PRECURSOR AND GENE STRUCTURE OF A GROWTH HORMONE-RELEASING HORMONE-LIKE MOLECULE AND PITUITARY ADENYLATE CYCLASE ACTIVATING POLYPEPTIDE FROM SOCKEYE SALMON BRAIN

David B. Parker
B.Sc., Simon Fraser University, 1978
M.Sc., Simon Fraser University, 1985

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

DOCTOR OF PHILOSOPHY

ACCEPTED
FACULTY OF GRADUATE STUDIES
DEAN
DATE 24 Apr 92

We accept this thesis as conforming to the required standard

Dr. N.M. Sherwood, Supervisor (Dept. of Biology)

Dr. G.O. Mackie, Departmental Member (Dept. of Biology)

Dr. R.D. Burke, Departmental Member (Dept. of Biology)

Dr. T.W. Pearson, Outside Member (Dept. of Biochemistry)

Dr. C.L. Hew, (External Examiner)

© DAVID B. PARKER, 1992

University of Victoria

All rights reserved. This dissertation may not be reproduced in whole or part, by photocopying or other means, without permission of the author

Supervisor: Dr. Nancy M. Sherwood

ABSTRACT

Growth hormone-releasing hormone (GHRH) is a neuropeptide which stimulates the synthesis and release of growth hormone (GH) from the pituitary gland. The primary structure of this peptide has been identified in 7 mammalian species while the gene has been isolated from only rat and human. GHRH is a member of the glucagon superfamily which includes vasoactive intestinal peptide (VIP), glucagon, secretin, peptide histidine methionine (PHM), gastric inhibitory peptide (GIP) and a recently identified peptide, pituitary adenylate cyclase activating polypeptide (PACAP). The evolutionary relationships of this superfamily are not well understood because the gene structure of these molecules has only been identified in mammals. This thesis presents immunological evidence of a GHRH-like molecule, and identifies a GHRH/PACAP precursor and gene that encode two peptides, a GHRH-like molecule structurally related to PACAP-related peptide (PRP) and PACAP, from sockeye salmon brain.

An antiserum directed against a topologically assembled epitope of human GHRH 1-44 (NH_2) was produced and used to develop a radioimmunoassay for detection of immunoreactive GHRH in brain extracts of salmon, guinea pig, mouse and

alligator. An immunoreactive GHRH from salmon brain extracts with a retention time on reverse phase high-performance liquid chromatography (HPLC) distinct from human GHRH was present. In alligator, the same antiserum also detected a GHRH-like molecule. During attempts to purify alligator GHRH, alligator brain neuropeptide Y (NPY) was identified. Alligator NPY is the first non-mammalian vertebrate to have 100% sequence identity to human NPY. The sequence identity between alligator and human NPY suggests that this sequence is the same as the ancestral amniote NPY.

Molecular biological techniques were used for the structural identification of the salmon GHRH-like molecule and another peptide. The salmon GHRH/PACAP precursor contains 173 amino acids and has dibasic and monobasic processing sites for cleavage of a 45 amino acid GHRH-like peptide with a free acid C-terminus and a 38 amino acid PACAP with an amidated C-terminus. The salmon GHRH-like peptide has 40% amino acid sequence identity with the human GHRH and 56% identity with human PACAP-related peptide (PRP). Salmon PACAP-38 is highly conserved (89%) with only 4 amino acid substitutions compared with the human, ovine and rat PACAP-38 peptides.

Nucleotide sequencing and use of the polymerase chain reaction show the exon/intron organization of the salmon GHRH/PACAP gene to be similar to the human PACAP gene. Unlike the mammalian PACAP genes, the salmon gene produces two precursor forms by post-transcriptional processing. One

form is similar to the mammalian PACAP precursors, while the second form is shorter due to the excision of exon 4. This deletion results in the loss of the first 32 amino acids of the GHRH-like peptide from the precursor.

The high sequence identity and structural organization between the GHRH(PRP)/PACAP and PHM(PHI)/VIP genes suggest a duplication event occurred in an ancestral gene after the divergence from the other glucagon superfamily members. GHRH in mammals may have arisen by gene duplication after the divergence of the tetrapods from the other vertebrate lines. Thus, GH in fish may be controlled by the two molecules, GHRH-like peptide and PACAP, located on a single GHRH/PACAP gene.

Examiners:

Dr. N.M. Sherwood, Supervisor (Dept. of Biology)

Dr. G.O. Mackie, Departmental Member (Dept. of Biology)

Dr. R.D. Burke, Departmental Member (Dept. of Biology)

Dr. T.W. Pearson, Outside Member (Dept. of Biochemistry)

Dr. C.L. Hew, External Examiner, (Depts. of Clinical Biochemistry and Biochemistry, University of Toronto)

TABLE OF CONTENTS

ABSTRACT....................ii

TABLE OF CONTENTS....................v

LIST OF TABLES....................vii

LIST OF FIGURES....................viii

LIST OF ABBREVIATIONS....................xi

ACKNOWLEDGEMENTS....................xii

CHAPTER 1: General Introduction....................1

CHAPTER 2: Evidence for a growth hormone-releasing hormone-like molecule in salmon brain, *Oncorhynchus keta* and O. *kisutch*....................35
- Introduction....................36
- Methods and Materials....................38
- Results....................45
- Discussion....................66

CHAPTER 3: Primary structure of neuropeptide Y from brains of the American alligator (*Alligator mississipiensis*)....................69
- Introduction....................70
- Methods and Materials....................71
- Results....................76
- Discussion....................89

CHAPTER 4: Two Salmon neuropeptides coded in one brain cDNA are structurally related to growth hormone-releasing hormone (GHRH) and pituitary adenylate cyclase activating polypeptide (PACAP)....................93
- Introduction....................94
- Methods and Materials....................97
- Results....................103
- Discussion....................135

CHAPTER 5: Gene sequence of sockeye salmon GHRH-like and PACAP molecules: Evolutionary relationship to the glucagon superfamily....................151
- Introduction....................152
- Methods and Materials....................153
- Results....................157
- Discussion....................185

CHAPTER 6: General Discussion....................192
- Evolution of the glucagon superfamily....................193
- Evolution of the PACAP and VIP genes....................197

The possibility of another GHRH gene without PACAP in salmon..205
Structure/function relationships in the evolution of the PACAP and VIP genes..........................206
The function of the salmon GHRH/PACAP gene..........209
Future Directions..................................211

Literature cited.......................................213

LIST OF TABLES

Table 1.1 Growth hormone-releasing hormones..............8

Table 2.1 Cross-Reactivity of Ovine and Rat GHRH with hGHRH 1-44 using Antiserum 8119 (1:45,000)..........51

Table 3.1 Yield of phenylthiohydantoin (PTH)-derivatized amino acids....................................78

Table 6.1 A comparison of the exon encoding the salmon GHRH-like peptide or human GHRH domains with the respective exons of the human PHM and PRP genes..208

LIST OF FIGURES

Chapter 1

Figure 1.1 A schematic diagram showing the interaction of GHRH, SS, and IGF-I on the control of growth hormone release in mammals..........12

Figure 1.2 A schematic diagram showing the exon/intron organization of the human growth hormone-releasing hormone gene.(GHRH) and its mRNA....27

Chapter 2

Figure 2.1 Standard curve of hGHRH 1-44 NH_2 using antiserum 8119 at a final dilution of 1:45,000..........52

Figure 2.2 Binding of hGHRH fragments with three antisera developed against hGHRH 1-44 NH_2..............54

Figure 2.3 Immunoreactive fractions of coho and chum salmon brain crude extracts after reverse-phase HPLC....................................56

Figure 2.4 Reverse-phase HPLC of pooled fractions of coho or chum irGHRH.............................58

Figure 2.5 Reverse-phase HPLC of immunoaffinity purified material from chum salmon brains..............60

Figure 2.6 Reverse-phase HPLC of immunoaffinity purified material from chum salmon brains..............62

Figure 2.7 Reverse-phase HPLC of pooled fractions of chum salmon irGHRH..............................64

Chapter 3

Figure 3.1 Binding of VIP (human, porcine and rat), PHI (porcine), secretin (chicken) and NPY (human) compared to the hGHRH(1-44 NH_2) standard curve.......................................79

Figure 3.2 Initial purification strategy of alligator brain immunoreactive GHRH.....................81

Figure 3.3 An analytical rpHPLC run of immunoreactive fractions.....................................83

Figure 3.4 Final purification of immunoreactive material on HPLC.......................................85

Figure 3.5 Coelution of alligator NPY with synthetic human NPY and NPY-OH using rpHPLC..................87

Chapter 4

Figure 4.1 A schematic diagram showing the position of the primers used in the polymerase chain reaction of sockeye salmon cDNA..............111

Figure 4.2 A schematic diagram of the strategy used in the polymerase chain reaction (PCR)..........113

Figure 4.3 Photograph of sockeye salmon and catfish PCR amplification products run on a 2% agarose gel..................................115

Figure 4.4 Photograph showing a Pvu II digest of miniprep plasmid DNA obtained from cloned PCR products..................................117

Figure 4.5 Nucleotide sequence and the corresponding amino acid sequence of the sockeye salmon GHRH/PACAP cDNA..............................119

Figure 4.6 Nucleotide sequence of sockeye salmon cDNA clones obtained by the RACE technique (SS/RACE 2 and SS/RACE 7)..............................121

Figure 4.7 Photograph of the restriction enyzme digests of the sockeye salmon GHRH/PACAP clones SS/RACE 2 and SS/RACE 4 with Not I and Nco I..123

Figure 4.8 The nucleotide sequences of the GHRH/PACAP short precursor forms 125

Figure 4.9 A comparison of all the GHRH/PACAP precursor clones that contained a 5′ untranslated region..................................127

Figure 4.10 A comparison of the sockeye salmon prepro-GHRH/PACAP with the three mammalian PACAP precursors...........................129

Figure 4.11 A comparison of the 3′ untranslated region of the salmon preproGHRH/PACAP (clone SS/PCR 4) with the beginning of the 3 ′untranslated regions of human, ovine and rat preproPACAPs................................131

Figure 4.12 A Southern blot analysis of sockeye salmon brain genomic DNA..............................133

Figure 4.13 Salmon GHRH-like peptide sequence compared to other members of the GHRH family.........138

Figure 4.14 The salmon GHRH-like amino acid sequence (A) and the salmon PACAP-38 sequence (B) compared to the other members of the glucagon superfamily....................................142

Chapter 5

Figure 5.1 A photograph of the DNA bands from the PCR amplification of the positively hybridizing bacteriophage lambda clones..................164

Figure 5.2 A photograph of the restriction enyzme digests of lambda bacteriophage clones PL4 (A) and PL1 (B) run on a 0.8% agarose gel...........166

Figure 5.3 A photograph of a Southern blot of the restriction enyzme digests of PL4 (A) and PL1 (B)...168

Figure 5.4 A photograph of an Xba I digest of the sockeye salmon GHRH/PACAP lambda clone PL1...........170

Figure 5.5 A photograph of a Hae III digest of the sockeye salmon GHRH/PACAP lambda clone PL1...........172

Figure 5.6 A schematic diagram of the sockeye salmon GHRH/PACAP genomic clone PL1 and subclones...174

Figure 5.7 A schematic diagram showing the structure of the sockeye salmon GHRH/PACAP gene and mRNA (cDNA). 176

Figure 5.8 A comparison of the exon/intron organization of the salmon GHRH/PACAP gene with the human PACAP gene.............................178

Figure 5.9 DNA sequence of the salmon GHRH/PACAP gene...180

Figure 5.10 A schematic diagram of the proposed exon/intron organization of the GHRH/PACAP gene and the processing of the RNA transcript to obtain the short preprohormone.....................183

Chapter 6

Figure 6.1 A hypothetical scheme for the evolution of the glucagon superfamily......................195

Figure 6.2 A schematic diagram of the PACAP and VIP genes.......................................198

Figure 6.3 A schematic diagram of the hypothetical evolution of the VIP, PACAP and GHRH genes...203

LIST OF ABBREVIATIONS

AMP:	adenosine monophosphate
$(Bu)_2$cAMP:	N^6,2'-O-Dibutyryladenosine 3':5'-cyclic monophosphate
cAMP:	cyclic (adenosine 3',5'-cyclic monophosphate)
cDNA:	complementary DNA
Denhardts solution:	(50 X) 1% (w/v) Ficoll, 1% polyvinylpyrrolidone, 1% (w/v) bovine serum albumin
DNA:	deoxyribonucleic acid
DTT:	dithiothreitol
dNTP:	deoxyribonucleoside triphosphate
EDTA:	ethylenediaminetetraacetic acid
GH:	growth hormone
GHRH:	growth hormone-releasing hormone
GIP:	gastric inhibitory peptide
HFBA:	heptafluorobutyric acid
HPLC:	high performance liquid chromatography
IBMX:	3-isobutyl-1-methylxanthine
ir:	immunoreactive
mRNA:	messenger RNA
NPY :	neuropeptide Y
PACAP:	pituitary adenylate cyclase activating polypeptide
PBS:	phosphate buffered saline
PEG:	polyethylene glycol
pfu:	plaque forming units
PHI:	peptide histidine isoleucine
PHM:	peptide histidine methionine
PRP:	PACAP related peptide
RIA:	radioimmunoassay
RNA:	ribonucleic acid
RNase:	ribonuclease
rpHPLC:	reverse phase HPLC
SDS:	sodium dodecyl sulfate
SSC:	saline sodium citrate
TAE:	tris acetate EDTA
TBE:	tris borate EDTA
TEAF:	triethylammonium formate
TEAP:	triethylammonium phoshpate
TFA:	trifluoroacetic acid
Tris:	2-Amino-2-(hydroxymethyl)-1,3-propanediol
VIP:	vasoactive intestinal peptide

ACKNOWLEDGEMENTS

I would like to extend my greatest appreciation to my supervisor and friend Dr. Nancy Sherwood, for her guidance and support of this research work, and her direction in honing my skills to do good science.

I thank Dr. George Mackie, Dr. Terry Pearson and Dr. Robert Burke for their roles as committee members. I am grateful to Dr. Gordon Dixon of the University of Calgary for his advice in using the polymerase chain reaction and collaborating with this project.

To my colleagues David Lovejoy and Imogen Coe, whom I started graduate work with, a special gratitude and respect for their collaboration over the years. I thank my other fellow graduate students: John McRory for his excellent work with neuropeptide Y and those long hours in the cold room, Robin Munro for her words of wisdom while pouring sequencing gels, and David Lescheid and Kris Von Schalburg for their assistance. I also thank Carol Warby for teaching me those initial steps and her continued assistance.

This research was funded, in part, by the Science Council of British Columbia and General Sea Harvest Corporation as a Graduate Research Engineering and Technology Award to Nancy Sherwood and David Parker. Funding was also supplied by the Department of Fisheries and Oceans, Canada.

Chapter 1

GENERAL INTRODUCTION

Releasing hormones (releasing factors) belong to the group of molecules known as neuropeptides, which are molecules synthesized and secreted from neurosecretory neurons (Norris, 1980). These molecules not only act as neurohormones, but may also function as neurotransmitters and neuromodulators (Joosse, 1986). The theory of neurosecretion was originally proposed by Speidel in 1919, and preliminary evidence of such a process was then observed by Scharrer (1928). Two years later Popa and Fielding (1930) proposed a portal system connecting the hypothalamus and the adenohypophysis, but it was not until 1947 that control of adenohypophysial hormone secretion by hypothalamic hormones (releasing factors), via this hypophysial portal link, was proposed by Green and Harris (1947), and demonstrated by Harris (1948), Green and Harris (1949) and others (see references in Harris, 1948). Finally, in the late 1960's and early 1970's the first releasing factor, thyrotropin releasing factor (TRF), was identified from porcine and ovine hypothalami (Boler *et al.*, 1969; Schally *et al.*, 1969; Burgus *et al.* 1970; Nair *et al.*, 1970). Within eleven years three other releasing factors were isolated and characterized from mammals: gonadotropin-releasing hormone (GnRH), which stimulates gonadotropin

release; corticotropin-releasing factor (CRF) which stimulates ACTH and ß-endorphin release; and one release-inhibitory factor, somatostatin, which inhibits growth hormone (GH) release. In contrast, the isolation of a factor that stimulates growth hormone release was more difficult. Reichlin (1960) first reported evidence of a growth hormone-releasing hormone in mammals. After 22 years of trying to isolate growth hormone-releasing hormone (GHRH) from brain tissue, researchers finally purified GHRH from a pancreatic tumor in sufficient quantity for sequence analysis (Esch *et al.*, 1982; Guillemin *et al.*, 1982; Rivier *et al.*, 1982). GHRH isolated from the human hypothalamus was shown to be identical to this pancreatic form and to be specific for GH release in mammals.

GHRH is a relatively new member of the neuropeptides and unlike the other hypothalamic releasing hormones, GHRH has not been identified in any non-mammalian vertebrates. The identification of GHRH in mammals opened the door to a new family of peptides that had yet to be investigated. It was at this juncture that I initiated a research program to determine whether GHRH was present in the non-mammalian vertebrates. My objective was to understand the evolution of the GHRH family and to elucidate its role in the lower vertebrates.

Polypeptide Precursors

From their studies on the vasopressin system, Sachs and Takabatake (1964) hypothesized that bioactive peptides are products of larger polypeptide precursors. It is now well established that most neuropeptides are derived from larger precursor proteins containing at least three distinct regions: a signal peptide, the biologically active peptide and a cryptic region. Processing of neuropeptide precursors during transport to the axon terminal was originally demonstrated in the rat neurohypophysial system (Gainer *et al.*, 1977). These polypeptide precursors are cleaved by proteolytic enzymes (see review by Schwartz, 1986) and undergo post-translational modifications during intracellular transport from the rough endoplasmic reticulum to the Golgi apparatus, with final maturation in the secretory granules (Kelly, 1985). Differences in preprohormones can occur at the level of transcription or translation and have been found to be tissue specific (Acher, 1981; Mains *et al.*, 1983; Schwartz, 1986; Rehfeld *et al.*, 1991). The array of biologically active peptides generated from polypeptide precursors has placed these preprohormones into at least five categories: 1) a precursor containing multiple copies of the same bioactive peptide, 2) a precursor with one or more homologous peptides that are not identical, 3) a precursor with a single bioactive peptide, 4) a precursor containing one bioactive peptide that can form a variety of extended forms and 5) a precursor

that is processed into several different biologically active peptides (Rehfeld *et al.*, 1991).

Peptide Families

Neuropeptides are grouped into families or superfamilies of molecules that are structurally related. Comparison of their amino acid and nucleic acid sequences as well as gene and precursor structure is used to group the members of a family or superfamily. Peptides within a family usually have similar physiological functions, whereas peptides among the superfamily may have different biological activities (Niall, 1982). Gonadotropin releasing hormone (GnRH) is an example of a peptide that belongs only to a family, whereas peptides such as insulin, glucagon and GHRH belong in superfamilies.

Peptide families and superfamilies are believed to arise by several mechanisms. One way a family of peptides arises is by duplication of the entire gene. An example is the GnRH family of peptides, in which more than one form of the molecule is expressed in all vertebrates except for recently evolved mammals (Sherwood *et al.*, in press). The multiple forms of GnRH are encoded on separate genes rather than by tandem repeats within one gene. Another mechanism by which a superfamily, such as the one containing insulin, may have evolved is by a shift in the intron\exon splice sites in the ancestral gene, followed by gene duplication (Chan, *et al.*, 1990). An insulin-like peptide in Amphioxus with amino acid

similarity to insulin and the insulin-like growth factors (IGF) may represent a transition point in the evolution of the IGF genes from an ancestral insulin gene (Chan *et al.*, 1990). The resulting families include insulin, insulin-like growth factors, relaxin, and several related peptides identified from invertebrates. Both single exon and gene duplication may be the underlying mechanism that created the glucagon superfamily (Bell, 1986). Included in this superfamily are glucagon, vasoactive intestinal polypeptide (VIP), gastric inhibitory peptide (GIP), secretin and growth hormone-releasing hormone (GHRH). The presence of two or more structurally related hormones within one precursor for some members, suggests that single exons were duplicated and remained in tandem. Other superfamily members may have evolved by an additional complete gene duplication. The glucagon gene contains glucagon and two glucagon-like peptides (Heinrich *et al.*, 1984). The VIP gene is processed into two distinct peptides (Bodner *et al.*, 1985; Giladi *et al.*, 1990; Lamperti *et al.*, 1991) while secretin (Kopin *et al.*, 1991), gastric inhibitory peptide (Inagaki *et al.*, 1989) and GHRH genes contain only one bioactive peptide. A recently identified member of this family, the pituitary adenylate cyclase activating polypeptide (PACAP) gene, encodes two distinct peptides with little sequence similarity.

Neuropeptides within a family or superfamily may also be derived in evolution by transcriptional and post-

transcriptional changes, as well as translational and post-translational modifications (Acher, 1981). Calcitonin and calcitonin gene-related peptide (CGRP) are examples of different peptide products produced by differential splicing of the primary transcript producing two distinct mRNAs (Amara *et al.*, 1982). Differential processing of polypeptide precursors often correlates with tissue specific expression. The precursor for calcitonin is found predominately in the thyroid, while CGRP is the bioactive product in the hypothalamus (Amara *et al.*, 1982).

Cholecystokinin (CCK) (Gubler *et al.*, 1984) and proopiomelanocortin (POMC) (Eipper *et al.*, 1986) are two precursors that demonstrate differential post-translation into different peptide products. Both CCK8 and CCK33 are produced in the gut while only CCK8 is found in the brain (Eng *et al.*, 1983). Processing of the POMC precursor in the anterior pituitary yields ACTH as the major product, but processing of the same precursor in the intermediate lobe produces α-melanotropin and corticotropin-like intermediate lobe peptide [ACTH 18-39] (see review by Eipper *et al.*, 1986).

Relatedness of bioactive peptides within a family or superfamily may be shown also by their affinity for the receptor(s) of another member (Laburthe *et al.*, 1983). Within the glucagon superfamily, PHI, secretin, PACAP and GHRH cross-react with the VIP receptor (Laburthe *et al.*, 1983).

The Growth Hormone-Releasing Hormone Family (GHRH)

The GHRH family of peptides belongs to the glucagon superfamily which includes glucagon, secretin, vasoactive intestinal peptide (VIP), peptide histidine methionine (PHM), pituitary adenylate cyclase activating polypeptide (PACAP) and gastric inhibitory peptide (GIP). Other structurally related molecules that have been placed in this superfamily are helodermin (Hoshino *et al.*, 1984), helospectin (Parker, *et al.*, 1984) and exendin-3 (Eng *et al.*, 1990), which were isolated from Gila monster venom. The gene organization of the glucagon superfamily is similar; all are 9-10 Kb in length and the different precursor domains are encoded by separate exons (Bell, 1986).

Mammalian Growth Hormone-Releasing Hormones

GHRH has been isolated and sequenced from the hypothalamus of seven different mammalian species. Originally, three bioactive forms of GHRH were isolated and characterized from human pancreatic (hp) tumors: a 44 residue peptide with an amidated carboxy terminus (Guillemin *et al.*, 1982), and two forms having 40 and 37 residues with a free acid carboxy terminus (Esch *et al.*, 1982; Guillemin *et al.*, 1982; Rivier *et al.*, 1982; Spiess *et al.*, 1982). Subsequently, human hypothalamic GHRH was found to contain two forms, a 1-40 (OH) and a 1-44 (NH_2), which are identical

in structure to the GHRH pancreatic forms (Bohlen *et al.*, 1983a). Except for rat and mouse GHRH, which have 43 and 42 amino acids, respectively, and do not have an amidated carboxy terminus, all of the known GHRH peptides are 44 residues with an amidated carboxy terminus. Sequence identity among the mammalian GHRH's has not been highly conserved (Table 1.1). While porcine, bovine, caprine and ovine GHRHs have relatively few amino acid differences in comparison to human GHRH, rat and mouse have 14 and 15, respectively. The most highly conserved region of the mammalian GHRHs is the first 29 amino acids which are required for full biological activity.

TABLE 1.1

GROWTH HORMONE-RELEASING HORMONES

HUMAN	YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGESNQERGARARL	NH_2
PIG	·································**R**···**Q**···**V**··	NH_2
GOAT/COW	···························**N**·····**R**···**Q**··**KV**··	NH_2
SHEEP	············**I**··············**N**·····**R**···**Q**··**KV**··	NH_2
RAT	**H**······**S**···**RI**····**Y**····**HEI**·**N**·····**R**···**Q**−−·**S**·**FN**	**OH**
MOUSE	**HV**·····**TN**···**L**·**S**··**Y**···**VI**····**NK**·−··**RI**··**Q**−−·····**S**	**OH**

Factors Controlling the Release of Growth Hormone

The control of GH release is extremely complex and

involves the direct and indirect action of numerous factors including neuropeptides, hormones, neurotransmitters, steroids, amino acids and glucose. Intrinsic factors such as sex and age, as well as environmental status also modify the pattern of GH release.

A. *The control of growth hormone release by GHRH, somatostatin and insulin-like growth factor I*

In mammals, the pulsatile secretion of GH from the somatotropic cells of the pituitary gland is directly controlled by the two hypothalamic peptides GHRH and somatostatin (Tannenbaum and Ling, 1984). GHRH is synthesized in the neurons of the arcuate nucleus; somatostatin is present in the periventricular nucleus of the hypothalamus. These factors are released from axon terminals in the median eminence and transported to the pituitary gland via the hypophyseal portal system. In rats, the secretion of GH has an endogenous ultradian rhythm of about 3 hours (Tannenbaum and Martin, 1976; Tannenbaum, 1985). Tannenbaum and Ling (1984) hypothesized that somatostatin and GHRH are released tonically with rhythmic surges of each peptide 180° out of phase, which accounts for the trough and peak periods of GH release. Somatostatin inhibits GH release in a non-competitive receptor-mediated manner (Patel and Sirkant, 1986; Seifert *et al.*, 1985). Using *in situ* hybridization studies, Zeitler *et al.* (1990) showed that the rhythmic pattern of GH release may be

dependent on the rate of transcription and/or translation of GHRH and somatostatin. The rate of GHRH synthesis was greatest within the trough period of GH release.

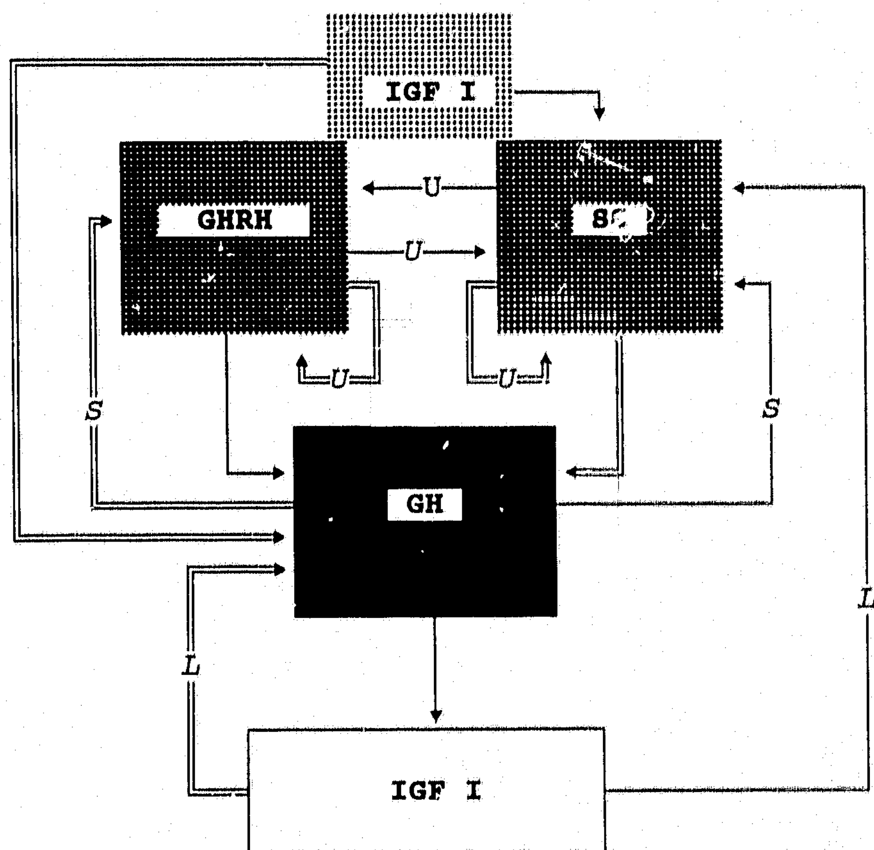
Insulin-like growth factor I (IGF-I) has also been implicated in having a direct effect on GH release. The origin of IGF-I acting at the level of the pituitary may be hypothalamic, systemic or both. It is not yet clear if hypothalamic IGF-I is secreted into the portal system to act directly on the somatotropic cells or if IGF-I acts indirectly by modulating somatostatin secretion within the median eminence (Wood *et al.*, 1991). IGF-I may act through both direct and indirect effects. The role of IGF-I in GH release may be a long-term inhibition (Berelowitz *et al.*, 1981), rather than a direct involvement in the rhythmic cycle. At least one study *in vitro* has shown a delayed effect of IGF-I inhibition on GH release from adenohypophyseal cells (Berelowitz *et al.*, 1981).

B. *Feedback regulation of growth hormone release*

The feedback regulation of GH secretion occurs at several levels: long, short and ultrashort feedback loops. In the long-loop feedback system, the insulin-like growth factors (IGFs) inhibit GH release by acting directly at the pituitary as well as at the hypothalamic level (Fig. 1.1). IGF I inhibits GH mRNA synthesis and GH release; it increases somatostatin and decreases GHRH mRNA levels. In the dwarf rat, the source of IGF I involved in hypothalamic

inhibitory and stimulatory effects appears to be from the central nervous system (CNS), not systemic circulation (Chomczynski *et al.*, 1988; Sato *et al.*, 1992). The role of IGF II is uncertain, but it may be involved in inhibiting the GHRH stimulated GH release. GH regulates its own secretion via a short-loop feedback system by stimulating the synthesis and release of somatostatin (Zeitler *et al.*, 1990), and inhibiting the synthesis (Chomczynski *et al.*, 1988; Sato *et al.*, 1992) and release of GHRH (Fernández *et al.*, 1991 in Devesa *et al.*, 1992) (Fig. 1.1). In hypophysectomized rats, however, GH replacement therapy neither depressed GHRH mRNA (Wood *et al.*, 1991) nor increased somatostatin mRNA levels to normal (Chomczynski *et al.*, 1988; Sato *et al.*, 1992). The reason for the discrepancy in the GH feedback regulation of GHRH mRNA levels is unclear, but may be related to the inability of experiments to reproduce the intrinsic GH secretory pattern (Wood *et al.*, 1991). However, infusion of GH did restore hypothalamic IGF I mRNA levels in hypophysectomized rats (Wood *et al.*, 1991). GH does not appear to feedback at the pituitary to inhibit its own release (Kraicer *et al.*, 1988). GHRH and somatostatin act in an ultra-short loop feedback system via autocrine or paracrine control (Lumpkin *et al.*, 1981, 1985) (Fig. 1.1). Somatostatin receptors have been shown on a subpopulation of GHRH neurons in the arcuate nucleus (Epelbaum *et al.*, 1989; Farhadi-Jou *et al.*, 1992). Binding of somatostatin to receptors on GHRH neurons is

Figure 1.1. A schematic diagram showing the interaction of growth hormone-releasing hormone (GHRH), somatostatin (SS) and insulin-like growth factor I (IGF-I) on the control of growth hormone (GH) release in mammals. Regions located within the brain are shown by stippling (▓ or ⁞). The pituitary gland is represented by the black box (█) and the liver by an open box. Single lines (|) represent stimulatory actions and double lines (‖) are inhibitory. The feedback regulation of GH secretion is denoted by *L* for long-loop, *S* for short-loop and *U* for ultrashort-loop.


IGF I
GHRH
U
U
U
U
S
S
GH
L
L
IGF I

greater during the GH peak, suggesting an involvement in the rhythmic cycle in which GH is released (Farhadi-Jou *et al.*, 1992).

C. *The control of GH release by thyrotropin releasing hormone (TRH)*

Thyrotropin releasing hormone (TRH) has been found to modulate GH release *in vivo* and *in vitro* in vertebrates. This effect on GH secretion is variable and depends on the mode of administration, type of *in vitro* assay, species, age of the animals, and type of anesthetic used. In humans with pathological disorders such as acromegaly, renal failure, anorexia nervosa and hypothyroidism, TRH stimulated GH release, although this was not observed in normal males (Giusti *et al.*, 1986). In contrast, TRH had no effect on GH levels in ether-anesthetized rats (Brown and Vale, 1975), but these observations have not been confirmed by others. TRH did produce a dose-related response in plasma GH levels when injected intravenously into urethane-anesthetized rats (Kato *et al.*, 1975) or into rats with hypothalamic lesions (Chihara *et al.*, 1976). Likewise, plasma GH levels increased in a dose-dependent manner after TRH was injected into the right lateral ventricle of anesthetized rats (Chihara *et al.*, 1976). A dose-dependent GH increase also occurs in intravenous injected conscious mediobasal hypothalamic lesioned rats, but not in normal conscious rats (Bluet-Pajat *et al.*, 1986). An explanation for the

differing response in anesthetized and conscious rats may have been the effect of anesthetics on other neurohormones that interfere with the release of GH. The response of animals with hypothalamic lesions to TRH suggests inhibitory factors may be present under normal conditions. Somatostatin has been found to attenuate TRH stimulated plasma GH levels in rats (Kato *et al.*, 1975) and ducks (Harvey *et al.*, 1988). Also, immunoneutralization of somatostatin elevates plasma GH levels in fowl (Harvey and Hall, 1987) and rats (Chihara *et al.*, 1976).

In vitro studies support the idea that TRH acts on the pituitary to regulate GH release. Szabo *et al.* (1984) found that TRH stimulated GH release in an anterior pituitary perfusion assay, but not in a monolayer culture. Hence TRH may have released a neurotransmitter or neuropeptide from neuron endings in the pituitary fragments. Bluet-Pajat *et al.* (1986) found that TRH stimulated GH release in incubated as well as perfused pituitary cells obtained from intact and hypothalamic-lesioned rats.

The mechanism by which TRH affects GH secretion has been studied by administering GHRH and TRH simultaneously, *in vitro* and *in vivo*. Additive effects of TRH and GHRH in stimulating GH release have been found *in vitro* (Perez *et al.* 1987) and *in vivo* (Woolny *et al.*, 1987). However, studies on the additive effects of releasing factors have been equivocal. In an experiment of multifactorial design, Wehrenberg *et al.* (1984) showed that the four releasing

factors, GHRH, CRF, TRF and GnRH had no interactive effects on the release of pituitary hormones *in vivo* and *in vitro*, in rat.

Other studies suggest that TRH acts on GH via a cAMP-independent but Ca^{2+}-dependent manner through different receptors than those used by GHRH, at least in rats. Thus, TRH and DBcAMP when administered together *in vitro* stimulated GH release in an additive manner, but calcium-blockers attenuated the GH stimulated release to TRH.

In fowl, TRH stimulated GH release in conscious and anesthetized chickens (Scanes and Harvey, 1988), while somatostatin inhibits the GH response to TRH (Harvey *et al.*, 1978). The interaction of human GHRH and TRH is also additive when administered to chickens (Harvey and Scanes, 1984). However, in ducks, intravenous infusion of TRH only stimulated GH plasma levels in young but not in old ducks (Harvey *et al.*, 1988).

The fact that hypothyroidism decreases GH release and plasma GH levels in rats (Coiro *et al.*, 1979) and increases the response of GH stimulation to TRH suggests a regulatory role of thyroxine. This may be only a permissive role in that the rate of metabolism in general depends on thyroxine. T_4 administration to hypothyroid rats returned the normal GH response to GHRH. In the anesthetized rat, intraperitoneal injection of T_3 or T_4 attenuated the plasma GH increase in response to TRH (Kato *et al.*, 1975). T_3 enhanced GH release of rat pituitary cell cultures to GHRH stimulation (Vale *et*

al., 1983). Szabo *et al.* (1984) suggested thyroid hormones may play a role in suppressing TRH receptors on somatotrophs. In contrast to rats, where T3 increased GH mRNA synthesis (Spindler *et al.*, 1982 in Brent *et al.*, 1988), GH mRNA accumulation was inhibited by T3 in bovine pituitaries (Silverman *et al.*, 1988). Rat and bovine GH promoters were highly responsive to T3, whereas the human GH promoter was not, in a chloramphenicol acetyltransferase (CAT) expression assay (Brent *et al.*, 1988). Increased GH synthesis and mRNA transcription have been found in all rat pituitary GH cell lines in response to thyroid hormone (Samuels *et al.*, 1989). A thyroid response element has been mapped to the 5' flanking region of the rat GH gene (see review by Samuels *et al.*, 1989; Karin *et al.*, 1990).

D. *Other factors that affect growth hormone release*

Other factors also regulate the control of GH secretion either directly or indirectly through the modulation of GHRH and somatostatin. Glucocorticoids have been found to increase GH transcription (Gertz *et al.*, 1987) and stimulate GH release. They also stimulate somatostatin release thereby inhibiting GH secretion. Many of the opposing effects of the glucocorticoids have been related to the timing of its application (Devesa *et al.*, 1992), the dose and the species used (Silverman *et al.*, 1988). Glucocorticoid response elements have been located on the human GH gene (Slater *et al.*, 1985), but not yet identified

in rat (Karin *et al.*, 1990). The human GH promoter activity was found to be enhanced by dexamethasone in a chloramphenicol acetyltransferase assay (CAT) while the rat GH promoter was not (Brent *et al.*, 1988).

The sex steroids have been implicated in the regulation of GH release because of the sexual dimorphism observed in the pulsatile patterns and amplitude of GH secretion in rats (reviewed by Jansson *et al.*, 1985 in Wehrenberg and Giustian, 1992). The role of androgens in GHRH synthesis and release is still not clearly understood. However, the available data, while conflicting, suggests that testosterone increases GHRH synthesis and secretion. Studies on the effects of estradiol on GHRH are even more ambiguous and show no conclusive pattern (Wehrenberg and Giustina, 1992).

Devesa *et al.* (1992) have recently reviewed the role that the neurotransmitters play in the control of GH release and concluded the adrenergic pathway acts mainly at the level of the hypothalamus by controlling somatostatin release. The stimulation of α2-adrenergic receptors inhibits somatostatin release whereas the activation of ß2-adrenergic receptors stimulates somatostatin release.

The effects of dopamine on GH release have been difficult to interpret. Devesa and colleagues (1992) suggest dopamine acts as a modulator by acting on the adrenergic system to affect the release of somatostatin.

The role of the cholinergic pathway in GH secretion seems

to be a positive modulation of the adrenergic pathway. High cholinergic activity causes a high catecholamine response, resulting in the inhibition of somatostatin release.

The Mechanism of Action of GHRH and Somatostatin Regulation of Growth Hormone Release

A. *A receptor mediated event*

The stimulation of growth hormone release from the anterior pituitary by GHRH is mediated by a class of specific receptors on bovine anterior pituitary cells (Velicelebe *et al.* 1985). High affinity binding sites for human GHRH, which do not bind VIP (Robberecht *et al.*, 1986) or PHI have also been demonstrated on rat anterior pituitary cells (Seifert *et al.*, 1985; Campbell *et al.*, 1991). In contrast, human GHRH and VIP bind to a VIP preferring receptor in the rat pancreas (Waelbroeck *et al.*, 1985) and liver, (Robberecht *et al.*, 1986; Christophe *et al.*, 1986). A similar observation was found for guinea pig exocrine pancreatic cells (Pandol *et al.*, 1984).

The possibility that GHRH 1-29 may form an amphiphilic secondary structure and that this π-helix may be important in receptor membrane binding had been proposed by Kaiser and Kedzy (1984). To test this hypothesis, Tou *et al.* (1986) synthesized four 1-29 amidated peptides with increased amphiphilicity and helix content. These peptides, which had up to 8 amino acid substitutions, had higher potency than

bovine GHRH 1-44 (NH_2) when injected intraventricularly into sheep. The increased potency of the D-analogue forms of GHRH 1-29 could be due, in part, to an increase in helix stability or amphiphilic content of the molecule. More recently, Campbell *et al.* (1991) found that increasing the amphiphilicity of GHRH 1-29, by replacing Gly^{15} with Ala, stabilized the helix character of the molecule. Furthermore, $[Ala^{15}]$hGHRH 1-29 had a higher binding affinity to rat anterior pituitary cells.

B. *Intracellular paths*

GHRH is one of a group of peptide hormones that bind to cell surface receptors and activate adenylate cyclase, which in turn stimulates cyclic AMP accumulation (Baxter *et al.*, 1979). *In vivo* and *in vitro* assays using indirect (inhibitors and non-GHRH agonists) and direct methods have shown GHRH to stimulate GH secretion, cAMP levels (Bilezikjian and Vale, 1983; Cronin *et al.*, 1984; Law *et al.*, 1985) and adenylate cyclase activity (Reyl-Desmars *et al.*, 1985a, 1985b). Cholera toxin, which inhibits the GTPase activity of the stimulatory guanyl-nucleotide-binding protein (*Gs* protein) resulting in continuous stimulation of adenylate cyclase, and forskolin, which stimulates the catalytic subunit of adenylate cyclase, have been found to increase cAMP levels and GH secretion (Cronin *et al.*, 1984; Guillemin, 1986). In a rat anterior pituitary cell culture, pertussis toxin, which inactivates the inhibitory G protein

(*Gi* protein) complex, enhanced hGHRH stimulated GH and cAMP release (Cronin *et al.*, 1984). Although many studies infer that GHRH-stimulated GH secretion is regulated by the adenylate cyclase-cAMP system, other pathways may also be involved. Higher concentrations of GHRH are required to stimulate adenylate cyclase activity than GH release in pituitary adenomas and rat pituitary cell cultures (Bliezikjian and Vale, 1983; Lewin *et al.*, 1983; Spada *et al.*, 1984). Bilezikjian and Vale (1983) suggest this difference in adenylate cyclase activity and GH release might be explained by the spare receptor hypothesis or compartmentalization of cAMP. Lewin *et al.* (1983) proposed an alternative mechanism of GHRH-stimulated GH exocytosis via phosphorylation of a receptor-linked cAMP dependent protein-kinase on secretory granules. This would fit the idea of compartmentalization of cAMP for immediate GH release.

Calcium is also required for GH release because the calcium blockers $CoCl_2$ and verapamil completely inhibited the GHRH-stimulated intracellular Ca^{2+} rise (Holl *et al.*, 1988). Also, $CoCl_2$ partially inhibits basal and stimulated GH release (Bilezikijian and Vale, 1983; Guillemin, 1986;). When anterior pituitary cells were incubated with the Ca^{2+} channel blocker verapamil, with or without hGHRH or BrcAMP, GH release was inhibited, but cAMP was not (Bilezikjian and Vale, 1983) These results suggest that Ca^{2+} is required for GH exocytosis but not the accumulation of cAMP. A calcium

ionophore was also found to increase GH release while not affecting cAMP levels (see review by Frohman and Jansson, 1986). This seems to contradict the mechanism of cAMP-mediated exocytosis of GH proposed by Lewin *et al.* (1983). Alternatively, both a cAMP-independent mechanism as well as a cAMP-dependent mechanism(s) exist.

There is a possibility that a calcium-calmodulin system regulates GH release. Bilezikjian and Vale (1983) showed that pimozide, which inhibits the Ca^{2+}-calmodulin dependent system, also inhibits both basal and GHRH-stimulated GH release but not cAMP levels. Schettini *et al.* (1983, 1984) found basal and secretagogue-stimulated GH release and cAMP accumulation were inhibited *in vitro* by calmodulin antagonists, penfluridol and a naphthalenesulfonamide derivative W7. However, the concentration of W7 was very high, 50-100 μM, suggesting the possibility of pharmacological effects. W7 attenuated hGHRH 1-40 stimulated adenylate cyclase activities, although basal levels were not affected. Apparently, W7 does not affect Ca^{2+} uptake, inferring that the Ca^{2+}-calmodulin complex is involved in the activation of the adenylate cyclase system.

C. *Cellular response*

GHRH 1-44 has been shown to differentially stimulate the release of stored and newly synthesized GH in rat anterior pituitary cell cultures and perfusion assays (Stachura *et al.* 1985, 1986). These authors postulated different

mechanisms by which GH is released: 1) hGHRH causes cell membrane depolarization, resulting in a rapid release of GH independent of cAMP and 2) a compartmentalization of cAMP results in higher local cAMP levels. These results confirm earlier work (see above) that suggested different pathways leading to GH release.

GHRH not only stimulates the release of GH but has been shown to affect GH synthesis (Barinaga *et al.*, 1983; Guillemin, 1986) and stimulate somatotropic cell proliferation (Frawley and Hoeffler, 1988). Increased mRNA levels were found when normal rat pituitary cells and GH3 cells were incubated with hGHRH 1-44 for 24 h (Guillemin, 1986). Barinaga *et al.* (1983) reported that GHRH regulates gene control as well as stimulating GH release in a rat pituitary cell culture and in rats *in vivo*. The induction of GH-gene transcription occurs within 15 min *in vivo*.

D. *An integrated model of GHRH and somatostatin intracellular action*

An integrated model of the control of GH release in rats by GHRH and somatostatin was recently presented (Lussier *et al.* 1991c). GHRH binds to its receptor, on somatotropic cells, stimulating cAMP which acts to increase Na^+ conductance. cAMP may also activate a protein kinase which in turn increases Na^+ conductance. The cell is depolarized allowing Ca^{2+} to move into the cell through voltage-sensitive channels (Holl *et al.*, 1988; Lussier *et al.*,

1991a,c). A *G* protein may also interact directly with the voltage channel to increase Ca^{2+} influx and/or a cAMP-dependent kinase may phosphorylate the calcium channels to increase the influx of calcium (Holl *et al.*, 1988). An increase in intracellular Ca^{2+} stimulates the calcium-calmodulin system and protein-kinase C which activates the Ca^{2+}-ATPase system to lower intracellular Ca^{2+}. High Ca^{2+} levels activate K^+ channels causing a K^+ efflux and repolarization of the cells. Furthermore, adenylate cyclase activity is inhibited at high Ca^{2+} concentrations thus reducing Ca^{2+} influx.

