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**Developmental expression and evolution of growth hormone-releasing hormone and pituitary adenylate cyclase-activating polypeptide in teleost fishes, rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*)**

by

Sandra Lea Krueckl  
B.A., University of Victoria, 1993

A Dissertation Submitted in Partial Fulfillment of the  
Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Biology

We accept this dissertation as conforming  
to the required standard

---

Dr. N.M. Sherwood, ~~Supervisor~~ (Department of Biology)

---

Dr. L. Page, ~~Departmental Member~~ (Department of Biology)

---

~~Dr. W. Hinz, Departmental Member~~ (Department of Biology)

---

Dr. T. Pearson, ~~Outside Member~~ (Department of Biochemistry)

---

Dr. P. Leung, External Examiner (University of British Columbia, Faculty of Medicine)

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University of Victoria

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Supervisor: Dr. N.M. Sherwood

## **ABSTRACT**

Growth hormone-releasing hormone (GRF) and pituitary adenylate cyclase-activating polypeptide (PACAP) are members of the PACAP/Glucagon superfamily. The family is proposed to have developed from an ancestral PACAP-like molecule in invertebrates. Through successive exon, gene and genome duplications the family has grown to include seven other members. In mammals GRF and PACAP are located on different genes, but in fish, amphibians and birds they are located on the same gene. The main function of GRF is the release of growth hormone (GH) from the pituitary. Also, during development GRF influences the fetal pituitary and stimulates GH release during late gestation. In contrast, the functions of PACAP are extremely varied. PACAP is the newest member of the superfamily and there is still much work to be done before its actions are well understood. Like GRF, PACAP is a releasing hormone acting on the pituitary and in addition, the adrenal gland, pancreas and heart, as well as other organs. Also, PACAP regulates smooth muscle in the vascular system, gut, respiratory tract and reproductive tract. During development PACAP affects proliferation, differentiation and apoptosis.

GRF and PACAP are expressed throughout development in fish, beginning during the blastula period in rainbow trout and at the end of gastrulation in zebrafish (earliest stage examined). In rainbow trout the *grf/pacap* gene is expressed as two transcripts, a short and a long transcript. The short transcript is produced by alternative splicing of the gene and does not include the fourth exon which codes for GRF. The long transcript includes the coding regions for both GRF and PACAP. By this means PACAP can be regulated separately from GRF. With the extensive role PACAP appears to play in development, separate regulation of the hormone may be necessary. Expression of the *grf/pacap* gene in zebrafish is widespread early in development and gradually becomes localized. Of

particular interest is the expression of the *grf/pacp* transcript in regions associated with the prechordal plate, an important organizing center in development. Although it is not yet confirmed, there is evidence to suggest GRF and PACAP are expressed in the prechordal plate and its derivatives in the gut and hatching gland. In addition, expression of the *grf/pacp* transcript is observed in the neuroectoderm (eye, brain and spinal cord) and the developing heart. Considering the expression pattern of GRF and PACAP, I propose that one of both of these hormones may be involved in patterning during vertebrate embryogenesis.

The evolution of gene families is thought to occur through successive exon, gene and genome duplications. Duplicate exons or genes become differentiated and eventually gain new functions or become functionless. During evolution of the *grf/pacp* lineage, several duplication events have occurred. Analysis of rainbow trout leads me to think that this fish and other salmonids possess two copies of the *grf/pacp* gene. This is not unexpected considering the tetraploid nature of salmonids. Present day mammals encode GRF and PACAP on separate genes. At some point during the evolution of this lineage a duplication event has occurred, possibly in early mammals or prior to the divergence of birds. The study of multigene families is a useful way to understand evolutionary processes. To this end I examined three members of multigene families from sockeye salmon. Therefore, in addition to the evolutionary mechanisms and pathways that directed *grf/pacp* gene evolution, I examined the ferritin-H subunit, the alpha-tubulin subunit and the beta-globin subunit. These cDNA sequences are similar to their counterparts in other teleost. The evolution of the ferritin gene family is particularly interesting because it involves the addition or deletion of DNA sequences that affect regulation and cytosolic location.

## Examiners:

~~Dr. N.M. Sherwood, Supervisor (Department of Biology)~~

~~Dr. L. Page, Departmental Member (Department of Biology)~~

~~Dr. W. Hintz, Departmental Member (Department of Biology)~~

~~Dr. T. Pearson, Outside Member (Department of Biochemistry)~~

~~Dr. P. Leung, External Examiner (University of British Columbia, Faculty of Medicine)~~

## TABLE OF CONTENTS

|                                                                                                    |      |
|----------------------------------------------------------------------------------------------------|------|
| ABSTRACT .....                                                                                     | ii   |
| TABLE OF CONTENTS .....                                                                            | v    |
| LIST OF TABLES .....                                                                               | vii  |
| LIST OF FIGURES .....                                                                              | viii |
| LIST OF ABBREVIATIONS .....                                                                        | x    |
| ACKNOWLEDGEMENTS.....                                                                              | xii  |
| DEDICATION .....                                                                                   | xiii |
| CHAPTER 1: General Introduction .....                                                              | 1    |
| I. Rationale .....                                                                                 | 2    |
| II. GRF.....                                                                                       | 3    |
| A. A hypophysiotropic regulator.....                                                               | 4    |
| B. A regulator of cell cycle and development .....                                                 | 5    |
| III. PACAP .....                                                                                   | 7    |
| A. Introduction.....                                                                               | 7    |
| B. PACAP receptors.....                                                                            | 8    |
| C. A regulator of cell cycle and development .....                                                 | 13   |
| D. A regulator of smooth and cardiac muscle.....                                                   | 24   |
| E. An immune system regulator.....                                                                 | 33   |
| F. A regulator of bone metabolism .....                                                            | 36   |
| G. An endocrine/paracrine regulator.....                                                           | 37   |
| H. An exocrine regulator.....                                                                      | 53   |
| I. A regulator in the nervous system .....                                                         | 54   |
| CHAPTER 2: Temporal expression pattern of grf/pacap during<br>fish embryogenesis .....             | 57   |
| Introduction .....                                                                                 | 58   |
| Methods and Materials .....                                                                        | 59   |
| Results.....                                                                                       | 62   |
| Discussion.....                                                                                    | 62   |
| CHAPTER 3: In situ localization of grf/pacap expression during<br>embryogenesis of zebrafish ..... | 71   |
| Introduction .....                                                                                 | 72   |
| Methods and Materials .....                                                                        | 73   |
| Results.....                                                                                       | 81   |
| Discussion.....                                                                                    | 103  |
| CHAPTER 4: Overexpression of the grf/pacap gene in several<br>salmonid species.....                | 111  |
| Introduction .....                                                                                 | 112  |
| Methods and Materials .....                                                                        | 113  |
| Results.....                                                                                       | 125  |
| Discussion.....                                                                                    | 143  |

|                                                                                              |     |
|----------------------------------------------------------------------------------------------|-----|
| CHAPTER 5: Gene copy number and evolution of grf/pacap from<br>invertebrates to mammals..... | 148 |
| Introduction .....                                                                           | 149 |
| Methods and Materials .....                                                                  | 150 |
| Results .....                                                                                | 154 |
| Discussion .....                                                                             | 154 |
| CHAPTER 6: General Discussion.....                                                           | 170 |
| Introduction .....                                                                           | 171 |
| GRF/PACAP and development.....                                                               | 172 |
| Overexpression of GRF/PACAP.....                                                             | 174 |
| Evolution of multigene families.....                                                         | 175 |
| REFERENCES.....                                                                              | 177 |

## LIST OF TABLES

|            |                                                                       |     |
|------------|-----------------------------------------------------------------------|-----|
| TABLE 1-1. | Nomenclature for PACAP receptors.....                                 | 9   |
| TABLE 4-1. | Percent Mortality of Control vs. Microinjected<br>Salmonid eggs ..... | 131 |

## LIST OF FIGURES

|              |                                                                                                                              |     |
|--------------|------------------------------------------------------------------------------------------------------------------------------|-----|
| FIGURE 2-1.  | RT-PCR results for alpha tubulin products from rainbow trout embryos.....                                                    | 63  |
| FIGURE 2-2.  | RT-PCR results for grf/pacap products from rainbow trout embryos.....                                                        | 65  |
| FIGURE 2-3.  | The grf/pacap cDNA sequence for rainbow trout.....                                                                           | 67  |
| FIGURE 3-1.  | A schematic of the zebrafish grf/pacap cDNA .....                                                                            | 74  |
| FIGURE 3-2.  | Labeling of grf/pacap in the bud stage zebrafish embryo (lateral view).....                                                  | 83  |
| FIGURE 3-3.  | Negative control. Labeling of grf/pacap (sense probe) of an 18-somite stage zebrafish embryo.....                            | 85  |
| FIGURE 3-4.  | Labeling of an 18 somite stage zebrafish embryo with grf/pacap .....                                                         | 87  |
| FIGURE 3-5.  | Negative control. Labeling of grf/pacap (sense probe) for the 18-somite, 24 hour, 36 hour and 48 hour zebrafish embryo ..... | 89  |
| FIGURE 3-6.  | Labeling of the hatching gland with a grf/pacap probe (antisense probe) in 18-somite and 24 hour zebrafish embryos.....      | 91  |
| FIGURE 3-7.  | Negative control. Labeling of grf/pacap (sense probe) in the 24 hour zebrafish embryo .....                                  | 93  |
| FIGURE 3-8.  | Labeling of grf/pacap (antisense probe) in the 24 hour zebrafish embryo .....                                                | 95  |
| FIGURE 3-9.  | Labeling of grf/pacap in the 36 hour zebrafish embryo.....                                                                   | 97  |
| FIGURE 3-10. | Labeling of the hatching gland with a grf/pacap probe in 36 hour and 48 hour zebrafish embryos .....                         | 99  |
| FIGURE 3-11. | Labeling of grf/pacap in the 48 hour zebrafish embryo.....                                                                   | 101 |
| FIGURE 4-1.  | The pit-grf/pacap construct .....                                                                                            | 115 |
| FIGURE 4-2.  | The hyp-grf/pacap construct .....                                                                                            | 117 |

|             |                                                                                                                 |     |
|-------------|-----------------------------------------------------------------------------------------------------------------|-----|
| FIGURE 4-3. | Diagram of a salmonid egg that is unfertilized or fertilized but not activated .....                            | 121 |
| FIGURE 4-4. | Tubulin PCR analysis of a representative subset of the electroporation experiments.....                         | 126 |
| FIGURE 4-5. | Pit-grf/pacap PCR analysis of a representative subset of the electroporation experiments.....                   | 128 |
| FIGURE 4-6. | Tubulin PCR analysis of a representative subset of the pit-grf/pacap microinjection experiments .....           | 135 |
| FIGURE 4-7. | Pit-grf/pacap PCR analysis of a representative subset of the microinjection experiments.....                    | 137 |
| FIGURE 4-8. | Tubulin PCR analysis of a representative subset of the hyp-grf/pacap microinjection experiments.....            | 139 |
| FIGURE 4-9. | Hyp-grf/pacap analysis of a representative subset of the microinjection experiments.....                        | 141 |
| FIGURE 5-1. | A Southern blot analysis of rainbow trout genomic DNA using a 239 bp PACAP/3' untranslated region probe.....    | 155 |
| FIGURE 5-2. | Schematic diagram of the grf/pacap gene in salmon and trout along with the pacap and grf genes in mammals ..... | 157 |
| FIGURE 5-3. | A diagram of proposed scheme 1 of the evolution of grf and pacap from invertebrates to mammals.....             | 159 |
| FIGURE 5-4. | Schematic diagram of the salmon grf/pacap gene.....                                                             | 162 |
| FIGURE 5-5. | A diagram of proposed scheme 2 of the evolution of grf and pacap from invertebrates to mammals.....             | 165 |
| FIGURE 5-6. | A diagram of proposed scheme 3 of the evolution of grf and pacap from invertebrates to mammals.....             | 167 |

## List of abbreviations defined in text

**$\alpha$ -MSH** -alpha-melanocyte stimulating hormone  
**AC** -adenylate cyclase  
**ACTH** -adrenocorticotrophic hormone  
**ANP** -atrial natriuretic peptide  
**AVP** -arginine vasopressin  
**BCIP** -5-bromo-4-chloro-3-indolyl-phosphate  
**Bmp** -bone morphogenic protein  
**CNS** -central nervous system  
**CREB** -cAMP response element binding protein  
**DEPC** -diethyl pyrocarbonate  
**DIG** -digoxigenin  
**EGL** -external granule cell layer  
**FSH** -follicle stimulating hormone  
**GH** -growth hormone  
**GIP** -glucose-dependent insulintropic polypeptide  
**GLP-1** -glucagon-like peptide-1  
**GLP-2** -glucagon-like peptide-2  
**GnRH** -gonadotropin-releasing hormone  
**grf** -the mRNA of gene encoding growth hormone-releasing hormone  
**GRF** -growth hormone-releasing hormone  
**IGL** -internal granule cell layer  
**IL** -interleukin  
**IP** -inositol phosphate  
**ir** -immunoreactive  
**LH** -luteinizing hormone  
**MAP** -mitogen-activated protein  
**NBT** -4-nitro-blue-tetrazolium  
**NGF** -nerve growth factor  
**NO** -nitric oxide  
**PAC<sub>1</sub>-R** -PACAP-1 receptor  
**PAC<sub>1</sub>-R-hip** -PACAP-1 receptor, hip variant  
**PAC<sub>1</sub>-R-hiphop1** -PACAP-1 receptor, hip and hop1 variant  
**PAC<sub>1</sub>-R-hiphop2** -PACAP-1 receptor, hip and hop2 variant  
**PAC<sub>1</sub>-R-hop1** -PACAP-1 receptor, hop1 variant  
**PAC<sub>1</sub>-R-hop2** -PACAP-1 receptor, hop2 variant  
**PAC<sub>1</sub>-R-s** -PACAP-1 receptor, short variant  
**PAC<sub>1</sub>-R-TM4** -PACAP-1 receptor, transmembrane IV variant  
**PAC<sub>1</sub>-R-vs** -PACAP-1 receptor, very short variant  
**pacap** -the mRNA or gene encoding PACAP  
**PACAP** -pituitary adenylyl cyclase-activating polypeptide  
**PBST** -phosphate-buffered saline/0.1% Tween-20  
**PHM** -peptide histidine-methionine  
**PKA** -protein kinase A  
**PLC** -phospholipase C  
**POMC** -pro-opiomelanocortin  
**PRL** -prolactin  
**PRP** -PACAP-related peptide  
**SCN** -suprachiasmatic nucleus  
**shh** -sonic hedgehog  
**SSC** -standard sodium citrate  
**TBS** -Tris-buffered saline

**UTR** - untranslated region  
**VIP** - vasoactive intestinal peptide  
**VPAC<sub>1</sub>-R** -VIP/PACAP-1 receptor  
**VPAC<sub>2</sub>-R** -VIP/PACAP-2 receptor

## **List of abbreviations not defined in text**

**cAMP** -3',5'-cyclic adenosine monophosphate  
**cDNA**- complementary DNA  
**DNA** -deoxyribonucleic acid  
**mRNA** -messenger RNA  
**PBS** -phosphate buffered saline  
**PCR** -polymerase chain reaction  
**RNA** - ribonucleic acid  
**RT-PCR** -reverse transcriptase polymerase chain reaction

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**This thesis is dedicated with love  
to my parents Bob & Carole  
and to my sister Jennifer.**

# **Chapter 1**

## **General Introduction**

A modified version of the following material is accepted for publication (N.M. Sherwood, S.L. Krueckl and J.E. McRory. 2000 The Origin and Function of the Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP)/Glucagon Superfamily. *Endocrine Reviews* 21: 619-670.

## **I. Rationale**

Growth and development are functions common to many living organisms. In plants and animals alike, these processes are triggered and directed in many cases by hormones. Two hormones involved in growth and development in vertebrates are growth hormone-releasing factor (GRF) and pituitary adenylate cyclase-activating polypeptide (PACAP). PACAP and GRF are members of the PACAP/Glucagon superfamily of peptides. The family contains nine members that are related in structure and function. The family members include: Glucagon, glucagon-like peptide -1 (GLP-1), GLP-2, glucose-dependent insulintropic polypeptide (GIP), peptide histidine-methionine (PHM), secretin and vasoactive intestinal peptide (VIP) (Sherwood et al., 2000). In mammals GRF and PACAP are encoded on separate genes. However, in fish the hormones are encoded on adjacent exons of the same gene. A proposed gene duplication early in the mammalian lineage led to two genes in mammals (Sherwood et al., 2000). In fish the *grf/pacap* gene is composed of 5 exons. Exon 1 encodes the 5' untranslated region (UTR), exon 2 the signal peptide, exon 3 the cryptic peptide, exon 4 GRF and exon 5 PACAP (Parker et al., 1993). Both hormones regulate growth by acting as hypophysiotropic factors that affect growth hormone (GH) release.

Little is known about the *in vivo* effects of GRF or PACAP expression in fish. Therefore, I began my research studying the growth-enhancing effects of these two hormones. To do this I introduced a DNA construct containing the *grf/pacap* coding region into various species of salmon and trout. With transgenic technology the effects of *grf/pacap* over-expression can be studied and inferences can be made regarding the *in vivo* function of these hormones. Although of high risk, I undertook this project because it had the potential to make a valuable contribution to our understanding of GRF and PACAP function.

My next question regarded the involvement of PACAP in embryonic development. Like PACAP, GRF appears to have a role in development. However, as will be discussed in the following pages, the main contribution of GRF in the regulation of development is postnatal and pubertal. In contrast, during the course of my research a number of studies were published on the role of PACAP in mammalian fetal development. To date, no work has been done on developmental expression patterns of the *grf/pacap* gene in fish. The developmental studies presented in this thesis began in rainbow trout in order to complement the over-expression study. However, I deemed zebrafish to be more appropriate for in situ hybridization studies, due to the rapid development and transparent nature of the embryo. Also, zebrafish have become an important vertebrate model for the study of development.

The following section reviews current research into the expression and function of GRF and PACAP, with a focus on development. The reason for the focus on PACAP is twofold. First, there is more evidence to suggest PACAP has an important role to play in embryonic development when compared to GRF. Second, I consider PACAP to be a very important member of the PACAP/Glucagon superfamily. It has been proposed by our laboratory that PACAP is an ancestral molecule in the superfamily due to high conservation of nucleotide and amino acid sequences across many species (Sherwood et al., 2000). Another striking similarity between PACAP and other family members is the overlap of function. As a newly discovered hormone, PACAP's functional aspects are just becoming clear and have rarely been summarized. For this reason I have given a more detailed summary of the current findings regarding PACAP's function.

## **II. GRF**

The GRF peptide was first discovered in human pancreatic tumors by two groups in 1982 (Guillemin et al., 1982; Rivier et al., 1982). It has since been found in the

hypothalamus of humans (Ling et al., 1984a), pigs (Bohlen et al., 1983), cattle (Esch et al., 1983), sheep and goats (Brazeau et al., 1984), rats (Bohlen et al., 1984) and carp (Vaughan et al., 1992). The cDNA sequence has been determined for humans (Mayo et al., 1983; Gubler et al., 1983), hamster (Ono et al., 1994), rats (Mayo et al., 1985b), mice (Suhr et al., 1989) and several fish species (Parker et al., 1993, 1997; McRory et al., 1995). The gene has also been cloned from humans (Mayo et al., 1985a); rats (Mayo et al., 1985b), chicken (McRory et al., 1997); sockeye salmon (Parker et al., 1997) and tunicates (McRory and Sherwood, 1997). The peptide ranges in size from 44 amino acids in humans, 43 in rats, 42 in mice, 43 or 46 in chickens, 45 in fish to 27 in tunicates. Like other members of the superfamily, the first 27-29 amino acids are the most conserved (Sherwood et al., 2000). The GRF peptide binds to a seven transmembrane receptor that is linked to a G protein. Also, this receptor is associated with cAMP accumulation (Lin et al., 1992). The receptor has been cloned from humans, pigs, rats and mice (Mayo, 1992; Lin et al., 1992; Gaylinn et al., 1993; Hsiung et al., 1993; Petersenn et al., 1998).

#### ***A. A hypophysiotropic regulator***

Immunoreactive (ir) GRF is found predominantly in the brain (particularly the hypothalamus) and pancreas. Low levels are found in the thyroid, lung, stomach, duodenum, ileum, colon, adrenals and the kidney (Shibasaki et al., 1984; Christofides et al., 1984; Bosman et al., 1984; Bruhn et al., 1985). The main action of GRF is hypophysiotropic. In mammals, it is produced in the hypothalamus, predominantly in the arcuate nucleus (Shibasaki et al., 1984; Bruhn et al., 1985; Guillemin 1986; Luo et al., 1989a, 1989b; Mayo et al., 1996). Immunoreactivity for GRF has also been found in the median eminence in axons originating from GRF-expressing neurons in the hypothalamus (Shibasaki et al., 1984; Mayo et al., 1996). From the median eminence GRF is released into the portal blood system and travels to the pituitary where it acts on GRF receptors found on the surface of the anterior pituitary (Lin et al., 1992). As a result of exposure to

GRF, growth hormone is released from somatotroph cells in the anterior pituitary (Ling et al., 1984b; Frohman and Jansson, 1986; Luo et al., 1990).

### ***B. A regulator of cell cycle and development***

Studies in mice indicate that GRF may regulate proliferation of somatotroph cells. Dwarf mice (dw/dw) suffer from pituitary hypoplasia. These animals do not express the GRF receptor or GH (Lin et al., 1992). Thus, the removal of a receptor that permits GRF activity results in hypoplasia. Transgenic mice that over-express human GRF suffer from the opposite disorder, hyperplasia of the pituitary. These mice have increased GRF expression and as a result the pituitary is enlarged due to selective proliferation of somatotrophs, (Mayo et al., 1988). Both of these studies indicate that GRF is a factor regulating the cell division of somatotrophs. In addition to its proliferative action in somatotrophs GRF expression has been noted in several cancers. GRF appears to increase proliferation in prostate cancer (Jungwirth et al., 1997a) and renal adenocarcinomas (Jungwirth et al., 1997b). Also, GRF is present in the following cancers: pancreatic tumors, insulinomas, glucinomas, bronchial carcinoids, gut carcinoids, gastrinomas, VIPomas, thymic carcinoids, medullary carcinomas of the thyroid, pheochromocytomas, ganglioneuroblastomas, and small cell carcinomas of the lung (Christofides et al., 1984; Asa et al., 1985).

There is considerable evidence for the presence of irGRF and GRF mRNA expression in the placenta of pigs (Farmer *et al*, 1997), rats (Margioris et al., 1990; Gonzalez-Crespo et al., 1991; Srivastava et al., 1995) and mice (Mizobuchi et al., 1991; Mizobuchi et al., 1995). However, GRF is not found in full and midterm human placentas. In the rat placenta, GRF has been observed on gestational days 13 through 19. The levels of GRF peak at days 16-17 (Meigan et al., 1988; Suhr et al., 1989). The presence of GRF in the placentas of rats and mice during development suggests that it may have a role in fetal and/or maternal physiology. Immunoreactive GRF is not detectable in

the rat fetus until gestational day 18 (Frohman and Jansson, 1986). Therefore, fetal GRF is not available to the fetus until the end of gestation. This puts limits on the role that it can have during development. There are further complications to fetal GRF being involved in pituitary development. First, it has been suggested that the concentration of GRF produced by fetal hypothalamic neurons is inadequate for eliciting an effect (Bloch et al., 1984). Second, the neurovascular link between the median eminence and the adenohypophysis may not be fully developed before the end of the second postnatal week (Glyclon et al., 1957). Thus, it appears that the most likely source of GRF for the developing fetus comes from the placenta (Margioris et al., 1990). In fact, rat placental extractions can stimulate growth hormone release from rat anterior pituitary cells (Baird et al., 1985).

The most significant developmental role for GRF may be postnatally through its effects on growth and reproductive maturity. In transgenic mice over-expressing human GRF, significant increases in size were observed in 9-week-old transgenic mice compared to control litter-mates. At maturity some of the transgenic mice were nearly twice the size of the controls (Hammer et al., 1985). In terms of reproductive maturity, irGRF is proposed to be a paracrine regulator of testicular function because it is found in rat interstitial cells from day 4 to adulthood. In addition, irGRF is found in the acrosomal region of early and intermediate spermatids but not in mature sperm, sertoli cells or late spermatids (Fabbri et al., 1995). GRF mRNA has also been isolated in rat testis, particularly germ cells (Srivastava et al., 1995; Monts et al., 1996). However, GRF mRNA was not detectable until postnatal day 2 (Berry et al., 1990). Although others would disagree (Fabbri et al., 1995), Monts et al., (1996) found GRF mRNA in Sertoli cells. He also found the GRF receptor expressed in all testicular cell types. GRF treatment of Sertoli cells increased accumulation of cAMP and expression of c-fos and Sertoli cell factor. The sertoli-cell-factor and the c-fos gene are important for normal germ cell development. GRF is not only expressed in male reproductive organs but in the female counterparts as well. GRF mRNA is found in rat ovaries and irGRF can be isolated from

ovarian extracts and rat granulosa cell culture medium. It is thought that GRF may promote follicular maturation by an autocrine or paracrine effect on granulosa cell function (Bagnato et al., 1992). Co-administration of GRF and follicle stimulating hormone (FSH) in the treatment of infertility promotes follicular maturation (Moretti et al., 1990).

Some other actions of GRF include the release of insulin from islet cells (Green et al., 1990) and promotion of mammary gland maturity and lactation (Kann et al., 1997). In addition, GRF may have an immune function. GRF-like peptide and mRNA are produced by human lymphocytes and the release of irGRF increases following lymphocyte activation (Stepanou et al., 1991).

### **III. PACAP**

#### ***A. Introduction***

It has now been just over a decade since the discovery of PACAP. It was first isolated in 1989 from sheep hypothalami. It exists in two forms, a 27 amino acid peptide and a 38 amino acid peptide (Miyata et al., 1989, 1990). PACAP is the newest peptide to be identified in the PACAP/Glucagon superfamily, but more importantly, it appears to be the ancestral molecule for the superfamily based on its conservation across 500-600 million years of evolution. For both of these reasons, the functions of PACAP are considered in great detail in this section. The tight conservation of PACAP suggests its functions may be essential for survival. PACAP is reported to have an array of functions that involve the nervous, endocrine, cardiovascular, muscular and immune systems. However, the puzzling aspect of PACAP is that the physiological event that triggers its release is not clear.

An overview of PACAP hormone and receptor expression shows that PACAP mRNA and protein production has been localized to the following areas: the CNS, especially the hypothalamus, brain stem and spinal cord (McRory et al, 1997a; Chartrel et

al., 1991; Parker et al., 1993, 1997; McRory et al., 1995; Wong et al., 1998; Montero et al., 1998; Matsuda et al., 1997; Koves et al., 1994; Ghatei et al., 1993; Legradi et al., 1994; Nielsen et al., 1998; Moller et al., 1993), the peripheral nervous system innervating the eye, pituitary gland, respiratory tract, salivary glands, gastrointestinal tract, reproductive tract, pancreas, urinary bladder and swimbladder (Arimura et al., 1991; Cardell et al., 1991; Chartrel et al., 1991; Sundler et al., 1991; Uddman et al., 1991a; Ghatei et al., 1993; Koves et al., 1993; Moller et al., 1993; Kimura et al., 1994; Olsson and Holmgren, 1994; Yada et al., 1994; Hedlund et al., 1995; Fahrenkrug and Hannibal, 1996; Matsuda et al., 1997; Parker et al., 1997; Werkstrom et al., 1997; Montero et al., 1998). PACAP is also produced in several non-neural tissues such as the adrenal gland, gonads, immune cells and pancreas (Arimura et al., 1991; Ghatei et al., 1993; Yon et al., 1993; Gaytan et al., 1994; Olsson and Holmgren, 1994; Shioda et al., 1994; Reid et al., 1995; Moller and Sundler, 1996; McRory et al., 1997; Yada et al., 1997; Nielsen et al., 1998). Upon its discovery PACAP was proposed to be a hypothalamic releaser of anterior pituitary hormones. This definition has since been expanded to include regulation of cell cycle, smooth muscle and cardiac muscle function, immune response, endocrine and paracrine secretions outside of the anterior pituitary and exocrine secretions. All studies cited in this chapter involving exogenous exposure to PACAP have been conducted at physiologically relevant concentrations ( $1 \times 10^{-7}$  M or less).

### ***B. PACAP receptors***

Several PACAP receptors have been identified to date. They are members of the secretin/glucagon subfamily of receptors that are seven transmembrane receptors coupled to a G protein (Segre and Goldring, 1993; Christophe, 1993). The PACAP receptors have traditionally been known as Type I receptors that bind PACAP with greater affinity (100-1000X) than VIP and Type II receptors that bind PACAP and VIP with equal affinity (Ishihara et al., 1992; Couvineau et al., 1994). Recently these receptors have been reclassified as PACAP-1 receptors ( $\text{PAC}_1\text{-R}$ ) and VIP/PACAP-1 or -2 receptors ( $\text{VPAC}_1\text{-R}$

**Table 1-1. Nomenclature for PACAP receptors**

| Receptor             | Alternative Nomenclature                                                                                                                                                                                            | Splice Variants                                                                                                                                                                                                                                                                                                         |
|----------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| PAC <sub>1</sub> -R  | <ul style="list-style-type: none"> <li>• Type I-binding site</li> <li>• PACAP Type-I receptor</li> <li>• PACAPR</li> <li>• PACAP/VIP receptor I (PVR1)</li> </ul>                                                   | <ul style="list-style-type: none"> <li>• PAC<sub>1</sub>-R-s</li> <li>• PAC<sub>1</sub>-R-vs</li> <li>• PAC<sub>1</sub>-R-hop1</li> <li>• PAC<sub>1</sub>-R-hop2</li> <li>• PAC<sub>1</sub>-R-hiphop1</li> <li>• PAC<sub>1</sub>-R-hiphop2</li> <li>• PAC<sub>1</sub>-R-hip</li> <li>• PAC<sub>1</sub>-R-TM4</li> </ul> |
| VPAC <sub>1</sub> -R | <ul style="list-style-type: none"> <li>• Type II-binding site</li> <li>• “Classic” VIP receptor</li> <li>• VIP-PACAP Type-II receptor</li> <li>• VIP<sub>1</sub>R</li> <li>• PACAP/VIP receptor 2 (PVR2)</li> </ul> | <ul style="list-style-type: none"> <li>• None identified</li> </ul>                                                                                                                                                                                                                                                     |
| VPAC <sub>2</sub> -R | <ul style="list-style-type: none"> <li>• Type II-binding site</li> <li>• “Helodermin”-preferring VIP receptor</li> <li>• VIP<sub>2</sub>R</li> <li>• PACAP/VIP receptor 3 (PVR3)</li> </ul>                         | <ul style="list-style-type: none"> <li>• None identified</li> </ul>                                                                                                                                                                                                                                                     |

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Modified from Rawlings and Hezareh (1996)

or VPAC<sub>2</sub>-R) (Harmar et al., 1998). Other names for the PAC<sub>1</sub> and the two VPAC receptors are listed in Table 1-1. The PAC<sub>1</sub> receptor is a most interesting example of a product from a single gene that has multiple signalling paths due to its variant forms. The wide distribution of this receptor and the receptors shared with VIP provide clear evidence that PACAP has many target sites and functions.

The PACAP-specific receptors (PAC<sub>1</sub>-R) are produced from alternative splicing of the transcript from a single gene and inclusion or exclusion of one or two cassettes, the hip and hop cassettes. The hip and hop cassettes are inserted in the third intracellular loop. Originally, six splice variants were isolated from rats (Hashimoto et al., 1993; Hosoya et al., 1993; Morrow et al., 1993; Ogi et al., 1993; Pisegna and Wank, 1993; Spengler et al., 1993; Svoboda et al., 1993). The splice variants are the short PAC<sub>1</sub>-R (PAC<sub>1</sub>-R-s), PAC<sub>1</sub>-R-hop1, PAC<sub>1</sub>-R-hop2, PAC<sub>1</sub>-R-hiphop1, PAC<sub>1</sub>-R-hiphop2 and PAC<sub>1</sub>-R-hip (Spengler et al., 1993). Although other species likely express these variants, to date only the human, bovine, chicken, frog and goldfish short form and bovine hop form have been isolated (Ogi et al., 1993; Miyamoto et al., 1994; Peeters et al., 1999; Hu et al., 2000). The PAC<sub>1</sub> receptors bind PACAP-27 and -38 and bind VIP with 100-1000 fold lower affinity. The variants, PAC<sub>1</sub>-R-s, PAC<sub>1</sub>-R-hop1 and PAC<sub>1</sub>-R-hop2 bind PACAP-38 with greater affinity than PACAP-27 (Arimura and Shioda, 1995; Basille et al., 1995; Rawlings and Hezareh, 1996). With the exception of the PAC<sub>1</sub>-R-hip variant, all PAC<sub>1</sub>-R variants trigger AC through G<sub>s</sub> with equal potency. Phospholipase C accumulation is triggered to varying degrees, through G<sub>q/11</sub>. PAC<sub>1</sub>-R-hip activates AC only (Spengler et al., 1993; Pisegna and Wank, 1993; Arimura and Shioda, 1995). A seventh splice variant has recently been cloned, the very short PAC<sub>1</sub>-R (PAC<sub>1</sub>-R-vs). This receptor has a 21 amino acid deletion in the amino-terminal extracellular domain. The PAC<sub>1</sub>-R-vs has approximately equal affinity for PACAP-27 and PACAP-38, unlike PAC<sub>1</sub>-R-s and PAC<sub>1</sub>-R-hop1/hop2. PACAP-27 and PACAP-38 were equally potent in the stimulation of both AC and PLC. Therefore, it

seems likely that the 21 amino acids in the extracellular domain are important for determining the selectivity of the receptor to PACAP-27 and PACAP-38, as well as determining the potency of the peptides in stimulating PLC activation (Pantaloni et al., 1996). In addition to alternative splicing in the translated region of the gene, there is also alternative splicing in the 5' untranslated region of the PAC<sub>1</sub> receptor (Chatterjee et al., 1997). The significance of the various transcripts may be to regulate mRNA stability, mRNA translation or tissue/cell specific expression (Chatterjee et al., 1997). The PAC<sub>1</sub>-R has been cloned in humans (Ogi et al., 1993), bovids (Miyamoto et al., 1994), rats (Hashimoto et al., 1993; Hosoya et al., 1993; Morrow et al., 1993; Pisegna and Wank, 1993; Spengler et al., 1993; Svoboda et al., 1993), chickens (Peeters et al., 1999) frogs (Hu et al., 2000) and goldfish (Wong et al., 1998). In humans the PAC<sub>1</sub>-R is found in most of the CNS, lung, liver, thymus, spleen, pancreas and placenta (Ogi et al., 1993). In rats the PAC<sub>1</sub>-R is found throughout the brain and in the spinal cord, anterior pituitary, lung, liver, pancreas, adrenal medulla and the testis (Buscail et al., 1990; Gottschall et al., 1990; Cauvin et al., 1991; Shivers et al., 1991; Masou et al., 1992; Hosoya et al., 1993; Svoboda et al., 1993; Moller and Sundler, 1996). There is limited information localizing the receptor outside of mammals. However, in goldfish the receptor has been identified in the brain, heart and pituitary (Wong et al., 1998).

The eighth variant in the PAC<sub>1</sub> receptor group is PAC<sub>1</sub>-R-TM4. This receptor is also a G protein-linked receptor, but unlike the PAC<sub>1</sub>-R and either of the VPAC receptors, the G protein is not linked to AC or phospholipase. This receptor appears to affect an L-type calcium channel instead. It differs from the PAC<sub>1</sub> receptor variants by amino acid substitutions and deletions in the II and IV transmembrane domains. The receptor has been cloned in the rat and is found along with other PAC<sub>1</sub>-R variants in the rat cerebral cortex, cerebellum, brainstem, vas deferens and lung. Interestingly, the PAC<sub>1</sub>-R-TM4 receptor is the only PACAP receptor expressed in rat pancreatic  $\beta$ -islet cells (Chatterjee et al., 1996).

