ACCUMULATION OF HYPOPHYSIOTROPIC HORMONE mRNAs IN SOMATOTROPH PITUITARY TUMORS: BIOLOGICAL, CLINICAL, AND PROGNOSTIC IMPLICATIONS

by

Kamal Thapar

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy, Graduate Department of Laboratory Medicine and Pathobiology, University of Toronto

© Copyright by Kamal Thapar 2000
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-49927-8
Accumulation of hypophysiotropic hormone mRNAs in somatotroph pituitary tumors: biological, clinical, and prognostic implications

Doctor of Philosophy, 2000

Kamal Thapar
Graduate Department of Laboratory Medicine and Pathobiology
University of Toronto

ABSTRACT
The behavior of somatotroph pituitary tumors greatly varies, however, the events/mechanisms underlying this variability remain poorly understood. These tumors arise from transformation of somatotrophs, the growth hormone (GH) producing pituitary cells. The secretory and proliferative capabilities of the normal somatotroph are governed by two hypothalamic (hypophysiotropic) hormones: growth hormone releasing hormone (GHRH) and somatostatin (SRIF). We have shown that the neoplastic somatotroph can, in a nonrandom fashion, assume the capacity to transcribe and translate genes encoding GHRH and SRIF, the mRNA levels of which are associated with prognostically relevant differences in tumor behavior. In 100 adenomas studied by in situ hybridization, the distribution of GHRH and SRIF mRNA transcripts was quantified, and their clinicopathologic correlates determined; protein translation was confirmed by Western blotting. GHRH transcripts were found to preferentially accumulate within aggressive tumors. Specifically, GHRH mRNA signal intensity was: (i) linearly correlated with Ki-67 tumor growth fractions; (ii) linearly correlated with blood GH levels; (iii) higher among invasive tumors; and (iv) highest in those tumors in which postoperative remission was not achieved. Alternatively, SRIF transcripts were found to preferentially accumulate among clinically favorable variants; the SRIF mRNA signal was: (i) inversely correlated with Ki-67 tumor growth fractions; (ii) inversely correlated with preoperative GH levels; (iii) higher in noninvasive tumors; and (iv) highest in tumors amenable to surgical remission. From the GHRH and SRIF mRNA signal intensities, several significant logistic models of postoperative outcome were fitted and validated in a second population of 30 somatotroph adenomas. The pattern of GHRH and SRIF transcript accumulation permitted tumors to be meaningfully grouped into aggressive and favorable variants; aggressive variants expressed high and low levels of GHRH and SRIF transcripts, respectively; favorable variants expressed high and low levels of SRIF and GHRH transcripts, respectively. That the latter clinically favorable state could be approximated pharmacologically with presurgical
therapy an SRIF analog. This agent was associated with an antiproliferative effect and reductions in GHRH mRNA and protein expression. The nonrandom and prognostically informative patterns of GHRH and SRIF transcript accumulation supports an autocrine/paracrine regulatory role for these hypophysiotropic hormones in this tumor system.
ACKNOWLEDGEMENTS

The work that follows is the product of many contributions from many people. Whereas some provided their guidance, others their technical expertise, and others still, provided encouragement, support and friendship, the contribution of each is reflected in every page of this thesis.

I was especially privileged and honoured in having Dr. Kalman Kovacs as my supervisor. He is the consummate teacher, having the wisdom and the ability to strike the perfect balance between providing guidance and encouraging creativity. He fully believed in our project and in my abilities to carry it out, and through that confidence I felt anything was possible. During our 6 year relationship, he wore many hats: supervisor, confidant, a second father, a part-time publicist and a full time friend. I will always be grateful for the many doors he has opened in my scientific and professional career. If I should hope to ever achieve any scientific legacy in my professional career, let it be that I conducted myself with the same integrity, enthusiasm, and passion for science as did this extraordinary teacher.

I am also very grateful to Dr. Lucia Stefaneanu for her supervision throughout this project. She introduced me to molecular science, in situ hybridization, and microscopy, and was always available for thoughtful discussion and advice. In particular, I am very appreciative for all the time she expended in reviewing my in situ hybridization slides, and helping me to discern fact from artifact.

I am very appreciative of the support of Dr. Charles Tator, past Chairman of the Division of Neurosurgery at the University for Toronto for providing me with the opportunity to pursue this degree in consort with my neurosurgical residency training.

Much appreciation goes out to Dr. D. Killinger, who provided me with much of the clinical data for the 100 tumors in our primary study population. Similarly, I thank Dr. Bernd Scheithauer and Dr. Edward Laws for their provision of tumor samples and clinical data of the Mayo Clinic patients studied in this thesis. As mentors, they provided much expertise, guidance, and friendship, and for this I will always be grateful.
To my committee members, Drs. J Minta, D. Sarma, E. Cutz, and J. Rutka, I thank them for their guidance and encouragement, but also for the latitude they extended to me in developing this project. I am very grateful to Dr. Rutka for allowing me ready access to his laboratory and to his technologist, Sherrilyn, for help with my Northerns, Westerns, and RTPCR.

I am grateful to Dr. Kovacs’ team at St. Michael’s Hospital, namely, Anca, Fabio, Mark, and Zi, for their technical help throughout this project. I truly owe a debt of gratitude to Anca, for she assumed the arduous task of counting, quite literally, millions of silver grains and quantifying the in situ hybridization studies. I am also very appreciative of Dr. Eva Horvath, in whose company I always found advice, suggestions, and encouragement. I also acknowledge the help of Kristin Latour of the SAS Institute, who helped me in writing SAS code for the logistic regression analyses. A special thanks goes out to Naomi Currie, Medical Illustrator, who prepared the illustrations for all my presentations and for this thesis.

The experience of this thesis was not without sadness. Certainly the lowest point for our laboratory occurred with the unexpected passing of our friend and fellow graduate student, Alina Tampanaru. Her intellect, presence, and friendship are greatly missed by us all.

Finally, I wish to acknowledge my family for all their love, encouragement, and sacrifice. My parents have always made great sacrifices for my education, and this degree was no exception. This thesis would not have been possible were it not for the love and support of my wife, Hazel. From the very first to the very last day of graduate school, and at every moment in between, it has been her love that has sustained me. All that I have achieved, I owe to her.

Support for this work was provided by a Research Fellowship from the Medical Research Council of Canada; grants from the Physicians Services Incorporated (PSI) Foundation and the St. Michael’s Hospital Research Foundation; and additional support from the Departments of Postgraduate Medicine and Surgery, University of Toronto.
# TABLE OF CONTENTS

Abstract  
Acknowledgements  
List of Tables  
List of Figures  
List of Appendices  
List of Abbreviations  

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>One</td>
<td>General Introduction</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Hypothesis</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Study Design</td>
<td>30</td>
</tr>
<tr>
<td>Two</td>
<td>GHRH mRNA transcript accumulation in somatotroph adenomas</td>
<td>31</td>
</tr>
<tr>
<td>Three</td>
<td>SRIF mRNA transcript accumulation in somatotroph adenomas</td>
<td>61</td>
</tr>
<tr>
<td>Four</td>
<td>GHRH and SRIF mRNA accumulation and somatotroph adenoma behavior: Multivariate modelling</td>
<td>80</td>
</tr>
<tr>
<td>Five</td>
<td>Antiproliferative effects of the SRIF analog, octreotide, in somatotroph adenomas</td>
<td>101</td>
</tr>
<tr>
<td>Six</td>
<td>Effects of the SRIF analog, octreotide, on GHRH and SRIF mRNAs</td>
<td>112</td>
</tr>
<tr>
<td>Seven</td>
<td>Conclusions and Relevance</td>
<td>120</td>
</tr>
<tr>
<td>APPENDIX 1</td>
<td>Model selection for multivariate analysis</td>
<td>124</td>
</tr>
<tr>
<td>References</td>
<td></td>
<td>128</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

### Chapter Two

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Summary of univariate logistic regression analysis for postoperative remission likelihood</td>
<td>49</td>
</tr>
<tr>
<td>2.2</td>
<td>Unreduced multivariate logistic regression model for postoperative remission likelihood</td>
<td>50</td>
</tr>
<tr>
<td>2.3</td>
<td>Contingency table analysis comparing actual remission status of the Mayo Clinic cohort with the predicted remission status</td>
<td>52</td>
</tr>
</tbody>
</table>

### Chapter Three

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Contingency table analysis comparing actual remission status of the Mayo Clinic cohort with the predicted remission status</td>
<td>73</td>
</tr>
</tbody>
</table>

### Chapter Four

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Clinicopathologic characteristics of tumor clustered on the basis of median GHRH and SRIF mRNA levels</td>
<td>83</td>
</tr>
<tr>
<td>4.2</td>
<td>Frequency of gross tumor invasion in tumors grouped on the basis of median GHRH and SRIF mRNA signal intensities</td>
<td>87</td>
</tr>
<tr>
<td>4.3</td>
<td>Frequency of postoperative remission in tumors grouped on the basis of median GHRH and SRIF mRNA signal intensities</td>
<td>87</td>
</tr>
<tr>
<td>5.1</td>
<td>Tumor pathologic features, treatment status, and tumor growth fractions in 32 study patients</td>
<td>105</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Chapter One

Figure 1.1 Whole mount axial section of a pituitary gland obtained at autopsy 4
Figure 1.2 Organization of the hypothalamic-pituitary axis 5
Figure 1.3 Normal pituitary gland 6
Figure 1.4 Regulation of the somatotroph 8
Figure 1.5 Intracellular signalling in the somatotroph 10
Figure 1.6 Multistep model of somatotroph tumorigenesis 21
Figure 1.7 Hypothesis 29
Figure 1.8 Flow diagram outlining the design of studies in this thesis 30

Chapter Two

Figure 2.1 In situ hybridization for GHRH mRNA 41
Figure 2.2 Northern analysis for GHRH, GHRH-R, and Western analysis for GHRH protein 43
Figure 2.3 Detection of GHRH transcripts by RTPCR analysis 44
Figure 2.4 Scatterplot analysis comparing GHRH mRNA signal intensities as determined by ISH versus Northern analysis 45
Figure 2.5 Comparison of GHRH signal intensities between the normal pituitary and different somatotroph adenoma subtypes 46
Figure 2.6  (A) Nuclear expression of the Ki-67 and (B) Scatterplot analysis of GHRH mRNA signal intensity versus Ki-67 LI  46

Figure 2.7  (A) Mean GHRH mRNA signal intensity in tumors stratified on the basis of size and invasion status. (B) Hardy classification of pituitary tumors  47

Figure 2.8  Scatterplot analysis showing the relationship between GHRH mRNA signal intensity and the mean preoperative serum GH level  48

Figure 2.9  Comparison of the distributions of GHRH mRNA signal intensities in tumors stratified on the basis of remission status using boxplot analysis  48

Figure 2.10  Plot of predicted postoperative remission probabilities versus the tumoral GHRH mRNA signal intensity.  51

Chapter Three

Figure 3.1  ISH for SRIF mRNA in the nontumorous pituitary gland  64

Figure 3.2  ISH for SRIF mRNA in a somatotroph adenoma  64

Figure 3.3  Boxplot analysis showing distribution of SRIF mRNA signal intensities in tumors, surgically removed and autopsy pituitary glands  65

Figure 3.4  ISH for SRIF mRNA in a somatotroph adenoma showing the border between neoplasm and normal gland.  65
| Figure 3.5 | Comparison of mean SRIF mRNA signal intensities between different morphologic types of somatotroph adenomas | 66 |
| Figure 3.6 | Northern and Western analyses for SRIF | 67 |
| Figure 3.7 | Scatterplot analysis showing significant inverse correlation between the SRIF mRNA signal intensity and the Ki-67 LI | 68 |
| Figure 3.8 | Mean SRIF mRNA signal intensities of tumors stratified on the basis of size and invasivenessiveness | 69 |
| Figure 3.9 | Cellular relationship between the distributions of SRIFmRNA immunoreactive GH in two somatotroph adenomas | 70 |
| Figure 3.10 | Scatterplot analysis showing a weak but statistically significant negative linear correlation between the serum GH level and the tumoral mRNA signal intensity | 70 |
| Figure 3.11 | Comparisons of the distributions of SRIF mRNA signal intensities in tumors stratified on the basis of postoperative remission using boxplot analysis | 71 |
| Figure 3.12 | Plot of predicted postoperative remission probabilities versus the tumor SRIF mRNA signal intensity. | 72 |

**Chapter Four**

| Figure 4.1 | Analysis of Ki-67 LI in tumors grouped on the basis of median GHRH and SRIF mRNA signal intensites. | 85 |
| Figure 4.2 | Analysis of preoperative GH levels of tumors grouped on the basis of median GHRH and SRIF mRNA signal intensities | 86 |
| Figure 4.3 | Fitting a multivariate model for prediction of Ki-67 LI | 89 |
| Figure 4.4 | Fitting a multivariate model for prediction of preoperative GH levels | 91 |
| Figure 4.5 | Output from SAS statistical software summarizing significance of overall outcome model and parameter estimates | 92 |
| Figure 4.6 | Outcome prediction in the Mayo Clinic population | 93 |
| Figure 4.7 | Scatterplot analysis of GHRH versus SRIF mRNA signal intensities showing a reciprocal linear relationship between these two variables | 95 |

**Chapter 5**

| Figure 5.1 | Comparisons of mean tumor growth fractions of tumors exposed to preoperative octreotide therapy and controls | 105 |
| Figure 5.2 | The effect of octreotide therapy on mean tumor growth fractions, as stratified by tumor subtype | 106 |
| Figure 5.3 | Cell cycle specific effects of octreotide and/or SRIF | 108 |

**Chapter 6**

| Figure 6.1 | Comparison of the distributions of GHRH mRNA signal intensities in tumors pretreated with octreotide and in untreated controls | 114 |
| Figure 6.2 | Western analysis for GHRH in octreotide treated somatoroph adenomas and in controls | 115 |
LIST OF APPENDICES

APPENDIX 1  Model selection for multivariate analysis  124
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>αSU</td>
<td>alpha subunit of glycoprotein hormones</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AgNORs</td>
<td>argyrophilic nucleolar organizing regions</td>
</tr>
<tr>
<td>ANCOVA</td>
<td>analysis of covariance</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>c</td>
<td>area under the receiver operator characteristic (ROC) curve</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine 3',5'-monophosphate</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin dependent kinase</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP responsive element binding protein</td>
</tr>
<tr>
<td>df</td>
<td>degrees of freedom</td>
</tr>
<tr>
<td>EGF/R</td>
<td>epidermal growth factor/receptor</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
</tr>
<tr>
<td>G-protein</td>
<td>guanosine triphosphate binding protein</td>
</tr>
<tr>
<td>G_i</td>
<td>inhibitory (adenylate cyclase inhibiting) G-protein</td>
</tr>
<tr>
<td>G_k</td>
<td>stimulatory G-protein coupled to opening of K⁺ channels</td>
</tr>
<tr>
<td>G_s</td>
<td>stimulatory (adenylate cyclase activating) G-protein</td>
</tr>
<tr>
<td>G_sα</td>
<td>alpha subunit of G_s</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>GHRH</td>
<td>growth hormone releasing hormone</td>
</tr>
<tr>
<td>GHRH-R</td>
<td>growth hormone releasing hormone receptor</td>
</tr>
<tr>
<td>GHRP</td>
<td>growth hormone releasing peptide</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>ICC</td>
<td>immunocytochemistry</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin-like growth factor 1</td>
</tr>
<tr>
<td>ISH</td>
<td>in situ hybridization</td>
</tr>
<tr>
<td>Ki-67 LI</td>
<td>Ki-67 labeling index</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>MEN-1</td>
<td>multiple endocrine neoplasia type 1 syndrome</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>OGTT</td>
<td>oral glucose tolerance test</td>
</tr>
<tr>
<td>$P_{remission}$</td>
<td>probability of remission</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PKCα</td>
<td>alpha isoform of protein kinase C</td>
</tr>
<tr>
<td>PRL</td>
<td>prolactin</td>
</tr>
<tr>
<td>r</td>
<td>Pearson correlation coefficient</td>
</tr>
<tr>
<td>RER</td>
<td>rough endoplasmic reticulum</td>
</tr>
<tr>
<td>ROC</td>
<td>receiver operator characteristic</td>
</tr>
<tr>
<td>RTPCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SRIF</td>
<td>somatostatin</td>
</tr>
<tr>
<td>SSTR</td>
<td>somatostatin receptor</td>
</tr>
<tr>
<td>SSTR1, SSTR2... etc.</td>
<td>somatostatin receptor subtype 1, subtype 2...etc.</td>
</tr>
<tr>
<td>TSH</td>
<td>thyroid stimulating hormone</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>chi squared statistic</td>
</tr>
</tbody>
</table>
CHAPTER 1: Introduction

Overview

The hypothalamus exercises a vigorous level of regulatory control over the pituitary gland. The vehicle for this regulation is a class of hypothalamic factors known as hypophysiotropic hormones. Produced in the hypothalamus and released into the portal circulation, hypophysiotropic hormones govern the secretory and proliferative capabilities of specific target cells in the pituitary gland. Insofar as a defining feature of the neoplastic phenotype is an ability to exploit, circumvent, or to otherwise disrupt physiologic regulatory mechanisms responsible for normal cellular growth, phenotypic maintenance, and proliferative restraint, hypophysiotropic hormones assume a logical culpability as having some role in the development and/or progression of pituitary tumors. Indeed, the ‘hypothalamic hypothesis’ of pituitary tumorigenesis, an early and once dominant concept, proposed that pituitary tumors arose as the downstream consequence of a stimulatory imbalance of hypophysiotropic hormones liberated from a dysfunctional hypothalamus. Whereas several lines of evidence had eventually exonerated the hypothalamus from having a primary role in pituitary tumor development, a potential role for hypophysiotropic hormones in the growth and progression of pituitary tumors remained too compelling to dismiss. The observation that pituitary tumors express receptors for and retain responsiveness to hypophysiotropic hormones sustained the plausibility of a hypophysiotropic component to pituitary tumor maintenance and/or progression, as did demonstrations of hypophysiotropic hormone mRNAs within some pituitary tumors. Furthermore, as concepts of autocrine and paracrine stimulation became increasingly applied to human neoplasia as potential mechanisms underlying tumor growth and progression, hypophysiotropic hormones assumed renewed implication as candidate autocrine/paracrine mediators of pituitary tumor progression. Still, and despite speculation concerning the existence of hypophysiotropic hormone mediated autocrine/paracrine circuits within pituitary tumors, data substantiating their existence, functionality, and biological relevance have been few.