Somatostatin may act by receptor coupling to the *Gi* protein that inhibits adenylate cyclase (Patel and Sirkant, 1986; Reyl-Desmars *et al.*, 1985a). The evidence is that pertussis toxin can reduce somatostatin inhibition of secretagogue-stimulated GH secretion and cAMP accumulation in a pituitary cell culture (Cronin *et al.*, 1984). However, somatostatin does not inhibit basal cAMP levels (Patel and Sirkant, 1986) and basal adenylate cyclase activity (Schettini *et al.*, 1984; Reyl-Desmars *et al.*, 1985b). These and other studies suggest somatostatin might inhibit GH secretion by both cAMP dependent and independent mechanisms, the latter of which might involve the inhibition of Ca^{2+} fluxes (Bilezikjian and Vale, 1983). Law *et al.* (1985) found that somatostatin inhibited GH release and cAMP accumulation in the rat, but elevated cAMP levels were not inhibited in sheep even though GH release was blocked. An

in vitro study showed that somatostatin could completely block GH secretion but only partially inhibit cAMP accumulation (Bilezikzici and Vale, 1983). Also, pertussis toxin attenuated stimulated GH levels more than cAMP activity (Cronin *et al.*, 1984). Further evidence supporting the cAMP independent effect was presented by Holl *et al.* (1988), who showed somatostatin could decrease intracellular calcium concentration and block the GHRH-stimulated increase in intracellular calcium. Another mechanism in which somatostatin may inhibit GH release is by stimulating phosphodiesterase activity. This, however, does not appear to be the case since somatostatin attenuated cAMP levels, stimulated by hGHRH, even in the presence of the phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine (IBMX) (Bilezikjian and Vale, 1983). Thus, stimulation of the *Gi* protein by somatostatin may result in interaction with the voltage sensitive calcium channel to decrease Ca^{2+} influx and activate K^{+} channels to hyperpolarize the cells, thus inhibiting GH release (Lussier *et al.*, 1991b,c).

The Gene and Preprohormone Structure of the Mammalian Growth Hormone-Releasing Hormones

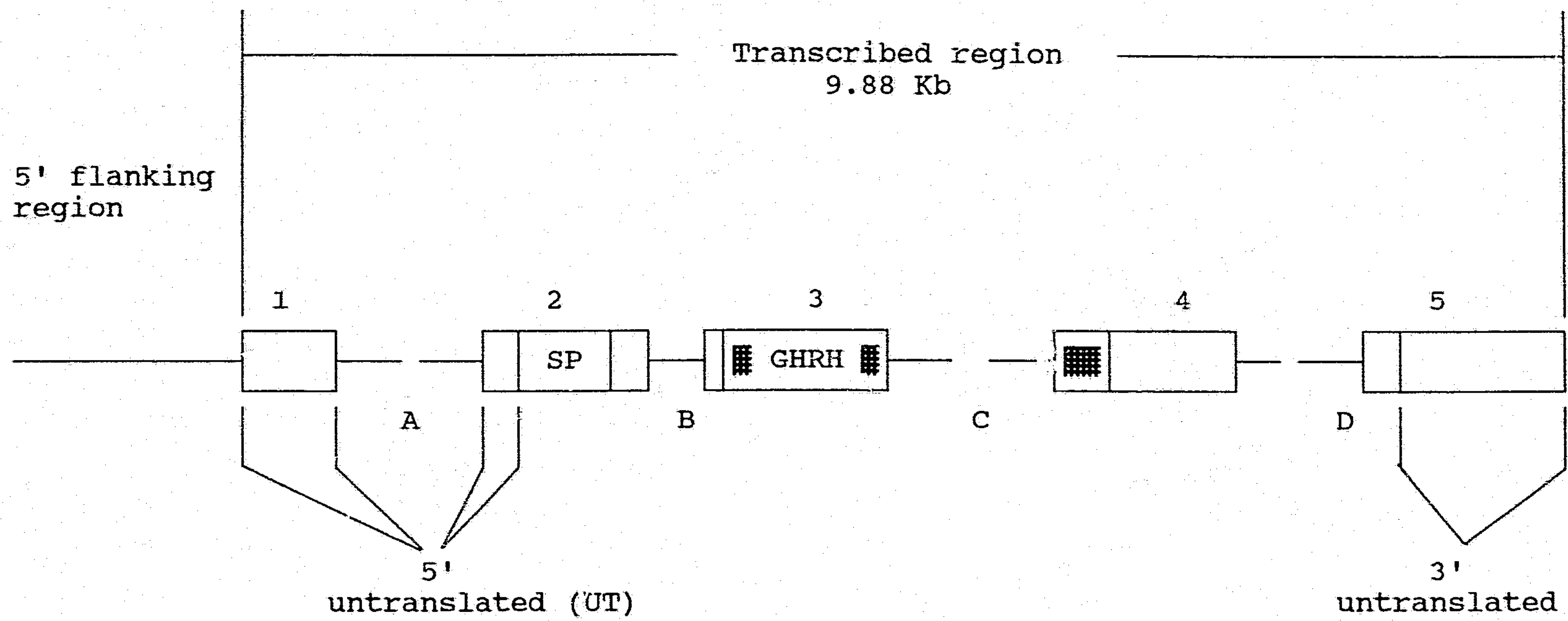
The GHRH cDNA has been identified for human (Gubler *et al.*, 1983; Mayo *et al.*, 1983), rat (Mayo *et al.*, 1985b) and mouse (Frohman *et al.*, 1989; Suhr *et al.*, 1989), and the gene for rat (Mayo *et al.*, 1985b) and human (Mayo *et al.*, 1985a). Both the human and rat have a single gene with a

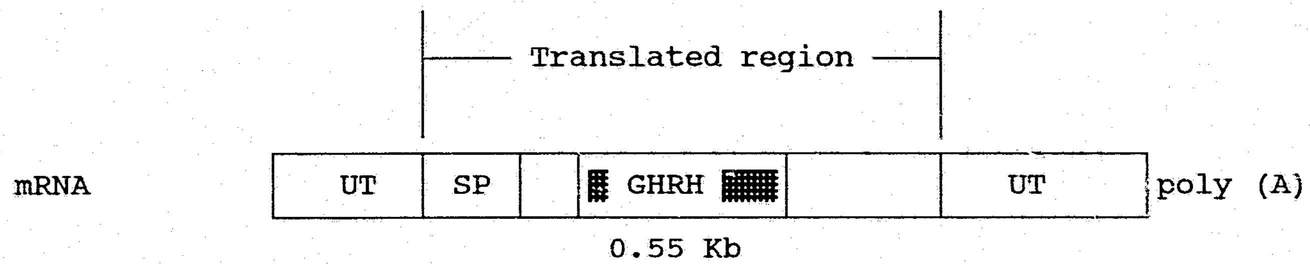
transcription region of approximately 10 Kb containing five exons. Within the transcribed region of the human GHRH gene, exon 1 contains most of the 5' untranslated (UT) region and exon 2 encodes the signal peptide and part of a small cryptic peptide (Fig. 1.2). Exon 3 encodes most of the mature GHRH peptide, with exon 4 encoding the carboxy terminus and the 30-31 residue peptide of unknown function (Fig. 1.2). The 3' UT region is encoded in exon 5 (Fig. 1.2). Comparative analysis of the human and rat genes has shown that the highest degree of identity (86%) lies in the 5'-flanking region (-23 to -109), which suggests the importance of this area in gene regulation (Mayo *et al.*, 1985b). Differences in the carboxy terminal end of the C-terminal peptide between rat and human are a result of changes in the donor splice site of intron D (Mayo *et al.*, 1985b). In the promoter region, both the human and rat GHRH hypothalamic genes contain a TATA box and CAAT region. However, the rat GHRH placental gene (see below), which uses an alternate promoter region, has no consensus TATA box. The only member of the glucagon superfamily lacking a TATA box is the PACAP gene.

The cDNA for hGHRH was isolated and sequenced from human tumor tissue using oligonucleotide probes and antibody screening techniques. Two forms of mRNA were identified, one coding for a precursor of 107 amino acids and the other for 108 residues. The 108 amino acid precursor has an additional serine residue located at position 103. The 107

Figure 1.2. A schematic diagram showing the exon/intron organization of the human growth hormone-releasing hormone (GHRH) gene and its mRNA. There are 5 exons (1-5) and 4 introns (A-D) in the gene. The region encoding the bioactive peptide (GHRH) is shown by the stippling. SP= signal peptide.

HUMAN GHRH GENE





and 108 precursors result from differential processing of the hnRNA at two alternative splice-acceptor sites resulting in two mRNAs. The human GHRH precursor is also unique among the mammalian GHRH precursors in that three peptide products, a 1-44 NH_2, 1-40 OH and 1-37 OH, are produced from post-translational processing.

Novel Locations and Functions

GHRH was originally thought to be restricted to the hypothalamus. However, GHRH in situ hybridization studies have detected mRNA in rat placenta (Margioris *et al.*, 1990), testis (Berry and Pescovitz, 1988, 1990) and ovary (Bagnato *et al.*, 1992). Rat placental mRNA and an immunoreactive GHRH extracted from placental tissue are of the same size as their hypothalamic counterparts. Hybridization studies have not detected a GHRH mRNA in human placenta. In testis and ovary, multiple mRNAs substantially larger than the hypothalamic GHRH mRNA were found in the rat. Testis and ovary contain a 1750 bp mRNA, as well as one larger form in the testis and two larger forms in the ovary, compared to 750 bp in the hypothalamus. In contrast to the testis, the ovary contains a translation product that is similar in size to the one in the hypothalamus (Bagnato *et al.*, 1992).

In mouse, the GHRH cDNA has been identified from placenta (Suhr *et al.*, 1989). Whereas the entire coding region of the cDNA for GHRH in the placenta and hypothalamus was found to be identical, the 5' untranslated regions were

completely different in nucleotides and length. This difference has been recently investigated by Mizobuchi *et al.* (1991). They found that the 5' untranslated region of the placental GHRH cDNA reported by Suhr *et al.* (1989) must have been a cloning artifact. Mizobuchi and coworkers (1991) report a different mouse placental 5' untranslated sequence as well as the cDNA for GHRH in rat placenta. Both 5' untranslated regions of the GHRH placental cDNAs differ from their respective hypothalamic cDNAs, but are similar to each other. These differences suggest that GHRH gene transcription is regulated differently in the placenta compared to the hypothalamus (Mizobuchi *et al.*, 1991). González-Crespo and Boronat (1991) have more clearly defined the 5' end of the rat placental GHRH cDNA. The sequence is identical to the rat cDNA isolated by Mizobuchi *et al.* (1991) except it extends further 5'. Analysis of the rat GHRH gene showed the placental 5' untranslated region to be located approximately 10 Kb upstream of the hypothalamic promoter region (González-Crespo and Boronat, 1991). This suggests that tissue specific regulation of the GHRH gene occurs in the rat.

It has been proposed that placental GHRH controls the release of GH in the embryo (Margioris *et al.*, 1990). This is based on two lines of evidence: 1) the fetal hypothalamus lags behind the development of the adenohypophysis, and 2) the hypophysial portal link is not complete before the second week of postnatal development.

Another function proposed for GHRH is in the ovary. Moretti *et al.* (1990) recently discovered that infertile women, resistant to gonadotropin therapy, can be treated with GHRH 1-29 (NH_2) and pure follicle stimulating hormone (FSH) to produce folliculogenesis and development of ovarian follicles. It was concluded that bioactive GHRH must help stimulate gonadotropic hormonal action on the ovaries. GHRH alone has no effect, but acts synergistically to increase granulosa cell responsiveness to gonadotropic stimulation.

Rat testicular GHRH may also be involved in spermatogenesis (Pescovitz *et al.*, 1990). This is implied by the localization of GHRH in the germ cells (Pescovitz *et al.*, 1990) and the low levels of testicular GHRH mRNA until the onset of puberty (Berry and Pescovitz, 1990).

Rationale

The evolution of a superfamily or family of peptides depends not only on gene duplication, but also on differential transcription, post-transcriptional processing, and tissue specific expression of these molecules. These mechanisms depend on specific sequences within the gene or mRNA. These regulatory regions were orginally thought to be located mainly in the 5' flanking region of the gene, but it has become evident that they are also within the introns, the 5' and 3'untranslated regions, and the 3'end (Brar *et al.*, 1989 in Gonzalez-Crespo and Boronat, 1991). To understand the regulation of transcription and therefore the

production of different peptide products, the sequence of the genes has to be determined. By studying the evolution of these molecules, our understanding of the principles of gene regulation is increased. Changes in the mRNA may also result in sequence substitutions within the precursor. An increase in the number of peptide products at the translational level is also related to the expression of the required processing factors in that tissue.

The evolutionary approach for identifying other members of a family of peptides also allows structure/function relationships to be elucidated. Conserved regions within a family of bioactive peptides may be related to receptor binding and/or activation. For the most part, the peptide coding region of the mRNA for a preprohormone is the most highly conserved region, whereas the cryptic and untranslated regions show greater sequence divergence (Sherwood and Parker, 1990). In the gene, introns have even greater sequence substitution among related molecules (Li *et al.*, 1985).

The GHRH family of peptides is a recently identified family and no information is available on the evolution of this group. Furthermore, even less is known about the transcriptional control of the genes. Novel functions and tissue locations for GHRH can also be identified by looking at different taxa. Therefore, the identification of GHRH in different vertebrate or invertebrate groups will add to the understanding of the evolution of this family. Salmon are

interesting to study for several reasons: 1) gene duplication in these tetraploid fish may allow for variations in gene structure and peptide products, 2) background studies on growth hormone in salmonids are available, and 3) novel functions for GHRH are apparent in that GH is implicated in the smoltification process and migration of the smolts to sea. This function of GH does not exist in mammals. Therefore, the main hypotheses of my research were that: 1) a GHRH or GHRH-like molecule is present in salmon, 2) the gene organization of salmon GHRH is similar to other members of the glucagon superfamily, and 3) GHRH releases GH in salmon.

The chapters in this thesis are presented in the chronological order in which the research was done. The data presented in chapter 2 shows the evidence for an immunoreactive GHRH in fish. At the commencement of this research the peptide structure of GHRH was known in 6 mammals and the DNA sequence of the encoding gene in 2. The lack of sequence identity among the GHRHs (i.e. human compared to rat) and the number of substitutions in the cryptic regions of other neuropeptide precursors (Sherwood and Parker, 1990), suggested that the best approach for identifying salmon GHRH was first to determine the structure of the peptide. Furthermore, to expand our knowledge of GHRH in the vertebrate groups, I attempted to identify GHRH in the American alligator. The identification of an immunoreactive GHRH in the alligator also resulted in the

purification of a coeluting peptide, neuropeptide Y. The purification and sequence of this molecule is presented in chapter 3. During the course of this research, advances made in molecular biological techniques, particularly the polymerase chain reaction (PCR), made it advantageous to pursue the salmon GHRH cDNA sequence. In 1988, Frohman *et al.* published a new technique called rapid amplification of cDNA ends (RACE). Using the PCR and RACE/PCR with modifications, I was able to obtain the sequence of a sockeye salmon GHRH-like cDNA which is presented in chapter 4. Once the cDNA had been obtained, a genomic library was screened to identify the salmon GHRH-like gene. This work is presented in chapter 5. In 1989, a new brain peptide, called pituitary adenlyate cyclase activating polypeptide (PACAP), of unknown function was isolated. One year later the cDNA structure was identified for PACAP revealing an upstream cryptic peptide that had partial sequence identity to the human GHRH. It was at this point I realized my cDNA clone encoded a salmon GHRH\PACAP precursor and that it had an even more interesting story than was first revealed.

Chapter 2

Evidence of a Growth Hormone-Releasing Hormone-like Molecule in Salmon brain, *Oncorhynchus keta* and *O. kisutch*

A version of this chapter has been published:

D.B. Parker and N.M. Sherwood, 1990. Evidence of a growth hormone-releasing hormone-like molecule in salmon brain, *Oncorhynchus keta* and *O. kisutch*. Gen. Comp. Endocrinol. 79:95-102.

INTRODUCTION

Growth hormone-releasing hormone (GHRH) has been isolated and sequenced from the hypothalamus of six different mammalian species (Guillimen, 1986). At present, only a carp GHRH-like molecule has been sequenced in non-mammalian vertebrates (Vaughan *et al.*, 1992). The carp GHRH sequence has 40% sequence identity to human GHRH. Three fractions from codfish brain-pituitary extracts, isolated using exclusion chromatography, were immunoreactive with human pancreatic (hp)GHRH antisera (Pan *et al.*, 1985). More recently, a GHRH-like extract from codfish brains was shown to elute with a similar retention time on high-performance liquid chromatography (HPLC) as hGHRH 1-44 NH_2 (Ackland *et al.*, 1989).

Direct and indirect evidence suggests GH release in fish is controlled by the dual action of GHRH and somatostatin. Carp GHRH has been shown to stimulate GH secretion in a dose dependent manner from rainbow trout pituitary cells (Luo *et al.*, 1990; Luo and McKeown, 1991). The release of GH, stimulated by carp GHRH-like peptide, is potentiated by thyroid hormone (T_3) and glucocorticoids (dexamethasone) (Luo and McKeown, 1991). Indirect evidence that GHRH and somatostatin have functional roles in fish similar to those in mammals has also been shown. Somatostatin inhibits growth hormone release *in vitro* in Tilapia pituitary glands (Fryer *et al.* 1979) and *in vivo* in goldfish (Cook and Peter,

1984) and coho salmon (Sweeting and McKeown, 1986). Peter *et al.* (1984) have also shown that synthetic hpGHRH (1-40) injected into goldfish stimulates growth hormone release.

In fish, immunocytochemical studies using antisera to the mammalian GHRH's have shown that immunoreactive GHRH cells are localized in the magnocellular and parvocellular perikarya of the preoptic nucleus of the brain (Marivoet *et al.*, 1988; Moons *et al.*, 1989; Olivereau *et al.*, 1990; Pan *et al.*, 1985). The GHRH immunoreactive nerve fibers project from the hypothalamus to the pituitary, where they terminate in close proximity to the growth hormone cells in cod and seabass (Moons *et al.*, 1989; Pan *et al.*,1985). In salmonids, GHRH immunoreactive fibers extend into the rostral neurohypophysis where they terminate on the capillary plexus or on the basement membrane of the neurohypophysis-pars distalis interface, but do not penetrate into the proximal pars distalis (Olivereau *et al.*, 1990). This implies that GHRH in salmonids is released into the neurohypophyseal capillary blood vessels, which connect to the adenohypophysis (Ball, 1981). The rostral neurohypophysis of teleosts is believed to be functionally analogous to the median eminence of tetrapods (Olivereau *et al.*, 1990).

To fully understand the neural regulation of GH secretion in fish it is important to identify the structure of fish GHRH. In the present study I report on the chromatographic and immunological behavior of a GHRH-like

molecule from salmon brain extracts using an antiserum with specificity for hGHRH 1-44 NH_2.

MATERIALS AND METHODS

Antibody production

Human growth hormone-releasing hormone (2.0 mg, hGHRH 1-44 NH_2, Sigma) was conjugated to bovine thyroglobulin (2.145 mg) using the carbodiimide method of Skowsky and Fisher (1972). Conjugated GHRH was diluted with phosphate-buffered saline (PBS, pH 7.4) to 100 $\mu g \cdot ml^{-1}$, aliquoted, and frozen at -70°C.

Two male New Zealand white rabbits (NZW) were injected with 100 μg of conjugated GHRH and one male NZW rabbit was injected with 100 μg of unconjugated GHRH, emulsified with an equal volume (1.0 ml) of Freund's complete adjuvant. Each rabbit received two 0.5 ml subcutaneous injections in the back and two 0.5 ml intramuscular injections, one in each hip. Subsequent booster shots of GHRH, emulsified with Freund's incomplete adjuvant, were administered every 3 weeks over 5 to 10 months. Rabbits were bled from the peripheral ear vein every 3 weeks, commencing 1 week after the third injection. Blood was allowed to clot and the serum was separated from the red blood cells by centrifugation. Serum obtained was frozen in 0.01% sodium azide at -70°C. Antisera were assayed for titer and

specificity by radioimmunoassay.

Radioimmunoassay (RIA)

Radioiodinated hGHRH 1-44 was obtained by incubating 5 μg of synthetic GHRH in 500 mM PBS, pH 7.5, with 0.75 mCi of ^{125}I (as sodium iodide, Amersham) and 10 μl (20μg) of chloramine-T (Greenwood *et al.*, 1963). After gentle agitation for 60 s the reaction was stopped by the addition of 50 μl (116.8 μg) of potassium metabisulfite, followed by 100 μl of 1% bovine serum albumin (BSA) in PBS. Free $Na^{125}I$ was separated from the protein-bound portion by eluting with PBS (0.2% BSA) from a Sephadex G-25 column. One-milliliter fractions were collected, of which 5 μl was counted on an LKB 1275 Minigamma Counter, and subsequently tested in an RIA to determine the position of the protein bound peak. The ^{125}I GHRH (1-44) was stored at 4°C and was stable for 4-6 weeks.

Standard concentrations of hGHRH ranged from 7.8 to 750 pg and were prepared in phosphate buffer containing 19 mM NaH_2PO_4, 81 mM Na_2HPO_4, 50 mM NaCl, 0.1% Triton X-100, 0.1% BSA, and 0.01% sodium azide (pH 7.4). Antibody solutions were prepared in a PBS/EDTA buffer containing 6.7 mM Na_2PO_4, 3.3 mM KH_2PO_4, 145 mM NaCl, 0.01% merthiolate, 62 mM EDTA, and adjusted to pH 7.0 with 5 N NaOH. Prior to use, normal rabbit serum (2%) was added to PBS/EDTA buffer. Nonspecific binding (blanks) and maximum binding tubes were prepared with the phosphate buffer/BSA. On Day 1, 100 μl of standard

or test sample and 100 μl of antiserum (final dilution 1:45,000) were pipetted into 12 X 75 mm borosilicate tubes. After a 16-18 h incubation at 4°C, ^{125}I-GHRH (10,000-15,000 cpm) in 100 μl was added and allowed to incubate for 24 h at 4°C. To separate the free from the bound GHRH a second antibody technique was used. To each tube, except the totals, 100 μl of 2% normal rabbit serum (NRS) in phosphate buffer and 1.0 ml of precipitating solution containing goat anti-rabbit gamma-globulin [IgG] plus polyethylene glycol (Diagnostic Products Corp.) were added, followed by a further incubation at 4°C for 6 h. The tubes were centrifuged in an IEC Centra-7R at 1600xg for 30 min and the pellets were counted on a gamma counter.

A computer database search was performed to find peptides with a similar amino acid sequence to hGHRH. These peptides were tested by RIA for cross-reactivity and specificity with our antiserum. Antisera cross-reactivity was determined against vasoactive intestinal peptide (VIP; human, porcine, rat), secretin (chicken), peptide histidine isoleucine (PHI) 1-27 (porcine), GHRH (ovine and rat), hGHRH 5-16 NH_2, hGHRH 17-28 NH_2, hGHRH 29-40 NH_2, and hGHRH 41-44 NH_2, at concentrations ranging from 0.01 to 50 ng. The four hGHRH fragments were a gift from Dr. Blair Fraser.

The RIA data were analyzed by spline function and log-logit methods. Intraassay variation was determined by measuring 10 replicates at 125 and 250 pg of synthetic GHRH. Interassay variation was determined by comparing the 50%

displacement point among assays.

Antibody purification

Proteins in antiserum 8119 were precipitated using 45% ammonium sulfate at 4°C overnight. The antiserum/ammonium sulfate mixture was centrifuged at 10,000xg for 30 min and the pellet was washed and centrifuged twice in 50% ammonium sulfate/PBS. The pellet was resuspended in PBS, desalted using a PD-10 column (Pharmacia), and diluted 1:1 with binding buffer, pH 9.0 (Affi-Gel Protein A MAPS II Kit, Bio-Rad). The protein solution was applied to a protein A column at 10 $ml \cdot h^{-1}$ and bound IgG was eluted with elution buffer (pH 3.0) at 17 $ml \cdot h^{-1}$. Fractions collected were neutralized with 1 M Tris-HCl, pH 8.6. Purified IgG was dialyzed against three changes (1:1000) of 0.1 M $NaHCO_3$, 0.5 N NaCl, pH 8.5 and frozen at -70°C.

Tissue extraction

Frozen chum salmon (188.8 g), coho salmon (176.6 g), guinea pig (206.4 g), or mouse (11.5 g) brains were pulverized to a fine powder with liquid nitrogen in a Waring blender. Brain powder was then extracted in cold acetone/1 N HCl (33:1) containing 10 $\mu g \cdot ml^{-1}$ pepstatin A and subsequently filtered through a Whatman No. 1 filter. The precipitate was re-extracted in acetone/0.01 N HCl (4:1) for 3 min, filtered, and the two filtrates combined. The filtrate was defatted with petroleum ether (30-60° BP, 20%

of total filtrate volume) five times and vacuum centrifuged to reduce the volume. These crude extracts were then directly applied to HPLC.

Affinity chromatography

Purified IgG was dialyzed against 0.1 M $NaHCO_3$, pH 8.5, and diluted in the same buffer to a final concentration of 2.41 $mg{\cdot}ml^{-1}$. Prior to coupling, an aliquot of the purified antibody was checked for binding by RIA. Antibody was coupled to 10 ml of Affi-Gel 10 activated support (Bio-Rad) at a concentration of 9.63 $mg{\cdot}ml^{-1}$ gel by agitating end over end for 2 h at room temperature, followed by 1 h with 1 M ethanolamine HCl (pH 8.0) to block remaining active esters. The gel was washed twice in phosphate buffer (20 mM sodium phosphate, 145 mM NaCl, 0.01% sodium azide (NaN_3), pH 7.4;, Bohlen *et al.*, 1983b) by rotating end over end in a 50 ml falcon tube. The immunoaffinity matrix was poured into a 1.5 X 20 cm column (Bio-Rad), allowed to settle and a flow-adaptor attached. The column was washed with alternating cycles of phosphate buffer (pH 7.4), 0.1 M sodium acetate/0.5 N NaCl (pH 4.0), 0.1 M $NaHCO_3$/0.5 N NaCl (pH 8.5) and 1 M acetic acid (pH 2.4).

Chum salmon brains were extracted as described above except for the addition of 0.5% ß-mercaptoethanol. The extracts were diluted 1:4 (extract:water) with HPLC grade water and adsorbed to 10 C-18 Sep-pak cartridges arranged in tandem. The columns were washed with HPLC grade water and

the bound material eluted in three steps: 1) 10% acetonitrile/90% water, 2) 60% acetonitrile/40% water and 80% acetonitrile/20% water. The fractions eluted with 60% acetonitrile contained most of the chum salmon immunoreactive (ir)GHRH. The eluates were then reduced in volume on a vacuum concentrator to remove the acetonitrile. The fractions were pooled and diluted 1:1 with 40 mM sodium phosphate/290 mM NaCl/0.02% NaN_3, adjusted to pH 7.4 with NaOH, and pumped through the affinity column at 8.8 $ml \cdot h^{-1}$. Tween 80 was added to extracts and phosphate buffers to a final concentration of 0.2%. The effluent was collected and the column washed with 10 times the bed volume using sodium phosphate buffer containing Tween 80, followed by 2 times the bed volume of buffer without Tween 80. The material bound to the column was eluted with either 1 M acetic or 0.5 N propionic acid/1 N NaCl (pH 3.5) followed by the addition of ß-mercaptoethanol (0.5%) to the immunoreactive fractions. The column was subsequently washed with starting buffer.

High performance liquid chromatography

Crude extracts

Crude extracts were filtered through a 0.22 μM Millex-GV filter and further purified by reverse-phase HPLC using a Varian 5000 liquid chromatograph, 2050 detector, and a 4290 integrator. The samples were eluted from a Supelco analytical LC-18 column (0.46 X 25 cm, particle size 5μM) equipped with a Supelco LC-18 guard column (0.46 X 2 cm,

particle size 5 μM) using a linear gradient of 1 % acetonitrile (AN)$\cdot$min^{-1} from 5 to 60%. The mobile phases were 0.25 M triethylammonium formate (TEAF; pH 6.5) and acetonitrile as the solvents. Fractions of 1.0 ml were collected at a flow rate of 1.0 ml$\cdot$min^{-1}; aliquots were dried on a vacuum concentrator and assayed for the presence of GHRH by RIA using antiserum 8119. Those fractions containing irGHRH were pooled, reduced in volume on a vacuum concentrator and chromatographed on the LC-18 column using a step program with a gradient elution (2% AN/min) at 5-15 min, and isocratic periods at 0-5 min (15% AN) and 15-35 min (35% AN) with the same mobile phase. Blank runs were done using an injection of TEAF (pH 6.5) before and after all sample runs; each blank HPLC fraction was assayed for GHRH.

Immunoaffinity purified extracts

All HPLC runs were done at a flow rate of 1 ml$\cdot$min^{-1}. Immunoaffinity-purified salmon irGHRH was adjusted to pH 7.0, adsorbed to a C-18 cartridge (Sep-Pak) and subsequently eluted with 60% AN/0.25 M TEAF (pH 6.5). Sep-pak fractions were loaded onto a Supelco analytical LC-18 column and eluted with 0.25 M TEAF and acetonitrile using a linear gradient (see above) or a step program of 15% AN for 10 min, followed by a linear gradient of 2% AN$\cdot$min^{-1} for 10 min, an isocratic elution at 35% AN for 20 min and a linear gradient to 60% AN over 15 min. Immunoreactive fractions from the previous HPLC linear gradient were pooled and

chromatographed on the same column using a gradient of 28 to 35% AN over 30 min. Those immunoreactive fractions from the first step program were pooled and rechromatographed using the same column. The program was changed to 5 min at 15% AN followed by a linear gradient of 4% AN·min^{-1} for 5 min and an isocratic elution of 35% AN for 20 min. Further purification of all salmon irGHRH was done on a Vydac C-18 column (0.46 X 25 cm, 5 μM particle size) using a linear gradient of 1% AN·min^{-1}. The second HPLC of the immunoreactive fractions was done using a gradient of 28-35% AN over 50 min using 0.05% trifluoroacetic acid (TFA) and 0.05% TFA in AN:water (80:20) as the mobile phases. The final purification run used the same column and mobile phases, except the gradient was changed to 24-32% AN over 50 min. ß-mercaptoethanol was added at a final concentration of 0.5% to all HPLC fractions.

RESULTS

Radioimmunoassay

Antiserum 8119 at a final dilution of 1:45,000 was used to produce a radioimmunnoassay for hGHRH 1-44 resulting in a standard curve as shown in Fig. 2.1. Assay sensitivity to hGHRH 1-44, defined as the minimum amount of unlabeled GHRH that can be distinguished from a zero concentration of unlabeled hormone, was 31.3 pg/ml (Student's *t* test, $P<0.05$). Intraassay variability at 125 and 250 pg was

V=4.4% and V=7.6% (V= coefficient of variation), respectively. Interassay variability, compared as the variation among the 50% binding points of five standard curves, was V=11.1%. The mean concentration at which 50% binding of the unlabeled hormone occurred was 138.4 ± 6.3 pg (mean ± SEM, n=5).

Antiserum 8119 (1:45,000) did not exhibit cross-reactivity with chicken secretin, porcine PHI, or human, porcine, and rat VIP (Fig. 2.1), but did show 70.4% cross-reactivity with ovine GHRH1-44 (NH2) and 11.7% with rat GHRH 1-40 (Table 2.1). Cross-reactivity of antisera 8118 and 8120 was not observed with the three GHRH fragments of 12 residues each nor with the C-terminal fragment of 4 residues (Fig. 2.2). However, the C-terminal fragment did show 32% displacement of radiolabeled hGHRH at a concentration of 1 ng with antiserum 8119 (Fig. 2.2). Nonparallel displacement with hGHRH 1-29 (NH2) was also observed with all antisera (Fig. 2.2); antiserum 8118 showed the highest cross-reactivity (CR = 50% displacement at 1 ng; deLauzon *et al.*, 1973) compared with antiserum 8119 and 8120 (CR = 42% and CR = 22.4% at 1 ng, respectively). Oxidized hGHRH 1-44 (NH2) showed parallel displacement with antiserum 8119 and 83% cross-reactivity (data not shown).

Crude extracts

The total amount of irGHRH in the acid/acetone crude extracts of coho salmon, chum salmon, guinea pig, and mouse

was 47.8, 26.0, 50.8 and 3.2 ng, respectively. Salmon irGHRH detected by radioimmunoassay had a similar retention time on HPLC as hGHRH 1-44 and guinea pig irGHRH (Fig. 2.3). hGHRH 1-29 (NH_2) eluted 2.5-3.0 min later than salmon irGHRH and hGHRH 1-44, while fragment hGHRH 5-16 eluted 6 min earlier. The other 12 amino acid fragments of hGHRH were not detected on HPLC at 280 nm due to the lack of aromatic structures in their primary sequence. After the first HPLC program, 17.18 ng of salmon irGHRH (fractions 40-49) was recovered from 176.6 g of coho salmon brains (100 brains) and 3.54 ng (fractions 41-44) from 188.6 g of chum salmon brains. Only 0.934 ng (fractions 43-45) of guinea pig irGHRH was recovered after the first HPLC. Mouse irGHRH was not detectable in a 300 μl subsample of the HPLC fractions. The yield of coho salmon irGHRH was 83.7% after the second HPLC purification step, while over 100% recovery of chum salmon irGHRH was obtained. This high recovery is most likely the result of a more purified fraction on the second HPLC elution, allowing for a more accurate quantification. An isocratic elution profile showed that coho and chum salmon irGHRH eluted within 1 min of hGHRH 1-44 (Fig. 2.4).

Immunoreactivity observed in fractions 4-12 of the first HPLC run is most likely nonspecific binding of interfering substances due to the crude nature of the extract. Immunoreactivity was not observed in fractions other than those eluting at a similar time to hGHRH 1-44 in the second HPLC run.

Immunoaffinity purified extracts

Immunoaffinity-purified salmon irGHRH eluted earlier than that in crude preparations of chum and coho salmon extracts on HPLC. Immunoaffinity purified irGHRH chromatographed on a isocratic step program eluted 2-3 min earlier than crude extract run on the same program (compare Figs. 2.4 and 2.5B). With a linear gradient, two prominent peaks were found on the first HPLC run eluting 10-11 min and 5-6 min earlier than the single peak observed from the crude extract (compare Figs. 2.3 and 2.6A).

Initially, there were two approaches in the strategy to purify the immunoreactive salmon GHRH using HPLC. In the first approach, step programs with isocratic periods were used in the first two HPLC runs of the immunoaffinity-purified material (Fig. 2.5A and B). However, immunoreactive material was not resolved from the major peaks of contaminating material. Therefore, subsequent immunoaffinity-purified material was applied with a linear gradient (1% AN$\cdot$min^{-1}) (Fig. 2.6A) on HPLC, followed by a second application with a reduced gradient of 0.2% AN$\cdot$min^{-1} (Fig. 2.6B). Furthermore, separation of the two peaks eluting at 19 and 23 min on the step program (Fig. 2.5A) was less than with a linear gradient (Fig. 2.6A).

The chromatogram in figure 2.5A shows most of the immunoreactive material eluted over fractions 19-24 on a step program. The immunoreactive material from fraction 23 was chromatographed using an isocratic program and eluted as

a major peak at 18 min (fraction 18, Fig. 2.5B). Subsequent HPLC purifications followed the same protocol as the immunoaffinity-purified material with linear gradient programs. Fraction 18 eluted as two major peaks when chromatograped on a Vydac C-18 column with a linear gradient (Fig. 2.6C).

In a second approach, immunoaffinity-purified GHRH was initially chromatographed using a linear gradient on HPLC. The immunoreactive material eluted as two distinct peaks, fractions 32 and 37 (Fig. 2.6A). Fraction 32 was chromatographed on a shallow gradient (Fig. 2.6B) and eluted as a single peak at 18 min. When fraction 37 was chromatographed on the same shallow gradient, the main immunoreactive peak eluted at 20 min (data not shown). Further purification of these two peaks on a Vydac C-18 column resulted in immunoreactive peaks at fractions 31 and 35 (Fig. 2.6C). These peaks were in the same elution positions as those obtained with immunoreactive material partially purified using the step program approach (see above). Purification of these two peak areas, fractions 30-32 and 35-36, was done separately. However, the chromatograms for both peak areas (30-32 and 35-36) showed a major immunoreactive peak at 23 min and a minor peak over 18-20 min (Fig. 2.6D). Further purification of fraction 23 (Fig. 2.6D) resulted in a single major peak eluting 12-13 min earlier than the native form of hGHRH 1-44 (NH2) and a minor peak at 18 min (Fig. 2.7). Chromatography of the

minor immunoreactive fractions 18-20, shown in Fig. 1.6D, on the same program as in Fig. 2.7, resulted in a peak at 18-19 min (data not shown). Therefore, changing the initial acetonitrile concentration did not appear to affect the elution position of this immunoreactive material.

To test whether multiple peaks might be due to oxidized and reduced forms of GHRH rather than two distinct structures or fragments, synthetic hGHRH 1-44 was applied in both its oxidized and reduced forms to HPLC. The results (Fig. 2.7) of applying oxidized and reduced GHRH on HPLC show that oxidized GHRH eluted 10 min before reduced CHRH.

TABLE 2.1

Cross-Reactivity of Ovine and Rat GHRH with hGHRH 1-44 using Antiserum 8119 (1:45,000)

	B/Bo (%) concentration (ng)							
Peptide	0.01	0.05	0.10	0.50	1.0	50	% cross-reactivity	ED50 (pmol)
hGHRH 1-44	94.3	93.9	89.1	36.5	--	--	100	0.0714
Ovine GHRH	96.1	94.1	89.6	51.6	24.5	5.2	70.4	0.1015
Rat GHRH	97.2	88.9	87.3	62.9	55.6	39.6	11.7	0.6116

Figure 2.1. Standard curve of hGHRH 1-44 NH_2 (·) using antiserum 8119 at a final dilution of 1:45,000. Vertical lines represent the standard error (SE), n=5. Binding to increasing concentrations of chicken secretin (■). porcine PHI (▲), and human, porcine and rat VIP (O) are shown.

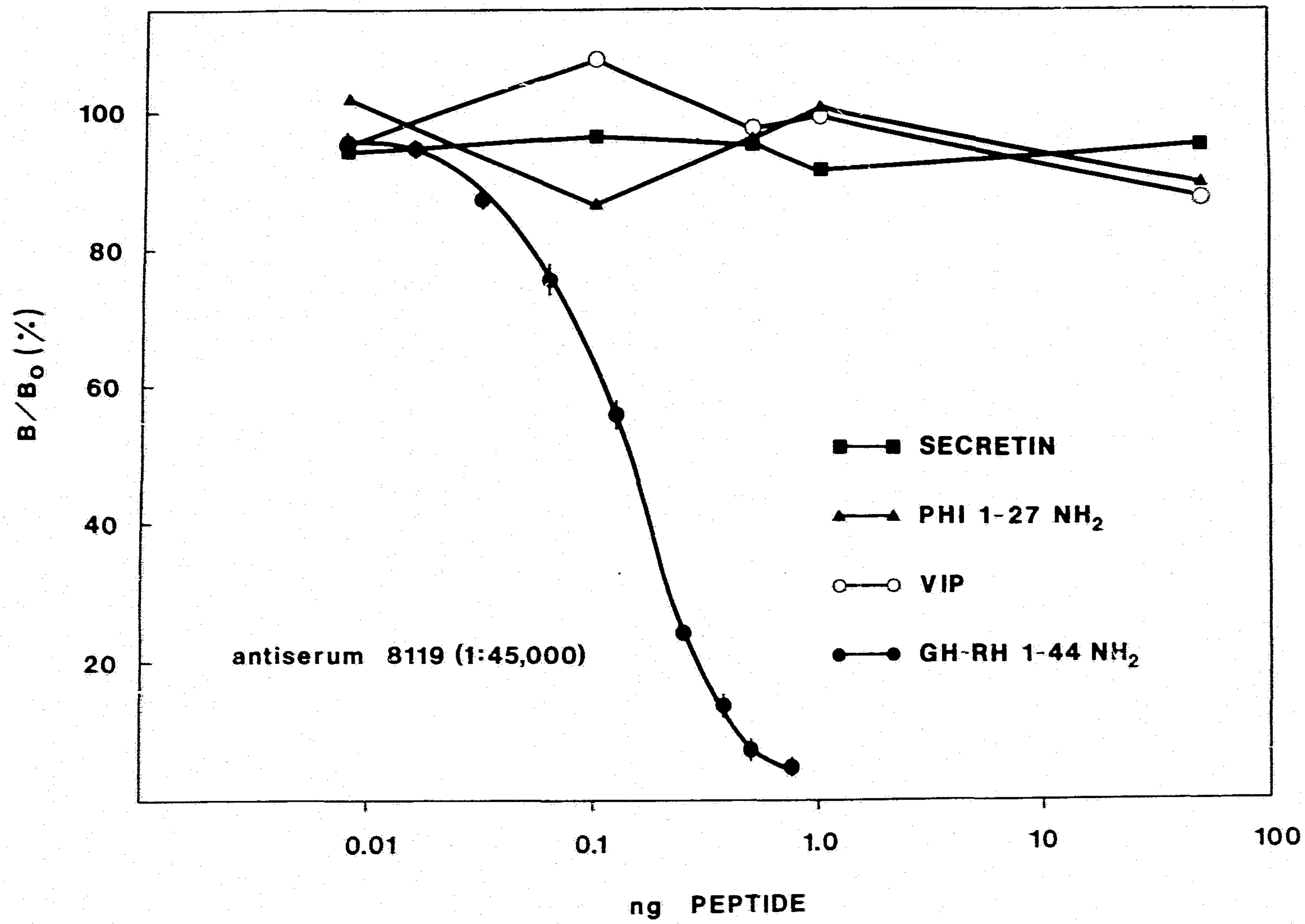

100
80
60
40
20
B/B$_o$ (%)
SECRETIN
PHI 1-27 NH_2
VIP
GH-RH 1-44 NH_2
antiserum 8119 (1:45,000)
0.01
0.1
1.0
10
100
ng PEPTIDE

Figure 2.2. Binding of hGHRH fragments with three antisera developed against hGHRH 1-44 NH_2. The ordinate (B/Bo) is the percentage of precipitated radioactivity in the presence of unlabeled GHRH (B) compared with that in the absence of unlabeled GHRH fragments (Bo).

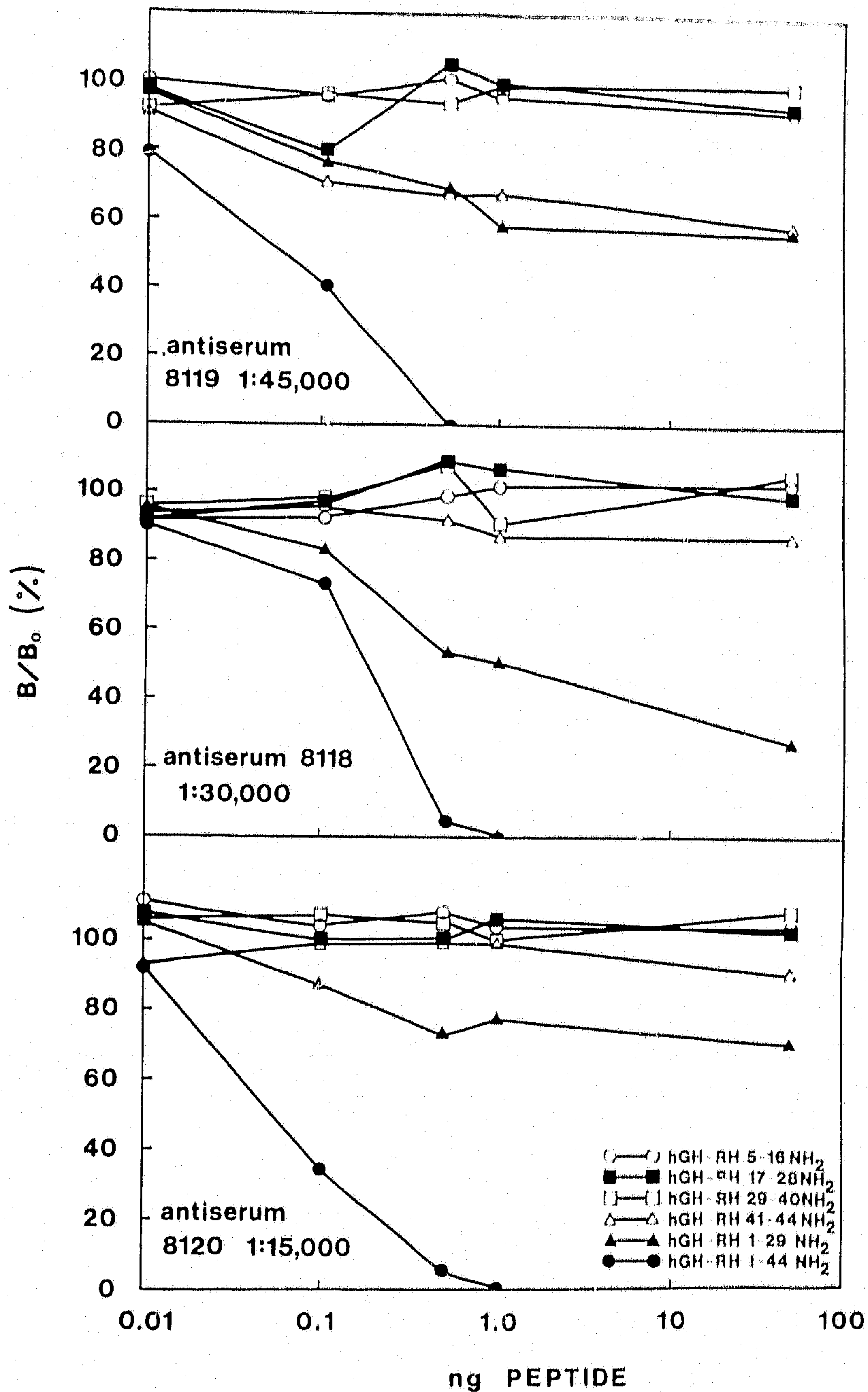

antiserum
8119 1:45,000
antiserum 8118
1:30,000
antiserum
8120 1:15,000
B/Bo (%)
ng PEPTIDE
hGH-RH 5-16 NH2
hGH-RH 17-28 NH2
hGH-RH 29-40 NH2
hGH-RH 41-44 NH2
hGH-RH 1-29 NH2
hGH-RH 1-44 NH2

Figure 2.3. Immunoreactive fractions of coho and chum salmon brain crude extracts after reverse-phase HPLC. Retention times of hGHRH 1-44, hGHRH fragments, and guinea pig irGHRH are shown by the vertical arrows.

