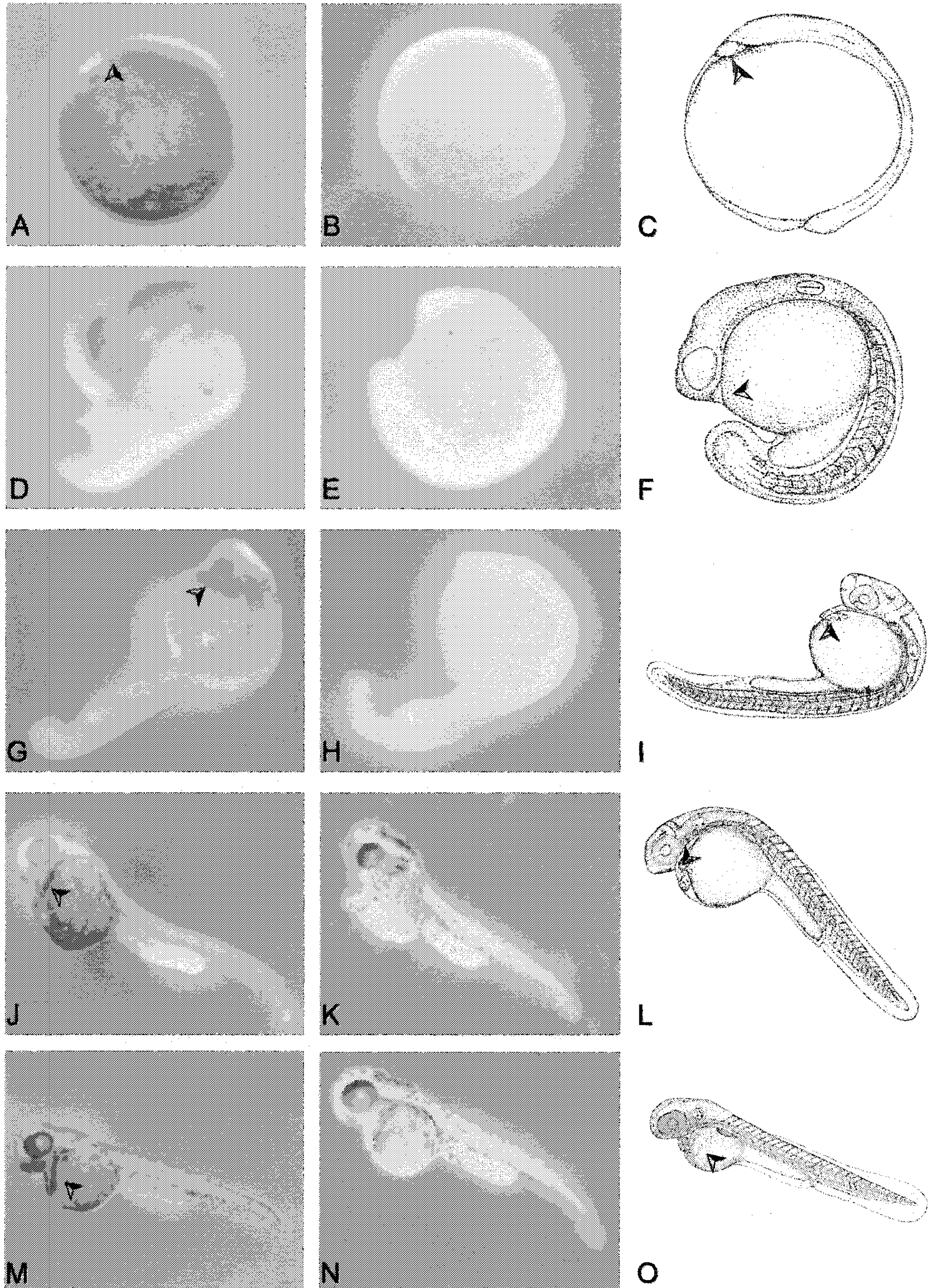
tcaaatgcgtctctctaaccatcaatgtacagctcctgtagcaagtcac 779