Activation of VPAC<sub>1</sub>-R by PACAP or VIP stimulates an increase in cAMP via adenylate cyclase (AC). The AC activation is likely achieved through coupling with a G<sub>s</sub> protein. Originally VPAC<sub>1</sub>-R activation was thought not to affect the inositol phosphate (IP) /phospholipase C (PLC) system (Sreedharan et al., 1991; Ishihara et al., 1992; Couvineau et al., 1994). Although not observed in normal tissues, the VPAC<sub>1</sub>-R also couples to a G<sub>i</sub> or G<sub>o</sub> protein when transfected in Chinese hamster ovarian (CHO) cells and does have a stimulatory effect on IP production in cells that express  $\beta_2$  and  $\beta_3$  PLC isoforms (Van Rampelbergh et al., 1997). In addition to its effects on the AC and PLC systems, in stably transfected CHO cells and HT29 human intestinal epithelial cells, VIP induces the VPAC<sub>1</sub>-R to increase intracellular calcium (Sreedharan et al., 1994). The VPAC<sub>1</sub>-R has been cloned in humans (Couvineau et al., 1994; Sreedharan et al., 1991; Gagnon et al., 1994) and rats (Ishihara et al., 1992). Distribution of the receptor in rats is as follows: lungs, small intestine, thymus, heart, aorta, liver, vas deferens, pancreas, kidney, adrenal gland, uterus and the brain (especially the cerebral cortex, hippocampus and several amygdaloid nuclei) (Sreedharan et al., 1991; Ishihara et al., 1992; Usdin et al., 1994). In frogs a VPAC-R with a sequence identity closest to the VPAC<sub>1</sub>-R and a distribution and binding pharmacology closest to VPAC<sub>2</sub>-R has been cloned. In goldfish a VPAC receptor has been sequenced (Chow et al., 1997) but has not been tested pharmacologically to determine if it is a VPAC<sub>1</sub>, VPAC<sub>2</sub> or a hybrid like the frog receptor. These studies may provide a clue that duplication of the VPAC receptors occurred in vertebrates.

The VPAC<sub>2</sub>-R has been cloned in humans (Svoboda et al., 1994; Adamou et al., 1995), rats (Lutz et al., 1993) and mice (Inagaki et al., 1994). Like the VPAC<sub>1</sub>-R, the VPAC<sub>2</sub>-R binds PACAP and VIP with equal affinity. Both VIP and PACAP stimulate, with approximately equal potency, the activation of AC (Lutz et al., 1993; Inagaki et al.,

1994; Svoboda et al., 1994; Usdin et al., 1994; Adamou et al., 1995). There is also some suggestion that this receptor is linked to the PLC-IP system (Inagaki et al., 1994; Cai et al., 1997). However, there are many instances where no IP turnover is stimulated (Lutz et al., 1995; Rawlings et al., 1995). A clear picture of the link to the PLC-IP system remains to be found. The VPAC<sub>2</sub>-R has been identified in human skeletal muscle, heart, pancreas, placenta and the brain (Adamou et al., 1995). In rats and mice it has been localized to the stomach, colon, spleen, kidney, thymus, adrenal gland, heart, lung, pancreas, testis, ovary, uterus, pituitary and the brain (especially the thalamus, hypothalamus, midbrain, brainstem and olfactory bulbs) (Lutz et al., 1993; Inagaki et al., 1994; Usdin et al., 1994).

### ***C. A regulator of cell cycle and development***

PACAP is reported to regulate cell division, cell cycle arrest, differentiation and cell death. These fundamental functions can affect development and cell cycle dysfunction. The following section surveys these actions of PACAP and how they relate to normal development, particularly of the CNS, and to abnormalities resulting from improper PACAP or PACAP receptor expression.

#### **1. Cell Division**

PACAP regulates division of several cell types. For example, PACAP stimulates proliferation of a folliculo-stellate -like cell line (Matsumoto et al., 1993), primordial germ cells (De Felici and Pesce, 1994; Pesce et al., 1996), chromaffin cells (Tischler et al., 1995), clonal lactotrope and gonadotrope cell lines (Schomerus et al., 1994; Lelievre et al., 1996), astrocytes (Moroo et al., 1998), and peripheral sympathetic neuroblasts (Lu and DiCicco-Bloom, 1998). In contrast, PACAP can inhibit proliferation of cerebral cortical precursors (Lu and DiCicco-Bloom, 1998), corticotrope cells (Braas et al., 1994) and murine splenocytes induced to divide by concanavalin A (Tatsuno et al., 1991a); PACAP in rats also inhibits DNA synthesis in aortic smooth muscle cultures stimulated by arginine vasopressin (AVP) (Oiso et al., 1993), growth-factor stimulated chromaffin cells (Frodin et

al., 1995; Tischler et al., 1995) and cortical precursors (Lu and DiCicco-Bloom, 1997).

Thus, it would appear that PACAP regulates two opposing actions, both the stimulation and inhibition of cell proliferation. To explain these actions several scenarios have been studied, three of which are related below.

The first explanation is that PACAP can use different receptors to facilitate opposing outcomes (Lu and DiCicco-Bloom, 1998). PACAP has been shown to stimulate proliferation in sympathetic neuroblasts and inhibit proliferation in cerebral cortical precursors by using different signalling pathways in these tissues. For example, sympathetic neuroblasts express PAC<sub>1</sub>-R-hop and have a measurable increase in cAMP and IP following PACAP stimulation, whereas cerebral cortical precursors express PAC<sub>1</sub>-R-s and to a much lesser extent PAC<sub>1</sub>-R-hop. Cerebral cortical precursors only exhibit an increase in cAMP following exposure to PACAP (DiCicco-Bloom et al., 1998; Lu and DiCicco-Bloom, 1998). Therefore, the distribution of PACAP receptors can dictate opposing functions in different tissues. Although both PAC<sub>1</sub>-R-s and PAC<sub>1</sub>-R-hop can stimulate cAMP and IP accumulation, a difference in intracellular signalling efficiency appears to exist depending on the cellular system employed.

The second explanation is that PACAP can selectively activate intracellular pathways through concentration differences. In cultured rat astrocytes, PACAP causes proliferation at concentrations that are below those that stimulate cAMP. At these low concentrations ( $10^{-14}$ - $10^{-12}$  M) PACAP activates mitogen-activated protein (MAP) kinase, which is associated with DNA synthesis and proliferation. In fact, a cAMP analog actually suppresses MAP kinase activation in cultured rat astrocytes (Moroo et al., 1998). Thus PACAP can trigger different intracellular pathways by concentration-dependent pathway selection.

Finally, PACAP can have both mitogenic and antimitogenic effects on the same tissue. For example, PACAP has mitogenic effects on adult rat chromaffin cell cultures but

has antimitogenic effects on NGF-stimulated proliferation of chromaffin cell cultures in rats (Frodin et al., 1995; Tischler et al., 1995). These effects do not seem to be regulated by either dosage effects or differential receptor activation. One possible explanation is that inhibition of the mitogenic effects of NGF by PACAP might be a mechanism by which neurally derived signals override growth factor stimulated proliferation during development. PACAP might accomplish this by commandeering portions of the same intracellular pathway utilized by the growth factor (Tischler et al., 1995).

In summary, the evidence points to an important role for PACAP in fine tuning the cell division of various neuronal and non-neuronal cell types.

## **2. Differentiation**

Not only does PACAP regulate proliferation, but it also appears to regulate differentiation. The presence of multiple PACAP receptor variants makes these opposing actions seem plausible. PACAP activation of the PAC<sub>1</sub>-R is known to stimulate neurite outgrowth, a sign of differentiation, in rat pheochromocytoma PC12 cells (Hernandez et al., 1995; Barrie et al., 1997), some human neuroblastoma cell lines (Hoshino et al., 1993), cortical precursor cells (Lu and DiCicco-Bloom, 1997), immature cerebellar granule cells (Gonzalez et al., 1997) and a corticotrope cell line (Braas et al., 1994). In addition, the coincident expression of both PACAP and the PAC<sub>1</sub>-R in the ovary (granulosa cells) and testis suggests that PACAP may be involved in germ cell maturation (Shivers et al., 1991; Spengler et al., 1993; Kononen et al., 1994; Shioda et al., 1994; Kotani et al., 1997; Li et al., 1998). In the testis PACAP is processed and expressed in maturing spermatids in a stage-specific manner at a critical point in spermatogenesis (Kononen et al., 1994; Shioda et al., 1994; Li et al., 1998). PACAP may also affect Cl<sup>-</sup> secretions in the epididymal epithelium. Cl<sup>-</sup> secretions are thought to help maintain a stable microenvironment, which is important for the promotion of maturation and storage of spermatozoa (Zhou et al., 1997).

### 3. Apoptosis

PACAP is involved in both protecting cells from apoptosis and in triggering apoptosis, depending on the system (Cavallaro et al., 1996; Campard et al., 1997; Gonzalez et al., 1997; Spengler et al., 1997; Gillardon et al., 1998; Vaudry et al., 1998). Apoptosis, or programmed cell death is a tightly controlled suicide program initiated by the cell. It has two main functions: to act as a developmental regulator and, to kill cells that have been damaged. The latter role prevents a potentially dangerous phenotype from being propagated. PACAP may affect apoptosis by regulation of gene transcription, perhaps by transactivation of cAMP response elements and/or through activation of particular PACAP receptor variants (Campard et al., 1997; Vaudry et al., 1998).

The neuroprotective role of PACAP has been studied in rat cerebellar cells. In these cells PACAP acts on the PAC<sub>1</sub>-R to induce the MAP kinase pathway via AC and protein kinase A (PKA) activation (Campard et al., 1997; Vaudry et al., 1998). This activation may be required to protect cells from apoptotic events ensuring proper cerebellar development. Cerebellar granule cells express PAC<sub>1</sub>-R-s and PAC<sub>1</sub>-R-hop, whereas cerebellar glial cells express only PAC<sub>1</sub>-R-s. PACAP stimulates c-fos gene expression in cerebellar granule cells through a cAMP/protein kinase A-dependent mechanism involving the PAC<sub>1</sub>-R. The protein kinase A pathway is a major mediator of the neurotrophic actions of PACAP. It is likely that both c-fos gene transcription and one or both of the PAC<sub>1</sub>-R variants are involved in the anti-apoptotic effects of PACAP on cerebellar granule cell cultures (Cavallaro et al., 1996; Campard et al., 1997; Gonzalez et al., 1997; Villalba et al., 1997; Vaudry et al., 1998). Also, the neuroprotective actions of PACAP have been noted in the induced cell death of the following: rat embryonic cortical neurons (Morio et al., 1996), hippocampal neurons (Uchida et al., 1996), sympathetic neurons (Chang and Korolev, 1997), rat thymocytes (Delgado et al., 1996c) and PC12 cells (Frodin et al., 1995). In contrast, in chick sympathetic neuroblasts PACAP rescues cells from death but does not act through the usual pathways. Instead PACAP appears to operate through an

unidentified receptor to decrease the concentration of a death protein by stimulating the destruction of the protein (Pryzwara et al., 1998). Although a direct link for protection from apoptosis has not been established, PACAP has been shown to promote cell survival in the following cultured primary neurons: cortical, hippocampal, septal cholinergic, mesencephalic dopaminergic and dorsal root ganglion (Lindholm et al., 1998). Clearly PACAP has a neuroprotective function in many neural and some non-neural cell types.

The ZAC1 protein, a recently discovered zinc-finger protein, induces apoptosis and G<sub>1</sub> cell cycle arrest in tumor cells. ZAC1 and the tumor suppressor, p53, inhibit tumor cell growth in vitro by different pathways. Interestingly, the two proteins also induce expression of the PAC<sub>1</sub>-R gene. Thus PACAP has a protective function in neurons but it appears to promote apoptosis in tumor cells (Spengler et al., 1997; Gillardon et al., 1998).

#### **4. Development**

There are several lines of evidence to suggest that PACAP may have a role in development of the nervous system and several other organs in mammals. The presence or effect of PACAP and its receptor has been examined in the developing CNS (Basille et al., 1993, 1994, 1995; Gonzalez et al., 1994; Masuo et al., 1994; Tatsuno *et al*, 1994; D'Agata et al., 1996; Sheward et al., 1996; Shuto et al., 1996; Lu and DiCicco-Bloom, 1997, 1998; Nielsen et al., 1998; Waschek et al., 1998), eye (Olianas et al., 1997) liver (El Fahhime et al., 1996), adrenal glands (Nielsen et al., 1998; Moller and Sundler, 1996) and pancreas (Le Meuth et al., 1991). The data to date do not necessarily agree on a common function for PACAP among these organs or even on a common function within different regions or cell types of the same organ. However, both cAMP and IP signalling pathways are activated by PACAP in developing tissues. The predominant receptor expressed during development in the tissues examined is the PAC<sub>1</sub>-R with the exception of rat fetal hepatocytes, which appear to express a VPAC-R, and human fetal retinal cells which express mRNA for both the PAC<sub>1</sub>-R and a VPAC-R (Le Meuth et al., 1991; Basille et al.,

1993, 1994, 1995; Gonzalez et al., 1994; Masuo et al., 1994; Tatsuno et al., 1994; D'Agata et al., 1996; El Fahime et al., 1996; Lu and DiCicco-Bloom, 1997, 1998; Orianas et al., 1997). In short, the functions of PACAP in development are not well understood but several examples are highlighted to demonstrate the possible significance of PACAP in the developmental process.

The role of PACAP in the developing nervous system has been examined in greater detail than in any other system during development. Evidence for the presence of PACAP and its receptor is found during embryogenesis in the autonomic and sensory ganglia and the spinal cord (Lindholm et al., 1998; Nielsen et al., 1998), glial and neuronal cells (Masou et al., 1994) and in the following brain regions: the neocortex, cortex, cortical plate, thalamic and hypothalamic nuclei, habenular nucleus, hippocampus, septum, trigeminal ganglion, amygdala, olfactory bulbs, inferior colliculus, solitary nucleus, inferior olive and other pontine nuclei, midbrain, hindbrain, particularly the cerebellum and neural tube (Basille et al., 1994, 1995; Gonzalez et al., 1994; Tatsuno et al., 1994; Sheward et al., 1996; Lindholm et al., 1998; Lu and DiCicco-Bloom, 1998; Sheward et al., 1998; Waschek et al., 1998). In the developing brain the PAC<sub>1</sub>-R is the dominant receptor, but there is differential distribution of the variants. In the postnatal rat brain, the PAC<sub>1</sub>-R-s variant is found in the cortex, hippocampus, cerebellum and hypothalamus, whereas the PAC<sub>1</sub>-R-hop variant is found only in the cerebellum and the hypothalamus, not the cortex and hippocampus. Differential distribution of PAC<sub>1</sub>-R variants is further confirmed by experiments revealing that PACAP-induced cAMP production occurred in all four of the brain regions examined, but [<sup>3</sup>H] inositol monophosphate accumulation occurred only in the cerebellum and hypothalamus (Basille et al., 1993, 1995; D'Agata et al., 1996; Sheward et al., 1996; Campard et al., 1997; Villalba et al., 1997). Although both PAC<sub>1</sub>-R-s and PAC<sub>1</sub>-R-hop have been shown to trigger IP turnover, it appears that PAC<sub>1</sub>-R-s is not linked to the inositol phosphate path in the cortex and hippocampus. The significance of this distribution pattern in the cerebellum is discussed below.

The cerebellum, a division of the hindbrain, is one of the first brain regions to express PACAP and the PAC<sub>1</sub>-R variants short and hop. The immature cerebellum is composed of 4 layers, the external granule cell layer (EGL), the molecular layer, the internal granule cell layer (IGL) and the medulla. Immature neurons are generated early in development in the EGL. These neurons migrate through the molecular layer to reach their destination in the IGL. The development of the cerebellum is a complex process that involves proliferation, differentiation, migration and massive cell death in both the EGL and IGL. PACAP immunoreactivity is present early in the cerebellum of postnatal rats (Masou et al., 1994; Tatsuno et al., 1994). At birth, (postnatal day 0, P0), PACAP receptors are present in the EGL and medulla. Later, these receptors disappear (P8-P25) in concert with the involution of the EGL. Concurrent with the involution of the EGL, PACAP receptors appear in the IGL and the molecular layer. Once the cerebellum matures, receptors appear only in the granule cell layer (Basille et al., 1994). This period of intense PACAP and PACAP receptor expression is coincident with a period of neurogenesis in the rat brain (Jacobson, 1991). The cerebellum has two PAC<sub>1</sub>-R variants, PAC<sub>1</sub>-R-s and PAC<sub>1</sub>-R-hop (Basille et al., 1993, 1994; Gonzalez et al., 1994; D'Agata et al., 1996; Sheward et al., 1996; Campard et al., 1997). The differential distribution of PACAP receptors in the immature brain may allow PACAP to play several roles in the postnatally developing cerebellum. Experiments on cerebellar neuroblasts in culture indicate that PACAP acts through PAC<sub>1</sub>-R to increase cell survival and differentiation (Gonzalez et al., 1997). The cell survival actions of PACAP appear to be mediated by the cAMP second messenger pathway. In addition, the PACAP-induced cAMP accumulation leads to the transcription of important regulatory factors such as, c-fos. C-fos acts as a transcription factor for many genes. Therefore, PACAP-induced expression of c-fos provides an indirect pathway for PACAP to promote the expression of an array of genes in these cells (Campard et al., 1997). The effects of PACAP on cell survival and differentiation have been confirmed in vivo (Vaudry et al., 1999). PACAP injections in P8 rats cause an increase in the thickness of the EGL, the molecular layer and

the IGL, but PACAP has no effect on the medulla. It is known that during the first 2 postnatal weeks the EGL undergoes apoptosis. Given the cell survival effects of PACAP *in vitro* it can be supposed that the increase in EGL thickness following *in vivo* PACAP administration likely results from apoptosis protection rather than proliferation. An increase in cells migrating through the molecular layer to the IGL and an increase in the number of neurites from the IGL projecting into the molecular layer has also been observed. These factors could account for the increase in the volume of the molecular layer and the IGL following PACAP injections. There is also some suggestion that PACAP accelerates the migration of granule cells to the bottom of the IGL. In summary, both the *in vivo* and *in vitro* findings indicate that PACAP has a role in protecting cerebellar cells from apoptosis and in promoting differentiation (i.e. neurite outgrowth) (Gonzalez et al., 1997; Vaudry et al., 1999). Given that we know the cerebellum expresses two PAC<sub>1</sub>-R variants, it will be interesting to see whether these variants are used to regulate the distinctly different effects of PACAP within this region of the developing brain.

Several studies indicate that PACAP may function in many other regions of the CNS during development. PACAP mRNA and PAC<sub>1</sub>-R mRNA are detected as early as embryonic day 9.5 (E9.5) in mice (Shuto et al., 1996) and at E14 in rats (Tatsuno et al., 1994). The following PACAP receptors have also been found: PAC<sub>1</sub>-R-s and a longer transcript (representing one or more of the variants, PAC<sub>1</sub>-R-hip, PAC<sub>1</sub>-R-hop1 and PAC<sub>1</sub>-R-hop2 (Sheward et al., 1996; Villalba et al., 1997). *In situ* studies in mice ages E9.5 and onwards localized the receptor and peptide mRNA to the neural tube, hindbrain, trigeminal ganglia, dorsal root ganglia and the developing sympathetic chain. PACAP mRNA was also found in the hypothalamus and the nuclei of the pons and medulla. The presence of labelled cells in the dorsal root ganglia and in autonomic structures suggests that neural crest derived structures may express PACAP and PAC<sub>1</sub>-R during development (Sheward et al., 1996; Waschek et al., 1998). A recent study suggests a role for PACAP in the

patterning of the nervous system (Waschek et al., 1998). PACAP and PAC<sub>1</sub>-R were present by in situ hybridization in the mouse neural tube on embryonic day 10.5. PACAP was expressed throughout the neural tube and PAC<sub>1</sub>-R mRNA was expressed in the alar and floor plate region of the underlying ventricular zone. Distribution of PACAP mRNA in two columns of cells in the ventromedial portion of the neural tube places PACAP in the same region as developing autonomic motoneurons. In addition, PACAP down-regulated expression of two PKA-dependent patterning genes (sonic hedgehog and gli-1) in cultured neuroepithelial cells (Waschek et al., 1998). Human fetal retinal cells also express mRNA for PACAP and PACAP receptors (PAC<sub>1</sub>-R, VPAC-R). In these cells PACAP stimulates AC activity, although, the exact function (i.e. proliferation, differentiation, apoptosis etc.) is not clear. (Olianas et al., 1997). Also, PACAP is involved in cerebral cortical neurogenesis through initiating inhibition of proliferation in cortical precursors. It is this action of PACAP through the PAC<sub>1</sub>-R-s receptor that is thought to elicit cell cycle exit and differentiation of the developing cerebral cortex (DiCicco-Bloom et al., 1998; Lu and DiCicco-Bloom, 1998).

PACAP is involved in development outside of the brain as well. In fetal rat hepatocytes PACAP stimulates an increase in cAMP levels through a VPAC-R. Also, exposure to PACAP results in an increase in corticosteroid-binding globulin mRNA suggesting it may participate in the regulation of gluconeogenesis during development (El Fahhime et al., 1996). In the adrenal gland of newborn rats, ir-PACAP nerve fibers have been observed on chromaffin cells in the adrenal medulla. In addition, mRNA for the PAC<sub>1</sub>-R has been localized to adrenal medullary cells by in situ hybridization (Moller and Sundler, 1996; Nielsen et al., 1998). In human fetuses at 14-20 weeks old, PAC<sub>1</sub>-R binding sites have been localized to chromaffin cells. PACAP induced a dose-dependent increase in cAMP production and a modest increase in IP formation in human fetal adrenal cell suspensions and in cultured cells (Yon et al., 1998). These data prove that a functional PAC<sub>1</sub>-R is present in the human adrenal medulla during a phase of organization. This

phase is characterized by the migration of chromaffin cells from the periphery to the central part of the gland (Yon et al., 1998). The presence of both PACAP and its receptor in the rat and human adrenal gland suggest a possible role for this peptide in adrenal gland development. Also, PACAP affinity studies have revealed the presence of a PACAP binding site in the postnatal calf pancreas and PACAP activates AC in this tissue suggesting a role in postnatal pancreatic function (Le Meuth et al., 1991).

## **5. Dysfunction**

PACAP and its various receptor forms are found in many cancers. PACAP is expressed as a protein or mRNA in pheochromocytomas (Takahashi et al., 1993), neuroblastomas (Suzuki et al., 1993a; Vertongen et al., 1996b; Waschek et al., 1997), human ovarian cancers (Odum and Fahrenkrug, 1998), nerves innervating parathyroid adenomas (Luts et al., 1995) and in gliomas (Vertongen et al., 1995a). The receptors (PAC<sub>1</sub>-R and/or VPAC-R) have an even wider distribution among cancerous tissues and can be found in glial tumors (Robberecht et al., 1994; Vertongen et al., 1995a), breast cancer (Waschek et al., 1995a), intestinal cancer (Waschek et al., 1995a), pancreatic cancer (Gourlet et al., 1991; Waschek et al., 1995a), non-small cell lung cancer (Moody et al., 1997), retinoblastomas (Olianas et al., 1996), lymphoid tumors (Waschek et al., 1995b), pituitary adenomas (Vertongen et al., 1995b), adenocarcinomas (Lelievre et al., 1998b), tumorous adrenal cells (Bodart et al., 1997), prostate cancer (Leyton et al., 1998), and neuroblastomas (Lelievre et al., 1996; Vertongen et al., 1996b; Waschek et al., 1997).

In these tissues PACAP is involved in both proliferation and differentiation, making study of this peptide useful for both determining how cancers develop and how proliferation can be hindered or stopped. In glioblastomas and some human colonic adenocarcinoma cell lines, PACAP reduces proliferation (Vertongen et al., 1996a ; Lelievre et al., 1998b) and in neuroblastomas (NB-OK-1), exposure to PACAP stimulates cAMP accumulation, arrests cell growth and induces morphological changes such as neurite

outgrowth (Hoshino et al., 1993; Olanas et al., 1996). PACAP stimulates proliferation in a pancreatic acinar tumor cell line (Morisset et al., 1995; Schafer et al., 1996), non-small-cell lung cancer cells (Zia et al., 1995) and a prostate cancer cell line (Leyton et al., 1998). Differences in receptor subtype expression may explain the different actions of PACAP listed above (Lelievre et al., 1998b). In addition, the presence or absence of retinoic acid may alter the mitogenic effects and binding capacity of PACAP (Waschek et al., 1997). The proliferative actions of PACAP may be regulated in some cancers through PACAP-induced transcription of c-fos and c-jun, two important nuclear oncogenes. The products of these two oncogenes heterodimerize to form the transcription factor AP-1 (Moody et al., 1993; Schafer et al., 1996; Leyton et al., 1998). Moreover, PACAP induces MAP kinase activity (Schafer et al., 1996). Given the above information PACAP's role in the cell cycle appears to extend to a role in cancer as well. However, at this point a major role in tumorigenesis cannot be supported by these data because tumor cells are known to express many peptides and receptors compared to normal cells.

Holoprosencephaly is a developmental dysfunction, which may in some cases be caused by chromosomal abnormalities affecting PACAP and PACAP receptor expression. The holoprosencephaly phenotype is characterized by incomplete cleavage of the forebrain and several facial abnormalities, which range from mild (microcephaly, mild hypotelorism, and single maxillary central incisor), to severe (cyclopia, a primitive nasal structure and sometimes midfacial clefting). Four loci have been identified as sites of chromosomal rearrangements leading to holoprosencephaly. One of the sites identified is associated with PACAP and involves a chromosomal rearrangement mapped to the location of the human PACAP gene at 18pter-q11 (Belloni et al., 1996). Another of the four loci maps to the human chromosomal region 7q36, which includes the site for the VPAC<sub>2</sub>-R (7q36.3). These genetic studies suggest that PACAP is an important gene involved in patterning of the midline ventral CNS (Belloni et al., 1996; Roessler et al., 1996).

The actions of PACAP on proliferation, differentiation and apoptosis present a complex and interesting story. Although studies on cell cycle regulation, development and dysfunction are now only scratching the surface, the data collected so far suggest there is an intricate balancing act between the death promoting, protective and proliferative actions of PACAP.

#### ***D. A regulator of smooth and cardiac muscle***

PACAP functions as a neurotransmitter or neuromodulator of smooth muscle tone. Consequently, PACAP may affect many systems in the body that are composed in part of smooth muscle. Its effects on the vascular system, respiratory system, digestive system and reproductive system are discussed below. The studies have been done primarily in mammals so that the evolutionary trends are not known.

##### **1. Effects on vascular system smooth muscle**

*General Circulation* - PACAP appears to play an important role in neural and hormonal regulation of systemic circulation. The primary action of PACAP on circulation may be accomplished through vasorelaxant effects on vascular smooth muscle. PACAP-ir nerve fibers have been found around blood vessels in the respiratory tract (Cardell et al., 1991; Uddman et al., 1991a) and PACAP can bind to the membranes of blood vessels (Nandha et al., 1991). However, localization of PACAP to vascular smooth muscle is not the only evidence of its action here. Intravenous or intra-arterial injection of PACAP into humans, sheep, dogs, cats and rats results in a decrease in blood pressure (Miyata et al., 1989; Miyata et al., 1990; Nandha et al., 1991; Minkes et al., 1992a, 1992b; Absood et al., 1992; Sawangjaroen et al., 1992; Naruse et al., 1993). However, different studies have shown PACAP causes a variety of other responses in the same animals. For example in cats and dogs, intravenous administration of PACAP induces a biphasic change in arterial pressure characterized by an initial decrease followed by an increase (Ishizuka et al., 1992; Minkes et al., 1992a, 1992b; Suzuki et al., 1993b). Other studies in rats and dogs

contradict the above results and show that following intracerebroventricular (rats) or intracisternal (dogs) injection of PACAP there is an increase in blood pressure (Murase et al., 1993; Seki et al., 1995). Although it is true that PACAP plays a role in regulation of the vascular system, it is evident from the literature that PACAP's role is unclear. Some of this confusion is likely due to the number of species studied, receptor differences, modes of administration of the peptide, concentration of the peptide and other aspects of the experimental methods used. In general, in the above *in vivo* studies it is difficult to separate the direct effects that PACAP has on vascular smooth muscle from its other possible actions on the vascular system. Therefore, *in vitro* studies have been attempted in order to isolate the direct actions of PACAP on vascular smooth muscle tone.

First, it is clear that PACAP directly causes relaxation of vascular smooth muscle. *In vitro* studies have revealed that PACAP is a potent vasorelaxant of arterial segments (Cardell et al., 1991; Warren et al., 1991; Absood et al., 1992; Huang et al., 1993; Cardell et al., 1997a). These vasorelaxant effects of PACAP appear to be mediated by the AC/cAMP signalling pathway (Warren et al., 1991; Absood et al., 1992). In porcine coronary arteries, the vasorelaxant effect of PACAP was equal to that of VIP, but in the rabbit aorta, PACAP was a 100-fold more potent vasorelaxant than VIP. In both cases the effects of PACAP and VIP were endothelium-independent. This is unlike the situation in humans and guinea pigs where the removal of the endothelium from the pulmonary arteries abolished the vasorelaxant effects of PACAP, but not those of VIP. This suggests some receptor or tissue differences may exist between the species (Cardell et al., 1991; Warren et al., 1991; Huang et al., 1993; Cardell et al., 1997a). Those in the "endothelium-dependent" group suggest that PACAP's vasorelaxant effect is mediated by nitric oxide (NO) synthase, which is released from the endothelium. Support for this theory is derived from the fact that a NO synthase inhibitor, N(G)-monomethyl-L-arginine, inhibits PACAP-induced vasorelaxation of endothelium-intact human and guinea pig pulmonary artery segments (Cardell et al., 1997a). In contrast, *in vivo* analysis of vascular responses in cats

indicates that N(omega)-nitro-L-arginine methyl ester (also a NO synthase inhibitor) has no effect on PACAP-induced vasorelaxation of the pulmonary vascular bed (Minkes et al., 1992b). Perhaps information on receptor distribution in tissue layers, among vessel types and between species will explain these results.

Second, a perplexing aspect of PACAP's action on vascular smooth muscle is the biphasic change in arterial pressure that sometimes occurs following PACAP administration. The initial decrease in blood pressure is logical considering the vasorelaxant properties of PACAP on vascular smooth muscle. The increase in blood pressure seen in the latter phase of the biphasic response may be due to less direct actions of PACAP. One of the key factors that may determine the vascular response is dosage. In cats and dogs, a low dose of PACAP administered into the external jugular vein (0.1 nmol/kg) or femoral vein (0.01 nmol/kg) induces a decrease in arterial pressure, and a higher dose (3.0 nmol/kg) triggers a biphasic change in arterial pressure (Ishizuka et al., 1992; Minkes et al., 1992a, 1992b). The initial phase probably results from the vasorelaxant effects of PACAP. The second phase is thought to result from a large increase in cardiac output and/or some central actions of PACAP (Ishizuka et al., 1992). Two studies examine the second phase of the response. In the first experiment, intracerebroventricular injection of PACAP caused a dose-dependent (0.1 - 0.5 nmol/rat) increase in mean arterial pressure in rats. This reaction can be explained in part because intracerebroventricular injection of PACAP raises plasma AVP, a vasoconstrictor (Murase et al., 1993). In a second experiment the same increase in AVP was found following intracisternal injection of PACAP in dogs (Seki et al., 1995). This effect of PACAP on AVP release appears to be mediated by both a VPAC and a PAC<sub>1</sub>-R in rats (Murase et al., 1993). However, the increase in mean arterial pressure is greater than can be accounted for by AVP alone, so it has been suggested that in addition, PACAP may influence the central cardiovascular control system through stimulation of the sympathetic nervous system (Murase et al., 1993; Seki et al., 1995). A recent study (Lai et al., 1997) confirms this

suggestion. Intrathecal injection of PACAP into anesthetized rats has an excitatory effect on sympathetic preganglionic neurons. One of the effects of this excitation is an increase in spinal sympathetic outflow, which in turn, leads to an increase in blood pressure. Like intracerebroventricular and intracisternal injections, intrathecal administration limits access of the peptide mainly to neurons in the spinal cord. It is also possible that the pressor response following intravenous injections of high doses may allow PACAP access to sympathetic neuron excitation. The receptor type mediating the effect on the spinal cord has not yet been determined, but immunocytochemical and mRNA studies have confirmed localization of PACAP to sympathetic preganglionic neurons (Chiba et al., 1996; Brandenburg et al., 1997).

Catecholamine release may be another factor that mediates the PACAP-induced pressor response. In cats, when PACAP is administered intravenously at a high dose (3.0 nmol/kg), vasorelaxation is followed by vasoconstriction in the hindquarter vascular beds. In this case the pressor response is thought to result from a PACAP-induced stimulation of the release of catecholamines from the adrenal gland or by the release of norepinephrine from adrenergic terminals in the vascular bed (Minkes et al., 1992b). Also, PACAP has been noted to cause an increase in plasma epinephrine following intracisternal administration in dogs (Seki et al., 1995).

The different pressor responses also raise questions regarding receptor variants. So far, studies in the cat suggest that two receptor types are present due to different responses to PACAP and VIP (Minkes et al., 1992a, 1992b). Also, the endothelium-dependent actions of PACAP versus the endothelium-independent actions of VIP in guinea pig and human pulmonary artery relaxation may reflect the use of PAC<sub>1</sub>-R and VPAC-R types. One receptor (likely a VPAC-R) appears to be present in the rat aorta, tail artery, iliac and femoral veins, guinea pig pulmonary artery, guinea pig aorta and porcine coronary artery (Nandha et al., 1991; Minkes et al., 1992b; Huang et al., 1993).

The studies summarized above identify PACAP as having a role in vascular regulation of the general circulatory system. Its primary action is vasorelaxation and its vasoconstrictor actions are likely to result from a secondary reaction and include sympathetic regulation of central cardiovascular control, differential receptor use, PACAP-induced AVP or catecholamine release. Other experiments have revealed that PACAP can have a more localized vasorelaxant effect on blood flow and hence affect the function of particular organs and systems, such as the respiratory system, digestive system, reproductive system, visual system, pancreas and brain.

*Reproductive tissue circulation* - In the reproductive system, PACAP can act as a vasorelaxant in the human-uteroplacental unit (Steenstrup et al., 1996), rabbit ovarian artery (Yao et al., 1996) and the vasculature of the female genital tract (Graf et al., 1995; Fahrenkrug and Hannibal, 1996). In males PACAP has been localized to nerve fibers in the human cavernous tissue where it may play a role in the vasorelaxant response necessary for engorgement of the penis with blood, leading to penile erection (Hedlund et al., 1995).

*Respiratory tissue circulation* - PACAP-immunoreactive nerve fibers have been localized around blood vessels found in the nose and tracheobronchial wall of the respiratory tract of the squirrel monkey, sheep, pig, ferret, guinea pig and rat (Uddman et al., 1991a) and in the esophagus of sheep and man (Uddman et al., 1991b). Localization of PACAP-ir fibers to the respiratory system suggests it may play a role in vascular regulation of these tissues.

*Digestive tissue circulation* - PACAP ( $10^{-11}$  to  $10^{-7}$  M) has vasorelaxant effects on endothelium-denuded rat mesenteric arteries (Huang et al., 1993) and causes an increase in blood flow to the submandibular gland (Mirfendereski et al., 1997). It decreases blood flow to the duodenum when administered at 10 nmol/kg bw (Carlsson et al., 1996). In addition, PACAP-ir nerve fibers innervate small arteries and arterioles of the fish and mammalian gastrointestinal tract, which suggests it may be involved in the regulation of

blood flow in the digestive system (Koves et al., 1993; Olsson and Holmgren, 1994). This also suggests that the vasorelaxant properties of PACAP may have arisen at least as early as the emergence of fish.