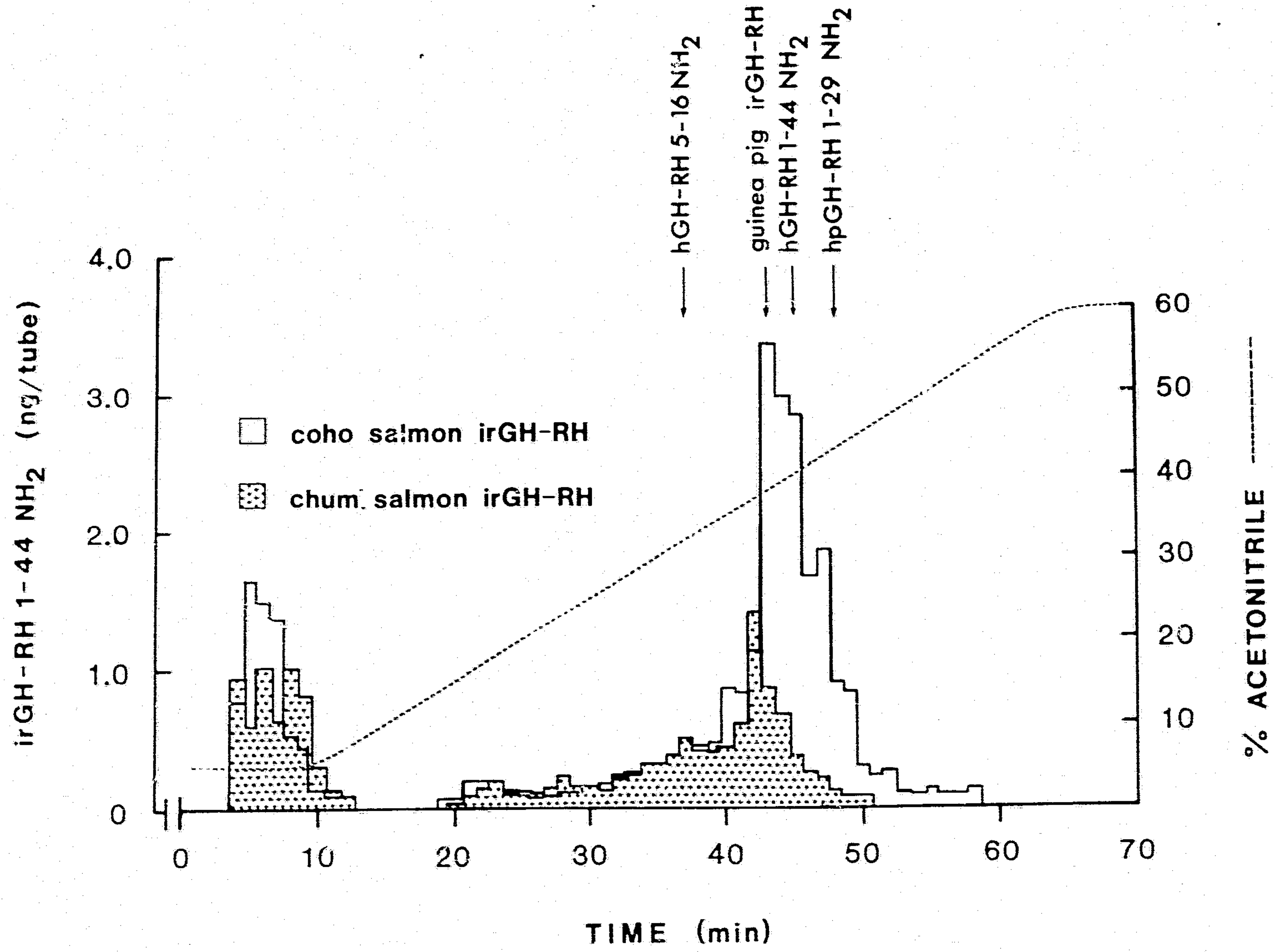

irGH-RH 1-44 NH2 (ng/tube)
4.0
3.0
2.0
1.0
0
coho salmon irGH-RH
chum salmon irGH-RH
hGH-RH 5-16 NH2
guinea pig irGH-RH
hGH-RH 1-44 NH2
hpGH-RH 1-29 NH2
60
50
40
30
20
10
% ACETONITRILE
0
10
20
30
40
50
60
70
TIME (min)

Figure 2.4. Reverse-phase HPLC of pooled fractions of coho or chum irGHRH from the first HPLC run. Retention times of hGHRH and two fragments are shown by the vertical arrows.

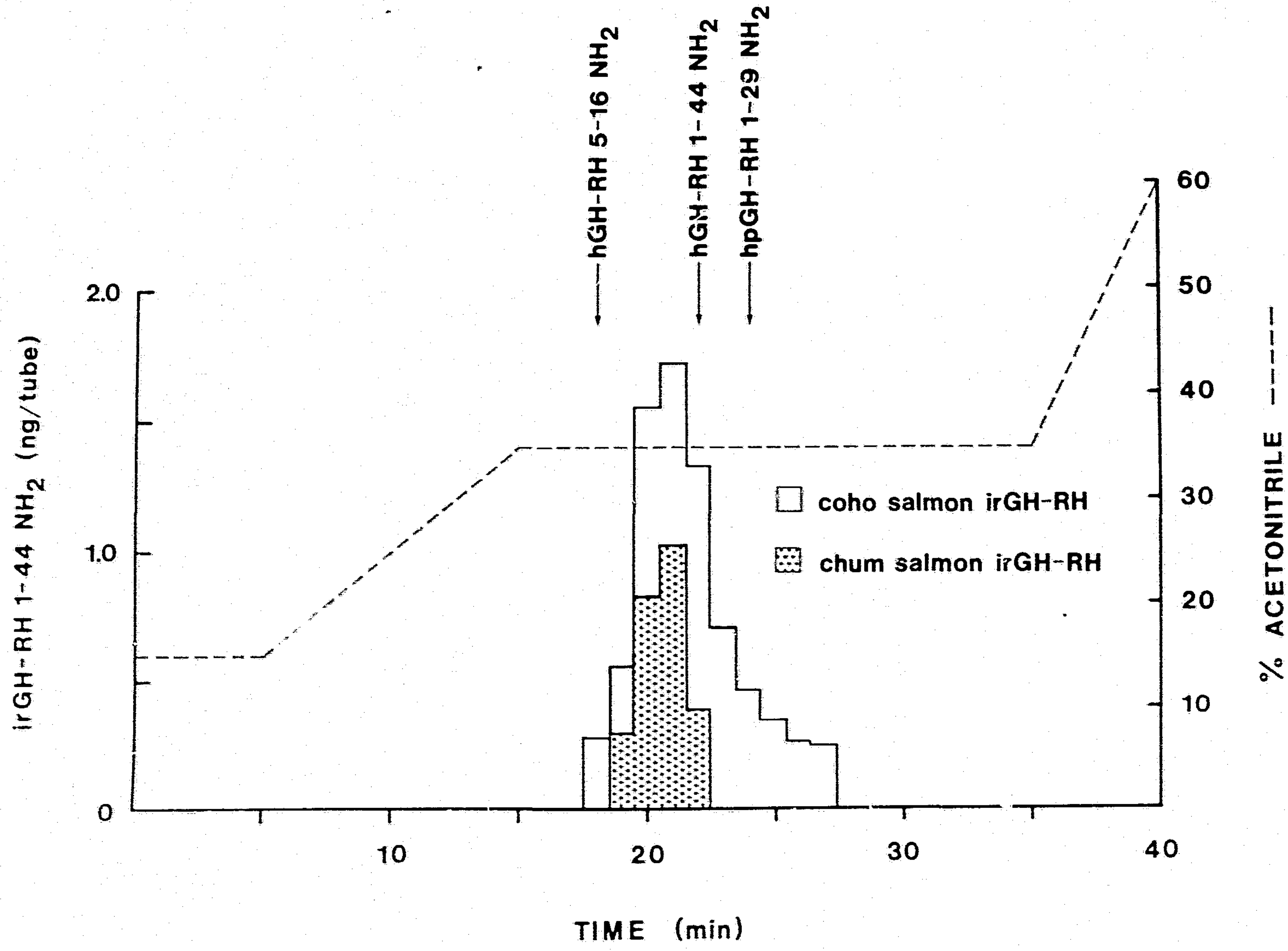
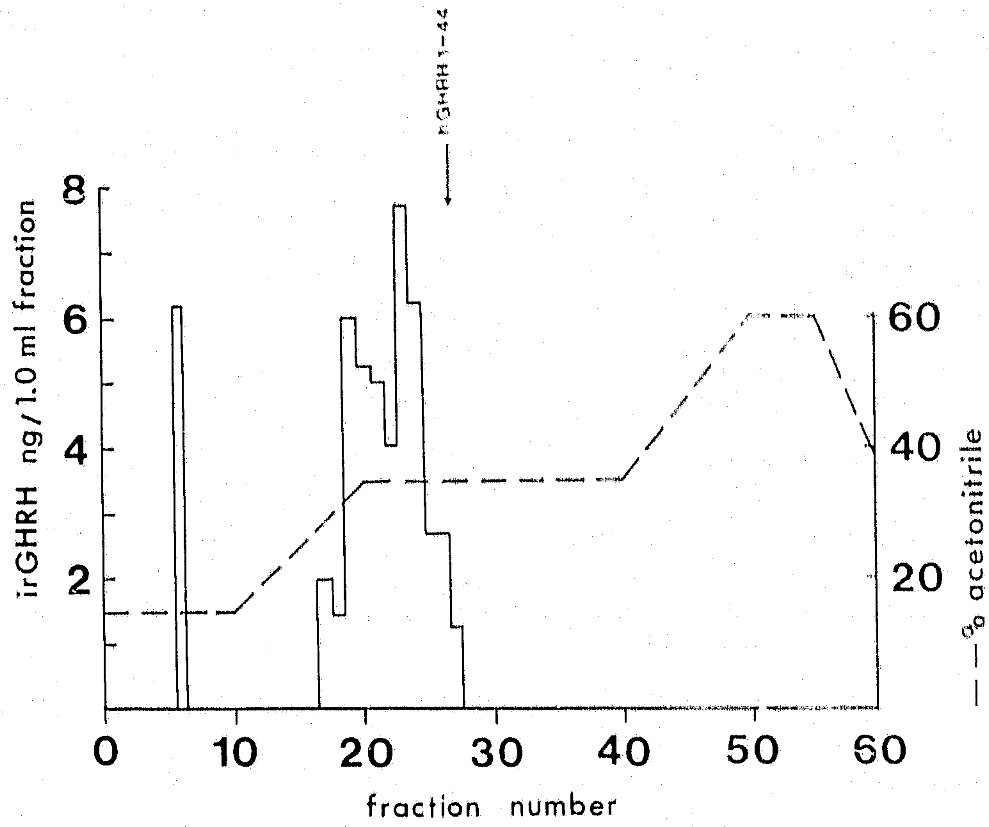

irGH-RH 1-44 NH2 (ng/tube)
0
1.0
2.0
hGH-RH 5-16 NH2
hGH-RH 1-44 NH2
hpGH-RH 1-29 NH2
coho salmon irGH-RH
chum salmon irGH-RH
10
20
30
40
50
60
% ACETONITRILE
TIME (min)

Figure 2.5. A) Reverse-phase HPLC of immunoaffinity purified material from chum salmon brains using a step program with an isocratic elution profile. B) Immunoreactive fractions from A were chromatographed on the same column, but initiation of the isocratic period was advanced 5 min. Mobile phases were 0.25 M TEAF and acetonitrile.

A.



B.

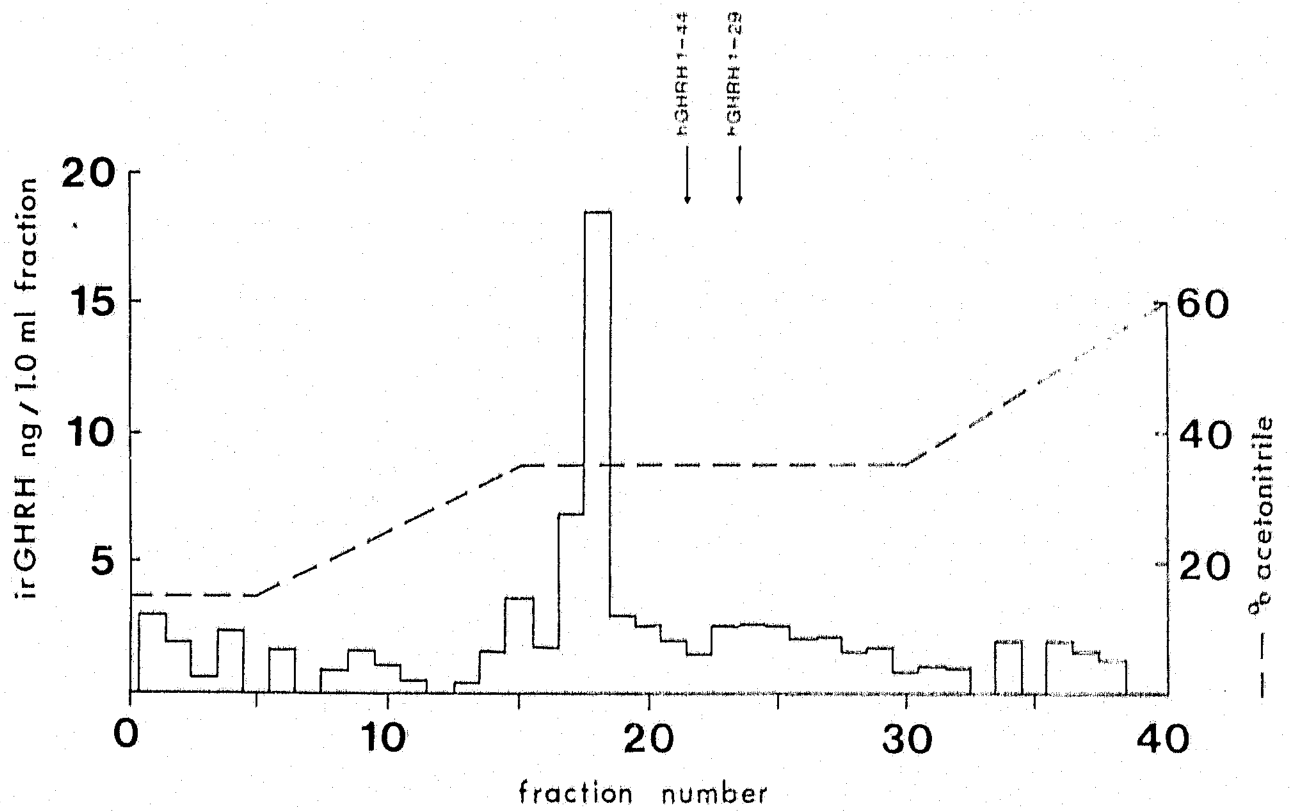


Figure 2.6. Reverse-phase HPLC of immunoaffinity purified material from chum salmon brains. A) A linear gradient of 1% acetonitrile·min^{-1}. The mobile phase was 0.25 M TEAF and acetonitrile. B) Chromatography of immunoreactive fractions 31-33 from program A using a gradient with an increase of 0.23% acetonitrile·min^{-1} and the same mobile phases. C) Chromatography of immunoreactive fractions from program B on a Vydac C-18 column using a gradient with an increase of 1% acetonitrile·min^{-1}. The buffers were 0.05% TFA and 0.05% TFA/acetonitrile. D) Immunoreactive fractions of the major peak of program C were chromatographed using the same column and buffers, but with a gradient increase of 0.144%·min^{-1}.

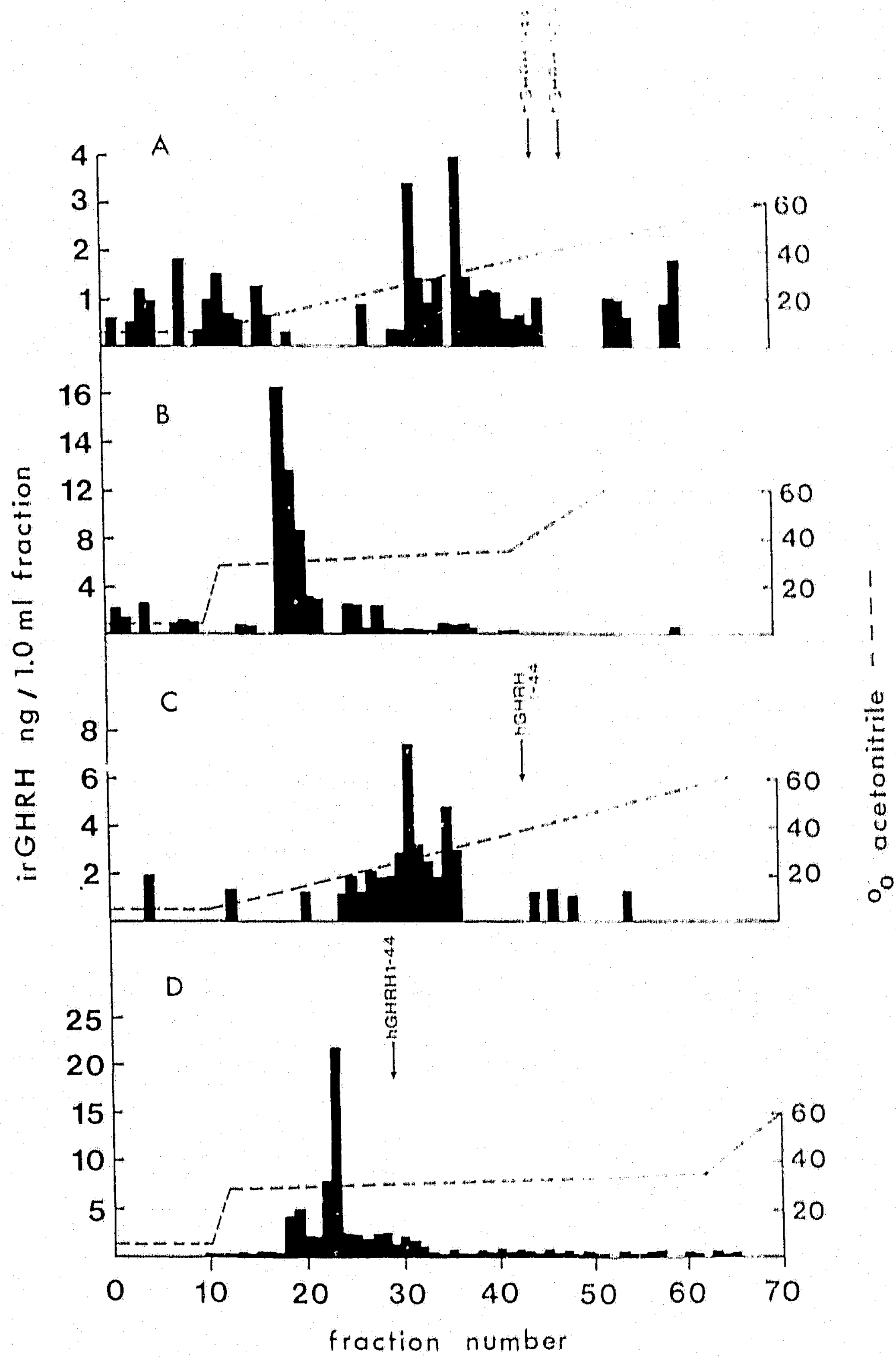
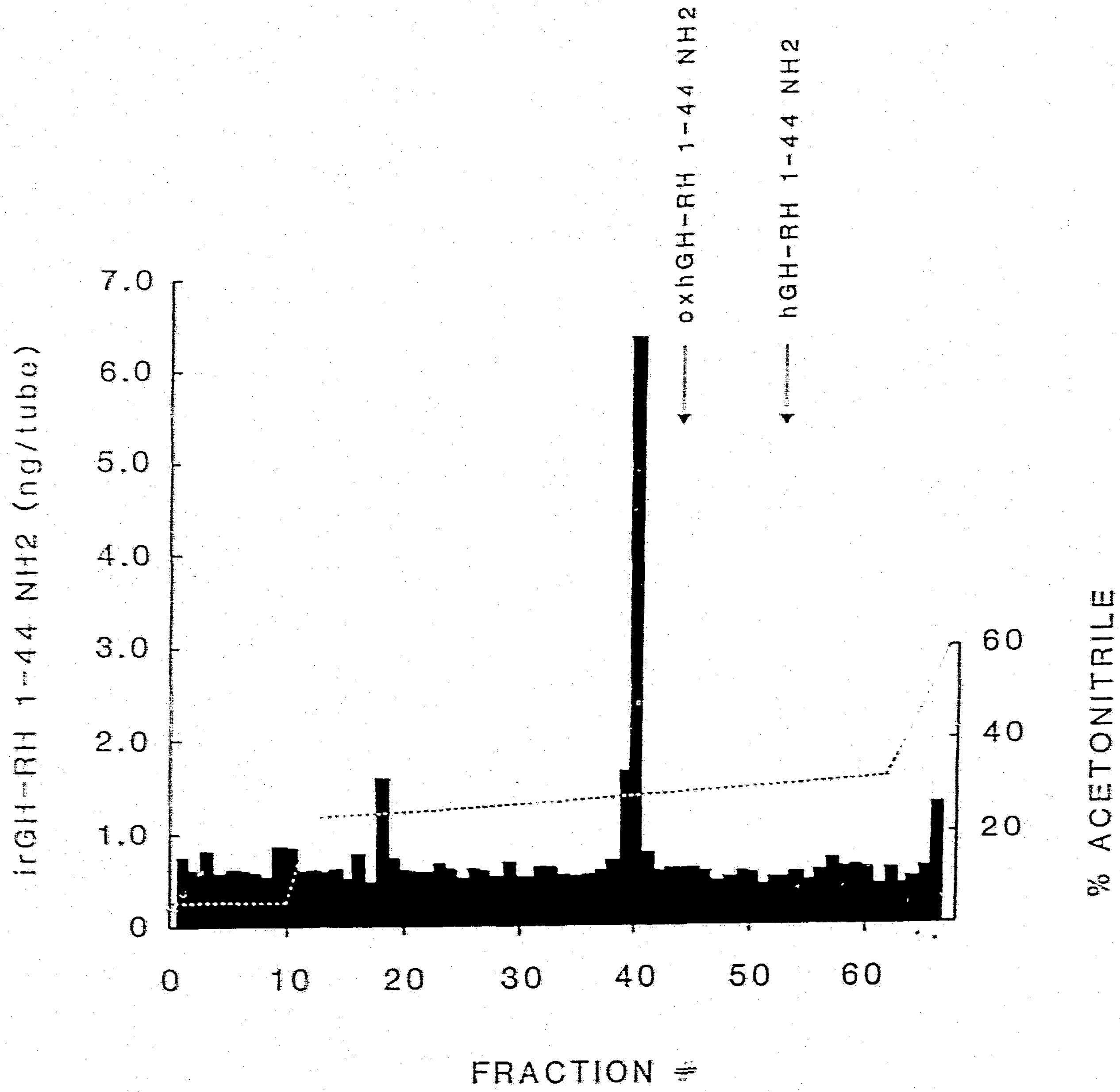

A
B
C
D
irGHRH ng / 1.0 ml fraction
% acetonitrile
hGHRH 1-44
hGHRH1-44
fraction number

Figure 2.7. Reverse-phase HPLC of pooled fractions of chum salmon irGHRH that had been previously applied to affinity chromatography and previous HPLC programs. Values of less than 0.9 ng/tube have a B/Bo greater than reference binding in the radioimmunoassay.


irGH-RH 1-44 NH2 (ng/tube)
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
0
10
20
30
40
50
60
FRACTION #
oxhGH-RH 1-44 NH2
hGH-RH 1-44 NH2
20
40
60
% ACETONITRILE

DISCUSSION

Antisera developed against hGHRH 1-44 (NH_2) in the present study appeared to bind a topographically assembled epitope rather than a linear one. Cross-reactivity was not observed with any of the synthetic fragments for residues 5-44 or with peptides of similar sequence to hGHRH. However, antiserum 8119 did show some recognition of the carboxy terminal end of hGHRH (40-44) (H-Arg-Ala-Arg-Leu-NH_2). This same antiserum (8119) was used to detect an immunoreactive GHRH in HPLC fractions of coho and chum salmon brains. Initial studies with crude extracts showed that a main peak of salmon irGHRH eluted in the same position from the HPLC with guinea pig irGHRH and 1-2 min earlier than hGHRH 1-44. However, affinity chromatography-purified salmon irGHRH was found to elute earlier than crude preparations using the same HPLC programs, suggesting an interference of the crude material on the elution behavior of salmon irGHRH. Alternatively, it is possible that some modification of the immunoreactive material occurred during affinity purification.

A 12-13 min difference in elution time from the HPLC between hGHRH 1-44 and salmon irGHRH in a near-isocratic HPLC program suggests that the latter molecule is more hydrophilic than the former. Although two immunoreactive peaks in the earlier stages of the purification procedure could be discerned, only one peak was prominent on the final

purification run. Several possibilities are that there is 1) a second form of salmon GHRH, 2) one form of GHRH in an oxidized or reduced form, or 3) an immunoreactive fragment in addition to the native form. Pan *et al.* (1985) have also shown chromatographic evidence of an immunoreactive GHRH from codfish brains. However, more recent work on codfish, also by Jackson's group (Ackland *et al.*, 1989), identified only one GHRH-like molecule, which had cross-reactivity with rat, but not with human GHRH antisera. Clear differences in the salmon and codfish GHRH-like molecules are indicated by the present study, in that a human GHRH antiserum did cross-react with salmon material. However, Jackson's group did immunocytochemical studies on codfish brains with antisera made to the human forms of GHRH and found immunoreactive neurons.

The low recovery of guinea pig and mouse irGHRH may be partially attributed to the low cross-reactivity of these molecules in a heterologous RIA. Guinea pig and mouse are likely to have a GHRH peptide with a sequence identity similar to rat GHRH (Spiess *et al.*, 1983), which exhibited low cross-reactivity in our hGHRH radioimmunoassay.

In a preliminary study, we found that salmon irGHRH, partially purified by HPLC, stimulated growth hormone (GH) release in a dose dependent manner in an *in vitro* rainbow trout pituitary cell culture assay (see Luo *et al.*, 1990). These data suggest that GHRH exists in salmonids and functions to stimulate GH release. Salmonid GHRH probably

operates in conjunction with somatostatin, which has been found to inhibit GH release in teleosts (Cook and Peter, 1984; Sweeting and McKeown, 1986).

Chapter 3

Primary structure of neuropeptide Y from brains of the American alligator (*Alligator mississippiensis*)

INTRODUCTION

Studies on the evolution of neuropeptides have focused on mammals and fish. Reptiles have been ignored in this regard, but are an important class of vertebrates because both birds and mammals are thought to have evolved from ancestral reptiles. In the course of our attempts to isolate GHRH-like material from alligator brains, we purified neuropeptide Y and were able to determine the primary structure. It was of interest to determine the relationship that allowed NPY to coelute with GHRH-like immunoreactive material in several purification steps.

Neuropeptide Y (NPY) is a peptide 36 amino acids in length originally isolated from porcine brain (Tatemoto, 1982; Tatemoto *et al.*, 1982). The sequence similarity of this molecule to pancreatic polypeptide (PP) and peptide YY (PYY) suggests that these molecules form a new family of peptides (Tatemoto, 1982). All of the family members are 36 amino acids in length and have an amidated carboxy terminus. NPY is found mainly in the central and peripheral nervous systems of mammals (McDonald, 1988; Dumont *et al.*, 1992), while PP and PYY are primarily localized in the pancreas and intestine, respectively (Larhammar *et al.*, in press). Other peptides with a more similar amino acid sequence to NPY than to PP, have been identified from the pancreas of several fish: eel (Conlon *et al.*, 1991b), sculpin (Conlon *et al.*, 1986), anglerfish (Andrews *et al.*, 1985; Balasubramaniam *et*

al., 1989), salmon (Kimmel *et al*., 1986), bowfin (Conlon *et al*., 1991b), gar (Pollock *et al*., 1987), skate (Conlon *et al*., 1991b) and dogfish (Conlon *et al*., 1991a).

NPY has been identified in 11 vertebrate species, but not from representatives of Agnatha and Reptilia. Although NPY immunoreactivity has been reported in the central nervous system of lizard and turtle, the primary structure of NPY has not been reported. Identification of the NPY molecule in all phylogenetic groups is required to fully understand the evolution of NPY and its relationship to the other family members.

MATERIALS AND METHODS

1) *Biological material*

Five hundred brains were obtained from American alligators of either sex at the Rockefeller Wildlife Refuge, Louisiana, in September 1988. Alligators were mature, but sexually quiescent. The brains were excised, frozen on dry ice and kept at -80 °C until extracted.

2) *Radioimmunoassay*

The human (h)GHRH (1-44NH_2) radioimmunoassay (RIA) used in this study was previously described in chapter 2 and in Parker and Sherwood (1990). In the present study binding of antiserum 8119-7, raised against human GHRH 1-44 NH_2, was

tested against human NPY, vasoactive intestinal peptide (VIP), secretin, PHI and hGHRH (1-44 NH_2) at concentrations ranging from 0.01 to 50 ng.

3) *Tissue extraction and Sep-Pak purification*

Tissue extraction was done by the procedure described in chapter 2 and in Parker and Sherwood (1990). Briefly, 500 alligator brains (1.37 Kg) were extracted in three batches with acetone/HCL containing 10 $\mu g \cdot ml^{-1}$ pepstatin A and 0.5% ß-mercaptoethanol. After filtration to remove the solids, each filtrate was extracted 5 times with petroleum ether (30-60° BP) and vacuum concentrated to 150-200 ml. Each concentrate was diluted 1:4 with HPLC-grade water, adjusted to pH 3.0 with 5 N NaOH and adsorbed to ten activated C18 Sep-Pak cartridges (Waters) connected in tandem. The columns were washed with 0.05% trifluoroacetic acid (TFA) prior to elution. Sep-Pak columns were connected to the HPLC and eluted with 0.05% TFA and acetonitrile(AN)/0.05% TFA (80:20) as the mobile phases. A linear gradient with an increase of 1% $AN \cdot min^{-1}$ from 0 to 80% at a flow rate of 1 $ml \cdot min^{-1}$ was used (see dotted line in Fig. 3.2A). A 30 μl aliquot from each fraction was analyzed by RIA for the presence of immunoreactive (ir) alligator GHRH. For the three extractions, the immunoreactive peaks (fractions 39-47, 41-48 and 40-45) were pooled and vacuum concentrated.

4) *High performance liquid chromatography (HPLC) purification*

HPLC was done on a Varian 5000 liquid chromatograph connected to a 2050 detector and 4290 integrator. Blank runs were done before each sample run.

The Sep-Pak concentrate was doubled in volume by the addition of 0.05% TFA to decrease the viscosity of the extract. The sample was centrifuged at 10,000xg to pellet particulate material. The supernatant was filtered (0.22 μm) and loaded onto a Vydac Semi-Preparative C-18 column (25cm x 10mm, 5 μm particle size) by multiple injections at a flow rate of 2 $ml \cdot ml^{-1}$. The buffer was 0.05% TFA. Adsorbed material was eluted with 0.05% TFA (buffer A) and AN/0.05% TFA (80:20) (buffer B) with a gradient of 1% $AN \cdot min^{-1}$ and flow rate of 2 $ml \cdot min^{-1}$. All subsequent analytical runs were done at a flow rate of 1 $ml \cdot min^{-1}$. Immunoreactive fractions 33-41 were pooled, reduced in volume and loaded onto a Supelco analytical LC-18 column (0.46 x 25cm, particulate size 5μm). The mobile phases were the same as for the semi-preparative column, except the sample was loaded at 6% buffer B. The sample was eluted on a program of 6% buffer B for 10 mins, 6% to 75% over 55 min (1% $AN \cdot min^{-1}$) and then 75% B for 5 min (Fig. 3.2C). Fractions (39, 41-47) from this analytical run were chromatographed on a Vydac C-18 column (0.46 x 25cm, 5μm) using a program of 6% B for 10 min, then 6% to 30% B in 2 min, followed by a gradient of 0.16% $AN \cdot min^{-1}$ for 50 min

(Fig. 3.2D). The mobile phase was the same as the two previous runs. Immunoreactive fractions (43-53) were chromatographed on the same Vydac C-18 column using 0.13% heptafluorobutyric acid (HFBA) (buffer A) and AN/0.13% HFBA (80:20) (buffer B). Samples were loaded at 6% B. Buffer B was changed from 6% to 35% (28% AN) over 2 min, then increased as a linear gradient of 0.5% AN$\cdot$min^{-1} over 32 min followed by an increase to 60% AN (Fig. 3.2E). Immunoreactive fractions were again chromatographed using the same program except the mobile phases were changed to 0.05% TFA and AN/0.05% TFA (80:20) (Fig. 3.2F). Most of the immunoreactive material was contained within 2 fractions which were chromatographed on the same Vydac analytical column using 0.25M TEAP (pH 6.5) and AN/0.25M TEAP as the mobile phases. The samples were loaded onto the column at 6% B and the program was run at 6% to 30% B over 5 min, 30% to 75% B over 46 min and 75% B for 7 min (Fig. 3.3).

Ten percent of the major cross-reactive material from fraction 28 (Fig. 3.3) was applied to a narrow bore HPLC system consisting of a Hewlett-Packard 1090L Liquid chromatograph equipped with a Vydac C18 column (2.1 x 150mm, pore size: 300 Å, 5 μm). The flow rate was 0.2 ml$\cdot$min^{-1} and detection was at 210 nm. Elution was achieved by increasing the acetonitrile concentration in the eluant (0.5% trifluoroacetic acid/water) from 10 to 50% within 50 min. This was followed by 90% acetonitrile for 10 min.

5) *Sequence analysis*

HPLC fractions were subjected to Edman degradation in an automated protein sequencer (ABI Model 470A) equipped with an on-line phenylthiohydantoin (PTH) amino acid analyzer. The manufacturer's standard programs were used. Amino acid sequencing was done in collaboration with Dr. W.H. Fischer at The Salk Institute for Biological Studies, Clayton Foundation Laboratories for Peptide Biology, La Jolla, California.

6) *Coeultion of alligator NPY with synthetic human NPY*

Approximately 100 ng of each peptide (alligator NPY, human NPY and human NPY-OH) was analyzed by HPLC on a Hewlett-Packard 1090L Liquid Chromatograph equipped with a Vydac C18 column (2.1 x 150 mm; particle size: 5 μm; pore size: 300 Å). The mobile phase was 0.05% TFA in water (solvent A) and 0.05% TFA in 90% AN and 10% water (solvent B). After 10 min of isocratic elution at 30% solvent B, a linear gradient to 55% solvent B within 15 min was applied. The flow rate was 0.2 $ml \cdot min^{-1}$ and the absorbance was detected at 210 nm. Synthetic human NPY free acid (hNPY-OH) was purchased from Peninsula Laboratories. The synthetic human NPY ($hNPY-NH_2$) was kindly provided by Dr. Jean Rivier and Mr. Ron Kaiser, The Salk Institute, La Jolla, California.

RESULTS

During our program to identify GHRH-like material in alligator brain, an abundant protein was found to co-purify with the major immunoreactivity. Our antiserum (8119-7) cross-reacted with hGHRH, but showed no cross-reactivity with human NPY, chicken secretin, porcine PHI or VIP (human, porcine and rat) at concentrations of 0.01-50 ng (Fig. 3.1).

Immunoreactive material eluted from the Sep-Pak columns over a wide range of fractions (Fig. 3.2A). The elution profile was similar from all three Sep-Pak columns; Fig. 3.2A represents the results from one of these runs. Chromatography of the Sep-Pak immunoreactive fractions on a semi-preparative HPLC column concentrated the material into 9 fractions (Fig. 3.2B). Further purification and concentration of the immunoreactive material was obtained by elution through an analytical column (Fig. 3.2C). Two peaks of immunoreactive material were separated from a coeluting red pigment using a shallow gradient of 0.16% AN$\cdot$min^{-1} (Fig. 3.2D). The second peak was chromatographed through three more analytical HPLC runs (Figs. 3.2E, 3.2F, and 3.3); this resulted in a major absorption peak in which one fraction contained the highest concentration of immunoreactive material (Fraction 28, Fig. 3.3), when compared with the other fractions.

Final purification was achieved by narrow-bore reversed phase HPLC. Three fractions, 14, 19 and 20 (Fig. 3.4), were

analyzed by protein sequencing. The initial yield in the sequence analysis of 30% of fraction 20 was 765 pmol. In subsequent cycles, PTH amino acids were identified at 32-570 pmol (Table 3.1). Fraction 20 allowed the determination of a 36 amino acid sequence that a data base search revealed to be identical to human NPY: Tyr-Pro-Ser-Lys-Pro Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Met-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr. Fraction 19 contained the same N-terminal sequence and an impurity for which no significant match could be found in a data base search. In fraction 14 a sequence corresponding to hNPY1-23 was identified. This is likely to be a C-terminally truncated or otherwise modified form of NPY.

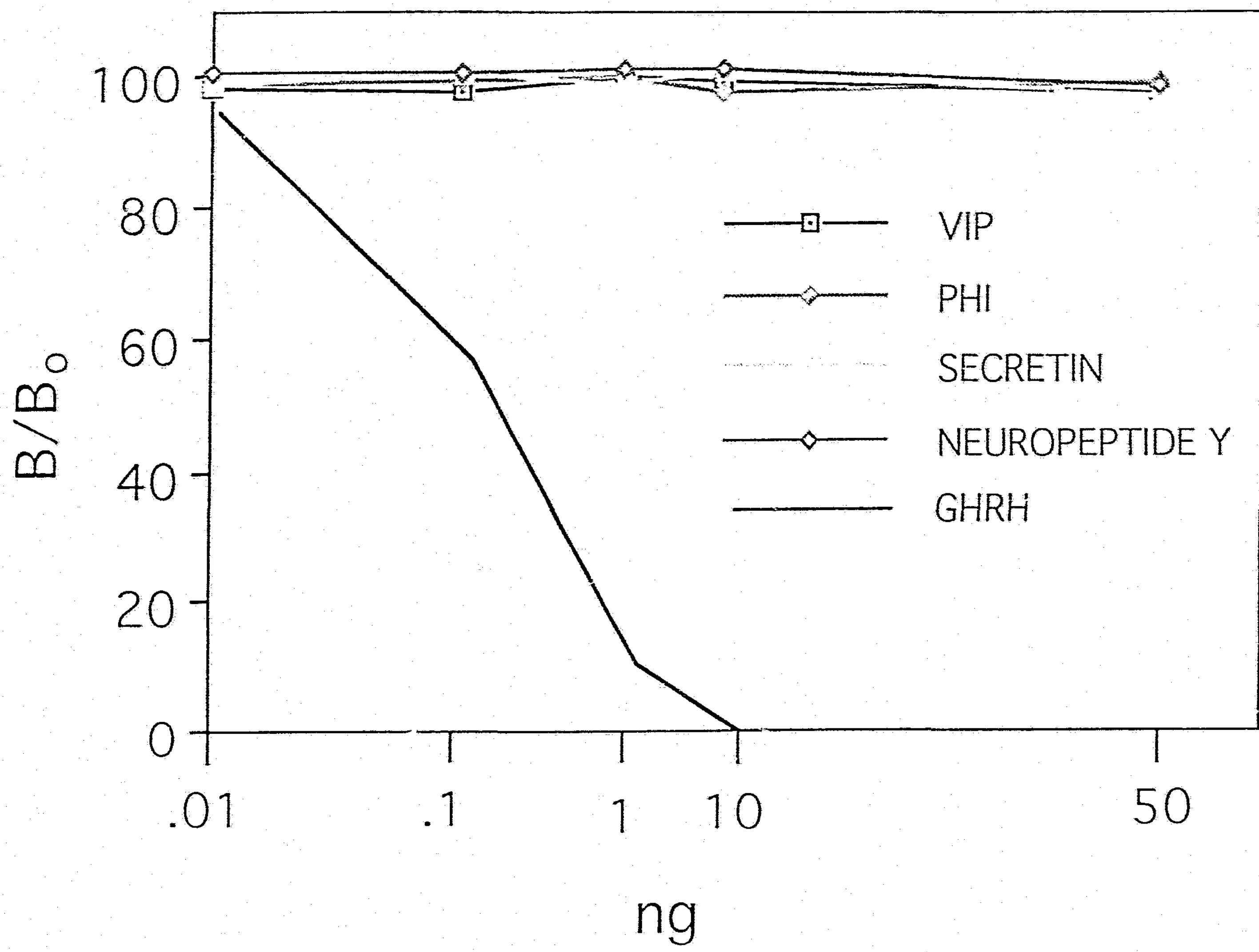
In the final HPLC run, immunoreactive GHRH was not separated from those peaks containing NPY. In fractions 19 and 20 (Fig. 3.4), immunoreactive GHRH represented less than 0.5% of the total amount of NPY. Fraction 19 contained the majority of the immunoreactive GHRH representing 1.13 pmole. Trace amounts of immunoreactive material were detected in fraction 14 and 20 (Fig. 3.4). In the sequence analysis of fractions 19 and 20, the amount of immunoreactive GHRH was not in sufficient quantity to show as a secondary sequence.

To confirm the presence of an amidated C-terminal tyrosine, the HPLC elution profile of alligator NPY was compared with those of synthetic human NPY and human NPY-OH. Alligator NPY coeluted with synthetic human NPY and eluted 0.92 min earlier than the synthetic human NPY-OH (Fig. 3.5).

TABLE 3.1: Yield of phenylthiohydantoin (PTH)-derivatized amino acids.

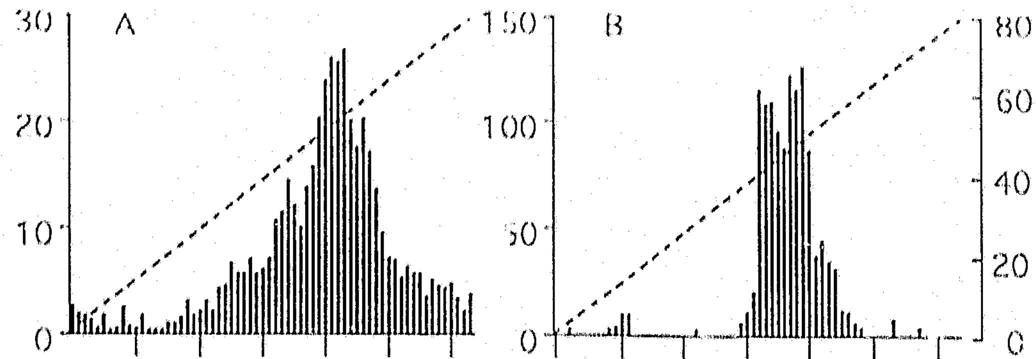
Cycle No.	amino acid	pmol
1	Tyr	765.3
2	Pro	481.3
3	Ser	120.9
4	Lys	560.9
5	Pro	328.2
6	Asp	286.4
7	Asn	252.2
8	Pro	244.3
9	Gly	229.0
10	Glu	233.7
11	Asp	230.5
12	Ala	422.4
13	Pro	244.3
14	Ala	570.0
15	Glu	147.9
16	Asp	157.3
17	Met	393.9
18	Ala	272.7
19	Arg	309.3
20	Tyr	270.1
21	Tyr	241.6
22	Ser	45.3
23	Ala	130.7
24	Leu	95.5
25	Arg	203.5
26	His	55.8
27	Tyr	135.4
28	Ile	89.5
29	Asn	65.6
30	Leu	80.7
31	Ile	87.8
32	Thr	31.6
33	Arg	154.6
34	Gln	56.8
35	Arg	131.9
36	Tyr	66.3

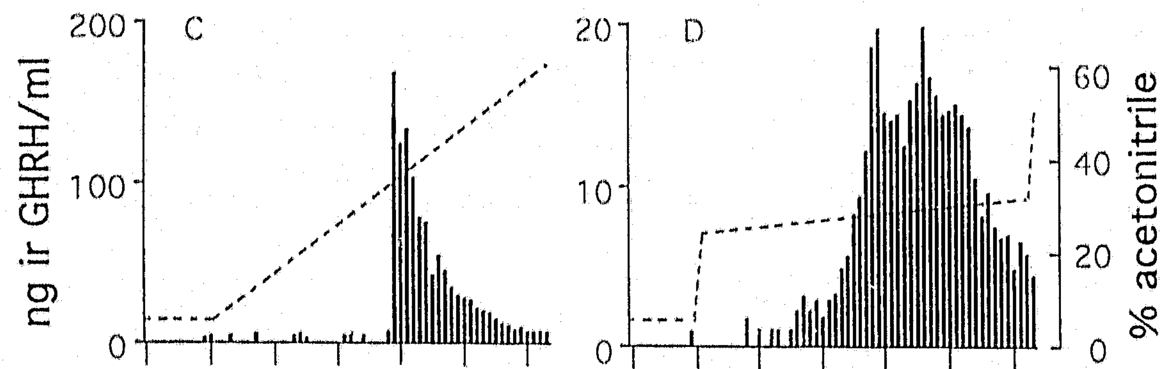
Figure 3.1. Binding of VIP (human, porcine and rat), PHI (porcine), secretin (chicken) and NPY (human) compared to the hGHRH (1-44 NH2) standard curve, using antiserum 8119-7 at a final dilution of 1:45,000.