tattaaggctatggacctgtgtcctttctaaacgtgtatttatgtattaag 830

taaagatattacaatgaatatttgataataatattgcttttcttttgtac 881

tgaccacctgacaatgcaat] 901

Figure 5.3 Wholemount *in situ* hybridization of zebrafish embryos from the bud stage (A,B,C), the 18 somite stage (D,E,F), the 24 hour stage (G,H,I), the 30 hour stage (J,K,L) and the 48 hour stage (M,N,O). The first column has embryos hybridized with the antisense (positive) GRF-PACAP probe. The second column has embryos hybridized with the sense (negative control) GRF-PACAP probe was hybridized. The third column is a schematic representation of embryos at each stage (adapted from Kimmel et al. 1995). Arrowheads indicate the polster (anterior prechordal plate) in the bud stage embryo and the hatching gland cells in the other stages.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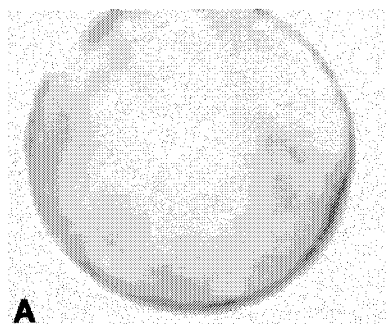
neural ectoderm of the bud stage embryo (Fig. 5.4C) and the neural tube in the 18 somite (Fig. 5.5C,D,E) and 24 hour (Fig. 5.6C,D,E,F) embryos. In the 18 somite embryo expression of the GRF-PACAP transcript is localized to the neural epithelium lining the inside and outside of the neural tube (Fig. 5.5C,D,E). In the 24 hour embryo, expression is predominantly localized at the midbrain/hindbrian boundary (Fig. 5.3G). At this stage the GRF-PACAP transcript is localized to neuroblasts throughout the neural tube, but highest levels of expression are seen in the germinative epithelium of the diencephalon and midbrain (Fig. 5.6C,D,E). By 30 hours after fertilization, when the major brain subdivisions are established, no expression of the transcript is detected in the nervous system (Fig. 5.3J).

In the developing retina the GRF-PACAP transcript is expressed during the segmentation period when this tissue is undergoing proliferation and morphogenic movement, prior to differentiation. At the 18 somite stage the GRF-PACAP transcript is widely expressed in the optic lobe (Fig. 5.5A,C). In the 24 hour embryo, the GRF-PACAP transcript is expressed in both the lens placode and the retinal neuroepithelia (Fig. 5.6A,D). At later stages of development when the eye is undergoing differentiation, no expression of the GRF-PACAP transcript is detected.

In non-neural tissues, PACAP is expressed in the prechordal plate and tissues derived from the prechordal plate. In the bud stage, the transcript is expressed throughout the embryo including the polster or prechordal plate at the anterior end of the neural plate (Fig. 5.3A). Expression of the GRF-PACAP transcript appears to be localized to the deeper cell layers and not to the enveloping layer of the bud stage embryo (Fig. 5.4). Later in development strong expression of the GRF-PACAP transcript is seen in the