Comprising the normal pituitary gland are five major cell types, each differing in structure and function. One of these is the somatotroph, a cell whose primary function is the secretion of
growth hormone (GH). The normal somatotroph is under the dual neuroendocrine control of two hypophysiotropic hormones: growth hormone releasing hormone (GHRH) and somatostatin (SRIF). The former is the positive regulator, the latter is the negative regulator, and together, in a highly coordinated fashion, the two govern the secretory and proliferative activities of the normal somatotroph. Neoplastic derivatives of pituitary somatotrophs are termed somatotroph adenomas. They secrete GH in excess and are responsible for the clinical phenotypes of acromegaly and gigantism. Whereas some somatotroph adenomas are amenable to curative resection, others can progress relentlessly, often despite maximal surgical, pharmacologic, and radiotherapeutic interventions. Insofar as the basis for this variability in biologic behavior is unknown, so too is the means to predict which tumors are destined for a clinically aggressive course from those having a more indolent profile.

This thesis links the dual objectives of evaluating the biologic significance of GHRH and SRIF mRNA transcript accumulation in somatotroph adenomas with the need to develop a prognostically informative strategy whereby the behavior of somatotroph adenomas can be predicted. First, the distribution of GHRH mRNA transcripts in a consecutive series of 100 somatotroph adenomas is described (Chapter 2). The relationship between GHRH transcript accumulation and a number of clinicopathologic parameters is evaluated, a statistical prognostic model is fitted and then tested and generalized to a secondary population of somatotroph adenomas. In Chapter 3, a similar analysis is performed for SRIF transcript accumulation in the same tumor population. To more comprehensively explore the relationship between GHRH and SRIF mRNAs to each other, as well as their simultaneous and combined statistical effects on pertinent aspects of tumor behavior, Chapter 4 uses multivariate methods to integrate data presented in preceding chapters. Finally, to better understand the inhibitory effects of SRIF on somatotroph adenomas, a series of somatotroph adenomas exposed to preoperative treatment with an analog of SRIF (octreotide) were investigated in the context of a randomized controlled trial. The effect of such therapy on tumor cell proliferation (Chapter 5) and on the levels of GHRH and SRIF mRNAs (Chapter 6) is presented.
GENERAL CONSIDERATIONS

Pituitary tumors are common lesions, accounting for 10 to 15% of all primary intracranial neoplasms [84]. Unified by their adenohypophyseal origin, pituitary tumors are an otherwise heterogeneous collection of neoplasms. Their individual diversity is reflected along a variety of fronts, including cytogenesis, morphology, endocrine activity, clinical presentation, biological behavior, and therapeutic responsiveness. Endowed with the secretory capacity of their parent adenohypophyseal cells, many pituitary tumors liberate physiologic hormones to pathologic excess, generating a full spectrum of metabolic aberrations and some of the most dramatic clinical syndromes known to medicine. Thus, pituitary tumors constitute a unique form of neoplasia which, in concept and in practice, differs fundamentally from virtually all other tumors of intracranial origin. The most obvious difference relates to the double-edged clinical problem posed by these lesions, featuring endocrine concerns on the one hand, complicated by neuro-oncologic issues on the other. Accordingly, the diagnostic and therapeutic imperatives that accompany pituitary tumors are correspondingly unique, reflecting the duality of the clinical problem. Kovacs and Horvath, in their classification of pituitary tumors have identified 17 different morphologic entities [84]. This thesis focuses on the neoplastic progression of one major class of pituitary tumors: those which secrete growth hormone (GH) in excess and result in the clinical syndromes of acromegaly and/or gigantism. This class of tumors, which itself consists of at least 6 morphologic variants, are broadly classified as somatotroph adenomas or somatotropinomas, a nomenclature which reflects their hypersecretion of GH (a hormone historically referred to as somatotropin), as well as their origin from and/or their presumed shared cytogenetic lineage with the normal GH producing cells of the pituitary (somatotrophs).

From biological, clinical, and prognostic standpoints, the behavior of somatotroph adenomas tends to be highly variable and generally defies reliable prediction. Whereas some somatotroph adenomas are amenable to curative surgical resection, others will progress, often in spite of maximal multimodal intervention [186, 190, 191]. The tendencies of some somatotroph adenomas toward aggressive, invasive, or recurrent growth, although neither reflected in nor predicted by the tumor's histologic or ultrastructural morphology, is presumably the result of specific subcellular events that promote neoplastic progression among aggressive variants, or conversely, are due to the preservation of protective events that forestall neoplastic progression.
among clinically favorable variants. To date, however, neither promoting nor protective events are well characterized in this tumor system. Whereas a number of genomic alterations have been identified in somatotroph adenomas, some of which have been linked with aggressive behavior, none occur with sufficient frequency, sensitivity, or specificity to account for the wide behavioural variability exhibited by these tumors [185]. This implies the presence of as yet unidentified genomic alterations and/or the presence of other growth promoting mechanisms independent of gene mutation/loss. Beyond its conceptual importance, uncertainty surrounding the determinants of neoplastic progression in these tumors also constitutes an important clinical problem for it undermines our ability to reliably predict the behavior of these tumors. Were it possible to distinguish those tumors destined for an aggressive clinical course from those having a more indolent profile, then the need for adjuvant postoperative therapies could be better anticipated, allowing for a more rational and an overall more comprehensive management plan. Accordingly, a clear need exists for the identification of prognostically informative and clinically relevant mechanisms and markers of neoplastic progression in this tumor system.

THE NORMAL PITUITARY GLAND

The human pituitary gland is a composite neuroendocrine structure composed of an anterior adenohypophyseal component in apposition with an embryologically, morphologically, and functionally distinct posterior neurohypophyseal component. This feature is grossly apparent from the gland’s bi-lobed appearance (Figure 1.1). All pituitary adenomas arise from the adenohypophysis. The neurohypophysis, which includes the posterior lobe of the pituitary

![Figure 1.1 Whole mount axial section of a pituitary gland obtained at autopsy. (A) The larger anterior lobe is readily distinguished from the smaller posterior lobe. A small pars intermedia cyst is present at the interface (H&E stain). (B) A GH immunostain has been used to identify somatotrophs. Topologically, somatotrophs are most abundant within, but not restricted to, the lateral wings of the gland.](image)
gland. is a direct extension of the central nervous system and is the site of storage and release of the hormones vasopressin and oxytocin. In that the neurohypophysis is not relevant to the development of somatotroph adenomas, it is not discussed further.

**The Adenohypophysis**

The adenohypophysis, which collectively includes the pars distalis (anterior lobe), pars intermedia (intermediate lobe), and the pars tuberalis (a funnel-shaped upward extension of anterior lobe cells on the anterior face of the pituitary stalk), is the site of meticulously regulated hormone synthesis and release. The anterior lobe is comprised of five principal secretory cell types, each distinct functionally and ultrastructurally, and each distributed in a fairly consistent topological arrangement within the gland. These five cell types are the somatotrophs, lactotrophs, corticotrophs, thyrotrophs, and gonadotrophs, and are distinguished functionally by their secretion of growth hormone (GH), prolactin (PRL), adrenocorticotropic hormone (ACTH), thyroid stimulating hormone (TSH), and the gonadotropins [luteinizing hormone (LH) and follicle stimulating hormone (FSH)], respectively. In a remarkably integrated fashion, the secretory and proliferative capabilities of these cells are governed by a precise and continuously regulated balance between stimulatory and suppressive hypothalamic influences and the negative feedback effects imposed by target organ hormones [195] (Figure 1.2). Although susceptibilities vary, any of these cell types may be subject to neoplastic transformation. The resulting adenoma generally retains the secretory capabilities, some of the morphologic characteristics, and nomenclature of the cell of origin [185].

Microscopically, the anterior pituitary exhibits a delicate acinar architecture, each acinus being comprised of an admixture of

---

*Figure 1.2 Organization of the hypothalamic-pituitary axis. Each of the 5 cell types in the anterior pituitary is regulated by one or more hypophysiotropic hormone. Each is regulated further by negative feedback effects of pituitary and/or target gland hormones. The somatotroph (arrow) is regulated by GHRH and SRIF.*
various secretory cell types (Figure 1.3). After staining with hematoxylin and eosin (H&E), pituitary cells appear either acidophilic, basophilic, or chromophobic. Historically, acidophils were regarded as being somatotrophs, basophils as being either corticotrophs, gonadotrophs, or thyrotrophs, and chromophobic cells being regarded as lactotrophs. It is now known that such tinctorial appearances are not a reliable means of distinguishing different secretory cell types. Of pituitary somatotrophs, however, most do tend to appear acidophilic on such stains (Figure 1.3A). When visualized in horizontal cross section, the anterior lobe is comprised of two lateral “wings” and a trapezoid-shaped “central mucoid wedge”. As shown in Figure 1.1B pituitary somatotrophs most heavily populate the lateral wings of the gland, although some can also be scattered within the central mucoid wedge of the gland. Correspondingly, somatotroph adenomas are generally assumed to arise at these intraglandular sites.

The normal somatotroph: Morphology

Somatotrophs represent the most common secretory cell type in the human pituitary, accounting for approximately 50% of all adenohypophyseal cells [84]. Although somatotrophs can be detected immunohistochemically within the fetal pituitary by the eighth gestational week [5, 130], their functionality, vis à vis GH secretion, has been demonstrated as early as the fifth week of fetal life [169]. In the newborn, and throughout life, the cellular density, intraglandular distribution, and morphology of pituitary somatotrophs remains remarkably constant, showing little deviation with gender, advancing age, the presence of systemic disease, or drug therapies.
Morphologically, somatotrophs are cells of medium size, with an ovoid cell body, spherical nucleus, and a well developed cytoplasm [183]. Their cytoplasm contains an abundance of GH-containing secretory granules. Accordingly, at the light microscopic level, they are most reliably detected in GH immunostained preparations, which functionally identifies somatotrophs on the basis of their diffuse cytoplasmic GH content (Figure 1.3B).

The ultrastructure of pituitary somatotrophs is highly characteristic [84]. In keeping with the high secretory demands imposed on these cells, their ultrastructure is remarkable for an abundant cytoplasm replete with a full complement of synthetic organelles. The nucleus is centrally located and typically bears a prominent nucleolus. Indicative of the somatotrophs high secretory potential, is its well developed secretory apparatus. There is an exuberance of rough endoplasmic reticulum (RER) which consists of slender ribosome studded cisternae, generously disposed at the cytoplasmic periphery. The Golgi apparatus is conspicuous, and typically contains secretory granules in varying stages of maturation. Mitochondria are frequent, their cristae assuming a lamellar configuration on the background of a moderately electron-dense matrix. Most conspicuous, however, are the numerous spherical or ovoid GH containing secretory granules. These electron dense secretory granules can vary considerably in size (150-800nm); the diameter of most measure in the 350-500nm range. Secretory granules can be seen throughout the cytoplasm, although they frequently accumulate at the cell periphery. In addition to their content of GH-containing secretory granules, immunoelectron microscopy has shown that a proportion of somatotrophs also contain the glycoprotein hormone alpha-subunit [67].

REGULATION OF THE NORMAL SOMATOTROPH

The singular function of the somatotroph is the secretion of GH. Human GH is a nonglycosylated, single chain, 191-amino acid, 22kd protein (reviewed in [195]). Aside from its transient secretion by the placenta during pregnancy, the somatotroph is the only physiologic biosynthetic site for GH secretion in the human. Although GH is not an essential hormone insofar as its congenital or acquired absence is compatible with life, GH does subserve a number of important physiologic functions in a variety of human tissues. Principal among these are induction of pre- and postnatal skeletal growth, as well as anabolic effects on carbohydrate, lipid, and protein
metabolism [195]. The mechanisms by which GH achieves these diverse effects are unsettled. According to the somatomedin hypothesis of Salmon and Daughaday, an indirect action of GH is proposed, with the GH dependent effects being mediated by somatomedins of hepatic origin, rather than by GH directly [37, 158]. The concept was broadened by Green, with his 'dual effector' theory, proposing that GH has some direct effects, but most are mediated by GH induced secretion of IGF-1 in target tissues [57].

In a highly coordinated and tightly regulated fashion, the secretory and proliferative capabilities of the normal pituitary somatotroph are governed by several tiers of regulatory control (Figure 1.4) [57a]. In general, GH secretion and somatotroph proliferation are physiologically coupled events. With few exceptions, both processes share the same basic regulatory cascades, intracellular signaling, and second messenger systems, with positive and negative regulators affecting both processes in a somewhat similar and simultaneous fashion. Through a series of interactive and coordinated feedback loops, the somatotroph is subject to two levels of regulatory control: hypothalamic and systemic. There are four essential regulatory elements involved in these loops: GHRH, SRIF, IGF-1, and GH itself. GHRH and SRIF are secretory products of the hypothalamus, GH is the secretory product of the somatotroph, and IGF-1 is the secretory product of the liver and other systemic tissues responsive to GH action; IGF-1 mediates the local trophic effects of GH (ie. the somatomedin hypothesis).

Hypothalamic regulation of the somatotroph is mediated by the hypophysiotropic hormones, GHRH and SRIF, the former being the positive regulator and the latter exercising inhibitory con-
trol. Produced in hypothalamic nuclei and released primarily in response to feedback effects of circulating GH and IGF-1 levels, but also modified by endogenous neural rhythms, external stimuli, and a host of central neurotransmitter and neuropeptide projections, GHRH and SRIF descend via the portal circulation to the pituitary somatotroph. Although each has opposite effects and each is released from the hypothalamus in independent secretory waves, the two interact to titrate the secretory and proliferative tone of the somatotroph.

Systemic regulation of somatotroph function is via 'long' feedback loops mediated by IGF-1 and GH. IGF-1 inhibits somatotroph secretion by stimulating hypothalamic SRIF release and inhibition of GH gene transcription within the somatotroph [13, 210]. Growth hormone also inhibits its own release, by way of stimulating SRIF release from the hypothalamus and inhibiting alpha adrenergic projections in the hypothalamus that normally stimulate GHRH release [151].

The various hypothalamic and system regulatory loops ultimately converge on the adenohypophysis, being coupled to several signal transduction and effector systems in the somatotroph (Figure 1.5). Included among these are stimulatory and inhibitory G-protein cascades [134, 185, 201], the adenylate cyclase-protein kinase A pathway [201], the phosphoinositide-protein kinase C pathway [96], and ion channel proteins governing GH secretion [73, 83, 107, 163, 206]. The regulatory pathways and their intermediaries are depicted in figure 1.5

**Growth hormone releasing hormone (GHRH)**

Growth hormone releasing hormone is the primary positive regulator of the somatotroph, stimulating both GH secretion and somatotroph proliferation [194a]. The existence of GHRH was first suspected in 1960 with the demonstration that rats with structural hypothalamic lesions suffered growth failure, and later in 1963, when hypothalamic extracts were shown to possess GH-releasing activity [149]. It was not until 1982, however, that GHRH was first isolated, initially from a GHRH producing pancreatic tumor [60, 156], and later from the hypothalamus [104]. Immunohistochemical studies localized GHRH to the arcuate and ventromedial hypothalamic nuclei, confirming previous physiologic studies that indicated these regions as being facilitatory in GH regulation and release [21, 22]. Shortly thereafter, the GHRH cDNA was identified and the gene was sequenced and localized to chromosome 20p12.1 [110, 112]. GHRH is produced
in precursor form and undergoes post-translational enzymatic cleavage to produce the mature hormone. The GHRH gene spans 18kb and contains 5 exons, four of which encode for the GHRH precursor protein; the fifth contains 5'nontranslated sequences. Alternative splicing of the gene produces two distinct mRNA transcripts, both approximately 750 kb in size, and each encoding the identical GHRH precursor protein. The precursor protein is approximately 103-108 amino acids in length, from which a mature 42-44 amino acid peptide is cleaved. Few data are available concerning the regulation of the GHRH gene in either health or disease.

Following its descent to the pituitary, GHRH binds to its receptor (GHRH-R) on the somatotroph cell surface. The structure of the GHRH-R has been recently characterized [54, 111]. Based on its cDNA, the receptor protein is 423 amino acids in length and has a predicted structure which includes seven hydrophilic domains with the potential to serve as membrane-spanning helices, the defining feature of G-protein coupled receptors. Ligand activated GHRH-R is thought to be coupled to several effector pathways, although details of downstream events have not been fully established (Figure 1.5). The most important is a classic stimulatory G-protein (G_s) pathway in which the following sequence of events is believed to occur [111]: i) activation of adenylate cyclase and accumulation of cAMP [90]; ii) cAMP leads to activation of protein kinase A; iv) protein kinase A in turn phosphorylates the cAMP responsive element binding protein.
(CREB)[177]; iii) CREB transactivates the pituitary-specific transcription factor (Pit-1) gene promoter [23, 113]; iv) Pit-1, a prototypical POU domain protein, transactivates the GH gene promoter and provides the final pathway in which GHRH stimulation leads to GH secretion [102, 103]. In addition, it is likely that Pit-1 also directly regulates the synthesis of the GHRH-R, in that the receptor is not expressed in the dw/dw variant dwarf mice. These mice lack functional Pit-1, and a cotransfected Pit-1 expression construct can activate the GHRH receptor gene promoter in transiently transfected CV1 cells [102, 103]. Although Pit-1 transcripts and protein are uniformly present in somatotroph and other types of pituitary adenomas, its role in somatotroph tumorigenesis, if any, is unsettled [105]. In agreement with the importance of this signaling system for normal growth, a transgene encoding a nonphosphorylatable mutant CREB protein, which blocks the function of the endogenous CREB protein, is able to cause somatotroph hypoplasia and dwarfism in mice when its expression is targeted to pituitary somatotrophs [177]. Moreover, human somatotroph adenomas uniformly express high levels of activated CREB, as compared with nonfunctioning pituitary adenomas, confirming that activated CREB is an important intermediate in the mechanism by which cAMP stimulates GH secretion and somatotroph proliferation [14].