100
80
60
40
20
0
B/B$_o$
.01
.1
1
10
50
ng
VIP
PHI
SECRETIN
NEUROPEPTIDE Y
GHRH

Figure 3.2. Initial purification strategy of alligator brain immunoreactive GHRH. A) Sep-Pak elution of the alligator brain crude extract using TFA and acetonitrile. This graph represents one of three runs. B) Pooled immunoreactive fractions from the three Sep-Pak runs were chromatographed on a semi-preparative reverse phase (rp)HPLC columun using TFA and AN as the buffers. C) A rpHPLC run of immunoreactive fractions from (B) on an analytical C-18 column with TFA and AN/TFA as the buffers. D) Separation of the immunoreactive material from a co-eluting red pigment was then achieved by using a linear gradient of 0.16% AN$\cdot$min^{-1} over 50 min with TFA and AN. E) Analytical rpHPLC run with HFBA and AN/HFBA. F) Analytical rpHPLC run with TFA and AN/TFA, using the same program as in (E).


A
B
% acetonitrile


ng ir GHRH/ml
C
D

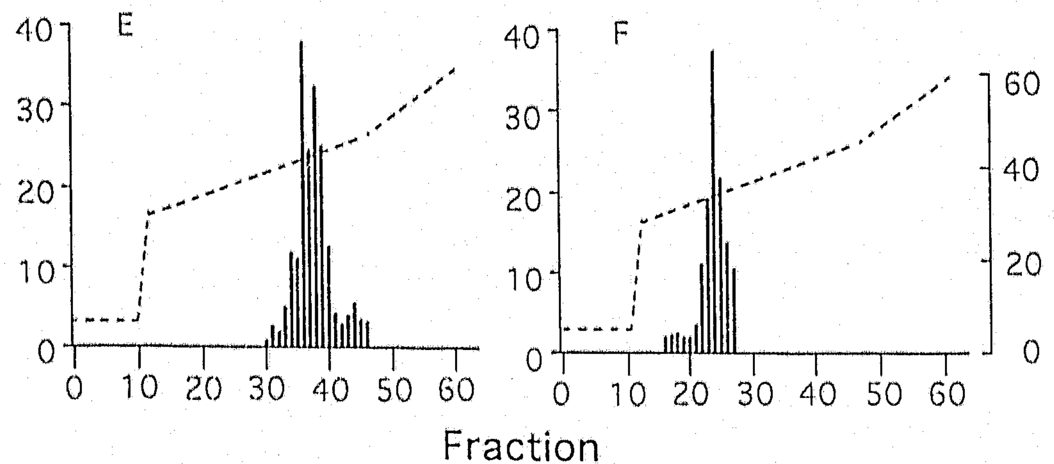

E
F
Fraction

Figure 3.3. An analytical rpHPLC run of immunoreactive fractions 24 and 25 (Fig. 2F) on a Vydac C18 column using TEAP (pH 6.5) and AN/TEAP as the mobile phases. A major immunoreactive peak was resolved from contaminating substances.

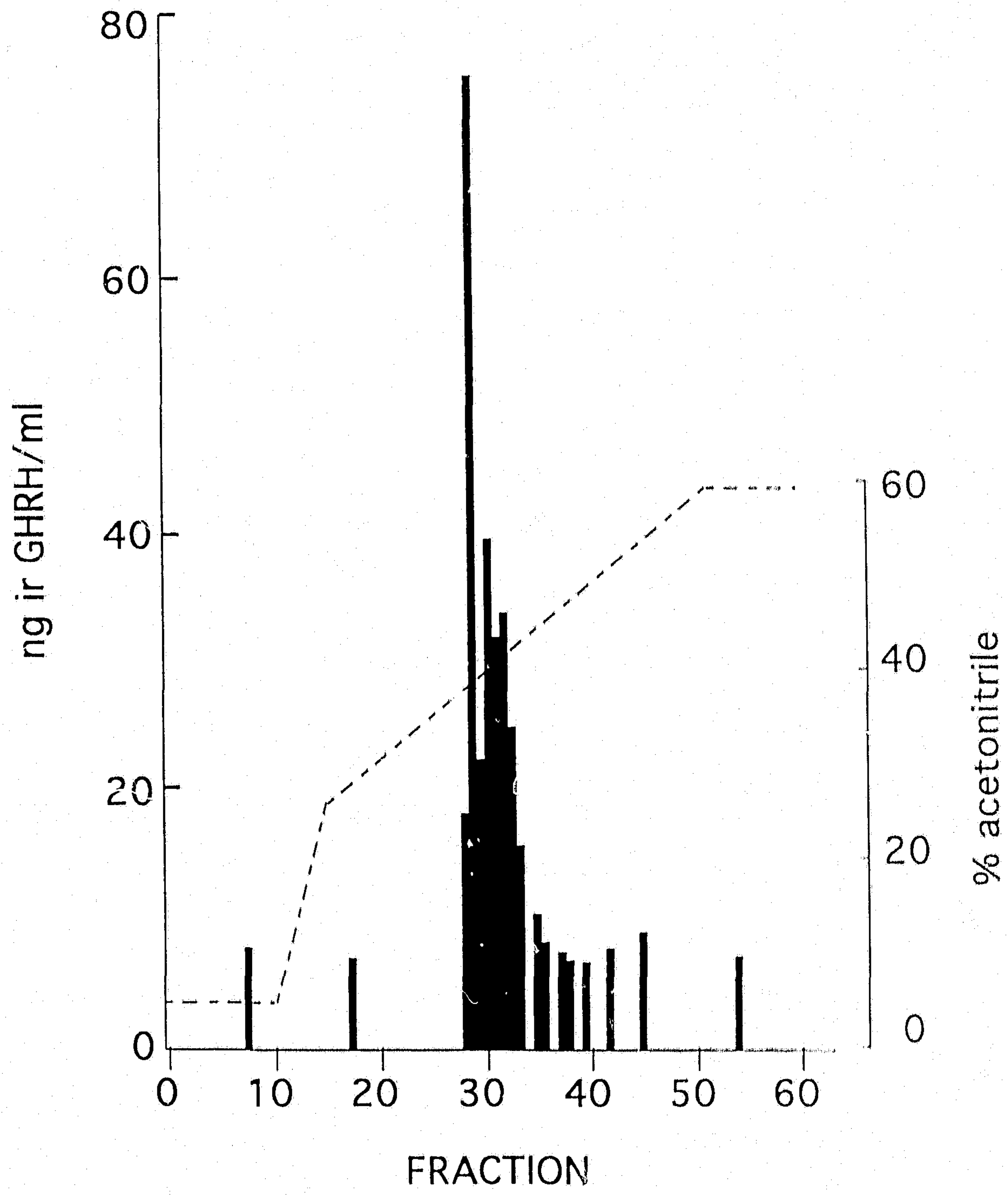

ng ir GHRH/ml
80
60
40
20
0
0
10
20
30
40
50
60
FRACTION
% acetonitrile
60
40
20
0

Figure 3.4. Final purification of immunoreactive material on HPLC using 0.5% TFA/water and AN as the mobile phases. The elution position of immunoreactive GHRH is represented by the horizontal bars.

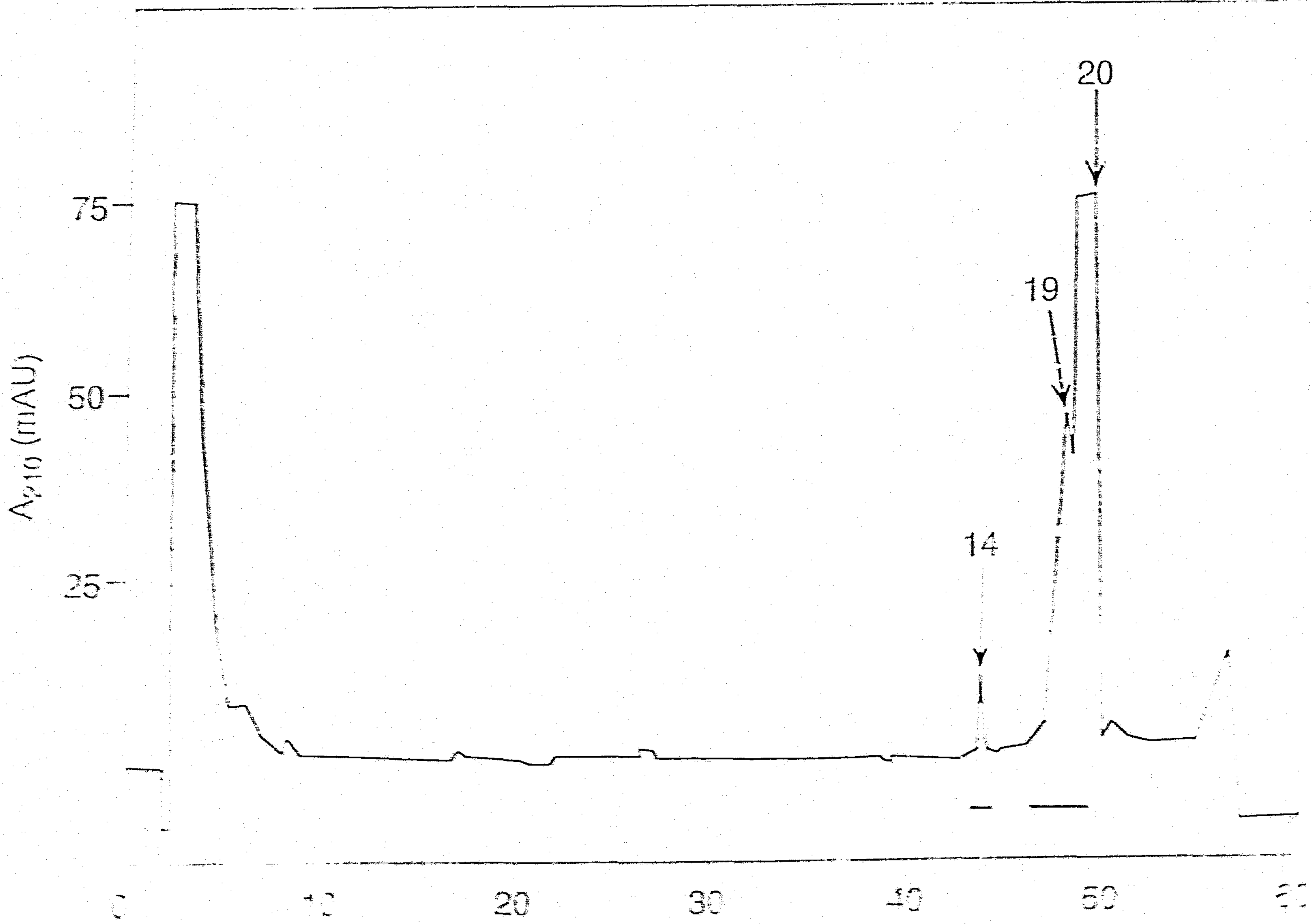
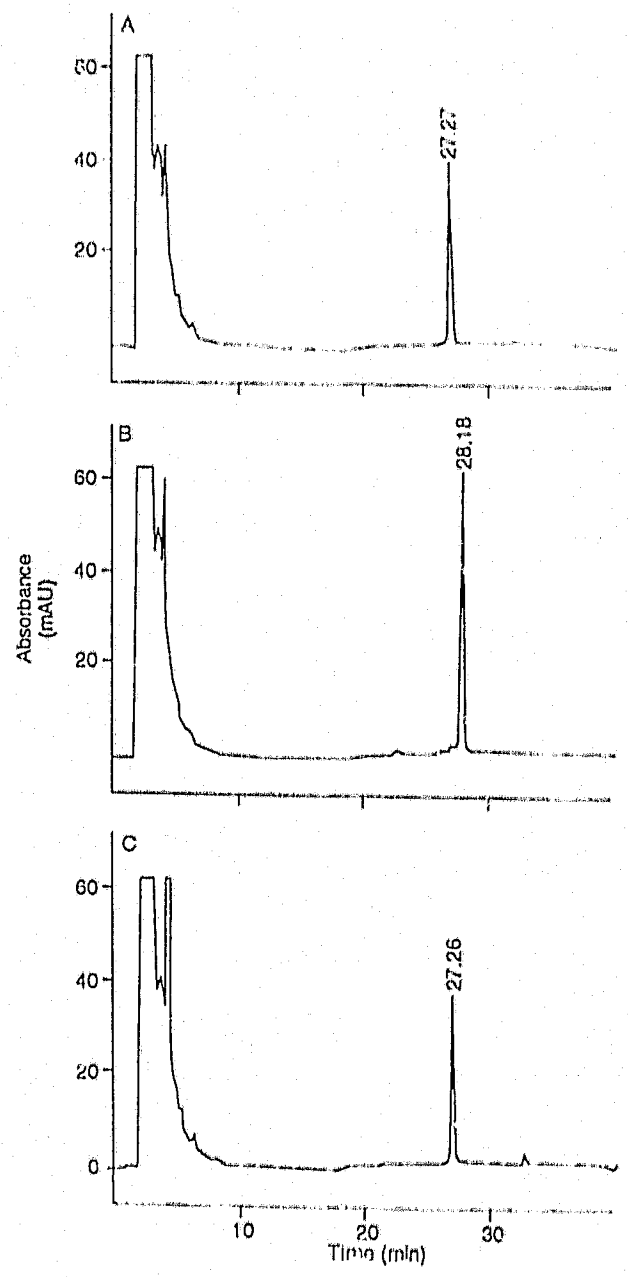
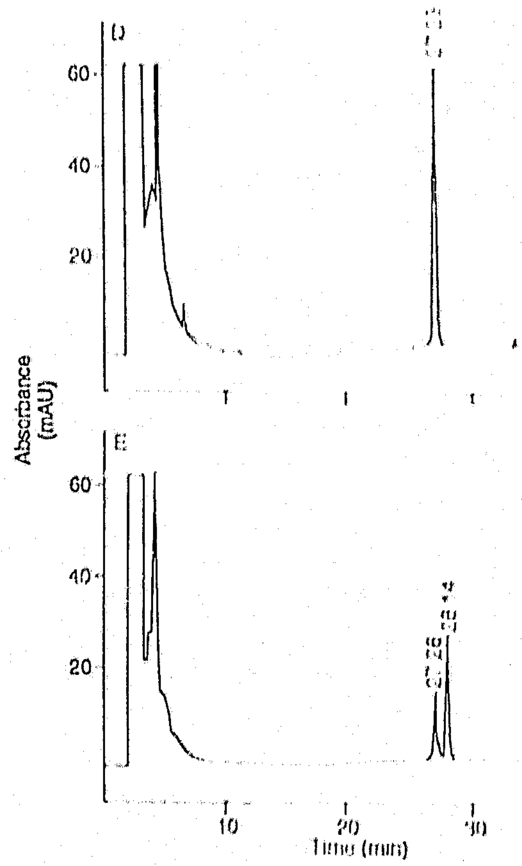

20
19
14
75
50
25
A_{210} (mAU)
20
30
40
50
Time (min)

Figure 3.5. Coelution of alligator NPY with synthetic human NPY and NPY-OH using rpHPLC (see materials and methods for HPLC parameters). A: synthetic human NPY, B: synthetic human NPY-OH, C: alligator NPY, D: alligator NPY + synthetic human NPY, E: alligator NPY + synthetic human NPY-OH


A
B
C
Absorbance
(mAU)
0
20
40
60
27.27
28.18
27.26
10
20
30
Time (min)


D
E
Absorbance
(mAU)
20
40
60
27.26
10
20
30
Time (min)

DISCUSSION

We have identified alligator NPY which is 36 amino acids in length and is identical to human NPY, the sequence of which was deduced from the cDNA (Minth *et al.*, 1984) and gene (Minth *et al.*, 1986). This is the first reptilian NPY to be identified, as well as the first non-mammalian vertebrate species to have 100% sequence identity to the human NPY. Our coelution studies showed alligator NPY to have a carboxy terminal amide, which is consistent with all NPY molecules identified to date (Larhammar *et al.*, in press).

Alligator pancreatic polypeptide (PP) (Lance *et al.*, 1984) is the only other member of the NPY, PP and PYY family of regulatory peptides that has been identified from the Class Reptilia. The present study confirms previous indirect evidence that NPY is present in reptiles; NPY immunoreactivity has been reported in the telencephalon of the turtle (Reiner and Oliver, 1987) and the spinal cord of the lizard (Marti *et al.*, 1990). In addition, Larhammar *et al.* (in press) showed that a rat NPY DNA probe hybridizes to crocodile genomic DNA.

Identification of alligator NPY in this study was unexpected in that the original intent was to isolate alligator GHRH. Therefore, the purification protocol was based on cross-reactivity with an antiserum to growth hormone-releasing hormone (GHRH). However, amino acid

analysis of one of the purified peaks (fraction 20) revealed a sequence identical to NPY. Two possibilties are that NPY cross-reacted with the human GHRH antiserum or that NPY coeluted with immunoreactive GHRH during purification. Subsequent tests showed that NPY in the range of 0.1-50 ng did not cross-react with the GHRH antiserum used in this study. Therefore, it appears that NPY and immunoreactive GHRH overlapped in their elution profiles. GHRH and NPY may have similar hydrophobicity, a property that is important in reverse phase HPLC elution position. Also, NPY has been found to oxidize easily causing a shift in its elution position on HPLC (O'Hare *et al.*, 1988), which may have resulted in its co-elution with irGHRH-like material. Steps taken to prevent peptide oxidation in this study, may not have been adequate for the large amount of NPY that was present. Also, the fact that NPY is the most abundant neuropeptide detected in the mammalian brain (Adrian *et al.*, 1983; Allen, *et al.*, 1983), suggests NPY is more likely to overlap in detectable amounts with peptides that elute in neighbouring fractions.

NPY has been reported to coexist with GHRH in the neurons of the arcuate nucleus of the rat (Ciofi *et al.*, 1987). These neurons are medially located and do not project into the median eminence, suggesting a neuromodulatory or neurotransmitter role rather than a direct effect on the pituitary (Ciofi *et al.*, 1987). The co-localization of GHRH and NPY in perikarya of the hypothalamus, not related to the

median eminence, is interesting because both peptides have been found to stimulate feeding behavior when injected into the hypothalamus of rats (see reviews by McDonald, 1988; Vaccarino, 1990). The interplay between NPY and GHRH in the physiology of growth, is further emphasized by the fact that NPY has been found to promote GH release from the pituitary cells of rats (Chabot *et al.*, 1988) and fish (Peng *et al.*, 1990).

Identification of alligator NPY is important for understanding the evolution of this family of molecules. NPY is a highly conserved peptide. This suggests the ancestral NPY structure for the tetrapods may have been similar to that of Torpedo NPY (Blomqvist *et al.*, 1992). In support of this idea, frog NPY (Chartrel *et al.*, 1991a) has only two substitutions compared to Torpedo NPY, while alligator and human have an additional change. Therefore, the substitutions of aspartic acid at position 11 and methionine at position 17, as seen in frog, alligator, and human NPY, may have occurred before the tetrapods arose. In tetrapods the divergence of the amphibians and amniotes resulted in a change in NPY from lysine at position 19 to arginine. Hence, only a single substitution separates NPY among frog, alligator and human.

The fact that NPY is identical in alligators and humans suggests that this sequence is the basic structure for the ancestral amniote NPY. This same sequence is found for NPY in rat (Larhammar *et al.*, 1987), guinea pig (O'Hare *et al.*,

1988), and rabbit (O'Hare *et al.*, 1988). One consequence of this is that one of the substitutions in ovine (Sillard *et al.*, 1989), bovine (Tatemoto, 1989) and porcine (Tatemoto, 1982) NPY are recent. The conservation of alligator-human NPY suggests that chicken NPY (Blomzvist *et al.*, 1992) acquired a serine in position 7 after birds and reptiles diverged from a common Archosaurian ancestor.

Chapter 4

Two Salmon Neuropeptides Coded in One Brain cDNA are Structurally Related to Growth Hormone-Releasing Hormone (GHRH) and Pituitary Adenylate Cyclase Activating Polypeptide (PACAP)

INTRODUCTION

The glucagon superfamily contains a number of members including growth hormone-releasing hormone (GHRH), glucagon, vasoactive intestinal polypeptide (VIP), peptide histidine methionine (PHM), peptide histidine isoleucine (PHI), secretin, gastric inhibitory peptide (GIP), helodermin and helospectin. Recently, two new candidates, pituitary adenylate cyclase activating peptide (PACAP) and PACAP-related peptide (PRP) were added for mammals. Structurally, they resemble VIP and GHRH.

Two forms of PACAP have been identified from ovine hypothalamus. One is a 38 residue peptide (Miyata *et al.*, 1989) and the other is a shorter form of 27 residues (Miyata *et al.*, 1990), but both have an amidated carboxy terminus. The cDNA has been cloned from ovine hypothalamus (Kimura *et al.*, 1990), human testis (Kimura *et al.*, 1990; Ohkubo *et al.*, 1992) and rat brain (Ogi *et al.*, 1990). As well, the human gene is known (Hosoya *et al.*, 1992). All three of the mammalian PACAP's are identical, while the recently identified frog brain PACAP has a single amino acid substitution (Chartrel *et al.*, 1991b). Human(h) PACAP has 68% sequence identity to hVIP as well as a limited identity to hGHRH. In tandem with PACAP in the precursor is the PRP region with 38% sequence identity to GHRH (Ohkubo *et al.*, 1992). PACAP has been placed as a member of the glucagon superfamily not only because of the close sequence identity

with VIP and GHRH (Ohkubo *et al.*, 1992), but also because of the similarity of the PACAP gene structure (Hosoya *et al.*, 1992) to that of the VIP gene (Bodner *et al.*, 1985; Giladi *et al.*, 1990). However, the evolution of this superfamily is not well understood. Neither the cDNA nor gene has been identified for PACAP, VIP or GHRH in a non-mammalian vertebrate. Indirect evidence of a GHRH molecule in fish (Chapter 2; Parker and Sherwood, 1990; Ackland *et al.*, 1989), suggests this molecule has been conserved. However, no information regarding the existence of PACAP in fish has yet been presented.

The physiological role of PACAP is not well understood. The highest concentrations of PACAP in the rat have been found in the hypothalamus, testis and adrenal gland (Arimura *et al.*, 1991). However, the distribution of PACAP also includes other tissues such as extrahypothalamic brain (Arimura *et al.*, 1991; Köves *et al.*, 1991), respiratory tract (Uddman *et al.*, 1991), and gut (Arimura *et al.*, 1991). Immunolocalization studies show PACAP nerve fibers extending throughout the mammalian brain and making contact with the portal vessels (Köves *et al.*, 1991; Vigh *et al.*, 1991; Köves *et al.*, 1990). Receptor binding assays have identified high affinity binding sites in the brain, pituitary, testis and adrenal gland, while peripheral tissues share low affinity sites with VIP (Robberecht *et al.*, 1991; Shivers *et al.*, 1991; Gottschall *et al.*, 1990; Lam *et al.*, 1990; Schäfer *et al.*, 1991; Suda *et al.*, 1991). These results imply that

PACAP may potentially function as a releasing factor, neurotransmitter or neuromodulator (Köves *et al.*, 1991; Köves *et al.*, 1990).

As a releasing factor, PACAP not only stimulated GH release and the number of GH secreting cells in a hemolytic plaque assay, but also acted through receptors distinct from those for GHRH (Goth *et al.*, 1992). Also, PACAP has been shown to stimulate the release of GH, prolactin, ACTH and LH in a rat pituitary cell perfusion, but not in a static cell culture (Miyata *et al.*, 1989; Culler *et al.*, 1991). PACAP was a weak stimulator of LH and FSH secretion and acted synergistically with GnRH to promote gonadotropin release from rat anterior pituitary cell cultures (Culler *et al.*, 1991).

To understand the evolution of the glucagon superfamily, and more specifically the GHRH and PACAP families, as well as the control of GH release, it is important that these molecules be identified in the non-mammalian vertebrates. Therefore, I have cloned the cDNA for sockeye salmon GHRH/PACAP precursor, which contains not only PACAP, but also an area of high sequence similarity with carp GHRH-like peptide and the mammmalian PRP region.

METHODS AND MATERIALS

mRNA purification and cDNA synthesis

Sockeye salmon (*Oncorhynchus nerka*) were anesthetized with carbon dioxide and the brains were immediately excised and frozen in liquid nitrogen. Total mRNA was made from sockeye salmon brains by extraction with acidic guanidinium thyiocyanate according to Chomczynski and Sacchi (1987). Poly $(A)^+$ mRNA was purified by oligo (dT) affinity chromatography (Pharmacia). Double stranded cDNA was synthesized from 5 μg of poly $(A)^+$ mRNA using Stratagene's cDNA synthesis kit, extracted with phenol-chloroform, precipitated with ethanol and dissolved in 50 μl of water.

For RNA work, HPLC grade water was treated with diethylpyrocarbonate (DEPC) and autoclaved. Glassware used for all RNA and DNA experiments was baked for 12 h at 220°C. Where applicable, DNase and RNase free, sterile disposable labware was used.

Amplification of cDNA

PCR samples were each prepared in a 100 μl volume containing 10 μl of double stranded cDNA, 100 pmol of primer CSC 33, 100 pmol of adaptor GAATTCT(dT)36, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01% gelatin, 0.05% NP 40, 0.05% Tween 20, 1.5 mM $MgCl_2$, 200 μM dNTPs and 2.5 units of Taq DNA polymerase (Perkin-Elmer Cetus). DNA was amplified using a step program of 1 cycle at 94°C for 3 min, 55°C for 2 min,

and 72°C for 4 min, followed by 29 cycles of 94°C for 1 min 30 s, 55°C for 2 min and 72°C for 4 min (DNA Thermal Cycler, Perkin-Elmer Cetus). The last cycle had an 8 min extension at 72°C. Ten microliters of PCR product were removed and reamplified under the same conditions. PCR products were chloroform extracted, ethanol precipitated at -20°C, and separated by electrophoresis on a 2.0% agarose gel (Bio 101). cDNA bands were recovered by binding to Glassfog™ using MERmaid (Bio 101). To increase the amount of cDNA for cloning, 50% of the cDNA recovered from these bands was reamplified using the same program conditions.

Amplification of the 5'end

The rapid amplification of cDNA ends (RACE) strategy designed by Frohman *et al.* (1988), with minor modifications, was used to amplify the 5'end of the cDNA. Two microliters of sockeye salmon poly $(A)^+$ mRNA (0.344ug/μl) were mixed with 10 pmol of NMS3 primer (5' TCGGTAGCGGCTGTAGCTATCTG 3') and 7 μl of DEPC treated water to a final volume of 10 μl, heated at 65°C for 3 min then cooled rapidly on ice. Single stranded cDNA was made by adding 10 μl of reverse transcriptase solution (4 μl 5X BRL buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$), 2 μl 0.1 M DTT, 2 μl 10 mM dNTP's, 1 μl RNA guard (Pharmacia), 1 μl M-MLV H^- RT (Superscript, BRL)) to the mRNA/primer mix and incubating for 2 h at 41°C. The reaction was stopped by heating to 75°C for 10 min, then diluted to 500 μl with TE pH 7.5 and stored at 4°C. The

primer was removed from the reaction mixture by centrifugation through a centricon-100 (Amicon) twice with 0.2X TE pH 7.5. First strand cDNA was vacuum concentrated to 12.5 μl, of which 10 μl was tailed with dATP at 37°C for 5 min in 4 μl 5X BRL buffer, 4 μl 1 mM dATP, 1 μl water and 1 μl TdT enzyme (BRL). The reaction mixture was heat denatured 5 min at 70°C and diluted to 200 μl with sterile water. The remaining 2.5 μl of first strand cDNA was sham tailed (no enzyme) under the same conditions.

Polymerase chain reactions were done in 50 μl volumes containing 20 pmol of primer NMS1 (5' TGACAGAGGCTCTGTGTC 3'), 20 pmol of adaptor #3(GGCTCGAGCCCGGGAATTCCG), 5 pmol of adaptor #2(GGCTCGAGCCCGGGAATTCCG-dT_{15}), 1X Promega buffer (50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100), 2.0 mM $MgCl_2$, 200uM dNTP's, and 2.5 units of Taq DNA polyermase (Promega). In the first round of the PCR a 5 μl aliquot of dATP tailed cDNA was amplified using a step program for 30 cycles (DNA Thermal Cycler, Perkin-Elmer Cetus). In the first cycle the DNA was denatured at 94°C for 3 min, annealed at 60°C for 2 min and extended at 72°C for 8 min. The following 29 cycles were 94°C, 58°C and 72°C, all at 1 min intervals except for a final 8 min extension at 72°C. The PCR products were separated on a 1.5% agarose gel (Seakem, FMC BioProducts). DNA bands were electroeluted in dialysis tubing (Spect/Por, Spectrum Medical Ind., Inc.) with 1X TAE, ethanol precipitated at -20°C and dissolved in 20 μl of water. Three microliters of this DNA was

reamplified using primers NMS1 and adaptor #3 with a lower annealing temperature and more cycles (1 cycle at 94°C, 3 min; 55°C, 2 min; 72°C 5 min; 41 cycles at 94°C, 1 min; 55°C, 1 min; 72°C 1 min). The PCR amplified DNA was extracted, gel purified and electroeluted as previously.

Another strategy used to determine the 5' end of the GHRH/PACAP precursor, was to use the PCR to screen a chum salmon cDNA library prepared in lambda ZAP II (Stratagene) by Imogen Coe (1992). To amplify the 5' erd, primer NMS 1 in combination with primer SK or KS (Stratagene) were used in the first round of the PCR (1 cycle at 94°C for 3 min, 45°C for 2 min, 72°C for 10 min; 41 cycles at 94° for 1 min, 45°C for 2 min, 72°C for 2 min; the last cycle had an 8 min extension at 72°C). SK and KS are 17 base sequencing primers within the multiple cloning region of the pBluescript II SK$^+$ phagemid (Stratagene). The phagemid is contained within the lambda ZAP II vector. These primers bind on opposite strands, on either side of the multiple cloning site. The PCR products were separated on a 2% agarose gel (Seakem) and the DNA bands retrieved as previously described. One fifth of this DNA was then reamplified with primers NMS2 and SK. The NMS2 primer was selected because it is internal to NMS1 and hence is specific for the region desired for amplification. The program used in the second round of the PCR was the same as the first, except the annealing and extension steps were 1 min 30 s. DNA bands obtained from the second round of the

PCR were reamplified (40 cycles at 94°C for 1 min, 45°C for 1 min and 72°C for 1 min) to produce enough DNA for cloning.

DNA cloning

DNA obtained from the PCR was blunt end ligated into pBluescript II KS+ (Stratagene), which had been cut with EcoR V and dephosphorylated. The PCR products were blunt ended and kinased in 1X ligase buffer (50 mM Tris-HCl pH7.6, 10 mM $MgCl_2$, 5% polyethylene glycol-8000), 1 mM ATP and 200 μM dNTP's. Klenow polymerase (Pharmacia) was added and the reaction mixture incubated at 12°C for 1 h, followed by the addition of T4PNK (Pharmacia) and an incubation at 37°C for 45 min. The reaction mixture was heat denatured at 75°C for 15 min, then placed on ice. The cDNA was blunt end ligated into pBluescript II at 22°C for 16-18 h by the addition 1 μl 5X ligase buffer, 2 μl 100 mM DTT, 0.5 μl 10 mM ATP, 0.5 μl vector and 1 unit of T4 ligase (BRL). Recombinant plasmids were identified by blue and white selection in XL-1 blue cells (Stratagene, La Jolla, CA). Miniprep plasmid DNA was prepared by the alkaline hydrolysis method (Birnboim, 1983) and digested with Pvu II to identify plasmids with inserts. Recombinant plasmids were then screened by the PCR using primers CSC33 and NMS1 to identify clones containing the GHRH-like cDNA.

Sequencing

Single and double stranded cDNA inserts were sequenced by the chain termination method (Sanger *et al.*, 1977) using ^{35}S- dATP (Dupont) and Sequenase Version 2.0 according to the manufacturer's instructions (US Biochemical Corp., Cleveland, OH). Sequencing products were run on 5% or 6% polyacrylamide/7 M urea wedge gels and the gels dried under vacuum at 80°C (Bio Rad). Gels were exposed to XAR-5 film (Kodak) for 16 to 24 h.

Southern and Northern Blots

Sockeye salmon genomic DNA (24 μg) was digested with either Hae III, Hind III, Pst I, or EcoR I and electrophoresed on a 1% agarose gel. DNA was capillary transferred to Zeta probe nylon membrane (Bio-Rad) and prehybridized in aqueous buffer (Church and Gilbert, 1984) at 60°C for 3 h. A 355 bp cDNA probe, which had been produced by digesting the salmon GHRH/PACAP cDNA with MSE I, was labeled with ^{32}P-dCTP (Dupont) by random priming and hybridized to the filter (5.6 X 10^6 cpm/ml) in an aqueous buffer at 60°C for 22 h. The filter was washed twice in 2X SSC/0.1% SDS for 15 min at room temperature, then twice in 1X SSC/0.1% SDS for 15 min at 60°C and twice again in 1X SSC/0.1% SDS for 10 min at 60°C. The filter was dried and exposed to XAR-5 film (Kodak) for 6 days.

Total RNA or poly $(A)^+$ mRNA was prepared as previously described (see above). Northern blots and hybridization

conditions were done according to Ausubel *et al.* (1987). Ten micrograms of brain poly $(A)^+$ mRNA was electophoresed on a formaldehyde denaturing agarose gel in 1X MOPS running buffer. RNA was capillary transferred with 10X SSC to a Zeta probe nylon membrane (Bio-Rad). The membrane was baked for 2 h at 80°C and then prehybridized for 4 h at 42°C with 15 ml of hybridization buffer (6X SSC/50% formamide/0.1% TWEEN 20/100 $\mu g \cdot ml^{-1}$/100 $\mu g \cdot ml^{-1}$ denatured sea urchin DNA) in a sealed plastic bag. The membrane was hybridized with a 588 bp cDNA probe for 18-20 h at 42°C in hybridization solution. The cDNA probe was made by amplification of the salmon GHRH/PACAP clone (Fig. 12) using primers SP1 (5'GGAATCATAATGCACTACAGTGTC 3') and NMS5 (5'GAACACAAGAGCGATCCACTGA 3'), and labeled with ^{32}P-dCTP by random priming.

RESULTS

GHRH and PACAP sequences from PCR

Degenerate primers were constructed based on conserved sequences in the human (Gubler *et al.*, 1983) and rat (Mayo *et al.*, 1985b) GHRH precursors (preprohormone). Initially, combinations of primers made to regions in the signal peptide (sense), GHRH (sense and antisense) and the C-terminal region (antisense) did not amplify any specific cDNA bands. This suggested that regions of the precursor

outside the peptide coding domain were not conserved in the salmon GHRH cDNA. New primers were designed using inosine in the third position of those codons where four nucleotides were possible. These primers were based on conserved regions of GHRH in human (Guillemin *et al.*, 1982), rat (Spiess *et al.*, 1983) and carp (J. Rivier, personal communication). Primer CSC33 was made to the first seven amino acids of GHRH and primer SC12 to amino acid positions 12-19. Primer SC12 required the insertion of five inosines compared with two for primer CSC33. This may have been the reason for the high nonspecific binding of primer SC12, which did not amplify a GHRH-like molecule. Since previous results suggested little if any conservation of the cryptic regions, we used an oligo (dT) as the 3' antisense primer.

A schematic diagram of the primers used in the PCR, in relation to their position on the preprohormone mRNA, are shown in Figure 4.1. Primer CSC33 and an oligo (dT) primer were used in the PCR to amplify a 425 bp cDNA fragment from sockeye salmon cDNA (Fig. 4.2A and 4.3). The cDNA was subsequently cloned into pBluescript KS II. Two clones, designated as SS/PCR 4 and SS/PCR 5 (SS=sockeye salmon) (see lanes 4 and 5, Fig. 4.4), were sequenced and each was found to contain a GHRH-like and PACAP sequence (Fig. 4.5A). These two clones differed at 13 bp positions and clone SS/PCR 5 had a deletion of 4 bases (Fig. 4.5A). Three of the nucleotide changes between clones SS/PCR4 and SS/PCR5 resulted in amino acid substitutions: serine in place of

threonine at position 123, serine in place of asparagine at position 165 and alanine for glycine at position 171 (Fig. 4.5A).

RACE/PCR amplification for 5' GHRH/PACAP cDNA

To identify the cDNA sequence 5' to the coding region of the GHRH-like region, the sequence-specific primer NMS 3 was used to initiate the reverse transcription of poly $(A)^+$ mRNA to produce single stranded cDNA (Fig. 4.2B). The cDNA was tailed with dATP and subsequently used in the PCR with anchor #2 (dT15), anchor #3 and the internal primer NMS1 (Fig. 4.2C). A cDNA fragment of approximately 400 bp was cloned and sequenced. Two of 32 plasmid preparations, identified with PCR, contained the 5' GHRH-like region. These two clones, SS/RACE 2 and SS/RACE 7 were different at 4 nucleotide positions, resulting in 4 amino acid changes within the coding region of the precursor (Fig. 4.6). Only the substitution of a cytosine at position 346, for an adenine, changes the primary structure of the GHRH-like peptide (Fig. 4.6). This substitution has not been confirmed by sequencing on both strands. The 5' untranslated regions of these two clones were very different in size and sequence. Thirty nine bases of the 5' untranslated region were identified in clone SS/RACE 2 and 84 bases in clone SS/RACE 7. Clone SS/RACE 7 has an an additional start codon (ATG) in the untranslated region at position -58 (Fig. 4.6), but, translation from this site

would end at an in frame stop codon 61 bases downstream (position 6, Fig. 4.6).

Full length GHRH/PACAP cDNA

A full length GHRH/PACAP cDNA was obtained by digesting the two clones, SS/RACE 2 and SS/PCR 4, with the restriction endonucleases Nco I (Fig. 4.5B) and Not I (Fig. 4.7). The latter enyzme cleavage site is located in the multiple cloning site of pBluescript II KS. After removal of the fragment from clone SS/PCR 4, the fragment obtained from clone SS/RACE 2 was ligated into clone SS/PCR 4 (Fig. 4.7). This resulted in a PACAP cDNA clone of 707 bp with an open reading frame of 173 amino acids (Fig. 4.5A).

PCR products obtained from screening the chum salmon (CS) cDNA library with primers NMS 2 combined with SK or KS, were assessed for GHRH/PACAP sequences. Two different 5' untranslated regions were identified in clones (CS/LIBa and CS/LIBb) containing the short precursor form (Fig. 4.8). Clones containing the 5' untranslated region, including these two short versions and the clones from sockeye salmon, were identical from position -9 through to -1, except for clone SS/RACE 7 which had an adenine at position -4 (Fig. 4.9). Upstream of position -9, the 5'untranslated regions were completely different in all the clones (Fig. 4.9). Like clone SS/RACE 7, clone CS/LIBb has an additional ATG codon in the 5' untranslated region and an in frame stop codon downstream (Fig. 4.9).

Short precursors lacking the GHRH coding region

From the PCR products amplified from the chum salmon library, six clones (CS/LIB) were identified that contained a shortened version of the GHRH/PACAP precursor. Four clones had a complete sequence from the NMS2 binding site through to the 5' untranslated region (Fig. 4.8). Two different 5' untranslated regions (CS/LIBa and CS/LIBb) were identified from these four clones (Fig. 4.8). The remaining two clones were truncated versions of the short precursor. In all CS/LIB clones the short precursor was formed by a deletion of 105 nucleotides at positions 234-338 (compare Fig. 4.8 with 4.5). Five of the short precursor CS/LIB clones were found to contain an adenine at position 232 like that of the longer form of clone SS/RACE 7 (compare Fig. 4.8 with 4.6). This results in a change in the reading frame of the short precursor to a serine at amino acid position 78 (Fig. 4.8). Following position 78 in the short precursor, the reading frame then commences at position 114 of the longer GHRH/PACAP precursor (Fig. 4.5A and 4.6) and remains unchanged. Clone SS/RACE 2 has a guanine at position 232, which results in the codon for glycine. Whether the remaining clone of the short precursor form have an adenine or a guanine, remains to be determined by sequence analysis.

Analysis of other clones from the RACE reaction also showed two clones (SS/RACE) that contained the same short precursor form as CS/LIB. Although these clones were identical in respect to the region deleted (bp positions

234-338), they had several nucleotide substitutions resulting in three amino acid changes (data not shown).

Organization of the GHRH/PACAP precursor

The salmon GHRH/PACAP precursor is 173 amino acids containing four distinct domains: a signal peptide, a cryptic peptide (following the signal peptide), the GHRH-like region and PACAP, which are 22, 59, 45 and 38 amino acids, respectively (Fig. 4.10). One clone (SS/RACE 7, Fig. 4.6) and the short precursor forms (Fig. 4.8) have a cysteine at position 22 in the signal peptide in place of a serine (shown in Fig. 4.10). The salmon GHRH-like peptide of 45 amino acids has a single arginine processing site at position 81 and a dibasic lysine-arginine processing site at positions 127 and 128 (Fig. 4.5A and 4.10). The salmon PACAP is preceded by a Lys-Arg processing site and has a Gly-Arg-Arg at its carboxy terminus. A peptide of 38 amino acids with an amidated carboxy terminus could result. There is a Gly-Lys-Arg processing site at positions 156-158 of the salmon precursor (Fig. 4.5A). This site lies within the salmon PACAP-38 and could result in a PACAP-27-NH2 identical with the ovine PACAP-27-NH_2 (Miyata et al., 1990).

The 3'untranslated region is shorter in salmon GHRH/PACAP than in the mammalian precursors. Both clones SS/PCR 4 and SS/PCR 5 appeared to be complete because a homopolymeric (dA) tail was present with a putative polyadenylation signal 16 bp upstream. The polyadenylation

signal, ATTAAA, is different from the ovine and human AGTAAA. However, the 3'UT regions are highly conserved between the salmon, human, sheep and rat (Fig. 4.11). There is 70% sequence identity (114 of 160 nucleotides, including gaps) of the 3' untranslated region between the salmon and mammals. The sequence AATC (590-594 bp, Figs. 4.5A and 4.11) is repeated in clone SS/PCR 4, but occurs only once in clone SS/PCR 5, and is absent in the ovine and human precursors (Fig. 4.11).

Southern Blots

A Southern blot analysis of sockeye salmon genomic DNA was done using a 355 bp probe which included the GHRH and PACAP coding regions and 91 bp of the 3' untranslated region (Fig. 4.12). Restriction enzyme digests resulted in two bands in lanes A2-4 under high stringency washing. These bands may represent different allelic forms or genes (Fig. 4.12). Digestion with Hae III resulted in a single band in lane A-1 which is about double the intensity compared with the bands in lanes A2-4; the band in A-1 may be two bands of similar length. Under low stringency washing conditions, additional bands in lanes B2 and B3 were present (Fig. 4.12). After washing under higher stringency conditions (A) these bands were removed. These bands may represent other members of the glucagon superfamily.

Northern Blots

Northern blots were done with a 588 bp probe that was amplified from the full length cDNA clone (see above). Studies of sockeye salmon and chinook salmon brain poly $(A)^+$ mRNA by northern hybridization detected a band which ranged from 2.2 to 2.7 Kb (Data not shown). The large band width suggested that mRNAs of varying length were present. Indeed, precursors of different sizes were obtained using the PCR. No hybridization signals were detected in chum salmon muscle, liver or heart total RNA.

Figure 4.1. A Schematic diagram showing the position of the primers used in the polymerase chain reaction of sockeye salmon cDNA. The arrows denote the 5' to 3' direction of the primers.

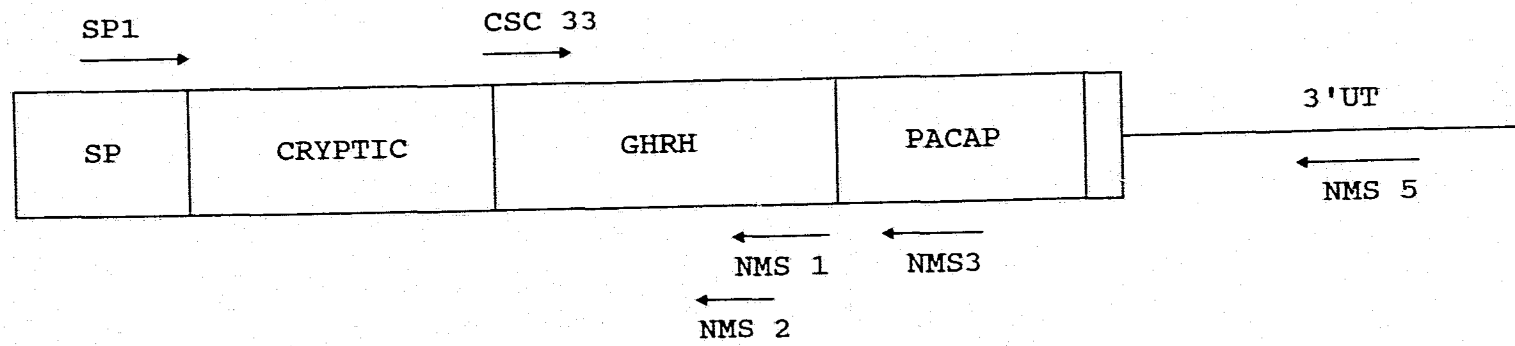
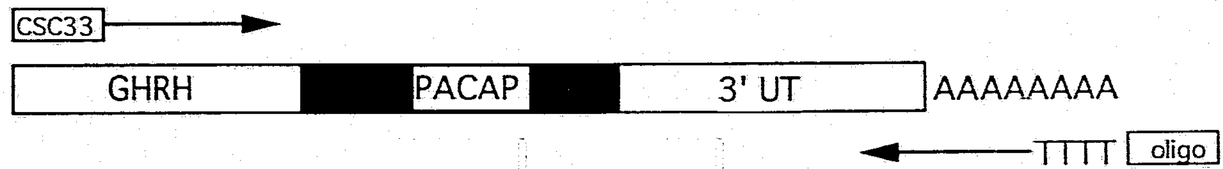
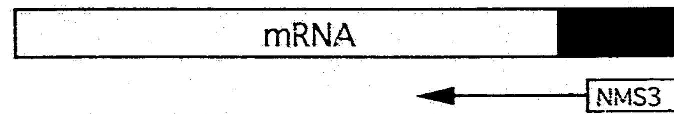

SP1
CSC 33
SP
CRYPTIC
GHRH
PACAP
3'UT
NMS 5
NMS 1
NMS3
NMS 2

Figure 4.2. A schematic diagram of the strategy used in the polymerase chain reaction (PCR) and rapid amplification of cDNA ends (RACE). A) Amplification of sockeye salmon double stranded cDNA. B) Reverse transcriptase using sequence specific primer NMS3 to obtain 5' region. C) Tailing of single stranded cDNA with dATP and subsequent amplification using the internal primer NMS 1, and primer #2 and #3. Primers: CSC33 5'CA(T/C)GCIGA (T/C)GGIATGTT(T/C)AA3'; oligo (dT) GAATTCT(dT)36; NMS1 5'TGACAGAGGCTCTGTGTC3'; NMS3 5'TCGGTAGCGGCTGTAGCTATCTG3'; #2 GGCTCGAGCCCGGGAATTCCG(dT)15; #3 5'GGCTCGAGCCCGGGAATTCCG3'. GHRH = growth hormone releasing hormone; PACAP = pituitary adenylate cyclase activating polypeptide; SP = signal peptide; UT = untranslated.