Figure 5.4 Wholemount *in situ* hybridization of the GRF-PACAP mRNA transcript in the bud stage zebrafish embryo. (A) Lateral view of whole embryo negative control hybridized with the antisense mRNA probe. (B) Lateral view of whole embryo negative control hybridized with the sense mRNA probe. (C) Saggital section of bud stage embryo hybridized with the antisense mRNA probe. (D) Saggital section of bud stage embryo hybridized with the sense mRNA probe. Abbreviations are: **dcl**, deep cell layers; **envl**, enveloping layer.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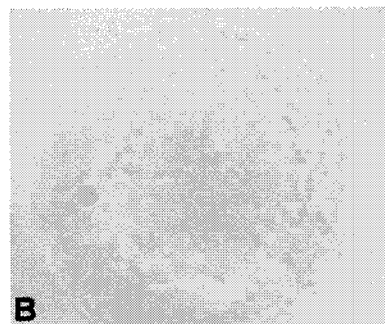
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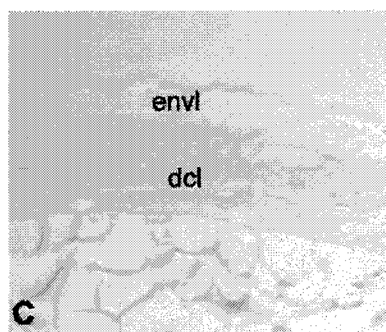
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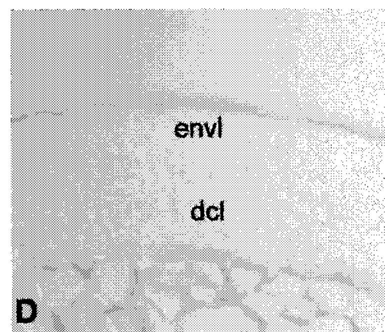
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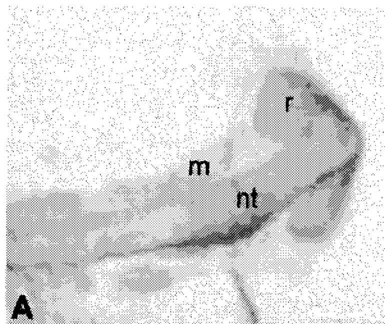
Figure 5.5 Wholemount *in situ* hybridization of the GRF-PACAP mRNA transcript in the 18 somite stage zebrafish embryo. (A) Dorsal view of whole embryo hybridized with the antisense mRNA probe. (B) Ventral view of whole embryo negative control hybridized with the sense mRNA probe. (C) Longitudinal section of the forebrain of an 18 somite stage embryo hybridized with the antisense mRNA probe. (D) Longitudinal section of the midbrain of an 18 somite stage embryo hybridized with the antisense mRNA probe. (E) Saggital section of the hindbrain of an 18 somite stage embryo hybridized with the antisense mRNA probe. (F) Longitudinal section of the midbrain of an 18 somite stage embryo hybridized with the sense mRNA probe, inset of retina. Abbreviations are: **m**, mesoderm; **nt**, neural tube; **r**, retinal neuroepithelia.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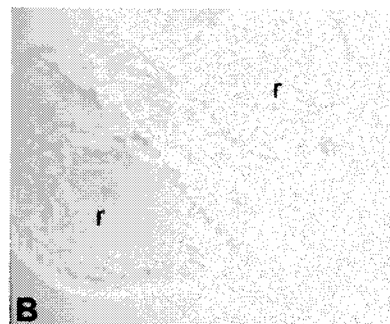
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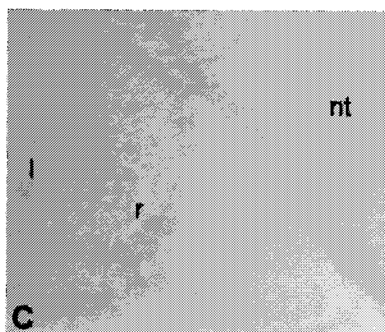
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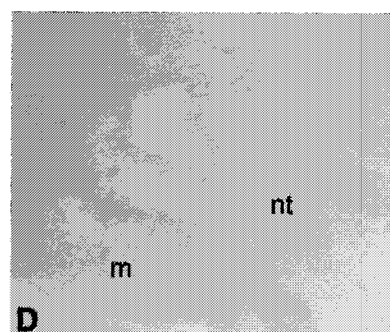