*Pancreatic tissue circulation* - PACAP increases pancreatic blood flow at lower doses ( $10^{-11}$  to  $10^{-9}$  M), but at higher doses ( $10^{-7}$  M) PACAP provokes a transient decrease of blood flow followed by a return to a basal flow rate. The effect of PACAP on pancreatic blood flow may be mediated by a VPAC receptor (Bertrand et al., 1996). In situ hybridization studies in the rat pancreas identify the VPAC<sub>2</sub>-R subtype in pancreatic blood vessels (Usdin et al., 1994). However, the vasoconstrictor effect of PACAP at high concentrations ( $10^{-7}$  M) and the failure of VIP to induce the same response indicate that PAC<sub>1</sub>-R receptors are involved as well. The increase in blood flow to the pancreas caused by PACAP results in redistribution of the blood flow in favor of the exocrine pancreas so as to stimulate blood perfusion and exocrine secretions for the gut (Carlsson et al., 1996).

*Ocular tissue circulation* - Intraocular tissues are supplied with nutrients from two vascular beds, the retinal and uveal blood vessels. In rabbits infusion of PACAP results in a decrease in uveal vascular resistance and an increase in blood flow to the uveal blood vessels of the choroid. PACAP receptors are present in the anterior uvea, choroid and retina. In the choroid, it is likely that these receptors are localized to blood vessels. Both PAC<sub>1</sub>-R and VPAC-R are present in the intraocular tissues although PAC<sub>1</sub>-R predominate in the choroid. Exposure to PACAP causes an increase in cAMP concentrations in these tissues. Also, PACAP induces an increase in blood flow to the eyelids and nictitating membrane (Nilsson, 1994a, 1994b).

*Cerebral circulation* - PACAP may also have a role as a vasorelaxant of canine cerebral arterioles, rat intracerebral arterioles, cat pial arteries and cerebral arterioles in newborn pigs (Anazi et al., 1995; Tong et al., 1993). Innervation of cerebral arteries by PACAP immunoreactive nerve fibers has been observed in cat pial arteries (Uddman et al.,

1993). Topical administration of PACAP through a cranial window in newborn pigs caused vasorelaxation and a dose-dependent increase in cerebrospinal fluid cAMP levels. Similar results were observed in the pial artery following intravenous administration of PACAP during hypoxia in the pig. (Tong et al., 1993; Wilderman and Armstead, 1997). Therefore it appears that PACAP is a regulator of vasorelaxation in the cerebral circulatory system.

## **2. Effects on non-vascular smooth muscle**

*Respiratory smooth muscle* - PACAP-ir fibers and receptor sites (possibly a VPAC-R and a PAC<sub>1</sub>-R) have been localized in the rat lung (Gottschall et al., 1990; Cardell et al., 1991; Shivers et al., 1991; Uddman et al., 1991a; Bitar and Coy, 1993). In particular, PACAP-ir fibers have been observed close to small bronchiole in squirrel monkeys, pigs, sheep, ferrets, guinea pigs and rats (Cardell et al., 1991; Uddman et al., 1991a). The current hypothesis is that PACAP is involved in bronchial relaxation as shown in primates and guinea pigs (Linden et al., 1995; Yoshihara et al., 1997).

Outside of the lung, PACAP-ir fibers are located throughout the respiratory tract. Immunoreactive fibers have been observed beneath the epithelial surface among bundles of smooth muscle in man, squirrel monkeys, pigs, sheep, ferrets, guinea pigs and rats (Cardell et al., 1991; Uddman et al., 1991a, 1991b). In vitro studies revealed that PACAP has a concentration-dependent relaxant effect on guinea pig and rabbit tracheal segments (Cardell et al., 1991; Kanemura et al., 1993; Conroy et al., 1995; Foda et al., 1995). Removal of epithelium from guinea pig tracheal segments reduced the relaxant properties of PACAP but did not entirely inhibit relaxation (Conroy et al., 1995). In rabbit tracheal segments PACAP-induced relaxation was followed by an increase in intracellular cAMP levels (Kanemura et al., 1993). The relaxant effects of PACAP may also involve activation of a Na<sup>+</sup>-K<sup>+</sup>-ATPase (Kanemura et al., 1993). These studies suggest that PACAP is a neurotransmitter/neuromodulator regulating smooth muscle tone in airways. PACAP's

relaxant effects may be useful for treating bronchial asthma. Studies in guinea pigs show that PACAP inhibits histamine-induced respiratory resistance (Saguchi et al., 1997).

*Digestive tract smooth muscle* - PACAP-ir fibers have been identified innervating smooth muscle in the gastrointestinal tract of humans, pigs, sheep, cats, ferrets, guinea pigs, hamsters, rats, mice, chickens and fish (Sundler et al., 1991; Koves et al., 1993; Olsson and Holmgren, 1994; Portbury et al., 1995). Several experiments have shown that PACAP induces a concentration-dependent relaxation of gastrointestinal tract smooth muscles in humans, cats, rabbits, guinea pigs and rats (Mungan et al., 1992; Huang et al., 1993; Schworer et al., 1993; McConalogue et al., 1995a, 1995b; Ny et al., 1995; Katsoulis et al., 1996; Kishi et al., 1996; Ekblad and Sundler, 1997; Parkman et al., 1997b; Zagorodnyuk et al., 1997). The actions of PACAP are mediated by a PAC<sub>1</sub>-R in the human sigmoid colon and rat distal colon (Schworer et al., 1993; Kishi et al., 1996), whereas inhibition of contractions is mediated by a VPAC-R in rabbit pyloric muscles and in gastric cells (Murthy et al., 1997; Parkman et al., 1997b). A study on rat ileal longitudinal muscles suggests three receptors induce relaxation, a PACAP-27 preferring receptor coupled to an apamin sensitive Ca<sup>++</sup>-dependent K<sup>+</sup> channel, a PACAP-27 and -38 preferring receptor and a VIP-specific receptor (Ekblad and Sundler, 1997). Neither the PACAP-27 preferring receptor nor the VIP preferring receptor have yet been cloned. However, other researchers have proposed the existence of a PACAP-27 preferring receptor (Cox, 1992). In addition, an apamin sensitive PACAP receptor coupled to potassium channels has been proposed to exist in gastrointestinal tract smooth muscle, but the preference for PACAP-27 has not been observed (McConalogue et al., 1995a, 1995b; Katsoulis et al., 1996). To date, studies have linked PACAP's relaxant effects to cAMP, NO synthase and K<sup>+</sup> channels in the digestive tract (Schworer et al., 1993; Ny et al., 1995; Kishi et al., 1996; Ekblad and Sundler, 1997; Murthy et al., 1997)

In addition to its relaxant effects, PACAP appears to mediate central nervous system effects on gastric smooth muscle. PACAP causes an increase in rat intragastric pressure via excitatory actions on the vagal nerve, located in the hindbrain. Thus, PACAP can have direct relaxant effects via local interaction with PACAP receptors in the gut smooth muscles or indirect effects via stimulation of excitatory neurons located in the brain. These neurons synapse with gastric smooth muscle and release excitatory neurotransmitters (Krowicki et al., 1997). Taken together, these studies suggest PACAP is a potent modulator of stomach muscle relaxation and contraction, colonic motility and motor activity in the gut. Also of note, PACAP has both excitatory and relaxant effects on guinea pig gallbladder muscles. The contractile effect in the gallbladder is mediated through a PAC<sub>1</sub>-R and the relaxant effect is mediated through a VPAC receptor (Parkman et al., 1997a).

*Reproductive tissue smooth muscle* - In the reproductive system PACAP has been localized to fibers associated with smooth muscle bundles in the rat genital tract (Fahrenkrug and Hannibal, 1996). PACAP has also been shown to induce relaxation of urethral smooth muscle in the female pig (Werkstrom et al., 1997).

### **3. Effects on cardiac muscle**

It is difficult to elucidate PACAP's direct effect on heart rate due to its concurrent effect on blood pressure. However, PACAP has been observed to increase heart rate in sheep and cats (Minkes et al., 1992b; Sawangjaroen et al., 1992), although an intravenous injection of PACAP into beagle dogs slowed heart rate following a transient increase. The changes in heart rate in dogs resulted in hypotension following transient hypertension (Ishizuka et al., 1992). In isolated and blood-perfused dog heart preparations, PACAP directly increased the sinus rate and atrial and ventricular contractile force (Yonezawa et al., 1996). The direct effects of PACAP on heart rate and contractile force have been investigated in vitro on rat cell cultures of cardiac myocytes. In these cultures PACAP stimulates cAMP production in a dose-dependent manner ( $10^{-12}$  to  $10^{-6}$  M) (Suzuki et al.,

1993b). These results suggest that in the first phase of the *in vivo* biphasic response PACAP directly stimulates cardiac myocytes to induce an increase in heart rate and contractile force. The second phase (decrease in heart rate and contractile force) is mediated by interaction of PACAP with cardiac parasympathetic nerves via the PAC<sub>1</sub>-R (Yonezawa et al., 1996; Hirose et al., 1997; Braas et al., 1998). PACAP immunoreactivity has been localized in the guinea pig heart to neuronal fibers and a subpopulation of intrinsic postganglionic cardiac neurons. In addition, cardiac ganglia expressed the PACAP transcript and PAC<sub>1</sub>-R transcripts. The predominant receptor expressed is the PAC<sub>1</sub>-R-*vs*, but low levels of the short and hop2 isoforms are also expressed. The majority of parasympathetic neurons were immunoreactive for PAC<sub>1</sub>-R. Exposure of atrial whole-mount preparations to PACAP results in depolarization of cardiac ganglia and increased neuronal excitability. These results support the evidence that PACAP and a PAC<sub>1</sub>-R modulate the parasympathetic-mediated inhibition of cardiac output (Braas et al., 1998).

#### ***E. An immune system regulator***

PACAP immunoreactive cells can be found in various tissues associated with the immune system in rats: bone marrow, thymus, spleen, lymph nodes and duodenal mucosa (Gaytan et al., 1994). PACAP receptors are also associated with many immune cells. VPAC receptors have been localized to leukocytes, mononuclear cells, lymphoblasts, lymphocytes, monocytes, lymphoid cells, macrophages, myeloma cells, T-cells and B-lymphocytes (Ganea, 1996). Several other papers have noted that PACAP receptor expression is mainly in lymphocytes, macrophages and astrocytes. In the lymphocytes of rats and mice, a VPAC<sub>1</sub>-R gene is expressed (Waschek et al., 1995b; Delgado et al., 1996a; Delgado et al., 1996b) and in rat macrophages both immunoreactive and mRNA data identify a VPAC-R and perhaps a PAC<sub>1</sub>-R (Segura et al., 1991; Delgado et al., 1996a, Pozo et al., 1997). Evidence also suggests the existence of a VPAC-R and perhaps a PAC<sub>1</sub>-R in human mononuclear cells (Guerrero et al., 1981). In rat astrocytes a PACAP

receptor exists, although the type of receptor has not yet been determined (Tatsuno et al., 1991b). The function of PACAP in these cell types is unclear but there are connections between PACAP and immune cell maturity, mobility and the inflammation response as described below.

### **1. Immune cell protection**

In rats, thymus lymphocytes (thymocytes) have increased cell survival when exposed to PACAP. The protection results from exposure to PACAP causing a dose-dependent ( $10^{-13}$  to  $10^{-6}$  mol/L) inhibition of spontaneous apoptosis and a decrease in proliferation (Tatsuno et al., 1991a; Delgado et al., 1996c). It has been suggested that PACAP is involved in T-cell maturation. As thymocytes reach maturity, proliferation decreases. However, one of the first processes in thymocyte maturity is rescue from negative selection by inhibition of apoptosis. Because PACAP can regulate inhibition of both thymocyte proliferation and apoptosis, it may be an important regulator of thymocyte maturity (Delgado et al., 1996c). This effect appears to be mediated by a VPAC<sub>1</sub>-R. The antiproliferative actions of PACAP have been linked to the stimulation of a cAMP-dependent protein kinase (Tatsuno et al., 1991a; Waschek et al., 1995b; Delgado et al., 1996c, 1996b). Unlike rats, mice do not have PACAP binding sites on thymocytes (Shivers et al., 1991).

PACAP is also involved indirectly in lymphocyte maturation by stimulating the release of interleukin-6 (IL-6) from folliculo-stellate cells in the pituitary. The release is triggered by a PACAP-induced dose-dependent increase in intracellular  $\text{Ca}^{++}$ . IL-6 stimulates B-cell growth and differentiation and increases synthesis and secretion of immunoglobulins by B-lymphocytes (Tatsuno et al., 1991c; Yada et al., 1993).

### **2. Immune cell phagocytosis**

When exposed to PACAP rat peritoneal macrophages were observed to increase both phagocytosis and production of superoxide anion (required for digestion of ingested

cells) in a dose-dependent manner. Phagocytosis and cell digestion are the two most characteristic functions of macrophages. This process appears to be mediated by PAC<sub>1</sub>-R in rat peritoneal macrophages via phosphokinase C stimulation (Delgado et al., 1996d). Recently, the presence of PAC<sub>1</sub>-R in these cells was confirmed (Pozo et al., 1997). At present the role of VPAC receptor types remains unclear, although VPAC<sub>1</sub>-R expression has been confirmed in rat peritoneal macrophages (Segura et al., 1991; Delgado et al., 1996a). In mice PACAP is also involved in increasing phagocytosis in peritoneal macrophages likely through a PAC<sub>1</sub>-R variant (Ichinose et al., 1995).

PACAP ( $10^{-13}$  to  $10^{-6}$  M) increases adherence of both macrophages and lymphocytes. In addition, it increases motility of macrophages while decreasing the motility of lymphocytes. The data suggest these processes are regulated by PAC<sub>1</sub>-R in macrophages via PLC activation and by VPAC-R in lymphocytes via AC activation (Delgado et al., 1995; Garrido et al., 1996).

### **3. Immune response**

PACAP is reported to both suppress and activate inflammation through regulation of certain interleukins (IL-6 and IL-10). The PACAP-induced inhibition of IL-10 production by lymphocytes is likely mediated via cAMP. PACAP appears to regulate transcriptional expression of IL-10. In turn, IL-10 suppresses the action of certain cytokines that are involved in the local immune response (Martinez et al., 1996). PACAP can inhibit IL-6 production from stimulated macrophages through the PAC<sub>1</sub>-R (Martinez et al., 1998b). This action of PACAP suppresses inflammation. In contrast, PACAP also has proinflammatory effects. In unstimulated macrophages PACAP acts through VPAC<sub>1</sub>-R and PAC<sub>1</sub>-R to upregulate IL-6 transcription and release (Martinez et al., 1998a). Also, PACAP stimulates IL-6 production in rat astrocytes (Gottschall et al., 1994). Thus, it appears that PACAP both initiates and inhibits the inflammatory response. The findings suggest that PACAP may be involved in immune system homeostasis in the absence of

stimulation. However, in the presence of intense stimulation or toxicity the PACAP-regulated inhibition of IL-6 transcription may help protect the tissue from excessive IL-6 release in order to reduce inflammation or shock (Martinez et al., 1998a, 1998b). Finally, PACAP induces extravasation in rat skin by a cAMP-independent mechanism. Part of the extravasation response may be mediated by PACAP-induced histamine release from mast cells in the skin, which also contributes to inflammation (Cardell et al., 1997b).

In the brain PACAP may modulate the immune response through regulation of outward potassium currents in microglial cells. The regulation of ion channels is a means by which PACAP may modulate microglia activity (Ichinose et al., 1998).

#### ***F. A regulator of bone metabolism***

Traditionally PACAP has not been thought of as a hormone that has direct effects on the skeletal system. Any effect of PACAP on bone and cartilage has been considered indirect through the release of GH and subsequently insulin-like growth factor II, which is involved in skeletal growth. However, recent evidence suggests PACAP has a direct modulatory role in bone tissue. PACAP has been identified by immunocytochemistry in nerve fibers innervating the cartilage canals of newborn pigs. Receptors for PACAP can be found in human osteoblast and osteosarcoma cells (VPAC<sub>1</sub>-R), rabbit stromal cells (VPAC<sub>2</sub>-R), and rat bone marrow-derived stromal cells (VPAC<sub>2</sub>-R >> PAC<sub>1</sub>-R or VPAC<sub>1</sub>-R mRNA) (Cai et al., 1997; Strange-Vognsen et al., 1997; Togari et al., 1997; Winding et al., 1997). In osteoblasts and osteoblast-enriched bone cultures, PACAP stimulates cAMP accumulation (Lerner et al., 1994; Kovacs et al., 1996). Several of these studies suggest PACAP may play a role in bone formation (Strange-Vognsen et al., 1997), bone resorption (Winding et al., 1997), and hematopoiesis through stimulation of IL-6 production in bone marrow-derived stromal cells (Cai et al., 1997). Although these findings are not conclusive, it seems that PACAP functions in some capacity as a neuroendocrine or paracrine regulator of bone metabolism.

**G. *An endocrine/paracrine regulator***

**1. Anterior pituitary secretions**

PACAP was discovered in ovine hypothalamic extracts because it was able to increase cAMP in static rat anterior pituitary cell cultures and cause the release of GH, prolactin (PRL), adenocorticotrophic hormone (ACTH) and luteinizing hormone (LH) from superfused rat pituitary cells (Miyata et al., 1989, 1990). This original work paved the way for numerous studies into the role of PACAP as a hypophysiotropic factor. Several criteria need to be met for a substance to be classified as a hypophysiotropic factor. These criteria are discussed in detail in an review on PACAP's actions on the anterior pituitary by Rawlings and Hezarah (1996). In summary, the substance must be 1) present in neurons in the hypothalamus that project to the hypophysial portal blood system, 2) present in the hypophysial portal blood, 3) able to interact with receptors on cells of the anterior pituitary, and 4) able to regulate anterior pituitary cell function. Ironically, sheep, the animal in which PACAP was discovered, appear not to use PACAP as a hypophysiotropic hormone. Unlike rats and humans, infusion of PACAP into sheep has no effect on the release of anterior pituitary hormones (Koves et al., 1990; Sawangjaroen and Curlewis, 1994; Sawangjaroen et al., 1997). However, PACAP is a hypophysiotropic factor in many other vertebrates.

First, PACAP is present in the hypothalamus. PACAP immunoreactivity has been demonstrated in the hypothalamus of primates (including humans), sheep, rats, frogs and fish (Koves et al., 1990, 1991; Arimura et al., 1991; Vigh et al., 1991; Yon et al., 1992; Masou et al., 1993, 1994; Tatsuno et al., 1994; Piggins et al., 1996; Matsuda et al., 1997; Montero et al., 1998; Wong et al., 1998). In humans and spider monkeys, the immunoreactive fibers were localized in the supraoptic and the paraventricular nuclei, the periventricular region and preoptic area. Both the supraoptic and paraventricular nuclei

send fibers to the anterior pituitary via the hypophysial portal blood vessels and the posterior pituitary. In monkeys PACAP-ir fibers were found in the external zone of the tuber cinereum around hypophysial portal capillaries, this region is close to the transition of the pituitary stalk (median eminence). A similar distribution of PACAP immunoreactivity has been observed in the rat, frog and fish (Yon et al., 1992; Piggins et al., 1996; Matsuda et al., 1997; Montero et al., 1998). PACAP is also detectable as mRNA in human and rat hypothalami (Ghatei et al., 1993). Together these results give evidence that PACAP is present in the hypothalami of several vertebrates with a distribution suitable for a hypophysiotropic factor.

Second, PACAP has been detected in the portal blood of rats at a concentration significantly higher than in the peripheral blood (Dow et al., 1994). The presence of PACAP in the portal blood is a crucial piece of evidence supporting the role of PACAP as a hypophysiotropic factor.

Third, PACAP interacts with the anterior pituitary. Immunoreactive PACAP is found in the human, rat, amphibian and fish anterior pituitary (Ghatei et al., 1993; Mikkelsen et al., 1995; Koves et al., 1998; Matsuda et al., 1998; Montero et al., 1998). mRNA studies reveal it is not synthesized in the anterior pituitary of humans; the presence of it here may be the result of transport via nerve terminals and portal blood from the hypothalamus (Ghatei et al., 1993). In fish PACAP-containing nerve terminals grow into the anterior pituitary from the brain. Several immunoassay and mRNA studies have identified PACAP receptors in rat anterior pituitary cells or whole pituitary extracts (Gottschall et al., 1990; Shivers et al., 1991; Morrow et al., 1993; Spengler et al., 1993; Vigh et al., 1993; Rawlings et al., 1995; Vertongen et al., 1995b; Hashimoto et al., 1996). PACAP receptors have also been identified in human pituitary adenomas (gonadotropin-producing, GH-producing and ACTH-producing adenomas) by binding assays (Robberecht et al., 1993). The most frequently identified PACAP receptor in the anterior

pituitary appears to be the PAC<sub>1</sub>-R (Gottschall et al., 1990; Shivers et al., 1991; Robberecht et al., 1993; Hashimoto et al., 1996). In frog pituitaries a PACAP-preferring receptor has been proposed that is pharmacologically different from mammalian PAC<sub>1</sub> receptors (Chartrel et al., 1995) and recently PAC<sub>1</sub> receptors have been cloned in chicken, frog and goldfish pituitaries (Wong et al., 1998; Peeters et al., 1999; Hu et al., 2000). Other studies also detect the presence of VPAC<sub>2</sub>-R and VPAC<sub>1</sub>-R (weakly) (Rawlings et al., 1995). The abundance of the receptor subtype mRNA in normal rat anterior pituitary cells is as follows: PAC<sub>1</sub>-R=VPAC<sub>2</sub>-R and VPAC<sub>1</sub>-R is expressed weakly (Rawlings et al., 1995). Although all the splice variant forms of the PAC<sub>1</sub>-R may be present in the anterior pituitary of rats, the dominant forms are PAC<sub>1</sub>-R-s and PAC<sub>1</sub>-R-hop (Rawlings et al., 1995).

Fourth, PACAP regulates anterior pituitary function. The rat anterior pituitary releases several pituitary hormones into the blood in response to intravenous PACAP administration (Leonhardt et al., 1992). Each pituitary cell type reacts to PACAP differently. In mammals there has been considerable debate as to whether PACAP is a modulator of pituitary hormone release or a true hypophysiotropic hormone. The picture should be clarified eventually in knockout mice. In the meantime several studies are outlined below that summarize what is known about PACAP's actions on anterior pituitary cells.

*Gonadotrope secretions* - PACAP affects LH and FSH synthesis/release on two levels. It can modulate the actions of GnRH at the level of the hypothalamus, or it can act alone or in concert with GnRH to modulate gonadotropes in the pituitary. A recent study on rats has localized PACAP to LH- and FSH-producing cells in the anterior pituitary (Koves et al., 1998). This suggests PACAP may also have some paracrine or autocrine effects on anterior pituitary cells. The following paragraphs will discuss the actions of PACAP on gonadotrope function both alone and in conjunction with GnRH exposure in

the anterior pituitary. The actions of PACAP on GnRH function in the hypothalamus are discussed in Section G.3.

Many of the experiments that have been done to assess gonadotrope response to PACAP have used the  $\alpha$ T3-1 clonal gonadotrope cell line. It is clear that exposure to PACAP has an effect on these cells, but it is important to note that this cell line is immortalized and may not reflect normal cell function (Schomerus et al., 1994). The  $\alpha$ T3-1 cell line was generated by targeted oncogenesis in mice. Like normal gonadotropes these cells express the  $\alpha$ -subunit gene and synthesize and secrete the  $\alpha$ -subunit protein, making them a good model. However, they do not express the  $\beta$ -subunit gene and therefore cannot synthesize LH or FSH (Windle et al., 1990).

PACAP regulates an increase in the release of gonadotropins and the  $\alpha$ -subunit. At physiological concentrations continuous PACAP exposure stimulates the release of LH and FSH from normal rat gonadotropes (Miyata et al., 1990; Culler and Paschall, 1991; Hart et al., 1992; Perrin et al., 1993; Schomerus et al., 1994; Tsujii et al., 1994). Following the initial stimulation, secretion of both LH and FSH drops. However, continued PACAP exposure causes a steady increase in LH release back to maximum levels, whereas FSH secretion gradually decreases (Tsujii et al., 1994). In addition, PACAP triggers the release of the  $\alpha$ -subunit protein from normal rat gonadotropes (Hart et al., 1992; Tsujii et al., 1994, 1995b). The  $\alpha$ -subunit protein is also released from the  $\alpha$ T3-1 clonal gonadotrope cell line in response to PACAP exposure (Schomerus et al., 1994; Tsujii et al., 1995a). If PACAP is administered in a pulsatile fashion, it likewise increases gonadotropin secretion in normal gonadotropes (Tsujii et al., 1995b).

In addition to its releasing powers, PACAP also elicits an increase in gonadotropin subunit transcription. Continuous exposure to PACAP causes 1) an increase in LH $\beta$  half life by increasing transcript length, 2) an increase in  $\alpha$ -subunit transcription and 3) a decrease in FSH $\beta$  subunit transcription (Schomerus et al., 1994; Tsujii et al., 1994, 1995a). These results correlate well with the measures of LH and FSH release noted above. Pulsatile delivery of PACAP increases the level of LH $\beta$  mRNA and  $\alpha$ -subunit mRNA, but has no effect on FSH $\beta$  mRNA. The native pattern of exposure is unknown but is thought to be continuous (Tsujii et al., 1995b). PACAP regulation of gonadotropin gene transcription appears to be regulated via the cAMP/PKA pathway (Ishizaka et al., 1993; Tsujii et al., 1995a), but the exact mechanism by which PACAP affects LH $\beta$  and  $\alpha$ -subunit gene transcription is not clear. However, the decrease in FSH $\beta$  mRNA appears to be correlated with a PACAP-induced increase in follistatin mRNA. Follistatin is produced by a subset of gonadotropes and folliculo-stellate cells. It is thought that PACAP (via cAMP/PKA) stimulates follistatin transcription, which in turn, neutralizes activin activity. Once neutralized, activin is no longer able to increase FSH $\beta$  mRNA and the level drops (Winters et al., 1997). Therefore, the action of PACAP on FSH $\beta$  mRNA transcription is probably indirect, mediated through PACAP's stimulation of follistatin gene transcription.

Long-term exposure to PACAP results in down regulation of PAC<sub>1</sub>-R concentration in the cell membrane and homologous desensitization of the AC and PLC pathways (Tsujii et al., 1994; McArdle and Forrest-Owen, 1997). PACAP dissociation from its receptor is slow and gonadotropes become desensitized to continuous PACAP exposure through changes in receptor-effector coupling and PAC<sub>1</sub>-R down-regulation at the cell surface

(McArdle and Forrest-Owen, 1997). The main effector of gonadotropin regulation is GnRH, but PACAP and GnRH synergistically increase gonadotropin release and regulate transcription differently and more efficiently than either hormone alone (Culler and Paschall, 1991; Tsujii et al., 1994).

PACAP is thought to regulate gonadotropin release through changes in cytosolic calcium (Canny et al., 1992; Perrin et al., 1993; Rawlings et al., 1993, 1995, 1996). Due to the presence of two PACAP receptor types (PAC<sub>1</sub>-R and VPAC<sub>2</sub>-R) in gonadotropes, PACAP can potentially regulate several intracellular signalling mechanisms (Rawlings et al., 1995). In rat gonadotropes PACAP alters gonadotropin mRNA levels as well as LH, FSH and  $\alpha$ -subunit release, likely via the PAC<sub>1</sub>-R (Tsujii et al., 1994, 1995a). Gene transcription is mediated by the cAMP/PKA pathway. However, cAMP has no effect on the observed PAC<sub>1</sub>-R-mediated changes in Ca<sup>++</sup> concentration (Rawlings et al., 1993, 1994, 1996). Typically, Ca<sup>++</sup> is an important molecule involved in regulation of exocytosis. It is thought that, as is the case with GnRH-induced gonadotropin secretion, the actions of PACAP on PLC and cytosolic calcium are involved in exocytosis of the gonadotropins (Stojilkovic et al., 1989).

Because PACAP and GnRH both affect gonadotropin secretion and production and, in some cases, use the same intracellular pathways, there is potential for cross talk and coincidence signalling (McArdle and Forrest-Owen, 1997; McArdle et al., 1994; McArdle and Counis, 1996). Together, continuous PACAP infusion in the presence of GnRH pulses stimulates LH and FSH release, increases  $\alpha$ -subunit and FSH $\beta$  mRNA and lengthens LH $\beta$  mRNA transcripts (Tsujii et al., 1994). Also, in humans infusion of PACAP causes no effect on gonadotropin release, even when administered along with GnRH pulses (Hammond et al., 1992; Chiodera et al., 1996). These differences between rats and humans may reflect species differences or experimental conditions. In addition,

PACAP may increase proliferation of  $\alpha$ T3-1 cells (Schomerus et al., 1994). Further research is required to clarify these results.

*Somatotrope secretions* - Somatotropes are growth hormone (GH) producing cells found in the anterior pituitary. In mammals the primary releaser of GH is GRF, but PACAP also appears to have a role in GH release. PACAP ( $10^{-11}$  to  $10^{-8}$  M) increases  $\text{Ca}^{++}$  concentrations in rat anterior pituitary cell cultures in a dose-dependent manner and some of the cells experiencing a rise in  $\text{Ca}^{++}$  concentration have been identified as somatotropes (Yada et al., 1993). PACAP also stimulates cAMP accumulation in rat static pituitary cell cultures (Wei et al., 1993). Several researchers have shown that PACAP induces GH release and synthesis (Goth et al., 1992; Hart et al., 1992; Jarry et al., 1992; Propato-Mussafiri et al., 1992; Wei et al., 1993; Velkeniers et al., 1994). The proposed mechanism is that PACAP increases  $\text{Ca}^{++}$  levels via a cAMP/PKA mediated pathway (Rawlings et al., 1993; Miyake et al., 1996) and that the release of GH by PACAP does not require PKC activation. This points to the involvement of cAMP in the process of GH release and not IP (Wei et al., 1993). Also, it has been suggested that PACAP activates a sodium-channel via an AC/PKA pathway (Miyake et al., 1996). The result is membrane depolarization and, in turn, calcium-channel activation that triggers the increase in cytosolic calcium necessary for GH release. PACAP stimulates GH release from  $\text{GH}_3$  and  $\text{GH}_4\text{C}_1$  cells through a  $\text{VPAC}_2$ -R (Rawlings et al., 1995; Murakami et al., 1996). However, in a rat GH-producing enriched cell population, the only PACAP receptor expressed, as determined by RT-PCR, is  $\text{PAC}_1$ -R-hop (Vertongen et al., 1995b). More work is required to determine if this is the only receptor that PACAP activates to cause GH release. In addition to stimulating GH release, PACAP induces an increase in rat GH gene transcription in static cell culture (Velkeniers et al., 1994). The effects of PACAP on GH may involve increasing the number of GH-secreting cells (Goth et al., 1992). It should be noted that PACAP infusion into human males does not cause any increase in serum GH

levels (Chiodera et al., 1996; Murakami et al., 1996). Although the actions of PACAP on GH release in mammals have not been fully elucidated, PACAP functions have been studied across vertebrates and an evolutionary perspective is clear. In fish at physiologically relevant concentrations, PACAP stimulates GH release from cultured pituitary cells (Parker et al., 1997; Montero et al., 1998; Wong et al., 1998). Therefore, it appears that, at least in some non-mammals, PACAP is a hypophysiotropic releaser of GH.

*Lactotrope secretions* - Lactotropes are prolactin (PRL) producing cells in the anterior pituitary. PRL-producing enriched cells from rat anterior pituitaries express both PAC<sub>1</sub>-R and VPAC<sub>2</sub>-R receptors. There are three PAC<sub>1</sub>-R splice variants expressed in these cells: PAC<sub>1</sub>-R-s, PAC<sub>1</sub>-R-hop and PAC<sub>1</sub>-R-hiphop (weakly expressed) (Vertongen et al., 1995b). The action of PACAP on PRL release is not yet clear. PACAP dose-dependently increases cAMP concentrations and also increases PRL mRNA in GH<sub>3</sub> cells and in total pituitary cell suspensions by direct action on the PRL promoter (Coleman and Bancroft, 1993; Velkeniers et al., 1994). The effects of PACAP on PRL gene transcription may be controlled by a cAMP-independent pathway (Coleman and Bancroft, 1993). In contrast, the action of PACAP on PRL release is not yet clear. In vitro experiments on dispersed anterior pituitary primary cell cultures indicate that PACAP dose-dependently ( $10^{-7}$  and  $10^{-6}$  M) inhibits PRL release (Jarry et al., 1992; Propato-Mussafiri et al., 1992). These results are obviously in conflict with the actions of PACAP on gene transcription. A theory explaining this paradox suggests that PACAP may trigger the secretion of a paracrine factor that stimulates PRL release (Jarry et al., 1992). In intact pituitaries the paracrine factor is more potent than PACAP and overrides PACAP's inhibitory action on PRL. In cell culture the paracrine factor is diluted by the medium and is unable to override the inhibitory effects of PACAP. These results have since been confirmed and the proposed paracrine factor is IL-6 (Benter et al., 1995). In vivo experiments in humans show that infusion of high doses of PACAP (4-10 pmol/kg/min) results in an increase in

plasma PRL. This effect appears to be mediated by both VIP and PACAP through a VPAC-R (Chiodera et al., 1996; Murakami et al., 1996).

*Corticotrope secretions* - Corticotrope cells are responsible for the release of several pro-opiomelanocorticotin (POMC) hormones, including ACTH. PACAP (0.1 - 100 nmol) causes an increase of ACTH release from dispersed rat pituitary primary cell cultures. The release of ACTH is only significant following a 24-hour time delay. The time delay between cAMP stimulation and ACTH release suggests other factors are involved in ACTH secretion, perhaps IL-6 (Hart et al., 1992). Also, PACAP induces a dose-dependent secretion of ACTH from an AtT-20 pituitary cell line and in human males infused with PACAP (Propato-Mussafiri et al., 1992; Chiodera et al., 1996; Aoki et al., 1997). The effect of PACAP in humans appears to be mediated by PACAP-specific receptors (not VPAC-R) (Chiodera et al., 1996). In contrast, in the mouse corticotrope tumor cell line (AtT-20), PACAP was found to stimulate POMC gene transcription, cAMP accumulation and cellular differentiation through the VPAC<sub>2</sub>-R (Braas et al., 1994; Aoki et al., 1997). These receptor differences may reflect changes between normal and tumorous cells or among species. PACAP can act alone or in combination with hypothalamic corticotropin-releasing hormone to regulate POMC transcription. Corticotropin-releasing hormone activity on POMC is PKA-dependent. In contrast, PACAP acts in a PKA-independent manner. These results suggest that POMC gene transcription can be induced by multiple signalling pathways, one of which is regulated by PACAP (Aoki et al., 1997).

*Folliculo-stellate secretions* – Folliculo-stellate cells are agranular cells that differ substantially from the other pituitary glandular cells. Folliculo-stellate cells have long processes and phagocytic ability. The majority of PACAP receptors in the anterior pituitary are found on folliculo-stellate cells (Vigh et al., 1993). PACAP is suspected to cause IL-6 release from these cells through AC stimulation (Tatsuno et al., 1991c).

## 2. Intermediate pituitary secretions

*Melanotrope secretions* - Melanotropes, located in the pars intermedia, are another cell type that transcribe the POMC gene. Alpha-melanocyte stimulating hormone ( $\alpha$ -MSH) production is associated with POMC transcription in these cells. In the rat PACAP-like immunoreactivity is exhibited by cells located in the pars intermedia of the pituitary (Kimura et al., 1994). Also, cells of the pars intermedia express PACAP and PAC<sub>1</sub>-R mRNA (Spengler et al., 1993; Rene et al., 1996; Tanaka et al., 1997). Mouse melanotropes express two isoforms of the PAC<sub>1</sub>-R (PAC<sub>1</sub>-R-s and PAC<sub>1</sub>-R-hop) and PACAP stimulates both cAMP and IP accumulation in melanotropes (Rene et al., 1996). In melanotropes, PACAP and CRH were additive in increasing cAMP levels. PACAP has also been reported to stimulate  $\alpha$ -MSH secretion in rats and mice (Koch and Lutz-Bucher, 1992; Rene et al., 1996; Tanaka et al., 1997). In rats, the signal transduction mechanism triggered by PACAP is PKA-activation of voltage-dependent Ca<sup>++</sup> channels and PKC-activation of nonselective cation channels (Tanaka et al., 1997). The expression of both PACAP and its receptor in the pars intermedia suggests that PACAP may be an autocrine/paracrine regulator of melanotrope secretion.