The precise signaling mechanisms used by GHRH to induce somatotroph proliferation is less clear. Several lines of evidence indicate that many of the components of the above described pathway for GH secretion are also operational in the context of somatotroph proliferation. First, in states of pathologic GHRH excess, such as those occurring in humans with GHRH producing tumors, or in mice bearing the human GHRH transgene, hyperplasia of pituitary somatotrophs is a regular feature [4, 106, 159, 175]. That the mitogenic signal is transduced by the same GHRH-R is suggested by the fact that in the little mouse (lit), a dwarf variant with an inactivating missense mutation of the GHRH-R, the pituitary is hypoplastic with a 10-fold reduction in the somatotroph population [103]. Evidence that the signaling underlying somatotroph proliferation involves coupling of the GHRH-R to a stimulatory G-protein stems from two sources, both of which associate somatotroph proliferation with constitutive activation of Gs. The first is the GSP1 oncogene, activating mutations of which are found in 40% of somatotroph adenomas [89]; this is discussed in detail later in this chapter. Second, a cholera toxin transgene able to transactivate the alpha chain of (Gsα), when targeted to pituitary somatotrophs induces somatotroph hyperplasia in mice [Burton, 1991 #870]. Further downstream events leading to cell prolifera-
tion are not well characterized, although penultimate stages of the process, vis a vis early response gene induction, has been shown in GHRH stimulated somatotrophs in vitro [19, 20].

**Somatostatin (SRIF)**

Somatostatin is the principal negative regulator for the somatotroph, inhibiting both GH secretion and somatotroph proliferation. It is a phylogenetically ancient protein, whose gene sequence, structure, and presence has been conserved through 400 million years of evolution, being detectable in species ranging from the simplest protozoans to all vertebrates. The existence of somatostatin was first suspected in 1968 when Krulich et al, trying to demonstrate GH-releasing activity in hypothalamic extracts, unexpectedly identified portions of hypothalamic tissue that inhibited GH release [85]. They made the then novel, but ultimately correct proposal that GH secretion was subject to dual regulation, involving both stimulatory and inhibitory hypothalamic factors. At first, this concept was not readily accepted, however, its credibility was later confirmed with the characterisation of somatostatin in 1973 [25].

The term somatostatin was originally applied to the 14 amino acid cyclic peptide that was first purified [25]. Somatostatin peptides are now known to constitute a family of related molecules. These include the originally identified peptide, designated SRIF-14, an amino-terminal-extended somatostatin (SRIF-28), several species-specific variants, and larger prohormone forms (reviewed in [134, 148, 151, 166, 168]). Although SRIF is widely distributed throughout the body, the peptide relevant to somatotroph regulation is produced in the anterior periventricular nuclei of the hypothalamus. It is secreted in prohormone form, undergoing posttranslational processing to produce SRIF-14 and SRIF 28; the former is the dominant form in the hypothalamus, although both species are demonstrable. In the hypothalamic-somatotroph axis, the major stimulus for SRIF release is an increase in GH and IGF-1 levels. In response, SRIF descends via the portal circulation to inhibit the somatotroph.

The mechanisms by which SRIF exercises inhibitory control over GH secretion and somatotroph proliferation are complex and only partly understood. Part of the complexity stems from the fact that a family of SRIF receptors (termed SSTRs) exist. Molecular cloning has revealed a family of five structurally related SSTR subtype genes, each encoding for a receptor with a dis-
tinct SRIF agonist binding profile and each appearing to differ in their postreceptor coupling to effector systems [9, 29, 58, 70, 118, 135, 147, 154, 155]. A common feature, however, is that each receptor protein is coupled to a pertussis toxin-sensitive inhibitory G protein [135]. Of the five SSTRs (termed SSTR1-SSTR5), SSTR-1, SSTR2, SSTR3, and SSTR5 are present in the pituitary [9, 26]. As reviewed by Patel, the effects of SRIF in the pituitary and other sites appear to involve at least five distinct effector pathways. The first is an inhibitory G-protein pathway (Gi) resulting in inhibition of adenyl cyclase, counteraction of GHRH- mediated GH release, and additional downstream events directly opposite to those described for GHRH. Three effector pathways appear to directly inhibit GH secretion [163]. The first involves a subset of K+ channels, which are directly coupled to the receptor, once again via a GTP-binding, pertussis-toxin-sensitive protein putatively termed Gk [83]. Receptor activation leads to an opening of K+ channels, efflux of K+ ions, membrane hyperpolarization, and a secondary reduction in intracellular Ca++. The second is a direct inhibitory effect on voltage dependent Ca++ channels, again reducing intracellular Ca and GH secretion [73]. The third involves a 'distal' inhibition in the secretory response, by inhibiting hormone exocytosis [107, 206]. A final effector system involves tyrosine phosphatase activity, leading to dephosphorylation and inactivation of tyrosine kinases [28]; this action is believed to contribute to the antiproliferative actions of somatostatin.

Other factors

In addition to direct neuroendocrine regulation provided by GHRH and SRIF, and feedback inhibition provided by GH and IGF-1, a variety of other neurogenic (adrenergic, dopaminergic, and cholinergic tone), metabolic (hypo/hyperglycemia, fatty acid levels, renal and liver function) and hormonal (estrogens, glucocorticoids, thyroid hormones) factors also contribute to the regulation of GH secretion. The extent to which these factors directly influence the somatotroph versus an effect mediated through GHRH and SRIF is unclear; both mechanisms are likely.

Growth hormone-releasing peptides (GHRPs). Whereas the regulatory factors discussed so far primarily converge on the adenylate cyclase second messenger system, accumulating evidence during the past decade supports the existence of a regulatory cascade independent of adenylate cyclase. Several peptide analogues of met-enkephalin stimulate rhythmic secretion of GH. The
first identified was the GH-releasing hexapeptide hexarelin (GHRP-6, His-DTrp-Ala-Trp-DPhe-Lys-NH2). Thereafter, various non-peptidic analogues were also developed. The subject has recently been reviewed by Bowers [reference 29a]. These analogues do not bind to the GHRH-R and do not act by suppressing SRIF secretion or by activating adenylate cyclase in the somatotroph. GHRPs potentiate the stimulatory effects of GHRH and inhibit SRIF action on GH release. Although the natural ligand for this receptor remains to be identified, the recent demonstration of GHRP receptors in the pituitary and hypothalamus suggests the existence of an endogenous GHRP that may be involved with the physiological regulation of GH secretion [69a]. The role of GHRP pathways, if any, in the maintainence or progression of the neoplastic somatotroph is unknown.

SOMATOTROPH ADENOMAS

Historical Considerations

Acromegaly, a dramatic clinical syndrome of disordered somatic growth and proportion, and attributable to pathologic growth hormone excess, has intrigued physicians since the beginnings of recorded history. Human folklore and legend is punctuated with repeated and almost mythical reference to acromegalic individuals, who, given their large stature and distinctive physical appearance, were revered as keepers of great strength and power. Among the earliest and most well known individuals now suspected of being acromegalic was Goliath of Biblical reference (1 Samuel 17: 40-50). His unlikely defeat by the callow youth David has been reconciled with the supposition that Goliath had a large somatotroph adenoma which impinged on his optic apparatus, rendering him partially blind and vulnerable to David’s somewhat meagre assaults. A more concrete example of acromegaly through the ages, relates to the Egyptian King Akenhaton who ruled in the 14th century BC. His well known effigy, immortalized in a stone tablet, reveals the chiselled facies of acromegaly, complete with prognathism, enlarged nose and maxilla, and coarsening of facial features.

The term acromegaly, first introduced by Pierre Marie in 1886, derives from the Greek akron
and megas, meaning extremities and great, respectively [108, 109]. Whereas, Marie is rightfully credited with providing name and the original clinical description of the acromegalic state, the causal relationship between the latter and a pituitary tumor was not recognized at that time; the enlarged pituitaries encountered at autopsy of these patients were simply regarded as another feature of the generalized hypertrophy constituting the acromegalic state. In the following year, Minkowski reported another patient with acromegaly, and despite having no anatomic information about the patients pituitary gland, he proposed that the condition was somehow related to the pituitary [119]. Following Marie’s report, there was widespread interest in acromegaly and many case reports ensued. A lack of consensus with regard to its cause remained, however, with the role of the pituitary in the genesis of acromegaly being a particularly contentious issue. This quandary would remain until the report of Benda who, in 1900, recognized the enlarged pituitaries of acromegalics as being primarily of eosinophilic adenohypophyseal cells, which he regarded as being both neoplastic and hyperfunctioning [10]. Subsequent clinicopathologic studies by Cushing [34], Davidoff, and Bailey (reviewed in [36]), together with demonstrations that surgical resection of these eosinophilic masses resulted in regression of acromegalic symptoms and signs, reinforced the causal role of the pituitary in acromegaly. Later, Evans and Long demonstrated that gigantism could be produced in rats with injection of anterior pituitary extracts, confirming that some pituitary factor was responsible for growth induction [40]. These studies served to inaugurate a new era in endocrinology, as the establishment of an unequivocal link between a hyperfunctioning pituitary adenoma and acromegaly was the earliest example of a pituitary disorder to be clinically and pathologically recognized and appropriately treated.

**Acromegaly: A disorder of pathologic GH excess**

Having an annual incidence of 3 per 100,000 population, acromegaly is an uncommon condition. In the overwhelming majority of cases (>99%), acromegaly is the direct result of GH-secreting pituitary tumor. Of the few instances that remain, rare extrapituitary GHRH producing tumors will be the cause, producing hyperplasia of pituitary somatotrophs, GH excess, and an acromegalic state that is phenotypically identical to that caused by a somatotroph adenoma.

Pathologic GH excess will assume one of two related clinical phenotypes. The first, and more
common of the two is acromegaly, the result of GH excess that begins or persists beyond puberty. Should GH excess manifest prior to epiphyseal closure, the result is excessive linear growth or gigantism. Genetic predisposition in the form of the MEN-1 syndrome is evident in fewer than 5 percent of cases [129, 162], thus acromegaly will most commonly present in a sporadic fashion.

The clinical features of acromegaly are referable to either tumor mass effects or the endocrinologic sequelae of GH excess. Local mass effects, due to compression of surrounding neural structures by an expanding tumor, are of particular concern in acromegaly because the relative proportion of larger tumors (ie. macroadenomas) is substantially higher than that observed with any other hormonally active pituitary tumor [186]. Features of local mass effects include headache, visual loss, obstructive hydrocephalus, and cranial nerve palsies due to compression of nerves within the cavernous sinus. Still, endocrine manifestations are the most conspicuous feature of the disease, and provide the usual basis for presentation. Because GH has so wide a spectrum of physiologic action, the endocrine manifestations of acromegaly are correspondingly diverse. Major areas of pathologic involvement include changes in skin and connective tissues, abnormalities of musculoskeletal, cardiovascular, and respiratory systems, impaired glucose tolerance, and an increased risk for the development of gastrointestinal cancers. The diverse clinical manifestations of acromegaly have been detailed in a recent review [182].

Pathology of somatotroph adenomas

The pituitary tumors underlying acromegaly and gigantism are a heterogeneous group, one unified by pathologic hypersecretion of GH, but distinguished by their relative incidence, immunohistochemical profiles, ultrastructural morphology, and relevant differences in biological behavior. Five distinct adenomas have been causally related to GH hypersecretion [84]. These include the sparsely and densely granulated GH cell adenomas, the mammosomatotroph adenoma, the mixed GH cell-PRL cell adenoma, and the acidophil stem cell adenoma. A sixth group of tumors, also associated with GH hypersecretion, are the plurihormonal adenomas which co-secrete GH along with other anterior pituitary hormones. Details of the pathology of somatotroph adenomas can be found in several recent reviews from our laboratory [181, 185].
Whereas somatotroph adenomas frequently exhibit varying degrees of expansile, destructive and/or invasive local growth, the overwhelming majority are histologically benign adenomas. Truly malignant pituitary tumors (ie. pituitary carcinomas), a designation strictly reserved for those anterior pituitary tumors with demonstrated metastatic dissemination, are exquisitely rare [139].

**Predicting the behavior of somatotroph adenomas: A clinical problem**

The reliable prediction of pituitary tumor behavior remains one of the most inscrutable aspects of pituitary tumor biology. Current inabilities to gage the aggressiveness of these lesion and predicting their clinical course and/or therapeutic responsiveness represent significant barriers to the development of rational and comprehensive management protocols for these lesions. Were it possible to "grade" the intrinsic aggressiveness, therapeutic responsiveness and recurrence likelihood of any given somatotroph adenoma at the time of initial surgery, the need for, and the timing of postoperative surveillance, adjuvant pharmacotherapy, radiation therapy, and future surgical interventions could be better anticipated. At present, however, no such means exists.

Unlike most human neoplasms wherein histopathologic appraisal provides some insight into tumor behavior, and in some instances, may also influence choice of therapy, the same in not true for pituitary tumors. Morphologic markers correlated with aggressiveness in most other tumor systems, namely, nuclear pleomorphism, cytologic atypia, high cellularity, mitotic activity, and even necrosis, are not informative of pituitary tumor behavior [186, 187, 192]. These features are neither sufficiently frequent in aggressive pituitary tumors nor sufficient rare in clinically favorable variants to be of prognostic use. Electron microscopy, while essential to the appropriate classification of pituitary tumors, it is not, nor is it intended to be, a means of predicting behavior.

Given these prognostic limitations of morphologic analyses, a variety of alternative strategies have been evaluated to predict the behavior of pituitary adenomas. Most have centered on evaluation of proliferative activity, as determined by flow cytometry and various proliferation markers (bromodeoxyuridine labeling [126], Ki-67 [82, 91, 187], proliferative cell nuclear antigen (PCNA) [71], and argyrophil nucleolar organizing regions (AgNORs) [174]. Whereas some of
these studies have shown some relationship between proliferative activity and invasive growth. None have shown any clear prognostic relationship between proliferative activity and patient outcome, either in the short- or long term. Although p53 mutations have not been identified in pituitary adenomas, nuclear accumulation of p53 protein has been demonstrated in some aggressive pituitary tumors and appears to be associated with higher proliferative activity; still, no definite prognostic relationship has been demonstrated between p53 expression and patient outcome [27, 193]. A clear need exists for the development of clinically applicable and prognostically informative means of gauging neoplastic progression and aggressive behavior in these tumors.

**Therapy for somatotroph adenomas**

Therapeutic options for somatotroph adenomas include surgical resection, pharmacologic control with somatostatin analogs, and radiation therapy (conventional and stereotactic). Whereas each mode of therapy has specific indications and benefits, each is also associated with very significant limitations [190]. Surgical resection, which is the intervention of choice for most somatotroph adenomas, can induce remission in only 50-70% of cases [47, 48, 93, 157, 190, 196]. Surgical success is heavily dependent on the biology of the tumor; remission rates decline with increasing tumor size, invasiveness, and with increased preoperative GH levels. Furthermore, successful surgery is accompanied by eventual tumor recurrence in 6-10% of cases [95, 191].

Insofar as somatotroph adenomas are still responsive to the physiologic inhibition exercised by native SRIF, analogs of SRIF (which are pharmacologically more stable than native SRIF) represent a promising additional option for the treatment of somatotroph adenomas. While the great majority of patients (80-90%) experience some clinical and biochemical response to SRIF analogs, only about half of all patients will have sufficient reduction in serum GH levels to forestall the morbidity and premature mortality that accompanies somatotroph adenomas [44, 203, 204].

Radiation therapy, in view of its many adverse effects on normal tissues and the risk of inducing secondary neoplasia, is generally considered an adjuvant option of last resort. It is used when surgery and pharmacologic intervention have failed, or in the setting of tumor recurrence.
Despite the various treatment options, there remains a significant proportion of acromegalic patients for whom current management protocols are still suboptimal. In the majority of instances, these refractory cases will represent lesions that have progressed beyond limits of surgical curability due to their size or invasiveness. Improvements in the management of these tumors will essentially rest upon understanding and then pharmacologically targeting those subcellular pathways responsible for their maintenance, growth, and progression.

**SOMATOTROPH TUMORIGENESIS: CURRENT KNOWLEDGE**

In keeping with contemporary paradigms of human tumorigenesis, the development of somatotroph adenomas appears to be a multistep and multicausal process. In its most abbreviated form, the process begins with a tumor initiation phase, being sustained thereafter by a growth promotion phase \([3a, 114, 115, 116a]\). Given the constraints of existing knowledge, the specific events required to accomplish either component of the process are only superficially understood. What is known, however, is that hereditary predisposition, the acquisition of specific somatic mutations, aberrant intracellular signaling, and hypothalamic hypothalamic and growth factors may all serve as contributing factors in varying proportions of somatotroph adenomas.

**Transformation in the pituitary: The ‘hypothalamic’ and ‘pituitary’ hypotheses**

One of the most fundamental issues surrounding pituitary tumorigenesis relates to whether transformation in the somatotroph is primarily the product of hypothalamic dysfunction or whether it is the result of a transforming mutation intrinsic to an isolated adenohypophyseal cell. In extension of the physiologic control normally exercised by the hypothalamus over the secretory and proliferative activities of adenohypophyseal cells, the hypothalamic hypothesis suggests that pituitary adenomas arise as the eventual, downstream, and seemingly passive consequence of aberrant trophic influences emanating from a dysfunctional hypothalamus. It implies an excess of stimulatory and/or a deficiency of inhibitory hypophysiotropic hormones which, in the case of somatotroph adenomas, would take the form of an excess of hypothalamic GHRH and/or a deficiency of hypothalamic SRIF, or an altered responsiveness to these hormones. Historically,
hypothalamic dysfunction was considered an important, if not a necessary mechanism of transformation in the pituitary, a view well supported by early animal experimentation [53] and one that continues to maintain some clinical and conceptual support [150]. Alternatively, the pituitary hypothesis suggests that pituitary adenomas arise as the direct result of an intrinsic pituitary defect (i.e., somatic mutation) occurring at the level of a single, susceptible adenohypophyseal cell, with neoplastic transformation occurring in relative autonomy of hypothalamic trophic influence. Whereas substantial clinical and experimental evidence exists in support of both possibilities, the denovo pituitary concept has been especially favored in view of the lack of peritumoral hyperplasia in association with pituitary adenomas and because some pituitary tumors can be definitively "cured" when completely removed surgically. Neither of these would be expected were hypothalamic overstimulation the dominant tumorigenic mechanism.