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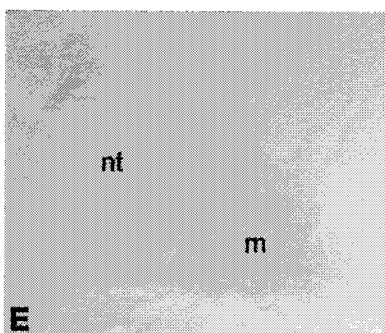
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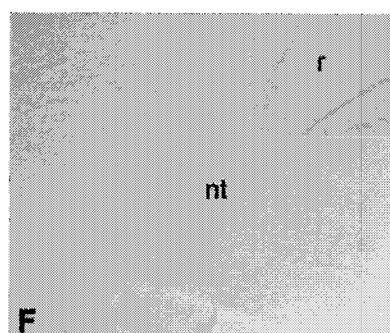
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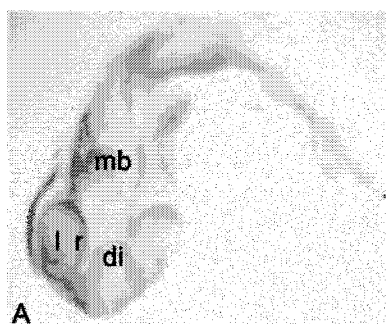
Figure 5.6 Wholemount *in situ* hybridization of the GRF-PACAP mRNA transcript in the 24 hour zebrafish embryo. (A) Dorsal view of whole embryo negative control hybridized with the antisense mRNA probe. (B) Saggital view of whole embryo hybridized with the sense mRNA probe. (C) Cross section of the forebrain of a 24 hour embryo hybridized with the antisense mRNA probe. (D) Cross section of the forebrain of a 24 hour embryo hybridized with the antisense mRNA probe. (E) Cross section of the midbrain of a 24 hour embryo hybridized with the antisense mRNA probe. (F) Cross section of the hindbrain a 24 hour embryo hybridized with the antisense mRNA probe. Abbreviations are: **di**, diencephalon; **h**, hypoblast; **l**, lens primordia; **mb**, midbrain; **m**, mesoderm; **nt**, neural tube; **r**, retinal neuroepithelia.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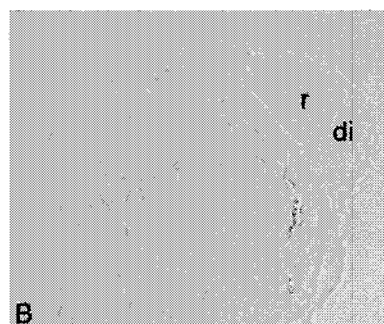
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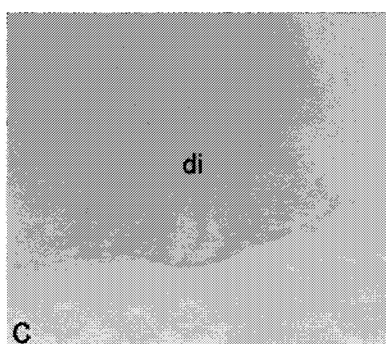
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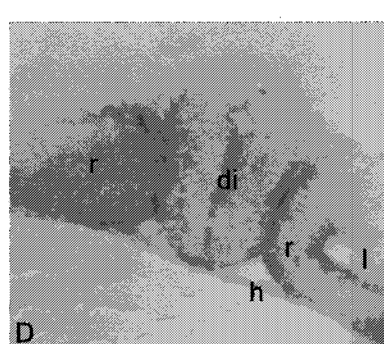
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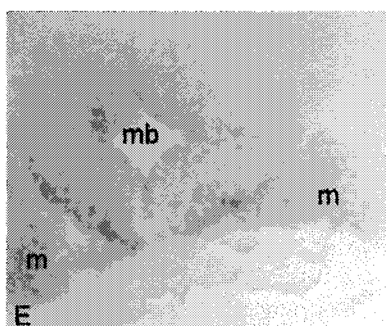
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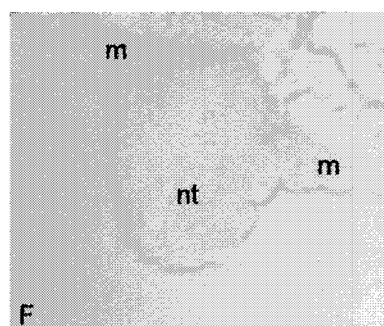
m
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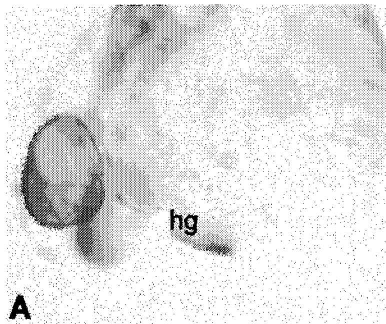
hatching gland, a derivative of the prechordal plate, in the 24 hour (Fig 5.3G), 30 hour (Fig. 5.7A,D) and 48 hour (Fig. 5.8A,C,D) embryos. In addition, in the 24 hour embryo the GRF-PACAP transcript is expressed in mesoderm cells along the neural tube and in the endoderm enveloping the yolk in the ventral region underlying the diencephalon of the embryo (Fig. 5.6).