## 3. Hypothalamic secretions

The presence of PACAP receptors in the hypothalamus suggests the existence of paracrine regulation and/or the possibility of a feedback loop. In hypophysectomized rats the rate of PACAP gene transcription and hormone levels decreased 1 to 2 weeks after hypophysectomy. Replacement therapy with GH, PRL, thyroxine and corticosterone significantly restored these levels suggesting a feedback loop between pituitary hormones or pituitary-dependent factors and PACAP gene transcription (Shuto et al., 1995). Regarding paracrine regulation, the GT1-7 neuronal cell line (an immortalized hypothalamic cell line) expresses PAC<sub>1</sub>-R splice variants and VPAC<sub>2</sub>-R mRNA. Both VIP and PACAP stimulate cAMP in these cells but neither affect IP turnover. Likewise, in the chick

hypothalamus PACAP stimulates cAMP accumulation, likely through the PAC<sub>1</sub>-R (Nowak et al., 1999). In addition, PACAP and VIP stimulate GnRH release from these cells likely by VPAC<sub>2</sub>-R action on cAMP (Olcese et al., 1997). Intracerebroventricular injections of PACAP into rats cause an increase in GnRH and somatostatin mRNA levels. However, intravenous administration causes a decrease in GnRH mRNA levels and no change in somatostatin mRNA expression. Although not conclusive, these results suggest that PACAP may be a neurotransmitter/neuromodulator of hypothalamic hormone secretion (Li et al., 1996).

PACAP also causes the release of AVP in human males and may have the same effect in rats (Murase et al., 1993; Chiodera et al., 1996). This action of PACAP may be involved in the control of osmolarity and blood pressure. Recently, PACAP has been localized to nerve terminals in the hypothalamus that innervate the AVP-containing neurons of the hypothalamic supraoptic nucleus. These AVP-rich neurons also express the PAC<sub>1</sub>-R. PACAP acts on AVP-containing neurons to increase cytosolic calcium via a cAMP/PKA pathway. This influx of calcium may trigger AVP secretion (Shioda et al., 1997).

#### **4. Gonadal secretions**

PACAP mRNA is found in the developing germ cells of the rat testis (Kononen et al., 1994; Shioda et al., 1994; Li et al., 1998). Both the PAC<sub>1</sub>-R and VPAC-R are expressed in the rat testis (Shivers et al., 1991; Spengler et al., 1993). In situ hybridization and Northern analysis reveal that PACAP mRNA is present in round spermatids in stages III - VII, spermatogonia and primary spermatocytes. PACAP was not found in mature spermatids, testicular spermatozoa or epididymal spermatozoa (Kononen et al., 1994; Shioda et al., 1994). PACAP's action in these cells is mediated through a VPAC<sub>2</sub>-R (Krempels et al., 1995). PACAP can regulate synthesis of secreted and intracellular proteins by spermatids and spermatocytes *in vitro*. However, the effects of PACAP on

these stages of maturing germ cells differ. In spermatocytes PACAP increases production of certain germ cell secreted proteins, whereas in spermatids PACAP inhibits production of the proteins (West et al., 1995).

Neither PACAP nor PACAP receptors have been found on Sertoli or Leydig cells (Shioda et al., 1994; Romanelli et al., 1997). However, it has been suggested that PACAP can stimulate cAMP accumulation and secretion of lactate, estradiol and inhibin in Sertoli cells (Heindel et al., 1992). In addition, PACAP expression in the rat testis may be positively regulated, at least in part, by FSH released from the pituitary. Pituitary gonadotropes, such as FSH directly affect other cells in the testis (Sertoli and Leydig cells), but not the germ cells. However, by affecting PACAP expression, FSH may have an indirect effect on germ cell function (Shuto et al., 1995).

Although the receptor has not been identified, PACAP causes a dose-dependent secretion of testosterone from rat Leydig cells. This effect is independent of  $\text{Ca}^{++}$  and cAMP, but is dependent on an influx of  $\text{Na}^+$  from the extracellular medium. These results suggest that PACAP may exert its effect through stimulation of a new PACAP receptor subtype that is  $\text{Na}^+$ -dependent and cAMP- and  $\text{Ca}^{++}$ -independent (Rossato et al., 1997). A different study suggests that the cAMP-independent actions of PACAP on testosterone are mediated by a known  $\text{PAC}_1\text{-R}$  variant. Also, this study determined that the presence of human chorionic gonadotropin is required for testosterone production (Rossato et al., 1997).

In the female PACAP causes a dose-dependent ( $10^{-11}$  to  $10^{-10}$  M) stimulation of cAMP accumulation, estradiol secretion and progesterone secretion from cultured rat ovarian granulosa cells. Also, PACAP may be involved in the induction of LH responsiveness in granulosa cells. These actions may be mediated by the  $\text{PAC}_1\text{-R}$  (Zhong and Kasson, 1994; Heindel et al., 1996). Both PACAP and  $\text{PAC}_1\text{-R}$  mRNA transcripts are found in the rat ovary, specifically granulosa and luteal cells (Kotani et al., 1997). The

splice variants found are PAC<sub>1</sub>-R-s, PAC<sub>1</sub>-R-hip or -hop (the hip and hop variants are not distinguishable by the methods employed in this experiment) and PAC<sub>1</sub>-R-hiphop. These expression data combined with the aforementioned study suggest that PACAP may be an autocrine and/or paracrine regulator of ovarian function (Scaldaferri et al., 1996; Kotani et al., 1997).

## **5. Adrenal gland secretions**

PACAP and PACAP binding sites have been detected in the adrenal gland of mammals, amphibians and fish by immunoreactive methods, binding assays and mRNA detection. In particular, chromaffin cells of the adrenal medulla are innervated by PACAP-ir nerve fibers (Gottschall et al., 1990; Arimura et al., 1991; Shivers et al., 1991; Watanabe et al., 1992; Ghatei et al., 1993; Spengler et al., 1993; Yon et al., 1993, 1994; Reid et al., 1995; Frodin et al., 1995;). In cultured chromaffin cells (PC12) PACAP increases cAMP production (Watanabe et al., 1990; Watanabe et al., 1992) and inhibits proliferation (Frodin et al., 1995). PACAP also increases cell survival, but does not significantly promote neurite outgrowth. PACAP may be involved in overriding growth factor mitogenic signals and maintaining the postmitotic state of chromaffin cells. During development (P8-P12) in the rat, innervation of the adrenal gland with preganglionic sympathetic cholinergic fibers occurs at about the same time as a decline in cell division (Frodin et al., 1995). Not only is PACAP expressed in fibers innervating the adrenal gland, but following sympathectomy PACAP is expressed in some adrenal chromaffin cells (Holgert et al., 1996). These results indicate that PACAP expression may be suppressed under normal conditions.

In the adrenal gland, PACAP is involved in the secretion of a catecholamine (adrenaline) from chromaffin cells of the adrenal medulla. The PACAP-induced secretion of adrenaline modifies cardiac function (Suzuki et al., 1993b). Also, release of catecholamines and leu-enkephalin from chromaffin cells is stimulated by PACAP, likely through a PAC<sub>1</sub>-R (Watanabe et al., 1990; Babinski et al., 1996; Isobe et al., 1996; Neri et

al., 1996). The PACAP-induced release of catecholamines is  $\text{Ca}^{++}$ -dependent (Watanabe et al., 1992).

Not only is PACAP involved in catecholamine release, it also causes an increase in the mRNA for tyrosine hydroxylase in a neuron-derived cell line (CATH.a) and in porcine chromaffin cell cultures. Also, the porcine chromaffin cultures increase dopamine  $\beta$ -hydroxylase gene expression in response to PACAP. Both tyrosine hydroxylase and dopamine  $\beta$ -hydroxylase are involved in catecholamine synthesis. PACAP activates tyrosine hydroxylase and dopamine  $\beta$ -hydroxylase transcription through cAMP accumulation and activation of PKA (Isobe et al., 1996; Marley et al., 1996; Muller et al., 1997).

PACAP is also active in the adrenal cortex. Another adrenal gland hormone, aldosterone, is synthesized by the adrenal cortex. PACAP stimulates aldosterone secretion directly through VPAC receptors in human tumorous adrenal cells, via  $\text{PAC}_1\text{-R}$ -hop or  $\text{PAC}_1\text{-R-s}$  in normal bovine adrenal cells and in unidentified PACAP-preferring receptors in frog adrenal cells (Yon et al., 1993; Bodart et al., 1997). PACAP-stimulated aldosterone secretion is inhibited by atrial natriuretic peptide (ANP) in both bovine and human cells. However, ANP acts via a different mechanism in the two cell types. This suggests that there may be species-specific factors involved in aldosterone production as regulated by PACAP (Bodart et al., 1997). In addition, PACAP stimulates the renewal of intracellular pools of the neuropeptides, ANP and brain natriuretic peptide. PACAP is probably involved in the biosynthesis of these peptides via the  $\text{PAC}_1\text{-R}$ . The process appears to involve the intracellular messengers, PKA and PKC (Babinski et al., 1996). Another study suggests that PACAP acts indirectly on aldosterone secretion. PACAP-induced catecholamine release stimulates aldosterone synthesis and secretion in humans by enhancing aldosterone synthase activity (Neri et al., 1996). In frogs PACAP induces an

elevation in cAMP and  $\text{Ca}^{++}$  in adrenal slices. A PACAP-preferring receptor is present on both chromaffin and adrenocortical cells. In addition to aldosterone the adrenocortical cells also secrete corticosteroid in response to PACAP ( $10^{-8}$ - $10^{-5}$  M) (Yon et al., 1994).

## 6. Pancreatic Secretions

The two main hormones involved in the regulation of blood glucose are insulin and glucagon. Surprisingly, PACAP regulates the secretion of both hormones. In fact, PACAP is a more potent releaser of insulin than of glucagon (Yada et al., 1994, 1997). In mammals PACAP-like immunoreactivity is found in pancreatic nerve fibers, islets and capillaries and PACAP mRNA has been localized to  $\beta$ -islet cells (Yada et al., 1994, 1997). In fish the insulin and glucagon cells are innervated by PACAP-containing fibers (Jonsson, 1995). In vitro experiments in the isolated perfused rat pancreas show that PACAP releases insulin in a glucose-dependent manner. When the pancreas is perfused with 8.3 mM glucose, PACAP ( $10^{-11}$  to  $10^{-10}$  M) elicits a concentration-dependent biphasic release of insulin. However, in the presence of non-stimulating glucose levels (2.8 mM) no change in basal insulin secretion is observed (Yada et al., 1994; Kulkarni et al., 1995; Bertrand et al., 1996; Komatsu et al., 1996). In contrast, glucagon secretion is stimulated by PACAP ( $10^{-10}$  to  $10^{-8}$  M) in the presence of non-stimulating glucose levels (2.8mM) but not in the presence of stimulating glucose levels (8.3 mM) (Bertrand et al., 1996). One proposal is that the effect of PACAP on insulin and glucagon secretion is mediated by a VPAC receptor (Bertrand et al., 1996) and involves PKA and PKC, but not  $\text{Ca}^{++}$  (Komatsu et al., 1996). However, recent findings suggest otherwise. In fact, the  $\text{PAC}_1\text{-R-TM4}$  variant is the only PACAP specific receptor expressed in the  $\beta$ -islet cells of the pancreas (Chatterjee et al., 1996). PACAP is known to stimulate insulin secretion from  $\beta$ -islet cells through activation of L-type  $\text{Ca}^{++}$  channels (Yada et al., 1994, 1997). The  $\text{PAC}_1\text{-R-TM4}$  is not coupled to a G protein and does not act on AC or PLC, but it is

coupled to L-type  $\text{Ca}^{++}$  channels (Chatterjee et al., 1996). Thus, PACAP appears to be a potent releaser of glucose-induced insulin secretion from  $\beta$ -islets cells through activation of the  $\text{PAC}_1\text{-R-TM4}$ . The action of PACAP on insulin has been confirmed in a knockout mouse in which the  $\text{PAC}_1\text{-R}$  was disrupted. The  $\text{PAC}_1\text{-R}$  null mice had reduced glucose-stimulated insulin secretion in vivo and in vitro (Jamen et al., 2000).

Unlike rats, sheep did not exhibit any effect on insulin or glucagon plasma concentration from a PACAP i.v. infusion (100 pmol/kg/min) whether the system was glucose stimulated or not. This may be due to species differences (Onaga et al., 1996).

## **7. Cardiac Secretions**

The heart is not traditionally thought of as an endocrine or paracrine organ but it does produce and secrete hormones. In cultured rat neonatal cardiomyocytes, PACAP is involved in the regulation of one of the hormones secreted by the heart, atrial natriuretic peptide. Incubation with PACAP causes a dose-dependent increase in cAMP content in cardiac myocytes and a concomitant dose-dependent increase in atrial natriuretic peptide content (Basler et al., 1995).

## **8. Gastrointestinal secretions**

The enterochromaffin-like cells of the gastrointestinal tract secrete histamine and pancreastatin in response to gastrin. However, PACAP has been shown to stimulate histamine and pancreastatin secretions from enterochromaffin-like cells with a greater potency than gastrin (Lindstrom et al., 1997). The distribution of PACAP in the gastrointestinal tract was discussed in Section D.2 on smooth muscle regulation. In addition, PACAP-ir endocrine cells are present in the chicken gut. This provides further evidence that PACAP may be a gut hormone or paracrine factor (Sundler et al., 1991)

### ***H. An exocrine regulator***

*Gastrointestinal secretions* - PACAP immunoreactive nerve fibers innervate the gut wall of humans, pigs, sheep, cats, ferrets, guinea pigs, hamsters, rats, mice and chickens (Sundler et al., 1991; Koves et al., 1993; Portbury et al., 1995). The following are examples of PACAP regulating exocrine secretions in the gut. Intravenous exposure to PACAP inhibits gastric acid secretions from the parietal cells. PACAP also inhibits both histamine-stimulated and pentagastrin-stimulated gastric acid secretions (Mungan et al., 1992; Mungan et al., 1995). Although VIP is also active in this system, PACAP-27 and not VIP or PACAP-38 inhibits pentagastrin-stimulated gastric acid secretion (Mungan et al., 1992). The receptors active in these responses have not been determined. Also, PACAP causes an increase in pepsinogen release from chief cells via a VPAC receptor (Felley et al., 1992).

*Pancreatic secretions* - PACAP not only increases blood flow in the exocrine pancreas and regulates insulin and glucagon, but PACAP also regulates exocrine function. VPAC<sub>2</sub>-R binding sites have been identified in the pancreatic acini of rats (Schmidt et al., 1993). PACAP and VIP have been shown to increase acinar lipase release, amylase release and cAMP accumulation (Raufman et al., 1991; Schmidt et al., 1993). In sheep, pancreatic juice flow and protein secretions increase following PACAP or VIP (1,3,10 pmol/kg/min) infusion. Bicarbonate secretion also increases due to PACAP or VIP (1, 3, 10 pmol/kg/min) infusion. The effect of PACAP and VIP on bicarbonate secretion may result from a direct action of the hormones on the duct. PACAP (1, 3, 10 pmol/kg/min), but not VIP, dose-dependently increases amylase output (Onaga et al., 1996). PACAP's action on protein and amylase secretion may be indirectly mediated via cholinergic nerve stimulation (Onaga et al., 1996). Similar results were observed in the dog exocrine pancreas following intravenous injection of PACAP (Naruse et al., 1993).

PACAP stimulates calcium signalling in AR-4-2J cells, a rat acinar-like pancreatic cell line. The calcium signalling has two phases. First, an initial peak is dependent on an influx of external calcium and mobilization of internal calcium. Second, a plateau phase is dependent on influx of external calcium only. The peak is PLC dependent. The above effects are evoked at levels that stimulate amylase release (Barnhardt et al., 1997). Perhaps the unique actions of PACAP that regulate amylase output are mediated by PAC<sub>1</sub> receptors. This would account for the inability of VIP to elicit a change in amylase secretion. It should be noted that, whereas PAC<sub>1</sub> receptors dominate in AR-4-2J cells, the VPAC<sub>2</sub>-R dominates in normal pancreatic acini (Schmidt et al., 1993).

*Seromucous secretions* - PACAP also appears to affect mucous secretions in various tissues. In several mammals PACAP and VIP were coexpressed in nerve fibers observed around seromucous glands in the nose, trachea and lungs. This suggests that along with VIP, PACAP may have a role in mucous secretions (Cardell et al., 1991; Uddman et al., 1991a). Also, in the human vagina PACAP and VIP were coexpressed in fibers running parallel to the mucosal epithelium. The distribution of the fibers suggests that these hormones may play a role in lubrication of the vagina during sexual stimulation (Graf et al., 1995).

## ***I. A Regulator in the nervous system***

### **1. Circadian rhythm**

The suprachiasmatic nucleus (SCN) is located in the hypothalamus and acts as the primary pacemaker in mammalian systems. The SCN is responsible for generating circadian rhythms. The actions of the SCN are linked to environmental cues such as light/dark cycles. Light/dark cues are transmitted from the eyes back to the hypothalamus via the retinohypothalamic tract. The SCN regulates synthesis and release of melatonin by the pineal gland and melatonin feeds back to the SCN (Fukuhara et al., 1997). PACAP

immunoreactivity has been observed in retinal ganglion cells that send axons to the SCN where PACAP-ir has also been observed (Hannibal et al., 1997). Through its action on the SCN, PACAP may stimulate melatonin synthesis and secretion in the pineal gland. In the SCN, PACAP regulates phosphorylation of the cAMP response element binding protein (CREB), likely through the cAMP intracellular signalling system. In turn, the CREB transcription factor stimulates melatonin synthesis. Melatonin appears to feed back and inhibit PACAP induced phosphorylation of CREB (Kopp et al., 1997; Schomerus et al., 1996; Simonneaux et al., 1997). The level of PACAP in retinal ganglion cells is low in the day and high at night. Thus, the presence and location of PACAP suggests it is a neurotransmitter/ neuromodulator of the retinohypothalamic tract (Fukuhara et al., 1997). Experiments show that PACAP acts as a neurotransmitter/neuromodulator late in the day (Hannibal et al., 1997; Kopp et al., 1997). The phase of the circadian rhythm can also be advanced by PACAP during the day but not at night. The phase-shift is mediated through the PAC<sub>1</sub>-R (Hannibal et al., 1997; Kopp et al., 1997). Also, in a recent publication PACAP and VIP were shown to directly stimulate melatonin synthesis in the rat pineal gland through the VPAC<sub>2</sub>-R (Simonneaux et al., 1998)

## **2. Autonomic and sensory nervous systems**

The neurotransmitter/neuromodulator actions of PACAP have already been discussed regarding smooth muscle regulation, hormonal secretions, exocrine secretions and circadian rhythm. Additionally, PACAP appears to be involved in regulation of autonomic functions associated with the medulla oblongata, otic ganglia, sphenopalatine ganglia and the jugular nodose ganglia (Legradi et al., 1994; Mulder et al., 1995). The involvement of PACAP in sensory neurotransmission has been suggested due to PACAP or PACAP receptor presence in the ciliary ganglion neurons, jugular nodose ganglia, dorsal horn neurons, spinal cord, mesencephalic trigeminal nucleus and the vagus nerve (Mulder et al., 1995; Zhang et al., 1995, 1997; Dickinson et al., 1997; Larsen et al., 1997; Reimer et al., 1998). Also, PACAP has been noted to have a role in synapse formation and

neurotransmission in the limbic system (Kondo et al., 1997; Kozicz et al., 1997). PACAP is involved in neurotransmission in several sympathetic neurons (Chiba et al., 1996; Brandenburg et al., 1997; Wu and Dun, 1997).

### **3. Behavior**

A limited number of studies have examined the effect of PACAP on behavior. Distribution of PACAP and VIP immunoreactive fibers in the rat forebrain and the formation of synapses with corticotropin-releasing factor immunoreactive neurons have led to the suggestion that PACAP may have a stress-related function (Kozicz et al., 1997). PACAP may also be involved in behavior associated with increased grooming, pain responses, reduced food intake, motor activity and body temperature (Morley et al., 1992; Masou et al., 1995, 1997; Narita et al., 1996; Zhang et al., 1996; Mizuno et al., 1998). Finally, PACAP may enhance REM sleep. This action of PACAP on REM may in part be mediated by PACAP-induced PRL release from the hypothalamus. PRL is also known to stimulate REM sleep (Bredlow et al., 1994; Fang et al., 1995).

## **Chapter 2**

**Temporal expression pattern of grf/pacap during fish embryogenesis**

## Introduction

The presence of PACAP and its receptor has been well documented in developing rats. Studies on the CNS have detected both the PACAP hormone and receptor(s) in autonomic and sensory ganglia, glial and neuronal cells, the spinal cord, the eye, numerous brain regions including the cortex, hypothalamus, hippocampus, olfactory bulbs and hindbrain (cerebellum and neural tube) (Basille et al., 1994; Masuo et al., 1994; Tatsuno et al., 1994; Basille et al., 1995; D'Agata et al., 1996; Orianas et al., 1997; Lu et al., 1998; Nielson et al., 1998; Waschek et al., 1998). In particular, the PAC<sub>1</sub>-R has been identified in the external granule layer of the cerebellum. The receptor is also expressed in a temporally changing pattern in the other cerebellar layers such as the medulla and the internal granule layer (Gonzalez et al., 1994; Basille et al., 1993, 1994). In addition, postnatal effects directed by PACAP have been shown in the liver, adrenal medulla and pancreas. For example, in fetal rat hepatocytes PACAP stimulates an increase in cAMP through a VPAC-R (El Fahime et al., 1996). Also, in the postnatal adrenal medulla of rats irPACAP fibers innervate chromaffin cells. In addition, PAC<sub>1</sub>-R mRNA has been found in adrenal medullary cells (Moller and Sundler, 1996). PACAP may also have a role in postnatal pancreatic function. Binding sites for PACAP have been identified in the postnatal calf pancreas (Le Meuth et al., 1991). The presence of PACAP and/or its receptor in these tissue was well established by the early to mid 1990s.

Many studies on the role of PACAP have focussed on the embryonic brain in recent years. To date, published studies concentrate on mice and rats. Studies in rat have detected irPACAP as early as embryonic day 14 (E14). In mice PACAP and PACAP receptor mRNA are present at E9.5 (Shuto et al., 1996). In situ hybridization studies in mice ages E9.5 and onwards localize the receptor and peptide mRNA to the neural tube, hindbrain, trigeminal ganglia, dorsal root ganglia and the developing sympathetic chain. At E10.5 and E11.5 the receptor is observed in the developing rhombencephalon (Sheward et al., 1996).

It has recently been proposed that PACAP functions in patterning of the nervous system (Waschek et al., 1998). PACAP is expressed in regions of the developing neural tube at a critical time and in a critical region to influence patterning. More importantly, PACAP down-regulates expression of two patterning genes, sonic hedgehog and *gli-1* in neuroepithelial cells (Waschek et al., 1998). For these reasons I think that PACAP may be an important regulator of neural development in vertebrates.

Unlike PACAP, in mammals GRF does not appear to have a significant role until postnatal development. In rats, GRF has been observed only late in gestation, just prior to birth at day 18 (Frohman and Jansson, 1986). Since both *grf* and *pacap* are encoded on the same mRNA transcript in non-mammalian vertebrates, *grf* may be expressed in early development in these groups.

There is a lack of information regarding the embryonic expression of GRF and PACAP protein or mRNA in animals other than mammals. Therefore, to begin answering questions about the role of these hormones in vertebrate development, I have traced the expression of the *grf/pacap* mRNA molecule throughout development in whole rainbow trout embryos.

## **Materials and Methods**

### ***Biological materials***

The rainbow trout eggs and milt used for this experiment were obtained from the Fraser Valley Trout Hatchery (Abbotsford, B.C.). Eggs and milt were transported on ice in a darkened and oxygen saturated environment to the Aquatics Facility at the University of Victoria. The eggs were fertilized in a glass beaker by the addition of a few drops of milt and left for approximately 1 minute. The eggs were then rinsed in sterile filtered Ringer's solution (6.5 g NaCl, 0.25 g KCl, 0.2 g NaHCO<sub>3</sub>, 0.4 g CaCl<sub>2</sub>·2H<sub>2</sub>O in 1 L sterile distilled water) and transferred to Heath trays where they were incubated at 11-13°C until sampled.

***RNA extraction***

Eggs were sampled daily throughout embryogenesis (E1 to E19). Samples of 3-5 eggs at each stage were pooled and immediately frozen in liquid nitrogen. The samples were stored at -80°C. I used a mortar and pestle to grind the frozen samples. The RNA was extracted in a guanidinium thiocyanate solution and mRNA was isolated from total RNA using an oligo dT-cellulose column. The reagents and columns used for this purification were supplied in a MicroPoly(A)Pure™ mRNA isolation kit (Ambion). Isolated mRNA was precipitated in 1/10 volume 5M ammonium acetate, glycogen (50µg/ml) and 2.5 volumes of 100% ethanol. The purified mRNA samples were stored in a -80°C freezer until needed for the reverse transcription reaction.

***Reverse transcriptase-polymerase chain reaction***

The mRNA samples were centrifuged at 12,000g for 25 minutes. The supernatant was removed and the pellet was resuspended in 70% ethanol and centrifuged at 12,000g for 15 minutes. The ethanol was removed and the pellet was rehydrated in 10-20 µl of RNase-free water.

The mRNA (9.5 µl) was incubated at 70°C for 5 minutes with 20µM oligo dT<sub>20</sub> primers. The samples were then cooled on ice for 2 minutes and centrifuged briefly. To the mRNA/primer solution were added 5µl of 5X 1st strand buffer (Gibco BRL), 2mM dNTPs, 8mM dithiothreitol, 16 units (0.5 µl) RNA Guard (Pharmacia) and 100 units (0.5 µl) Superscript II reverse transcriptase (Gibco BRL). The reagents were mixed and incubated at 42°C for 1.5 hours. The reverse transcriptase enzyme was then heat inactivated at 95°C for 10 minutes and the cDNA was ethanol precipitated and stored at -20°C. A negative reverse transcriptase reaction was performed according to the above directions. However, no mRNA template was added. This negative reaction was carried through to the polymerase chain reaction and used as a negative control.

The cDNA was used in the polymerase chain reaction. Two PCR analyses were done on each cDNA sample (E1 to E19). The primers used in the alpha-tubulin PCR were tub1 (5' CAGGTGTCCACGGCTGTGGTG 3') and tub2 (5' AGGGCTCCATCGAAACGCAG 3'). These primers were designed against chum salmon alpha-tubulin (Coe et al., 1992). The conditions for the PCR were as follows, 5 µl 10 X PCR buffer (Gibco BRL), 4 µl 25 mM MgCl (Gibco BRL), 0.2 mM dNTPs, 0.4 mM primer tub1, 0.4 mM primer tub2, 5 units Taq polymerase (Gibco BRL), 1 µl reverse transcriptase cDNA product and 29 µl sterile filtered water. The PCR reactions were run for 35 cycles on a DNA thermal cycler. Each cycle consisted of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. An upstream primer to exon 1 (5'EX1) and a downstream primer for exon 5 (3'EX5) of the grf/pacap gene were also used for PCR analysis. The sequence for 5'EX1 is 5' AATGCAGATCTCTCCACACGGTAATAGCAGG 3'. The sequence for the downstream primer, 3'EX5, is 5' CATGTTTGGG[ACGT]AGAACACA[ACGT]GAGCG 3'. The conditions for the grf/pacap PCR were 5 µl 10 X PCR buffer (Gibco BRL), 4 µl 25 mM MgCl (Gibco BRL), 0.2 mM dNTPs, 0.4 mM primer 5'EX1, 0.4 mM primer 3'EX5, 5 units Taq polymerase (Gibco BRL), 2 µl reverse transcriptase cDNA product and 28 µl sterile filtered water. The reaction was put through 35 cycles in a DNA thermal cycler. The conditions for a single cycle were as follows, 94°C for 30 seconds, 60°C for 30 sec and 72°C for 30 seconds. For both the alpha-tubulin and grf/pacap polymerase chain reactions a negative control was done. The control contained the same ingredients as the other reactions except that the negative reverse transcriptase reaction was substituted for the cDNA. Following the completion of the reaction, 10 µl aliquots of the alpha-tubulin and grf/pacap PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide for visualization.

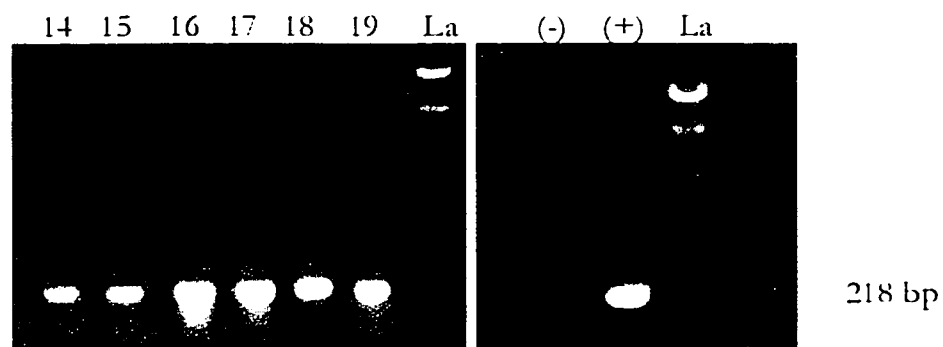
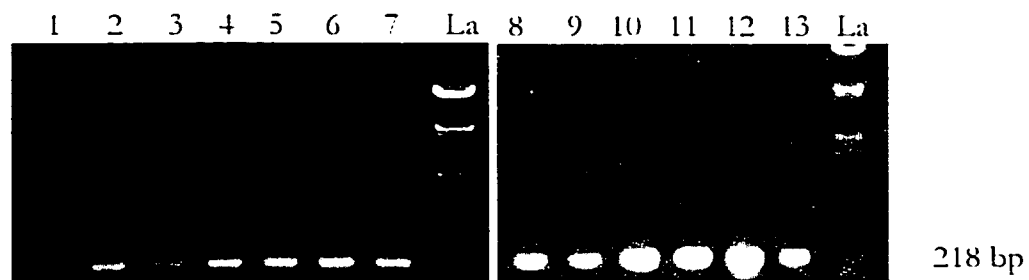
## Results

The RNA extractions, reverse transcriptase reactions and PCR analyses done in this chapter were each performed multiple times (at least 3X each). The cDNA used in the RT-PCR analysis of grf/pacap expression was confirmed as intact and free from genomic contamination by using a control PCR reaction with alpha-tubulin primers (figure 2-1). The alpha-tubulin primers flank an intron. Therefore, PCR analysis with these primers enables the detection of genomic contamination. Once the cDNA was determined to be free from genomic DNA, it was used for analysis of grf/pacap expression. Figure 2-2 depicts the result of the grf/pacap RT-PCR analysis of a rainbow trout embryonic series (E1 to E19). Expression of the grf/pacap transcript can be detected by day E4 and remains expressed through to hatching at day E19. The primers used in the PCR analysis amplify a section of the grf/pacap cDNA between the 5' untranslated region and the 3' untranslated region. Two products can be observed, a 540 bp molecule and a 646 bp molecule. Sequencing (figure 2-3) of the two bands from RT-PCR analysis of an E10 embryo confirm that the smaller PCR product does not encode exon 4, the GRF coding region. The larger PCR product encodes all 5 exons, including both GRF and PACAP.

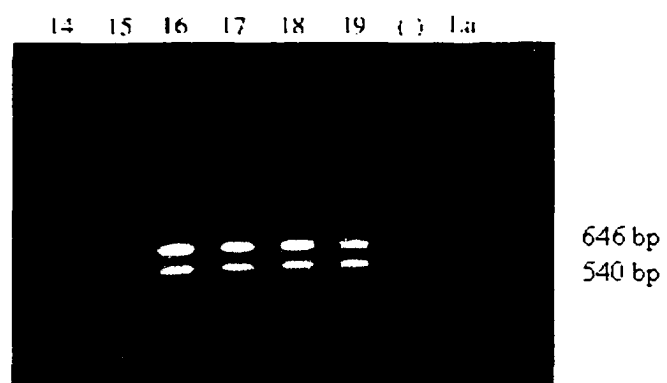
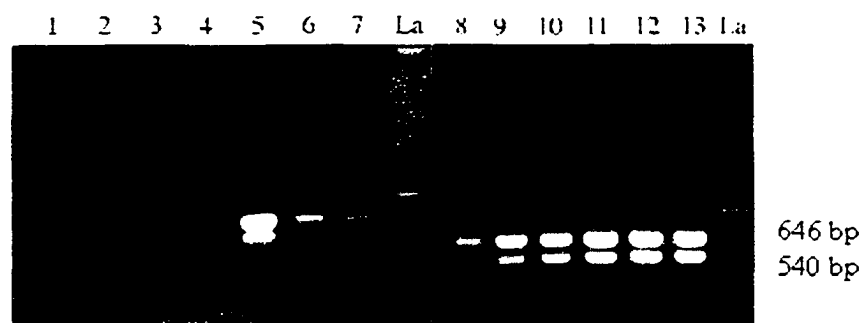
## Discussion

The evidence presented through RT-PCR analysis of rainbow trout embryos shows that pacap/grf mRNA is expressed early in embryogenesis. Two mRNA transcripts are expressed. The long transcript encodes both GRF and PACAP. The shorter transcript encodes PACAP only. This has also been shown in the adult salmon (Parker et al., 1997). The results indicate that expression of PACAP can occur independently of GRF in fish. In mammals the two hormones are encoded on separate genes making expression independent because each gene is controlled by its own promoter. In contrast, with the hormones encoded on the same gene in fish, a mechanism of alternative splicing has been employed to

**Figure 2-1.** RT-PCR results for alpha-tubulin products from rainbow trout embryos. All 19 embryonic days were analyzed and are represented by **1-19**. The negative PCR control (-) was done on the negative reverse transcriptase reaction. The DNA ladder (**La**) used in this experiment is a 123 bp ladder. The PCR products were generated using primers for alpha-tubulin (tub1 and tub2). The alpha-tubulin product is **218 bp**.



**Figure 2-2.** RT-PCR results for grf/pacap products from rainbow trout embryos. All 19 embryonic days were analyzed and are represented by **1-19**. The PCR negative control (-) was done on the negative reverse transcriptase reaction. The DNA ladder (**La**) used in this experiment is a 123 bp ladder. The PCR products were generated using primers for exon 1 or grf/pacap (5'EX1) and exon 5 of grf/pacap (3'EX5). The shorter product is **540 bp** and does not encode GRF. The longer product is **646 bp** and encodes both GRF and PACAP.



**Figure 2-3.** The grf/pacap cDNA sequence for rainbow trout. The sequence begins at the 3' end of exon 1 and ends near the 3' end of exon 5. Two sequences are shown, the longer sequence includes **exon 4** and the shorter sequence is an alternative transcription product that omits **exon 4**. These sequences are based on PCR products generated using the primers **5'EX1** and **3'EX5**.



facilitate independent PACAP expression. To date there is no evidence to suggest that GRF can be independently expressed in fish. With the exception of releasing GH, PACAP performs many functions that are independent of and quite different from GRF in mammals (e.g. vasoregulation, immune system regulation, digestive tract regulation etc). If some or all of the functions performed in mammals are also regulated by PACAP in fish development, then independent regulation of PACAP expression is necessary. It is of particular interest that PACAP can be regulated separately from GRF in development in light of recent findings that suggest PACAP is an important regulator of neural development in mammals.