A.



B.



C.

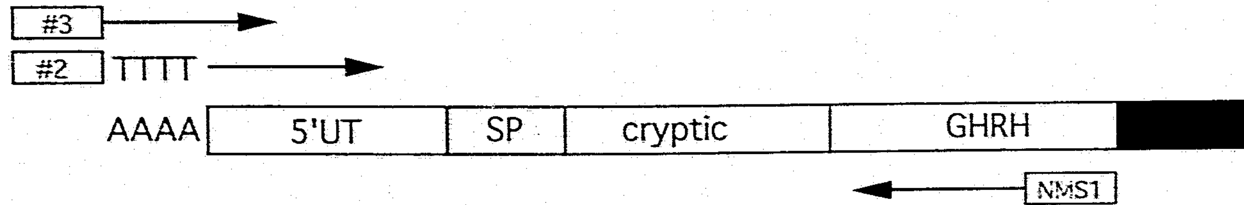


Figure 4.3. Photograph of sockeye salmon and catfish PCR amplification products run on a 2% agarose gel. Double stranded DNA was amplified using primers CSC33 and an oligo (dT). The PCR conditions are described in the text. Lane 1 and 2 represent cDNA from catfish, and lane 3 represents cDNA from sockeye salmon. The band enclosed by], in lane 3, was cut out and cloned into pBluescript II KS. DNA markers are shown in Kb at the left side.

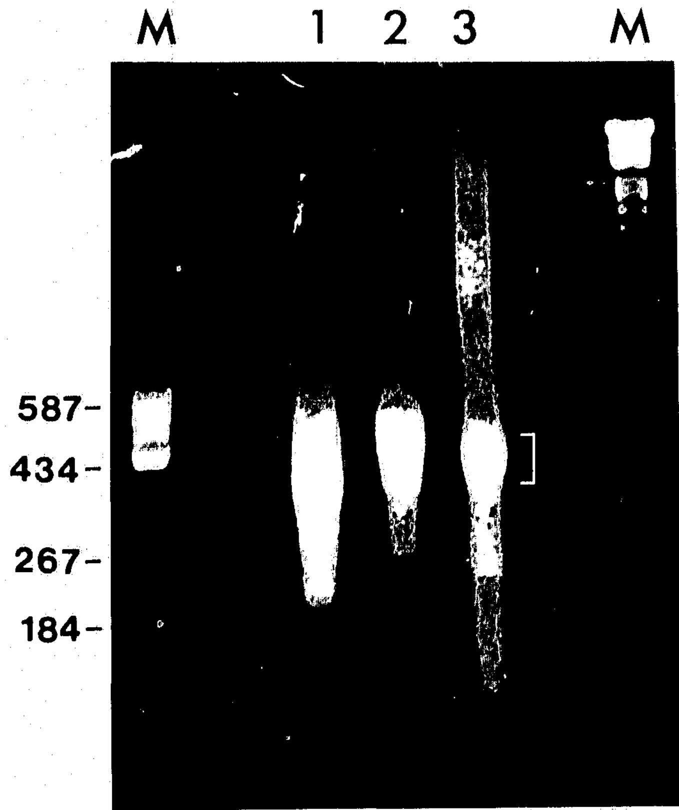

M
1
2
3
M
587-
434-
267-
184-

Figure 4.4. Photograph showing a Pvu II digest of miniprep plasmid DNA obtained from cloned PCR products. Sockeye salmon GHRH clones were identified in lane 4, SS/PCR 4, and lane 5, SS/PCR 5. The band of approximately 900 bp (lane 4 and 5) contains 448 bp of the plasmid as well as the insert DNA. The larger band in lane 4 and 5 represents the remaining plasmid DNA. Plasmid preps in lanes 1, 2, and 3, did not contain GHRH recombinant clones.

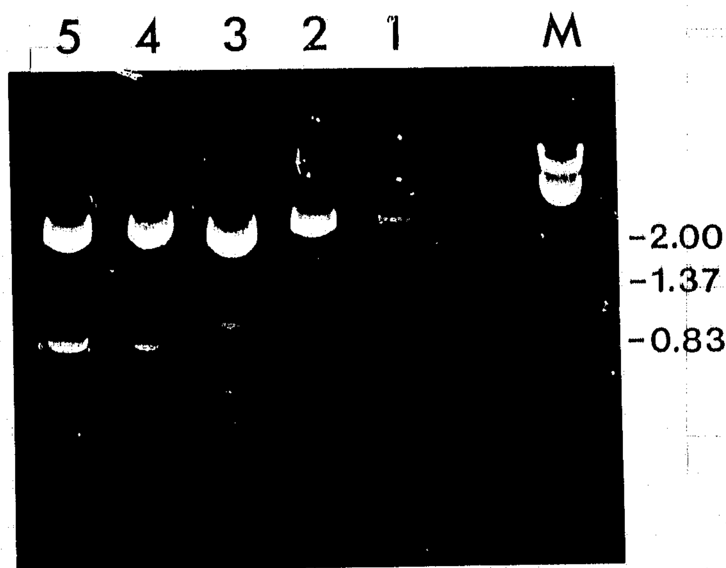

5
4
3
2
1
M
-2.00
-1.37
-0.83

Figure 4.5. A) Nucleotide sequence and the corresponding amino acid sequence of the sockeye salmon GHRH/PACAP cDNA. Overlapping cDNA clones SS/PCR 4 and SS/RACE 2 were used to obtain the full length sequence. The putative signal peptide and polyadenylation signal (ATTAAA) are underlined. The GHRH-like peptide, amino acid positions 82-126 and PACAP38, position 129-166, are boxed. Nucleotide substitutions and amino acid changes in clone SS/PCR 5 and SS/RACE 7 are shown below the the main reading frame. B) Restriction map of the GHRH/PACAP cDNA showing the unique restriction sites (DNA Strider^{TM}).

A

```
                                                                                        -84
                                                                                         CGTTTTTCCTCAGTCTGACTGTGGAAATGTTAGATAGCCTTCGCA
                                            +1
GACATCCAGCTTGTCTCTCCACACGGTAATAGCAGGACA ATG TCT AGT AAA GCG ACT TTA GCC TTA CTC ATC TAT GGA ATC ATA ATG CAC TAC AGT    57
C TT AAC T GTGA A TTT TTCCCC C      A   Met Ser Ser Lys Ala Thr Leu Ala Leu Leu Ile Tyr Gly Ile Ile Met His Tyr Ser
                                         1                                  10

GTC TAC AGC TCA CCT CTC GGG CTT AAC TAT CCT AAC CTT AGA CTT GAA AAT GAG GTT TAT GAC GAG GAT GGG AAT TCG TTA CCG GCC   144
Val Tyr Ser Ser Pro Leu Gly Leu Asn Tyr Pro Asn Leu Arg Leu Glu Asn Glu Val Tyr Asp Glu Asp Gly Asn Ser Leu Pro Ala
 20     T                               30                                      40
        Cys

TTG GCT TTT GAC AGC GAT CAA ATT GCT ATA AGA AGT CCC CCG TCT GTG GCT GAC GAT TTA TAC ACT TTA TAC TAC CCA CCG GAG AAA   231
Leu Ala Phe Asp Ser Asp Gln Ile Ala Ile Arg Ser Pro Pro Ser Val Ala Asp Asp Leu Tyr Thr Leu Tyr Tyr Pro Pro Glu Lys
    50                                      60 T                                    70
                                               Ser

                <------------------------------------------------------ GHRH-like peptide ----------------------
GGA ACG GAA AGG CAT GCA GAC GGA ATG TTT AAT AAA GCC TAC AGG AAA GCG CTG GGT CAG TTA TCA GCA AGA AAA TAT CTT CAT TCT   318
Gly Thr Glu Arg His Ala Asp Gly Met Phe Asn Lys Ala Tyr Arg Lys Ala Leu Gly Gln Leu Ser Ala Arg Lys Tyr Leu His Ser
A       80                                  90                                          100            C
Arg

------------------------------------------------------------------------------>        <-----------------------
CTG ATG GCA AAG CGT GTA GGT GGA GGG AGC ACC ATG GAA GAC GAC ACA GAG CCT CTG TCA AAG CGA CAT TCG GAT GGG ATC TTC ACA   405
Leu Met Ala Lys Arg Val Gly Gly Gly Ser Thr Met Glu Asp Asp Thr Glu Pro Leu Ser Lys Arg His Ser Asp Gly Ile Phe Thr
            110                                     120     T                              C 130
                                                            Ser

--------------------------------------------------- PACAP -----------------------------------------------------------
GAT AGC TAC AGC CGC TAC CGA AAG CAA ATG GCA GTC AAG AAA TAC CTG GCG GCA GTC CTT GGG AAA AGG TAT AGA CAG AGA TAT AGA   492
Asp Ser Tyr Ser Arg Tyr Arg Lys Gln Met Ala Val Lys Lys Tyr Leu Ala Ala Val Leu Gly Lys Arg Tyr Arg Gln Arg Tyr Arg
 C              140                             150                                         160

------->
AAC AAA GGA CGC CGG CTA GGC TAT CTG TAG CGTTGCTAACCCAAACTACCATGTGTGTACAGCCCAGATCAAGTCATTTTGAGATAACTGAACAATCAATCAGTGG   598
Asn Lys Gly Arg Arg Leu Gly Tyr Leu ***                                                         A                ----G
 G                  170  CG      T
Ser                      Ala

ATCGCTCTTGTGTTCTTTAAACATGTATTTATGTATGAAGTAAAGCCATTAAAATGAATATTTTGATAAT                                                668
                                                  T       C
```

B

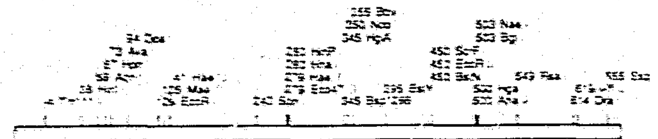


Figure 4.6. Nucleotide sequence of sockeye salmon cDNA clones obtained by the RACE technique (SS/RACE 2 and SS/RACE 7). Nucleotides and amino acids shown in bold represent differences between the two clones. The region coding for the GHRH-like peptide is underlined. The nucleotides in italics represent the region bound by the antisense primer NMS 1. Position of the nucleotides are represented by the numbers in the right margin. Nucleotides are numbered from the beginning of the start coding.

```
     -84
SS 7   C[illegible]TTTTCCTCAGTCTGACTGTGGAAATGTTAG[illegible]TAGCCTTCGCACATT
SS 2                                                   GACA
                                             +1
TAACGTTGTGATATTTTCTTCCCCACAGCAGAACA ATG TCT AGT AAA GCG    15
TCCAGCTTGTCTCTCCACACGGTAATAGCAGGACA ATG TCT AGT AAA GCG


ACT TTA GCC TTA CTC ATC TAT GGA ATC ATA ATG CAC TAC AGT    57
ACT TTA GCC TTA CTC ATC TAT GGA ATC ATA ATG CAC TAC AGT

        Cys
GTC TAC TGC TCA CCT CTC GGG CTT AAC TAT CCT AAC CTT AGA    99
GTC TAC AGC TCA CCT CTC GGG CTT AAC TAT CCT AAC CTT AGA
        Ser

CTT GAA AAT GAG GTT TAT GAC GAG GAT GGG AAT TCG TTA CCG   141
CTT GAA AAT GAG GTT TAT GAC GAG GAT GGG AAT TCG TTA CCG

                                                    Ser
GCC TTG GCT TTT GAC AGC GAT CAA ATT GCT ATA AGA AGT TCC   183
GCC TTG GCT TTT GAC AGC GAT CAA ATT GCT ATA AGA AGT CCC
                                                    Pro


CCG TCT GTG GCT GAC GAT TTG TAC ACT TTA TAC TAC CCA CCG   225
CCG TCT GTG GCT GAC GAT TTA TAC ACT TTA TAC TAC CCA CCG


        Arg
GAG AAA AGA ACG GAA AGG CAT GCA GAC GGA ATG TTT AAT AAA   267
GAG AAA GGA ACG GAA AGG CAT GCA GAC GGA ATG TTT AAT AAA
        Gly

GCC TAC AGG AAA GCG CTG GGT CAG TTA TCA GCA AGA AAA TAT   309
GCC TAC AGG AAA GCG CTG GGT CAG TTA TCA GCA AGA AAA TAT

                                                Arg
CTT CAT TCT CTG ATG GCA AAG CGT GTA GGT GGA GGG CGC ACC   351
CTT CAT TCT CTG ATG GCA AAG CGT GTA GGT GGA GGG AGC ACC
                                                Ser

ATG GAA GAC GAC ACA GAG CCT CTG TCA                       378
ATG GAA GAC GAC ACA GAG CCT CTG TCA
            ————————NMS 1 Primer
```

Figure 4.7. Photograph of the restriction enyzme digests of the sockeye salmon GHRH/PACAP clones SS/RACE 2 and SS/PCR 4 with Not I and Nco I. Bands marked by the arrows (←-) were cut out and electroeluted. The band from lane 2 was ligated into the band from lane 4 to obtain a full length GHRH/PACAP cDNA.

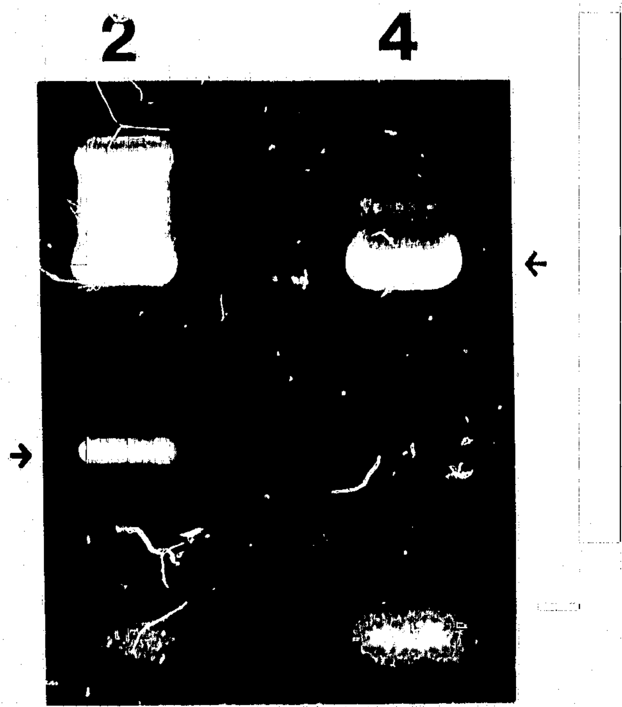

2
4

Figure 4.8. The nucleotide sequences of the GHRH/PACAP short precursor forms. Nucleotides in the 5' untranslated regions different between the two clones are shown in bold. A putative ATG start codon in the 5' untranslated region of clone CS/LIBb is shown by the double underline. A Cys in position 22, represented by ***, is identical to the full length clone SS/PCR 7, but different from that of SS/PCR 2 (compare to Fig. 4.5).

```
                                        -51
CS/LIBa                                   CACGAGCCGGATCCGATACAGCGTCTAT
CS/LIBb                                          GCACGAGACAGGCTTGGGTAC

                                   1
                              -1 Met Ser Ser Lys Ala Thr Leu Ala Leu
TTCGACACTGGAATAGCAGGACA ATG TCT AGT AAA GCG ACT TTA GCC TTA     27
TTTAGAATGTTTGGAGCAGGACA ATG TCT AGT AAA GCG ACT TTA GCC TTA

 10                                          20      ***
Leu Ile Tyr Gly Ile Ile Met His Tyr Ser Ile Tyr Cys Ser Pro
CTC ATC TAT GGA ATC ATA ATG CAC TAC AGT GTC TAC TGC TCA CCT     72
CTC ATC TAT GGA ATC ATA ATG CAC TAC AGT GTC TAC TGC TCA CCT

                        30
Leu Gly Leu Asn Tyr Pro Asn Leu Arg Leu Glu Asn Glu Val Tyr
CTC GGG CTT AAC TAT CCT AAC CTT AGA CTT GAA AAT GAG GTT TAT    117
CTC GGG CTT AAC TAT CCT AAC CTT AGA CTT GAA AAT GAG GTT TAT

 40                                          50
Asp Glu Asp Gly Asn Ser Leu Pro Ala Phe Gly Phe Asp Ser Asp
GAC GAG GAT GGG AAT TCG TTA CCG GCC TTG GCT TTT GAC AGC GAT    162
GAC GAG GAT GGG AAT TCG TTA CCG GCC TTG GCT TTT GAC AGC GAT

                        60
Gln Ile Ala Ile Arg Ser Pro Pro Ser Val Ala Asp Asp Leu Tyr
CAA ATT GCT ATA AGA AGT CCC CCG TCT GTG GCT GAC GAT TTG TAC    207
CAA ATT GCT ATA AGA AGT CCC CCG TCT GTG GCT GAC GAT TTG TAC

 70                                           80
Thr Leu Tyr Tyr Pro Pro Glu Lys [Ser] Gly Gly Ser Thr Met Glu
ACT TTA TAC TAC CCA CCG GAG AAA [AGT] GGA GGG AGC ACC ATG GAA  252
ACT TTA TAC TAC CCA CCG GAG AAA [AGT] GGA GGG AGC ACC ATG GAA

Asp Asp Thr
GAC GAC ACA                                                    261
GAC GAC ACA
```

Figure 4.9. A comparison of all the GHRH/PACAP precursor clones that contained a 5' untranslated region. Identical nucleotides among the different clones are in bold and underlined (single). Additional start codons (ATG) are double underlined. Nucleotides are numbered negatively from the start codon.

-9

CLONE SS/RACE 7
CGTTTTTCCTCAGTCTGACTGTGGAAATGTTAGATAGCCTTCGCACATTTAACGTTGTGATA**T**TTTCTTCCCCAC**AGCAG**A**ACA**

CLONE SS/RACE 2 GACATCCAGCTTGTCTC**T**CCACACGGTAAT**AGCAGGACA**

CLONE SS/LIBa CACGAGCCGGATCCGATACAGCGTCTATT**T**CGACACTGGAAT**AGCAGGACA**

CLONE SS/LIBb GCACGAAGACAGGCTTGGGTACT**T**TAGAATGTTTGG**AGCAGGACA**

Figure 4.10. A comparison of the sockeye salmon preproGHRH/PACAP (shown in Fig. 4.5A) to the three mammalian PACAP precursors. Amino acids different from the salmon precursor are shown, while identical residues are not. The signal peptide of the salmon precursor is underlined and the peptide regions are boxed.

```
                <----------------------------------- Signal peptide
SALMON          Met Ser Ser Lys Ala Thr Leu A'a Leu Leu Ile Tyr Gly Ile Ile Met His Tyr Ser Val Tyr
HUMAN   Met Thr     Cys     Gly     Arg                     Val                         Ser
OVINE   Met Thr     Cys     Gly     Arg                     Val             Leu         Ser
RAT     Met Thr     Cys     Gly     Arg                     Val                         Asn         Ser

-->
Ser Ser Pro Leu Gly Leu Asn Tyr Pro Asn Leu Arg Leu Glu Asn Glu Val --- --- Tyr Asp Glu Asp Gly Asn
            Ala Ala Ala Gly Leu Arg Phe Pro Gly Ile Arg Pro Glu Glu Glu Ala     Gly
Gly         Ala Ala Ser Gly Leu Arg Phe Pro Gly Ile Arg Pro Glu Asn Glu Ala
Cys         Ala Ala --- Gly Leu Ser Phe Pro Gly Ile Arg Pro Glu Glu Glu Ala         Gln

Ser Leu Pro Ala Leu Ala Phe Asp Ser Asp Gln Ile Ala Ile Arg Ser Pro Pro Ser Val Ala Asp Asp Leu Tyr
Pro         Asp Phe Gly --- Gly     Glu Pro Pro Gly Ala Gly         Ala     Ala Pro Arg Ala Ala Ala
Pro Gln Gln Asp Phe Tyr ---         Glu Pro Pro Gly Val Gly         Ala     Ala Leu Arg     Ala
Pro     Gln Asp Phe Tyr ---     Trp     Pro Pro Gly Ala Gly         Ala     Ala Leu Arg     Ala

                                                 <--------------------------------------------------
Thr Leu Tyr Tyr Pro Pro Glu Lys Gly Thr Glu Arg | His --- Ala Asp Gly Met Phe Asn Lys Ala Tyr Arg Lys
Ala Trp     Arg     Ala Gly --- --- --- Arg     | Asp Val     His     Ile Leu     Glu
Ala                 Ala     --- --- ---         | Asp Val     His     Ile Leu Asp
Ala                 Ala Asp --- --- --- Arg     | Asp Val     His Glu Ile Leu     Glu

------------------------------------------ GHRH-like peptide -------------------------------------
Ala Leu Gly Gln Leu Ser Ala Arg Lys Tyr Leu His Ser Leu Met Ala Lys Arg Val Gly Gly --- --- --- ---
Val     Asp                 Gly     His     Gln         Val     Arg Gly             Ser Leu Gly Gly
Val     Asp                     Arg         Gln Thr                 Gly Leu         Thr Pro Gly Gly
Val     Asp                                 Gln     Met Val     Arg Gly Met     Glu Asn Leu Ala Ala

----------------------------------------------->     <--------------------------------------------
                            Ser
Gly Ser Thr Met Glu Asp Asp Thr Glu Pro Leu Ser | Lys Arg | His Ser Asp Gly Ile Phe Thr Asp Ser Tyr Ser
    Ala --- --- Gly         Ala                 |
    Ala --- --- Asp         Ser                 |
Ala Ala --- --- Val         Arg Ala         Thr |

------------------------------------------------ PACAP --------------------------------------------
Arg Tyr Arg Lys Gln Met Ala Val Lys Lys Tyr Leu Ala Ala Val Leu Gly Lys Arg Tyr Arg Gln Arg Tyr Arg
                                                                                Lys         Val Lys
                                                                                Lys         Val Lys
                                                                                Lys         Val Lys

------>
Ser
Asn Lys | Gly Arg Arg Leu Gly Tyr Leu
                      Ile Ala
                      Ile Pro
                      Ile Ala
```

Figure 4.11. A comparison of the 3' untranslated region of the salmon preproGHRH/PACAP (clone SS/PCR 4) with the beginning of the 3' untranslated regions of human, ovine and rat preproPACAPs. Nucleotides identical to the salmon for any of the three mammalian forms are shown by an *. Where a nucleotide is not identical to the salmon, but at least one of the mammalian forms is, this nucleotide is in bold. Gaps (-) were inserted for maximum sequence alignment. The salmon polyadenylation site (ATTAAA) is underlined. Substitution changes in clone SS/PCR 5 are shown above the salmon clone SS/PCR 4 sequence.

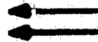
```
                                   A
SALMON  CGTTGCTAACCCAAACTACCATGTGTGTACAGCCCAGAT-------CAAGTCATTTTGAGATA---
        ** **     ***     ** ***** ******** **         ***** ****   * *
HUMAN   CGATGGGTTACCAGCTACCC-TGTGTATACAGCCCTGACGCAATGAAAAGTCGTTTT-CCAAACTG
OVINE   CGACGAGTTACCAGCTATCC-TGTGTATACAGCCCTGACACAATGAGAAGTCGTTTTTCCCAACTG
RAT     CGATGAGTTGCCAGCTACCG TGTGTAT-----------AAAATGAAAAGTCGTTTT-CCAAATTG

           ----G
ACTGAACAATCAATCAGTGG-ATCGCTCTTGTGTTCTTT--AAACATGTATTTATGTA-TGAAGTAAA
******** ***         ******* **********  ***************** *********
ACTCAACAGT----------CATCGCTCGTGTGTTCTATCCAAACATGTATTTATGTAATGAAGTAAA
ACTGAACTGT----------CATCGCTGCTGTGTTCTGTCCC-ACATGTATTTATGTA-TGAAGTCAA
ACTGACCAGTCAT-------CACTCA---TGTGTTCTTTCCAAACATGTATTTATGTA-TCAAGTAAA

           T       C
GCCATTAAAATGAATATTTTGATAAT
********* ****************
GCCATTAAA-TGAATATTTTGATAAT
GCCATTAAA-TGAATATTTTGATAAT
GCCATTAAA-TGACTATTTTGATAAT
```

Figure 4.12. A Southern blot analysis of sockeye salmon brain genomic DNA using a 355bp MSE I digested cDNA probe. The filter was washed 30 min in 2X SSC/0.1% SDS at 22°C. The filter was then washed in 1X SSC/0.1% SDS at 60°C for 15 min (A) or 30 (B) min. Lane 1) Hae III, 2) Hind III, 3) Pst I and 4) EcoR I. Molecular weight markers are shown at the left. The arrows at the right indicate two bands in lane 4.

A
B
1 2 3 4
1 2 3 4
21
5.1
2.0
1.6
1.3



DISCUSSION

A sockeye salmon GHRH/PACAP precursor which contains two putative hormones, a GHRH-like peptide and a PACAP molecule, has been identified. The presence of two dibasic enzyme processing sites and a single arginine processing site may result in the cleavage of the precursor into a PACAP-38 with an amidated carboxy terminus and a GHRH-like molecule of 45 amino acids with a free hydroxyl carboxy terminus. The salmon GHRH/PACAP precursor has an open reading frame of 173 amino acids compared to 176 for the ovine and human PACAP precursors, and 175 for the rat. The precursor organization of salmon GHRH/PACAP is similar to that of the mammalian PACAP precursors. Four distinct tandem regions are present within the salmon precursor: a signal peptide, followed by a cryptic peptide and then two regions giving rise to the GHRH-like and PACAP-38 mature peptides.

The high conservation (92%) of PACAP-38 between such divergent groups as salmon and humans suggests this molecule has an important biological function(s). Neuropeptide Y is possibly the only peptide of comparable length with such stringent sequence conservation (Larhammar *et al.*, in press). The function of PACAP has been tested primarily in mammalian systems. At the level of the pituitary, PACAP may play a role in growth and reproduction. Like GHRH, PACAP stimulates adenylate cyclase and cAMP accumulation in pituitary cells. PACAP also induces an increase in

cytosolic calcium ion concentration in rat somatropic and gonadotropic cells (Canny *et al.*, 1992), and in a subpopulation of frog pituitary GH and prolactin cells (Gracia-Navarro *et al.*, 1992). *In vitro* studies show that PACAP stimulates GH, prolactin, LH and ACTH release from superfused rat pituitary cells in a dose-dependent manner over the concentration range of 10^{-10} to 10^{-8} M (Miyata *et al.*, 1989). However, these authors did not find the same effect in a static pituitary cell culture. Using a reverse hemolytic plaque assay, Goth *et al.* (1992) showed that PACAP-38 stimulates rat pituitary GH secretion in a dose dependent manner and, like GHRH, PACAP-38 increased the number of active somatropic cells as well as the amount of GH secreted. In contrast, Culler and Paschall (1991) did not find that PACAP significantly increased GH secretion in rat anterior pituitary cells. Whether PACAP is involved in the control of GH release in fish has yet to be determined.

GH release is stimulated by PACAP-38 via distinct receptors for PACAP. High affinity binding sites that are specific for PACAP-38 or PACAP-27 do not crossreact with VIP on anterior pituitary cells (Gottschall *et al.*, 1990).

The signal peptide of the salmon GHRH/PACAP precursor has a hydrophobic core and is two amino acids shorter than the signal peptide of the ovine (Kimura *et al.*, 1990), human and rat (Ogi *et al.*, 1990) PACAP precursors (Fig. 4.10). At the amino acid level there is 77% sequence identity between the salmon and human PACAP signal peptides. A cysteine at

position 22 of the signal peptide in some of the salmon GHRH/PACAP clones is identical to that of the rat, while the serine in clone SS/PCR 4 is the same as the human cDNA (Fig. 4.10). The salmon 5'UT region has an adenine at position -3 from the initiation codon ATG, which appears to be required for efficient translation (Kozak, 1986; Kozak, 1991). The salmon precursor unlike the mammalian PACAP precursors does not appear to have two possible methionines for the initiation of translation (Fig. 4.10). Following the signal peptide is a cryptic region of 59 amino acids with 28% sequence similarity with human, 36% with sheep, and 31% with rat (Fig. 4.10).

The salmon GHRH-like peptide would be processed to a free acid carboxy terminus like those of carp (Vaughan *et al.*, 1992), rat (Bohlen *et al.*, 1984) and mouse (Frohman *et al.*, 1989; Suhr *et al.*, 1989) GHRH peptides (Fig. 4.13A). Salmon GHRH-like peptide is identical in length to the carp hypothalamic GHRH and has 91% amino acid sequence identity (Fig. 4.13A). There is only 40% sequence identity between the salmon GHRH-like peptide and human GHRH (Fig. 4.13A). However, the GHRH family is not highly conserved. Rat (Bohlen *et al.*, 1984) and mouse (Frohman *et al.*, 1989; Suhr *et al.*, 1989) have only 66% and 61% sequence identity to human GHRH, respectively. The first 28 residues of the salmon GHRH-like peptide and human GHRH are 57% identical. The higher conservation of the amino terminus probably reflects the fact that first 29 amino acids of the carp and

Figure 4.13. A) Sockeye salmon GHRH-like peptide sequence compared with all other members of the GHRH family. B) Salmon GHRH-like peptide sequence compared with the mammalian PRP peptides. Amino acids different from the salmon molecule are in bold and identical residues are shown by the dots (·). Bars (-) represent spaces where the sequence was shifted to obtain maximum alignment of the peptides. Percent sequence identity to the salmon is shown on the right. The * shows all possible amino acids of the mammalian PRPs that are identical to the salmon. GHRH = growth hormone releasing hormone; PRP = PACAP (pituitary adenylate cyclase activating polypeptide)-related peptide.

A.

GROWTH HORMONE-RELEASING HORMONES

	1 … 45		
SALMON	HADGMFNKAYRKALGQLSARKYLHSLMAKRVGGGSTMEDDTEPLS	(OH)	
CARP	·······················T·········MI···N····	(OH)	91%
MOUSE	·V·AI·TTN···L·S··Y···VIQDI·N·Q--·ERIQEQ--RAR··	(OH)	38%
RAT	···AI·TSS··RI····Y···L··EI·NRQQ·ERNQEQ--RSRFN	(OH)	38%
SHEEP	Y··AI·TNS···I········L·QDI·NRQQ·ERNQEQGAKVR·	(NH_2)	40%
GOAT	Y··AI·TNS···V········L·QDI·NRQQ·ERNQEQGAKVR·	(NH_2)	40%
COW	Y··AI·TNS···V········L·QDI·NRQQ·ERNQEQGAKVR·	(NH_2)	40%
PIG	Y··AI·TNS···V········L·QDI·SRQQ·ERNQEQGARVR·	(NH_2)	40%
HUMAN	Y··AI·TNS···V········L·QDI·SRQQ·ESNQERGARAR·	(NH_2)	40%

B.

SALMON GHRH VS MAMMALIAN "Big PRP'S"

sGHRH	H-ADGMFNKAYRKALGQLSARKYLHSLMAKRVG———GGSTMEDDTEPLS	
OVINE	DV·H·ILD·····V·D····R··QT····GL·GTPG··A--D··S····	28/48= 58%
HUMAN	DV·H·IL·E····V·D····G·H·Q··V·RG··GSLG··A--G··A····	27/48= 56%
RAT	DV·HEIL·E····V·D········Q·MV·RGM·ENLAAAA--V··RA··T	22/48= 46%
	* * ****** * ******** ***** ** ** ** ****	32/48 =71%

human GHRHs are required for full biological activity.

The PACAP precursors of human, sheep and rat contain a region of 29 amino acids designated as PRP which has 48-52% sequence identity to human GHRH. The PRP peptide has not been isolated from any tissue, but has been shown to be processed and secreted from Chinese hamster cells, transfected with an expression vector containing the human PACAP cDNA (Okazaki *et al.*, 1992). No function has yet been determined for the PRP molecule. The salmon GHRH/PACAP precursor contains a Lys-Arg processing site within the GHRH-like peptide region, which could produce a 28 residue peptide with 59% sequence identity to the human, ovine and rat PRP peptides.

In theory, a big PRP of 48 amino acids could also be processed from the mammalian precursors (Okazaki *et al.*, 1992). The amino acid sequence alignments in figure 4.13B show 58%, 56% and 46% sequence identity between the salmon GHRH-like peptide and the big PRP region of the PACAP precursor from sheep, human and rat, respectively. The sequence identity of the salmon GHRH-like peptide to the mammalian PRP's is 71%, if identity is defined as a match of the salmon residue with at least one of the mammalian residues (Fig. 4.13B). A big peptide histidine-valine (PHV-42) has also been identified from an adrenal pheochromocytoma (Yiangou *et al.*, 1987), which is a homologous to the salmon GHRH-like peptide and big PRP. PHV-42 is a product of peptide histidine-methionine (PHM)

and the intervening peptide in the VIP/PHM precursor. PHV-42 is expressed in the urogenital tract, nasal mucosa and stomach, but not the brain, intestine and lung (Yiangou *et al.*, 1986). Although there is greater sequence identity between salmon GHRH-like peptide and the mammalian big PRPs, than there is between salmon and human GHRHs, the salmon GHRH45-like peptide retains an aromatic residue at position 1, which is required for biological activity in mammamlian GHRH's (Guillemin *et al.*, 1982; Coy *et al.*, 1987). Like rat and mouse GHRH, salmon GHRH45-like peptide has a histidine at position 1, while all other known mammalian GHRH's have a tyrosine (Bohlen *et al.*, 1984). In contrast, the mammalian PRP peptides have aspartic acid at position 1. Both salmon GHRH-like peptide and PACAP have a phenylalanine at position 6, which is conserved in all of the superfamily members and is also essential for biological activity of the mammalian GHRH peptides (Coy *et al.*, 1987).

The salmon GHRH-like peptide is also closely related to the other glucagon family members with 67% sequence identity to PHM-27 and 59% to peptide histidine-isoleucine (PHI-27), but a decreasing identity to VIP, glucagon and secretin (Fig. 4.14A).

It appears that the salmon GHRH/PACAP precursor has had a tight structural constraint imposed on it, compared with the mammalian PACAP precursors, since the putative duplication of their ancestral gene. The N-terminus (first

Fig. 4.14. The salmon GHRH-like peptide (A) and the salmon PACAP-38 (B) compared to the other members of the glucagon superfamily. Residues identical to the salmon sequence are shown by the dots (·) and amino acids different from the salmon are in bold. Percent amino acid identity of the glucagon superfamily members, compared to the salmon peptides, is shown on the right. GHRH = growth hormone releasing hormone; PHM-27 = peptide histidine-methionine; PHI-27 = peptide histidine-isoleucine; VIP = vasoactive intestinal peptide; GLP = glucagon-like peptide; GIP = gastric inhibitory peptide; PACAP = pituitary adenylate cyclase activating polypeptide.

A.

SALMON GHRH VS GLUCAGON SUPERFAMILY

GHRH salmon	HADGMFNKAYRKALGQLSARKYLHSLMAKRVGGGSTMEDDTEPLS (OH)	
PHM-27	····V·TSDFS·L······K···E··· (NH_2)	67%
PHI-27 rat	····V·TSD·SRL···I··K···E··I (NH_2)	59%
VIP dogfish	·S·AV·TDN·SRIRK·MAVK··IN··L· (NH_2)	36%
VIP human	·S·AV·TDN·TRLRK·MAVK···N·ILN (NH_2)	32%
GLUCAGON salmon	·SE·T·SND·S·YQEERM·QDFVQW··NS	28%
GLP salmon	····TYTSNVSTY·QDQA·KDFVSW·KSG·A	26%
SECRETIN human	·S··T·TSELSRLREGARLQRL·QG·V (NH_2)	22%
GIP human	Y·E·T·ISD·SI·MDKIHQQDFVNW·L·QKGKKNDWKHNI·Q (OH)	19%

B.

SALMON PACAP VS GLUCAGON SUPERFAMILY

PACAP salmon	HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYRQRYRNK (NH2)	
VIP dogfish	···AV···N···I·········INSL·A (NH_2)	68%
VIP human	···AV···N·T·L··········NSI·N (NH_2)	68%
PHM-27	·A··V··SDF·KLLG·LSA····ESLM (NH_2)	41%
SECRETIN human	····T··SEL··L·EGARLQRL·QGLV (NH_2)	37%
GHRH salmon	·A··M·NKA·RKALG·LSAR···HSLMA··VGGGSTMEDDTEPLS (OH) 9/27	29% 33%
GLP salmon	·A··TY·SNV·T·LQDQ·A·DFVSWLKSG·A 8/27	29% 30%
GLUCAGON salmon	··E·T·SND··K·QEERMAQDFVQWLMNS	24%
GIP human	YAE·T·ISD··IAMDKIHQQDFVNWL·AQKGKKNDWKHNITQ (OH) 5/27	12% 19%

28 amino acids) of PACAP in both salmon and human have the same sequence identity to human VIP, while the N-terminus of salmon GHRH has 23% greater sequence identity to PHM than does the human PRP region; salmon GHRH45-like peptide and PACAP have 67% and 68% sequence identity to PHM and VIP (Fig. 14B), respectively, while there is only 44% sequence identity of human PRP to PHM.

The GHRH/PACAP families represent an interesting structure/function relationship in evolution. The stimulation of GH release in mammals is thought to be controlled through the interplay of GHRH and PACAP, peptides processed from separate genes. However, in salmon, GH release may be controlled by the two peptides GHRH45 (PRP region) and PACAP, both processed from the same gene and/or by another GHRH-like molecule on a separate gene. A dose dependent response of GH release was found when carp GHRH-like molecule was used in a rainbow trout pituitary monolayer cell culture (Luo *et al.*, 1990). In this same study human GHRH either showed no stimulatory response or the response was not dose dependent. The control of growth in fish may be regulated differently in that GH has additional or different physiological functions, such as smoltification. The relationship of GHRH and PACAP to NPY may also be important in growth. However, NPY which coexists with GHRH in some mammalian neurons may be more important in stimulating appetite than GH release.

The detection of a short precursor that would process

only PACAP is of considerable interest in a functional sense. This short precursor form was obtained from the RACE reaction and the chum salmon cDNA library, suggesting it is not a cloning artifact of the type that can occur in the making of a cDNA library. The short precursor form may be due to problems of secondary structure in the mRNA during the reverse transcriptase reaction, but, it is also possible that the short precursor was generated by differential post-transcriptional processing. Exon 4 has been deleted in the short form, inferring differential processing. The two exons encoding the cryptic peptide (exon 3) and PACAP (exon 5) are joined, resulting in a shorter mRNA (see chapter 5 Fig. 5.11). This would result in a precursor without GHRH 1-32 that could process only PACAP. Shorter transcript forms have been observed for another member of the glucagon superfamily. Different transcriptional processing of secretin RNA results in the loss of exon three and a shorter precursor (Kopin *et al.*, 1991). The cDNA in the present study was obtained from whole salmon brains for both the library and the RACE reaction. Therefore, cell specific processing of both precursors may have occurred in the brain. It is also possible that the shorter precursor is transcribed from a different gene. This would suggest different regulatory elements on the two genes. The sequence differences among clones containing the short precursor form may also be allelic polymorphisms.

Different lengths of the salmon GHRH/PACAP mRNA shown by

Northern hybridization could have been due to varying lengths of the poly (A) region and/or different termination sites. The rat VIP/PHI gene, which is homologous to the PACAP gene, produces two different sized mRNAs (Lamperti *et al.*, 1991). One of these messages is a rare, small VIP/PHI mRNA with a 3' untranslated region of approximately 100 base pairs, and the other is a more abundant, long mRNA with a 3' untranslated region of approximately 800 base pairs (Lamperti *et al.*, 1991). Quality of the mRNA could also account for the wide band width. The size of the salmon mRNA by Northern analysis is in the same range (3 Kb) as the rat, human, and ovine cDNAs. However, the 5' untranslated regions of the salmon GHRH/PACAP cDNAs are much shorter than the same region of other GHRH (Gubler *et al.*, 1983; Mayo *et al.*, 1983,1985b; Frohman *et al.*, 1989) and PACAP cDNAs (Kimura *et al.*, 1990; Ogi *et al.*, 1990), suggesting these salmon GHRH/PACAP clones may be incomplete (Fig. 4.7). The length of the 5' UT region is unknown in any of the PACAP cDNAs, although it is at least 500-600 bp in rat and sheep. The 3' UT region in the mammalian cDNAs is estimated to be 1400-1600 bp, although the poly (A) tail has not been found. This 3' UT region is quite different in length compared to 146 bp in the 3' untranslated region of the salmon, where the poly (A) tail has been located. The size of the salmon mRNA detected by Northern blot (2.2-2.7) does not agree with the size of the cDNA clone obtained by the PCR (0.73). This may be due to several reasons: 1) a mRNA of the same size as

the PCR clone was present but not in sufficient quantity to be detected by Northern hybridization (this would be similar to the rare short VIP/PHI mRNA, see above) 2) the quality of the Northern blots was not good enough to rule out a band of smaller size and 3) differential expression of the mRNA may have occurred because the mRNA was obtained from different pools of salmon brains, collected at different times.

The 3' untranslated region of the salmon GHRH/PACAP precursor is unique in that it has a high sequence identity to the mammalian forms. While sequence identity occurs in untranslated regions between mRNAs of different fish species, these regions are usually different from their mammalian counterparts (Sorokin *et al.*, 1982). Conservation of 3' untranslated sequences of other neuropeptide gene families is restricted to a only few small motifs (Morley *et al.*, 1991). Considering the evolutionary distance between fish and mammals, approximately 400 million years, the question of functional importance is raised for this highly conserved 3' untranslated region in preproGHRH/PACAP. This conservation is probably related to the function of the 3' untranslated region in interactions of the mRNA with proteins that affect translational control (Kwon *et al.*, 1991).

Two clones (SS/PCR 4 and SS/PCR 5) were identified that differed by 3 amino acids in the coding region. The sequence heterogeneity of the coding and 3' untranslated regions between these two clones was greater than the

expected error rate of Taq polymerase (Tindall and Kunkel, 1988; Saiki *et al.*, 1988). Therefore these two clones may represent allelic polymorphisms. However, salmonids are known to be tetraploid (Ohno *et al.*, 1968; Allendorf and Thorgaard, 1984), and hence the clones may have come from two non-allelic genes. Furthermore, two hybridization bands in 3 of 4 lanes of the Southern blot (Fig. 4.12) indicate more than one gene copy. Differences in nucleotide sequence in the coding and 3' untranslated regions as well as additions within the latter have been reported for chum salmon for the two vasotocin cDNAs (Heierhorst *et al.*, 1990), melanin-concentrating hormone (MCH) genes (Ono *et al.*, 1988; Takayama *et al.*, 1989) and the two clones of proopiomelanocortin (POMC) (Nishizawa *et al.*, 1984), postulated to be different precursors. Two genes have also been recently identified in white sucker for corticotropin-releasing factor (CRF) that differ by a single amino acid change within the peptide coding region (Morley *et al.*, 1991); there are also nucleotide substitutions within the cryptic regions as well as deletions or additions in the untranslated regions (Morley *et al.*, 1991).

Differences in the 5' untranslated region are not unusual and the polymorphisms observed in this study may be due to differences in the regulation of the molecule. IGF I in the rat has different 5' untranslated regions which are connected to an identical precursor coding region (Lowe *et al.*, 1987). The position at which a discrepancy occurs in

the 5' UT region of the salmon GHRH/PACAP cDNA corresponds to a putative intron/exon splice site (see Chapter 5). Therefore, the different 5' UT regions observed in this study may also be a result of differential post-transcriptional processing. An additional AUG codon in each of the 5'untranslated regions of two clones was terminated by a downstream stop codon. The 5' AUG start site would produce translation products of 12 and 21 amino acids, respectively. However, reinitiation at the next AUG codon could result in an in-frame precursor starting 16 amino acids downstream of the original initiator. Reinitiation in eukaryotic cells requires that the preceding open reading frame is short (Hunt, 1985; Kozak, 1991). Whether these upstream AUG codons can be used for efficient translation is not known at present. The use of an AUG codon for initiation is not only dependent on an optimal context, but also on the proximity of secondary structures and the length of the 5' leader (Kozak, 1991). If the upstream triplet codons are not the most favourable, then few if any translation products would be initiated at this site.

In conclusion, I have identified the salmon GHRH/PACAP precursor that possesses structural features allowing it to be processed into two mature peptides, a PACAP and a GHRH-like peptide. It is not yet known if there is an additional GHRH gene in fish or if this gene arose after the divergence of the tetrapods. The cDNA from this study was used to isolate the gene from sockeye salmon, which is presented in

chapter 5 and should further elucidate this evolutionary ts story.

Chapter 5

Gene Sequence of Sockeye Salmon GHRH-Like and PACAP Molecules: Evolutionary Relationship to the Glucagon Superfamily

INTRODUCTION

As genes evolve, mutations may result in changes in the protein products, thereby creating structurally related molecules with similar or distinct functions. In the glucagon superfamily, the different members have evolved different functions, although there is binding to the receptors of other family members in certain tissues. However, evolution of the structure of bioactive peptides and proteins is not the only way functions arise or disappear. Changes in the regulation of these genes may be as important as changes in the molecules (Wilson, 1976). Therefore, changes in the expression of a molecule can result in a functional difference without a structural change (Marx, 1988). A protein or peptide may be expressed in different tissues, at different times in development or in different amounts. For example, the crystallins play a structural role in the lens of the eye, but function as an enzyme in a variety of other tissues (Marx, 1988).