DISCUSSION

Isolation of the GRF-PACAP mRNA transcript

I have isolated a partial cDNA from the zebrafish brain encoding both the GRF and PACAP peptides. The isolated partial cDNA is identical to the coding region of the previously isolated GRF-PACAP gene (Fradinger and Sherwood 2000). The GRF and PACAP peptides share high amino acid sequence identity with the PHM and VIP peptides that are encoded by a single gene as well (Sherwood et al. 2000). Nucleotide sequence comparison with the VIP gene revealed that the regions of the zebrafish transcript encoding GRF and PACAP show approximately 65% to 75% identity with mammalian PHM and VIP, whereas the other regions of the transcript had negligible identity with the VIP gene. This indicated that to avoid any cross reactivity with the VIP gene in the zebrafish, the regions encoding GRF and PACAP should not be used as a probe for the analysis of GRF-PACAP gene expression in the zebrafish embryo. Therefore, the probe used for *in situ* hybridization studies in the zebrafish embryo spanned from the 5'untranslated region to the cryptic peptide and did not include the coding regions for the peptides.

Figure 5.7 Wholemount *in situ* hybridization of the GRF-PACAP mRNA transcript in the 30 hour zebrafish embryo. (A) Lateral view of whole embryo hybridized with the antisense mRNA probe. (B) Saggital section of 30 hour embryo negative control hybridized with the sense mRNA probe. (C) Saggital section showing the base of the yolk of a 30 hour embryo hybridized with the antisense mRNA probe. (D) Saggital section showing the hatching gland of a 30 hour embryo hybridized with the antisense mRNA probe. Abbreviations are: **e**, epidermis; **hg**, hatching gland.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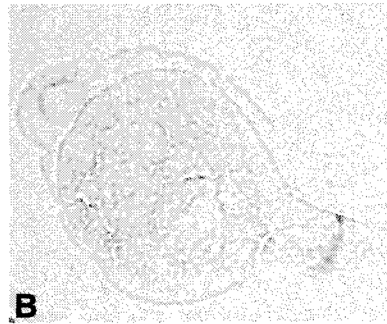
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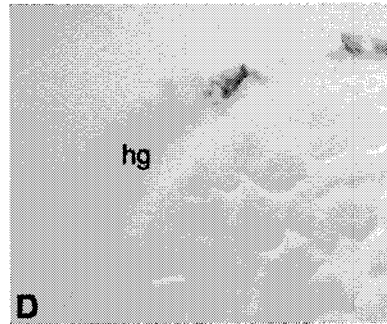
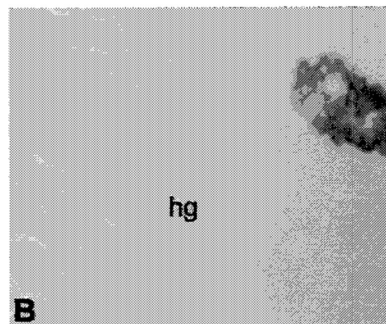

hg
D

Figure 5.8 Wholemount *in situ* hybridization of the GRF-PACAP mRNA transcript in the 48 hour zebrafish embryo. (A) Lateral view of whole embryo hybridized with the antisense mRNA probe. (B) Saggital section of the head and hatching gland region of a 48 hour embryo hybridized with the sense mRNA probe. (C) Saggital section showing the head and hatching gland of a 48 hour embryo hybridized with the antisense mRNA probe. (D) Saggital section showing the hatching gland of a 48 hour embryo hybridized with the antisense mRNA probe. Abbreviations are: **a**, mandibular and hyoid arches; **hg**, hatching gland.

hg
A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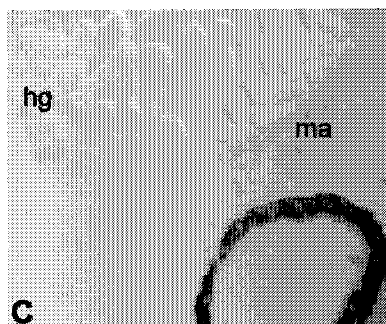
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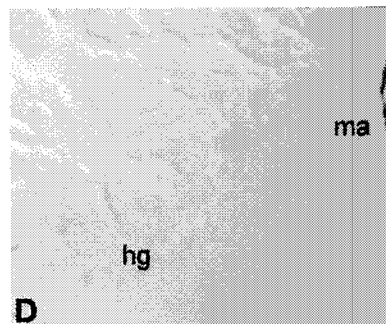
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Developmental expression of the GRF-PACAP mRNA transcript

The GRF-PACAP transcript is widely expressed in the zebrafish embryo indicating that GRF and PACAP may modulate the developmental program. In particular, the transcript is predominantly expressed during the segmentation period encompassing the bud, 18 somite and 24 hour stages, whereas only limited expression is seen during the pharyngula period encompassing the 30 hour and 48 hour stages. Gastrulation results in the formation of the three germ layers, endoderm, mesoderm and ectoderm. During the segmentation period individual populations of cells from these layers undergo extensive proliferation, morphogenesis and differentiation to form the rudimentary organs. During the segmentation period much of the embryo is in cell cycle 16 and many cells are exiting the cell cycle and starting to differentiate (Kane 1999). By the end of the segmentation period, most of the major systems of the embryo are developed (Kane 1999). During the pharyngula period, neural crest derived structures including the jaw and endodermal derived structures of the gut and circulatory system develop in preparation for hatching (Kimmel et al. 1995). The present evidence coupled with the literature suggests that the GRF-PACAP transcript is predominantly expressed in undifferentiated cells and that expression is turned off as cells become differentiated. The widespread expression of the GRF-PACAP gene during the segmentation period may indicate that GRF and PACAP are involved in promoting cell cycle exit prior to differentiation. Determining which PAC_1 receptor isoforms are present would be key in elucidating the function of PACAP in these cells because activation of the PAC_1 short receptor isoform stimulates cell cycle exit and activation of the PAC_1 hop receptor isoform stimulates proliferation in neuroblast cultures (Nicot and DiCicco-Bloom 2001).