Further strengthening the idea that pituitary adenomas, including somatotroph adenomas, result form somatic mutations that occur at the level of a single, susceptible, adenohypophyseal cell have been reports concerning their clonal composition [18, 66, 74, 164]. Using the strategy of allelic X-chromosome inactivation analysis which assesses restriction fragment length polymorphisms and differential methylation patterns in various X-linked genes, several independent laboratories have confirmed a monoclonal composition for virtually all pituitary adenomas. Validation of the monoclonal nature of pituitary adenomas has been an important conceptual advance for it has established pituitary adenomas as monoclonal expansions of a single, somatically mutated, and transformed adenohypophyseal cell. Were abnormal hypothalamic influences the dominant initiating event, then a population of anterior pituitary cells should simultaneously be affected and a polyclonal tumor would be the expected result; this has not been the case.

Whereas the finding of monoclonality in somatotroph adenomas argues against a primary hypothalamic role in somatotroph transformation, it does not eliminate a role for hypophysiotropic hormones in somatotroph adenomas, particularly with regard to tumor maintenance and/or progression. In this context, it may be too confining and too simplistic to accept one hypothesis over the other in a mutually exclusive fashion. Elements of both hypotheses may be applicable to different aspects of somatotroph tumorigenesis. This concept is further developed in chapter 2.
Multistep model of somatotroph tumorigenesis

Having established a monoclonal composition for somatotroph adenomas, attention has since turned to characterizing the responsible mutations underlying initiation and progression in this tumor system. A number of genomic alterations have been identified to date. These include activating mutations of one oncogene (GSPT1), inactivation of one tumor suppressor gene (MEN-1), as well as several alterations at other genomic loci. Some of these are regarded as initiating events, whereas others appear to be associated with neoplastic progression. An overview of the process is illustrated in Figure 1.6.

![Diagram](image)

**Figure 1.6 Multistep model of somatotroph tumorigenesis.** The events currently considered important to somatotroph tumorigenesis and their presumed temporal occurrence are depicted. This theoretical model assumes a linear progression from indolence to aggressive behavior, but this may not be the case. Biologically aggressive tumors may be aggressive from the outset and may not require staged escalation from indolence. Still, the model is useful in providing some context to data from the literature. If hypothalamic hormones contribute to the process, they most likely do so in the growth/progression of the transformed clone.

Initiating events

To date, only two genomic alterations have been identified as being consistently transforming in the human somatotroph. The first is a deactivating mutation of the multiple endocrine neoplasia (MEN-1) tumor suppressor gene and the second is activating mutations of the GSPT1 onco-
gene. A third event, also appearing early in somatotroph tumorigenesis relates to functional inactivation of the CDK inhibitor p16.

**MEN-1 tumor suppressor gene** Genetic predisposition to pituitary tumor development is restricted to a single and uncommon condition, the MEN-1 (multiple endocrine neoplasia type 1) syndrome. Approximately 3 percent of all pituitary adenomas occur in this context. An autosomal dominant condition, the MEN-1 syndrome is characterized by the development of tumors involving the parathyroid glands, pancreatic islet cells, and the pituitary. As the disorder is variably penetrant, only 25 percent of patients develop pituitary tumors, the majority being macroadenomas associated with GH and/or PRL hypersecretion [129, 162]. The nature of the genetic defect in MEN1 involves allelic loss of a putative tumor suppressor gene at the 11q13 locus [30, 61]. In its recessive behavior, the MEN-1 gene is typical of a tumor suppressor gene, with susceptible individuals inheriting a germ line mutation of one of the two 11q13 alleles. Subsequent spontaneous mutation, inactivation or deletion of the remaining normal 11q13 locus in susceptible endocrine tissues ultimately leads to tumor formation in the involved tissue. Loss of heterozygosity at the MEN-1 locus has been demonstrated in the majority of parathyroid tumors removed from MEN-1 patients [49], in at least 25% of sporadic parathyroid adenomas [65], and in pancreatic islet cell tumors [92].

Once believed to be a genetic defect accounting for pituitary adenomas occurring exclusively in the context of MEN-1, several recent studies have also demonstrated loss of the 11q13 locus in seemingly sporadic pituitary adenomas as well. In the earliest of these, allelic deletions of 11q13 were found in two of three sporadic prolactin-producing pituitary adenomas [30]. Subsequently, four of 12 sporadic somatotroph adenomas were found to have deletions involving the 11q13 locus [180]. More recently, allelic deletions of chromosome 11 were found in 18% of pituitary adenomas of all major types, including 16% of somatotroph adenoma [24]. Collectively, these data suggest that the 11q13 locus may be the site of an important tumor suppressor gene, the inactivation of which may be of pathogenetic relevance to the development of some sporadic and MEN-1 related somatotroph adenomas. Of addition relevance was the recent observation of Bates et al (1997), wherein loss of heterozygosity at the 11q13 locus was seen to be significantly more frequent among invasive as compared to noninvasive pituitary adenomas. This finding suggests that allelic loss of 11q13 may not only be relevant to the genesis of pituitary tumors,
but it also may have some role in the progression of some pituitary tumors as well. Again, it remains to be determined whether the precise locus on 11q13 is the MEN-1 gene or is simply adjacent to it; recent evidence favors the latter [5a, 49a].

**GSPN1 oncogene:** The only consistent evidence favoring oncogene activation as a transforming mechanism in the pituitary stems from the discovery and characterization of the GSPT1 oncogene, an oncogene first identified in somatotroph adenomas [89, 90, 201]. The signal transduction cascades governing the secretory and proliferative functions of pituitary somatotrophs converge on the adenylate cyclase second messenger system. In the normal state, the hypothalamic hormone GHRH, is the principal positive regulator of somatotroph proliferative and secretory functions. After binding to its membrane receptor on the somatotroph cell surface, the GHRH proliferative signal is coupled to a stimulatory heterotrimeric G-protein (Gs) that binds GTP and activates adenylate cyclase. Resultant intracellular cAMP elevations initiate a series of downstream events that ultimately lead to GH secretion and somatotroph proliferation (Figure 1.5). Because one structural component of Gs, known as the alpha chain (Gsα), maintains intrinsic GTPase activity, adenylate cyclase activation is normally a self-limiting, transient, and tightly regulated event. After transducing the signal, Gsα hydrolyzes GTP and returns Gs to its inactive state, terminating the trophic signal. Activating mutations of GSPT1 are the result of point mutations in the Gsα gene, involving either arginine 201 (replaced by cysteine or histidine) or glutamine 227 (replaced with arginine or leucine). These residues are critical to normal GDP/GTP binding. Mutations at these sites result in a protein with deficient GTPase activity, one that constitutively activates Gsα, converting the latter to an oncogene (GSPT1). Such mutant forms of Gsα stabilize Gs in its active configuration, mimicking the trophic effects of persistent GHRH action. Since somatotrophs bearing this mutation bypass the normal somatotrophs requirement for GHRH ligand-mediated receptor activation, chronic elevations of cAMP ensue, providing an autonomous capacity for cell proliferation, GH secretion, and ostensibly, neoplastic transformation.

Whereas in North American and European studies, activating mutations of GSPT1 have been reported in approximately 40% of somatotroph adenomas, in Japan, such mutations are rare events. [212]. In neither geographic setting, however, do they appear to confer any significantly distinctive clinical, behavioural, biochemical, or radiological characteristics to the tumor. In
one report, tumors exhibiting GSPT1 mutations occurred in older patients, were smaller, and had lower basal GH levels than wild type tumors, although this has not been uniformly observed [89, 116]. They may also be more responsive to SRIF analog therapy [6a].

**p16:** The cyclin-dependent kinase (CDK) inhibitor p16, by binding to or sequestrating CDK4, prevents the phosphorylation of Rb (reviewed in [140-143]). Loss of p16 results in a shift in the phosphorylation status of pRb, negating its ability to regulate the cell cycle. Woloschak et al studied mRNA and protein levels of p16, the specific inhibitor of CDK4, in a series of 25 human pituitary tumors and 10 normal pituitary specimens [208]. Whereas p16 protein was readily detected in all normal pituitary specimens by Western analysis, it was undetectable in all pituitary tumors. Correspondingly, mRNA levels were low in pituitary tumors as compared to the normal gland. Reduction in p16 expression was not the result gene loss or mutation. Instead, methylation within the exon 1 5’CpG island of p16 appears to be the mechanism by which the gene is silenced in pituitary tumors [207], although this appears infrequent among somatotroph adenomas [169a]. In that relatively few somatotroph tumors have been studied to date, the role of p16 in somatotroph tumorigenesis is unsettled. Still, since its diminished expression is present in all pituitary tumors, both aggressive and indolent, suggests that it is an early event.

A second negative regulator of the cyclin-CDK complex is p27. Targeted disruption of the p27 coding region in transgenic mice resulted in p27 knockout mice that were larger, had multiorgan hyperplasia, and hyperplasia of the pars intermedia of the pituitary which, and after 10 weeks, also developed benign intermediate lobe pituitary tumors; none of these tumors were somatotrop in nature and serum GH and IGF-1 levels were comparable to controls [50, 79, 127]. At the present time, the role of p27 inactivation in human pituitary tumors is unknown, although reduction in its expression has been demonstrated in somatotroph adenomas [5b].

**Growth promoting events**

Several genomic alterations in somatotroph adenomas have been reported as being associated with neoplastic progression. Their common feature is that they all tend to occur more frequently among grossly invasive adenomas, as compared to noninvasive adenomas. Whereas these alterations are regarded as ‘intermediate’ or ‘late’ events in somatotroph tumorigenesis, the pre-
one report, tumors exhibiting GSPT1 mutations occurred in older patients, were smaller, and had lower basal GH levels than wild type tumors, although this has not been uniformly observed [89, 116]. They may also be more responsive to SRIF analog therapy [6a].

**p16:** The cyclin-dependent kinase (CDK) inhibitor p16, by binding to or sequestrating CDK4, prevents the phosphorylation of Rb (reviewed in [140-143]). Loss of p16 results in a shift in the phosphorylation status of pRb, negating its ability to regulate the cell cycle. Woloschak et al studied mRNA and protein levels of p16, the specific inhibitor of CDK4, in a series of 25 human pituitary tumors and 10 normal pituitary specimens [208]. Whereas p16 protein was readily detected in all normal pituitary specimens by Western analysis, it was undetectable in all pituitary tumors. Correspondingly, mRNA levels were low in pituitary tumors as compared to the normal gland. Reduction in p16 expression was not the result gene loss or mutation. Instead, methylation within the exon 1 5'CpG island of p16 appears to be the mechanism by which the gene is silenced in pituitary tumors [207], although this appears infrequent among somatotroph adenomas [169a]. In that relatively few somatotroph tumors have been studied to date, the role of p16 in somatotroph tumorigenesis is unsettled. Still, since its diminished expression is present in all pituitary tumors, both aggressive and indolent, suggests that it is an early event.

A second negative regulator of the cyclin-CDK complex is p27. Targeted disruption of the p27 coding region in transgenic mice resulted in p27 knockout mice that were larger, had multiorgan hyperplasia, and hyperplasia of the pars intermedia of the pituitary which, and after 10 weeks, also developed benign intermediate lobe pituitary tumors; none of these tumors were somatotropin in nature and serum GH and IGF-1 levels were comparable to controls [50, 79, 127]. At the present time, the role of p27 inactivation in human pituitary tumors is unknown, although reduction in its expression has been demonstrated in somatotroph adenomas [5b].

**Growth promoting events**

Several genomic alterations in somatotroph adenomas have been reported as being associated with neoplastic progression. Their common feature is that they all tend to occur more frequently among grossly invasive adenomas, as compared to noninvasive adenomas. Whereas these alterations are regarded as 'intermediate' or 'late' events in somatotroph tumorigenesis, the pre-
cise temporal occurrence in the evolution of somatotroph adenomas has not been established. The most important of these include loss of heterozygosity of 11q13 (distinct from the MEN-1 gene), 13q12-14 (distinct from the Rb locus), and 10q26 [8, 24, 137]. In a recent series of 89 pituitary adenomas, of which 11 were somatotroph adenomas, one of more of these alterations was identified in 27% of tumors [8].

Although less intensely studied, two additional alterations associated with neoplastic progression in somatotroph adenomas have been described. Alvaro et al. described a conserved point mutation of the alpha isoform of the protein kinase-C gene (PKCα), an alteration they believed to be specific to invasive adenomas [1, 2]. The second involved expression studies of the purine-binding factor (nm23) gene. A putative tumor suppressor gene, nm23 is expressed at reduced levels in several high grade human malignancies, including metastasizing carcinomas of breast, colorectal, and hepatic origin [17]. Messenger RNA expression of the gene was recently studied in a series of 22 pituitary adenomas. In this report, expression of the H2 isoform and HR protein were significantly reduced in invasive adenomas, suggesting a possible relationship between altered expression of this gene and aggressive tumor behavior. In none of these tumors, however, were structural alterations of the nm23 gene demonstrable [178].

During the past decade, there has been a flurry of reports promoting a role for various growth factors in pituitary tumorigenesis (reviewed in [167, 185]). Most of the better known growth factors, including the family of fibroblast growth factors, EGF/EGFR, and transforming growth factor alpha have been implicated to varying degrees in pituitary tumor development, however, no conclusive link between these or any other growth factor system and somatotroph adenomas has been demonstrated.

**HYPOPHYSIOTROPIC HORMONES AND SOMATOTROPH ADENOMAS**

From the foregoing it is clear that a variety of genomic alterations can occur in human somatotroph adenomas. In actual fact, however, only a minority of somatotroph adenomas will harbour any of the aforementioned genomic alterations. Aside from the 40% frequency of activating mutations of GSP1, no other genomic changes have been identified with any regularity in sporadic somatotroph adenomas. This suggests the presence of additional, and as yet unidenti-
fied genomic alterations, and/or the occurrence of other subcellular events and tumorigenic mechanisms independent of gene mutation/loss.

From the standpoint of neoplastic maintenance and progression, one especially attractive mechanism, given the functionality and endocrine nature of somatotroph adenomas, has been that these tumors may be subject to aberrant autocrine/paracrine regulation [77, 138]. Indeed, the potential existence of stimulatory autocrine/paracrine regulatory circuits within these tumors has been repeatedly invoked, with numerous growth factors, hormones, cytokines, and trophic factors having been proposed as potential autocrine/paracrine regulators (reviewed in [181, 185]. Still, conclusive proof of aberrant autocrine/paracrine signalling that would be of biologic, clinical, or prognostic relevance has not been forthcoming in this tumor system.

Were aberrant autocrine/paracrine regulation a mechanism in somatotroph tumorigenesis, its most plausible role would be during the growth promotion phase of an already transformed clone. Given their preeminent role in the regulation of the normal somatotroph, the hypophysiotropic hypothalamic hormones GHRH and SRIF would represent especially strong candidates as potential autocrine/paracrine mediators/modifiers of neoplastic progression in these tumors. In extension of their physiologic functions in regulating the normal somatotroph, several lines of evidence indicate that hypophysiotropic hormones may have some role in somatotroph tumorigenesis. The earliest direct evidence for such a role stemmed from the observation that in states of pathologic GHRH excess, such as those occurring with rare GHRH producing tumors (eg. pancreatic islet cell tumors, carcinoid tumors), chronic GHRH stimulation leads to hyperplasia of pituitary somatotrophs, GH hypersecretion, and clinical acromegaly. Depending on the duration of exposure to GHRH excess, progression from somatotroph hyperplasia to adenomatous transformation has been documented in some instances [159]. Along the same lines, the finding that rare GHRH-containing hypothalamic gangliocytomas have been identified in association with a somatotroph adenomas, raises the possibility of an inductive effect of the former on the development of the latter, presumably by way paracrine GHRH stimulation [3, 68, 181, 200]. A parallel, but more consistent phenomenon has been demonstrated in transgenic mice overexpressing the human GHRH transgene. That these animals develop gigantism, elevated GH levels, somatotroph hyperplasia, and eventually, somatotroph adenomas, provide compelling and conclusive evidence of the tumor promoting properties of GHRH [4, 106, 175].
Although less rigorously studied, there is also support for SRIF as a negative autocrine regulator under experimental conditions [146, 155].

In proposing a possible autocrine/paracrine role for GHRH and SRIF in somatotroph adenoma progression, two minimum criteria must be fulfilled: (i) evidence of intratumoral GHRH and SRIF synthesis; and (ii) evidence of tumoral expression and functionality of the corresponding receptors. Both appear to be fulfilled in this tumor system. First, several investigators have, in a relatively small number of cases, demonstrated the presence of GHRH and SRIF mRNA transcripts and/or protein within somatotroph adenomas [78, 98, 100, 109a, 124, 132, 133, 145, 205]. Second, abundant evidence indicates that, even when transformed to the neoplastic phenotype, the somatotroph retains receptors for, and responsiveness to GHRH and SRIF stimulation. With regard to the former, patients with somatotroph adenomas continue to release GH in response to repeated GHRH injection whereas normal patients do not; among the latter, a marked refractoriness to repeat GHRH provocation is encountered [171]. The same lack of receptor desensitization has also been demonstrated by somatotroph adenomas in vitro [170]. Evidence for preservation of receptor functionality is even stronger for SRIF. Not only, can SSTRs be demonstrated in somatotroph adenomas in vivo using SSTR scintigraphy, but functional preservation of the SRIF pathway is the basis for pharmacologic control of these tumors, in the form of SRIF-analogs [6, 9, 42-44, 47, 55, 58, 59, 86, 87, 134, 135, 147, 154, 155, 209]. Having fulfilled these criteria, it would appear that the neoplastic somatotroph is, at the very least, sufficiently configured to engage in GHRH and SRIF mediated autocrine/paracrine regulation.

Whereas the foregoing observations do suggest a potential role for GHRH and SRIF mediated autocrine/paracrine regulation in somatotroph adenomas, neither the clinicopathologic nor the prognostic significance of these circuits have been systematically examined. To a large extent, this reflects inherent difficulties in studying functional circuits in a human tumor for which neither a representative experimental model nor a replication competent in vitro assay system exist. Despite these constraints, a number of fundamental questions can be answered concerning the presence of GHRH and SRIF mRNA transcripts/protein in somatotroph adenomas. Foremost among these are the biologic, clinicopathologic, and prognostic correlates of GHRH and SRIF expression within human somatotroph adenomas. These questions are comprehensively
addressed in this thesis. A key objective is to determine whether differences in the biological behavior of these tumors are reflected in the intratumoral accumulation of GHRH and SRIF mRNA transcripts, and if so, whether the latter can be used to predict the former in a manner that is meaningful, biologically and clinically relevant, and most importantly, one that is generalizable to other populations of somatotroph adenomas. Finally, to better understand the mechanism of action and subcellular effects of SRIF on somatotroph adenomas, both as a therapeutic agent and as a potential autocrine/paracrine mediator, the effects of the preoperative SRIF-analog therapy on the tumoral proliferative activity and GHRH and SRIF mRNAs are evaluated.
HYPOTHESIS

Alterations in the patterns of GHRH and SRIF mRNA transcript accumulation in human somatotroph adenomas are associated with clinically and prognostically relevant differences in tumor behavior.