Given the information provided by the RT-PCR expression data, it seems possible that GRF and PACAP regulate fish development. However, unlike the expression in mice and rats, fish GRF and PACAP are expressed much earlier (E4). In mice PACAP mRNA and PACAP receptor mRNA have been detected at E9.5. This is approximately the midpoint of development during advanced somitogenesis (mice are born at 21 days) (Hogan et al., 1994). At E4 in fish the embryo is at the blastula stage (stages 7-8) and will not reach early somitogenesis (stage 10) for a few more days (approximately E6) (Ballard, 1973). PACAP mRNA expression has not been examined in murine embryos earlier than E9.5, but receptor mRNA expression was examined at E8.5 and found to be undetectable by in situ hybridization analysis (Sheward et al., 1996; Shuto et al., 1996). At E9.5 in mouse development, the neural tube has formed and PACAP and its receptor are expressed in the neural tube. GRF mRNA cannot be detected in rats until E18 (Frohman and Jansson, 1986). Of course it is possible that both PACAP and GRF expression may be too weak to detect earlier than the published results indicate in rats and mice. At embryonic day 4 of rainbow trout development (stage 7-8), the embryo is still at the blastodisc stage (Ballard, 1973). Therefore the stages when PACAP and GRF are expressed in mice and rats compared to fish are not equivalent. If these hormones are expressed earlier in fish than in rats and mice, it presents an interesting contrast between teleost and mammalian

development. In rats GRF is derived maternally from the placenta during development (Srivastava et al., 1995; Gonzalez-Crespo et al., 1991; Margioris et al., 1990). Therefore the embryo does not need to produce its own GRF until after birth. In non-placental animals such as teleost fish, the source of GRF must come from the developing embryo. This may be one reason for the much earlier expression of GRF during development in fish compared to mammals. Also, PACAP is a regulator of the cell cycle, regulating such events as proliferation, cell cycle exit and differentiation. It is my hypothesis that PACAP is functioning as a general cell cycle regulator early in fish development. This would necessitate expression of PACAP independently from GRF (e.g. alternative splicing). To further understand PACAP function in early development, future studies localizing PACAP expression to particular tissues during development and functional information for PACAP is necessary. In addition, more information regarding PACAP expression in mammalian development is required to answer questions regarding different roles for PACAP in teleosts vs mammalian development.

## **Chapter 3**

### **In situ localization of grf/pacap expression during embryogenesis of zebrafish**

This study reports the preliminary results of a larger more comprehensive study being done with Erica Fradinger in Dr. Sherwood's laboratory and in collaboration with Dr. Cecelia Moens (Fred Hutchison Cancer Research Center, Seattle, WA)

## Introduction

Development of multicellular organisms is a complex and intricate process that is directed by a multitude of factors. For an organism to develop from a single cell to a juvenile or adult its cells must multiply and gain new or different functions than their progenitors. Hormones have a vast array of effects on development. For example, thyroid hormone can regulate proliferation, differentiation, synapse formation and myelination of neural cells during rat brain development (Trentin et al., 1995; Munzo et al., 1991; Piosik et al., 1995; Cabacungan et al., 1991). Also, the sex steroids have long been implicated in several aspects of development in the central nervous system. They are involved in sexual differentiation of the mammalian brain in part through effects on proliferation and differentiation of specific brain regions (Kinel et al., 1965; Toran-Allerand et al., 1976)

In recent years research has revealed that PACAP is involved in mammalian embryogenesis, particularly neural development. GRF has a different role in mammals, with effects on postnatal and pubertal development rather than embryogenesis. The majority of this work has been done in rats and mice. In rats, GRF is expressed late in development on day E18 (Frohman and Jansson, 1986); birth is on E21. GRF triggers the release of growth hormone and affects the growth rate in mammals (Mayo et al., 1988; Lin et al., 1992). PACAP expression has been observed in mice on day E9.5 and in rats on day E14 (Shuto et al., 1996; Tatsuno et al., 1994). Expression of PACAP or its receptor(s) has been noted in the neural tube and throughout the central nervous system (e.g. spinal cord, eye, cortex, hypothalamus, hippocampus, olfactory bulb and the cerebellum) (Masuo et al., 1994; Basille et al., 1995; Orianas et al., 1997; D'Agata et al., 1996; Lu et al., 1998; Tatsuno et al., 1994; Basille et al., 1994; Waschek et al., 1998). A detailed account of the actions of GRF and PACAP is presented in Chapter One.

The earliest reported stage (E9.5) for PACAP detection in mice corresponds with somitogenesis. At E9.5 there are 21-29 somites and the forelimb bud is developing (Hogan

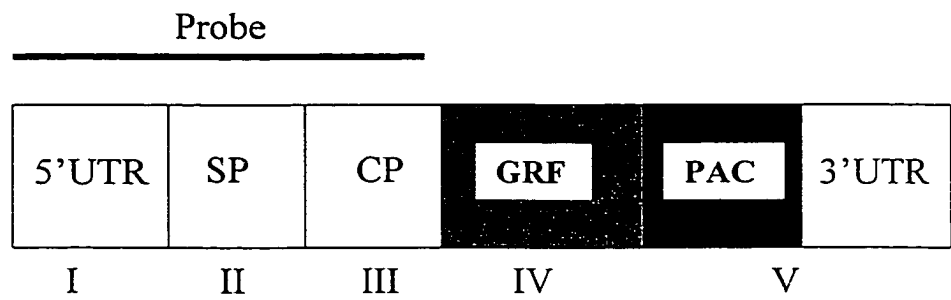
et al., 1994). This is approximately the midpoint of embryogenesis. The data presented in Chapter 2 are the first indication that either PACAP and/or GRF acts earlier in vertebrate development. The results of the RT-PCR experiment led me to ask the question, where is PACAP expressed in the fish embryo? Unfortunately, rainbow trout is a poor developmental model because eggs are available only twice a year. Also, the embryos are large for wholemount work making penetration of the probe more challenging. To my knowledge there is only one group doing in situ hybridization studies involving rainbow trout wholemounts and they are located in France. Trouble-shooting a new protocol requires access to numerous embryos. Considering these drawbacks, I decided that rainbow trout embryos were not a good choice for this project. Therefore, I chose to switch to zebrafish (*Danio rerio*). Zebrafish are an excellent and important developmental model. The time from fertilization of the egg to hatching is 72 hours. This makes it possible to work on a developmental series and do repeated experiments in a relatively short time frame compared to rainbow trout. The zebrafish embryos are small (approximately 1 mm in diameter with chorion intact) and essentially transparent. These features make them ideal for developmental work, especially wholemount studies. Also, the recent cloning of the zebrafish grf/pacap gene in our lab (Fradinger and Sherwood, 2000) makes it possible for wholemount studies to be conducted using an exact match RNA probe.

## Materials and Methods

### *Probe design*

A digoxigenin (DIG) labeled probe made from the zebrafish grf/pacap cDNA was used for this experiment. The probe (412bp) was made against exons 1, 2 and 3 (encoding the 5'UTR, signal peptide and cryptic peptide). The cDNA structure and probe region is identified in figure 3-1. The probe template DNA sequence was PCR amplified off the zebrafish grf/pacap cDNA provided by Erica A. Fradinger (Dr. Sherwood's laboratory).

**Figure 3-1.** A schematic of the **zebrafish grf/pacap cDNA**. The probe was designed to exons **I**, **II** and **III**. Exon **I** encodes the **5'UTR**. Exon **II** encodes the signal peptide (**SP**). Exon **III** encodes the cryptic peptide (**CP**). Exon **IV** encodes **grf** and exon **V** encodes the carboxy terminus of **GRF**, and the entire **PACAP (PAC)** and **3'UTR** coding regions



Zebrafish *grf/pacap* cDNA

Following amplification, the probe template DNA was cloned into pGEM-T vector (Promega) and sequenced for orientation. The probe was cloned in the sense direction. A stock of the probe template vector was prepared using the Qiaprep® Spin Miniprep kit (50) and the vector DNA was stored at 4°C in RNase free elution buffer until needed.

Two probes were generated from the template, a sense and an antisense probe. Before use as a template the vector containing the probe sequence was made linear. The sense probe was generated from a vector digested with Sal I according to the following recipe: 50 µg probe template vector, 10 µl One-Phor-All Buffer PLUS, 10X concentration (Pharmacia), 10 µl Sal I (5000 units/ml, Pharmacia) and twice-autoclaved, distilled water up to 100 µl. For the antisense probe, the template vector was digested by Sac II according to the above recipe, substituting Sac II (10,000 units/ml, pharmacia) for Sal I. The restriction digests were allowed to proceed for 2-3 hours at 37°C. Following this incubation period the reactions were heat denatured at 65°C for 15 minutes. An aliquot of each digest and an aliquot of the corresponding undigested vector was electrophoresed on a 1% TAE-agarose gel at 50 volts for 1.5 hours. The gel was visualized by ethidium bromide staining and photographed. If the linearization was deemed successful, the vector was used in the following reactions. The restriction digests were incubated overnight with proteinase K (10 mg/ml) at 50°C. From this point on only RNase-free solutions were used. Two phenol:chloroform:isoamyl alcohol (50:48:2) extractions were done on the digests and the organic layer was back-extracted with twice-autoclaved, distilled water in order to retrieve the maximal amount of DNA. The DNA was ethanol precipitated in 2.5X 100% ethanol and 1/10th volume 5M ammonium acetate. Following centrifugation at 12,000 g for 20 minutes, the linear probe template was washed in 70% ethanol and centrifuged again for 10 minutes at 12,000 g. The pellet was air-dried and dissolved in 10 µl of twice-autoclaved, distilled water. An aliquot of the DNA was used to determine the concentration by taking an absorbance reading.

One microgram of linearized DNA was required for the transcription reaction. To the 1 µg of Sal I digested template the following was added: 10 µl NTP DIG-UTP labeling mix (10 mM ATP, CTP, GTP, 6.5 mM UTP, 3.5 mM Dig-11-UTP), 10 µl transcription buffer (10X concentration), 5 µl RNase inhibitor (20 units/µl), 10 µl T7 RNA polymerase (20 units/µl) and enough twice-autoclaved, distilled water to bring the reaction up to 100 µl. All solutions used in the transcription reaction were from Boehringer Mannheim. The same reaction was set up for the Sac II digested template, except that 10 µl of SP6 RNA polymerase (20 units/µl, Boehringer Mannheim) was used in place of the T7 RNA polymerase. The reactions were incubated at 37°C for 4-5 hours. Following this incubation 45 µl of twice-autoclaved, distilled water and 5 µl of RNase-free DNase I was added to the mix. The mixture was incubated at 37°C for 15 minutes to degrade the template DNA. Then, the transcription reaction was precipitated with 15 µl of 0.2 M EDTA, 9.5 µl of 8 M LiCl and 450 µl of 100% ethanol and stored for 30 minutes at -80°C. Next, the solution was centrifuged at 12,000 g for 15-30 minutes, the ethanol solution was removed and the RNA pellet was resuspended in 70% ethanol and centrifuged again for 15 minutes at 12,000 g. The 70% ethanol was removed leaving enough behind to coat the RNA pellet. The pellet was immediately dissolved in 50 µl of diethyl pyrocarbonate-treated (DEPC-treated) water and 1 µl RNase inhibitor, 20 units/µl (Boehringer Mannheim). The RNA solution was incubated at 37°C to fully allow resuspension. The probe was stored at -80°C until further use.

After the transcription reaction was completed, the suitability of the probe was assessed in two ways. First, an aliquot of the probe was electrophoresed on a 1% ethidium bromide-TAE-agarose gel at 75 volts for 25-30 minutes. The RNA was visualized by trans-illumination to check that the probe transcripts were of uniform size. Second, a spot assay was done to determine the concentration of labeled probe. Serial dilutions were made of each probe (1:50, 1:500, 1:5000, 1:50,000, 1:500,000) and compared to a DIG-UTP labeled

RNA standard series (1 ng/ $\mu$ l, 100 pg/ $\mu$ l, 10 pg/ $\mu$ l, 1 pg/ $\mu$ l, 0.1 pg/ $\mu$ l, 0.01 pg/ $\mu$ l - Boehringer Mannheim). The experimental probe and control dilutions were spotted on a nylon membrane (Colony/Plaque Screen™ Hybridization Transfer membrane, NEN) in 1  $\mu$ l amounts. The ribonucleotides were fixed to the membrane by baking for 30 minutes at 120°C. Then, the membrane was briefly washed in washing buffer (Tris-buffered saline (TBS) and 0.3% Tween-20). TBS is composed of 150 mM NaCl and 50 mM Tris-HCl (pH 7.5) in water. Next, the membrane was incubated at room temperature for 30 minutes in blocking solution (TBS, 0.3% Tween-20, 10% rabbit serum). An anti-DIG-alkaline phosphatase conjugated antibody (Boehringer Mannheim, 150U) was used to label the DIG-RNA. The antibody was diluted 1:5000 in blocking solution and incubated with the membrane for 30 minutes at room temperature. After this incubation the membrane was washed twice with washing buffer for 15 minutes each wash. Detection solution for the color reaction was made fresh for each spot assay. The detection solution consisted of 100 mM Tris-HCl, pH 9.5 and 100 mM NaCl. The membrane was incubated in detection solution for 2 minutes. A color substrate solution was made fresh by mixing 45  $\mu$ l 4-Nitro blue tetrazolium chloride (NBT -75 mg/ml, Boehringer Mannheim) and 35  $\mu$ l 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP- 50 mg/ml, Boehringer Mannheim) in 10 ml detection buffer. The detection buffer/color substrate solution was added to the membrane and stored in the dark to develop at room temperature. The reaction was allowed to proceed overnight and stopped in the morning by washing twice briefly with sterile water. The spot intensities were compared and the concentration of the experimental probe was estimated.

### ***Zebrafish embryo fixation and storage***

The zebrafish used in this experiment were provided by Dr. C. Moens of the Fred Hutchison Cancer Research Center (Seattle, WA). The embryos were harvested at various developmental stages and fixed in 4% paraformaldehyde/PBS for 5 hours at room temperature or overnight at 4°C. Following fixation the embryos were washed for 10

minutes in 100% methanol and stored at -20°C in 100% methanol until needed. The embryos were shipped on dry ice in 100% methanol to Dr. N. Sherwood's laboratory at the University of Victoria.

### ***In situ hybridization***

#### **Permeabilization**

The solutions used in the following procedures were all RNase-free solutions. Each stage of development was processed separately in batches of 20-25 embryos per 1.5 ml RNase-free eppendorf tube. The embryos were rehydrated through a methanol series in PBST (75% methanol, 50% methanol, 25% methanol). PBST was made from PBS (phosphate-buffered saline) and 0.1% Tween-20. The PBS was made according to the recipe in The Zebrafish Book (1995). Following the rehydration series the embryos were washed 5 times for 5 minutes each at room temperature in PBST. The embryos were permeabilized with proteinase K (10 µg/ml) in PBST for various intervals depending on the developmental stage (bud -- 1 min, 5-7 somites -- 1-2 min, 18 somites -- 4 min, 24 hour embryo -- 11 min, 36 hr embryo -- 20 min, 48 hr embryo -- 24 min). The proteinase K reaction was stopped by rinsing the embryos quickly in PBST and refixing in 4% paraformaldehyde/PBS for 20 minutes. Then, the embryos were rinsed 5 times for 5 minutes each in PBST.

#### **Prehybridization and hybridization**

The embryos were incubated in prehybridization solution for 1-2 hours. The prehybridization solution consisted of 50% formamide, 5 X SSC, 50 µg/ml heparin, 500 µg/ml tRNA (yeast), 0.1% Tween-20, 0.1 M citric acid and DEPC-treated water. After the prehybridization was completed, the sense and anti-sense probes were diluted to 100 ng/200 µl in prehybridization solution. Then, 200 µl of this solution was added to the embryos. The embryos were incubated overnight at 65°C.

## **Washes**

For each probe and developmental stage, different batches of embryos were washed according to a low stringency and a high stringency protocol.

**Low stringency.** The following washes were done at 65°C using pre-warmed solutions. The embryos were washed for: 5 minutes in 66% hybridization solution / 33% 2 X SSC ; 5 minutes in 33% hybridization solution / 66% 2 X SSC; 5 minutes in 2 X SSC; 2 X 20 minutes in 0.2 X SSC and 0.1% Tween-20; 10 minutes in 0.1 X SSC and 0.1% Tween-20. Next, the embryos were washed at room temperature in: 66% 0.2 X SSC / 33% PBST for 5 minutes; 33% 0.2 X SSC / 66% PBST for 5 minutes; PBST for 5 minutes.

**High stringency.** Pre-warmed (65°C) solutions were used for the following washes: 5 minutes in 66% hybridization solution / 33% 2 X SSC ; 5 minutes in 33% hybridization solution / 66% 2 X SSC; 5 minutes in 2 X SSC; 20 minutes in 0.2 X SSC and 0.1% Tween-20; 2 X 20 minutes in 0.1 X SSC and 0.1% Tween-20. Next, the embryos were washed at room temperature in: 66% 0.2 X SSC / 33% PBST for 5 minutes; 33% 0.2 X SSC / 66% PBST for 5 minutes; PBST for 5 minutes.

## **Antibody reaction**

The embryos were incubated at room temperature for 1 hour in blocking solution: PBST, 2% rabbit serum (Sigma), 2 mg/ml bovine serum albumin (Boehringer Mannheim). Next, the anti-DIG-alkaline phosphatase antibody was diluted 1:5000 in blocking solution and incubated with the embryos for 2 hours with mild shaking at room temperature. Following antibody incubation the embryos were washed 5 X 15 minutes in PBST and left at 4°C in the fifth wash overnight.

## **Coloration reaction**

The embryos were washed twice for 15 minutes each in PBST. Meanwhile, coloration buffer was made (100 mM Tris-HCl, pH 9.5; 50 mM Mg Cl<sub>2</sub>; 100 mM NaCl; 0.1% Tween-20 and sterile water up to 50 ml). The embryos were washed 4 X for 5

minutes each in coloration buffer. The color substrate was made up with 45  $\mu$ l of NBT and 35  $\mu$ l of BCIP in 10 ml coloration buffer. This was added to the embryos and they were allowed to develop in the dark for 2-4 hours until a purple color reaction was visible. The reaction was stopped by washing the embryos 2 X in sterile water and then the embryos were stored in PBST.

### **Microscopy and photography**

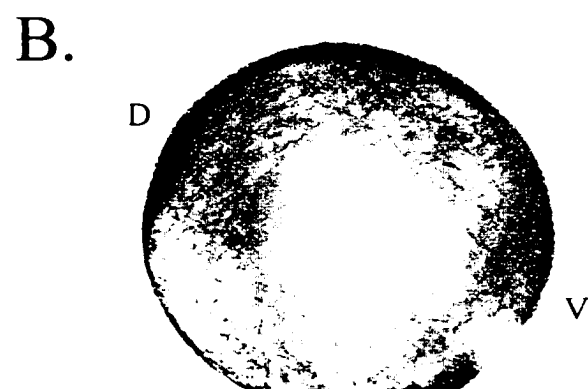
Some embryos were stored in PBST. These embryos were observed and photographed in solution using a stereoscope (Wild M420) at 40X magnification. Other embryos were dehydrated through an increasing methanol / PBS series (30%, 50%, 70%, 100%) for 5 minutes each. The embryos were then cleared in 66% benzyl alcohol / 33% benzyl benzoate and photographed using modified Nomarski optics (Zeiss Ultraphot II) at 125X and 312.5X magnifications. Some of the embryos were mounted in permount and others were photographed in solution. Photographs of the embryos were scanned using a Polaroid SprintScan 35 scanner and formatted for presentation in Adobe photoshop.

### **Results**

Various stages of zebrafish wholemounts (bud, 18-somites, 24-hour, 36-hour and 48 hour) were labeled with a DIG-UTP grf/pacap probe. Both sense and antisense probes were used. The sense probe was used as a negative control and the antisense probe was used to label grf/pacap mRNA. All the embryonic cells of the bud stage embryo appear to be labeled, but without sectioning I cannot distinguish whether the outermost cell layer (the enveloping layer) is labeled (figure 3-2). The extra-embryonic cells of the yolk syncytial layer underlie and surround the yolk surface. This layer cannot be seen through the darkly labeled embryo without sectioning. In the 18-somite embryo there is labeling in the epidermal ectoderm or periderm that lies over the brain, spinal cord and tailbud. These regions are labeled along with mesodermal cells that are lateral to the brain and spinal cord (figures 3-3 and 3-4). Also, the first sign of staining in the hatching gland appears at the

18-somite stage (figure 3-5 and figure 3-6). At 24 hours the hatching gland is obviously labeled (figure 3-5 and figure 3-6). There is still labeling in the head, especially the eye and ventral brain regions. There is no longer labeling down the length of the spinal cord but there is labeling in the primordial gut, along the surface dorsal to the yolk extension (figures 3-7 and 3-8). At 36 hours the hatching gland is still prominent (figure 3-5, 3-9 and 3-10). Also, there is labeling in the developing heart (figure 3-5 and 3-10). In the 48 hour embryo there continues to be labeling in the hatching gland and primordial heart (figure 3-5, 3-10 and 3-11). Also, the ventral surface of the yolk extension is labeled (figure 3-9).

**Figure 3-2.** Labeling of grf/pacap in the bud stage zebrafish embryo (lateral view). Images were photographed using modified Nomarski optics. A). Negative control (sense) - magnification is 125X. B). Labeling of grf/pacap (antisense) - magnification is 125X. C). Embryo in B) at 312.5X magnification. Abbreviations: **D**-dorsal, **V**-ventral.



**Figure 3-3.** Negative control. Labeling of grf/pacap (sense probe) of an 18-somite stage zebrafish embryo using modified Nomarski optics. A). Dorsal view - magnification is 125X B). Lateral view - magnification is 125X. Abbreviations: optic primordia (**op**), yolk (**y**), tailbud (**tb**).

A.

op

y

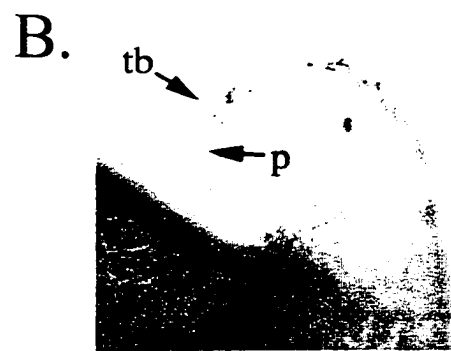
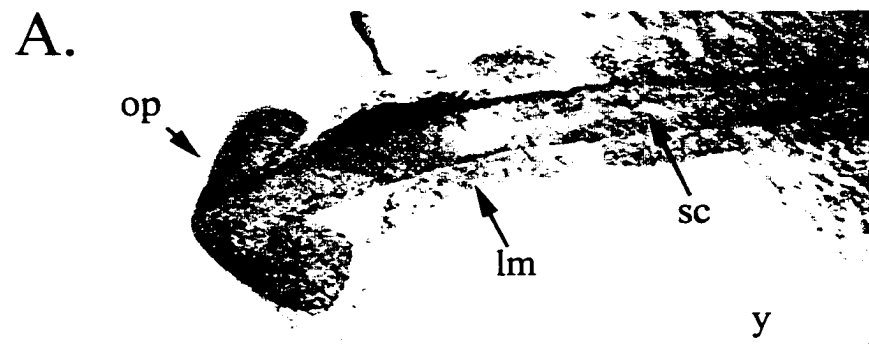
B.

y

tb

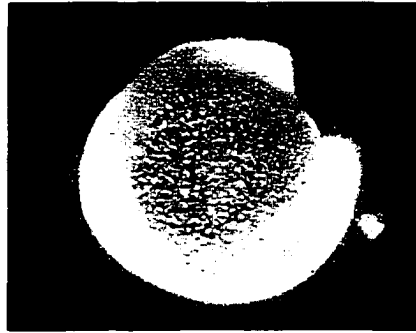


**Figure 3-4.** Labeling of an 18 somite stage zebrafish embryo with grf/pacap using modified Nomarski optics. A). Dorsal view - magnification is 125X. B). Tail bud - magnification is 312.5X. C). Dorsal view - magnification is 125X. Abbreviations: optic primordia (**op**), spinal cord (**sc**), lateral plate mesoderm (**lm**), yolk (**y**), periderm (**p**) and tailbud (**tb**).

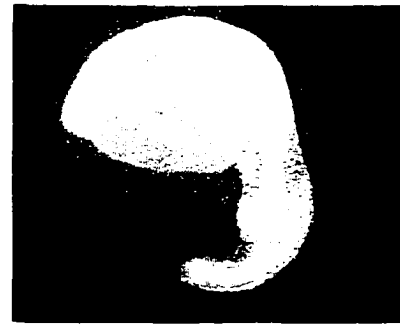


**Figure 3-5.** Negative control. Labeling of grf/pacap (sense probe) for the 18-somite, 24 hour, 36 hour and 48 hour zebrafish embryo. The image is magnified 40 times (stereoscope).

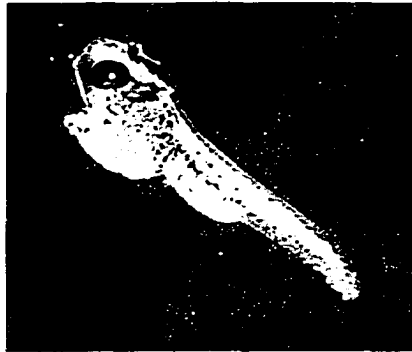
**18 somites**



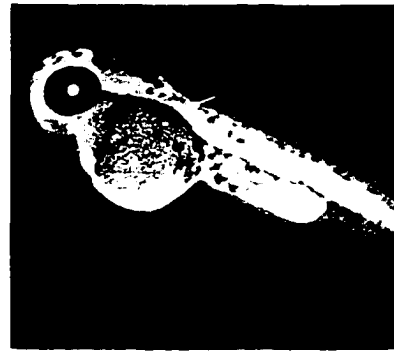
**24 hours**



**36 hours**



**48 hours**



**Figure 3-6.** Labeling of the hatching gland with a grf/pacap probe (antisense probe) in 18-somite and 24 hour zebrafish embryos. The image is magnified 40 times (stereoscope). Abbreviations: hatching gland (**hg**), lateral plate mesoderm (**lm**), midbrain (**mb**) and hindbrain (**hb**).

**18 somites**

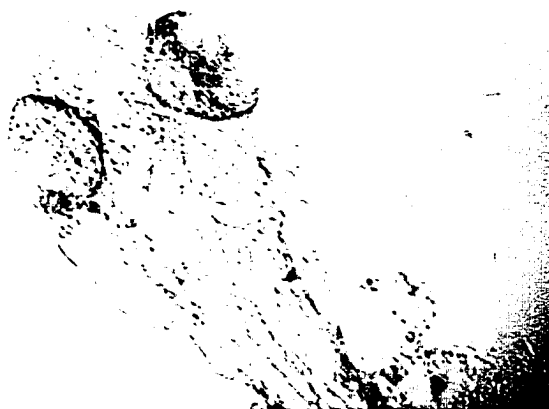


**24 hours**



**Figure 3-7.** Negative control. Labeling of grf/pacap (sense probe) in the 24 hour zebrafish embryo using modified Nomarski optics. A). Dorsal view - magnification is 125X. B). Lateral view - magnification is 125X.

A.

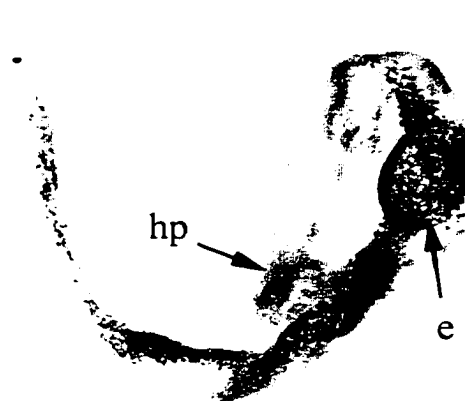


B.

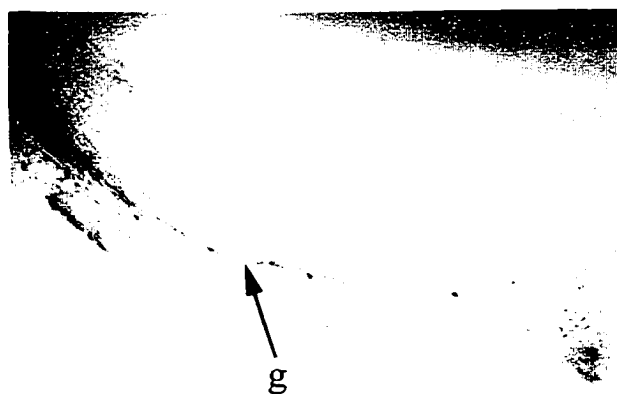


**Figure 3-8.** Labeling of grf/pacap (antisense probe) in the 24 hour zebrafish embryo (ventral view - A and lateral view - B, C) using modified Nomarski optics. Magnification for A). is 125X and 312.5X for C).. Abbreviations: eye (**e**), heart primordia (**hp**), gut (**g**).

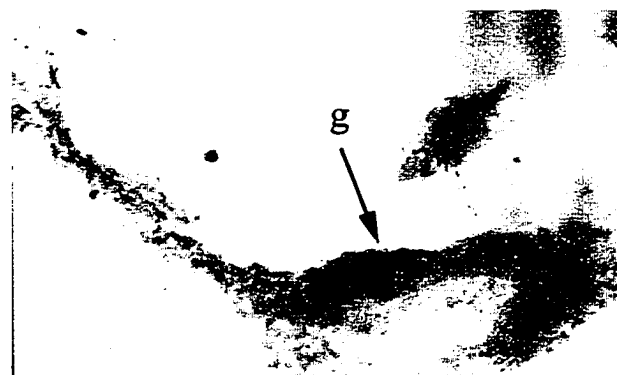
A.



B.



C.

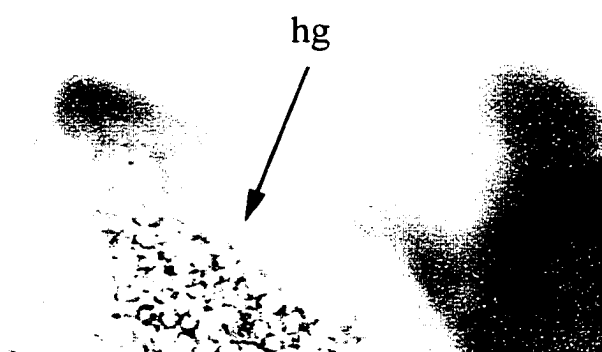


**Figure 3-9.** Labeling of grf/pacap in the 36 hour zebrafish embryo (dorsal view - A and ventral view - B) using modified Nomarski optics. A). Labeled with sense probe (negative control). Magnification is 125X. B). Labeled with antisense probe at 125X magnification. D). Abbreviations: hatching gland (**hg**), yolk (**y**).

A.

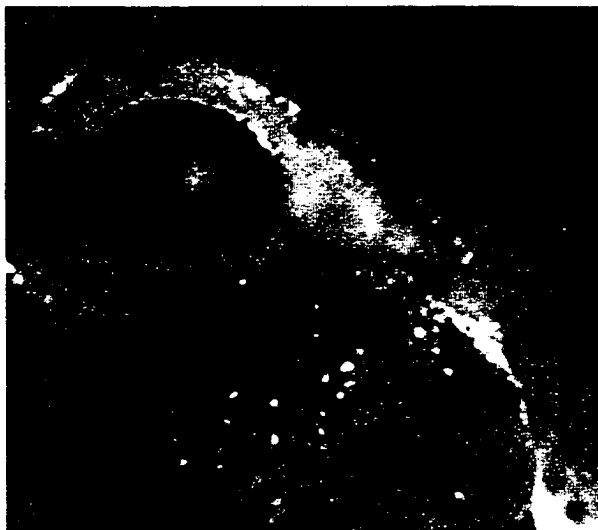


B.



**Figure 3-10.** Labeling of the hatching gland with a grf/pacap probe in 36 hour and 48 hour zebrafish embryos. The image is magnified 40 times (stereoscope). Abbreviations: hatching gland (**hg**), heart primordia (**hp**).

**36 hours**



**48 hours**



**Figure 3-11.** Labeling of grf/pacap in the 48 hour zebrafish embryo (ventral view - A) and lateral view (B and C) using modified Nomarski optics. A). Labeled with sense probe (negative control). Magnification is 125X. B). Labeled with antisense probe. Magnification is 125X. C). Labeled with antisense probe. Magnification is 312.5X. D). Labeled with antisense probe. Magnification is 312.5X. Abbreviations: hatching gland (**hg**), heart primordia (**hp**), yolk extension (**ye**).

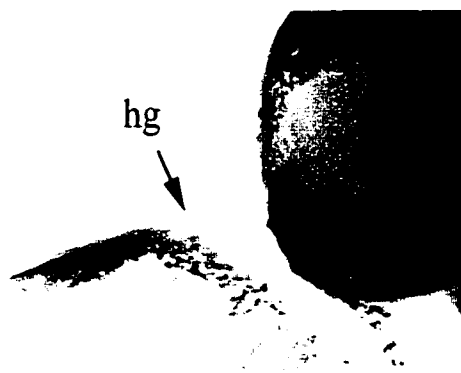
A.



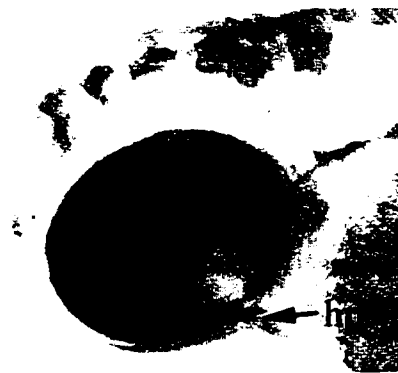
B.



C.



D.



## Discussion

The study reported in this chapter is part of a larger and ongoing project. The next phase in this work is to section wholemount zebrafish embryos labeled for grf/pacap to determine the cross-sectional location of grf/pacap mRNA expression. Therefore, the results presented herein are definitive only from one angle. At this point I can only propose the regions in which the labeling appears and note the other options when a single location cannot be determined. Also, I cannot completely rule out hybridization between the grf/pacap probe and other family members. VIP is the closest relative in nucleotide sequence. However, VIP has not been cloned in any fish. The grf/pacap gene sequence was recently cloned (Fradinger and Sherwood, 2000) in zebrafish. A BLAST alignment search of the sequence revealed no regions of overlap between the probe (exons 1-3) of zebrafish grf/pacap and other known VIP sequences (amphibians, birds and mammals). I have limited cross-hybridization with VIP by using high stringency washes (see Materials and Methods) and in choosing a probe sequence possessing little to no similarity with VIP. Therefore, I think cross hybridization with VIP can be ruled out.

An interesting contrast between grf/pacap expression in zebrafish and in salmonids is the lack of alternative splicing of the transcript in zebrafish (Fradinger and Sherwood, 2000). The adult short transcript (missing exon 4 - grf) which is seen in salmonids (Chapter 2, Parker et al., 1997) is absent in the zebrafish. The expression pattern of the short and long transcripts has not been studied in the zebrafish embryo. If we use the salmonids as a guide it would suggest that the adult and embryonic stages use the same means of separately regulating grf and pacap transcription. If the zebrafish embryo emulates the adult, it is unlikely to use alternative transcription. However, the evidence I have already cited in rats and mice does suggest that PACAP is more involved in development than GRF. Therefore, in contrast to the adult, the zebrafish embryo may need to use alternative splicing as a means of producing PACAP without GRF. The possibility

of a difference between the adult and embryonic stages cannot be ruled out until RT-PCR or Northern blot experiments have been done on zebrafish embryos.