Expression of GRF-PACAP in the developing brain

The GRF-PACAP transcript is expressed in the developing brain throughout the segmentation period. At the beginning of the segmentation period or the bud stage, the brain rudiment is a slight swelling at the anterior end of the embryo and no morphological subdivisions are evident. During the first half of the segmentation period distinctive swellings develop and by the 18 somite stage four major subdivisions can be distinguished: the telencephalon, diencephalon, mesencephalon and hindbrain. At the end of the segmentation period, subdivision of the hindbrain results in a prominent cerebellum primordium (Kimmel et al. 1995). In the bud stage embryo, the transcript is expressed throughout the embryo including the anterior region that will later become the brain. Similarly, in the 18 somite stage embryo the GRF-PACAP transcript is expressed along the length of the neural tube and is localized to the germinative neuroepithelia and cells in the perimeter of the neural tube. At this stage the neural tube is only one to two cells thick, although nuclei are distributed throughout the tube giving it a multicellular appearance (Cooper et al. 1999). In the 24 hour embryo, expression of the GRF-PACAP transcript is found predominantly in the forebrain, midbrain and cerebellum at the mid-hindbrain barrier. The transcript is expressed throughout the neural tube, but higher levels of expression are found in the germinative epithelium. This indicates that PACAP could be involved in promoting proliferation or in signaling for cell cycle exit in the developing neural tube.

In the embryonic rat brain at 13 days after fertilization PACAP mRNA was distributed in similar regions as found in the zebrafish indicating that the expression of PACAP is conserved throughout vertebrate evolution. Strong expression of the PACAP

transcript was found in neuroepithelium of the telencephalon, diencephalon, myelencephalon (medulla) and metencephalon (pons and cerebellum) in the rat embryo. In these regions the PACAP transcript was preferentially detected in the mitotic layer of the neuroepithelium (Skoglosa et al. 1999). In support of a developmental role for PACAP in these regions, PACAP binding sites were found in germinative areas of the myelencephalon and metencephalon of the embryonic rat at 14 days after fertilization (Basille et al. 2000).

PACAP may also be involved in promoting dorsal patterning of the neural tube. In mice, PACAP was found to down regulate genes involved in ventral patterning at early developmental stages (Waschek et al. 1998). In addition, the strong expression of PACAP in the midbrain and hindbrain in the 24 hour embryo suggests that PACAP may regulate the formation of the midbrain-hindbrain boundary. However, further work is required to determine whether GRF and PACAP are involved in patterning or cell cycle control in the neural tube. In addition, characterization of the PAC_1 receptor isoforms will aid in elucidating PACAP's role during the development of the vertebrate brain.

Expression of GRF-PACAP in the developing retina

The GRF-PACAP transcript is highly expressed in the developing retina during the segmentation period. In the zebrafish, the eye develops as a thickening of the anterior end of the neural tube forming the optic lobes. The optic lobes partially separate from the diencephalon and the attachments later become the optic stalk. Next, an invagination forms in the external surface of the optic lobe transforming it into the optic cup and the lens rudiment begins to develop from the overlying epithelium. Retinal cells become post mitotic and begin to differentiate during the pharyngula period after the last

morphological movements are complete (Malicki 1999). Therefore, the GRF-PACAP transcript is expressed in the developing retinal neuroepithelium and lens placode when these tissues are undergoing proliferation and morphogenic movement. PACAP and its receptor were present in human foetal retinas from 17-18 week embryos (Olianas et al. 1997). In cultured retinal neuroepithelial cells from these embryos, PACAP caused a dose-dependent increase in cAMP (Olianas et al. 1999). Therefore, the expression of the GRF-PACAP transcript in the developing zebrafish retina suggests that these peptides may be involved in regulating the growth of this tissue.

Expression of GRF-PACAP in non-neural tissues

The most significant site of non-neural GRF-PACAP gene expression is in the prechordal plate at the bud stage and in the hatching gland at later stages. The polster located at the anterior most tip of the neural tube, is a cell mass of the prechordal plate hypoblast and gives rise to the hatching gland (Inohaya et al. 1997). Therefore, the hatching gland cells undergo proliferation and then migrate toward their final location in the epidermis of the yolk sac. In the hatching gland the GRF-PACAP transcript is expressed predominantly in the pharyngula period when the cells are no longer dividing and have finished migrating (Kane et al. 1999). The hatching gland does not have a mammalian counterpart; it contains enzymes that promote eclosion of embryos. The physiological role of the GRF-PACAP gene in the hatching gland is unknown.

The prechordal plate is involved in patterning of the neural tube. Some mutants exhibiting a loss of the prechordal plate exhibit a ventralized phenotype; others with defects in the prechordal plate show developmental changes in the ventral forebrain (Mullins 1999). Interestingly, the dorsal marker gene *goosecoid* that was found to be up-

regulated by PACAP in the frog (Otto et al. 2000) is expressed in the prechordal plate (Schulte-Merker et al. 1994). The expression of the GRF-PACAP transcript in mesoderm along the neural tube may indicate that it is involved in patterning as well. Therefore, the role of PACAP in neural tube patterning should be investigated further.