Specific aims

1. To determine the biologic, clinicopathologic and prognostic significance of GHRH mRNA transcript accumulation in human somatotroph adenomas (Chapter 2).

2. To determine the biologic, clinicopathologic and prognostic significance of SRIF mRNA transcript accumulation in human somatotroph adenomas (Chapter 3).

3. To develop univariate and multivariate models of postoperative outcome based on the levels of GHRH and SRIF mRNA transcripts in somatotroph adenomas and test these models in a secondary population of acromegaly patients (Chapters 2-4).

4. To evaluate the effects of presurgical SRIF analogs on i) tumor cell proliferation (Chapter 5); and ii) on GHRH and SRIF mRNAs in somatotroph adenomas (Chapter 6).

Figure 1.7 Hypothesis. Somatotroph adenomas assume the capacity to produce GHRH and SRIF, the mRNA levels of which are associated with clinical and prognostically relevant differences in tumor behavior.
Study design

Primary study population
100 surgically treated somatotroph adenomas
(consecutive series, Wellesley Hospital)

Part 1

GHRH analysis
1. Evaluate GHRH mRNA distribution by ISH (n = 100)
2. Confirm results with Northern/RTPCR
3. Confirm protein translation (ICC / western blotting)
4. Evaluate relationship between distribution of GHRH mRNA by ISH with cellular localization of immunoreactive GH
5. Quantify GHRH mRNA signal by densitometry
6. Determine Ki-67 tumor growth fraction
7. Determine clinicopathologic correlates of GHRH transcript accumulation

SRIF analysis
1. Evaluate SRIF mRNA distribution by ISH (n = 100)
2. Confirm results with Northern analysis
3. Confirm protein translation (ICC / western blotting)
4. Evaluate relationship between distribution of SRIF mRNA by ISH with cellular localization of immunoreactive GH
5. Quantify SRIF mRNA signal by densitometry
6. Determine clinicopathologic correlates of SRIF transcript accumulation

Develop univariate and multivariate outcome models

Test models in
Secondary test population
random sample of 30 surgically treated somatotroph adenomas
(Mayo Clinic)

Part 2

32 somatotroph adenomas
(Part of multicenter randomized trial of presurgical SRIF-analog therapy)

Octreotide treated (n = 16)
No octreotide (n = 16)

In each group:
1. evaluate Ki-67 tumor growth fraction (n = 16)
2. evaluate GHRH and SRIF mRNAs by ISH (n = 10)
3. evaluated GHRH protein by Western analysis in SRIF analog treated group (n = 4)

Figure 1.8 Flow diagram outlining the study design.
CHAPTER 2  GHRH mRNA transcript accumulation in somatotroph adenomas

Summary
The clinical behavior of growth hormone (GH) producing pituitary tumors is known to vary greatly; however, the events underlying this variability remain poorly understood. Herein we demonstrate that tumoral accumulation of the growth-hormone releasing hormone (GHRH) mRNA transcripts is one prognostically informative event associated with the clinical aggressiveness of somatotroph pituitary tumors. Accumulation of GHRH mRNA transcripts was demonstrated in 91 of a consecutive series of 100 somatotroph tumors by in situ hybridization; these findings were corroborated by northern analysis, reverse transcriptase polymerase chain reaction, and protein translation was confirmed by Western blotting. By comparison, transcript accumulation was absent or negligibly low in 30 normal pituitary glands. GHRH transcripts were found to preferentially accumulate among clinically aggressive tumors. Specifically, GHRH mRNA signal intensity was: (i) linearly correlated with ki-67 tumor growth fractions \( r = 0.71, p < 0.001 \); (ii) linearly correlated with preoperative serum GH levels \( r = 0.56, p = 0.01 \); (iii) higher among invasive tumors \( p < 0.001 \); and (iv) highest in those tumors in which postoperative remission was not achieved \( p < 0.001 \). Using multivariate logistic regression, a model of postoperative remission likelihood was derived wherein remission was defined by the single criterion of suppressibility of GH levels to less than 2ng/ml during an oral glucose tolerance test. In this outcome model, GHRH mRNA signal intensity proved to be the most important explanatory variable overall, eclipsing any and all conventional clinicopathologic predictors as the single most significant predictor of postoperative remission; increases in GHRH mRNA signal were associated with marked declines in remission likelihood. The generalizability of this outcome model was further validated by the model's significant performance in predicting postoperative remission in a random sample of 30 somatotroph tumors treated at another institution. These data indicate that GHRH transcript accumulation is an event associated with the neoplastic progression and clinical aggressiveness of somatotroph adenomas. More generally, these data merge essential elements of the "hypothalamic" and "pituitary" hypotheses of pituitary tumorigenesis, providing for a more unified concept of neoplastic progression in the pituitary.
INTRODUCTION
The pituitary tumors underlying acromegaly, although unified by their pathologic hypersecretion of growth hormone (GH), are an otherwise heterogeneous group of lesions. From biological, clinical, and prognostic standpoints, the behavior of these tumors tends to be highly variable and generally defies reliable prediction. Whereas some somatotroph adenomas are amenable to curative resection, others will progress relentlessly, often in spite of maximal surgical, pharmacologic, and radiotherapeutic intervention [186]. It is recognized that some 80% of GH secreting adenomas will have progressed to a macroadenoma stage when detected, and half of these will be grossly invasive of surrounding neurovascular or bony structures [120, 125, 161]. Curative resections can be achieved by experienced surgeons in only 55-65% of all somatotroph adenomas [48, 94, 157]. In the remainder, it is usually tumor invasiveness which precludes complete excision, and for these tumors symptomatic regrowth and persistent hormone hypersecretion are virtually guaranteed [95, 182, 191]. The tendencies of some somatotroph adenomas toward aggressive, invasive, or recurrent growth, although neither reflected in nor predicted by the tumor’s histologic or ultrastructural morphology, is presumably the result of specific subcellular events that promote neoplastic progression among aggressive variants. To date, however, prognostically informative determinants of neoplastic progression remain poorly characterized in these tumors.

Growth hormone-releasing hormone (GHRH), a hypothalamic peptide and mitogen, is the principal positive regulator for GH-producing cells (somatotrophs) of the pituitary [111, 195]. Following its release from hypothalamic nuclei and subsequent descent to the anterior pituitary via the portal circulation, GHRH binds to its receptor (GHRH-R) on the somatotroph cell surface, stimulating both the proliferation of these cells and their secretion of GH. A logical extension of such trophic physiologic activity has been the implication that excessive GHRH stimulation may play a role in somatotroph tumorigenesis, particularly from the standpoint of neoplastic progression. In support of this possibility has been a growing body of evidence indicating that somatotroph adenomas may themselves be a local source of GHRH production. In this regard, several investigators have documented the presence of GHRH mRNA transcripts and/or immunoreactive GHRH within, as well as in-vitro GHRH secretion by somatotroph adenomas [78, 98, 124, 145, 205]. Whereas these observations raise the possibility of GHRH mediated autocrine/paracrine stimulatory loops within somatotroph adenomas, neither the clinicopatho-
logic nor prognostic significance of such local GHRH expression has been systematically examined. Moreover, the important question of whether locally produced GHRH can promote neoplastic progression in somatotroph adenomas and account for the aforementioned variability in their clinical behavior remains unresolved.

The present work evaluates the hypothesis that tumoral overexpression of the GHRH gene represents an event in the progression of GH-producing pituitary tumors, one associated with aggressive endocrinologic and oncologic behavior, and a poorer surgical outcome.

**MATERIALS AND METHODS**

**Overview of research design**
A consecutive series of 100 GH-producing pituitary tumors were screened for expression of the GHRH gene by in situ hybridization (ISH). To determine the biologic and clinical relevance of such expression, the degree of GHRH transcript accumulation was quantified and correlated with pertinent clinicopathologic parameters including, tumor invasion, preoperative GH level, tumor morphology, tumor growth fraction, and postoperative remission status. To explore further the relationship between GHRH mRNA transcript accumulation and surgical outcome, particularly as it compared to other clinicopathologic predictors, multivariate modelling was used to fit a logistic regression model of postoperative remission likelihood. The stability and reproducibility of this model were then tested in a second, randomly selected, and comparable cohort of 30 acromegaly associated pituitary tumors treated at another institution.

**Clinical material**
Of 114 consecutive acromegaly-associated pituitary tumors operated upon at the Wellesley Hospital (Toronto, Canada) between 1974 and 1991, tumor samples and clinical data of 100 cases were available for inclusion in this study. Included were 59 men and 41 women with a median age of 44.5 years (range 17-70 years). All patients had been subject to a uniform management protocol. Each had been evaluated by a single endocrinologist (DK), operated upon by a single neurosurgeon (HS), and each having had their tumors pathologically characterized by a single pathologist (KK). These 100 patients and their tumors comprised the primary study set upon which ISH analysis, clinicopathologic correlations, and statistical modelling were per-
formed. The derived outcome model was then tested in a comparable cohort of 30 randomly selected acromegalic patients managed at Mayo Clinic, Rochester, Minnesota between 1980-1987. Each patient had been subjected to a uniform endocrine evaluation, was operated upon by the same neurosurgeon (ERL), and each tumor was pathologically characterized by the same team of pathologists (BWS,KK).

Tumor samples and control tissues

Three categories of pituitary tissue were used in this study:

(i) The main group of tumors studied were those from the primary (Toronto) and secondary (Mayo) study populations. Consisting of 100 and 30 somatotroph adenomas, respectively, all tumors were fully characterized on the basis of their histology, immunohistochemical profile, and ultrastructure. All subtypes of growth-hormone producing adenomas were represented. These archived tissues, all of which had been formalin-fixed and paraffin embedded at the time of surgery, were used for ISH studies.

(ii) As control tissues for ISH, formalin fixed and paraffin embedded specimens of normal pituitary gland were used; these were obtained from two sources. The first included 20 normal autopsy pituitary glands, all of which were obtained from patients who died of non-endocrine causes and in whom glands could be retrieved within 12 hours of death. Because of potential mRNA degradation associated with the delay in procuring autopsy pituitaries, 10 surgical nontumorous pituitary specimens were included as a secondary control group. These specimens consisted of morphologically normal peritumoral tissue adjacent to either corticotroph adenomas (n=6), somatotroph adenomas (n=2), or prolactin cell adenomas (n=2).

(iii) A third group of pituitary tissues, each consisting of a freshly frozen fragment and a formalin-fixed fragment, were also studied. Included in this group were 10 somatotroph adenomas and 1 autopsy pituitary gland (obtained within 2 hours of death). In these specimens, ISH, northern analysis, reverse transcriptase polymerase chain reaction (RT-PCR), western analysis, and GHRH immunohistochemistry were all performed in each case. The purpose of these analysis was to: (i) confirm the results of ISH with other methods of detecting GHRH mRNA transcripts; (ii) to verify that the probe used for ISH was identifying mRNA transcripts of appropriate size;
(iii) to evaluate and quantify the relationship between GHRH mRNA levels as determined by ISH and northern analysis; and (iv) to demonstrate GHRH protein, thereby confirming translation of GHRH mRNA transcripts.

**In-situ hybridization (ISH)**

**GHRH probe** The human GHRH probe used for both ISH and Northern analysis was a 30mer antisense oligonucleotide probe (5-GTT GGT GAA GAT GGC ATC TGC ATA CCG CCG-3') derived from exon 3 (nucleotides 177-206) of the consensus cDNA sequence [112]. It was radiolabeled by the 3'-end labeling method using 35S deoxyadenosine 5'-triphosphate and a commercially available kit (Dupont, Missisauga, Canada). Radiolabeled antisense oligonucleotides were purified by affinity chromatography, from which labeled probes of high specific activity were eluted. The optimal specific activity for the probe, determined by a series of preliminary dilution experiments, was 1.0 x 10^6 cpm.

**In situ hybridization protocol** ISH was performed on 5μm slide mounted tissue sections. Details of the technique, including specifics of probe labeling and purification, prehybridization, overnight hybridization, and posthybridization treatments have been outlined in previous reports from our laboratory [176, 184, 194]. After liquid emulsion autoradiography (Kodak NTB2 emulsion) and a 1-week exposure time, slides were developed, fixed, rinsed, stained with hematoxylin, dehydrated, and coverslipped.

To evaluate the relationship between GHRH mRNA distribution and GH content at the level of the individual tumor cell, combined ISH for GHRH and immunohistochemistry for GH were performed on the same tissue section. The streptavidin-biotin peroxidase-complex method was used, being performed after posthybridization washes.

**Quantification of GHRH mRNA signal** In all adenomas, GHRH mRNA signal intensity was quantified by manual densitometry. The number of silver grains in all tumors cells present in each of 20 randomly selected high power fields were counted. Within each field, the silver grain content of every cell was specifically enumerated using oil immersion microscopy (x1000). A signal intensity index, representing the mean number of silver grains per cell, was
determined in each case. All counts were performed by a single experienced cytotechnologists blinded to clinical or other details of the case.

In quantifying the ISH signal in the nontumorous control glands there were additional methodologic considerations. The objective was to specifically enumerate the number of silver grains in normal somatotrophs. In the autopsy pituitary glands, this was facilitated by using horizontal sections and only evaluating those acidophilic cells in the lateral wings of the gland, a region primarily occupied by somatotrophs. In peritumoral surgical fragments, such topographic orientation was not possible, and serial GH immunostained sections were required to facilitate somatotroph localization.

**ISH control procedures** To exclude the possibility of nonspecific probe binding, 2 standard control procedures were performed in tandem with the ISH protocol. The first involved predigestion of tissue sections in 100 μg/mL of RNAse, and the second was a competitive hybridization assay wherein 20 fold excess unlabelled probe was added to the hybridization mixture. By both methods, the hybridization signal was effectively eliminated or reduced to negligible background level (< 5 silver grains / cell). One or both of these control procedures were performed in all tumors.

In total, 171 specimens were studied by quantitative ISH (ie. 100 tumors of the Toronto series; 30 tumors of Mayo Clinic series; 20 autopsy pituitaries; 10 surgical nontumorous pituitaries; and 11 specimens for corroboration with Northern / Western / PCR analysis). Once the experimental conditions were established, ISH was performed in a batch fashion, with a series of 9 separate batches or "runs" being required to complete all specimens. To ensure that batch-to-batch variability was not a confounding factor, 6 specimens (3 autopsy control pituitaries and 3 somatotroph adenomas) served as internal controls, a slide of each having been included in all 9 batches. After GHRH mRNA signal intensity was quantified for each of these specimens in each of 9 batches, batch-to-batch variability was assessed in two ways. First, a coefficient of variation was determined for each control sample. Individually, the value of these coefficients ranged from 0 to a maximum of 6.9%, indicating strong consistency from one batch to the next for each of the controls. Second, when the mean GHRH mRNA signal intensity of all controls in each of 9 batches were compared, no significant differences were found between batches (one way
ANOVA, P = 0.95).

Northern analysis

This analysis was performed primarily to verify that transcripts detected by ISH were of appropriate size. In 10 cases wherein freshly frozen tissue was available, total RNA was extracted using the single-step acid-quanidinium-thiocyanate-phenol-chloroform method [33]. In a similar fashion, RNA from an autopsy pituitary gland was also extracted, after removal of the posterior pituitary. RNA was fractionated on 0.8% agarose / 2.2% formaldehyde gels, transferred to nylon membranes, and cross-linked by UV irradiation. The same GHRH oligonucleotide probe used for ISH was \(^{32}\)P end-labeled to high specific activity (1.0 \times 10^6 \text{cpm/ml}) and membrane hybridized overnight at 42°C. Blots were washed under high stringency conditions, and exposed at to Kodak XAR-5 film for 5 days at -80°C.

The same blots probed for GHRH were sequentially reprobed for GHRH-receptor. A full length 1.6kbp GHRH-R cDNA probe had been previously cloned by us (BG, MT) [54] and inserted into a bluescript vector. Using an in vitro transcription kit, \(^{32}\)P labeled riboprobes of high specific activity were generated and subsequently purified by affinity chromatography (Boehringer Mannheim, Indianapolis, IN). Hybridization conditions and posthybridization treatments were identical to those described above for the GHRH probe.

As a loading control, all membranes were sequentially probed for their content of 18 s ribosomal RNA using the following probe sequence: (5'-CGG CAT GTA TTA GCT CTA GAA TTA CCA CAG-3'). Probe labeling and hybridization were identical to that described for the GHRH, although only a six hour exposure was required.

GHRH autoradiograms were subjected to densitometric quantification using the PDI system (Huntington Station, NY). Band densities were recorded in arbitrary densitometric units and were internally standardized for variations in the amount of RNA loaded into each lane. Specifically, the densities of the GHRH bands were divided by the densities of their respective 18s bands; this ratio was then multiplied by a factor of 100 to achieve whole number values (ie relative densitometric units).
Reverse transcriptase polymerase chain reaction (RT-PCR)

As a secondary means of demonstrating GHRH transcript accumulation, RT-PCR was performed in the same 11 cases studied by Northern analysis using an established protocol with minor modifications [205]. Briefly, an aliquot (5 μg) of total RNA was reverse transcribed using 10U avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim, Indianapolis, IN) from a 3'-primer derived from exon 5 of the consensus GHRH cDNA sequence. Ten microliters of the cDNA reaction mixture were used for the PCR amplification reaction, being carried out in a total volume of 100μl with a primer pair at 1μmol/L in 50 mmol/L KCl; 10mmol/L Tris-HCl (pH 8.3); 1.5 mmol/LMgCl2; 200 μmol/L dNTP, and 2U of AmpliTaq DNA polymerase (Perkin Elmer-Cetus, Emeryville, CA). PCR of 30 cycles was performed, consisting of denaturation (94°C, 1 min.), annealing (55°C, 1 min), and extension (72°C, 3min) in an automated DNA thermal cycler. The primer pair used to generate a specific GHRH 235bp fragment was the following: sense (5'-TAT GCA GAT GCC ATC TTC AC-3'); antisense (5'-T-TCA-TCC-CTG-GGA-GTT-CCT-G-3'). The PCR-product was phenol extracted, ethanol precipitated, and electrophoresed on an ethidium bromide containing 2% agarose gel. As a negative control, total RNA extracted from a corticotroph pituitary adenoma was used.