It is clear that early in zebrafish development (bud stage - 10 hours) grf/pacap is strongly expressed. Expression is found throughout the embryo at this stage. Without sectioning it cannot be determined whether labeling is present in the outer-most monolayer (the enveloping layer) or the inner-most monolayer (the yolk syncytial layer) (figure 3-2). Once gastrulation is complete the enveloping layer exists as a flattened monolayer referred to as the periderm (Kimmel et al., 1990, 1995). The bud stage occurs at 100% epiboly. At this point gastrulation is complete and the cells of the embryo (excluding the periderm and yolk syncytial layer) continue to divide rapidly. The embryo now exists as two tissue layers, the epiblast and hypoblast. The epiblast, which is located superior to the hypoblast, will become the ectoderm. The hypoblast will give rise to two germ layers, the mesoderm and endoderm. At this stage these three layers are beginning to differentiate morphologically (Kimmel et al., 1990, 1995, Warga and Nüsslein-Volhard, 1999). In many instances PACAP has been cited as having a role in proliferation and differentiation. PACAP's proliferative effects have been noted in folliculo-stellate-like cells, primordial germ cells, chromaffin cells, lactotopes, gonadotopes, astrocytes and peripheral sympathetic neuroblasts (Matsumoto et al., 1993; De Felici and Pesce, 1994; Schomerus et al., 1994; Tischler et al., 1995; Lelièvre et al., 1996; Pesce et al., 1996; Lu et al., 1998; Moroo et al., 1998). The proliferative actions of GRF have been cited in the stimulation of somatotroph proliferation (Lin et al., 1992; Mayo et al., 1988). Also, PACAP has been regarded as a factor involved in differentiation and cell survival in neural and non-neural cell types (Hoshino et al., 1993; Braas et al., 1994; Barrie et al., 1997; Campard et al., 1997; Gonzalez et al., 1997; Hernandez et al., 1997; Vaudry et al., 1998). Similarly GRF may be involved in somatotroph differentiation (Lin et al., 1992; Mayo et al., 1988). Consequently, it may be presumed that PACAP and/or GRF act in proliferation or differentiation of these cell types at this point and in later stages. Considering the early stage of development and

the rapid cell divisions ongoing at this stage it seems more likely that one or both of these hormones are acting as a regulator of proliferation rather than differentiation. As development progresses, the expression of grf/pacap mRNA becomes more restricted. In the following paragraphs I will discuss the progression of grf/pacap mRNA expression in the other embryonic stages.

### ***Periderm and ectoderm***

At 18-somites grf/pacap mRNA is observed in the outer-most cell layer(s). Its strong expression here obscures the view of deeper tissues. The tissue layer(s) overlying the eye, brain and spinal cord are labeled (figure 3-4A). It is not clear whether the labeling here is in the periderm or the underlying epidermal ectoderm. Factors belonging to the Bmp (bone morphogenic protein) family, involved in patterning of the nervous system can be found in the epidermal layer. The cell fates of dorsal spinal cord cells are thought to be directed by Bmp signaling from the epidermal ectoderm and the dorsal neural tube (Dickinson et al., 1995; Liem et al., 1995; Liem et al., 1997). From this cell layer PACAP and/or GRF may influence development of the dorsal CNS.

In addition, expression of grf/pacap is detected throughout the CNS at this point. It is observed in the eye, brain and spinal cord (figure 3-4C). This is in agreement with labeling of pacap in the neural tube and hindbrain of mice beginning on day E9.5 (somites 21-29) (Sheward et al., 1996; Waschek et al., 1998). In the 24 hour embryo, grf/pacap expression is more restricted to ventral neuroectoderm and the eyes (figure 3-6B and figure 3-8A). In figure 3-6B labeling is also present at the midbrain/hindbrain boundary. However, this labeling is not consistent and may result from overstaining. In the adult brain of mammals and fish, PACAP has been localized to many regions in the CNS, particularly the hypothalamus, brainstem and spinal cord (Wong et al., 1998; Montero et al., 1998; Matsuda et al., 1997; Koves et al., 1994; Legradi et al., 1994; Moller et al., 1993) and it has been observed in the peripheral nervous system innervating the eye (Moller et al., 1993). It

is interesting that as development progresses expression of *grf/pacap* persists the longest in the ventral brain, where the hypothalamus is located, and in the eye. At 36 and 48 hours of development expression is no longer apparent in these regions. Sectioning may help to determine if any labeling remains in isolated neurons of the CNS. It is possible that *grf/pacap* expression disappears transiently and then is expressed again in the adult.

It is not clear whether PACAP has a role in proliferation or differentiation in these tissues. An important regulatory molecule for patterning of the neuroectoderm is sonic hedgehog (*shh*). Certain prechordal plate mutants (*cyclops* mutant and *one-eyed pinhead* mutant) mis-express this factor and have severe malformations in the developing neuroectoderm (Krauss et al., 1993; Schier et al., 1997). Interestingly, PACAP down-regulates expression of *shh* in cultured mammalian neuroepithelial cells (Waschek et al., 1998). Therefore, PACAP appears to be a regulator of *shh* expression in neuroectoderm and consequently, may have a role in patterning of the nervous system. In addition, a developmental dysfunction called holoprosencephaly displays various forebrain and facial abnormalities including cyclopia. This dysfunction has been linked to chromosomal abnormalities in the region coding for the PACAP gene in humans (18pter-q11) (Belloni et al., 1996). However, in PACAP null mice there is no evidence of holoprosencephaly or other brain/facial midline deformities (personal communication with Sarah Leary). Perhaps PACAP's function in midline patterning overlaps with other patterning genes so that targeted disruption of the *pacap* gene in mice does not result in a severe phenotype. Regardless of the interpretation, these pieces of evidence do suggest an involvement of PACAP in patterning of anterior neuroectoderm.

Labeling of the periderm is evident in the tailbud (18-somite stage (arrow), figure 3-4B). This region contains the tailfin primordium. Labeling extends to the yolk sac where the yolk extension is beginning to form as the tail begins to extend. There appears to be uniform labeling throughout the tailbud; sectioning will reveal whether this labeling extends

to more internal tissue layers (e.g., epidermis, mesoderm and spinal cord). In the 48 hour embryo, labeling is again seen in the periderm surrounding the yolk extension. This region is the location of the developing median fin (Kimmel et al., 1995). Given the localization of *grf/pacap* to both tailfin primordia and median fin primordia it may be proposed that one or both of these hormones are involved in fin development.

### ***Prechordal plate***

The prechordal plate is a structure formed during gastrulation. By the bud stage it is located at the anterior end of the embryo ventral to the developing brain. This structure is the anterior extension of the notochord. Together, the prechordal plate and the notochord are known as the axial mesoderm (Kimmel et al., 1990, 1995). The prechordal plate in zebrafish is difficult to identify without using prechordal plate mutants. However, it appears to consist primarily of mesoderm cells and some endoderm cells (Schier et al., 1997; Warga and Nüsslein-Volhard, 1999). It is a source of dorsalizing signals that pattern the dorsoventral axis of the adjacent mesoderm and of the anterior neuroectoderm (Schier et al., 1997). The prechordal plate of zebrafish gives rise to the hatching gland, and gut endoderm (Schier et al., 1997; Warga and Nüsslein-Volhard, 1999). In mutants that possess no prechordal plate (*one-eyed pinhead* mutant) or reduced prechordal plate (*cyclops* mutant), several structures are reduced or absent: the gut, hatching gland, head mesoderm and ventral neuroectoderm. The embryos also have severe cyclopia (the eyes are fused). Interestingly, it is in many of these regions that *grf/pacap* is expressed in the zebrafish embryo. In the bud, labeling is found throughout the embryo (figure 3-2B, C). Consequently, the prechordal plate cells cannot be distinguished. In the 18-somite embryo the dense staining of the outermost layer makes it difficult to identify prechordal plate cells without sectioning. However, in the 24 hour embryo (figures 3-6B and 3-8A) there is considerable staining in the ventral brain/ventral mesoderm of the head. It is from this prechordal region that endodermal gut cells and mesodermal hatching gland cells migrate. Labeling of *grf/pacap* is evident in the gut and hatching gland of the developing embryo. The gut is labeled in the

24 hour embryo. It appears as a duct dorsal to the yolk extension (figure 3-8B, C). The hatching gland secretes enzymes that digest the proteinaceous chorion at hatching (Vogel and Gerster, 1997). The gland is first labeled at the 18-somite stage and persists through to the oldest stage examined (48 hour embryo) (See figures 3-6A, B, 3-9B, 3-10A, B and 3-11C). It would obviously be beneficial to determine conclusively that *grf/pacap* is expressed in the prechordal plate. This work is currently underway. The prechordal plate is absent or reduced in *cyclops* and *one-eyed pinhead* mutants. Therefore, in situ hybridization studies in these mutants should reveal whether *grf/pacap* is expressed in an altered fashion. Given the distribution of *grf/pacap* in prechordal plate derivatives, it seems probable that expression will be confirmed in this region.

### ***Heart***

The zebrafish heart is derived from precursors in the anterior lateral plate mesoderm, adjacent to the hindbrain. As somitogenesis progresses, the cardiac precursors migrate toward the midline and ventral to the hindbrain. By 24 hours of development, the cardiac cone begins to form in this ventral midline location. Eventually, the venous end of the cardiac tube migrates to a position on the pericardium. The pericardium is located on the yolk sac, dorsal to the hatching gland and ventral to the anterior margin of the head (Kimmel et al., 1995; Stainier et al., 1993). In the 18-somite embryo, two strips of labeled tissue can be observed on either side of the hindbrain and spinal cord (figure 3-4A and 3-6). It is difficult to say without sectioning whether the overlying epidermis or the underlying mesoderm is labeled. However, regardless of the tissue in which *grf/pacap* is expressed, it is conceivable that one or both hormones could have an effect on the lateral mesoderm. In addition, the 24 hour embryo is labeled for *grf/pacap* in a region ventral to the hindbrain (figure 3-8A). Again, sectioning will be valuable to confirm that the labeling is within the cardiac precursor cells. The strongest evidence for labeling of cardiac cells is found in the 36 hour and 48 hour embryos. Figure 3-10 (36 hour embryo) and figure 3-11D (48 hour) show labeling in the pericardium ventral to the head and dorsal to the hatching gland. The

positive identification of this region in the 36 and 48 hour embryos gives greater support to the labeling seen in the 18-somite stage and the 24 hour embryo. The role of GRF and PACAP in the cardiac tissues cannot be determined from this data. Perhaps PACAP or GRF are necessary for the proper development and function of cardiac myocytes. It has been noted in rats that PACAP acts on cardiac myocytes to increase heart rate and contractile force (Suzuki et al., 1993b). Also, in the guinea pig, PACAP immunoreactivity has been localized to neuronal fibers and a subpopulation of intrinsic postganglionic cardiac neurons. The cardiac ganglia express PACAP and PAC<sub>1</sub>-R mRNA transcripts. In addition, most of the parasympathetic nerves innervating the region are immunoreactive for the PAC<sub>1</sub>-R. When atrial wholemount preparations were exposed to PACAP, depolarization was observed in cardiac ganglia along with increased neuronal excitability (Braas et al., 1998). These results suggest that PACAP may regulate both cardiac myocytes and neuronal control of heart function. Coincidentally, some PACAP null mice die suddenly, without evidence of prior illness. Cardiac dysfunction is currently being examined as a cause of death (personal communication with Sarah Leary).

### ***Conclusions and future work***

The results in this section strongly suggest a role for PACAP in development. In fact, PACAP may be an important regulator of vertebrate patterning. To confirm and enhance the information in this chapter, sectioning of the wholemount embryos is currently being done. As well, experiments in *cyclops* and *one-eyed pinhead* mutants, are underway. Once these experiments have been completed, it would be valuable to examine the effects of *grf/pacap* in vivo and in vitro. Microinjection of *grf/pacap* mRNA transcripts into embryos would allow us to examine the effects of overexpression. Tissue culture work would be helpful for determining the role of either hormone in proliferation and/or differentiation. In addition, expansion of the work to include stages of epiboly and earlier somitogenesis would help to complete the picture. Clearly, one or both of the hormones affects

development; however, the targeted cells and physiological effects remain to be clarified.

## **Chapter 4**

**Over expression of the grf/pacap gene in several salmonid species**

## Introduction

GRF and PACAP, belonging to the PACAP/Glucagon superfamily, show a high degree of similarity. The human GRF (amino acids 1-27) and PACAP (amino acids 1-27) peptides have 33% sequence identity. In fish the peptides are encoded on separate exons of the gene and share 26-33% sequence identity (Sherwood et al., 2000). In addition to sequence similarity, these two peptides share functional characteristics. GRF, which was first characterized in human pancreatic tumors, was shown to have GH-releasing activity (Guillemin et al., 1982; Rivier et al., 1982). The identification of PACAP followed in 1989, and it too was observed to have GH-releasing activity in mammals (Miyata et al., 1989; Hart et al., 1992). The development of transgenic mice containing a grf transgene has helped to further our understanding of *in vivo* GRF peptide function. In these mice the level of GH in blood plasma was raised, pituitaries were enlarged due to selective proliferation of somatotrophs and growth rates were accelerated (Hammer et al., 1985, Mayo et al., 1988). These studies in grf transgenic mice are important because they confirm the results that *in vitro* studies have suggested regarding the GH-releasing activity of the GRF peptide. In fish, no grf transgenic studies have been done. However, experiments in cultured rainbow trout pituitaries have revealed that carp GRF causes a dose-dependent release of GH (Luo et al., 1990). GRF has also been identified by immunoreactivity in the hypothalamus of several salmonid species (rainbow trout, chum and coho salmon) (Luo et al., 1989a; Luo et al., 1989b). More recently, in our laboratory synthetic sockeye salmon GRF and PACAP have been shown to stimulate GH release from cultured coho salmon pituitary cells (Parker et al., 1997). To date, studies involving the *in vivo* over expression of PACAP using transgenic technology have not been done in any animal. In fish, transgenic techniques have been used to generate growth-enhanced salmon and carp that overexpress GH (Zhang et al., 1990; Du et al., 1992; Devlin et al., 1994, 1995). The microinjection technique used in the above experiments resulted in a 4-6% frequency of successful transgene incorporation. The

experiment in carp produced fish that were on average 22% larger than controls at 3 months of age. The experiments in salmon yielded fish that were approximately 10-11 fold heavier than controls at 14-15 months of age.

To examine the role of GRF and PACAP in fish physiology, I have used transgenic technology to introduce extra copies of “all-salmonid” constructs encoding GRF and PACAP. Two constructs were used. The first construct, *pit-grf/pacap*, was designed with the Chinook salmon *gh* promoter/5'UTR in front of the sockeye salmon *grf/pacap* cDNA. The construct was engineered in this manner so as to ensure expression of *grf/pacap* in the pituitary rather than the hypothalamus (prepared by Dr. N. Sherwood in the laboratory of Dr. C.L. Hew). Expression of the transgene in the pituitary was desired in order to circumvent possible negative regulation of the gene induced by its overexpression in the hypothalamus. The second construct is the native sockeye salmon *grf/pacap* gene including 645 base pairs of the *grf/pacap* promoter region (*hyp-grf/pacap*) (prepared by S. Krueckl). This construct was designed to be expressed along with the naturally expressed gene in the hypothalamus. Using electroporation or microinjection I introduced the constructs into cutthroat trout (*Salmo clarki*), coho salmon (*Oncorhynchus kisutch*), steelhead trout (*Oncorhynchus mykiss*), rainbow trout (*Oncorhynchus mykiss*), and Chinook salmon (*Oncorhynchus tshawytscha*).

## Materials and Methods

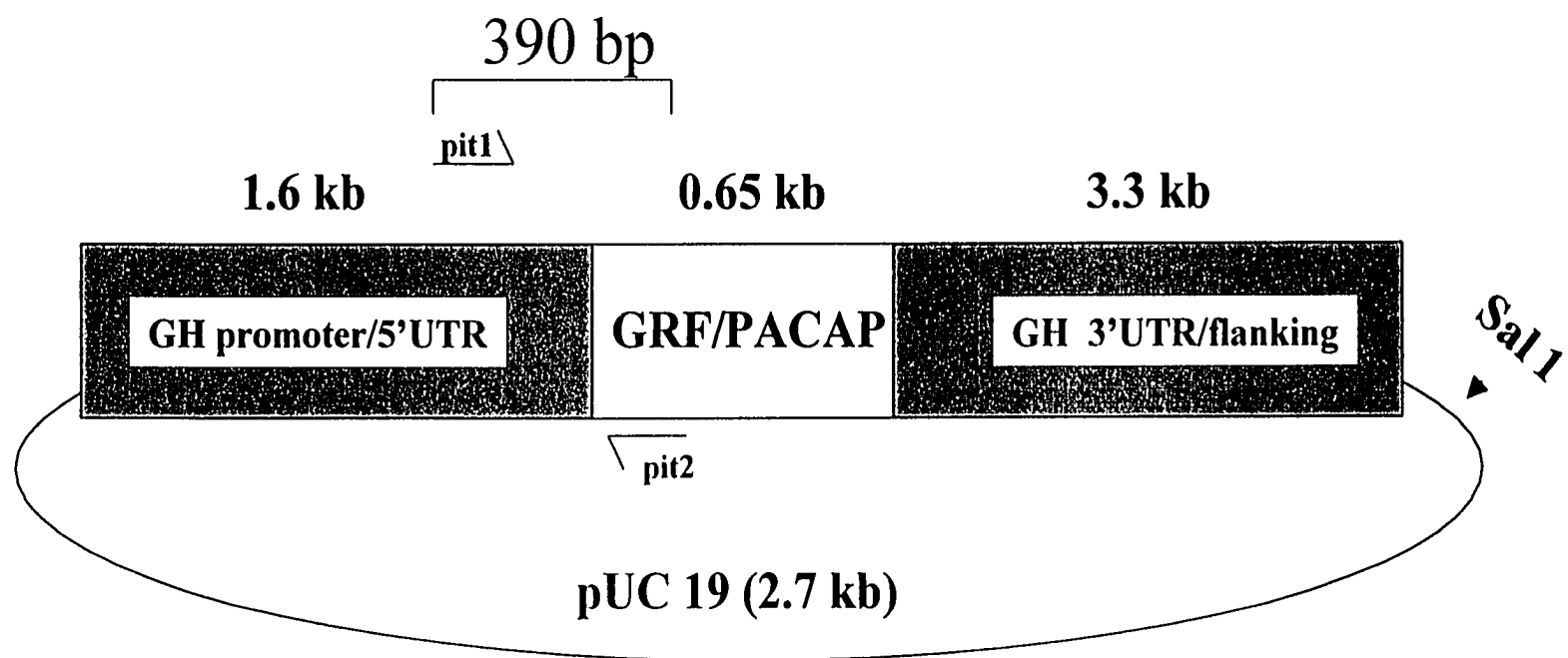
### *Biological Materials*

The eggs and milt used in these experiments were obtained from various hatcheries on Vancouver Island and the lower mainland of British Columbia. Once collected from the hatcheries, the eggs and milt were stored on ice and maintained in an oxygen saturated environment for same day transport to the University of Victoria.

### ***Preparation of transgene***

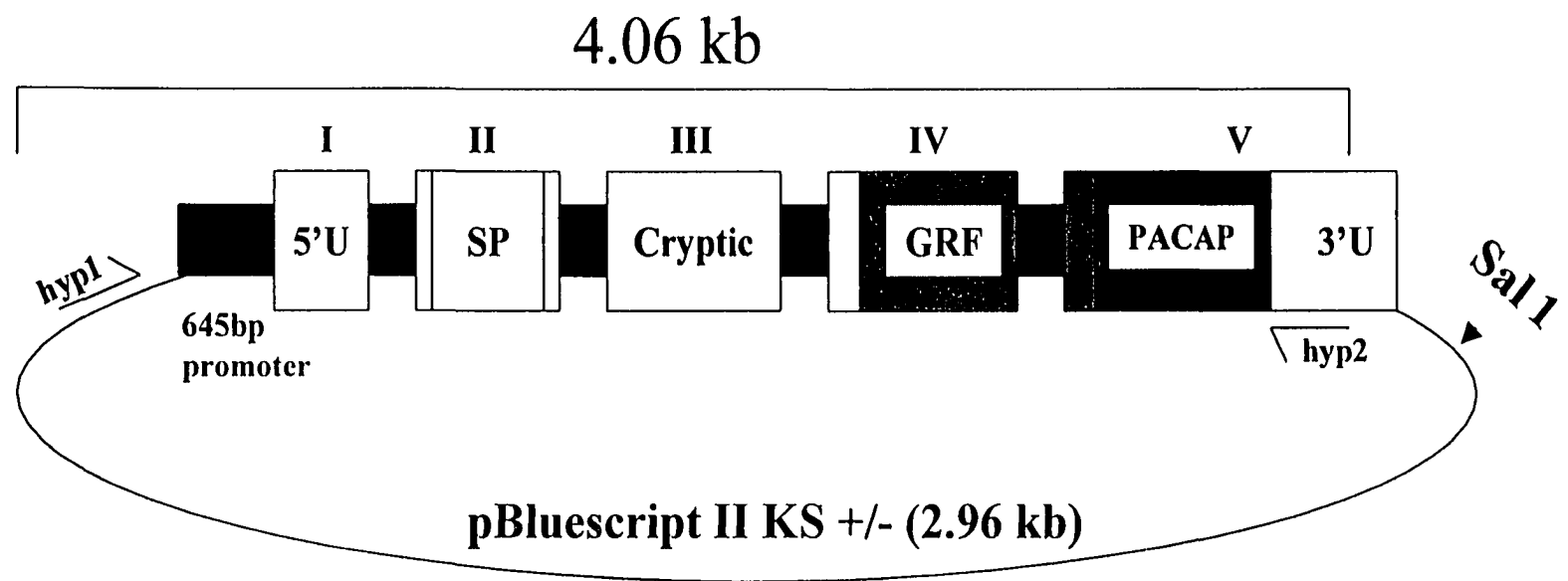
*E. coli* XL-2 Blue MRF' cells (Stratagene) were transformed with the desired transgene construct. Clones were identified by antibiotic resistance and blue/white selection. The plasmid constructs were extracted from the bacterial colonies by an alkaline hydrolysis method (Birnboim et al., 1983). Following plasmid DNA extraction an aliquot of the plasmid DNA was digested with an appropriate restriction enzyme to determine if the expected constructs were cloned. The results of the digest were observed by electrophoresis of the samples on a 1% agarose gel. The amplification of the appropriate clone was also confirmed by PCR analysis of the plasmid construct using primers specific for the appropriate construct being amplified (see below for primer sequences). If the results were as expected, the remaining plasmid DNA was digested with a restriction enzyme that cut in only one place to linearize the construct. The constructs for pituitary expression (pit-grf/pacap) and for hypothalamic expression (hyp-grf/pacap) were both digested with Sal I (figure 4-1 and figure 4-2). An aliquot of the digest was electrophoresed on a 1% agarose gel against undigested plasmid to determine whether the linearization was successful. The linearized plasmid DNA was extracted with an equal volume of phenol-chloroform-isoamyl (1X), chloroform-isoamyl (1X) and then ethanol precipitated. For the microinjection experiments the linearized plasmid was brought up in microinjection solution (1mM Tris-HCl, 0.1mM EDTA, 0.5% phenol red; pH 7.2) and the concentration was adjusted to approximately  $1 \times 10^6$  copies of the construct/5 nl. For electroporation the plasmid DNA was brought up in sterile filtered Ringer's solution to a concentration of 100  $\mu$ g/ml. Ringer's solution is composed of: 6.5 g NaCl, 0.25 g KCl, 0.2 g NaHCO<sub>3</sub>, 0.4 g CaCl<sub>2</sub>-2H<sub>2</sub>O in 1 L sterile distilled water. Neither pCMS-EGFP (Clontech) or pCMV $\beta$  (Clontech) were linearized. These two constructs were used as markers indicating either introduction of the transgene into the cell, or critical levels of DNA toxicity caused by injection of DNA into the cell.

**Figure 4-1.** The **pit-grf/pacap** construct. The construct was engineered in a **pUC19** vector (NEB). The gray boxes represent Chinook salmon gene sequences that encode **GH**; the white box is the sockeye salmon **grf/pacap** cDNA. The site for linearization is identified (**Sal I**). The primers used in PCR analysis are also identified, **pit1** and **pit2**. The PCR fragment generated by these primers is 390 base pairs.



**Pit-grf/pacap**

**Figure 4-2.** The **Hyp-grf/pacap** construct. The construct was engineered in **pbluescript II KS +/-** (Stratagene). The boxes represent **exons I-V** of the **grf/pacap** gene and they are labeled for the peptides they encode. Exons IV and V encode **GRF** and **PACAP** peptides respectively. The site for linearization is identified (**Sal 1**). The primers used in PCR analysis are also identified, **hyp1** and **hyp2**. The PCR fragment generated by these primers is 4.06 kilobases.



**Hyp-grf/pacap**

### ***Gamete collection***

Eggs and sperm from cutthroat trout, coho salmon, steelhead trout, rainbow trout and Chinook salmon were collected from various hatcheries, and brought to the University of Victoria Aquatics Facility. The samples were stored separately in whirl-pak bags in an oxygen saturated environment. The bags were kept on ice in the dark for 0-5 days, during which time some of the gametes were used in the experiment. The stored samples were supplied with fresh oxygen daily. Care was taken during collection and storage to avoid activation of the gametes through exposure to fresh water.

### ***Sperm viability***

The viability of sperm samples was tested by placing a drop of milt on a glass slide. The sample was observed under a compound microscope at 200 X magnification. After the addition of water to the milt, the sperm should spiral rapidly for about 30 seconds if they are viable. Only viable samples were used in electroporation or microinjection experiments.

### ***Electroporation***

Electroporation of cutthroat trout sperm was carried out in cuvettes (Bio-Rad Gene Pulser cuvettes - 0.2 cm electroporator gap) using a (Bio-Rad) Gene Pulser. The volume of sperm used was 0.5 ml. The sperm were diluted 4X in Ringer's solution. Both treated and untreated sperm were kept cool on ice in Ringer's solution throughout the procedure. The sperm were mixed with the DNA solution (100 µg/ml in Ringer's) and electroporated at field strengths ranging from 400-1000 volts/cm, 0.25 µF, a time constant of 0.1 msec and 3 pulses. Following electroporation the unelectroporated sperm, sham electroporated sperm and DNA-electroporated sperm were left on ice in the Ringer's solution for 10 minutes to allow uptake of DNA into the treated sperm. Then, the sperm were mixed with approximately 100 eggs. The eggs were left for 1 minute and transferred to a fresh water

environment in Heath trays at the University of Victoria Aquatics Facility. Unfertilized or dead eggs were removed every 2-3 days.

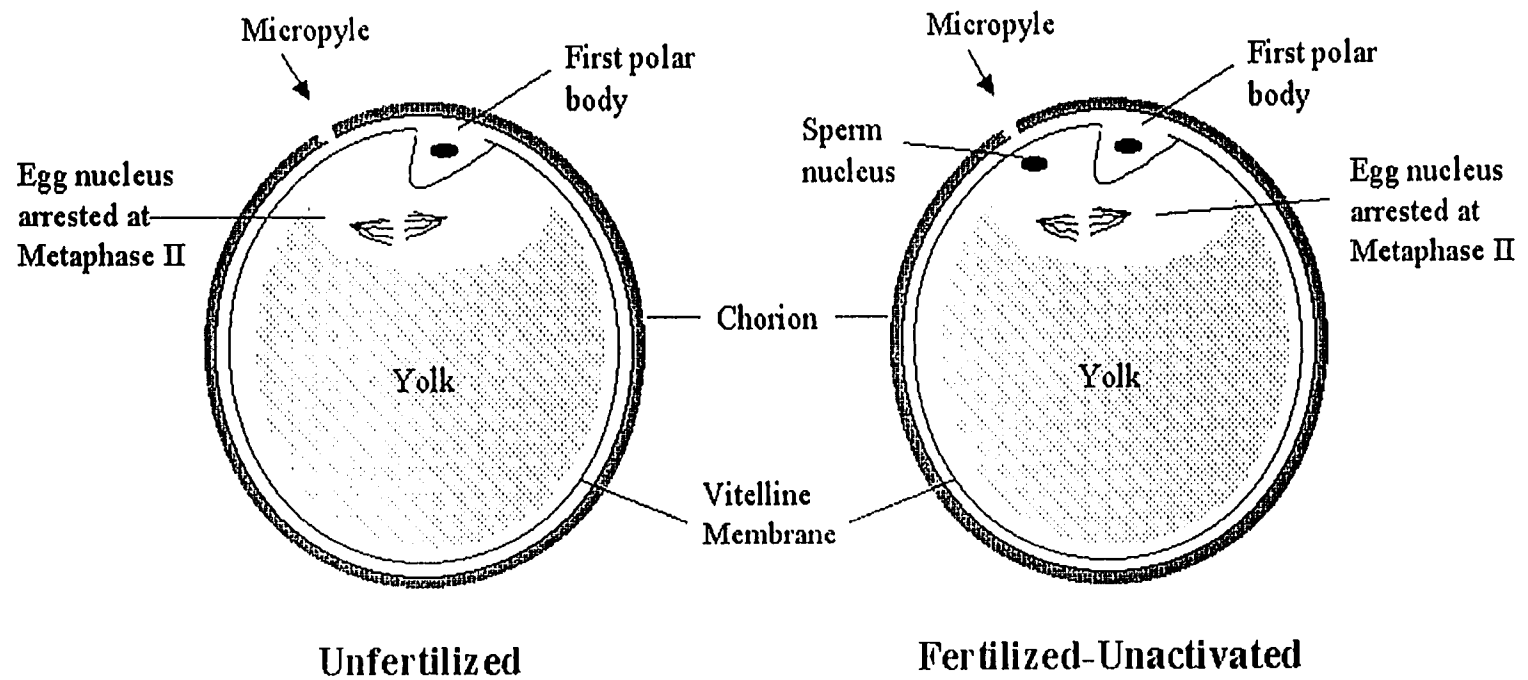
### ***Microinjection***

The microinjection apparatus was driven by a nitrogen pump. An ASI MPP1-2 pressure injector (Applied Science Inc.) was attached to a holder which housed a glass needle filled with the DNA/microinjection solution. The needle holder was mounted onto a micromanipulator. The N<sub>2</sub> cylinder was set at a pressure of 30 p.s.i.. The injection pressure was controlled by the pump which was set to administer short bursts of N<sub>2</sub> at 200 kPa. The power supply delivered a pulse of 100-1000 milliseconds.

The microinjection needles were made from borosilicate omega dot capillary tubes (Frederick Haer & Co.) (1 mm O.D., 0.5 mm I.D. x 4 in). A vertical needle puller (model 700C, David Kopf Instruments) was used to make needles from the capillary tubes. Once pulled, the needles were beveled under a dissecting scope to a diameter of approximately 5  $\mu$ m.

Prior to microinjection, eggs were fertilized but maintained in an unactivated state (i.e., male and female pronuclei have not yet fused - See figure 4-3). This was accomplished by the addition of a few drops of milt to 100-200 eggs in a glass beaker. The eggs/milt mixture was gently swirled and left for 1 minute. Following fertilization the eggs were rinsed with Ringer's solution to remove excess sperm. The eggs were kept in Ringer's on ice in a darkened container until they were ready to be activated through exposure to a hypotonic solution (fresh water). The eggs were injected within 2 hours post fertilization. The eggs were observed and microinjected under a compound microscope (Nikon SMZ-2B) at 20 X magnification using Nikon MKII fiber optics light source

**Figure 4-3.** Diagram of a salmonid egg that is unfertilized or fertilized but not activated.



directed at a 45° angle to the eggs. For the purpose of microinjection, eggs were transferred 5 at a time to a petri dish containing a sponge saturated with ice cold Ringer's solution. The sponge was prepared with a groove the appropriate size for an egg. The eggs were bathed in ice cold Ringer's throughout the microinjection procedure. Using a pair of forceps with curved plastic tips, I gently rotated the eggs until the micropyle (approximately 1  $\mu\text{m}$  in diameter) was found. The micropyle could be identified because light reflected differently in the pit surrounding the micropyle making the area appear slightly opaque. The micropyle was then oriented so that it was on top and slightly back of center of the egg. Once the micropyle was identified the micromanipulator was used to position the tip of the glass needle above the micropyle. The needle was inserted vertically into the opening and approximately 5nl of the DNA or saline solution was injected. Because the chorion surrounding the egg is very tough, the needle cannot penetrate the egg surface without breaking, except at the micropyle. Following microinjection the eggs were transferred to a freshwater environment in Heath trays. The eggs were incubated in Heath trays at 11-13°C until hatching. Dead eggs were removed from the trays every 2-3 days.

### ***Genomic Extraction***

Fish from the injected (control and experimental) groups, were given a lethal overdose of anesthetic and tissue samples were taken from the fin, liver, gonad, heart and brain/pituitary. DNA was extracted from the tissue for use in the polymerase chain reaction. For each tissue a small sample was incubated at 50°C for 30 minutes in a mixture of 5% analytical grade chelex 100 resin (Bio-Rad), 0.1% tween-20 and 0.1mg/ml proteinase K. This was followed by a 15 minute incubation at 95°C to inactivate the proteinase K. The extract was mixed and 1-2  $\mu\text{l}$  of the crude extract was used for analysis by PCR.

### ***Polymerase Chain Reaction Analysis***

The extracted DNA was used in the polymerase chain reaction. Two PCR analyses were done on each DNA sample. For each sample a tubulin PCR analysis was run. The primers used in the tubulin PCR were tub1 (5' CAGGTGTCCACGGCTGTGGTG 3') and tub2 (5' AGGGCTCCATCGAAACGCAG 3'). The conditions for the PCR were as follows, 1 X PCR buffer (Gibco BRL), 2 mM MgCl (Gibco BRL), 0.2 mM dNTPs, 0.4 mM primer tub1, 0.4 mM primer tub2, 5 units Taq polymerase (Gibco BRL), 1 µl DNA extract and 29 µl sterile water. The PCR reactions were run for 35 cycles on a DNA thermal cycler. Each cycle consisted of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. A gh promoter primer (pit 1) and a grf/pacap primer (pit2) for the pit-grf/pacap construct was used for PCR analysis (figure 4-1). The sequence for pit1 is 5' TGTTCGTCATCACATAAA 3'. The sequence for pit2 is 5' ATAGCAATTTGATCGCTGTC 3'. The conditions for the pit-grf/pacap PCR were, 1 X PCR buffer (Gibco BRL), 1.5 mM MgCl (Gibco BRL), 0.2 mM dNTPs, 0.4 mM pit1 primer, 0.4 mM pit2 primer, 5 units Taq polymerase (Gibco BRL), 2 µl DNA extract and 29 µl sterile water. The reaction was put through 35 cycles in a DNA thermal cycler. The conditions for a single cycle were as follows, 94°C for 30 seconds, 58°C for 30 sec and 72°C for 30 seconds. For PCR analysis of the hyp-grf/pacap construct the hyp1 primer (5' AATACGACTCACTATAG 3') was used upstream and hyp2, 5' CATGTTTGGA[ACGT]AGAACACA[ACGT]GAGCG 3' was used downstream (figure 4-2). The reaction conditions were 1 X PCR buffer (Gibco BRL), 2 mM MgCl (Gibco BRL), 0.2 mM dNTPs, 0.4 mM hyp1 primer, 0.4 mM hyp2 primer, 5 units Taq polymerase (Gibco BRL), 2 µl DNA extract and 28 µl sterile water. The reaction was put through 35 cycles in a DNA thermal cycler. The conditions for each cycle were 94°C for 1.5 minutes, 55°C for 1.5 minutes and 72°C for 1.5 minutes. Following the completion of the reactions,

20 µl aliquots of the tubulin, pit-grf/pacap and hyp-grf/pacap PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide for visualization.