During the pharyngula period, neural crest cells undergo differentiation to form the jaw. In the 48 hour embryo, expression of the GRF-PACAP gene is seen in a region corresponding to the mandibular and hyoid arches that give rise to the jaw. The timing of expression indicates that PACAP may be involved in stimulating cell cycle exit prior to the differentiation and morphogenesis of the jaw.

In conclusion, the GRF-PACAP gene is expressed in all three germ layers indicating that it may be involved in the development of organs in which it is found in the adult. In addition, the gene is highly expressed during the segmentation period. During this period cells are undergoing cell cycle exit and differentiating to form the embryo rudiment. Therefore, the timing of the expression of the GRF-PACAP gene indicates that it may, in general, regulate cell cycle exit during development. Similarly, the widespread expression of the gene is also indicative that the GRF-PACAP gene may be involved in controlling the cell cycle and not more specific events. However, PACAP has also been implicated in dorsal-ventral patterning and proliferation of some brain areas. Therefore, PACAP may have more than one function in the developing embryo. Full characterization of the receptors and signaling pathways for GRF and PACAP is required to elucidate the physiological role of the GRF-PACAP gene during development.

CHAPTER 6

GENERAL CONCLUSIONS

Growth and development are complex processes that require the coordinated actions of a variety of factors throughout the life of an organism. The developmental program of individual cells is determined by what genes are activated within the cell and cells in its environment. Therefore, two key questions in developmental biology are 1) what genes are expressed during development and 2) at what time are they being expressed.

The PACAP peptide is highly conserved indicating that it may have an important physiological role. In contrast, the GRF peptide is only moderately conserved, but its primary function appears to have been conserved throughout evolution. GRF causes the release of growth hormone from the pituitary in all species studied to date (Sherwood et al. 2000). However, examining gene function in fish can be complicated due to the presence of multiple copies of the gene in question. The salmon is tetraploid and was shown to possess two copies of the GRF-PACAP gene (Krueckl and Sherwood 2001). Therefore, the zebrafish provides a simpler fish model for studying the physiological role of GRF and PACAP, because its genome contains only one copy of the GRF-PACAP gene.

The GRF-PACAP mRNA transcript is widely expressed in the adult zebrafish and the transcript encodes both the GRF and PACAP peptides. In contrast, alternative splicing of the GRF-PACAP genes results in the generation of two transcripts in the salmon and catfish (Parker et al. 1993 and Small and Nonneman 2001). This difference in gene regulation may be due to the presence of two copies of the GRF-PACAP gene in these fish. However, in the zebrafish during mid-gastrulation, alternative splicing of the GRF-PACAP gene results in the generation of two transcripts. The significance of the

increased ratio of PACAP to GRF at this stage may have interesting functional implications. Alternative splicing of the GRF-PACAP gene is not found at any other developmental stage or in any adult tissue studied.

To examine the functional significance of the co-expression of the GRF and PACAP peptides, I identified the GRF and PACAP receptors from the zebrafish and characterized their expression pattern. Three transcripts were isolated from zebrafish brain cDNA library encoding the GRF receptor, the PAC_1 receptor and the $VPAC_1$ receptor. The PAC_1 receptor is the PACAP specific receptor and the $VPAC_1$ receptor is a shared VIP/PACAP receptor. The second shared VIP/PACAP receptor, the $VPAC_2$ receptor, was not isolated from the zebrafish and is hypothesized to have arisen later in evolution (Vaudry et al. 2000). All three receptors are widely expressed in the adult zebrafish and in most tissues the GRF and PACAP receptors are co-localized. The overlap between the tissue distributions of the GRF and PACAP receptors suggests that the two peptides could be functionally related. In addition, the GRF and PACAP receptors were expressed in tissues expressing the GRF-PACAP transcript. This suggests that GRF and PACAP may act in an autocrine or paracrine manner in these tissues. Of the nine isoforms of the PAC_1 receptor found in mammals, three were isolated in the zebrafish, the PAC_1 short, hop1 and hop2 isoforms. In addition, a novel isoform was isolated from the gill. The PAC_1 short receptor isoform is always expressed, whereas the hop1, hop2 and novel PAC_1 receptor isoforms are expressed in a tissue-specific manner. The different PAC_1 receptor isoforms are differentially coupled to the cAMP and phospholipase C signaling pathways (Spengler et al. 1993), indicating that the differential expression of the isoforms may modulate the physiological actions of PACAP in different

tissues. The coupling of the receptor isoforms to distinct intracellular signaling pathways may explain the tissue specific effects of PACAP. In addition, GRF, PACAP and their receptors are expressed in tissues that are developmentally derived from all three germ layers.