Therefore, when looking at the evolution of a family or superfamily of peptides, it is not only important to study the structure of the molecule, but also its regulation. Salmonids are a particularly good study organism in this respect. Variation in gene regulation should be greater in salmonids because in duplicated loci, one locus is free to diverge provided the other continues to express a product for normal functioning of the fish (Allendorf and Thorgaard, 1984). To understand the regulation of a peptide, the

structure of the gene, including the promoter and 5' flanking regions, is required. Furthermore, an understanding of the evolution of gene structure and/or regulation may help identify how novel functions arose.

In the glucagon superfamily, only genes for mammals have been identified, and in some cases in only one species. The genes for secretin (Kopin *et al.*, 1991), gastric inhibitory polypeptide (GIP) (Inagaki *et al.*, 1989) and pituitary adenylate cyclase activating polypeptide (PACAP) (Hosoya *et al*, 1992) have only been identified in the human. Clearly, there is a lack of comparative information within this superfamily. In this chapter, I present the identification of the GHRH/PACAP gene in sockeye salmon.

METHODS AND MATERIALS

Screening of sockeye salmon genomic library

A sockeye salmon genomic library in lambda Fix II (Stratagene), kindly provided by Dr. R. Devlin, was plated out at 25,000 plaque forming units (pfu) per plate. Phage were diluted in SM buffer and incubated with 200 μl of LE 392 cells (O.D.260=0.5) for 5 min at room temperature, then 15 min at 37°C. Ten milliters of NZYM top agarose (55°C) was added to the phage/cell suspension and the mixture plated out on 150 mm NZYM agar plates. The plates were incubated for 10-12 h at 37°C then placed at 4°C for 1 h. Duplicate filter lifts of each plate were done using 137 mm

Nylon membranes (Colony\Hybridization filters, NEN, Dupont). The first filter was placed on the plate for 1 min and the second filter for 1 min 30 s. Filters were alkaline denatured for 1 min in 1.5 N NaCl/0.5 N NaOH, neutralized for 5 min in 1.5 N NaCl/0.5 M Tris-HCL (pH 8.0) and then placed in 2x SSC for 5 min. The filters were air dried prior to baking for 2 h at 80°C.

Filters were soaked in 6X SSC for 5 min and then prehybridized in 100 ml of prehybridization buffer (6X SSC, 5X Denhardt's, 0.5% SDS, 50 μg/ml denatured sea urchin DNA) at 55°C for 4 h. Hybridization was carried out in 6X SSC/0.5% SDS containing 50 μg/ml sea urchin DNA, at 55°C for 18-20 h using a ^{32}P-dCTP labeled 135 bp sockeye salmon cDNA probe. The filters were washed four times for 5 min each at room temperature in 2X SSC/0.1% SDS, two times at 55°C for 20 min in 1X SSC/0.1% SDS and then 20 min at 55°C in 0.2X SSC/0.1% SDS. Filters were air dried and exposed to XAR-5 film (Kodak) for 5 days.

Regions of the plate containing positive plaques were cored using the wide end of a 1 ml sterile pipette tip. Agar plugs were placed in 15 ml culture tubes containing 2 ml of SM buffer/100 μl of chloroform and stored at 4°C. Plugs were titered by plating a 10,000 x dilution of the phage on 100 mm NZYM plates using the same procedure as for screening. Positive plugs were rescreened four times until a single isolated plaque (no other plaque within 0.5 cm) was obtained. Hybridization and washing stringencies were

increased in subsequent screenings. The final wash was done at 60°C in 0.1X SSC/0.1% SDS.

Plate lysates of single isolated plaques were done by plating 50,000 pfu/plate on NYZM agarose plates and growing at 37°C until confluent lysis occurred. The plates were flooded with 12 ml of lambda diluent (10 mM Tris-HCl/ 10 mM $MgSO_4$, pH 7.5) and placed at 8°C for 9 h. The lysate soluion was removed from the plates and the plates washed with an additional 3 ml of lambda diluent for 15 min. The wash was added to the lysate solution to which a few drops of chloroform was then added. The lysate was centrifuged for 10 min at 4000 xg at 4°C and the supernatant stored at 4°C.

Identification of positive clones using PCR

Polymerase chain reactions were done in 50 μl volumes containing 1X Promega buffer (50 mM KCl, 10 mM Tris-HCl, 0.1% Trition x-100, 1.5mM MgCl2), 200 μM dNTP's, and 2.5 units Taq DNA polyermase (Promega). Prior to rescreening the first round positive clones, 10 μl of phage solution was amplified using 40 pmol of primers CSC33 and NMS1. DNA was amplified using a step program of 1 cycle at 94°C for 3 min, 50°C for 2 min and 72°C for 5 min, followed by 36 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min 30 s (Perkin-Elmer Cetus). The last cycle had a final extension of 5 min at 72°C.

Purification of bacteriophage lambda and extraction of DNA

Purification of bacteriophage lambda and extraction of the DNA was done according to Sambrook *et al.* (1989) with minor modifications. Ten milliliters of plate lysate was incubated for 30 min at 37°C with 1μ/ml RNase A and 5μl/ml DNase I. An equal volume of 20% PEG 8000/2M NaCl in lambda diluent was added and the solution placed on ice for 1 h. PEG/lysate solution was centrifuged for 25 min at 10,000 xg at 4°C. The supernatant was decanted and the tubes inverted to remove all traces of PEG. The phage were resuspended in 500 μl TE pH 8.0, transferred to a 1.5 ml eppendorf and incubated in proteinase K/0.5% SDS/20 mM EDTA for 30 min at 55°C. The solution was extracted with an equal volume of phenol/chloroform/isoamyl alcohol, chloroform/isoamyl alcohol and the aqueous layer precipitated with 3 M sodium acetate (pH 7.0) (10% by volume) and an equal volume of isopropanol. The DNA was redissolved in 100 μl sterile water and incubated at 37°C with 50 μg/ml RNase A. Purified lambda recombinant DNA was digested with Sal I or Not I and separated on a 0.5 % agarose gel to determine the size of the insert.

Subcloning

Hae III, Sac I and Xba I restriction fragments of lambda recombinant positive clones were subcloned into pBluescript KS+ II by blunt end ligation as previously described (Methods and Materials, Chapter 4).

Sequencing

Double stranded DNA was sequenced by the dideoxy chain termination method using Sequenase Version 2.0 according to the manufacturer's instructions (U.S. Biochemical Corp., Cleveland, OH). Sequencing products were run on 5% polyacrylamide/7 M urea wedge and normal gels (Bio-Rad). Gels were dried under vacuum at 80°C and then exposed to XAR-5 film (Kodak) for 16-24 h. Sequence specific primers were synthesized at the Regional DNA Synthesis Laboratory, University of Calgary, Calgary.

RESULTS

Isolation of the genomic clones and restriction enyzme analysis

Approximately 300,000 plaques from a sockeye salmon genomic library, constructed in lambda Fix II, were screened with a radiolabeled 135 bp PCR fragment of the GHRH cDNA. The probe was amplified using the PCR with primers CSC33 (sense) and NMS 1 (antisense), and contained the entire coding sequence of the GHRH-like region from position 243 to 378 (Fig. 4.5A, Chapter 4). In the first round of screening 28 positive plaques were identified of which four were very strong. Use of the PCR revealed that 5 (including the 4 very strong positives) of the 28 positive plugs contained a bacteriophage clone with a 700 bp region coding for the GHRH-

like sequence (Fig. 5.1). The amplified DNA from four of the five 5 positive PCR reactions was subcloned into pBluescript II KS and sequenced. The plasmid subclones contained the GHRH sequence plus an intron. One of the four clones had 2 nucleotide substitutions. All four lambda bacteriophage clones were rescreened until single isolated plaques were obtained, of which two of these clones (PL1 and PL4) were analyzed by restriction enyzme digestion, Southern blotting and sequencing.

The strategy used in this study was first to identify for each bacteriophage lambda clone, the restriction fragments containing exons coding for the peptide sequences. The gene sequence was then compared to the cDNA sequence to determine the size and position of the introns. Restriction analysis of clones PL1 and PL4 with one enzyme or combinations of two enyzmes, in conjunction with Southern blotting, was used to identify fragments for subcloning (Figs. 5.2A and B, 5.3). A Not I restriction analysis of clones PL1 and PL4 showed these clones to contain genomic DNA inserts of approximately 12 Kb and 14 Kb, respectively (Fig. 5.2A and B, lane 1). A Sal I digest of PL1 and PL4 showed the former to contain an additional fragment of approximately 2.2-2.4 Kb (compare Fig. 5.2A lane 3 with B lane 3). The restriction enyzme EcoR I did not digest either of the two lambda clones (Fig. 5.2A and B, lane 6). This is interesting because the GHRH/PACAP cDNA had an EcoR I site within exon 3 (see Fig. 4.5B, Chapter 4). It was

possible that the gene sequence was different from the cDNA or that an EcoR I site is present and was not cut. If the latter is assumed correct, the resulting fragments may have been 18-22 Kb in length (including the lambda arms) and therefore did not separate on the gel. Restriction analysis with either Sac I, Xba I and the combination of the two, showed that both PL1 and PL4 contain the same size DNA fragments encoding the GHRH-like region (Figs. 5.2A and B, 5.3, 5.4). This suggested these two clones were identical. Confirmation that lambda PL1 and PL4 were identical was done by partial sequence analysis of the 5' end, directly from the lambda clones prior to subcloning. Therefore, only restriction digest fragments of PL1 were subcloned and their complete sequences obtained.

An Xba I digest of lambda clone PL1 resulted in 4 bands (Fig. 5.4). Southern blotting identified that the 2.5 Kb fragment (band 2) contained the coding region of the GHRH-like peptide (Figs. 5.3 and 5.4). A Southern blot of a Sac I digest showed that the 4.2 fragment contained the GHRH-like region (Figs. 5.2 and 5.3). In Chapter 4, the Southern blot of sockeye salmon genomic DNA revealed that a Hae III digestion fragment of approximately 1.5 Kb had the GHRH-like region. Digestion of the lambda clone PL1 with Hae III resulted in four main bands (Fig. 5.5). These four DNA fragments were cloned into the pBluescript phagemid. The 1.5 Kb fragment corresponds to band 2, Fig. 5.5; sequencing showed this fragment contained exons 4 and 5. The results

of the restriction digest analysis were used to construct a partial restriction map of the lambda PL1 clone and subclones (Fig 5.6). The subclone containing the Sac I fragment codes for exons 2-5 (Fig. 5.6). Whether exon 1 is contained within the Sac I fragment has not been established.

Organization of the GHRH/PACAP gene

Structural organization of the GHRH/PACAP gene was determined by comparison to the cDNA sequence presented in Chapter 4. Four exons have been identified in the salmon GHRH/PACAP gene (Fig. 5.7). Each exon encodes a distinct domain of the precursor (Fig. 5.7). The position and identification of exon 1 has not been established, however, comparison to the human PACAP gene suggests this exon codes for the 5' untranslated region. Exon 2 encodes 10 bp of the 5' untranslated region, the signal peptide and part of the 5' cryptic peptide. The majority of the 5' cryptic peptide is encoded on exon 3. Exon 4 encodes the last 3 amino acids of the cryptic peptide and the first 32 amino acids of the GHRH-like peptide. Exon 5 encodes the remaining 13 amino acids of the GHRH-like peptide, PACAP and the 3' untranslated region.

The number of exons and the exon/intron organization of the GHRH/PACAP gene is similar to the human PACAP gene (Fig. 5.8), although some incongruencies are apparent. Exon 4 of the salmon gene is 6 nucleotides longer than the respective

exon of the human PACAP gene. The 5' of exon 5 in the salmon gene appears to have a deletion of 6 nucleotides, or alternatively, they have been inserted in the human gene. The main difference in the structural organization of the sockeye salmon gene compared to the human, is the smaller introns in the former.

Partial sequence analysis of the GHRH/PACAP gene

The salmon GHRH/PACAP gene sequence is shown in figure 5.9. The sequence of the gene (lambda clone PL1) was identical to the cDNA sequence of clone SS/PCR 5 except at two positions in the 3' untranslated region. Only one of these substitutions was different from both cDNA clones SS/PCR 4 and SS/PCR 5 (Fig. 4.5A, chapter 4). Since only lambda clone PL1 was sequenced in the regions of discrepancy with the cDNA clones, one of the other lambda clones may contain the other form(s) of the cDNA(s). Although exons 1, 2 and 3 of the gene have not been completely sequenced, the consensus sequence donor- and acceptor-splice sites, GT and AG, respectively, were found in the exon/intron junctions sequenced (exon 4 and 5) (Breatnach and Chambon, 1981). Whether an intron (intron 1) exists within the 5' untranslated region of the salmon GHRH/PACAP gene has not been determined. However, all releasing hormones in which the genes have been sequenced, contained an intron in their 5' untranslated regions (Hosoya *et al.*, 1992). The human PACAP gene has an intron/exon splice site 7 bases upstream

of the first ATG codon (Hosoya *et al.*, 1992). This site corresponds closely to position -9 of the salmon cDNA 5' untranslated region, where changes in the sequence start between the different cDNA clones (see Fig. 4.9 chapter 4). Therefore it is assumed that the salmon gene will contain an intron within the 5' untranslated region. Intron 2 and 3 were only partially sequenced, therefore the size of these two introns was determined by using the PCR. Intron 2 is the largest intron of 1.2-1.3 Kb. Intron 3 was estimated as 0.4-0.5 Kb. Intron 4 was completely sequenced and has 0.58 Kb.

Sequence analysis of the 3' region of the gene revealed two additional polyadenylation signals (Fig. 5.9). In the salmon gene, downstream of the first polyadenylation signal, were found several (U)TG rich elements believed to be involved in polyadenylation signal recognition and 3' cleavage. A TTTTTCT element is located 7 bp downstream from the end of the mRNA cleavage site. Two other (T)UG motifs, usually found as a bipartite structure, were found 5 and 14 bp downstream of the mRNA termination site (Fig. 5.9). The first element has the sequence TGTTTT and the second TTTGTA. The first of these two elements encompasses part of the previous 7 bp motif.

Alternate splicing

A short cDNA precursor was identified in chapter 4. The structure of the gene shows this short precursor could be

the product of alternate processing of the mature RNA (Fig. 5.10). The region deleted in the short cDNA precursor corresponds to the excision of exon 4 containing the GHRH domain of the gene (Fig. 5.10). This exon codes for the last three amino acids of the cryptic peptide and the first 32 amino acids of the GHRH-like molecule. The gene structure shows exon/intron splice sites at nucleotide positions 233-234 and 338-339 in the full length cDNA (Fig. 5.10).

Figure 5.1. A photograph of the DNA bands from the PCR amplification of positively hybridizing bacteriophage lambda clones. Clones were obtained from the first round of screening of the sockeye salmon genomic library. DNA was amplified in the GHRH-like region using primers CSC33 and NMS1. PCR products were run on a 1.8% agarose gel. Bands 1-5 were cut out and electroeluted as described in the text.

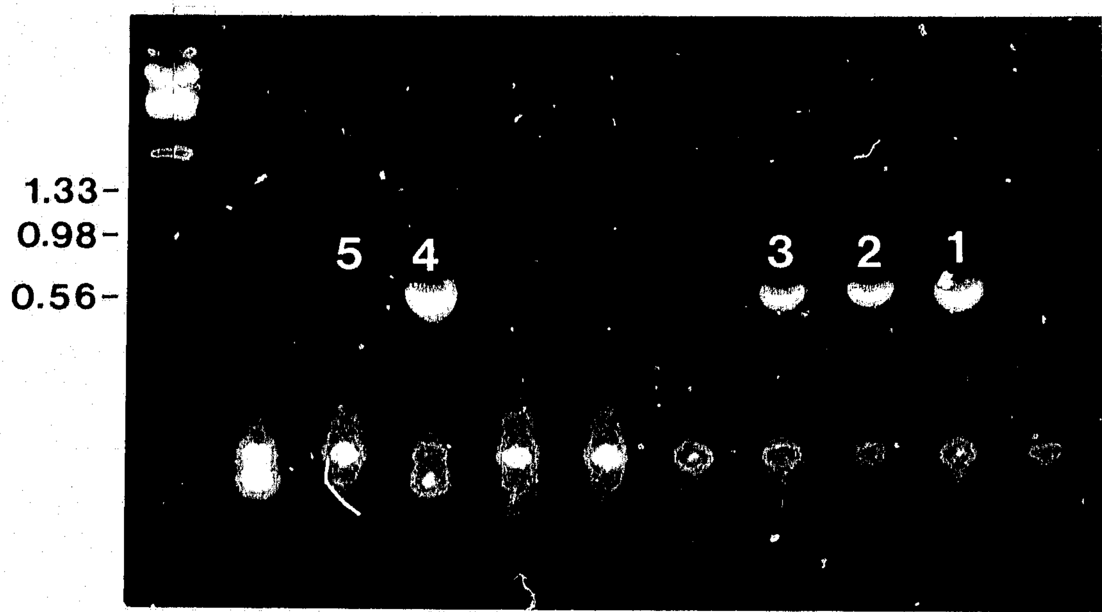

M
1.33-
0.98-
0.56-
5
4
3
2
1

Figure 5.2. A photograph of restriction enyzme digests of lambda bacteriophage clones PL4 (A) and PL1 (B). Samples were run on a 0.8% agarose gel. Lanes 1-8: Not I, Xho I, Sal I, Xba I, Sac I, EcoR I, Sal I+ EcoR I and Xba I+ Sac I. The arrows in lanes 4 and 5 show the DNA fragments coding for the GHRH and PACAP regions. DNA markers are shown in Kilobases at the sides .

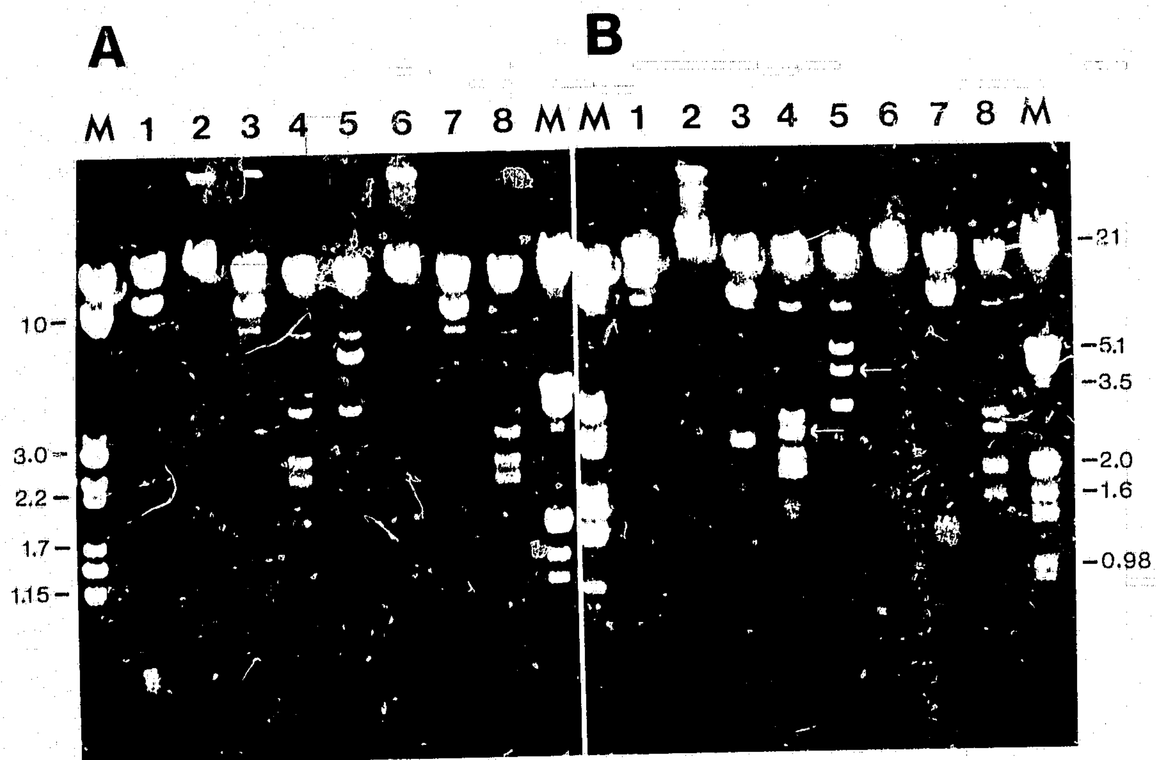

A
B
M 1 2 3 4 5 6 7 8 M
M 1 2 3 4 5 6 7 8 M
10
3.0
2.2
1.7
1.15
21
5.1
3.5
2.0
1.6
0.98

Figure 5.3. A photograph of a Southern blot of the restriction enyzme digests of PL4 (A) and PL1 (B)(see Fig. 5.2). Membranes were hybridized to a Mse I cut 355 bp salmon GHRH/PACAP cDNA probe. See text for hybridization and washing conditions. Lanes 1-8: Not I, Xho I, Sal I, Xba I, Sac I, EcoR I, Sal I + EcoR I, and Xba I + Sac I. The arrows in lanes 4 and 5 show the DNA fragments coding for the GHRH and PACAP regions (compare to Fig. 5.2). DNA markers are shown in Kilobases at the sides.

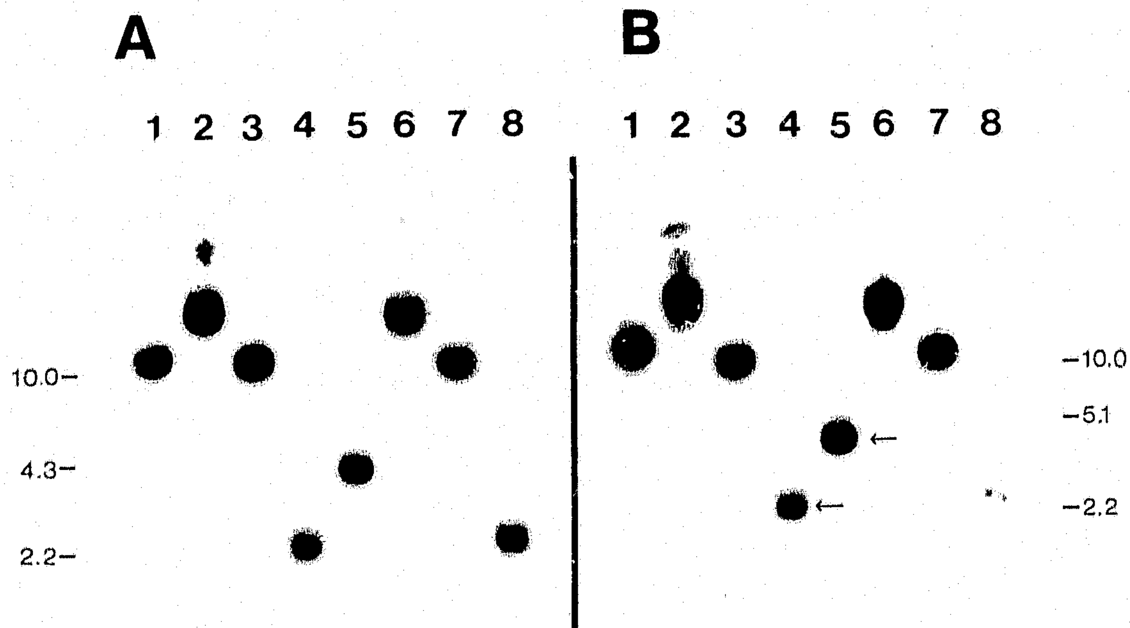

A
1 2 3 4 5 6 7 8
10.0
4.3
2.2
B
1 2 3 4 5 6 7 8
10.0
5.1
2.2

Figure 5.4. A photograph of an Xba I digest of the sockeye salmon GHRH/PACAP lambda clone PL1. The DNA was electrophoresed on a 1.0% agarose gel (replicate lanes). All four DNA bands were cut out, electroeluted and subcloned into pBluescript. Band 2 (marked by the arrow) contains the DNA coding for the GHRH and PACAP peptides.

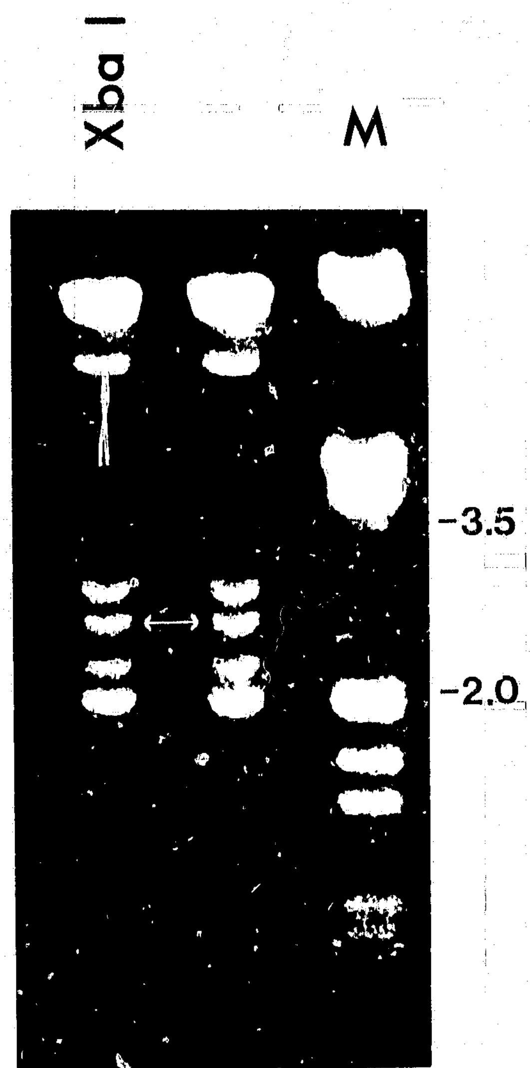

Xba I
M
-3.5
-2.0

Figure 5.5. A photograph of a Hae III digest of the sockeye salmon GHRH/PACAP lambda clone PL1. The DNA was electrophoresed on a 1.2% agarose gel. DNA bands 1-3 were cut from the gel and electroeluted. Band 2 (marked by the arrow) contains the DNA coding for the GHRH and PACAP peptides.

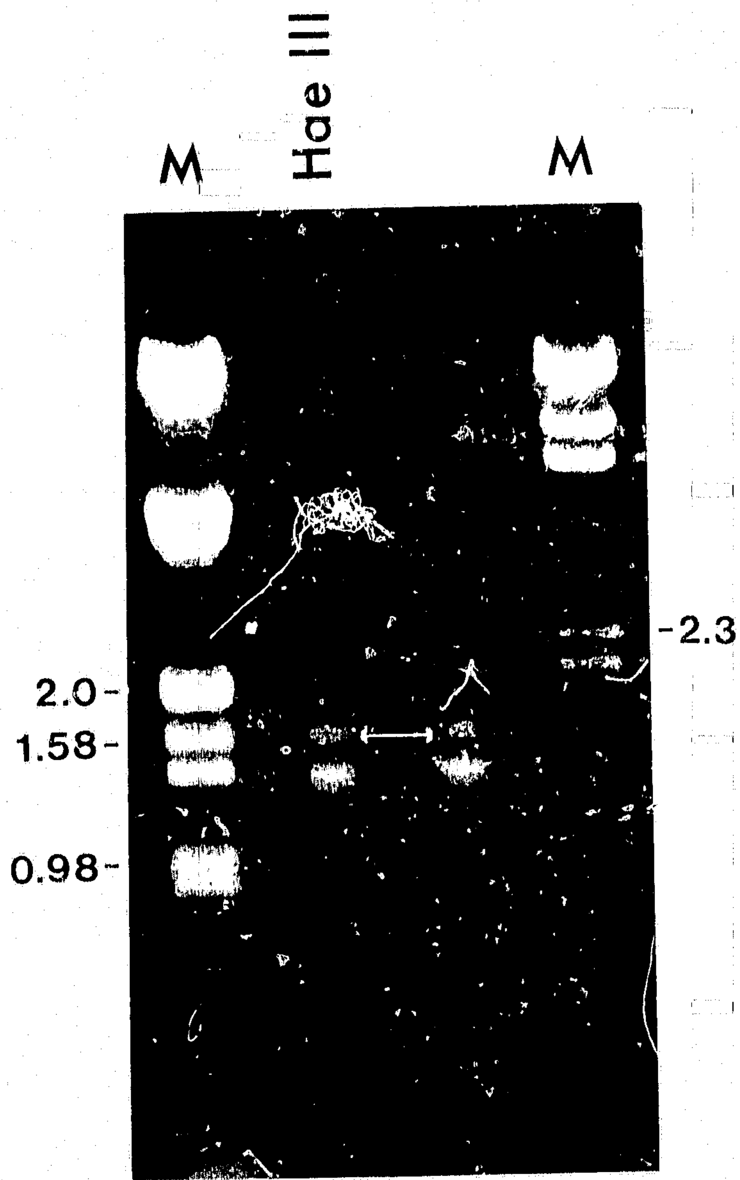
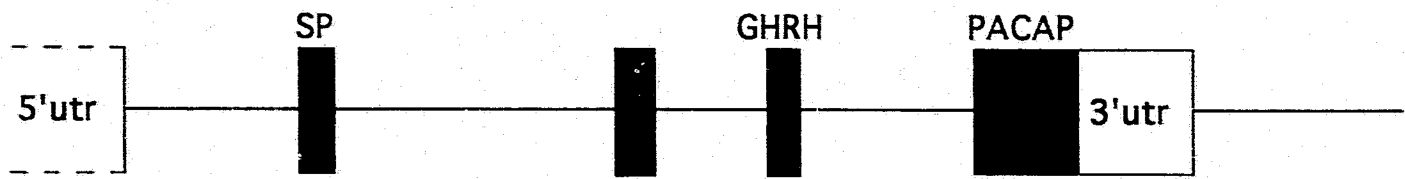

M
Hae III
M
2.0-
1.58-
0.98-
-2.3

Figure 5.6. A schematic diagram of the sockeye salmon GHRH/PACAP genomic clone PL1 and subclones. The black boxes represent the exons and the lines indicate introns. The three overlapping subclones used for sequence analysis are shown below the gene structure. The restriction enyzmes Sac I, Xba I and Hae III used to generate the subclones are shown by the vertical lines. Length and location of the subclones within the gene are indicated by the black lines. The direction and extent of DNA sequencing is indicated by the arrows.


SP
GHRH
PACAP
5'utr
3'utr

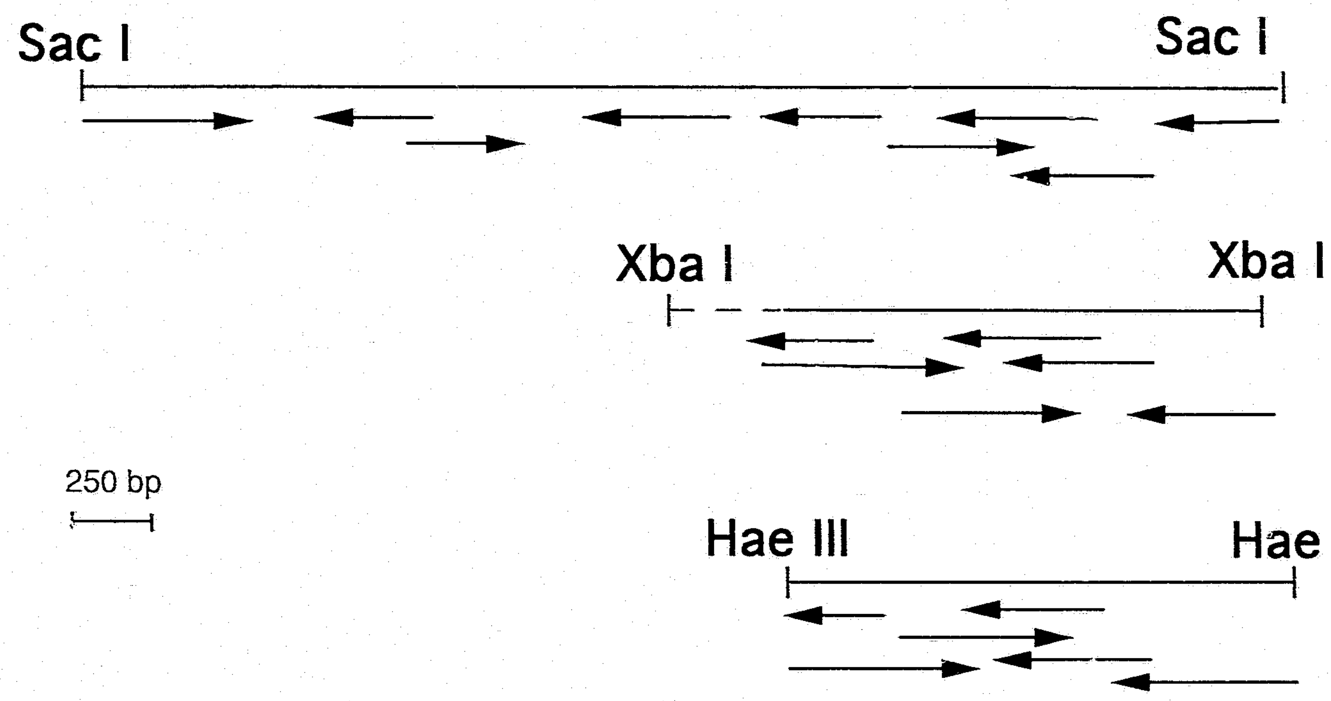

Sac I
Sac I
Xba I
Xba I
250 bp
Hae III
Hae III

Figure 5.7. A schematic diagram showing the structure of the sockeye salmon GHRH/PACAP gene and mRNA (cDNA). Open boxes represent the exons and thin lines the introns. A vertical line in exon 5 shows the 3' termination point after the first polyadenylation signal. Other polyadenylation signals further downstream would increase the size of the 3' untranslated region (UT) as shown in the mRNA. The thin black lines connecting the genomic DNA with the mRNA show the relationship between the two. SP= signal peptide, GHRH=growth hormone-releasing hormone, PACAP= pituitary adenylate cyclase activating polypeptide, UT= untranslated region. Cryptic= a sequence that is translated in a protein with unknown function. Note the scales are different between genomic DNA and mRNA.

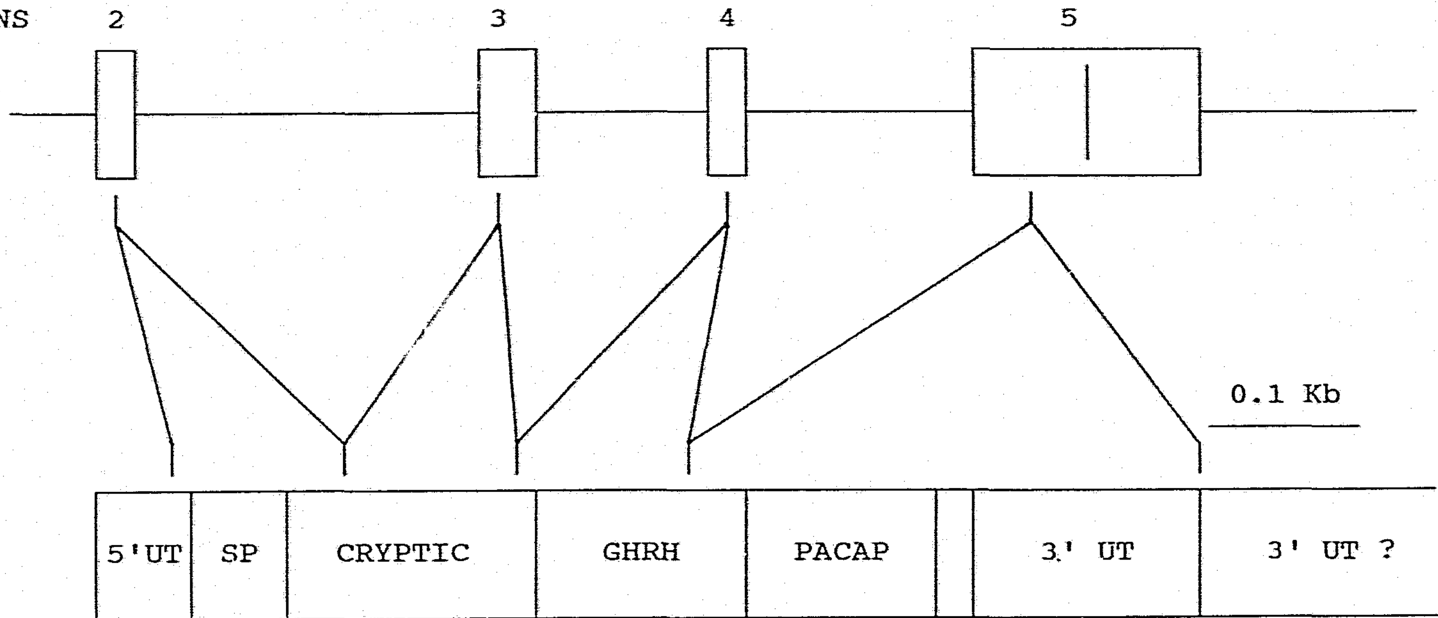
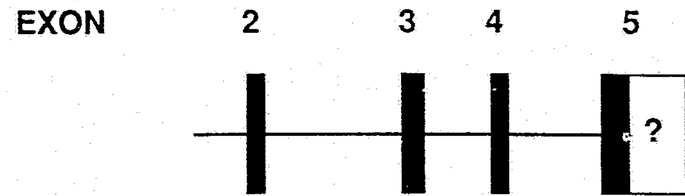

0.2 Kb
GENOMIC DNA
EXONS
2
3
4
5
mRNA
0.1 Kb
5'UT
SP
CRYPTIC
GHRH
PACAP
3' UT
3' UT ?

Figure 5.8. A comparison of the exon/intron organization of the salmon GHRH/PACAP gene with the human PACAP gene. Exons are indicated by the solid black boxes and introns by the thin lines. The open box for the salmon and human genes indicate that alternative 3' untranslated regions are possible. The position of exon 1 of the salmon gene has not been confirmed. The length of exon 1 is not certain in the human gene. The size and position of exon 3 and the flanking introns were determined by the PCR.

SALMON GHRH/PACAP



500 bp

HUMAN PACAP

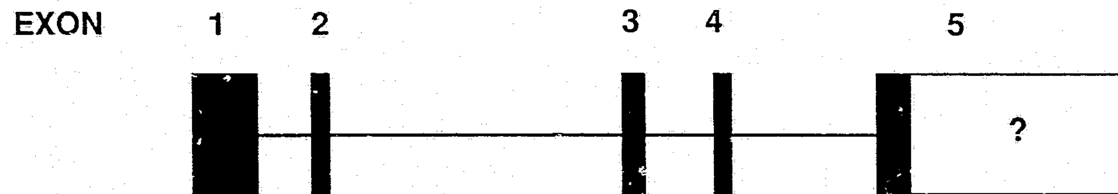


Figure 5.9. DNA sequence of the salmon GHRH/PACAP gene. Exons are shown in upper case and introns in lower case letters. The regions coding for the peptide products, GHRH-like peptide and PACAP, are shown in bold. Arrows indicate the start of an exon or the poly (A) region. Potential polyadenylation signals (ATTAAA and AATAAA) are doubly underlined. Sequences encoding elements thought to be involved in polyadenylation and 3' termination, as well as RNA instability are underlined. The dashed line indicates a another sequence motif used in 3' termination recognition. The boxed sequence represents a putative branch point recognition element.

Intron 3
taaatgtactgtaccattaataatcccattttgttatttaatctttagatggaaatacatagggg

ggaaaaaatgagactttaaaataacaagcttcccacattttcattgcagcacgatagactatggt

tggtagcctagtattcaaacgaagataatcggaaagcctataagcgaactaaaacagaacgagac

acttttaatataaaccggccatttccaaagtcatgaaattcaccagaatagcattgtccactttt

cagcccacattttggaaagcagcccacatgacgcgttggatttggtaggctaaatacattatgtt

Exon 4
accacttacgtgtgatcatttattgtttggcgttttatcatcccagAACGGAAAGGCATGCAGAC
yThrGluArgHisAlaAsp

GGAATGTTTAATAAAGCCTACAGGAAAGCGCTGGGTCAGTTATCAGCAAGAAAATATCTCCAT
GlyMetPheAsnLysAlaTyrArgLysAlaLeuGlyGlnLeuSerAlaArgLysTyrLeuHis
GHRH-like peptide

TCTCTGATGGCAAAGCGTGTAGGgtaaggacatcttcttagttctcgtctcttattttgtgatct
SerLeuMetAlaLysArgValGl Intron 4

tagttattttggtgttcgtttttcttctaactcagattttccgaatactcactattcattctat

gtttcgtcaaattgtccggtgtgtctatgtgtgtgttcttccgacttgaatttgacgccttcagg

cacaga?tgggttgacttgataaa?tctccgacagcgcaaatccgggtgcg?catgatttagatc

aa?tcggtggatctcagtgctgatattgtctctttaagtatgcaaagccccaggatcccacgtgg

atgaatacatttcattttcattattaatacttgatggtggtagggggttagtccaagggttgaag

ataaatgtaggcttcaattaccctacatcctccaccagccagcctaaccatgatgatatcgaaat

aaaaatcaatttgggatgattgattaaaagttgaagagatgagaaaatgaagagctgtgcagtgc

agtctatgggctacattccgaggcagggtaggcgtc tcctaac agtgaaaactatcctgctct

Exon 5
ccccctttcttag**TGGAGGGAGCACCATGGAAGACGACTCAGAGCCTCTGTCA**AAGCAG**CACTCG**
yGlyGlySerThrMetGluAspAspSerGluProLeuSerLysArg**HisSer**

GATGGGATCTTCACAGACAGCTACAGCCGCTACCGAAAGCAAATGGCAGTCAAGAAATACCTG
AspGlyIlePheThrAspSerTyrSerArgTyrArgLysGlnMetAlaValLysLysTyrLeu
PACAP

GCGGCAGTCCTTGGGAAAAGGTATAGACAGAGATATAGAAGCAAAGGACGCCGGCTAGCGTAT
AlaAlaValLeuGlyLysArgTyrArgGlnArgTyrArgSerLysGlyArgArgLeuAlaTyr

TTGTAGCGTTGCTAAACCAAACTACCATGTGTGTACAGCCCAGATCAAGTCATTTTGAGAAAACT
Leu***

GAACAATCGGTGGATCGCTCTTGTGTTCTTTAAACATGTATTTATGTATGAAGTAAAGCCATTAA

Poly A

AATTAATATTTTGATAATAATATTGTTTTTCTTTTGTACTTAAGCACTTGAGAACGCGCAGATCT

ACTTTGTGGACCAATCCTTTTGTTCAACCTAAATATAGCCTATATTTATTTATTTTTAGACCTAC

GTAGACAAGAAGATTAAATGATGTGCACATGATCAATATGCTCTTTACCCTGTCTTTTAAAGATT

TAAATCTAGATATGAATGCGACGGAAATAAATAGTTTAAAAGACAGTCAATGTATTGAATGTTAC

TTTGTGGCAACTGAAATTAAGAGCTCAGTGTCTTTATTTACAACAGCCCTGAAGACTTAGGTTTT

CACCATATTCTACAAGACATAGACTTTGATGCCTACCGGATGGGGATGAGTTTGAGGCTATTTTG

GGAGACTGGCTGAAACAGTTCTCTCCAATTTCCGGTGAGTTCCAGGCTCTTTCCCGAGG

Figure 5.10. A) A schematic diagram of the proposed exon/intron organization of the GHRH/PACAP gene and the processing of the RNA transcript to obtain the short preprohormone. B) The nucleotide and amino acid sequences of the exon/intron splice sites. Nucleotides in bold represent those on each exon boundary

SP CRYPTIC GHRH PACAP

SP CRYPTIC PACAP ?

233 339

Tyr Pro Pro Glu Lys .... GHRH .... Gly Gly Ser Thr Met Glu Asp
TAC CCA CCG GAG AAA **AG**........ ....**T** GGA GGG AGC ACC ATG GAA GAC

Tyr Pro Pro Glu Lys **Ser** Gly Gly Ser Thr Met Glu Asp
TAC CCA CCG GAG AAA **AGT** GGA GGG AGC ACT ATG GAA GAC

DISCUSSION

Based on the high sequence identity of the prohormone and a similar exon/intron structure, the salmon GHRH/PACAP gene is placed in the PACAP family and glucagon superfamily. The salmon GHRH/PACAP gene is the first non-mammalian gene to be identified within the glucagon superfamily. Structural orga[illegible]ation of the salmon gene, with 5 exons and 4 introns, is the same as the human PACAP gene. Conservation of the number of exons is found within many protein families (Breathnach and Chambon, 1981). Each exon of the salmon gene encodes a distinct domain of the GHRH/PACAP preprohormone mRNA, which is characteristic of the other members of the glucagon superfamily (Bell, 1986).

The salmon and human PACAP genes have greater sequence divergence in exons of the non-peptide coding regions and introns like that of other gene families (Heilig *et al.*, 1980; Li *et al.*, 1985). An exception to this paradigm is the strong conservation of the 3' untranslated region among species as discussed in chapter 4.

The size and sequence identity of the exons encoding peptide domains is well conserved between the salmon and human PACAP genes. Insertion and deletion events have occurred in exon 4 and 5, which are the peptide coding regions. There is 58% sequence identity of exon 4, excluding insertions and deletions, between the salmon and human genes. Excluding the first 18 nucleotides of exon 5

in the salmon gene, there is 84% sequence identity of the translated region from exon 5 between the salmon and human genes. The lower conservation of exon 4 suggests sequence drift has occurred in this region during the evolution of this molecule. This implies that a greater functional constraint exists on PACAP than the GHRH-like peptide. It is also possible that the tetraploid nature of salmon has allowed this GHRH-like region to diversify. On one of the duplicated genes the sequence remains conserved, whereas on the other copy the sequence is free to mutate. Gene duplication events followed by point mutations can allow for one of the genes to evolve new peptide functions (Niall, 1982). An additional 6 nucleotides at the 5' end of exon 5 in the human PACAP gene adds 2 amino acids to the big PRP peptide. This, plus a change in the cleavage site at the amino terminus in the precursor, accounts for the longer length of big PRP compared to the GHRH-like peptide. The structural difference between the two genes may be expressed as a functional difference.