## Results

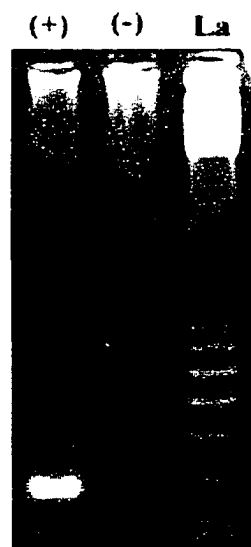
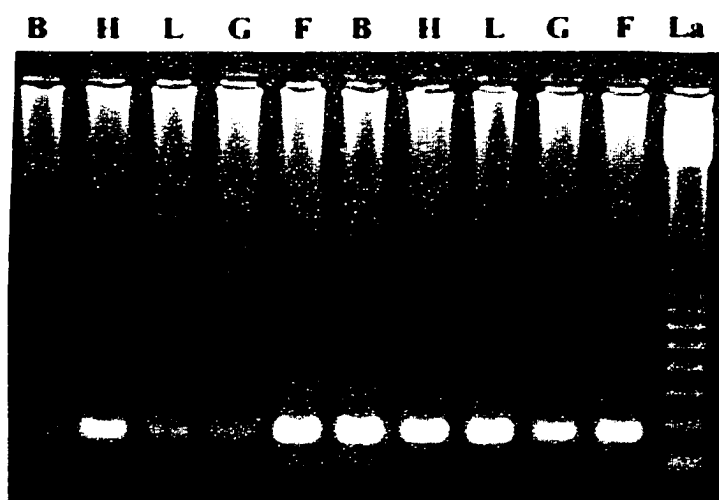
### *Electroporation Experiment.*

In February of 1994, the pit-grf/pacap construct (figure 4-1) was used in an electroporation experiment. The sperm samples were divided into seven groups. Group 1 sperm received 3 pulses at 400 volts/cm in the presence of the pit-grf/pacap construct. Group 3 received 3 pulses of 800 volt/cm and group 5 received 3 pulses of 1000 volt/cm. Groups 2 (3 pulses of 400 volts/cm), 4 (3 pulses of 800 volts/cm) and 6 (3 pulses of 1000 volts/cm) were sham electroporations (i.e. DNA construct not present during electroporations). Group 7 sperm received no treatment at all and was used for a normal fertilization event. The sperm samples were used to fertilize cutthroat trout eggs as outlined in the Materials and Methods. One hundred eggs were fertilized per sperm sample. The fish embryos were divided according to treatment groups within the Heath tray. The original containers for treatment groups provided insufficient isolation and some mixing of the groups occurred. Therefore no data is available regarding percent mortality of the different treatment groups. All of the fish were tested for presence of the transgene in five tissues, but none were found to contain the gene. A selection of PCR analyses from two fish (2 x 5 tissues) are displayed in figures 4-4 and 4-5 to represent the findings. The following season I decided to use microinjection as the technique for introduction of the transgene. Papers being published at the time indicated that successful incorporation could be accomplished by microinjection of DNA into the fertilized salmonid eggs.

**Figure 4-4.** Tubulin PCR analysis of a representative subset of the electroporation experiments. The tissues examined were brain/pituitary (**B**), heart (**H**), liver (**L**), gonad (**G**) and fin (**F**). PCR analysis of two fish (**A** and **B**) are shown. The ladder (**La**) is a 123 bp ladder. A negative control (-) and a positive (+) control was done with each PCR reaction. The tubulin PCR product is **218 bp**.

**A**

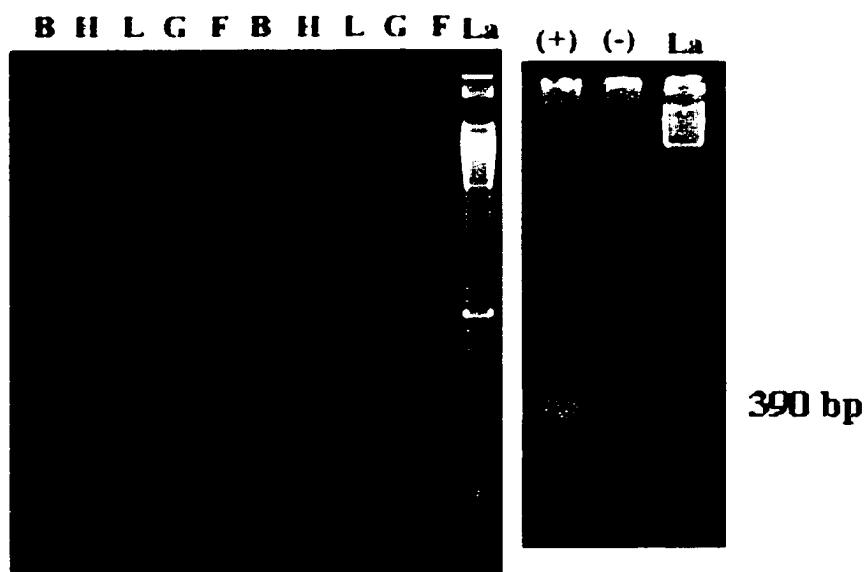
**B**



**Figure 4-5.** Pit-grf/pacap PCR analysis of a representative subset of the electroporation experiments. The tissues examined were brain/pituitary (**B**), heart (**H**), liver (**L**), gonad (**G**) and fin (**F**). PCR analysis of two fish (**A** and **B**) are shown. The ladder (**La**) is a 123 bp ladder. A negative control (-) and a positive (+) control was done with each PCR reaction. The pit-grf/pacap PCR product is **390 bp**.

A

B



### ***Microinjection Experiments***

The two main constructs used in the microinjection experiments are pictured in figures 4-1 and 4-2. The following paragraphs describe the microinjection experiments that were conducted. This information is also summarized in Table 4-1.

November 1994 - Fertilized-unactivated coho eggs were microinjected with the pit-grf/pacap construct, the hyp-grf/pacap construct and microinjection solution alone (sham microinjection). All microinjected embryos died before hatching. A total of 347 eggs were injected with one of the three injection solutions. One hundred controls were fertilized and 37 died. The percent mortality of the injected eggs was 100%. The normally fertilized control eggs had a percent mortality of 63%.

March 1995 - Fertilized-unactivated steelhead trout eggs were microinjected with the pit-grf/pacap construct. Seventy-three eggs were injected and 30 survived until hatching ( 59% mortality). One hundred control eggs were fertilized and 75 survived until hatching (25% mortality).

December 1995 - Fertilized-unactivated rainbow trout eggs were microinjected with the green fluorescent protein (CMS- EGFP) construct. A total of 88 eggs were injected and 2 survived until hatching (98% mortality). Following this the eggs were injected with a mixture of  $0.5 \times 10^6$  copies of pit-grf/pacap and  $0.5 \times 10^6$  copies of the CMS-EGFP construct. One hundred eggs were injected and 7 survived until hatching (93% mortality). In addition 100 eggs were injected with microinjection medium only (sham microinjection). Of the 100 eggs injected 16 survived until hatching (84% mortality). Finally, 100 control eggs were fertilized and 33 survived until hatching (67% mortality).

**Table 4-1.** Percent Mortality of Control vs. Microinjected Salmonid eggs

| Date      | Species         | Treatment                  | Sample number | % Mortality |
|-----------|-----------------|----------------------------|---------------|-------------|
| Nov 1994  | coho salmon     | pit-grf/pacap**            | 100           | 100         |
|           |                 | hyp-grf/pacap**            | 200           | 100         |
|           |                 | sham                       | 47            | 100         |
|           |                 | none                       | 100           | 63          |
| Mar 1995  | steelhead trout | pit-grf/pacap**            | 73            | 59          |
|           |                 | none                       | 100           | 25          |
| Dec 1995  | rainbow trout   | PCMS-EGFP**                | 88            | 98          |
|           |                 | pit-grf/pacap & pCMS-EGFP* | 100           | 93          |
|           |                 | sham                       | 100           | 84          |
|           |                 | none                       | 100           | 61          |
| Apr 1996  | steelhead trout | pit-grf/pacap**            | 372           | 100         |
|           |                 | OnMTGH1**                  | 78            | 100         |
|           |                 | none                       | 100           | 100         |
| May 1996  | rainbow trout   | hyp-grf/pacap**            | 550           | 85          |
|           |                 | none                       | 416           | 22          |
| June 1996 | rainbow trout   | hyp-grf/pacap**            | 654           | 100         |
|           |                 | none                       | 482           | 100         |
| Oct 1996  | rainbow trout   | pit-gfp/pacap**            | 875           | 72          |
|           |                 | none                       | 451           | 43          |

| Date      | Species        | Treatment       | Sample number | % Mortality |
|-----------|----------------|-----------------|---------------|-------------|
| Nov 1996  | chinook salmon | pit-grf/pacap** | 1193          | 83          |
|           |                | hyp-grf/pacap** | 548           | 100         |
|           |                | sham            | 142           | 100         |
|           |                | none            | 155           | 17          |
| June 1997 | rainbow trout  | CMV $\beta$ *   | 250           | 98          |
|           |                | CMV $\beta$ **  | 275           | 87          |
|           |                | CMV $\beta$ *** | 260           | 75          |
|           |                | sham            | 275           | 91          |
|           |                | none            | 800           | 73          |

\*The DNA concentration was  $10^5$  copies/5nl.

\*\*The DNA concentration was  $10^6$  copies/5nl

\*\*\*The DNA concentration was  $10^7$  copies/5nl

\*The DNA concentration was  $0.5 \times 10^6$  copies/5nl of pit-grf/pacap and  $0.5 \times 10^6$  copies/5nl of pCMS-EGFP

April 1996 – Fertilized-unactivated steelhead trout eggs were microinjected with the pit-grf/pacap construct or a GH expressing construct (OnMTGH1). Three hundred and seventy-two eggs were injected with pit-grf/pacap, 78 were injected with OnMTGH1 and 100 control eggs were fertilized. All eggs, including controls, died before hatching (100% mortality).

May 1996 – Fertilized-unactivated rainbow trout eggs were microinjected with the hyp-grf/pacap construct. At this point we stopped doing sham injections, unless we had an abundance of eggs and milt, because microinjection opportunities were limited and we needed to maximize the eggs injected with the transgene. Five hundred and fifty eggs were injected with the DNA construct and 81 survived until hatching (85% mortality). Four hundred and sixteen control eggs were fertilized and 323 survived until hatching (22% mortality).

June 1996 – Fertilized-unactivated rainbow trout eggs were microinjected with hyp-grf/pacap. Six hundred and fifty-four eggs were injected and none survived to hatching (100% mortality). Four hundred and eighty-two control eggs were fertilized and none survived to hatching (100% mortality).

Oct 1996 – Fertilized-unactivated rainbow trout eggs were injected with the pit-grf/pacap construct. Eight hundred and seventy-five eggs were injected and 244 survived to hatching (72% mortality). Four hundred and fifty-one eggs were fertilized as controls and 259 survived to hatching (43% mortality).

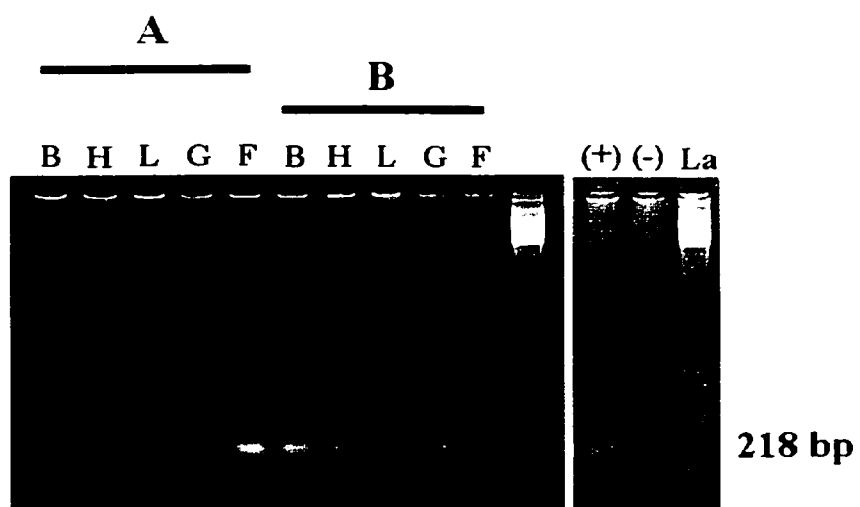
Nov 1996 – Fertilized-unactivated Chinook salmon eggs were injected with pit-grf/pacap construct or hyp-grf/pacap. One thousand one hundred and ninety-three eggs were injected (pit-grf/pacap) and 139 survived until hatching (88% mortality). One hundred and forty-two eggs were injected with microinjection solution and no eggs survived until hatching (100% mortality). Five hundred and forty-eight eggs were injected with hyp-

grf/pacap and no eggs survived until hatching (100% mortality). One hundred and fifty-five eggs were fertilized as controls and 128 survived to hatching (17% mortality).

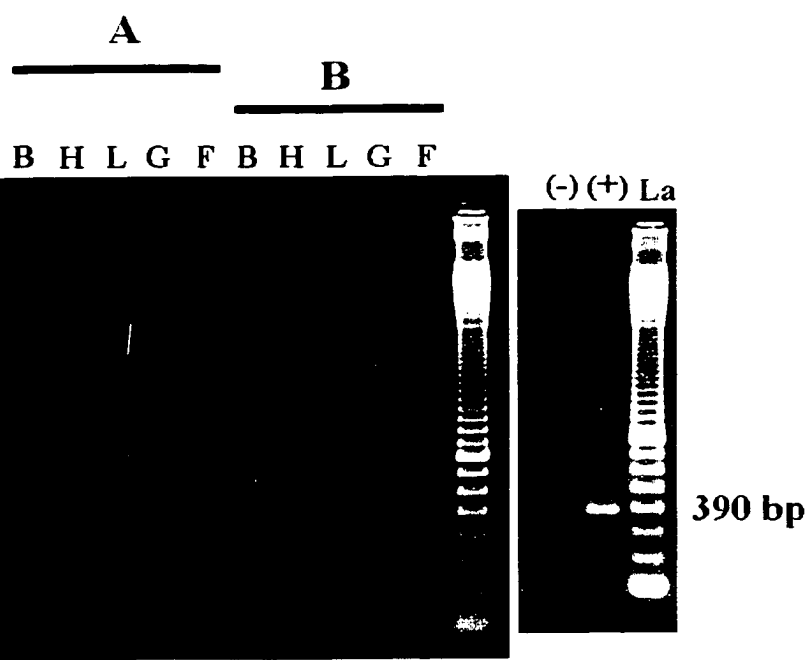
June 1997 – The following microinjection experiment was done to access the difference in mortality rate due to DNA toxicity. Fertilized-unactivated rainbow trout eggs were injected with pCMV- $\beta$ -gal or microinjection solution alone (sham microinjection). Two hundred and seventy-seven eggs were injected with microinjection solution and 24 eggs survived until hatching (91% mortality). Two hundred and fifty eggs were injected with  $10^5$  copies of the pCMV- $\beta$ -gal construct and 6 survived until hatching (98% mortality). Two hundred and seventy-five eggs were injected with  $10^6$  copies of the construct and 37 survived until hatching (87% mortality). Two hundred and sixty eggs were injected with  $10^7$  copies of the construct and 64 survived until hatching (75% mortality). Eight hundred control eggs were fertilized and 220 survived until hatching (73% mortality).

All of the fish that survived from these treatment groups were euthanized in 1997 and PCR analysis was performed on brain/pituitary, liver, heart, gonad and fin. None of the fish tested positive for a 390bp band for the pituitary construct or a 4.06 kb band for the hypothalamic construct. The total number of PCR analyses done on these tissues was approximately 7,500. A sample of the PCR analyses is displayed in figures 4-6 to 4-9).

**Figure 4-6.** Tubulin PCR analysis of a representative subset of the pit-grf/pacap microinjection experiments. The tissues examined were brain/pituitary (**B**), heart (**H**), liver (**L**), gonad (**G**) and fin (**F**). PCR analysis of two fish (**A** and **B**) are shown. The ladder (**La**) is a 123 bp ladder. A negative control (-) and a positive (+) control was done with each PCR reaction. The tubulin PCR product is **218 bp**.



**Figure 4-7.** Pit-grf/pacap PCR analysis of a representative subset of the microinjection experiments. The tissues examined were brain/pituitary (**B**), heart (**H**), liver (**L**), gonad (**G**) and fin (**F**). PCR analysis of two fish (**A** and **B**) are shown. The ladder (**La**) is a 123 bp ladder. A negative control (-) and a positive (+) control was done with each PCR reaction. The pit-grf/pacap PCR product is **390 bp**.

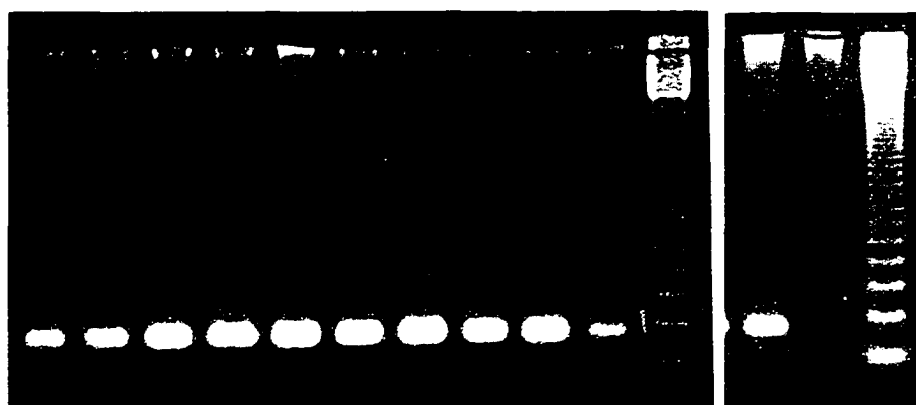


**Figure 4-8.** Tubulin PCR analysis of a representative subset of the hyp-grf/pacap microinjection experiments. The tissues examined were brain/pituitary (**B**), heart (**H**), liver (**L**), gonad (**G**) and fin (**F**). PCR analysis of two fish (**A** and **B**) are shown. The ladder (**La**) is a 123 bp ladder. A negative control (-) and a positive (+) control was done with each PCR reaction. The tubulin PCR product is **218 bp**.

**A**

**B**

**B H L G F B H L G F La (+) (-) La**



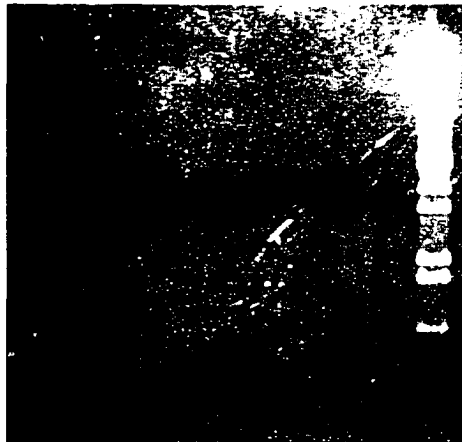
218 bp

**Figure 4-9.** Hyp-grf/pacap PCR analysis of a representative subset of the microinjection experiments. The tissues examined were brain/pituitary (**B**), heart (**H**), liver (**L**), gonad (**G**) and fin (**F**). PCR analysis of two fish (**A** and **B**) are shown. The ladder (**La**) is a EcoRI/HindIII lambda ladder. A negative control (-) and a positive (+) control was done with each PCR reaction. The hyp-grf/pacap PCR product is **4.06 kb**.

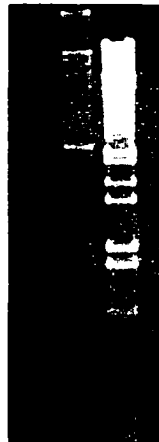
**A**

**B**

**B H L G F B H L G F L a**



**(-)(+)La**



**4060 bp**

## Discussion

Transgenic technology provides researchers with a means of examining the effects of gene expression in a living system. This sort of *in vivo* work is a valuable complement to *in vitro* work; it helps to provide a more comprehensive understanding of gene function. Three of the most important reasons for producing a line of grf/pacap transgenic salmon are: First, due to the industry-related importance of salmon, this work is not only useful in terms of basic science, but it also contributes to applied science. Growth-enhanced transgenic salmon should be able to reach market size earlier and be of interest to the fisheries industry (Devlin et al., 1994; Devlin et al., 1995). Also, there have been many growth studies done in salmon to provide a backdrop on which to superimpose the transgenic results. Second, others have successfully made transgenic salmon that overexpress different genes, such as GH (Zhang et al., 1990; Du et al., 1992; Devlin et al., 1994, 1995) and antifreeze protein (Fletcher et al., 1988). Third, several GH-transgenic fish have been noted to have acromegaly-like features (e.g., overproduction of cartilage in the cranium, jaw and operculum (Devlin et al., 1995). My proposal was that the constructs would allow for more natural growth enhancement with less pronounced acromegaly and normal levels of fertility compared to GH transgenic fish. However, during the course of this experiment several problems arose. First and foremost, the mortality rate for the control fertilization events was unpredictable and routinely too high (17-100%). An acceptable mortality rate is around 20% (Shears et al., 1992) but this level of mortality was approached only three times (25% in Mar 1995, 22% in May 1996 and 17% in November 1996). The remaining microinjection experiments had unacceptably high mortality rates for the controls (43-100%). There are three reasons for the high mortality rate. First, our facility was unable to provide an ideal flow rate for water in the Heath trays. An inadequate flow rate means metabolites are not readily removed from the developing eggs and the oxygen supply to the eggs is reduced. Both of these factors affect the survival and normal development of

the fish eggs. Second, the eggs and milt we used in these experiments were supplied from government hatcheries (Fraser Valley Trout Hatchery, Abbotsford BC) and we were not guaranteed the best quality because the hatchery was supplied first. The quality of the eggs and milt affects the success of a fertilization event. Third, eggs and milt were stored for 0-5 days before fertilization. Viability decreases the longer the gametes are stored (personal observations). However, due to the time required for microinjection there is no choice but to store the gametes for up to 5 days while the experiment is ongoing. Throughout the experiment, attempts were made to increase flow rate and improve the rearing conditions. The other two factors could not be controlled or changed. Of these three factors, I judge the quality of eggs or sperm to be the most important.

Another problem encountered during these experiments has to do with the technique for introducing the transgene. Originally we tried electroporation but skepticism within the scientific community persuaded us to try microinjection, a more established technique (personal communication with Dr. Bob Devlin). Microinjection has been used previously on salmon with success (Du et al., 1992; Shears et al., 1992; Devlin et al., 1994, 1995). Before starting this project we conferred with Dr. Garth Fletcher and Dr. Bob Devlin, two experts in the field. Following conversations with them and examination of their publications we decided to use the parameters for DNA concentration, injection volume and injection method that they found to be successful. Also, I spent time in Dr. Devlin's lab to learn the technique and to determine the equipment required. Due to space constraints within our Aquatics Facility we could not rear large numbers of salmon/trout. Therefore, our objective was not to produce mass numbers of transgenic fish but to generate a few transgenic salmon/trout to use in physiological and morphological studies. Microinjection is a very challenging technique, especially in salmon and trout. A tough chorion surrounds the egg and makes penetration with a fine tipped (5  $\mu\text{m}$ ) needle impossible. The only point on the egg's surface through which the needle can be inserted is the micropyle (1-3  $\mu\text{m}$

diameter) (figure 4-1). The micropyle is very difficult to see and can only be observed as a minute spot on the egg surface that reflects light differently than the rest of the egg. The needle is inserted using a micromanipulator into the micropyle and the DNA solution is dispensed into the cytoplasm of the egg which is only 40-100  $\mu\text{m}$  deep. Insertion of the needle into the correct spot on the egg and at the correct depth is very important. If this is done incorrectly the egg will die. Examination of the percent mortality rates, particularly on the dates when control mortality was low (17-25% in March 1995, May 1996 and November 1996), indicates that the technique irreparably damaged the eggs. An acceptable mortality rate for microinjected eggs is around 20-30% (Shears et al., 1992). Mortality rates of DNA-injected eggs for the three microinjection dates were 59%, 85% and 83% respectively. The other microinjection dates that had high mortality in the controls (>25%) also had high mortality of eggs injected with DNA (72-100%). In addition, the mortality rates of all sham microinjections (84-100%) were high, when compared to the controls (17-73%). Sham microinjections had around the same mortality rates as eggs injected with DNA (84-100%). Due to the difficulty of this technique, I think that it is necessary to inject about 10,000 eggs. If large numbers of eggs were injected one to two times a year for the first few years, good technical skills could be developed. We injected 6180 eggs over the entire 3 year period. Unfortunately, without access to broodstock at our convenience and with limited space for rearing, it was impossible to inject more eggs. I conclude that preparation of transgenic salmon is best done in a large aquatic facility such as those at the Department of Fisheries and Oceans (DFO). Government facilities have better water flow, more rearing space and access to broodstock. Apart from the technical complications experienced during this experiment, I think that salmonids are a good model for transgenic work. However, they are more difficult. The yearly spawning season greatly reduces the number of times the experiment can be tried and the rate of success is low, even with experienced technicians (2-6%) (Shears et al., 1992).

The microinjection experiment conducted with the pCMS-EGFP construct was done to introduce a visible marker into the fish. Green fluorescent protein (GFP) gives off a green fluorescence when observed at 488 nm wavelength. However, examination of the hatchlings did not yield any fluorescence above background. The DNA toxicity tests using the pCMV $\beta$  involved injecting DNA at three concentrations to determine a DNA toxicity response. The concentrations injected were  $10^5$ ,  $10^6$  and  $10^7$  copies/5nl. All concentrations had very high mortality (75-98%) and the sham microinjections had one of the highest mortality rates (91%). It is clear that other mortality issues prevented either experiment from yielding meaningful results.

Interestingly, when this experiment began we did not know PACAP was expressed during development in fish (see chapter 2 and chapter 3). In fact, data regarding the involvement of PACAP in mammalian development had not yet been published. If PACAP does regulate fish development, there is a possibility that overexpression of the gene in fish may be lethal. Therefore, even if the technique was perfected, this method may not be suitable for studying PACAP function. Other researchers experienced in this technique have tried to make transgenic fish using another gene involved in development, IGF-I, and were unsuccessful (personal communication with Dr. Bob Devlin).

### ***Future Work***

In order for an experiment such as this to work it must be done on a larger scale. The injection of more fish would allow the technique to be mastered and provide sufficient numbers of transgenic animals. To accomplish this a facility that can handle the rearing of large numbers of fish is required. Also, before attempting the experiment again in salmonids, it would be valuable to do experiments in mice or zebrafish where transgenic technology has been more successful (Constantini and Lacy, 1981; Gordon and Ruddle, 1985; Palmiter and Brinster, 1986; Stuart et al., 1988). By this means any problems related

to the actions of GRF and PACAP on development may be determined.

## **Chapter 5**

**Gene Copy number and evolution of grf/pacap from invertebrates to mammals**

## Introduction

During evolution of the PACAP/Glucagon superfamily, several gene and exon duplication events have led to the generation of a large family of 9 peptides (Sherwood et al., 2000). Two of the peptides, GRF and PACAP are related in sequence and function. In sockeye salmon and several other vertebrates, both hormones are involved in growth hormone release from the pituitary (Parker et al., 1997; Sherwood et al., 2000). In frogs, growth hormone secretion has not been examined. However, PACAP does cause a dose-dependent increase in cAMP concentrations in the frog pituitary (Chartrel et al., 1991). In fish these two peptides are found in tandem on the same gene (Parker et al., 1993). However, at some point in evolution a gene duplication event has occurred leading to the separation of the grf and pacap genes in mammals. The grf/pacap gene and/or cDNA has been sequenced in several species: sockeye salmon, coho salmon, chinook salmon, rainbow trout, Atlantic salmon, catfish, and zebrafish (See Chapter 2; Parker et al., 1993, 1997; McRory et al., 1995; Fradinger and Sherwood, 2000). In fish, amphibians (Hu et al., 2000b) and birds (McRory et al., 1997) grf and pacap are encoded on the same gene. The gene contains five exons encoding 4 peptides: the signal peptide, a peptide of unknown function - the cryptic peptide, GRF and PACAP. The gene for amphibian grf/pacap has not yet been isolated. The situation in mammals is different; GRF and PACAP are encoded on two separate genes. The grf gene has been identified in humans (Mayo et al., 1983, Gubler et al., 1983, Mayo et al., 1985a), hamsters (Ono et al., 1994) and mice (Frohman et al., 1989, Suhr et al., 1989). It has 5 exons and codes for three peptides: the signal peptide, grf and a C-terminal peptide. The mammalian DNA sequence for pacap has been identified in humans (Hosoya et al., 1992, Ohkubo et al., 1992), sheep (Kimura et al., 1990), rats (Ogi et al., 1990) and mice (Okazaki et al., 1995, Yamamoto et al., 1998). This gene also has 5 exons and codes for a signal peptide, PACAP-related peptide (PRP) and PACAP. Interestingly, in fish and chickens where GRF and PACAP are encoded on the same gene, a mechanism of alternative splicing of the grf/pacap gene allows for the production of two

transcripts; one encodes both GRF and PACAP and the other skips the exon that codes for the bioactive core of GRF and produces a PACAP-containing transcript (See Chapter 2; Parker et al., 1997, McRory et al., 1995, 1997a). This allows for independent production of the PACAP peptide. In mammals regulation of the two hormones has been separated by putting the genes under the control of independent promoters.

Due to a genome duplication event in the teleost lineage, salmon are tetraploid (Ohno et al., 1968). Therefore, it is likely that they will have more than one copy of many genes. The existence of a second *grf/pacap* gene has been proposed in sockeye salmon. In this chapter the issue of gene copy number in rainbow trout is addressed and discussed in relation to evolution of the GRF/PACAP lineage.

## **Materials and Methods**

### ***Genomic extraction***

A rainbow trout liver (2 mg) was ground to a fine powder in liquid nitrogen. The powder was suspended in 20 mls of digestion buffer (10 mM Tris-HCl, pH 8.0; 1% SDS; 5 mM EDTA; 100 µg/ml proteinase K). The mixture was incubated overnight at 37°C to allow for cell lysis and digestion of proteins. The total volume was extracted once with an equal volume of phenol and centrifuged for 3 min at 3000 rpm. The aqueous phase was decanted and divided into two tubes. The two crude DNA suspensions were then extracted two times with phenol:chloroform:iso-amyl alcohol (25:24:1) and centrifuged at 3000 rpm for 3 minutes to separate the layers. The aqueous layer was removed following centrifugation and subjected to a second phenol:chloroform:iso-amyl alcohol extraction. Then, 8 mls of sterile distilled water was added to each of the two crude DNA suspensions in order to reduce viscosity. A third phenol:chloroform:iso-amyl alcohol extraction was performed. The aqueous layer was removed and the two tubes were further divided into 4 tubes. The NaCl concentration was adjusted to 0.3 M and the DNA was precipitated out of solution by the addition of 2.5 volumes 100% ethanol. One of the 4 tubes was centrifuged

(10 minutes 2-3000 rpm). The resultant DNA pellet was rinsed by swirling in 70% ethanol and air dried. Then, the DNA pellet was suspended in 5 mls TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). NaCl was added to a final concentration of 100 mM and the DNA was incubated with 100 µg/ml of DNase-free RNase A at 37°C for 3 hours. Finally, the DNA was precipitated with 0.3 M NaCl (final concentration) and 2.5 volumes 100% ethanol. The solution was mixed by inversion and centrifuged for 10 minutes at 2,500 rpm. The DNA pellet was then resuspended in 2mls TE and the concentration of the DNA was determined. An aliquot of the genomic DNA was electrophoresed on a 1% TAE-agarose gel to confirm that the DNA was high molecular weight.

### ***Probe design***

The probe for use in the Southern blot was designed to the 5th exon encoding a region which includes PACAP and the 3'UTR (239 bp). The PCR primers Pa-1 (5' CACTCGGATGGGATCTTCACAGACAG) and 3'Pa (5' CATGTTTGGG[ACGT]AGAACA[ACGT]GAGCG) were used to amplify exon 5 of a partial rainbow trout grf/pacap cDNA cloned into pGEM-T vector (Promega). Three 50 µl PCR reactions were performed according to the following PCR parameters: 5 µl 10X PCR buffer (Promega), 2 µl 50 mM MgCl (Promega), 0.2 mM dNTPs, 0.4 mM Pa-1 primer, 0.4 mM 3'Pa primer, 5 units Taq polymerase (Promega), 1 µl rainbow trout grf/pacap clone and sterile filtered water to bring the volume up to 50 µl. The reaction was put through 35 cycles in a DNA thermal cycler. The conditions for a single cycle were as follows: 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds. Once amplified the 3 PCR reactions were electrophoresed on a 1% TAE-agarose gel and eluted from the gel using a millipore ULTRAFREE-MC column. The DNA was subsequently precipitated with 1/10th volume 3 M sodium acetate, pH 5.2 and 100% ethanol and stored at -20°C overnight. The probe DNA was centrifuged at 12,000 g for 25 minutes, then washed in 70% ethanol and centrifuged again for 15 minutes at 12,000 g. Finally the DNA pellet was brought up in 5

μl sterile distilled water and allowed to go into solution. The probe was labeled with  $\alpha^{32}$ -P-dCTP (NEN) using the Gibco BRL random primers DNA labeling system. The reaction was incubated at room temperature for 1.5 hours. Following incubation free radioactive nucleotides were removed by running the probe through a NAP 5 sephadex column (Amersham Pharmacia). The probe was washed off the column with 1 ml TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) and the probe was analyzed to be 43,086.5 cpm/μl

### ***Southern blot***

The genomic DNA was digested overnight in 50 μl reactions. Four enzymes were chosen for the digests: Eco RI (Pharmacia), HindIII (NEB), Sam I (NEB) and Xho I (NEB). In each restriction digest 3 μg of DNA was used. Following digestion a 5 μl aliquot of each digest was electrophoresed on a 1% TAE-agarose minigel to check the success of the digests. Once I confirmed that the digests were successful, the remaining 4 digests were electrophoresed for 9 hours on a large 1% TAE-agarose gel at 80 volts. After electrophoresis was completed the gel was covered in a dilute acid solution (0.25N HCl) and agitated for 10 minutes. The electrophoresed DNA was then denatured by soaking the gel in 0.4 N NaOH for 30 minutes. Meanwhile a nylon membrane (GeneScreen Plus, NEN) was placed in distilled water and equilibrated in 0.4 N NaOH for 15 minutes. A transfer pyramid was then set up using 0.4 N NaOH as the transfer solution to facilitate capillary action transfer of the DNA from the gel to the nylon membrane. The DNA was allowed to transfer overnight. The following morning the transfer pyramid was disassembled and the membrane was rinsed in 6 X SSC (standard sodium citrate) for 5 minutes. The membrane was allowed to dry at room temperature for 1.5 hours. The agarose gel was stained with ethidium bromide to check for success of the DNA transfer to the membrane. Once dry, the membrane was incubated at 50°C in pre-hybridization solution (6 X SSC, 5 X Denhardt's solution (USB), 0.5% SDS (sodium dodecyl sulfate) and 150 mg blocking DNA (sheared sea urchin sperm DNA). The blocking DNA was boiled for 5 minutes, then cooled on ice prior to adding to the pre-hybridization solution.

Likewise, the probe (1 ml) was boiled for 5 minutes and cooled on ice before adding to the hybridization solution (6 X SSC, 5 X Denhardt's solution (USB), 0.5% SDS). Following pre-hybridization the membrane was transferred to the hybridization solution plus probe ( $4.3 \times 10^7$  cpm/ml) and incubated overnight at 42°C.

After hybridization was complete the membrane was taken through a series of increasingly stringent washes at 42°C. It was washed twice in 2 x SSC and 0.1% SDS for 40 minutes each wash, twice in 1 X SSC and 0.1% SDS for 30 minutes each and three times in 0.5 X SSC and 0.1% SDS for 30 minutes each. Finally the temperature was increased to 45°C and the membrane was washed three more times at 0.5 X SSC and 0.1% SDS for 30 minutes each wash. For a slightly higher stringency the wash temperature was increased to 47°C and the membranes were washed according to the procedure above.

#### ***Visualization and photography***

Following washes the membrane was air dried and wrapped in saran wrap. The membrane was then exposed overnight to a phosphorimager screen at room temperature. The exposed image was scanned into a Storm 860 scanner (Molecular Dynamics). The image was adjusted for optimal contrast using two software programs, ImageQuant 5.0 and Adobe photoshop.