GHRH immunocytochemistry and Western blotting

GHRH immunostaining Five micron sections of formalin-fixed and paraffin embedded tissues were mounted onto glass slides. Immunostaining was performed using the avidin-biotin-peroxidase complex method of Hsu et al. [72]. To enhance protein detection, antigen retrieval was performed as previously described, using a 0.01 mmol/L sodium citrate retrieval buffer (pH 6.0) and tissue microwaving [179]. Polyclonal GHRH antisera (Peninsula Laboratories, Belmont CA) was used at a 1:300 dilution.

Western blotting Protein extraction, SDS/PAGE, and immunodetection were performed according to a standard protocol with minor modification [64]. Briefly, snap frozen tissues were homogenized and protein extracted in a lysis buffer consisting of 1%NP40, 20mmol Tris pH 7.4, 150 mmol NaCl, 5mmol EDTA, 1mmol sodium orthovanadate, 10 μg/ml aprotinin, 10μg/ml leupeptin, 10μg/ml phenylmethylsulfonfyl fluoride. The concentration of soluble protein was determined using the Bradford assay (Bio-Rad Laboratories, Richmond CA). Proteins were resolved on SDS/PAGE on a 15% acrylamide separating gel at constant current (35mA). Following elec-
trophoresis, proteins were transferred to Immobilon-PSQ polyvinylidene membranes (Millepore) using the Bio-Rad semi-dry transfer electroblotting apparatus. After incubation in a blocking solution consisting of 5% skim milk and 0.1% Tween-20 in PBS (0.01M sodium phosphate and 0.14 M NaCl, pH 7.4) for 60 minutes, membranes were incubated in a rabbit-derived polyclonal GHRH antibody (Peninsula Laboratories) at a final concentration of 1:500 for 2 hours. After 3 successive washes in the 5% skim milk-0.1% Tween-20/PBS mixture, the membrane was incubated in a goat anti-rabbit IgG-horseradish peroxidase conjugate. Peroxidase activity was detected by chemiluminescence using a standard kit (ECL Western blotting kit, Amersham, Arlington Heights, IL). As a positive control, a lane containing synthetic GHRH peptide (Peninsula Laboratories) was run with the tissue samples.

**Determination of tumor growth fractions**

Tumor growth fractions were determined in each case by Ki-67 immunolabeling using the MIB-1 monoclonal antibody (AMAC Inc. Westbrook, ME). Details of the technique have been described in a recent publication by the author [187]. After immunostaining was performed, a mean tumor growth fraction was determined in each case by counting the number of Ki-67 immunostained nuclei in each of 20 high power fields. The growth fraction or Ki-67 labeling index was expressed as the percentage ratio of Ki-67 labeled nuclei to total nuclei.

**Statistical analysis and outcome criteria**

Several statistical procedures were used to evaluate these data. In comparing the mean GHRH mRNA signal intensity between tumor groups, a one way analysis of variance (ANOVA) was used, followed by either pairwise comparisons using Bonferroni corrected p-values or linear orthogonal contrasts, depending on the nature of the comparison. To test for linear association between continuous variables, scatterplots were constructed and the Pearson correlation coefficient (r) was derived. To determine the prognostic relevance of tumor GHRH mRNA signal intensity and other clinicopathologic parameters in predicting remission likelihood, a logistic regression model was fitted. For this analysis, surgical outcome was considered a dichotomous outcome variable, being categorized as either "remission" or "no remission" and defined exclusively on the basis of postoperative dynamic endocrine testing. Postoperative remission was defined on the basis of the single, stringent, and widely accepted criterion of suppression of serum GH levels to less than 2ng/ml during an oral glucose tolerance test, performed one month
postoperatively [48, 80, 116, 195]. Values above this threshold were considered surgical failures, regardless of the degree of lowering of basal GH levels. Given the era in which the majority of these patients had been treated, the currently preferred criterion of a normalized serum insulin-like growth factor-1 level was not routinely unavailable. Of particular methodological importance is the fact that a single surgeon had operated upon all cases in the primary study population and a single surgeon also operated upon all tumors in the secondary test population. Since both surgeons have specific expertise with pituitary surgery, the occurrence of an unsuccessful outcome should be viewed as a reflection of the aggressiveness of the tumor which precluded its complete removal, rather than technical inexperience on the part of the surgeon. Once the outcome model was derived, its generalizability in predicting the remission status of the secondary (Mayo Clinic) test population was studied; a chi squared analysis was used to compare predicted versus actual surgical outcomes.

For all statistical analysis, two-tailed probability values less than 0.05 were designated as significant. All mean values are reported as mean ± standard error of the mean (SEM). Statistical analyses were performed using SAS system software version 6.10 (SAS Institute, Cary, NC).

RESULTS

GHRH mRNA transcripts in nontumorous control pituitaries
In all nontumorous control pituitaries, the GHRH mRNA signal was low (mean 3.31 ± 0.43 silver grains/cell; range: 0-8.5), most examples exhibiting only background levels of signal (Figure 2.1 A). The mean GHRH mRNA signal was comparable in both the autopsy and surgical controls (2.72 ± 0.45 versus 4.52 ± 0.85), values within the range of background signal (ie. < 5 silver grains/cell). In a small subgroup of nontumorous control specimens (3 of 20 autopsy glands and 4 of 10 surgical nontumorous pituitaries), the GHRH signal in somatotrophs, although still low, did exceed background levels (mean=7.04 ± 0.54 silver grains/cell). In some of these specimens, the signal was not confined to somatotrophs only, as low level signal could also be seen in occasional basophilic and chromophobic cells; no signal was evident in the posterior lobe.
Figure 2.1 ISH for GHRH mRNA (A) In the nontumorous pituitary gland, only a background level of signal is seen (hematoxylin; magnification x400). (B) This contrasts with the strong hybridization signal diffusely present in the somatotroph adenoma (hematoxylin; magnification x400). (C) Section of a somatotroph adenoma that also contains a tongue of nontumorous tissue (arrow). Note the selective localization of the hybridization signal only within tumor cells and not within the entrapped nontumorous pituitary (hematoxylin; magnification x200). (D) ISH for GHRH mRNA was combined with immunohistochemistry for GH. Note that cells having the most intense GHRH mRNA hybridization signal are also those most strongly immunoreactive for GH (GH immunostain; magnification x 200).

GHRH mRNA transcripts in somatotroph adenomas

In contrast to the nontumorous pituitary wherein the GHRH mRNA signal was absent or low, transcript accumulation was detectable in 91 of 100 somatotroph adenomas. Moreover, the mean GHRH signal intensity among tumors was 18.17 ± 1.45 silver grains/cell (range: 0-56.7), a value significantly higher than that observed in the control groups (one-way ANOVA, post hoc linear contrast, F-ratio= 15.63, p<0.001) (Figure 2.1B). Differences in the GHRH signal intensity between the tumor and normal glandular tissue were especially obvious in some surgical specimens wherein the border between tumor and surrounding normal gland was present (Figure 2.1C).
Co-localization of GHRH mRNA and immunoreactive GH
When ISH for GHRH mRNA was combined with immunohistochemistry for GH, the signal was co-localized with the immunoreaction in the same tumor cells. In some cases, tumor cells having the highest GHRH mRNA signal intensity were seen to also exhibit the strongest immunoreactivity for GH (Figure 2.1D).

Northern analysis
A single and appropriately sized GHRH transcript of approximately 0.75kb was detected in 9 of 10 somatotroph adenomas tested, although some variability in the level of expression was noted (Figure 2.2A). The level of GHRH message was higher among invasive adenomas as compared to noninvasive adenomas, and in those adenomas with higher growth fractions. No GHRH message could be detected in the normal gland. When blots were reprobed with a GHRH-receptor probe, all somatotroph adenomas also expressed the receptor mRNA. A full sized 4.0 kb GHRH-receptor mRNA transcript was identified in all tumors and in the autopsy pituitary control (Figure 2.2B). The level of GHRH-receptor message was fairly similar in cases tumors, suggesting a constitutive level of expression. In particular, there was no evidence of GHRH-receptor downregulation even when the level of GHRH message was high.

RT-PCR verification of GHRH gene expression
In 10 of 10 cases studied, RT-PCR resulted in selective amplification of the predicted 235 base pair fragment of the GHRH gene, being represented as a discrete band of expected size (Figure 2.3). No amplification was seen in either the normal gland, or in the negative control (corticotroph adenoma).

GHRH immunohistochemistry and Western blot analysis
Of the 30 control nontumorous pituitaries, all of which were formalin-fixed and paraffin-embedded, GHRH immunoreactivity could not be demonstrated in a signal instance despite application of vigorous antigen retrieval methods [179]. Even in the 7 examples wherein low level GHRH transcript accumulation was demonstrated by ISH, all cells were uniformly immunonegative for GHRH. Of 20 somatotroph adenomas studied by immunohistochemistry, all of which expressed high levels of GHRH message, conclusive cellular localization of GHRH protein could be demonstrated in only 2 cases.

(Text continued on page 44)
Figure 2.2 Northern analysis for GHRH (A); GHRH-receptor (B); and Western analysis for GHRH (C) in 10 somatotroph adenomas and in 1 autopsy nontumorous pituitary gland (NT). For each tumor, the ISH GHRH mRNA signal intensity, Ki-67 labeling index, and the radiological (Hardy) grade are noted. (A) Northern analysis for GHRH reveals a single, appropriately sized transcript of 0.75 kb in 9 of 10 tumors, but not in the autopsy pituitary gland. The GHRH band intensity is clearly higher among invasive tumors and in those with higher Ki-67 labeling indices. As a loading control, the membrane was probed for the 18s ribosomal RNA fraction. (B) When probed for GHRH-receptor, a single transcript of ~4.0kb was identified in all of 6 tumors and in the normal gland. Note that little variation is seen in the band intensity between cases, suggesting a constitutive level of expression. (C) Western analysis for GHRH reveals the presence of an appropriate sized (~5kDa) band having the same migrational characteristics as synthetic GHRH peptide [positive control (+)] in 9 of 10 somatotroph adenomas. In addition, secondary bands in the 6-16 KDa range are also seen in 7 of 10 cases, corresponding to the expected size of the proGHRH precursor protein. In the autopsy pituitary (NT), neither precursor nor mature proteins are seen. Strong, but not absolute concordance is seen between results of ISH/Northern analysis and Western analysis in detecting GHRH transcripts and protein, respectively. The quantitative relationship between ISH and Northern analysis in these cases is more precisely depicted in Figure 2.4.
In 9 of 10 somatotroph adenomas, western blotting revealed a dominant band of approximately 5kDa, corresponding to the size of the mature GHRH peptide and having the same migrational characteristics as synthetic GHRH peptide (positive control) (Figure 2.2C). In 7 specimens, secondary bands between 6-16kDa was also seen, corresponding the size range expected of the pro-GHRH precursor protein. In the autopsy control, neither the mature GHRH peptide nor the pro-GHRH precursor was present. Qualitatively, the amount of GHRH protein appeared higher in some invasive adenomas (cases 5, 6, and 10). As the purpose of Western analysis was purely to demonstrate protein translation, and given the small number of samples available for study, protein quantification and formal comparisons were not undertaken.

**Concordance between ISH and other methods of GHRH mRNA and protein detection**
In 10 tumors and 1 autopsy pituitary gland, ISH, Northern analysis, RT-PCR, Western analysis, and GHRH immunohistochemistry were performed (Figures 2.2 & 2.3). In detecting GHRH message, ISH, Northern analysis, and RT-PCR were comparable, concordant results being observed in all but one instance (case 2) wherein transcripts were demonstrable by RT-PCR but not by the other two methods. Furthermore, the GHRH mRNA signal intensities as determined...
by ISH and Northern analysis were quantitatively related, a significant linear relationship being present the two (*r* = 0.78; 95% CI: 0.33-0.94; *P* < 0.01) (Figure 2.4). In all of 9 samples wherein GHRH message was detected by these methods, GHRH protein was also demonstrable by Western analysis, confirming translation of GHRH transcripts. Whereas GHRH could be readily detected on Western analysis, it could not be detected by immunohistochemistry on formalin-fixed sections in any of these cases, despite the use of vigorous antigen retrieval.

**GHRH mRNA signal intensity and tumor pathology**

Some variability in the mean GHRH mRNA signal intensities was noted between different somatotroph adenoma subtypes (one-way ANOVA, *F* ratio = 2.54, *P*=0.033) (Figure 2.5). The mixed somatotroph-lactotroph adenomas had the highest signal intensity (25.41±3.20), differing significantly from the unclassified somatotroph adenomas which had the lowest (9.10 ± 2.60; Bonferroni correction, *P* <0.05).
GHRH mRNA signal intensity and Ki-67 labeling index (tumor growth fraction)

The MIB-1 antibody, conclusively discriminating proliferating from quiescent cells on the basis of nuclear expression of the Ki-67 antigen, permitted reliable quantification of the proportion of cycling cells and the derivation of a Ki-67 labeling index or tumor growth fraction (Figure 2.6A).

In comparing GHRH mRNA signal intensity with Ki-67 derived tumor growth fractions, a highly significant and positive linear correlation was observed ($r = 0.71$, $P < 0.001$) (Figure 2.6B).
**GHRH mRNA signal intensity and tumor size/invasion status**

All tumors were graded according to size, invasion status, and radiological appearance according to the Hardy classification [62]. Of grades 0-I, II, III, IV, the primary study group was represented by 9, 42, 31, and 18 tumors, respectively. The mean GHRH signal intensity among invasive adenomas (Grades III-IV) was significantly higher than that of noninvasive adenomas (Grades 0-II) (23.36 ± 2.0 versus 13.19 ± 1.84. ANOVA, F-ratio 5.02, post hoc linear orthogonal contrast, t-statistic = 3.56, P = 0.001) (Figure 2.7).

The mean GHRH mRNA signal intensity of microadenomas (grades 0-I) was lower than that of macroadenomas (grades II-IV). (11.05 versus 18.88 silver grains per cell). This difference, although of conceptual importance, fell short of statistical significance (post hoc linear orthogonal contrast, t-statistic = 1.97, P = 0.08). Given that there were only 9 microadenomas in this series, there was insufficient statistical power to ascribe significance to this trend.

![Figure 2.7](image)

**Figure 2.7** (A) Mean GHRH mRNA signal intensity in tumors stratified on the basis of size and invasiveness according to the radiologic classification of Hardy. The mean GHRH mRNA signal of invasive tumors (grades III, IV) was significantly higher than that of noninvasive tumors (grades 0, I, II) (ANOVA, F-ratio = 5.02, post hoc linear orthogonal contrast, p <0.001). The mean GHRH signal was higher in macroadenomas (II-IV) as compared to microadenomas (0-I), however, this difference did not reach statistical significance (p = 0.08.).

(B) **Hardy classification of pituitary tumors.**

- **Grade 0:** Intrapituitary microadenoma; normal sellar appearance
- **Grade 1:** Intrapituitary microadenoma; focal bulging of sellar wall
- **Grade 2:** Intraseellar macroadenoma; diffusely enlarged sella; no invasion
- **Grade 3:** Macroadenoma; localized sellar invasion and/or destruction.
- **Grade 4:** Macroadenoma; extensive sellar invasion and/or destruction.
**GHRH mRNA signal intensity and preoperative serum GH levels**

In all patients, multiple basal determinations of the preoperative serum GH level had been made, the mean of which was used for comparison. A significant positive linear correlation was noted between the mean preoperative GH level and tumoral GHRH mRNA signal intensity ($r = 0.56$, $P<0.01$).

**Figure 2.8** Scatterplot analysis showing the relationship between GHRH mRNA signal intensity and the mean preoperative serum GH level. A clear linear correlation can be seen between the two ($r = 0.56; 95\%CI: (0.42,0.67); P <0.01$).

**GHRH mRNA signal intensity and surgical outcome**

Response to surgical therapy was considered a dichotomous outcome variable defined solely on the basis of postoperative suppressibility of the serum GH level to less than 2 ng/ml during an oral glucose tolerance test (OGTT). Based on this criterion, remission was achieved in 43 of 100 patients. Although nearly all of the remaining 57 patients experienced substantial declines in basal GH levels, their failure to suppress below the established threshold placed them in the "no remission" category. The mean GHRH mRNA signal intensity observed in tumors wherein remission was achieved was markedly lower than in those in which it was not (8.79 ± 1.4 versus 25.2 ± 1.8, two sample t-test for independent samples, t-statistic = 7.13, $p < 0.001$) (Figure 2.9).