In mammals, both GRF and PACAP have been implicated in development. In fish, the GRF-PACAP transcript is expressed very early in development in both the salmon (Krueckl and Sherwood 2001) and the zebrafish. In addition, I have shown that the receptors for GRF and PACAP are expressed throughout the developmental period. In particular, strong expression of GRF, PACAP and their receptors is seen during mid-gastrulation when the embryonic axis is forming. Wide expression of the GRF-PACAP transcript is seen during the segmentation period in the zebrafish. During this period, cells stop dividing and begin to differentiate to form the embryonic body plan. Also, during the segmentation period differential expression of the PAC_1 receptor isoforms is seen. In mammals, the hop isoform was shown to promote cell proliferation and the short isoform was shown to promote cell cycle exit in cortical neuroblasts (Nicot and DiCicco-Bloom 2001). Therefore, the stage specific expression of PAC_1 receptor isoforms indicates that PACAP may be regulating these two processes in the developing zebrafish.

During development the expression of the GRF-PACAP transcript goes from diffuse to specific in the transition between the segmentation and subsequent pharyngula periods. In the segmentation stage the transcript is expressed in all three germ layers, the endoderm, ectoderm and mesoderm. In the ectoderm, the transcript is widely expressed in undifferentiated neuroblasts throughout the neural tube. In the chick, PACAP causes a dose dependent increase in cAMP in neuroblasts, indicating that PACAP may modulate

brain development (Erhardt et al. 2001). Also, the GRF-PACAP transcript is expressed in mesodermal tissues alongside the neural tube and in the prechordal plate. In particular, the transcript is expressed in the polster or anterior prechordal plate at the bud stage and the hatching gland that is derived from this region. The timing of GRF-PACAP gene expression and the expression of the PAC_1 short receptor isoform indicates that these peptides are involved in regulating cell cycle exit in the developing zebrafish embryo. However, further work is required to fully elucidate the developmental role of GRF and PACAP.

I have shown that mRNA expression of GRF, PACAP and their receptors is widely distributed in the adult and embryonic zebrafish. However, it is important to note that the transcription of a gene does not necessarily suggest that translation is occurring. Therefore, protein localization is needed to confirm whether the GRF-PACAP transcript is translated to yield both peptides in the tissues of the adult zebrafish and the developing embryo. In addition, although the mRNA transcript is present at the blastula/gastrula transition, the synthesis of mature peptide requires approximately one hour. Therefore, the earliest that GRF and PACAP may have a physiological action is during mid-epiboly. However, this still corresponds to a possible role for GRF and PACAP in early patterning events and cell cycle regulation.

Future Directions

A full characterization of the function of GRF and PACAP during development is an important direction. The work I have completed provides a strong background for functional studies in the zebrafish. It will be important to localize the expression of the

GRF and PACAP receptors during development. In addition, the expression of the PAC_1 receptor isoforms and their activation of the different intracellular signaling pathways must be elucidated.

The sequencing of the zebrafish genome is in progress and will make it possible to identify molecules that are structurally related to PACAP and GRF. As well, gene array systems can be constructed for examining the developmental role and intracellular pathways of the GRF and PACAP peptides. New methods for genetic manipulation, such as gene disruption, are now available. To date, a PACAP gene knockout can only be done in the mouse. Our laboratory constructed a PACAP knockout mouse and found that obvious changes occur after birth, but not during early development. However, subtle developmental changes are difficult to detect since the embryos are not accessible. In fertilized zebrafish eggs, microinjection of antisense GRF-PACAP morpholinos may provide a mechanism for examining the effects of reduced gene expression during early development. Morpholinos bind to a target mRNA transcript and prevent its translation. In addition, knockdown of the GRF, PAC_1 and $VPAC_1$ receptors alone or in combination will allow us to investigate whether the GRF, PACAP and VIP peptides compensate for each other during development. The functional and evolutionary significance of the co-expression of GRF and PACAP in fish, and the structural relationship between VIP and PACAP remain to be elucidated.

The zebrafish is an excellent developmental model system for examining gene function. The zebrafish is particularly well suited for gene expression studies, localization by *in situ* hybridization and knockdown studies. Therefore, the zebrafish will be useful in furthering our understanding of the role of GRF and PACAP in modulating

the formation of the embryonic axis and in the development of the embryonic body plan. Studies in the zebrafish will also allow us to answer evolutionary questions regarding the developmental role of these two peptides.

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