The introns of the salmon GHRH/PACAP gene contain the consensus GT/AG splice donor/acceptor sites, a pyrimidine rich region just upstream of the 3' splice site and a branch point recognition sequence, all associated with intron splice recognition. The branch point sequence TCCTACC is located 34 nucleotides upstream of the 3' splice acceptor site in intron 4. Intron 3 does not contain the same branch point sequence, but a variant of this, CACTTAC, which

conforms to the relaxed arrangement of the branch point sequences of mammalian introns (Green, 1986; Zhuang *et al.*, 1989). Introns of the salmon gene are smaller than the respective introns of the human PACAP gene. The Pacific (Coe, 1992) and Atlantic (Klungland *et al.*, 1992) salmon GnRH genes also have smaller introns compared to their mammalian counterparts. A survey of a number of genes from organisms encompassing the fungi to vertebrates showed a trend toward larger introns in genes of higher organisms (Hawkins, 1988). Although this does not always hold true, as exemplified by the Atlantic salmon GH gene which has a larger intron than the mammalian GH genes, but smaller introns 2 and 3 (Johansen *et al.*, 1989). Differences in intron size are also apparent between related peptide family members. NPY, PP and PY in both rat and human have the same exon/intron organization, but the introns are substantially smaller in PP and PY compared to those of NPY (Larhammar *et al.*, in press). The functional significance of intron size differences is not really understood. Smaller introns (51-80 bases) in *Drosophila* genes were found to lack pyrimidine rich regions upstream of the 3' splice site (Mount *et al.*, 1992). This difference is believed to reflect changes in the recognition of branch points because the points are closely related to the 5' splice site in these shorter introns (Mount *et al.*, 1992). Whether regulatory differences exist in the shorter introns of fish in comparison to mammals is not yet known.

Identification of the salmon GHRH/PACAP gene confirmed that a short precursor could be obtained by alternating splicing of the RNA transcript. It is interesting that a short precursor was found in the salmon (Chapter 4) that has not been identified in the mammals. This means that GHRH and PACAP need not be release in equimolar quantities as expected from cleavage of the long precursor, but that additional quantities of PACAP only could be released from the short precursor. Alternate splicing in the salmon gene may be regulated by a number of factors associated with the intron, including: specific consensus sequences, secondary structures, and purine and pyrimidine concentrations. It is also possible that sites within the exons regulate alternate splicing. AU-rich regions are characteristic of plant (Goodall and Filipowicz, 1989) and some non-mammalian animals introns. In plants, AU-rich regions in the intron are required for intron recognition and splicing, regardless of their position or the presence of GC-rich regions (Goodall and Filipowicz,1989). Furthermore, the pyrimidine rich region associated with intron splicing in animals, while present in plants, is not required for intron processing in the latter. A composition analysis of the first 100 nucleotides from the splice donor/acceptor sites of intron 4 in the salmon gene showed a high AT(U)-concentration adjacent to the 5'donor site. The AT(U) content of this region (100 nucleotides) is higher than that of the upstream exon and the comparable intron in the human

PACAP gene. A complete survey of all fish genes in comparison to mammalian genes, as well as experimental studies would be required to discern if this difference has any validity. It is also possible that neuropeptide genes may be regulated differently from other genes.

Identification of the salmon GHRH/PACAP gene confirmed the cDNA sequence to be that of clone SS/PCR 5 presented in Chapter 4. At present only one GHRH/PACAP gene has been identified. Southern blot analysis suggested more than one gene or different allelic polymorphisms. Since salmon are tetraploid they contain duplicates of their genes, however, what is not known is at what stage in the diploidization processs the GHRH/PACAP gene has evolved (Allendorf and Thorgaard, 1984). Two genes for melanin concentrating hormone (MCH), and two precursors for vasotocin and corticotropin releasing factor (Morley *et al.*, 1991) have been identified in salmon. The evolution of the GHRH/PACAP gene in teleosts may parallel that of somatostatin. The two somatostatin genes of anglerfish and catfish are believed to have arisen after the divergence of the teleosts from the tetrapod line, but prior to the separation of the two fish groups (Su *et al.*, 1988). Since the salmonids are believed to have emerged after catfish evolved (Carroll, 1988), duplication events in catfish are thought to be present in salmon. Salmon have also undergone polyploidy which may double these already duplicated genes (Ohno *et al.*, 1968).

The polyadenylation signal AUUAAA is the most commonly

used alternative to AAUAAA in mRNA (Manley, 1988). The polyadenylation signal is required for poly (A) formation and cleavage at the 3'end. It has also become evident that cis-acting sequence motifs are sometimes required for efficient polyadenylation and/or cleavage (McLauchlan *et al.*, 1985; Manley, 1988). These motifs are usually GU rich and are required to be within a minimum distance of the polyadenlation signal or the 3' termination site for optimal function (Manley, 1988). In the salmon gene, twenty-four base pairs downstream of the polyadenylation signal, is a 7 bp motif (TTTTTCT) identical to a transcription termination signal in viruses (Zhou *et al.*, 1991). This 7 bp sequence was also found 26 bp downstream of the polyadenylation signal in the 3'-flanking region of the ocean pout antifreeze protein gene (Du *et al.*, 1992). Two other GT rich elements in the salmon gene have sequence identity and similar spatial arrangement to the bipartite structure found in the 3' region of the mouse beta globin gene (Chen and Nordstrom, 1992). The two GT rich segments in the salmon gene are within the same relative distance, +5-10 and +14-19, of the 3' cleavage site as those in the mouse beta globin, +5-10 and +17-22.

Destabilizing sequences that increase mRNA degradation have been found within the 3' untranslated region of some mRNAs (Shaw and Kamen, 1986; Wilson and Treisman, 1988). These sequences contain the motifs 'AUUUA' or A(U)n', where 'n' is 3 or greater. There are two of these sequence motifs

distal to the 3' termination site in the GHRH/PACAP gene, but before the second polyadenylation signal. Use of the alternative polyadenylation signals in the salmon GHRH/PACAP RNA, creating longer mRNAs, may result in instability and therefore lower expression. Whether there is any selective pressure to synthesize a shorter mRNA in the salmon is not known. The role of destabilizing sequences in the mRNA is not completely understood, but may represent another level at which protein expression is regulated.

Chapter 6

GENERAL DISCUSSION

In this thesis research, the sockeye salmon GHRH/PACAP mRNA (cDNA) and gene have been identified, and the identity of the precursor and peptide structures deduced from the DNA sequence. Sequence identity of the peptides, and structural organization of the precursor and gene, place this molecule as a member of the glucagon superfamily. The glucagon superfamily is composed of glucagon, GHRH, GIP, secretin, VIP, PHM(PHI), PACAP, helodermin and helospectin (Bell, 1986). This superfamily is believed to have arisen from an ancestral progenitor gene by exon and gene duplications (Bell, 1986). There has been extensive sequence divergence and rearrangement in the exon/intron positions of the superfamily members since the original ancestral gene.

The salmon PACAP precursor shows greater sequence identity and structural organization to the human PACAP precursor than the other fish precursors of this superfamily compared with their human counterparts. There is also greater sequence identity between the signal peptides and cryptic regions of the salmon and human PACAP precursors than has been found for several other neuropeptides such as CRF, somatostatin, GnRH and vasotocin (see review by Sherwood and Parker, 1990). High sequence conservation of

the PACAP precursor suggests that structural and/or functional constraints have been imposed on this molecule during its evolution. PACAP is unique in comparison to the other glucagon superfamily members in that it has neither an intron located in the carboxy terminal peptide nor an intron in the 3' untranslated region. Inconsistencies of intron location have been found within the actin, insulin, and trypsin families (Craik *et al.*, 1983).

Given the lengths of PACAP and neuropeptide Y, they are two of the most highly conserved neuropeptides, next to corticotropin-releasing factor, identified to date. Somatostatin-14 is the most highly conserved neuropeptide in all vertebrates with only one substitution in the lamprey molecule (see review Sherwood and Parker, 1990). Alligator NPY was found to be identical to human NPY (chapter 3) while in the amphibian there is one substitution. In chapter 3, I suggested this substitution occurred after the divergence of the amphibians and reptiles. The recent identification of frog PACAP shows one amino acid difference from the mammalian PACAPs. One can predict that the reptilian PACAP will be identical or very close in structure to the mammalian PACAPs. The real intrigue is to determine the structural changes in PRP, a more rapidly evolving molecule.

Evolution of the glucagon superfamily

The structural organization of the precursors suggests that two lines within the superfamily may have evolved, one

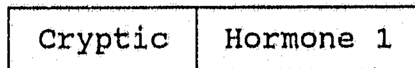
containing PACAP, VIP and glucagon, the other consisting of GHRH, GIP and secretin. The glucagon group contains two or three distinct peptides in the precursor, while the GHRH group has only one. The gene organization shows that the glucagon group contains tandem sequences coding for two or more functional peptides, whereas in the other group a cryptic sequence without known function results (Fig. 6.1). The pathway to this diversification may have initially involved exon amplification with duplication of the full gene later (Heinrich *et al.*, 1984; Hosoya *et al.*, 1992) (Fig. 6.1). The progenitor gene may have had an exon containing at least 100 nucleotides which encoded the first 27-30 amino acids of the bioactive peptide. This assumption is based on the fact that the first 27 amino acids are the most highly conserved between the superfamily members and that this region is encoded on a separate exon. However, through point mutations, additions and deletions of nuceolotides, different exon lengths in genes have evolved. Many protein superfamilies and families, including immunoglobulins (Rogers *et al.*, 1980), globins (Breathnach and Chambon, 1981) , growth hormone and prolactin (reviewed by Kawauchi *et al.*, 1990), vasopressin and oxytocin (Acher, 1981), actins, P450 genes (Nebert and Gonalez, 1987) and ovalbumins, have evolved by gene duplication and divergent evolution from an ancestral gene. A classical example is the evolution of the constant (C) and variable (V) regions

Figure 6.1. A hypothetical scheme for the evolution of the glucagon superfamily. █ = Bioactive peptide, c = cryptic sequences

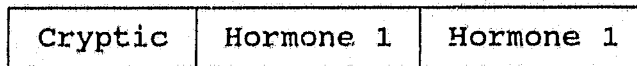
PRECURSORS

Derivation:

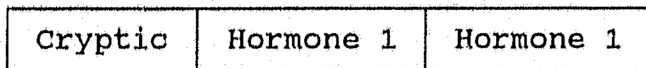
Ancestral gene

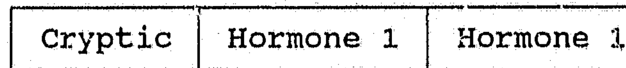


Exon duplication

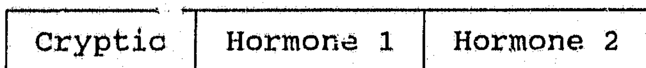


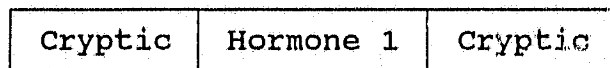
Gene duplication



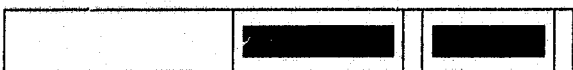


Base substitutions

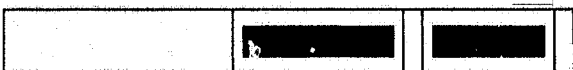




salmon GHRH-45 PACAP-38



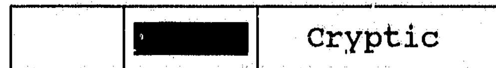
human PRP-48 PACAP-38



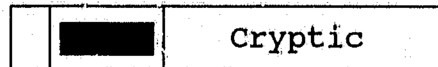
human GHRH-44

human PHM-27 VIP-28

human GIP-42



human Secretin-27



Gene duplication (2 copies in fish)

fish Gluc-29 GLP1-34

Exon duplication

human Gluc-29 GLP1-37 GLP2-33

of the immunoglobulin genes, which are believed to have arisen from duplication of an exon containing both the primordial Ig domain and some of its 3'flanking region (Rogers *et al.*, 1980). Duplication of the whole gene transcript would then have given rise to the genes for light and heavy chains. The duplicated exon domains are thought to remain in tandem by RNA splicing (Rogers *et al.*, 1980).

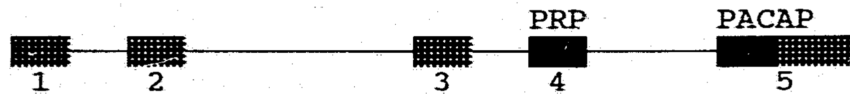
Evolution of the PACAP and VIP genes

Sequence identity and gene organization of PACAP is most closely related to VIP suggesting these two molecules diverged more recently than other members of the superfamily. VIP has two extra exons; one contains part of the 3' cryptic peptide and the other has the 3'untranslated region (Fig. 6.2). It is not yet clear whether an intron gain or loss occurred between the VIP and PACAP genes (Fig. 6.2).

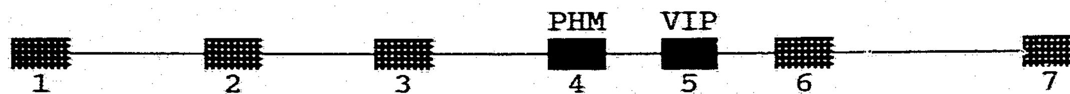
If introns were lost in the PACAP gene, it may have occurred during or after a gene duplication event in a manner similar to the precise exision of an intron from the insulin I gene in rat (Cordell *et al.*, 1979: Lomedico *et al.*, 1979) or the gene conversion event of the α-3 globin-like gene in mouse (Nishioka *et al.*, 1980). However, unlike these two genes, there is no sequence identity between VIP and PACAP in the cryptic peptide coding regions and 3' untranslated regions surrounding these introns. This is not surprising since gene conversion in PACAP would have

Figure 6.2. A schematic diagram of the PACAP and VIP genes. The filled boxes represent the bioactive peptides. Note the introns between exons 4-5 and 5-6, do not interrupt the coding of the two bioactive peptides in the VIP gene. During the evolution of the PACAP and VIP genes there was either an insertion or a deletion of an exon and two introns after a duplication event of the ancestral gene.

SALMON GHRH/PACAP
HUMAN PRP/PACAP



HUMAN PHM/VIP



occurred prior to the divergence of fish and tetrapods, whereas this event is much more recent in rat and mouse. The α-3 gene does have deletions and insertions causing out of frame reading and in frame stop codons (Nishioka *et al.*, 1980) compared to the globin genes, suggesting changes occurred after the gene conversion event. It is also likely that following a gene conversion event, mutations in the non-peptide coding regions of PACAP occurred much faster than in the peptide coding domains. The intron loss in the insulin I and α-3 genes occurred at consensus GT/AG splice sites resulting in a sequence identical to the mRNA. In this respect it is interesting that the sequence identity between the VIP and PACAP genes is highly conserved up to the exon/intron splice site at the 3' end of exon 5 in the VIP gene. There is also a potential donor-splice site (GT) in the PACAP gene within this region. After the gene conversion event, PACAP-38 appears to have evolved with a new function. However, VIP and both PACAP27 and PACAP-38 have maintained a moderate binding affinity between receptors. Another possible scenario is that the introns were simply deleted during the evolution of the PACAP gene. The earliest ancestral organisms are believed to have had split genes, and therefore, evolution proceeded mainly through intron loss rather than insertion (Gilbert, 1985).

Alternatively, VIP may have gained two introns instead of PACAP losing them. The ancestral gene organization may have been similar to PACAP. After gene duplication one of

the genes would be free to acquire mutations without harm to the organism. Mutations within the PACAP coding region may have given rise to an exon/intron splice site resulting in an exon containing VIP or a PACAP-27. A splice site may have been acquired within the exon at first, with insertion of an intron later. A second intron is hypothesized to be inserted in the 3' untranslated region at some point in time. However, it is less likely that an intron insertion occurred in the peptide coding region of the PACAP gene, since this is not a well documented event in the evolution of vertebrates (Darnell *et al.*, 1990).

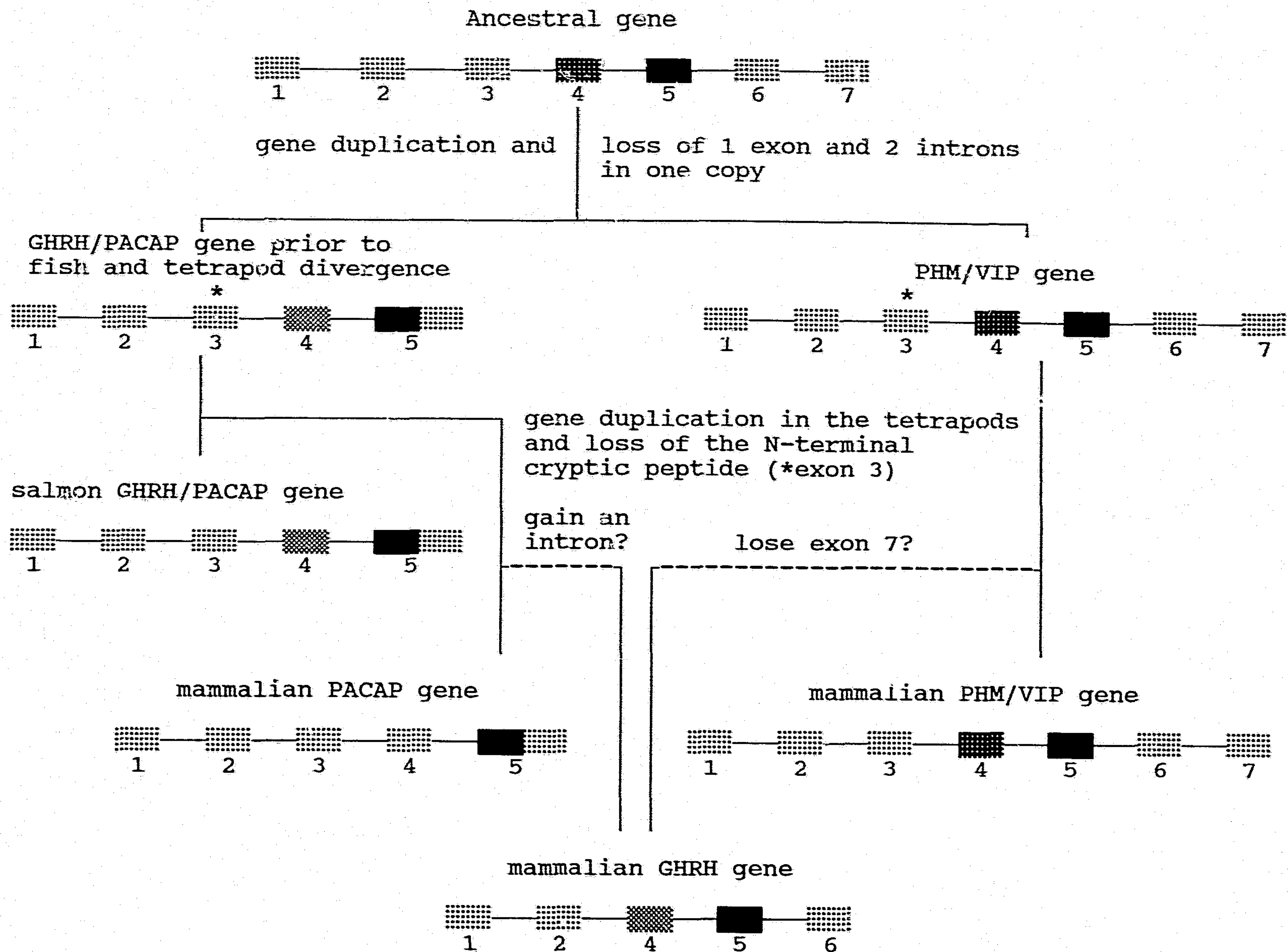
Another explanation for the evolution of the PACAP and VIP genes is that there was a loss or gain of DNA during the initial duplication event. Evidence for this hypothesis is the lack of sequence identity between the two genes after the VIP/PACAP27 coding region.

It is also possible that a second duplication event in one or both of these molecules occurred resulting in two different gene structures within the family. While only one copy of the PACAP and VIP genes is present in humans, the results in chapter 4 suggest that two PACAP genes exist in fish (chapter 4). A cDNA encoding only PACAP-27 has been identified in a teleost fish in Dr. Sherwood's laboratory by John McRory. Whether this PACAP-27 is located on a separate gene or is a result of alternate RNA splicing is not known yet. This implies that a function may exist for both PACAP-27 and -38 in fish.

There is not enough information to estimate the time at which the VIP and PACAP genes diverged. Isolation of dogfish VIP suggests the VIP gene was present in both ancestral cartilaginous and bony fish. Identification of the salmon PACAP gene places its emergence before fish and tetrapods diverged.

One of the most interesting aspects of the evolution of the VIP and PACAP genes is the consideration of the point at which the GHRH gene arose. The low sequence identity between GHRH, PRP and PHM, suggests that divergence of GHRH from the latter two molecules occurred prior to the divergence of VIP from PACAP. However, it is also possible that GHRH arose some time after the divergence of the tetrapods from fish (Fig 6.3). GHRH may have undergone rapid changes in its sequence as well as acquiring the main function of GH release. The low sequence identity between the rat and human GHRHs implies this molecule evolved at a rapid rate. What is not clear is why the GHRH region of the salmon PACAP gene is more closely related (18/27=67%) to human PHM, than it is to the PRP region of the human PACAP gene (16/27=59%) or than the comparison of human PRP with human PHM (14/27=52%). Either convergent evolution occurred in the salmon gene or the salmon gene is closer in sequence to the ancestral gene. This implies that the ancestral gene was structurally closer to VIP than PACAP. This also supports the idea that the GHRH gene arose after the fish and tetrapods diverged. The emergence of a gene with GHRH

Figure 6.3. A schematic diagram of the hypothetical evolution of the VIP, PACAP and GHRH genes. An ancestral gene may have contained two bioactive peptides, represented by ▦ and █. Gene duplication resulted in two copies. The exon/intron organization of the VIP gene remained the same as the ancestral gene, while the PACAP gene lost 1 exon and 2 introns. The structural organization of PACAP gene remained the same between salmon and humans. However, in the salmon PACAP gene, exon 4 (▒) either evolved or continued the function of a GHRH, but in the mammalian gene this function was lost (exon 4=┆┆┆). After the divergence of the tetrapods, a duplication event in either the PACAP or VIP genes resulted in the GHRH gene.



Ancestral gene
1 2 3 4 5 6 7
gene duplication and loss of 1 exon and 2 introns in one copy
GHRH/PACAP gene prior to fish and tetrapod divergence
1 2 3 4 5
PHM/VIP gene
1 2 3 4 5 6 7
gene duplication in the tetrapods and loss of the N-terminal cryptic peptide (*exon 3)
salmon GHRH/PACAP gene
1 2 3 4 5
gain an intron?
lose exon 7?
mammalian PACAP gene
1 2 3 4 5
mammalian PHM/VIP gene
1 2 3 4 5 6 7
mammalian GHRH gene
1 2 4 5 6

in tetrapods means the PRP region would be free to mutate without affecting the survival of the organism. To unravel this evolutionary story, identification of the PACAP and VIP genes in all vertebrate classes, especially the non-mammalian vertebrates, is required. To date, a GHRH peptide with structural similarity to the mammalian molecules has not been identified in birds, reptiles or amphibians.

The possibility of another GHRH gene without PACAP in salmon.

It is important to consider whether salmon have an additional GHRH that has not been identified, but like the mammalian form is encoded on a separate gene, which does not contain PACAP. This molecule should be structurally related to the mammalian GHRHs and also related to the GHRH-like peptide presented in this study. A comparison of the salmon GHRH-like peptide to the mammalian GHRHs shows the highest conservation in the amino terminus (1-29) of the molecules. Except for leucine at the carboxy terminal end, glycine in position 32 is the last conserved residue. This position also correlates to the exon/intron splice site in both the PACAP and GHRH genes. All of the mammalian GHRHs begin with a tyrosine or, in the case of rat and mouse, a histidine, which is required for receptor binding. The following 6 amino acids (positions 2-7) of the mammalian GHRHs are invariant except for a conservated substitution in position 2 of mouse GHRH. In the salmon and carp GHRH-like peptides,

both have identical substitutions at positions 4, 5, and 7, compared with the mammalian molecules. An unidentified salmon GHRH may be identical to the mammalian forms at these three positions. If so, this molecule would not have amplified by the PCR using primer CSC33. This primer was made to the first 7 amino acids of the carp GHRH.

Screening of the salmon genomic library revealed twenty-eight positive clones in the first round. Five of these clones were identified (chapter 5). The remaining 23 positives, 4 were classified as hybridizing strongly, while all other clones showed weak hybidization. These 4 clones were rescreened at a lower washing stringency. This evidence suggests these clones are not the same as the GHRH/PACAP clone. The probe contained only the region encoding the GHRH-like peptide, which suggests it hybridized to a GHRH related gene or another superfamily member.

Structure/function relationships in the evolution of the PACAP and VIP genes

The evolution of proteins from exons encoding domains of specific structure or function separated by introns has been suggested (Blake, 1979; Traut, 1988). While this hypothesis is not substantiated by all genes, there are some good examples. The exon/intron organization of the triose phosphate isomoerase gene in maize and chicken is conserved within the region coding for the structural domains of the protein (see Gilbert, 1985; Darnell *et al.*, 1990). The

signal peptides for secreted molecules are often encoded on a separate exon (Gilbert, 1985). A survey of several neuropeptide families including somatostatin, GHRH, GnRH, CRF and vaspressin/oxytocin shows conservation of specific domains within the precursors (Sherwood and Parker, 1990). The structure of the genes for some of the family members shows these domains are located on a single exon. The salmon GHRH/PACAP gene displays the same correlation in which a structural/functional element is encoded on a single exon. Conservation of exon length may be also linked to structural and/or functional significance of the encoded domain. In this regard exon 4 of the salmon GHRH/PACAP gene, which encodes the first 32 amino acids of the GHRH-like peptide, is the same length as the exon encoding the same region for both human GHRH and human PHM (Table 3). Furthermore, sequence identity of this exon is modestly closer between the domains of human PHM and salmon GHRH-like peptide (Table 6.1). In both the human GHRH and PHM/VIP genes, this exon codes for part or all of a functional peptide. PHM is encoded entirely within this exon, whereas only the first 32 amino acids of human GHRH are encoded on this exon. However, human 1-29 has full biological activity in stimulating GH release. On the other hand, exon 4 encoding the PRP region of the human PACAP gene is 6 nucleotides shorter than the related exons of the salmon GHRH/PACAP gene, and human PHM/VIP and GHRH genes (Table 6.1). The function of PRP is not known. The conservation

TABLE 6.1: A comparison of the exon encoding the salmon GHRH-like peptide or human GHRH domains with the respective exons of the human PHM and PRP genes. Percent identity of nucleotides (nt) or amino acids (aa). Length of the exon is in brackets.

		EXON DOMAIN			
exon length (nt)		sGHRH (105)	hGHRH (105)	hPRP (99)	hPHM (105)
sGHRH	nt	100%	56%	58*%	66%
	aa	100	51	54*	63
hGHRH	nt	--	100	58*	51
	aa	--	100	46*	43

*Gaps introduced for maximum alignment.

of the length and sequence of exon 4 in the salmon GHRH-like gene, compared with the human GHRH and PHM/VIP genes, may mean the salmon gene encodes for part or all of a functional molecule. An internal Lys-Arg site at positions 29-30 of the salmon precursor may be a cleavage site that produces a 28 residue peptide. Carp GHRH 1-29 was shown to have biological activity comparable to carp GHRH 1-45 (Luo *et al*,. 1990).

The function of the salmon GHRH/PACAP gene

The identification of the salmon GHRH/PACAP precursor with suitable processing sites for the production of a GHRH-like molecule presents a unique situation in the evolution of this family. GH release in salmon may be controlled by the two peptides, GHRH and PACAP, encoded in a single gene. This is in contrast to the mammalian situation where GH release is controlled by one and possibly both peptides encoded on separate genes.

The carp GHRH-like peptide is the only other non-mammalian GHRH that has been identified, although the cDNA and gene have not been studied. The sequence similarity of the salmon GHRH-like peptide to the carp peptide suggests the salmon molecule may also be a post-translational product. No other GHRH-like peptide has yet been identified in fish, even though a number of studies have reported the presence of an immunoreactive GHRH.

Physiological studies suggest that a GHRH-like molecule

stimulates GH release in teleost fish. Carp GHRH-like peptide was shown to stimulate the release of GH from rainbow trout and goldfish pituitaries *in vitro*, and goldfish *in vivo*. Human GHRH 1-44 or 1-29 administered in several ways *in vivo* did not stimulate growth in salmon (Sherwood and Donaldson, personal communication). However, human GHRH did stimulate GH release *in vivo* in goldfish (Peter *et al.*, 1984) and *in vitro* in rainbow trout, although in the latter it was not dose dependent. The problem with these bioassays is that they are heterologous, although the use of a teleost GHRH-like peptide in fish is an improvement. Our laboratory has begun *in vivo* and *in vitro* studies with salmon GHRH and PACAP, but these tests are still in progress. While a teleost GHRH-like peptide was used to show GH release in fish these bioassays are still heterologous. Somatostatin has been shown to inhibit GH release *in vitro* and *in vivo* in a number of teleost species, including salmon (Luo *et al.*, 1990), Tilapia (Fryer *et al.*, 1979: Helms *et al.*, 1987) and goldfish (Cook and Peter, 1984; Marchant *et al.*, 1987). However, the data to date do not provide enough information to make conclusive remarks regarding the control of GH release by the dual action of somatostatin and a GHRH-like molecule. Further testing using a homologous assay, as well as PACAP is required. GH release in teleosts may be under the control of several factors and may also be different between fish species. Both GnRH (Marchant *et al.*, 1987; Chang and Leeuw, 1990) and

NPY (Peng *et al.*, 1990) have been shown to stimulate the release of GH at certain concentrations from goldfish pituitary cells. Salmon are anadromous and GH is implicated in smoltification (Clarke *et al.*, 1977). Studies on the release of GH in fish will have to be interpreted with caution, since the stage in their life history could dramatically affect the results. In some respects these studies will parallel those of the mammalian system, where GH response is affected by sex, age and the timing in which the stimuli are delivered.

Future directions

Identification of the GHRH/PACAP gene in salmon presents the opportunity to study the physiological roles of the peptide products. Both salmon GHRH 1-45 and PACAP 1-38 have been synthesized for us by Dr. J. Rivier at the Salk Institute, La Jolla, California. We have begun *in vivo* and *in vitro* studies with these synthetic salmon peptides, but the experiments may prove to be difficult, as did the carp studies, due to the number of methionines in the molecule. Oxidation of the methionine residues results in almost complete loss of biological activity. In chapter 2, HPLC analysis of an immunoreactive salmon GHRH-like molecule showed multiple peaks. These peaks may have represented different oxidative forms of the molecule.

Sequence analysis of the 5' flanking region will provide information on consensus promoter and enhancer sites.

Factors regulating gene transcription can be identified and tested. Isolation of the PACAP cDNA and gene will also allow the construction of transgenic fish. Studies on transgenic fish may answer questions related to tissue specific expression, precursor processing and to the effects of over expression of GHRH and PACAP outside the central nervous system. Increased growth in transgenic fish carrying the salmon GHRH/PACAP construct would support the idea that these peptides are involved in physiological growth. Transgenic mice carrying the metallothionein-hGHRH fusion gene were found to be fertile, unlike transgenic mice expressing the GH gene which were infertile (Hammer *et al.*, 1985). Transgenic Altlantic salmon carrying a salmon GH gene have an increased growth rate although their reproductive status is not yet known (Du *et al.*, 1992). However, two transgenic carp species carrying the human GH gene were fertile and produced offspring carrying the transgene (Zhu *et al.*, 1992).

LITERATURE CITED

Acher, R. 1981. Evolution of neuropeptides. Trends in Neuroscience 4(9):225-229.

Ackland, J.F., Wu, P., Bruhn, T.O. and Jackson, I.M. 1989. Partial purification and characterization of a novel growth hormone-releasing factor (GRF) from teleost brain related to the rat hypothalamic peptide. Peptides 10:15-19

Adrian, T.G., Allen, J.M. and Polak, J.M. 1983. Neuropeptide Y distribution in human brain. Nature 306:584-586.

Allen, Y.S., Adrian, T.E., Allen, J.M., Tatemoto, K., Crow, T.J., Bloom, S.R. and Polak, J.M. 1983. Neuropeptide Y distribution in the rat brain. Science 22:877-879.

Allendorf, F.W. and Thorgaard, G.H. 1984. Tetraploidy and the evolution of salmonid fishes. In: Evolutionary Genetics of Fishes (J. Turner, ed.), Plenum Press, New York, pp. 1-53.

Amara, S.G., Jonas, V., Rosenfeld, M.G., Ong, E.S. and Evans, R.M. 1982. Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. Nature 298:240-244.

Andrews, P.C., Hawkes, D., Shively, J.E. and Dixon, J.E. 1985. A nonamidated peptide homologous to porcine peptide YY and neuropeptide Y. Endocrinology 116:2677-2681.

Arimura, A., Somogyvari-Vigh, A., Miyata, A., Mizuno, K., Coy, D.H. and Kitada, C. 1991. Tissue distribution of PACAP as determined by RIA: Highly abundant in the rat brain and testes. Endocrinology 129:2787-2789.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. Current Protocols in Molecular Biology. Greene Publishing Associates and Wiley-Interscience, New York.

Bagnato, A., Moretti, C., Ohnishi, J., Frajese, G. and Catt, K. 1992. Expression of the growth hormone-releasing hormone gene and its peptide product in the rat ovary. Endocrinology 130:1097-1102.

Ball, J.N. 1981. Review. Hypothalamic control of the pars distalis in fishes, amphibians, and reptiles. General and Comparative Endocrinology 44:135-170.

Balasubramaniam, A., Andrews, P.C., Renugopalakrishnan, V. and Rigel, D.F. 1989. Glycine-extended anglerfish

peptide YG (aPY) a neuropeptide Y (NPY) homologue may be a precursor of a biologically active peptide. Peptides 10:581-585.

Barinaga, M., Yamonoto, G., Rivier, C., Vale, W., Evans, R. and Rosenfeld, M.G. 1983. Transcriptional regulation of growth hormone gene expression by growth hormone-releasing factor. Nature 306:84- .

Baxter, J.D. and Funder, J.W. 1979. Hormone receptors. The England Journal of Medicine 301(21):1149-1160.

Bell, G.I. 1986. The glucagon superfamily: precursor structure and gene organization. Peptides 7(1):27-36.

Berelowitz, M., Szabo, M., Frohman, L.A., Firestone, S. and Chu, L. 1981. Somatomedin-C mediates growth hormone negative feedback by effects on both the hypothalamus and the pituitary. Science 212:1279-1281.

Berry, S.A. and Pescovitz, O.H. 1988. Identification of a GHRH-like substance and its messenger RNA in rat testis. Endocrinology 123:661-663.

Berry, S.A. and Pescovitz, O.H. 1990. Ontogeny and pituitary regulation of testicular growth hormone-releasing hormone-like messenger ribonucleic acid. Endocrinology 127:1404-1411.

Bilezikjian, L.M. and Vale, W.W. 1983. Stimulation of adenosine 3',5'-monophosphate production by growth hormone-releasing factor and its inhibition by somatostatin in anterior pituitary cells *in vitro*. Endocrinology 113:1726-1731.

Birnboim, M. 1983. A rapid alkaline extraction method for the isolation of plasmid DNA. Methods in Enzymology 100:243-255.

Blake, C.C.F. 1978. Do genes-in-pieces imply proteins-in-pieces? Nature 273:267.

Blomqvist, A., Söderberg, C., Lundell, I., Milner, R.J. and Larhammar, D. 1992. Strong evolutionary conservation of neuropeptide Y: Sequences of chicken, goldfish, and *Torpedo marmacrata* DNA clones. Proceedings of the National Academy of Sciences, U.S.A. 89, in press.

Bluet-Pajot, M-T., Durand, D., Drouva, S.V., Mounier, F., Pressac, M. and Kordon, C. 1986. Further evidence that thyrotropin-releasing hormone participates in the regulation of growth hormone secretion in the rat. Neuroendocrinolgy 44:70-75.

Bodner, M., Fridkin, M. and Gozes, I. 1985. Coding sequences for vasoactive intestinal peptide and PHM-27 peptide are located on two adjacent exons in the human genome. Proceedings of the National Academy of Sciences, U.S.A. 82:3548-3551.

Bohlen, P., Brazeau, P., Ling, N., Gaillard, R. and Guillemin, R. 1983a. Human hypothalamic growth hormone releasing factor (GRF): Evidence for two forms identical to tumor derived GRF-44-NH_2 and GRF-40. Biochemical and Biophysical Research Communications 114(3):930-936.

Bohlen, P., Esch, F., Brazeau, P., Ling, N. and Guillemin, R. 1983b. Isolation and characterization of the porcine hypothalamic growth hormone releasing factor. Biochemical and Biophysical Research Communications 116(2):726-734.

Bohlen, P., Wehrenberg, W.B., Esch, F., Ling, N., Brazeau, P.and Guillemin, R. 1984. Rat hypothalamic growth hormone-releasing factor: isolation, sequence analysis and total synthesis. Biochemical Biophysical Research Communications 125(3):1005-1012.

Boler, J., Enzmann, F. and Folkers, K. 1969. The identity of chemical and hormonal properties of the thyrotropin releasing hormone and pyroglutamyl-histidyl-proline amide. Biochemical and Biophysical Research Communications 37(4):705-710.

Breathnach, R. and Chambon, P. 1981. Organization and expression of eucaryotic split genes coding for proteins. Annual Review of Biochemistry 50:349-383.

Breitbart, R.E., Andreadis, A. and Bernardo, N.-G. 1987. Alternative splicing: A ubiquitous mechanism for the generation of multiple protein isoforms from single genes. Annual Review of Biochemistry 56:467-495.

Brent, G.A., Harney, J.W., Moore, D.D. and Larsen, P.R. 1988. Multihormonal regulation of the human, rat, and bovine growth promoters: Differential effects of 3',5'-cyclic adenosine monophosphate, thyroid hormone, and glucocorticoids. Molecular Endocrinology 2:792-798.

Brown, M. and Vale, W. 1975. Growth hormone release in the rat: Effects of somatostatin and thyrotropin-releasing factor. Endocrinology 97:1151-1156.

Burgus, R., Dunn, T.F., Desiderio, D., Ward, D.N., Vale, W. and Guillemin, R. 1970. Characterization of ovine hypothalamic hypophysiotropic TSH-releasing factor. Nature (London) 226:321-325.

Chabot, J.G., Enjalbert,A., Pelletier, G., Dubois, P.M. and Morel, G. 1988. Evidence for a direct action of neuropeptide Y in the rat pituitary gland. Neuroendocrinology 47:511-517

Campbell, R.M., Lee, Y., Rivier, J., Heimer, E.P., Felix, A.M. and Mowles, T.F. 1991. GRF analogs and fragments: Correlation between receptor binding, activity and structure. Peptides 12:569-574.

Canny, B.J., Rawlings, S.R. and Leong, D.A. 1992. Pituitary adenylate cyclase-activating polypeptide specifically increases cytosolic calcium ion concentration in rat gonadotropes and somatotropes. Endocrinology 130:211-215.

Carroll, R.L. 1988. Vertebrate paleontology and evolution. W.H. Freeman and Company, U.S. pp. 698.

Chan, S.J., Cao, Q.-P. and Steiner, D.F. 1990. Evolution of the insulin superfamily: Cloning of a hybrid insulin/insulin-like growht factor cDNA from amphio[illegible] Proceedings of the National Academy of Sciences, U.S.A. 87:9319-9323.

Chang, J.P. and de Leeuw, R. 1990. *In vitro* goldfish growth hormone responses to gonadotropin-releasing hormone: Possible roles of extracellular calcium and arachidonic acid metabolism. General and Comparative Endocrinology [illegible]0:155-164.

Chartrel, N., Conlon, J.M., Danger, J-M., Fournier, A., Tonon, M-C., and Vaudry, H. 1991a. Characterization of melanotropin-release-inhibiting factor (melanostatin) from frog brain: Homology with human neuropeptide Y. Proceedings of the National Academy of Sciences, U.S.A. 88:3862-3866.

Chartrel, N., Tonon, M-C., Vaudry, H. and Conlon, J.M. 1991b. Primary structure of frog pituitary adenylate cyclase-activating polypeptide (PACAP) and effects of ovine PACAP on frog pituitary. Endocrinology 129:3367-3371.

Chen, J.S. and Nordstrom, J.L. 1992. Bipartite structure of the downstream element of the mouse beta globin (major) poly(A) signal. Nucleic Acids Research 20:2565-2572.

Chihara, K., Kato, Y., Ohgo, S., Iwasaki, Y., Abe, H., Maeda, K. and Imura, H. 1976. Stimulating and inhibiting effects of thyrotropin-releasing hormone on growth hormone release in rats. Endocrinology 98:1047-1053.

Chomczynski, P., Downs, T.R. and Frohman, L.A. 1988. Feedback regulation of growth hormone (GH)-releasing hormone gene expression by GH in rat hypothalamus. Molecular Endocrinology 2:236-241.

Chomczynski, P. and Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry 162:156-159.

Christophe, J., Svoboda, M., Lambert, M., Waelbroeck, M., Winand, J., DeHaye, J.-P., Vandermeers-Piret, M.-C., Vandermeers, A. and Robberecht, P. 1986. Effector mechanisms of peptides of the VIP family. Peptides 7:101-107.

Church, G.M. and Gilbert, W. 1984. Genomic sequencing. Proceedings of the National Academy of Sciences, U.S.A. 81:1991-1995.

Ciofi, P., Croix, D. and Tramu, G. 1987. Coexistence of hGHRF and NPY immunoreactivities in neurons of the arcuate nucleus of the rat. Neuroendocrinology 45:425-428.

Clarke, W.C., Farmer, S.W. and Hartwell, K.M. 1977. Effect of teleost pituitary growth hormone on growth of *Tilapia mossambica* and on growth and seawater adaptation of sockeye salmon (*Oncorhynchus nerka*). General and Comparative Endocrinology 33:174-178.

Coe, I.R. 1992. Molecular evolution of neuropeptides isolated from the brain of the Pacific salmon (*Oncorhynchus spp.*). Ph.D. Thesis, University of Victoria, Victoria, B.C. pp. 231.

Conlon, J.M., Balasubramaniam, A. and Hazon, N. 1991a. Structural characterization and biological activity of a neuropeptide Y-peptide from the dogfish, *Scyliorhinus canicula*. Endocrinology 128:2273-2279.

Conlon, J.M., Bjenning, C., Moon, T.W. Youson, J.H. and Thim, L. 1991b. Neuropeptide Y-related peptides from the pancreas of a teleostean (eel), holostean (bowfin) and elasmobranch (skate) fish. Peptides 12:221-226.

Conlon, J.M., Schmidt, W.E., Gallwitz, B., Falkmer, S., and Thim, L. 1986. Characterization of an amidated form of pancreatic polypeptide from the daddy sculpin (*Cottus scorpius*). Regulatory Peptides 16:261-268.

Cook, A.F. and Peter, R.E. 1984. The effects of somatostatin on serum growth hormone levels in the goldfish,

Carassius auratus. General and Comparative Endocrinology 54:109-113.

Coiro, V., Braverman, L.E., Christianson, D., Fang, S-L. and Goodman, H.M. 1979. Effect of hypothyroidism and thyroxine replacement on growth hormone in the rat. Endocrinology 105:641-646.

Cordell, B., Bell, G., Tischer, E., DeNoto, F.M., Ullrich, A., Pictet, R., Rutter, W.J. and Goodman, H.M. 1979. Isolation and characterization of a cloned rat insulin gene. Cell 18:533-543.

Coy, D.H., Murphy, W.A. and Lance, V.A. 1987. Observations on the chemistry and biology of growth hormone releasing factor. In: Growth Hormone, Growth Factors, and Acromegaly (K. Lüdecke and G. Tolis, eds.) Raven Press, New York, pp. 13-20.

Craik, C.S., Rutter, W.J and Fletterick, R. 1983. Splice junctions: Association with variation in protein structure. Science 220:1125-1129.

Cronin, M.J., Hewlett, E.L., Evans, W.S., Thorner, M.O. and Rogol, A.D. 1984. Human pancreatic tumor growth hormone (GH)-releasing factor and cyclic adenosine 3',5'-monophosphate evoke GH release from anterior pituitary cells: The effects of pertussis toxin, cholera toxin, forskolin, and cycloheximide. Endocrinology 114:904-913.

Culler, M.D.and Paschall, C.S. 1991. Pituitary adenylate cyclase-activating polypeptide (PACAP) potentiates the gonadotropin-releasing activity of luteinizing hormone-releasing hormone. Endocrinology 129:2260-2262.

Darnell, J., Lodish, H. and Baltimore, D. 1990. Molecular Cell Biology. Second edition, Scientific American Books, Inc. pp. 1105.

Devesa, J., Lima, L. and Tresguerres, J.A.F. 1992. Neuroendocrine control of growth hormone secretion in humans. Trends in Endocrinology and Metabolism 3(5):175-183.

deLauzon, S., Cittanova, N., Desfosses, B. and Jayle, M.F. 1973. A new approach for quantitative evaluation of cross-reactivity of steriods with an antiserum by radioimmunoassay: Application to highly specific antiestriol. Steriods 22:747-761.

Du, J.S., Gong, Z., Fletcher, G.L., Shears, M.A., King, M.J., Idler, D.R. and Hew, C.L. 1992. Growth enhancement in transgenic Atlantic salmon by the use of

an "all fish" chimeric growth hormone gene construct. Bio/Technology 10:176-181.

Dumont, Y., Martel, J-C., Fournier, A., St-Pierre, S. and Quirion, R. 1992. Neuropeptide Y and Neuropeptide Y receptor subtypes in brain and peripheral tissues. Progress in Neurobiology 38:125-167.

Eipper, B.A., Mains, R.E. and Herbert, E. 1986. Peptides in the nervous system. Trends in Neurosciences 9:463-468.