**Figure 2.9** Comparison of the distributions of GHRH mRNA signal intensities in tumors stratified on the basis of remission status using boxplot analysis. For each population, the 10th, 25th, 50th, 75th, and 90th percentile of the GHRH mRNA signal are represented in the box and whiskers format. Note the distribution of GHRH mRNA signal intensities in tumors in which remission was achieved is shifted to the left, as compared to those not experiencing remission. In addition, the mean GHRH mRNA signal between these groups differs significantly (two sample t-test for independent samples, $p<0.001$).
To delineate more precisely the relationship between GHRH mRNA transcript accumulation and remission likelihood, particularly as compared to other predictors currently used in clinical practice, an outcome model was developed by means of logistic regression. First, univariate analysis was performed to assess the prognostic relevance of the following predictors: patient age, sex, tumor pathology, tumor size and invasion status (Hardy grade), mean preoperative GH level, Ki-67 labeling index, and GHRH mRNA signal intensity. As shown in Table 2.1, GHRH mRNA signal intensity and the Ki-67 labeling index were the most significant prognostic variables, although the preoperative GH level, radiological grade, and patient age were also variably significant predictors.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>Remission prevalence</th>
<th>df</th>
<th>$\chi^2$</th>
<th>Odds ratio$\dagger$</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td>1</td>
<td>4.0222</td>
<td>1.430</td>
<td>(1.016, 2.055)</td>
<td>0.0449</td>
</tr>
<tr>
<td>Sex</td>
<td>female$*$</td>
<td>43.9%</td>
<td>1</td>
<td>0.0231</td>
<td>0.940</td>
<td>(0.420, 2.111)</td>
<td>0.8792</td>
</tr>
<tr>
<td></td>
<td>male</td>
<td>42.4%</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pathological subtype</td>
<td></td>
<td></td>
<td>5</td>
<td>9.2179</td>
<td></td>
<td></td>
<td>0.1007</td>
</tr>
<tr>
<td>Unclassified GH cell$*$</td>
<td>44.4%</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acidophil stem cell</td>
<td>33.3%</td>
<td></td>
<td>1</td>
<td>0.1133</td>
<td>0.625</td>
<td>(0.024, 9.157)</td>
<td>0.7364</td>
</tr>
<tr>
<td>Densely granulated</td>
<td>60.0%</td>
<td></td>
<td>1</td>
<td>0.6408</td>
<td>1.875</td>
<td>(0.402, 9.300)</td>
<td>0.4234</td>
</tr>
<tr>
<td>Mammosomatotroph</td>
<td>66.7%</td>
<td></td>
<td>1</td>
<td>1.0177</td>
<td>2.500</td>
<td>(0.431, 16.158)</td>
<td>0.3131</td>
</tr>
<tr>
<td>Mixed GH-PRL</td>
<td>29.6%</td>
<td></td>
<td>1</td>
<td>0.6564</td>
<td>0.526</td>
<td>(0.109, 2.603)</td>
<td>0.4178</td>
</tr>
<tr>
<td>Sparsely granulated</td>
<td>29.2%</td>
<td></td>
<td>1</td>
<td>0.6769</td>
<td>0.515</td>
<td>(0.103, 2.609)</td>
<td>0.4107</td>
</tr>
<tr>
<td>Radiologic grade</td>
<td></td>
<td></td>
<td>3</td>
<td>10.9898</td>
<td></td>
<td></td>
<td>0.0118</td>
</tr>
<tr>
<td>Hardy grade 0-1$*$</td>
<td>66.6%</td>
<td></td>
<td>1</td>
<td>0.2753</td>
<td>0.667</td>
<td>(0.127, 2.895)</td>
<td>0.5998</td>
</tr>
<tr>
<td>Hardy grade II</td>
<td>57.1%</td>
<td></td>
<td>1</td>
<td>3.1801</td>
<td>0.238</td>
<td>(0.043, 1.094)</td>
<td>0.0745</td>
</tr>
<tr>
<td>Hardy grade III</td>
<td>32.3%</td>
<td></td>
<td>1</td>
<td>5.8912</td>
<td>0.100</td>
<td>(0.013, 0.582)</td>
<td>0.0152</td>
</tr>
<tr>
<td>Hardy grade IV</td>
<td>16.7%</td>
<td></td>
<td>1</td>
<td>7.1317</td>
<td>0.793</td>
<td>(0.658, 0.925)</td>
<td>0.0076</td>
</tr>
<tr>
<td>Preop GH level</td>
<td></td>
<td></td>
<td>1</td>
<td>19.9018</td>
<td>0.546</td>
<td>(0.409, 0.698)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Ki-67Labeling index</td>
<td></td>
<td></td>
<td>1</td>
<td>23.2279</td>
<td>0.296</td>
<td>(0.172, 0.464)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* for categorical variables, the asterisk represents the reference category to which other members of the group were compared.
$\dagger$ the odds ratios for age, preop GH and GHRH mRNA signal intensity are given for a 10 unit increase in the variable.

Table 2.1 Summary of univariate logistic regression analysis revealing the relationship between various clinicopathologic predictors and the likelihood of postoperative remission.
Using both forward selection and backward elimination in delineating the most stable model possible, the significance of these predictors was evaluated. Regardless of the modelling strategy used, only GHRH mRNA signal intensity (p < 0.005) proved to be a consistently significant predictor in every model having satisfactory goodness-of-fit. Conventional clinicopathologic predictors such as Ki-67 labeling index, the mean preoperative GH level, and the tumor size/invasion status, although each being of variable significance as univariate predictors, lost their explanatory contribution once GHRH mRNA signal intensity was entered into the model. In the saturated multivariate model, wherein all relevant predictors from univariate analysis were present, only GHRH mRNA signal intensity retained predictive significance (Table 2.2). The likelihood ratio \( \chi^2 \) statistic for the saturated model was 51.27, whereas that of a univariate model containing only GHRH mRNA signal intensity was 39.31. This implies that the GHRH mRNA signal intensity alone represented 77% (i.e., 39.31/51.27 x 100%) of the prognostic information contained in the full model. That GHRH mRNA signal intensity was, itself, the overwhelmingly dominant predictor, containing most of the prognostic information provided by other clinicopathologic parameters, it was justifiable to reduce the final fitted model to a univariate model containing GHRH mRNA signal intensity as the sole explanatory variable. In doing so, it also allowed the statistical effect of GHRH mRNA signal intensity on postoperative outcome to be clearly isolated. The final fitted model, where \( P_{\text{remission}} \) is the probability of remission, was as follows:

\[
\text{Logit} \left( \frac{P_{\text{remission}}}{1 - P_{\text{remission}}} \right) = 1.6327 - 0.1217(GHRH)
\]

\[
P_{\text{remission}} = \frac{1}{1 + e^{-[1.6327 - 0.1217(GHRH)]}} \quad (\text{Equation 2.1})
\]
As illustrated in Figure 2.10, the effect of GHRH transcript accumulation was strongly adverse; increases in GHRH mRNA signal intensity were associated with precipitous declines in remission likelihood. For example, an increment in GHRH mRNA signal intensity of 10 silver grains per cell was associated with more than a three-fold reduction in the odds favoring remission (odds ratio = 0.30; 95%CI:0.18 - 0.49).

To validate the adequacy with which the derived logistic model represented our data, two standard goodness-of-fit criteria were evaluated [69]. First, a Hosmer-Lemeshow statistic was calculated ($X^2=2.89$, 8df, $p=0.94$); its lack of significance legitimized our acceptance of adequate model fit. Second, the area under the receiver operator characteristic (ROC) curve for representing this model was high ($c=0.84$), indicating both good fit and high predictive accuracy for this model. As discussed below, a third validation of good model fit was provided by the model's satisfactory performance in a secondary patient population.

Given that all clinical data had been collected retrospectively and that patients were referred back to their primary physicians for ongoing care, the only endpoint consistently shared by all patients was the 1 month postoperative check, at which point a determination of remission status was made by one of us (DK). Whereas this fulfilled the immediate objectives of this study, it was not possible to determine from the available data, the long term prognostic relevance of GHRH mRNA transcript accumulation from such standpoints as tumor recurrence or relapse free survival. Of patients in whom remission was achieved, all patients were referred back to their primary care physicians, and many were lost to follow up. Of patients in whom surgical remis-
sion was not achieved, postoperative management was not uniform. Virtually all refractory patients received one or more forms of adjuvant therapy (somatostatin analogs, radiation therapy, dopamine agonists, or repeat surgery). This lack of uniformity in postoperative management among the surgical failures made assessment of tumor regrowth and endocrine relapse problematic, particularly when attempting to isolate the effect of GHRH signal intensity from the effects of postoperative adjuvant therapies.

**Outcome prediction in a secondary test population**

In evaluating tumor samples from the secondary population, the investigators were blinded to all information except the pathologic subtype of the tumor. In each case, ISH for GHRH mRNA was performed and the signal intensity quantified as described. Based only on the GHRH mRNA signal intensity, the derived outcome model (Equation 2.1) was applied and the probability of surgical remission was predicted for each case. A "cut-off" probability for remission of 0.5 was selected. Accordingly, when the model predicted a remission probability of greater than 0.5, the case was designated as a predicted remission; alternatively, values of 0.5 or less were designated as predicted failures. Predicted results were compared to actual results using a contingency table analysis (Table 2.3). The model correctly predicted the actual surgical outcome in 22 of 30 (73.3%) of cases (continuity adjusted $X^2 = 5.17$, 1 df, $p=0.023$). Correctly predicting 13 of 19 successes and 9 of 11 failures the model had a sensitivity and specificity in predicting remission of 68% and 82%, respectively.

<table>
<thead>
<tr>
<th>Actual remission status (Mayo clinic patients)</th>
<th>Predicted remission status</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>remission</td>
<td>no remission</td>
</tr>
<tr>
<td>remission</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td>no remission</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Totals</td>
<td>19</td>
<td>11</td>
</tr>
</tbody>
</table>

**Table 2.3** Contingency table analysis of actual remission status of the secondary test population (Mayo Clinic) versus the remission status predicted by the outcome model (Equation 2.1). The model correctly predicted outcome in 22 of 30 cases (73%). A significant association between predicted and actual outcomes is present (continuity corrected Chi-square = 5.17, 1 df, $p=0.023$).
DISCUSSION

The biologic behavior of pituitary adenomas is known to vary greatly. Some adenomas, such as those found in up to 20% of unselected autopsies, show neither a capacity for growth nor for hormone secretion, and are thus relegated to a subclinical existence as incidental autopsy findings [186]. Others will manifest clinically, however, their noninvasive nature, limited growth capacity, and overall indolent character lend themselves to curative resection and a durable endocrinologic remission. Finally, there are those adenomas which assume a more aggressive phenotype, being so prone to invasive, destructive growth and recurrence, that they defy any and all therapeutic intervention. In terms of the multistep model of human tumorigenesis, the problem can be viewed as one of neoplastic progression, however, the actual events underlying the process are poorly understood [115, 185]. Of the various pituitary adenoma subtypes, the problem of neoplastic progression appears especially relevant to GH-producing adenomas, of which a disproportionately large number are already invasive macroadenomas at the time of presentation [161].

Herein, we have identified accumulation of GHRH mRNA transcripts as a potentially important event in the progression of GH-producing pituitary tumors. In the normal nontumorous pituitary, GHRH mRNA transcripts were either absent (23/30) or present at very low levels (7/30) only. In contrast, transcript accumulation was evident in 91 of 100 consecutive GH-producing pituitary adenomas, the mean signal intensity being more than five fold higher than that observed in normal pituitary specimens. Of greatest importance, however, was the significant relationship between transcript accumulation and tumor behavior. Our finding of a positive correlation between the GHRH mRNA signal intensity and Ki-67 labeling index, together with the observation that the mean GHRH mRNA signal among invasive adenomas was almost twice that of noninvasive adenomas, indicates preferential accumulation of GHRH transcripts in tumors capable of growth and invasion. That GHRH transcript accumulation is also associated with endocrine aggressiveness was evidenced by the positive linear correlation between tumoral GHRH mRNA signal intensity and mean preoperative GH levels. Finally, when evaluated together with other conventional parameters of tumor aggressiveness, including tumor size and invasiveness, Ki67 labeling index, tumor pathology, and preoperative GH levels in a multivariate model of surgical outcome, the degree of GHRH transcript accumulation eclipsed all other
parameters as the single most significant explanatory variable. In fact, once GHRH mRNA signal intensity was entered into the logistic model, all remaining variables failed to make additional explanatory contribution. The statistical effect of GHRH mRNA transcript accumulation on outcome was strongly adverse, increments in GHRH mRNA signal intensity being associated with precipitous declines in remission likelihood. Finally, the reproducibility of the derived outcome model was validated by the model’s significant performance in predicting the outcomes of a second population of patients with somatotroph adenomas treated at another institution, suggesting that these methods can be generalized to other populations of acromegalic patients.

As outlined previously, the only endpoint consistently shared by all patients was the one month postoperative check, at which point a determination of remission status was made on the basis of suppressibility of the GH level to < 2.0 ng/ml during an OGTT. While it is true that this outcome criterion is a short term one, it is nonetheless a robust outcome criterion. Historically, when less stringent criteria were applied (ie: normalization of basal GH levels or suppressibility of GH levels to <5 ng/ml on OGTT), rates of ‘recurrence” were high, indicating that these patients did not in fact achieve a true remission; recurrence in this situation was the result of reconstitution / regrowth of residual tumor cells. In more recent surgical series, wherein suppressibility of GH to <2 ng/ml has been used as the remission criterion, tumor recurrence has been infrequent and patients typically experience a durable and disease-free remission over the long term [48]. While this does not diminish the need for follow data concerning the relationship between GHRH mRNA transcript accumulation and long term outcome, it does indicate that the outcome criterion employed in this study, in spite of its short term and front-ended nature, does have some long term prognostic relevance.

The culpability of GHRH as a potential contributor to neoplastic progression derives from several lines of physiologic, pathologic, and experimental evidence. First, GHRH is the principal positive regulator for pituitary somatotrophs, stimulating both their secretory function and proliferative activity. [51, 111, 195]. Acting through specific membrane receptors, GHRH stimulates GH secretion and somatotroph proliferation through a variety of potent effector mechanisms that include (i) signal transduction by a classic stimulatory G-protein pathway; (ii) activation of the protein kinase-A/adenylate cyclase second messenger cascades; (ii) recruitment of the inositol triphosphate/protein kinase C cascade; and (iv) the induction of early response genes such as c-
Given that GHRH is the most important physiologic stimulator of pituitary somatotrophs, the potential oncologic implications of pathologic GHRH excess are readily apparent. In states of pathologic GHRH excess, such as occurs in association with rare GHRH-producing tumors (e.g., pancreatic endocrine tumors, carcinoid tumors), chronic GHRH stimulation leads to somatotroph hyperplasia, GH hypersecretion, and clinical acromegaly. In some instances, progression from somatotroph hyperplasia to adenomatous transformation have been well documented [45, 159]. A parallel phenomenon has been demonstrated in transgenic mice bearing the human GHRH transgene. These animals develop hypersomatotropism, elevated GH levels, somatotroph hyperplasia, and eventually, GH-producing pituitary tumors - compelling and conclusive evidence of the tumor promoting potential of GHRH excess [4, 106, 175]. Finally, the discovery and characterization of the human oncogene, GSPT1, has further elucidated the tumor promoting effect of a chronically activated GHRH stimulatory pathway [90, 201]. Identified in up to 40% of somatotroph adenomas, mutations of this gene involve the alpha chain of the stimulatory GTP binding protein that normally transduces the GHRH signal. The result is a constitutively activated G-protein that mimics a persistent GHRH stimulatory signal, culminating in adenomatous transformation of the affected cell. Activating mutations of GSPT1 are regarded as transforming events only; for unknown reasons, their presence does not appear to confer aggressive behavior [89, 115].

Collectively, the above studies support the hypothesis that aberrant GHRH activity may play a role in the progression of somatotroph adenomas. Whereas recent demonstrations of GHRH gene and protein expression in somatotroph adenomas support this concept [76, 78, 98, 145, 205], the clinical and biologic implications of these findings had, until now, not been explored. The present study, in demonstrating accumulation of GHRH mRNA transcripts within a large series of somatotroph adenomas and systematically correlating their presence with clinically and biologically relevant differences in tumor behavior further strengthen this link. Furthermore, it strongly suggests that somatotroph adenomas are themselves both a local source of GHRH synthesis and a target for GHRH action. Our demonstration that both GHRH and GHRH-R mRNA transcripts are co-expressed in somatotroph adenomas points to GHRH-mediated autocrine and/or paracrine stimulation as one mechanism adversely associated with and/or contributing to their proliferative capacity, secretory activity, invasive potential, and surgical responsiveness. Crucial to the plausibility of such a mechanism is the fact that chronic exposure to GHRH
appears unassociated with receptor desensitization in the neoplastic somatotroph. As reviewed by Spada and Lania, patients with somatotroph adenomas continue to release GH after repeated GHRH injection, whereas normal subjects do not; among the latter, a marked refractoriness to repeated GHRH provocation is seen [171]. The same lack of receptor desensitization has also been demonstrated by neoplastic somatotrophs in vitro [170]. In the present study, there was no GHRH-R downregulation at the transcriptional level, as Northern analysis revealed a fairly constant and seemingly constitutive level of GHRH mRNA expression in all cases, including those in which the level of GHRH message was high. Whereas autocrine and/or paracrine stimulation are frequently invoked as somewhat generic tumor promoting mechanisms in endocrine neoplasia, this is first comprehensive demonstration of the biologic and clinical consequences that might accompany putative activity of this type in the pituitary. Implicit in this supposition of autocrine/paracrine stimulation are two fundamental requirements, both of which appear to be fulfilled in this tumor system: (i) increased local GHRH production; and (ii) a concomitant lack of GHRH-R downregulation.

Our correlations have been based solely on levels of GHRH message as determined by ISH. The significant linear relationship demonstrated between ISH and Northern analysis does legitimize this approach as a valid means of quantifying the level of GHRH message. Of greater importance, however, is the confirmation that overexpressed GHRH transcripts are in fact translated into protein. That this does occur was evident from the observed concordance between the results of Western blotting and ISH. In addition to demonstration of the mature (≈5 KDa) immunoreactive form of GHRH, the frequent presence of a second larger (6-16 KDa) immunoreactive species consistent with the expected size range of the pro-GHRH precursor molecule further confirms endogenous GHRH production by these tumors [128a]. The presence of the pro-GHRH precursor in these tumors was first demonstrated by Rauch et al. who, not only demonstrated a 10kDa pro-GHRH precursor in these tumors by Western analysis, but also quantified pro-GHRH content using size-exclusion chromatography, and also demonstrated GHRH immunoreactive cells within some pituitary adenomas [145]. Our attempts at GHRH immunohistochemistry on formalin-fixed specimens, despite application of antigen retrieval, yielded negative results in all but 2 cases, including those in which the levels of GHRH mRNA was high by ISH. Even in the 9 examples wherein GHRH was demonstrable by Western blotting, immunohistochemistry was uniformly negative. This suggests that technical limitations and/or
insufficiently sensitive antibodies rather than a failure of protein translation probably account for the observed negativity of GHRH immunohistochemistry in those tumors expressing high levels of GHRH message by ISH.

The significance of our observation that 7 of 30 nontumorous pituitaries expressed low levels of GHRH mRNA is uncertain. This finding is, however, consistent with prior demonstrations of proGHRH precursor within the normal pituitary, as well as the release of mature GHRH peptide by normal somatotrophs in vitro [12, 76, 145]. The particular finding of Joubert et al, that normal pituitaries release less GHRH in vitro than do somatotroph adenomas is in keeping with the significantly higher levels of GHRH mRNA we observed in somatotroph adenomas as compared to nontumorous pituitaries. Still, the physiologic significance GHRH production by the normal pituitary is unclear. It has been proposed that GHRH may, in consort with the competing hypophysiotropic hormone somatostatin, exert some degree of local neuroendocrine control over normal pituitary function [76, 100].