## Results

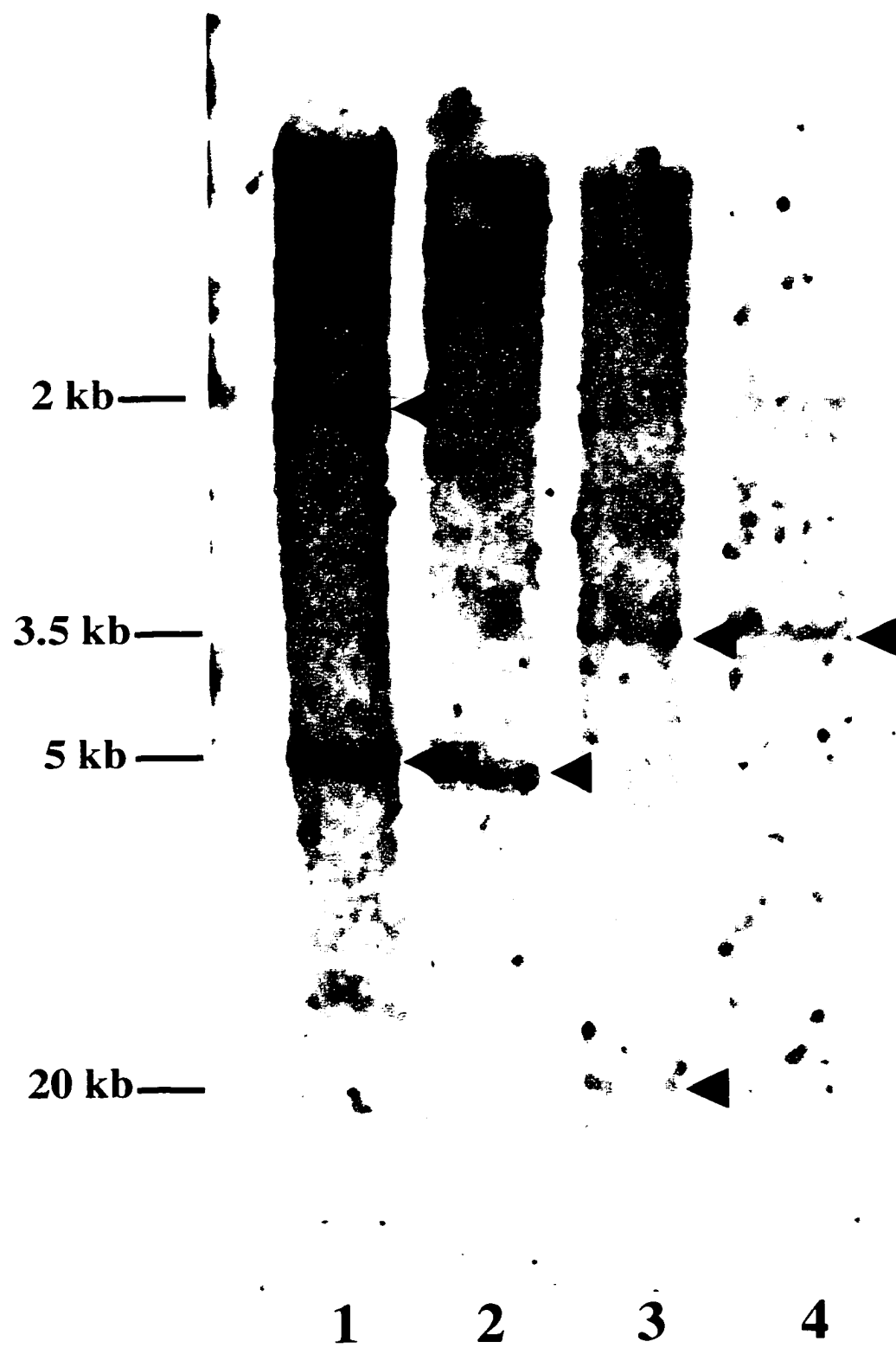
The Southern blot of rainbow trout genomic DNA with a 239 bp probe designed to PACAP and the 3' untranslated region revealed the presence of two bands in lanes 1 and 3. Lanes 2 and 4 had only one band. The second band indicates the presence of a second grf/pacap gene or possible allelic polymorphisms (figure 5-1). Both bands disappeared in higher stringency wash conditions.

## Discussion

Presently we can only speculate as to when the gene duplication event took place that led to the separation of the grf and pacap genes in mammals. The gene structure of grf/pacap in fish and the pacap gene in mammals is very similar (figure 5-2). Both genes have five exons encoding four peptides. The amino acid sequence identity between PACAP in fish and PACAP in mammals is 89-92% and 100% within the first 27 amino acids, which constitute the bioactive core of the peptide. The grf gene in mammals has undergone further modifications and appears to have lost exon 3 (the cryptic peptide). Also, the fifth exon, which encodes PACAP in fish, has greatly diverged in mammals and no longer resembles PACAP (the C-terminal peptide) (figure 5-2). Aside from structural similarities, evidence of promoter usage supports the theory that these two mammalian genes evolved from a gene duplication of grf/pacap. In the testes of rats, both the grf and pacap genes use alternative upstream promoters. Both promoters generate transcripts that include a portion of the 5'UTR (Srivastava et al., 1995; Hurley et al., 1995). Given the sequence evidence obtained to date combined with Southern analysis, I propose three different evolutionary pathways for the grf and pacap lineage.

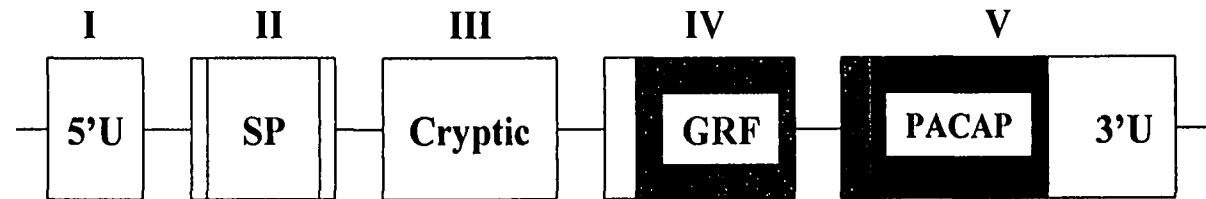
The first scheme I present (figure 5-3) is the most conservative of the three. This scheme relies more closely on confirmed sequence data. It begins with two grf/pacap

**Figure 5-1.** A Southern blot analysis of rainbow trout genomic DNA using a 239 bp PACAP/3'untranslated region probe. Lane 1) Eco RI, 2) Hind III, 3) Sma I, 4) Xho I. Approximate molecular weight markers are shown on the left.

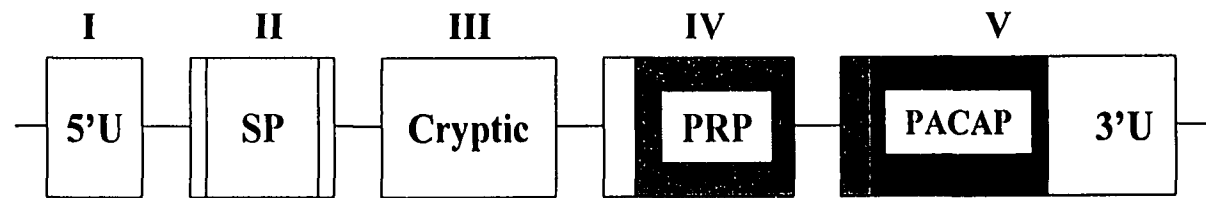


**Figure 5-2.** Schematic diagram of the grf/pacap gene in salmon and trout along with the pacap and grf genes in mammals. The roman numerals (**I-V**) indicate exon numbers 1-5. **5'U** and **3'U** refer to the 5' untranslated region and 3' untranslated region, respectively. Exon **III** of the grf gene in mammals is thought to have been deleted during evolution and therefore the grf coding exon is labeled as **III**. **C-peptide** refers to the C-terminal peptide.

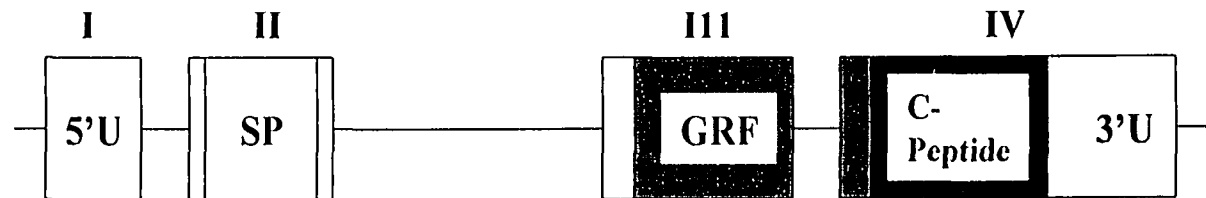
**grf/pacap gene**  
Salmon/Trout



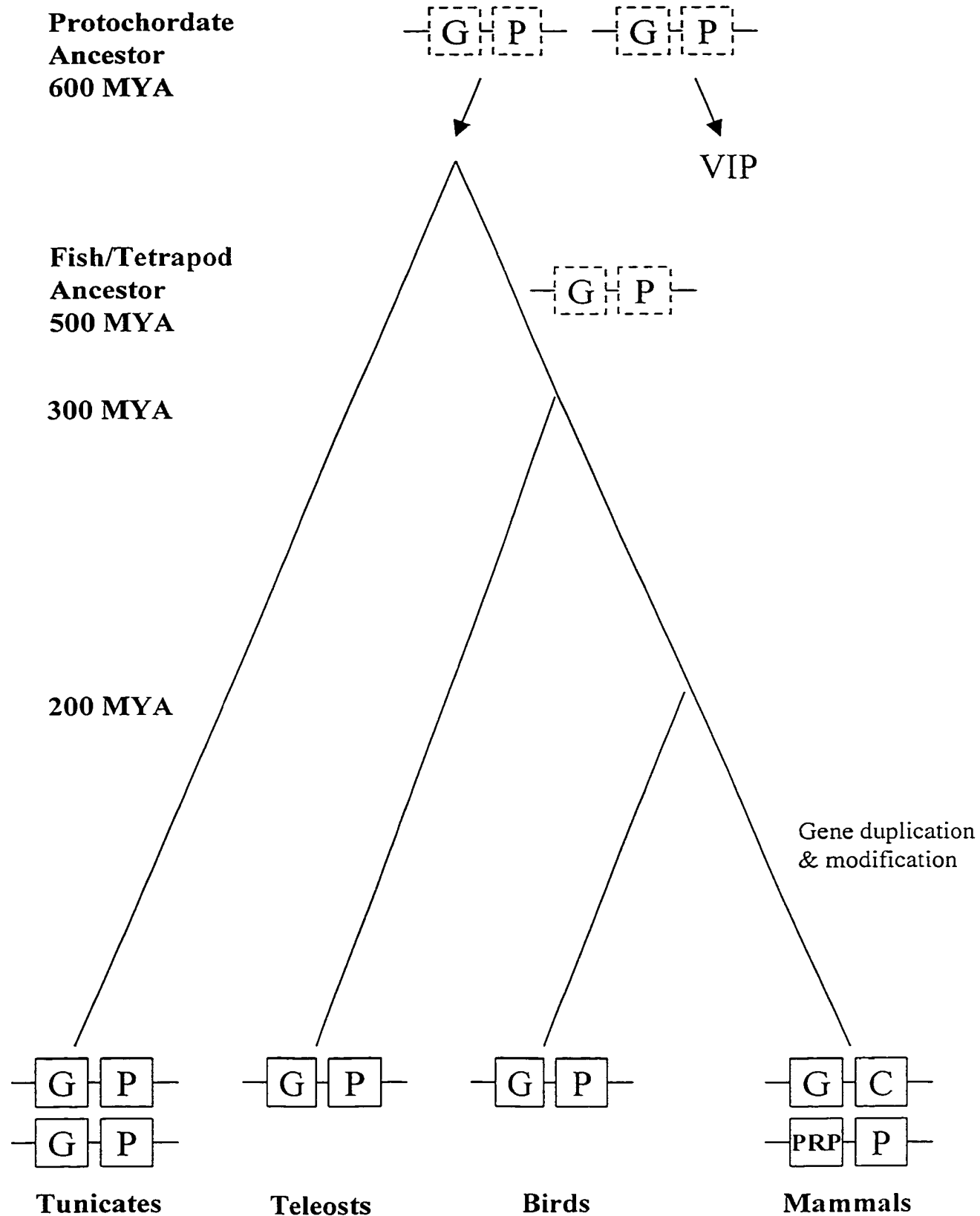
**prp/pacap gene**  
Mammals



**grf gene**  
Mammals



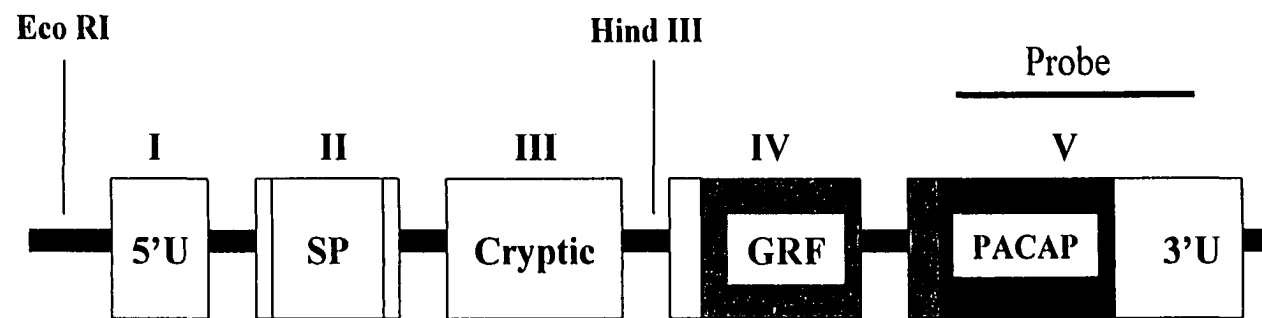
**Figure 5-3.** A diagram of proposed scheme 1 of the hypothetical evolution of grf and pacap exons from invertebrates to mammals. Exons are represented by boxes. Solid lines indicate that the nucleotide sequence has been identified and dashed lines indicate no nucleotide sequence is available. **G** represents the grf exon, **P** represents the pacap exon, **PRP** represents the pacap-related peptide exon, **C** represents the C-terminal peptide exon and **VIP** represents the vasoactive intestinal peptide exon.



genes in protochordates, based on gene/cDNA findings in tunicate (McRory and Sherwood, 1997). Both fish and birds have only one grf/pacap gene (based on sequence data) (Parker et al., 1997; McRory et al., 1997). A gene duplication event needs to occur early in the mammalian lineage, after the divergence of birds. This duplication event leads to separate grf and pacap genes (Sherwood et al., 2000). Compared to the third scheme I will present this scheme provides a shorter time frame for duplication and modification of the two grf and pacap genes in mammals.

Evidence in teleost fish suggests there may be two copies of the grf/pacap gene. Salmon and many other teleosts (e.g., carp, goldfish) are tetraploid (Ohno et al., 1968). Therefore it seems logical that two grf/pacap genes exist in these fishes. The Southern analysis of rainbow trout presented in this chapter supports this view (Figure 5-1). Figure 5-4 shows that the probe was designed to exon 5 (PACAP and 3'UTR). The genomic DNA was digested with four enzymes, Eco RI, Hind III, Sma I and Xho I. Based on the sockeye salmon sequence the enzymes Sma I and Xho I do not cut within the gene. Eco RI cuts in the promoter and Hind III cuts twice (promoter and intron 3). All cut sites within the gene are outside of exon 5 and therefore do not cut within the probe (figure 5-4). Figure 5-1 shows that there are two bands in lane 1 (Eco RI) and although fainter, there are two bands in lane 3 (Sma I). This indicates the sequences vary and the probe is probably binding to two regions in the genome. The probe (239 bp) used in this analysis aligned with 53 bp in the chicken VIP sequence. There is a risk that the second band is VIP. However, the wash conditions were stringent (low salt) and increasing the stringency (by an increase in temperature) resulted in the loss of both bands at the same time. Therefore, I think the bands represent two grf/pacap genes. These results are in agreement with a Southern done in sockeye salmon that also identified two grf/pacap bands (Parker et al., 1993). Results based on Southern analysis cannot rule out allelic polymorphisms. However, the findings in both species (Parker et al., 1993) support the theory that a genome duplication event happened at some point in the teleost lineage. Without evidence

**Figure 5-4.** Schematic diagram of the salmon grf/pacap gene. The roman numerals (**I-V**) indicate exon numbers 1-5. **5'U** and **3'U** refer to the 5' untranslated region and 3' untranslated region respectively. The locations of the **probe** and the two enzymes that cut within the gene (**Eco RI** and **Hind III**) are indicated.

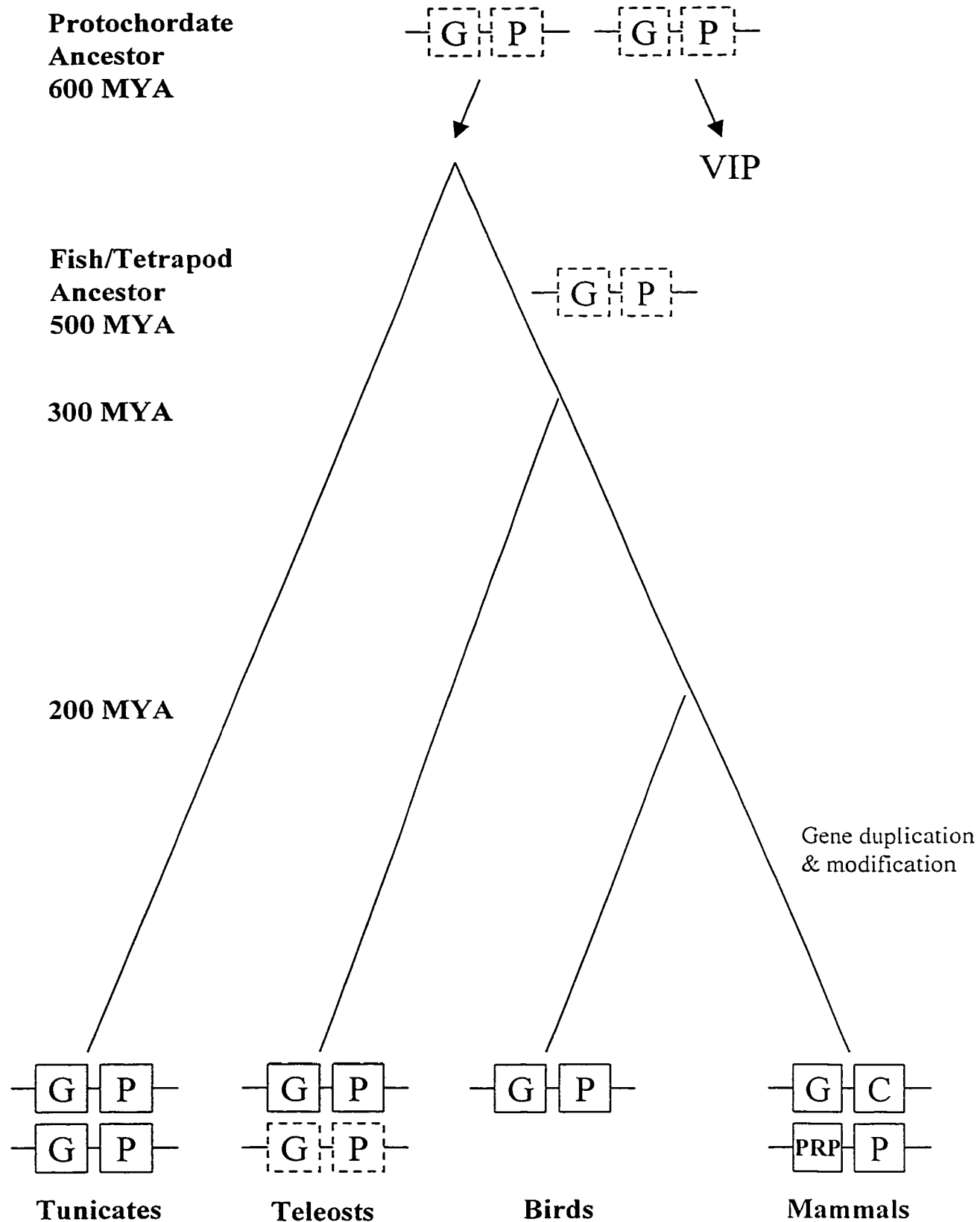


**Sockeye Salmon *grf/pacap* gene**

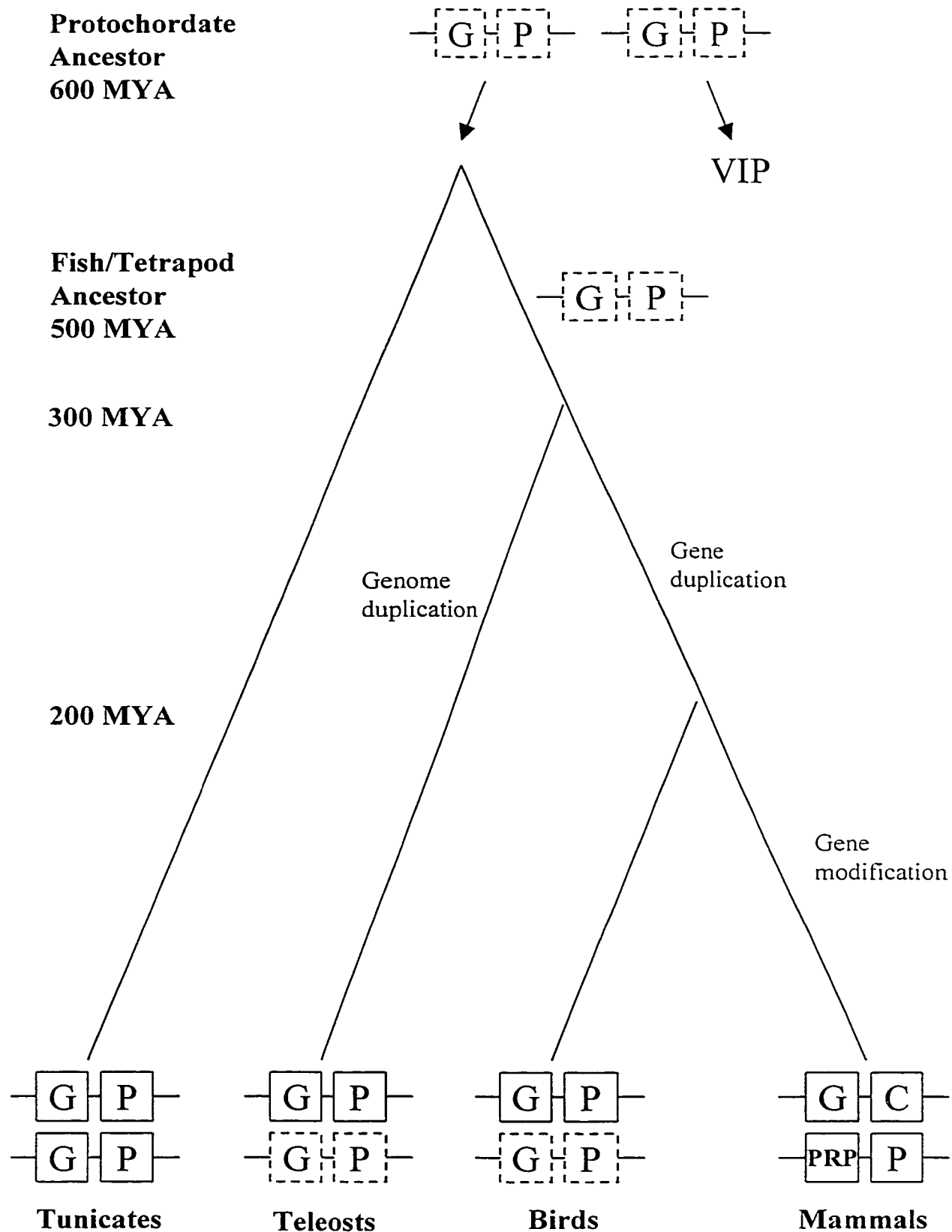
that duplicate grf/pacap genes exist in amphibians, reptiles or birds, the duplication event must be put after the divergence of ray-finned and lobe-finned fishes (approximately 500 million years ago). This precludes it from being the duplication event that led to the grf and pacap split in mammals. To date a second grf/pacap gene has not been found in birds, and only one grf/pacap gene cDNA sequence has been determined for amphibians (Hu et al., 2000b). Figure 5-5 shows this second scheme. As in the first scheme presented this scenario indicates a shorter evolutionary time-frame for gene duplication and divergence in mammals compared to the following scenario.

A third and final scenario suggests that there has been a gene duplication event prior to the divergence of birds (figure 5-6). In this case a genome duplication event occurs in fish leading to tetraploidization in some teleosts and consequently two grf/pacap gene copies. This is the same as the second scenario (figure 5-5). Then, a gene duplication occurs in birds or perhaps even earlier in amphibians or reptiles, leading to two grf/pacap genes. This duplication event is supported by a Southern analysis done in chicken (McRory et al., 1997). A Southern blot of chicken genomic DNA with a PACAP probe revealed the presence of two genes. The second gene was proposed to be a closely related family member, VIP (McRory et al., 1997). VIP and PACAP have 89% nucleotide sequence identity over a 56 base pair region contained within the probe; therefore cross-hybridization is possible. However, the remaining nucleotides of the 294 base pair probe have only 31% identity to VIP. Therefore, this does not exclude the possibility that the second band observed in the Southern blot is a duplicate grf/pacap gene. Also, high stringency washes did not remove the second band. I propose that this duplication event gives rise to the second gene in mammals (figure 5-6). This final scenario would allow more time for the modifications that eventually lead to separation of the grf and pacap in mammals.

**Figure 5-5.** A diagram of proposed scheme 2 of the hypothetical evolution of grf and pacap exons from invertebrates in mammals. Exons are represented by boxes. Solid lines indicate that the nucleotide sequence has been identified and dashed lines indicate no nucleotide sequence is available. **G** represents the grf exon, **P** represents the pacap exon, **PRP** represents the pacap-related peptide exon and **VIP** represent vasoactive intestinal polypeptide.



**Figure 5-6.** A diagram of proposed scheme 3 of the hypothetical evolution of grf and pacap exons from invertebrates in mammals. Exons are represented by boxes. Solid lines indicate that the nucleotide sequence has been identified and dashed lines indicate no nucleotide sequence is available. **G** represents the grf exon, **P** represents the pacap exon, **PRP** represents the pacap-related peptide exon and **VIP** represents vasoactive intestinal peptide.



At this time all three of the evolutionary schemes leading to separation of grf and pacap in mammals are speculative. In the future it would be valuable to fill in the gaps by determining the second gene hybridizing to PACAP probes in fish and chicken. Also, the grf/pacap gene sequence has not been determined in amphibians or reptiles. Identifying gene or cDNA sequences at key evolutionary points would greatly aid in our understanding of the evolutionary pathway leading to grf and pacap separation.

## **Chapter 6**

### **General Discussion**

## Introduction

The primary focus of my research has been to examine the expression of the neuropeptides, GRF and PACAP in fish. To accomplish this goal I have used RT-PCR analysis to examine expression of grf/pacap mRNA throughout various stages of rainbow trout development. Localization of grf/pacap expression was studied in an important developmental model, the zebrafish. In addition, overexpression studies were attempted in various salmonids.

A second area of interest to me is the evolution of the grf/pacap gene. Due to the tetraploid nature of salmonids I felt it was necessary to analyze rainbow trout to assess the number of copies of the gene. This information was compared with analysis of grf and pacap gene expression in other organisms and three evolutionary pathways for grf/pacap evolution were proposed.

GRF and PACAP are members of the PACAP/glucagon superfamily. The family is proposed to have developed from an ancestral PACAP-like molecule in invertebrates. Through successive exon, gene and genome duplications the family also includes seven other members, glucagon, GLP-1, GLP-2, GIP, PHM, secretin and VIP. In mammals GRF and PACAP are located on different genes, but in fish and birds they are located on the same gene in different exons (Sherwood et al., 2000).

The functions of GRF include the release of GH from the pituitary and the stimulation of somatotroph proliferation and differentiation. Through stimulation of GH, GRF is linked to growth. GRF expression has been localized to the brain, particularly the hypothalamus and it is found in the gonads, placenta, pancreas and gastrointestinal tract. During development in mammals, the action of GRF on GH secretion seems confined to late gestation and after birth (Chapter one and Sherwood et al., 2000). In contrast, the actions of PACAP are extremely varied. PACAP is expressed throughout the CNS, particularly in the hypothalamus, brainstem and spinal cord. PACAP is also found in the

peripheral nervous system innervating several organs (e.g. eye, pituitary, gastrointestinal tract, reproductive tract, heart, and pancreas). Outside of the nervous system PACAP is expressed in the adrenal glands, gonads, immune cells and pancreas. As to function, endocrine/paracrine secretions by the pituitary, adrenal gland, pancreas and heart as well as other organs are regulated by PACAP. PACAP also regulates smooth muscle in the vascular system, gut, respiratory tract and reproductive tract. During development in rats and mice, PACAP has been examined in the brain, especially the cerebellum and neural tube. It has been proposed to have a role in proliferation, differentiation and apoptosis during development (Chapter 1 and Sherwood et al., 2000).

### **GRF/PACAP and development**

My research examined expression of grf/pacap in rainbow trout and zebrafish embryos. In rainbow trout, RT-PCR analysis revealed that grf/pacap was first detected on embryonic day 4 (Chapter 2). At this stage in development the embryo is termed a blastodisc (stage 7-8); this is during the blastula period, before gastrulation (Ballard, 1973). Expression of grf/pacap may occur earlier but remain undetected by RT-PCR. The earliest zebrafish stage studied was the bud stage (Chapter 3). This is a later stage of development than the E4 rainbow trout embryo because at the bud stage gastrulation is complete (Kimmel et al., 1990, 1995). Therefore, if grf/pacap expression is parallel in rainbow trout and zebrafish, I expect that expression of grf/pacap probably occurs in earlier stages of zebrafish development (e.g. gastrulation). Unlike mammals, fish and birds encode GRF and PACAP on the same gene. Therefore, in order to separately regulate expression of the two hormones, a means of skipping the grf exon (alternative splicing) has been employed in salmonids and chickens (Chapter 2, Parker et al., 1997; McRory et al., 1997). The data presented in chapter two are the first indication that the embryo employs the same mechanism to separately transcribe pacap. The preferential expression of pacap over grf seems to make sense considering the wide array of functions attributed to PACAP. In

addition, PACAP seems to have a more extensive role in development than does GRF, at least in mammals. For these reasons I expected to see expression of the short transcript (encoding PACAP) early in development with the addition of the long transcript (encoding GRF and PACAP) later during embryogenesis. However, in rainbow trout, both transcripts were expressed continuously beginning on embryonic day 4. This leads me to speculate that, contrary to the situation in mammals, GRF is important in early development. Interestingly, in zebrafish, there is no evidence of alternative splicing of the *grf/pacap* gene in the adult (Fradinger and Sherwood, 2000). RT-PCR analysis of the zebrafish embryo has not yet been done, so it is not clear whether the embryo uses alternative splicing. In rats GRF is first detected toward the end of development at embryonic day 18 (Frohman and Jansson, 1986). GRF is involved in growth, proliferation and differentiation of somatotrophs (Mayo et al., 1985, 1988; Lin et al., 1992). Therefore it is not inconceivable that GRF could have the same array of effects on other tissues during development.

In situ hybridization studies in the zebrafish embryo revealed that *grf/pacap* is widely expressed (Chapter 3). It appears to be expressed ubiquitously early on in development (bud stage - 10 hours), and as development proceeds its expression becomes more localized. PACAP can downregulate an important patterning gene, *shh* (Waschek et al., 1998), and it is expressed in the mouse neural tube midway through development at day E9.5 (Sheward et al., 1996; Waschek et al., 1998). Considering the early expression of *grf/pacap* in mice and rainbow trout, I felt it was important to localize expression of *grf/pacap* and determine whether it is expressed in a fashion consistent with other patterning genes. Although the in situ hybridization work is preliminary, it does suggest that one or both of the hormones are involved in patterning. Other patterning genes such as *Bmp* and *shh* appear to share some regions of expression with *grf/pacap* (Dickensen et al., 1995; Liem et al., 1995, 1997; Krauss et al., 1993; Schier et al., 1997). Also, developmental mutants such as *one-eyed pinhead* and *cyclops* have disruption of prechordal plate derived structures such as the gut and hatching gland (Shier et al., 1997; Warga et al., 1999). The

grf/pacp transcript is expressed in these regions and in the ventral neuroectoderm which is influenced by prechordal plate gene expression. This suggests that grf/pacp may be expressed in the prechordal plate, an important organizing center during development. In addition the role of grf/pacp in heart development could render this gene important in the study of congenital heart defects.

### ***Future directions***

My work presents a strong case for further research into the possible patterning effects of grf/pacp on vertebrate development. This work is already being followed up with sectioning of the wholemount material. This will make it possible to specifically define the regions of grf/pacp expression. Also, work in prechordal plate mutants (*cyclops* and *one eyed pinhead*) will make it possible to assess whether grf/pacp is expressed in the prechordal plate, an important organizing center. Finally, examination of more developmental stages, particularly during epiboly and somitogenesis will make the study complete.

## **Overexpression of GRF/PACAP**

I believe overexpression of GRF and PACAP is a valuable tool for understanding the functions of these hormones. However, for a transgenic experiment in salmonids to work, I think it is necessary to have regular access to large numbers of fish. The technique is difficult to learn and must be done frequently in order for an individual to become proficient. Also, the low rate of success associated with transgenic work in salmonids (2-6%) makes a large sample size desirable (Devlin et al., 1994; Shears et al., 1992).

### ***Future directions***

First, it might be more valuable to attempt the transgenic experiments in mice where the technique is more reliable. GRF transgenic mice have been developed. They show a marked increase in growth rate compared to controls (Hammer et al., 1985). PACAP

transgenic mice have not yet been attempted. Second, zebrafish might be an easier fish model because they do not have a tough chorion, making microinjection more feasible. Also, microinjection techniques have been worked out in zebrafish. Overexpression studies with either the gene or mRNA should yield the desired information. Also, studies that allow for separate injection of grf and pacap could be attempted to try and unravel the individual actions of these hormones in fish.

### **Evolution of multigene families**

GRF and PACAP belong to the PACAP/glucagon superfamily. PACAP is the ancestral molecule in this family (Sherwood et al., 2000). The lineage leading to the GRF/PACAP subfamily has undergone exon, gene and genome duplications to enable the PACAP progenitor to produce two separate GRF and PACAP genes in mammals. In chapter 4, I presented three possible evolutionary scenarios for the GRF and PACAP lineage (figures 4-3, 4-4 and 4-5). It is thought that in invertebrates a single PACAP progenitor existed. Prior to tunicates a exon duplication gave rise to the grf and pacap exons (Sherwood et al., 2000). In tunicates two grf/pacap genes exist (McRory and Sherwood, 1997). Between tunicates and the fish/tetrapod ancestor two genome duplications led to the generation of other family members. In teleosts an additional genome duplication gave rise to a second grf/pacap gene (Chapter 4). A subsequent gene duplication in the tetrapod lineage allowed for the modification of the genes into separate grf and pacap genes in mammals (Sherwood et al., 2000). I believe this genome duplication occurred prior to the divergence of birds and mammals and that birds possess two grf/pacap genes. Evidence to support this theory was presented in chapter 4.

### ***Future work***

Studying multigene families and filling in the gaps within a gene family phylogeny is a valuable undertaking. It allows one to examine and test existing theories regarding the

mechanisms of molecular evolution, as well, it may give insight into new theories. The molecular evolution of the PACAP/glucagon superfamily is the least understood of the four families I have discussed. It would be beneficial to determine conclusively whether there is a second *grf/pacap* gene in fish and birds. Also, the gene sequences of other superfamily members are scarce in non-mammals. It is important to continue filling in these gaps with sequences from various phyla so that one day the evolutionary history of the superfamily may be understood. With each sequence that is added we gain clearer insight into the mechanisms of molecular evolution.

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