Eng, J., Andrews, P.C., Kleinman, W.A., Singh, L. and Raufman, J.-P. 1990. Purification and structure of exendin-3, a new pancreatic secretagogue isolated from *Heloderma horridum* venom. The Journal of Biological Chemistry 265(33):20259-20262.

Eng, J., Shiina, Y., Pan, Y.-C.E., Blacher, R., Chang, M., Stein, S. and Yalow, R.S. 1983. Pig brain contains cholecystokinin octapeptide and several cholecystokinin desoctapeptides. Proceedings of the National Academy of Sciences, U.S.A. 80:6381-6385.

Epelbaum, J., Agid, F., Agid, Y., Beaudet, A., Bertrand, P., Enjalbert, A., Heidet, V., Kordon, C., Krantic, S., Leonard, J.F., Musset, F., Moyse, E., Peillon, F., Slama, A. and Videau, C. 1989. Somatostatin receptors in brain and pituitary. Hormone Research 31:45-50.

Esch, F., Bohlen, P., Ling, N., Brazeau, P., Wehrenberg, W., Thorner, M., Cronin, M. and Guillemin, R. 1982. Characterization of a 40 residue peptide from a human pancreatic tumor with growth hormone releasing activity. Biochemical and Biophysical Research Communications 109(1):152-158.

Farhadi-Jou, F., Beaudet, A. and Tannenbaum, G.S. 1992. Ultradian variation of somatostatin binding in rat arcuate nucleus. The Endocrine Society, 74th Annual Meeting, Abstract #1374, pg 395.

Frawley, L.S. and Hoeffler, J.P. 1988. Hypothalamic peptides affect the ratios of GH and PRL cells: Role of cell division. Peptides 9:825-828.

Frohman, M.A., Downs, T.R., Chomczynski, P. and Frohman, L.A. 1989. Cloning and characterization of mouse of growth hormone-releasing hormone (GRH) complementary DNA: increased GRH messenger RNA levels in the growth hormone-deficient lit/lit mouse. Molecular Endocrinology 3:1529-1536.

Frohman, M.A., Dush, M.K. and Martin, G.R. 1988. Rapid production of full-length cDNAs from rare transcripts:

amplification using a single gene-specific olignucleotide primer. Proceedings of the National Academy of Sciences, U.S.A. 85:8998-9002.

Frohman, L.A. and Jansson, J-O. 1986. Growth hormone-releasing hormone. Endocrine Reviews 7(3):223-253.

Fryer, J.N., Nishioka, R.S. and Bern, H.A. 1979. Somatostatin inhibition of teleost growth hormone secretion. General and Comparative Endocrinology 39:244-246.

Gainer, H., Sarne, Y,, and Brownstein, M.J. 1977. Neurophysin Biosynthesis: Conversion of a putative precursor during axonal transport. Science 195:1354.

Gertz, B.J., Gardner, D.G. and Baxter, J.D. 1987. Glucocorticoid control of rat growth hormone gene expression: Effect on cytoplasmic messenger ribonucleic acid production and degradation. Molecular Endocrinology 1:933-941.

Giladi, E., Shani, Y., and Gozes, I. 1990. The complete structure of the rat VIP gene. Molecular Brain Research 7:261-267.

Gilbert, W. 1985. Genes-in-Pieces-Revisted. Science 228:823-824.

Giusti, M., Lomeo, A., Mazzocchi, G., Torre, R., Monachesi, M., Sessarego, P., Attanasio, R. and Giordano, G. 1986. Effect of simultaneous administration of GHRH (1-40) and TRH on GH, PRL, and TSH secretion in normal man. Journal of Endocrinological Investigations 9:497-501.

Goodall, G.J. and Filipowicz, W. 1989. The AU-rich sequences present in the introns of plant nuclear pre-mRNAs are required for splicing. Cell 58:473-483.

Gonzàlez-Crespo, S. and Boronat, A. 1991. Expression of the rat growth hormone-releasing hormone gene in placenta is directed by an alternative promoter. Proceedings of the National Academy of Sciences, U.S.A. 88:8749-8753.

Goth, M.I., Lyons, C.E., Canny, B.J. and Thorner, M.O. 1992. Pituitary adenylate cyclase activating polypeptide, growth hormone (GH)-releasing peptide and GH-releasing hormone stimulate GH release through distinct pituitary receptors. Endocrinology 130:939-944.

Gottschall, P.E., Tatsuno, I., Miyata, A. and Arimura. A. 1990. Characterization and distribution of binding sites for the hypothalamic peptide, pituitary adenylate cyclase-activating polypeptide. Endocrinology 127:272-

277.

Gracia-Navarro, F., Lamacz, M., Tonon, M-C. and Vaudry, H. 1992. Pituitary adenylate cyclase-activating polypeptide stimulates calcium mobilization in amphibian pituitary cells. Endocrinology 131:1069-1074.

Green, J.D. and Harris, G.W. 1947. The neurovascular link between the neurohypophysis and adenohypophysis. Journal of Endocrinology 5:

Green, J.D. and Harris, G.W. 1949. Observation of the hypophysio-portal vessels of the living rat. Journal of Physiology 108:359-361.

Green, M.R. 1986. Pre-mRNA splicing. In: Annual Review of Genetics (A. Campbell, I. Herskoxitz and L.M. Sandler, eds.). Annual Reviews Inc., California, USA, Vol. 20, pp.671-708.

Greenwood, F.C. Hunter, W.M. and Glover, J.S. 1963. The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Journal of Biochemistry 89:114-123.

Gubler, U., Monahan, J.J., Lomedico, P.T., Bhatt, R.S., Collier, K.J., Hoffman. B.J., Bohlen, P., Esch, F., Ling, N., Zeytin, F., Brazeau, P., Poonian, M.S. and Gage, L.P. 1983. Cloning and sequence analysis of cDNA for the precursor of human growth hormone-releasing factor, somatocrinin. Proceedings of the National Academy of Sciences, U.S.A. 80:4311-4314.

Gubler, U., Chua, A.O., Hoffman, B.J., Collier, K.J. and Eng, J. 1984. Cloned cDNA to cholecystokinin mRNA predicts an identical preprocholecystokinin in pig brain and gut. Proceedings of the National Academy of Science, U.S.A. 81:4307-4310.

Guillemin, R. 1986. "The Sherrington Lectures. XVIII. Hypothalamic Control of Pituitary Functions: The Growth Hormone Releasing Factor." Liverpool University Press, Liverpool, pp. 1-73.

Guillemin, R., Brazeau, P., Bohlen, P., Esch, F., Ling, N. and Wehrenberg, W. 1982. Growth hormone releasing factor from a human pancreatic tumor that caused acromegaly. Science 218:585-587.

Hammer, R.E., Brinster, R.L., Rosenfeld, M.G., Evans, R.M. and Mayo, K.E. 1985. Expression of human growth hormone-releasing factor in transgenic mice results in increased somatic growth. Nature 315:413-416.

Harris, G.W. 1948. Neural control of the pituitary gland. Physiological Reviews 28:139-179.

Harvey, S., Foltzer-Jourdainne, C., Karmann, H. and Mialhe, P. 1988. Thyrotropin-releasing hormone-induced growth hormone secretion in ducks: independence of peripheral plasma somatostatin, insulin, and glucagon. General and Comparative Endocrinology 70:374-381.

Harvey, S. and Hall, T.R. 1987. Inhibition of growth hormone secretion in anaesthetized fowl: hypothalamic participation. General and Comparative Endocrinology 65:264-268.

Harvey, S. and Scanes, C.G. 1984. Comparative stimulation of growth hormone secretion in anaesthetized chickens by human pancreatic growth hormone-releasing factor (hpGRF) and thyrotropin-releasing hormone (TRH). Neuroendocrinology 39:314-320.

Harvey, S., Scanes, C.G., Chadwick, A. and Bolton, N.J. 1978. The effect of thyrotropin-releasing hormone (TRH) and somatostatin (GHRIH) on growth hormone and prolactin secretion *in vitro* and *in vivo* in the domestic fowl (*Gallus domesticus*). Neuroendocrinology 26:249-260.

Hawkins, J.D. 1988. A survey on intron and exon lengths. Nucleic Acids Research 16:9893-9908.

Heierhorst. J., Mahlmann, S., Morley, S.D., Coe, I.R., Sherwood, N.M. and Richter, D. 1990. Molecular cloning of two distinct vasotocin precursor cDNAs from chum salmon (Oncorhynchus keta) suggests an ancient gene duplication. Federation of European Biochemical Societies Letters 260:310-304.

Heilig, R., Perrin, F., Gannon, F., Mandel, J.L. and Chambon, P. 1980. The ovalbumin gene family: Structure of the X gene and evolution of duplicated split genes. Cell 20:625-637.

Heinrich, G., Gros, P. and Habener, J.F. 1984. Glucagon gene sequence. Journal of Biological Chemistry 259:14082-14087.

Helms, L.M.H., Grau, G., Shimoda, S.K., Nishioka, R.S. and Bern, H.A 1987. Studies on the regulation of growth hormone release from the proximal pars distalis of male Tilapia, *Oreochromis mossambicus, in vitro*. General and Comparative Endocrinology 65:48-55.

Holl, R.W., Thorner, M.O. and Leong, D.A. 1988. Intracellular calcium concentration and growth hormone

secretion in individual somatotropes: Effects of growth hormone-releasing factor and somatostatin. Endocrinology 122:2927-2932.

Hoshino, M., Yanaihara, C., Hong, Y.-M., Kishida, S., Katsumaru, Y., Vandermeers, A., Vandermeers-Piret, M.-C., Robberecht, P., Christophe, J. and Yanaihara, N. 1984. Primary structure of helodermin, a VIP-secretin-like peptide isolated from Gila monster venom. Federation of European Biochemical Societies 178(2):233-239.

Hosoya, M., Kimura, C., Ogi, K., Ohkubo, S., Miyamoto, Y., Kugoh, H. Shimizu, M., Onda, H., Oshimura, M., Arimura, A.and Fujino, M. 1992. Structure of the human pituitary adenylate cyclase activating polypeptide (PACAP) gene. Biochimica et Biophysica Acta 1129:199-206.

Hunt, T. 1985. False starts in translational control gene expression. Nature 316:580-581.

Inagaki, N., Seino, Y., Takeda, J., Yano, H., Yamada, Y., Bell, G.I., Eddy, R.L., Fukushima, Y., Byers, M.G., Shows, T.B. and Imura, H. 1989. Gastric inhibitory polypeptide: Structure and chromosomal localization of the human gene. Molecular Endocrinology 3:1014-1021.

Johansen, B., Johnsen, O.C. and Valla, S. 1989. The complete nucleotide sequence of the growth-hormone gene from Atlantic salmon (*Salmo salar*). Gene 77:317-324.

Joosse, J. 1986. Neuropeptides: Peripheral and central messengers of the brain. In: Progress in Clinical and Biological Research, Comparative Endocrinology Developments and directions (C.L. Ralph, ed.), Alan R. Liss, Inc., New York, N.Y., Vol. 205 pp. 13-32.

Kaiser, E.T. and Kèzdy, F.J. 1984. Amphiphilic secondary structure: Design of peptide hormones. Science 223:249-255.

Karin, M., Theill, L., Castrillo, J.-L., McCormick, A. and Brady, H. 1990. Tissue-specific expression of the growth hormone gene and its control by growth hormone factor-1. Recent Progress in Hormone Research 46:43-59.

Kato, Y., Chihara, K., Maeda, K., Ohgo, S., Okanishi, Y. and Imura, H. 1975. Plasma growth hormone responses to thyrotropin-releasing hormone in the urethane-anesthetized rat. Endocrinology 96:1114-1118.

Kawauchi, H., Yasuda, A. and Rand-Weaver, M. 1990. Evolution of prolactin and growth hormone family. Progress in

Comparative Endocrinology, pp. 47-53.

Kelly, R.B. 1985. Pathways of protein secretion in eukaryotes. Science 230:25-32.

Kimmel, J.R., Plisetskaya, E.M., Pollock, H.G., Hamilton, J.W., Rouse, J.B., Ebner, K.E. and Rawitch,. A.B. 1986. Structure of a peptide from coho salmon endocrine pancreas with homology to neuropeptide Y. Biochemical Biophysical Research Communications 141:1084-1091.

Kimura, C., Ohkubo, S., Ogi, K., Hosoya, M., Itoh, Y., Onda, H., Miyata, A., Jiang, L., Dahl, R.R., Stibbs, H.H., Arimura, A., and Fujino, M. 1990. A novel peptide which stimulates adenylate cyclase: Molecular cloning and characterization of the ovine and human cDNAs. Biochemical Biophysical Research Communications 166:81-89.

Klungland, H., Lorens, J.B., Andersen, O., Kisen, G.O. and Alestrom, P. 1992. The Atlantic salmon prepro-gonadotropin releasing hormone gene and mRNA. Molecular and Cellular Endocrinology 84:167-174.

Kopin, A., Wheeler, M.B., Nishitani, J., McBride, E.W., Chang, T.-M., Chey, W.Y. and Leiter, A.B. 1991. The secretin gene: Evolutionary history, alternative splicing, and development regulation. Proceeding of the National Academy of Sciences, U.S.A. 88:5335-5339.

Köves, K., Arimura, A., Gorcs, T.G. and Somogyvari-Vigh, A. 1991. Comparative distribution of immunoreactive pituitary adenylate cyclase activating polypeptide and vasoactive intestinal polypeptide in rat forebrain. Neuroendocrinology 54:159-169.

Köves, K., Arimura, A., Somogyvari-Vigh, A., Vigh, S. and Miller, J. 1990. Immunohistochemical demonstration of a novel hypothalamic peptide, pituitary adenylate cyclase-activating polypeptide, in the ovine hypothalamus. Endocrinology 127:264-271.

Kozak, M. 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell 44:283-292.

Kozak, M. 1991. Structural features in eukaryotic mRNAs that modulate the initiation of translation. Journal of Biological Chemistry 266(30):19867-19870.

Kraicer, J., Lussier, B., Moor, B.C. and Cowan, J.S. 1988. Failure of growth hormone (GH) to feedback at the level of the pituitary to alter the response to the somatotrophs to GH-releasing factor. Endocrinology

122:1511-1514.

Kwon, Y.K. and Hecht, N.B. 1991. Cytoplasmic protein binding to highly conserved sequences in the 3' untranslated region of mouse protamine 2 mRNA, a translated region of mouse protamine 2 mRNA, a translationally regulated transcript of male germ cells. Proceedings of the National Academy of Sciences, U.S.A. 88:3584-3588.

Laburthe, M., Amiranoff, B., Boige, N., Rouyer-Fessard, C., Tatemoto, K. and Moroder, L. 1983. Interaction of GRF with VIP receptors and stimulation of adenylate cyclase in rat and human intestinal epithelial membranes. Federation of European Biochemical Societies Letters 159:89-92.

Lam, H-C., Takahashi, K., Ghatei, M.A., Kanse, S.M., Polak, J.M. and Bloom, S.R. 1990. Binding sites of a novel neuropeptide pituitary-adenylate-cyclase-activating polypeptide in the rat brain and lung. European Journal of Biochemistry 193:725-729.

Lamperti, E.D., Rosen, K.M. and Villa-Komaroff, L. 1991. Characterization of the gene and messages for vasoactive intestinal polypeptide (VIP) in rat and mouse. Molecular Brain Research 9:217-231.

Lance, V., Hamilton, J.W., Rouse, J.B., Kimmel,J.R., and Pollock, H.G. 1984. Isolation and characterization of reptilian insulin, glucagon, and pancreatic polypeptide: Complete amino acid sequence of alligator (*Alligator mississippiensis*) insulin and pancreatic polypeptide. General and Comparative Endocrinology 55:112-124.

Larhammar, D., Ericsson, A. and Persson, H. 1987. Structure and expression of the rat neuropeptide Y gene. Proceedings of the National Academy of Sciences, U.S.A. 84:2068-2072.

Larhammar, D., Söderberg, C. and Blomqvist, A.G. Evolution of the neuropeptide Y family of peptides. In C. Wahlestedt and W.F. Colmers (Eds.), Neuropeptide Y and related peptides. Contemporary Neurosciences Series. Humana Press, in press.

Law, G.J., Ray, K.P. and Wallis, M. 1985. Effects of growth hormone-releasing factor and somatostatin on growth hormone secretion and cellular cyclic AMP levels. Federation of European Biological Sciences Letters 179(1):12-16.

Lewin, M.J.M., Reyl-Desmars, F. and Ling, N. 1983. Somatocrinin receptor coupled with cAMP-dependent

protein kinase on anterior pituitary granules. Proceedings of the National Academy of Science U.S.A. 80:6538-6541.

Li, W.-H., Luo, C.-C. and Wu, C.-I. 1985. Evolution of DNA sequences. In: MacIntyre, R.J. (ed.), Molecular Evolutionary Genetics, Plenum Press, New York, pp.1-94.

Lomedico, P., Rosenthal, N , Efstratiadis, A., Gilbert, W., Kolodner, R. and Tizard, R. 1979. The structure and evolution of the two nonallelic rat preproinsulin genes. Cell 18:545-558.

Lowe, W.L., Roberts, C.T., Lasky, S.R. and Roith, D. 1987. Differential expression of alternative 5' untranslated regions in mRNAs encoding rat insulin-like growth factor I. Proceedings of the National Academy of Sciences, U.S.A. 84:8946-8950.

Lumpkin, M.D., Negro-Vilar, A. and McCann, S.M. 1981. Paradoxical elevation of growth hormone by intraventricular somatostatin: Possible ultrashort-loop feedback. Science 211:1072-1074.

Lumpkin, M.D., Samson, W.K. and McCann, S.M. 1985. Effect of intraventricular growth hormone-releasing hormone on growth hormone release: Further evidence for ultrashort loop feedback. Endocrinology 116:2070-2074.

Luo, D., McKeown, B.A., Rivier, J. and Vale, W. 1990. *In vitro* responses of rainbow trout (*Oncorhynchus mykiss*) somatotrophs to carp growth hormone-releasing factor (GRF) and somatostatin. General and Comparative Endocrinology 80:288-298.

Luo, D. and McKeown, B.A. 1991. The effect of thyroid hormone and glucocorticoids on carp growth hormone-releasing factor (GRF)-induced growth hormone (GH) release in rainbow trout (*Oncorhynchus mykiss*). Comparative Biochemistry and Physiology 99A:621-626.

Lussier, B.T., French, M.B., Moor, B.C. and Kraicer, J. 1991a. Free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and growth hormone release from purified rat somatotrophs. I. GH-releasing factor-induced Ca^{2+} influx raises $[Ca^{2+}]_i$. Endocrinology 128:570-582.

Lussier, B.T., Wood, D.A., French, M.B., Moor, B.C. and Kraicer, J. 1991b. Free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and growth hormone release from purified rat somatotrophs. II. Somatostatin lower $[Ca^{2+}]_i$ by inhibiting Ca^{2+} influx. Endocrinology 128:583-591.

Lussier, B.T., French, M.B., Moor, B.C. and Kraicer, J. 1991c. Free intracellular Ca^{2+} concentration and growth hormone (GH) release from purified rat somatotrophs. III. Mechanism of action of GH-releasing factor and somatostatin. Endocrinology 128:592-603.

Mains, R.E., Eipper, B.A., Glembotski, C.C. and Dores, R.M. 1983. Strategies for the biosynthesis of bioactive peptides. Trends in Neuroscience 6(6):229-235.

Manley, J.L. 1988. Review. Polyadenylation of mRNA precursors. Biochimica et Biophysica Acta 950:1-12.

Marchant, T.A., Fraser, R.A., Andrews, P.C. and Peter, R.E. 1987. The influence of mammalian and teleost somatostatins on the secretion of growth hormone from goldfish (Carassius auratus L.) pituitary fragments *in vitro*. Regulatory Peptides 17:41.

Margioris, A.N., Brockmann, G., Bohler, H.C.L., Grino, Jr. M., Vamvakopoulos, N. and Chrousos, G.P. 1990. Expression and localization of growth hormone-releasing hormone messenger ribonucleic acid in rat placenta: *In vitro* secretion and regulation of its peptide product. Endocrinology 126:151-158.

Marivoet, S., Moons, L. and Vandesande, F. 1988. Localization of growth hormone releasing factor-like immunoreactivity in the hypothalamo-hypophyseal system of the frog (*Rana temporaria*) and the sea bass (*Dicentrarchus labrax*). General and Comparative Endocrinology 72:72-79.

Marti, E., Bello, A.R., Lancha, A., and Batista, M.A.P. 1990. Neuropeptide tyrosine (NPY) and its C-terminal flanking peptide (C-PON) in the developing and adult spinal cord of a reptile. Journal of Anatomy 172:149-156.

Marx, J.L. 1988. Evolution's link to development explored. Science 240:880-882.

Mayo, K.E., Cerelli, G.M., Lebo, R.V., Bruce, B.D., Rosenfeld, M.G. and Evans, R.M. 1985a. Gene encoding human growth hormone-releasing factor: structure, sequence, and chromosomal assignment. Proceedings of the National Academy Sciences, U.S.A. 82:63-67.

Mayo, K.E., Cerelli, G.M., Rosenfeld, M.G. and Evans, R.M. 1985b. Characterization of cDNA and genomic clones encoding the precursor to rat hypothalamic growth hormone-releasing factor. Nature 314:464-467.

Mayo, K.E., Vale, W., Rivier, J., Rosenfeld, M.G. and Evans,

R.M. 1983. Expression-cloning and sequence of a cDNA encoding human growth hormone-releasing factor. Nature 306:856-88.

McDonald, J.K. 1988. NPY and related substances. Critical Reviews in Neurobiology 14:97-135.

Minth, C.D., Bloom, S.R., Polak, J.M. and Dixon, J.E. 1984. Cloning, characterization, and DNA sequence of a human cDNA encoding neuropeptide tyrosine. Proceedings of the National Academy of Sciences, U.S.A. 81:4577-4581.

Minth, C.D. et al., 1986. Characterization, sequence, and expression of the cloned human neuropeptide Y gene. Journal of Biological Chemistry 261:11974-11979.

Miyata, A., Arimura, A., Dahl, R.R., Minamino, N., Uehara, A., Jiang, L., Culler, M.D. and Coy, D.H. 1989. Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. Biochemical Biophysical Research Communications 164(1):567-574.

Miyata, A., Jiang, L., Dahl, R.D., Kitada, C., Kubo, K., Fujino, M., Minamino, N. and Arimura, A. 1990. Isolation of a neuropeptide corresponding to the N-terminal 27 residues of the pituitary adenylate cyclase activating polypeptide with 38 residues (PACAP38). Biochemical Biophysical Research Communications 170(2):643-648.

Mizobuchi, M., Frohman, M.A., Downs, T.R. and Frohman, L.A. 1991. Tissue-specific transcription initiation and effects of growth hormone (GH) deficiency on the regulation of mouse and rat GH-releasing hormone gene in hypothalamus and placenta. Molecular Endocrinology 5:476-484.

McLauchlan, J., Gaffney, D., Whitton, J.L. and Clements, J.B. 1985. The consensus sequence YGTGTTYY located downstream from the AATAAA signal is required for efficient formation of mRNA 3' termini. Nucleic Acids Research 13:1347-1368.

Moons, L., Cambre, M., Ollevier, F. and Vandesande, F. 1989. Immunocytochemical demonstration of close relationships between neuropeptidergic nerve fibers and hormone-producing cell types in the adenohypophysis of the sea bass (*Dicentrarchus labrax*). General and Comparative Endocrinology 73:270-283.

Morley, S.D., Schönrock, C., Richter, D., Okawara, Y. and Lederis, K. 1991. Corticotropin-releasing factor (CRF) gene family in the brain of the teleost fish *Catostomus*

commersoni (white sucker): Molecular analysis predicts distinct precursors for two CRFs and one urotensin I peptide. Molecular Marine Biology and Biotechnology 1:48-57.

Moretti, C., Bagnato, A., Solan, N., Frajese, G. and Catt, K.J. 1990. Receptor-mediated actions of growth hormone releasing factor on granulosa cell differentiation. Endocrinology 127:2117-2126.

Mount, S.M., Burks, C., Hertz, G., Stormo, G.D., White, O. and Fields, C. 1992. Splicing signals in *Drosophila*: intron size, information content, and consensus sequences. Nucleic Acid Research 20:4255-4262.

Nair, R.M.G., Barrett, J.F., Bowers, C.Y. and Schally, A.V. 1970. Structure of porcine thyrotropin releasing hormone. Biochemistry 9:1103-1106.

Nebert, D.W. and Gonzalez, F.J. 1987. P450 gene: Structure, evolution, and regulation. Annual Review of Biochemistry 56:945-993.

Niall, H.D. 1982. The evolution of peptide hormones. Annual Review of Physiology 44:615-624.

Nishioka, Y., Leder, A. and Leder, P. 1980. Unusual α-globin-like gene that has cleanly lost both globin intervening sequences. Proceedings of the National Academy of Sciences U.S.A. 77:2806-2809.

Nishizawa, T., Kitahara, N., Nanami, H., Hara, N., Kotake, C., Okazaki, H., Andoh, T. and Soma, G-I. 1984. Heterogeneity of 3' nontranslated regions in proopiomelanocortin (POMC) precursor mRNA of chum salmon Onchorynchus keta: polymorphism of the gene. Biochemical and Biophysical Research Communications 122(2):556-562

Norris, D.O. 1980. Vertebrate Endocrinology, Lea and Febiger, Bailliere Tindall, London, pp. 524.

Ogi, K., Kimura, C., Onda, H., Arimura, A. and Fujino, M. 1990. Molecular cloning and characterization of cDNA for the precursor of rat pituitary adenylate cyclase activating polypeptide (PACAP). Biochemical Biophysical Research Communications 173:1271.

O'Hare, M.M.T., Tenmoku, S., Aakerlund, L., Hilsted, L., Johnsen, A. and Schwartz, T.W. 1988. Neuropeptide Y in guinea pig, rabbit, rat and man. Identical amino acid sequence and oxidation of methionine-17. Regulatory Peptides 293-304.

Ohkubo, S., Kimura, C., Ogi, K., Okazaki, K., Hosoya, M., Onda, H., Miyata, A., Arimura, A. and Fujino, M. 1992. Primary structure and characterization of the precursor to human pituitary adenylate cyclase activating polypeptide. DNA and Cell Biology 11:21-30.

Ohno, S., Wolf, U. and Atkin, N.B. 1968. Evolution from fish to mammals by gene duplication. Herditas 59:169-187.

Okazaki, K., Kimura, C., Kosaka, T., Watanabe, T., Ohkubo, S., Ogi, K., Kitada, C., Onda, H. and Fujino, M. 1992. Expression of human pituitary adenylate cyclase activating polypeptide (PACAP) cDNA in CHO cells and characterization of the products. Federation of European Biochemical Sciences Letters 298(1):49-56.

Olivereau, M., Olivereau, J. and Vandesande, F. 1990. Localization of growth hormone-releasing factor-like immunoreactivity in the hypothalamo-hypophysial system of some teleost species. Cell Tissue Research 259:73-80.

Ono, M., Wada, C., Oikawa, I., Kawazoe, I. and Kawauchi, H. 1988. Structures of two kinds of mRNA encoding the chum salmon melanin-concentrating hormone. Gene 71:433-438.

Pan, J.X., Lechan, R.M., Lin, H.D., Sohn, J., Reichlin, S. and Jackson, I.M. 1985. Multiple forms of human pancreatic growth hormone releasing factor-like immunoreactivity in teleost brain and pituitary. Endocrinology 116(4):1663-1665.

Pandol, S.J., Seifert, H., Thomas, M.W., Rivier, J. and Vale, W. 1984. Growth hormone-releasing factor stimulates pancreatic enzyme secretion. Science 225:326-328.

Parker, D.B. and Sherwood, N.M. 1990. Evidence of a growth hormone-releasing hormone-like molecule in salmon brain, *Oncorhynchus keta* and *O. kisutch*. General and Comparative Endocrinology 79:95-102

Parker, D.S., Raufman, J.-P., O'Donohue, T.L., Bledsoe, M., Yoshida, H. and Pisano, J.J. 1984. Amino acid sequences of helospectins, new members of the glucagon superfamily, found in Gila monster venom. The Journal of Biological Chemistry 259(19):11751-11755.

Patel, Y.C. and Sirkant, C.B. 1986. Somatostatin mediation of adenohypohysial secretion. Annual Review of Physiology 48:551-567.

Peng, C., Huang, Y.-P. and Peter, R.E. 1990. Neuropeptide Y stimulates growth hormone and gonadotropin release from the goldfish pituitary *in vitro*. Neuroendocrinology 52:28-34.

Perez, F.M., Malamed, S. and Scanes, C.G. 1987. Growth hormone secretion from chicken adenohypophyseal cells in primary culture: effects of human pancreatic growth hormone-releasing factor, thyrotropin-releasing hormone, and somatostatin on growth hormone release. General and Comparative Endocrinology 65:408-414.

Pescovitz, O.H., Berry, S.A., Laudon, M., Ben-Jonathan, N., Martin-Myers, A., Hsu, S.-M., Lambros, T.J. and Felix, A.M. 1990. Localization and growth hormone (GH)-releasing activity of rat testicular GH-releasing hormone-like peptide. Endocrinology 127:2336-2342.

Peter, R.E., Nahorniak, C.S., Vale, W.W. and Rivier, J.E. 1984. Human pancreatic growth hormone-releasing factor (hpGRF) stimulates growth hormone release in goldfish. Journal of Experimental Zoology 231:161-163.

Pollock, H.G., Kimmel, J.R., Hamilton, J.W., Rouse, J.B. Ebner, K.E., Lance, V. and Rawitch, A.B. 1987. Isolation and structures of alligator gar (*Lepisosteus spatula*) insulin and pancreatic polypeptide. General and Comparative Endocrinology 67:375-382.

Popa, G. and Fielding, U. 1930. A portal circulation from the pituitary to the hypothalamic region. Journal of Anatomy (London). 65:88-91

Rehfeld, J.F., Bardram, L., Blanke, S., Cantor, P., Friis-Hansen, L., Hilsted, L., Johnsen, H., Monstein, H.-J., Wouter Van Solinge, W., Odum, L. and Orskov, C. 1991. The expression of peptide hormones in normal cells and tumor cells. Acta Oncologica 30:429-433.

Reichlin, S. 1960. Growth and the hypothalamus. Endocrinology 67:760-773.

Reiner, A. and Oliver, J.R. 1987. Somatostatin and neuropeptide Y are almost exclusively found in the same neurons in the telencephalon of turtles. Brain Research 426:149-156.

Reyl-Desmars, F., Baird, A. and Zeytin, F.N. 1985a. GRF is a highly potent activator of adenylate cyclase in the normal human, bovine and rat pituitary: interaction with somatostatin. Biochemical and Biophysical Research Communications 127(3):977-985.

Reyl-Desmars, F. and Zeytin, F. 1985b. Somatostatin inhibits

growth hormone-releasing factor-stimulated adenylate cyclase activity in GH_3 cells. Biochemical and Biophysical Research Communications 127:986-991.

Rivier, J., Spiess, J., Thorner, M. and Vale, W. 1982. Characterization of a growth hormone-releasing factor from a human pancreatic islet tumor. Nature 300:276-278.

Robberecht, P., Waelbroeck, M., Coy, D., Neef, P.D., Camus, J.-C. and Christophe, J. 1986. Comparative structural requirements of thirty GRF analogs for interaction with GRF-and VIP receptors and coupling to adenylate cyclase in rat adenopituitary, liver and pancreas. Peptides 7:53-59.

Robberecht, P., Gourlet, P., Cauvin, A., Buscail, L., De Neef, P., Arimura, A. and Christophe, J. 1991. PACAP and VIP receptors in rat liver membranes. American Journal of Physiology 260:G97-G102.

Rogers, J., Early, P., Carter, C., Calame, K., Bond, M., Hood, L. and Wall, R. 1980. Two mRNAs with different 3' ends encode membrane-bound and secreted forms of immunoglobulin μ chain. Cell 20:303-312.

Sachs, H. and Takabutake, Y. 1964. Evidence for a precursor in vasopressin biosynthesis. Endocrinology 75:943-948.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487-491.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular cloning--A laboratory manual, 2nd ed., Cold Spring Harbour Laboratory Press.

Samuels, H.H., Forman, B.M., Horowitz, Z.D. and Ye, Z.-S. 1989. Regulation of gene expression by thyroid hormone. Annual Review of Physiology 51:623-639.

Sanger, F., Nicklen, S. and Coulson, A.R. 1977. DNA sequencing with chain terminating inhibitors. Proceeding of the National Academy Sciences, U.S.A. 74:5463-5467.

Sato, M. and Frohman, L.A. 1992. Differential effects of central and peripheral administration of GH and IGF-I on hypothalamic GH-releasing hormone (GRH) and somatostatin (SRIH) gene expression in the GH-deficient dwarf (dw) rat. The Endocrine Society, 74th Annual Meeting, Abstract 1580.

Scanes, C.G. and Harvey, S. 1988. Growth hormone secretion induced by thyrotropin-releasing hormone in adult chickens: evidence of dose-dependent induction of either refractoriness or sensitization. Neuroendocrinology 47:369-373.

Schäfer, R., Schwarzhoff, R., Creutzfeldt, W. and Schmidt, W.E. 1991. Characterization of a guanosine-nucleotide-binding-protein-coupled receptor for pituitary adenylate-cyclase-activating polypeptide on plasma membranes from rat brain. European Journal Biochemistry 202:951-958.

Schally, A.V., Redding, T.W., Bowers, C.Y. and Barrett, J.F. 1969. Isolation and properties of porcine thyrotropin-releasing hormone. Journal of Biological Chemistry 244(15):4077-4088.

Scharrer, E. 1928. Die Lichtempfendlichkeit blinder Elritzen (Untersuchungen über das Zwischenhirn der Fische). Zeitschrist für vergleichende Physiologie 7:1-38.

Schettini, G., Cronin, M.J., Hewlett, E.L., Thorner, M.O. and MacLeod, R.M. 1984. Human pancreatic tumor growth hormone-releasing factor stimulates anterior pituitary adenylate cyclase activity, adenosine 3',5'-monophosphate accumulation, and growth hormone release in a calmodulin-dependent manner. Endocrinology 115(4):1308-1314.

Schettini, G., Judd, A.M. and MacLeod, R.M. 1983. Penfluridol decreases secretagogue-induced TSH, GH, and LH secretion *in vitro*: A possible role for calcium-calmodulin. Neuroendocrinology 37:229234.

Schwartz, T.W. 1986. Review-Hypothesis. The processing of peptide precursors. 'Proline-directed arginyl cleavage' and other monobasic processing mechanisms. Federation of European Biochemical Societies. 200:1-9.

Seifert, H., Perrin, M., Rivier, J. and Vale, W. 1985. Binding sites for growth hormone releasing factor on rat pituitary cells. Nature 313:487-489.

Shaw, G. and Kamen, R. 1986. A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell 46:659-667.

Sherwood, N.M., Lovejoy, D.A. and Coe, I.R. Origin of mammalian gonadotropin-releasing hormones. Endocrine Reviews (in press).

Sherwood, N.M. and Parker, D.B. 1990. Neuropeptide families:

An evolutionary perspective. The Journal of Experimental Zoology Supplement 4:63-71.

Shivers, B.D., Gorcs, T.J., Gottschall, P.E. and Arimura, A. 1991. Two high affinity binding sites for pituitary adenylate cyclase-activating polypeptide have different tissue distributions. Endocrinology 128:3055-3065.

Sillard, R., Agerberth, B., Mutt, V. and Jörnvall, H. 1989. Sheep neuropeptide Y. A third structural type of a highly conserved peptide. Federation of European Biochemical Societies 258:263-265.

Silverman, B.L., Kaplan, S.L., Grumbach, M.M. and Miller, W.L. 1988. Hormonal regulation of growth hormone secretion and messenger ribonucleic acid accumulation in cultured bovine pituitary cells. Endocrinology 122:1236-1241.

Skowsky, W.R. and Fisher, D.A. 1972. The use of thyroglobulin to induce antigenicity to small molecules. Journal of Laboratory and Clinical Medicine 80:134-144.

Slater, E.P., Rabenau, O., Karin, M., Baxter, J.D. and Beato, M. 1985. Molecular and Cellular Biology 5:2984-2992.

Sorokin, A.V., Petrenko, O.I., Kavsan, V.M., Kozlov, Y.I., Debabov, V.G. and Zlochevskij, M.L. 1982. Nucleotide sequence analysis of the cloned salmon preproinsulin cDNA. Gene 20:367-376.

Spada, A., Vallar, L. and Gainnattasio, G. 1984. Presence of an adenylate cyclase dually regulated by somatostatin and human pancreatic growth hormone (GH)-releasing factor in GH-secreting cells. Endocrinology 115:1203-1209.

Speidel, C.C. 1919. Gland-cells of internal secretion in the spinal cord of the skates. Carnegie Institution Publication No. 281. In: Carnegie Institution of Washington, Dept. of Marine Biology, D.C., vol. 8, pp. 1-31.

Spiess, J., Rivier, J., Thorner, M. and Vale, W. 1982. Sequence analysis of a growth hormone releasing factor from a human pancreatic islet tumor. Biochemistry 21:6037-6040.

Spiess, J., Rivier, J. and Vale, W. 1983. Characterization of rat hypothalamic growth hormone-releasing factor. Nature 303:532-535.

Stachura, M.E., Tyler, J.M. and Farmer, P.K. 1985. Human pancreatic growth hormone-releasing factor-44 differentially stimulates release of stored and newly synthesized rat growth hormone *in vitro*. Endocrinology 116(2):698-706.

Stachura, M.E., Tyler, J.M. and Kent, P.G. 1986. Effect of growth hormone-releasing factor-44 upon release of concurrently synthesized hormone by perifused rat pituitary tissue. Endocrinology 119(3):1245-1253.

Su, C.-J., White, J.W., Li, W.-H., Luo, C.-C., Frazier, M.L., Saunders, G.F. and Chan, L. 1988. Structure and evolution of somatostatin genes. Molecular Endocrinology 2:209-216.

Suda, K., Smith, D.M., Ghatei, M.A., Murphy, J.K. and Bloom, S.R. 1991. Investigation and characterization of receptors for pituitary adenylate cyclase-activating polypeptide in human brain by radioligand binding and chemical cross-linking. Journal of Clinical Endocrinology and Metabolism 72:958-964.

Suhr, S.T., Rahal, O.J., Mayo, K.E. 1989 Mouse growth hormone-releasing hormone: precursor structure and expression in brain and placenta. Molecular Endocrinology 3:1693-1700

Sweeting, R.M. and Mckeown, B.A. 1986. Somatostatin reduces plasma growth hormone levels during seawater adaptation in coho salmon (*Oncorhynchus kisutch*). Canadian Journal of Zoology 64(9):2062-2063.

Szabo, M., Stachura, M.E., Paleologos, N., Bybee, D.E. and Frohman, L.A. 1984. Thyrotropin-releasing hormone stimulates growth hormone release from the anterior pituitary of hypothyroid rats *in vitro*. Endocrinology 114:1344-1351.

Takayama, Y., Wada, C., Kawauchi, H. and [illegible], M. 1989. Structures of two genes coding for melanin-concentrating hormone of chum salmon. Gene 80:65-73.

Tannenbaum, G.S. 1985. Physiological role of somatostatin in regulation of pulsatile growth hormone secretion. In: Somatostatin (Y.C. Patel and G.S. Tannenbaum, eds.), Plenum Press, N.Y. pp. 229-260.

Tannenbaum, G.S. and Martin, 1976. Evidence for an endogenous ultradian rhythm governing growth hormone secretion in the rat. Endocrinology 98:562.

Tannenbaum, G.S. and Ling, N. 1984. The interrelationship of growth hormone (GH)-releasing factor and somatostatin

in generation of the ultradian rhythm of GH secretion. Endocrinology 115(5):1952-1957.

Tatemoto, K. 1982. Neuropeptide Y: Complete amino acid sequence of the brain peptide. Proceedings of the National Academy of Science U.S.A. 79:5485-5489.

Tatemoto, K. 1989. Neuropeptide Y: Isolation, structure and function. In: Neuropeptide Y (V. Mutt, K. Fuxe, T. Hökfelt, and J.M. Lunderg, eds.), Raven Press, New York, pp. 13-21.

Tatemoto, K., Carlquist, M. and Mutt, V., 1982. Neuropeptide Y- a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. Nature 296:659-660.

Tindall, K.R. and Kunkel, T.A. 1988. Fidelity of DNA synthesis by the *Thermus aquaticus* DNA polymerase. Biochemistry 27:6008-6013.

Tou, J.S., Kaempfe, L.A., Vineyard, B.D., Buonomo, F.C., Della-Fera, M.A. and Baile, C.A. 1986. Amphiphilic growth hormone-releasing factor (GRF) analogs: Peptide design and biological activity *in vitro*. Biochemical Biophysical Research Communications 139(2):763-770.

Traut, T.W. 1988. Do exons code for structural or functional units in proteins? Proceedings of the National Academy of Sciences, U.S.A. 85:2944-2948.

Uddman, R., Luts, A., Arimura, A. and Sundler, F. 1991. Pituitary adenylate cyclase-activating peptide (PACAP), a new vasoactive intestinal peptide (VIP)-like peptide in the respiratory tract. Cell Tissue Res 265:197-201.

Vale, W., Vaughan, J., Yamamoto, G., Spiess, J. and Rivier, J. 1983. Effects of synthetic human pancreatic (tumor) GH releasing factor and somatostatin, triiodothyronine and dexamethasone on GH secretion *in vitro*. Endocrinology 112(4):1553-1555.

Vaccarino, F.J. 1990. Growth hormone-releasing factor and feeding. Behavioral evidence for direct central actions. In: A decade of neuropeptides: Past, present, and future (G.F. Koob, C.A. Sandman and F.L. Strand, eds.), Annals of the New York Academy of Sciences, The New York Academy of Sciences, New York, New York, Vol. 579, pp. 227-232.

Vaughan, J.M., Rivier, J., Spiess, J., Peng, C., Chang, J.P., Peter, R.E. and Vale, W. 1992. Isolation and characterization of hypothalamic growth hormone-

releasing factor from Common carp, *Cyprinus carpio*. Neuroendocrinology 56:539-549.

Velicelebi, G., Santacroce, T.M. and Harpold, M.M. 1985. Specific binding of synthetic human pancreatic growth hormone releasing factor (1-40-OH) to bovine anterior pituitaries. Biochemical and Biophysical Research Communications 126(1):33-39.

Vigh, S., Arimura, A., Koves, K., Somogyvari-Vigh, A., Sitton, J., and Fermin, C.D. 1991. Immunohistochemical localization of the neuropeptide, pituitary adenylate cyclase activating polypeptide (PACAP), in human and primate hypothalamus. Peptides 12:313-318.

Waelbroeck, M., Robberecht, P., Coy, D.H., Camus, J.-C., Neef, P.D. and Christophe, J. 1985. Interaction of growth hormone-releasing factor (GRF) and 14 GRF analogs with vasoactive intestinal peptide (VIP) receptors of rat pancreas. Discovery of (N-Ac-Tyr^{1},D-Phe^{2})-GRF(1029)-NH_2 as a VIP antagonist. Endocrinology 116:2643-2649.

Wehrenberg, W.B., Baird, A, Ying, S.Y., Rivier, J., Ling, N. and Guillemin, R. 1984. Multiple stimulation of the adenohypophysis by combination of hypothalamic releasing factors. Endocrinology 114:1995-2001.

Wehrenberg, W.B. and Giustina, A. 1992. Basic Counterpoint: Mechanisms and pathways of gonadal steroid modulation of growth hormone secretion. Endocrine Reviews 13:299-308.

Wilson, A.C. 1976. Gene regulation in evolution. In: Molecular Evolution (F.J. Ayala, ed.), Sinauer, Sunderland, Massachusetts, pp. 225-234.

Wilson, T. and Treisman, R. 1988. Removal of poly(A) and consequent degradation of c-fos mRNA facilitated by 3' AU-rich sequences. Nature 336:396-399.

Wood, T.L., Berelowitz, M., Gelato, M.C., Roberts, C.T., Jr, LeRoith, D., Millard, W.J. and McKelvy, J.F. 1991. Hormonal regulation of rat hypothalamic neuropeptide mRNAs: Effect of hypophysectomy and hormone replacement on growth-hormone-releasing factor, somatostatin and the insulin-like growth factors. Neuroendocrinology 53:298-305.

Woolny, C., Fleet, I.R., Davis, A.J., Goode, J.A., Mason, W.T. and Heap, R.B. 1987. Growth hormone release after arterial injection on bovine growth hormone-releasing factor (GRF), GRF 1-29 and thyrotrophin-releasing factor (TRH) in lactating and pregnant cows. Abstract

146. Journal of Endocrinology 112.

Yiangou, Y., Marzo, V.D., Spokes, R.A., Panico, M., Morris, H.R. and Bloom, S.R. 1987. Isolation, characterization, and pharmacological actions of peptide histidine valine 42, a novel prepro-vasotive intestinal peptide-derived peptide. The Journal of Biological Chemistry 262:14010-14013.

Yiangou, Y., Requejo, F., Polak, J.M. and Bloom, S.R. 1986. Characterization of a novel prepro VIP derived peptide. Biochemical and Biophysical Research Communications 139:1142-1149.

Zeitler, P., Vician, L., Chowen-Breed, J.A., Argente, J., Tannenbaum, G.S., Clifton, D.K. and Steiner, R.A. 1990. Regulation of somatostatin and growth hormone-releasing hormone gene expression in the rat brain. Metabolism 39:46-49.

Zhou, J., McIndoe, A., Davies, H., Sun, X.-Y. and Crawford, L. 1991. The induction of cytotoxic T-lymphocyte precursor cells by recombinant vaccinia virus expressing human papillomavirus type 16 L1. Virology 181:203-210.

Zhu, Z. 1992. Generation of fast growing transgenic fish: Methods and mechanisms. In: Transgenic Fish (C.L. Hew and G.L. Fletcher, eds.) World Scientific Publishing Co. Pte. Ltd., Singapore, pp. 92-119.

Zhuang, Y., Goldstein, A.M. and Weiner, A.M. 1989. UACUAAC is the preferred branch site for mammalian mRNA splicing. Proceedings of the National Academy of Sciences, U.S.A. 86:2752-2756.