In calling attention to a possible role for locally generated hypothalamic hormones in the progression of somatotroph adenomas, these data provide the basis of a new paradigm from which to view the biology and pathogenesis of these neoplasms. Historically, the development and progression of pituitary tumors have been the subject of two conceptually opposing theories. The “hypothalamic” hypothesis, a once dominant concept which engendered substantial experimental, clinical, and conceptual support, proposed that pituitary adenomas arose as the downstream consequence of a stimulatory imbalance of hypothalamic hormones emanating from a dysregulated hypothalamus [46, 114, 115, 150, 185]. With the demonstration that most human pituitary adenomas are monoclonal neoplasms, neither preceded nor accompanied by a phase of pituitary hyperplasia, this view has been subordinated in favor of the “pituitary hypothesis” that suggests pituitary adenomas to be the result of subcellular alterations intrinsic to a single adenohypophysial cell. Although these theories have been considered mutually exclusive, the present data provide an important link between the two. In proposing that locally produced GHRH may modify the behavior of established adenomas, this model effectively merges pertinent components of the traditional “hypothalamic hypothesis” with the more contemporary “pituitary hypothesis”, and does so without violating the underlying essence of either. What emerges is a unified hypothesis that not only highlights the merits of existing theories but also addresses the problems
of biological behavior and neoplastic progression, issues not readily reconciled by prior hypotheses.

Whereas our data suggest an unrecognized but biologically relevant level of regulatory control exercised by somatotroph adenomas and mediated via GHRH, it is equally probable that other locally generated hypophysiotropic hormones may exert comparable effects upon their respective tumor types. For example, mRNA transcripts for gonadotrophin hormone-releasing hormone [117], thyrotropin-releasing hormone [131], and corticotropin-releasing hormone [99] have been identified in various types of pituitary adenomas. Although the clinical effects of such expression are unknown, the fact that hypophysiotropic hormones are expressed in so broad a spectrum of pituitary tumor types suggests a more unifying and pervasive role of such hormones in pituitary tumor biology than is currently appreciated. Furthermore, since most normal adeno-hypophyseal cells are subject to dual coordinated regulation by both stimulatory and inhibitory hypophysiotropic hormones, aberrant activity of stimulatory hormones would appear to be only a part of the equation. Thus, it is equally plausible that locally generated inhibitory hypothalamic hormones may also modify the behavior of pituitary adenomas, perhaps in a clinically favorable way. In this regard, locally produced somatostatin may be of particular importance in modifying the behavior of somatotroph adenomas. Several investigators have demonstrated somatostatin gene and/or protein expression in somatotroph adenomas [99-101]. The pioneering studies of Peillon and colleagues are of special relevance in this context, as they have shown an inverse relationship between somatostatin mRNA levels and GH secretory activity in somatotroph adenomas [132], as well as a tendency for noninvasive adenomas to contain higher amounts of somatostatin precursor, as compared to invasive adenomas [100].

Having demonstrated that GHRH is overexpressed in aggressive pituitary tumors, the mechanisms responsible for this overexpression remain to be elucidated. The human GHRH gene has been localized to chromosome 20p12.1. The structure of its promoter region, including the transcriptional start site have been characterized, but virtually nothing is known of the regulation of GHRH gene transcription in either health or disease [111]. The 5' flanking region upstream of the transcription start site contains fairly typical TATA and CCAAT-like elements, ones devoid of any obvious vulnerability toward transcriptional activation, and thus, provides few clues to the accumulation of GHRH transcripts described herein.
CONCLUSIONS

The present work comprehensively draws upon morphologic, molecular, cell kinetic, and clinical data in an effort to evaluate the role of GHRH in the progression of GH-producing pituitary tumors. Using ISH, northern analysis and RT-PCR, GHRH transcript accumulation was demonstrated within the majority of somatotroph adenomas tested and protein translation was confirmed by Western blotting. In contrast, expression was absent or low in the nontumorous pituitary gland. As evidenced by the significant associations between GHRH mRNA signal intensity and proliferative activity, invasiveness, and preoperative GH levels, GHRH transcripts appear to preferentially accumulate among aggressive tumors not subject to surgical cure. That this is the case was further confirmed by our analysis of surgical outcome wherein GHRH mRNA signal intensity was not only a highly significant negative predictor of postoperative remission, but it also out-performed all currently known clinicopathologic predictors of aggressive behavior. Finally, the generalizability of these conclusions were validated by the significant performance of our outcome model in a second population of somatotroph adenomas from another institution. Collectively, these results provide strong molecular, clinicopathologic, and statistical evidence that GHRH transcript accumulation is a prognostically relevant event associated with the neoplastic progression of GH producing pituitary tumors. As such, it is among the first statistically validated demonstrations of a prognostically informative marker/alteration in this tumor system. In light of the spectrum of other hypophysiotropic hormones known to be expressed by pituitary tumors, our findings suggest that pituitary adenomas can, in the course of their evolution, assume the capacity for local hypophysiotropic hormone production which may, in turn, serve to modify tumor behavior. Whereas the objective of this report was to assess the mechanistic relevance of GHRH mediated autocrine/paracrine stimulatory activity, and the data provided herein do support a relationship between the latter and aggressive behavior, there is the eventual possibility that determinations of GHRH gene and/or protein expression may also be of clinical utility as a prognostic marker, essentially gauging the inherent aggressiveness of somatotroph adenomas. At present, however, the diagnostic role of routine GHRH determinations in predicting the behavior of an individual somatotroph adenoma behavior is unclear. In that quantitative GHRH mRNA determinations by ISH or Northern analysis are both labor intensive and costly, routine clinical application of these techniques, as currently performed, would be difficult to justify, particularly in the absence of prospective data validating the long term prognostic significance of such determinations. Certainly, the eventual availability of sensitive
antisera that would permit immunohistochemical detection of GHRH protein should increase the feasibility of such determinations as routine diagnostic procedures, although the need for prospective data affirming the long term prognostic significance of such expression would still be required before its diagnostic potential could be endorsed as a routine practice. Still, the current retrospective data, in showing a definitive and adverse relationship between GHRH over-expression and tumoral growth kinetics, invasiveness, secretory activity, and the likelihood of immediate postoperative remission, provide the necessary foundation for future studies designed to assess long term prognostic correlates of local GHRH mRNA / protein expression and to clarify the potential diagnostic utility of GHRH determinations. Until that time, however, such determinations are best regarded as investigative tools only.

An important corollary to the mechanistic relationship demonstrated herein between local GHRH expression and tumor behavior, is the possibility that GHRH and/or its downstream effectors may come to represent cellular targets amenable to therapeutic manipulation. The recent design and synthesis of GHRH-antagonists as potential pharmacologic agents for the adjuvant treatment of acromegaly certainly support such a view, especially in the context of the findings reported here [33a, 78c, 160a, 204a, 213]. In analogy to analogs of the hypophysiotropic hormone, somatostatin, which have emerged as successful therapeutic adjuvants for acromegaly associated tumors, GHRH-antagonists may harbour similar therapeutic potential allowing for a more comprehensive approach to the postoperative management of these frequently aggressive neoplasms.
CHAPTER 3  

SRIF mRNA transcript accumulation in somatotroph adenomas

Summary

Somatostatin is the principal physiologic inhibitor of the somatotroph. Even when transformed to the neoplastic phenotype, the somatotroph retains its responsiveness to the inhibitory effects of SRIF and its analogs. Whereas recent demonstrations of SRIF mRNA transcripts within somatotroph adenomas raises the possibility that SRIF may represent an autocrine/paracrine regulator for somatotroph adenomas, the clinical correlates of SRIF transcript accumulation are unknown.

In this chapter, the significance of SRIF mRNA transcript accumulation in 100 somatotroph adenomas was determined. Accumulation of SRIF transcripts was identified in 76% of somatotroph adenomas by in situ hybridization; these findings were corroborated by Northern analysis and protein translation was confirmed by Western blotting. By comparison, SRIF transcript accumulation was absent or present at low levels only in 30 nontumorous pituitary glands. The accumulation of SRIF transcripts was not a random phenomenon; preferential accumulation was found among more clinically indolent tumors. Specifically, SRIF mRNA signal was: (i) inversely correlated with Ki-67 tumor growth fractions ($r = -0.64, P < 0.001$); (ii) was significantly higher in smaller ($P < 0.01$), noninvasive tumors ($P < 0.001$); (iv) was weakly inversely correlated with preoperative GH levels ($R = -0.31, P < 0.01$); and (iv) was higher in tumors that were amenable to surgical remission ($P < 0.001$). A logistic regression model for postoperative surgical remission was derived based only on the SRIF mRNA signal intensity; increases in SRIF mRNA signal intensity were associated with an increased remission likelihood. The model was then applied to a second population of 30 somatotroph adenomas from a different institution. The model correctly predicted 70% of the postoperative surgical outcomes, although model performance fell short of statistical significance ($P = 0.10$). These data indicate that SRIF mRNA transcript accumulation is a marker and/or potential mechanism that defines a clinically favorable clinical phenotype, providing further support for SRIF as a negative autocrine/paracrine regulator in this tumor system.
INTRODUCTION

Through its secretion of GHRH and SRIF, the hypothalamus exercises a powerful level of regulatory control over the normal somatotroph. This regulatory paradigm, although one of the most classic examples of neuroendocrine regulation, is neither restricted to the normal somatotroph, nor does it require a hypothalamic source for hypophysiotropic hormones. In the preceding chapter, evidence was provided in support of the concept that the neoplastic somatotroph can serve as both a source for GHRH synthesis and target for GHRH action. It is this putative autocrine/paracrine stimulatory loop that most readily explains the significant associations observed between GHRH mRNA transcript accumulation and GH secretion, proliferative activity, invasiveness, and reduced surgical responsiveness in somatotroph adenomas. Whereas accumulation of GHRH transcripts appears to represent one event associated with neoplastic progression in somatotrophs adenomas, it alone cannot fully explain the spectrum of biological and clinical behavior exhibited by these tumors, indicating the presence of additional regulatory mechanisms.

Recalling that the normal somatotroph is itself subject to dual regulation, wherein the stimulatory effects of GHRH are balanced by the inhibitory effects of hypothalamic SRIF, it is plausible the SRIF may also exercise regulatory control over the neoplastic somatotroph. In this regard, several investigators have demonstrated accumulation of SRIF mRNA transcripts and/or immunoreactive SRIF precursor (pro-SRIF) within somatotroph adenomas [98, 101, 132, 133]. In addition, others have documented SRIF release from somatotroph adenomas in vitro [77, 100, 124]. These observations, taken together with the fact that somatotroph adenomas express functional SRIF receptors (SSTRs), point to the possibility that somatotroph adenomas may be subject to SRIF mediated autocrine/paracrine regulation [9, 26, 86, 87]. In keeping with the physiologic inhibition exerted by SRIF on normal somatotrophs is the expectation that endogenous SRIF of tumoral origin may exert a comparable and clinically relevant inhibitory effect on the behavior of somatotroph adenomas. In this chapter, we address this hypothesis, comprehensively correlating accumulation of SRIF mRNA transcripts with clinical and biological behavior in a large series acromegaly associated pituitary tumors.
MATERIALS AND METHODS

Each of the 100 tumors in the primary study population, the 30 nontumorous pituitary glands, and each of the 30 tumors in the secondary study population that were evaluated for GHRH mRNA transcripts (Chapter 2) were studied for their expression of SRIF mRNA transcripts. Identical protocols were used for ISH, ISH control procedures, ISH signal quantification, and Northern analysis. The hSRIF 30mer antisense oligonucleotide probe used for both northern analysis and ISH was derived from nucleotides 1-30 (5'-GAA AGT CTT CCA GAA GAA ATT CTT CGA GCC-3') of the published hSRIF cDNA [165]. Probe labeling procedures and specific activities for hybridization (both ISH and Northern analysis) were identical to that described for GHRH.

Immunohistochemistry, antigen retrieval, and Western blotting were also performed following protocols established in described in chapter 2. The hSRIF antibody, which recognizes both SRIF-14 and SRIF-28, that was used for immunohistochemistry and Western blotting was a commercial rabbit-derived polyclonal SRIF antibody (Peninsula Laboratories, Belmont CA) For immunohistochemistry, antibody concentrations ranging from 1:25 to 1:1000 were tested and used. For Western blotting, a 1:500 concentration was satisfactory.

All statistical methods (one way ANOVA, Bonferroni corrected post hoc comparisons, bivariate correlation, logistic regression, Chi-squared analyses), outcome criteria, and overall research design were all identical to those employed in Chapter 2.

RESULTS

SRIF mRNA transcripts in nontumorous pituitary glands
In nontumorous control pituitaries, SRIF mRNA transcript levels were generally low, most examples having either no signal or background levels of signal only (Figure 3.1A). The mean SRIF mRNA signal intensity of 30 control glands was 4.36 ± 0.57 silver grains/cell (range: 0 - 11.1). No difference was noted in the mean SRIF mRNA signal intensity between autopsy and surgical nontumorous controls, the mean SRIF mRNA signal intensity being similar in both groups (4.05 versus 4.96, two-sample t-test for independent samples, p= 0.46). Among 12 of 30
nontumorous glands (7 of 20 autopsy glands and 5 of 10 surgical specimens), however. SRIF mRNA transcripts were demonstrable at levels above thresholds attributable to background signal (5 silver grains/cell). In these specimens, the mean SRIF signal intensity was 7.62 ± 0.53 silver grains/cell (range: 5.5 -11.1). SRIF transcripts were evident within all adenohypophyseal cell types, including acidophilic, basophilic, and chromophobic cells. In some nontumorous specimens, SRIF transcripts appeared to preferentially accumulate in small chromophobic cells (lactotrophs) as compared to the larger acidophilic cells which are typically somatotrophs.

(Figure 3.1B).

**SRIF mRNA transcripts in somatotroph adenomas**

Accumulation of SRIF mRNA transcripts was demonstrated in 76 of 100 somatotroph adenomas (Figure 3.2). Overall, the mean SRIF mRNA signal intensity was 18.26 ± 1.37 silver grains/ cell, a level significantly higher than the mean signal present in the nontumorous control tissues (two sample t-test for independent samples, t-statistic =5.49, p <0.001) (Figure 3.3). Differences in the SRIF signal

*Figure 3.1 ISH for SRIF mRNA in nontumorous pituitary gland.* In the majority of cases, as in example (A), the nontumorous pituitary gland showed only background levels of SRIF mRNA signal (hematoxylin, magnification x400). In a minority of cases, however, low to moderate levels of SRIF mRNA signal was seen. When present, the latter was more abundantly distributed among small chromophobic cells (lactotrophs) rather than within the large acidophilic somatotrophs (hematoxylin, magnification x400).

*Figure 3.2 ISH for SRIF mRNA in a somatotroph adenoma.* In the majority of cases, as in this example, the hybridization signal was diffusely distributed. Note only background levels of signal within the blood vessel (hematoxylin, magnification x400).
intensity between the tumor and normal glandular tissue were especially obvious in some surgical specimens wherein the border between tumor and normal gland was present (Figure 3.4). Considerable variability was noted in the SRIF mRNA signal intensity between individual tumors, as evidenced by the wide range in signal intensities (0 - 50.6 silver grains/cell).

Figure 3.3 Boxplot analysis showing distribution of SRIF mRNA signal intensities in tumors, surgically removed and autopsy nontumorous pituitary glands. For each group, the 10th, 25th, 50th, 75th, and 90th percentiles of the SRIF signal intensity distribution are depicted in box and wiskers format. The mean SRIF mRNA signal intensity of tumors (18.3±1.4) is significantly higher than that of the nontumorous pituitary glands (4.4±0.6): (Two-sample t-test for independent sample, t-statistic = 5.49, P <0.001)

Some variability was also present in the mean signal intensities between the different somatotroph adenoma subtypes (oneway ANOVA, F-ratio = 3.37, p < 0.01). As a group, the
mammosomatotroph adenomas had the highest mean signal intensity (26.41 ± 3.51), differing significantly from the mixed lactotroph-somatotroph adenomas which had the lowest (11.97 ±2.08) (posthoc pairwise comparison, Bonferroni correction, p<0.05). Pairwise comparisons between all other pathologic subtypes did not reveal significant differences.

![Figure 3.5](image)

**Figure 3.5**
Comparison of mean SRIF mRNA signal intensities between different morphologic types of somatotroph adenomas. Mean values for each group are indicated and error bars indicate 95% confidence intervals around the mean. The only groups which differed significantly were the mammosomatotroph adenomas (group 3) which had significantly higher levels of message than the mixed GH-PRL cell adenomas (group 4). (One way ANOVA, F-ratio = 3.37, p< 0.05, Bonferroni correction).

**Northern analysis**
In all of six tumors studied by northern analysis, as well as in one nontumorous pituitary gland a single, appropriately sized transcript of approximately 0.8 kb was demonstrated (Figure 3.6 A).
**Western analysis**

In all of 6 somatotroph adenomas studied by western analysis, and in one autopsy control pituitary gland, a discrete band of 3-5kDa that co-migrated with purified SRIF-28 was detected (Figure 3.6B) Qualitatively, the amount of immunoreactive SRIF was greater among tumors than in the autopsy pituitary in all but a single instance (Case#6). Although the tumor with the lowest level of SRIF also had a high growth fraction (also Case #6), there were too few cases and too little variability to permit clinicopathologic correlation. As the purpose of the Western analysis was purely to confirm protein translation, and given the small number of samples available for study, quantification and formal comparisons between cases were not undertaken.

![Western analysis diagram](image)

**Figure 3.6** Northern analysis for SRIF (A) and Western analysis for SRIF (B) in 6 somatotroph adenomas and in 1 autopsy nontumorous pituitary gland (NT). For each tumor, the Ki-67 labeling index and radiologic (Hardy) grade are noted. A: Northern analysis reveals a single, appropriately sized transcript of 0.8kb in all of 6 somatotroph adenomas and in the nontumorous pituitary gland. As a loading control, the 28s ribosomal fraction on the ethidium bromide containing gel is shown. The band intensity was higher in some noninvasive adenomas (Grade II), however, this was neither a strong nor consistent phenomenon in these cases. B: Western analysis for SRIF reveals an appropriately size band of approximately 5 KDa that co-migrated with synthetic SRIF-28. No SRIF precursor proteins were identified. Case numbers refer to cases studied for GHRH (Figure 2.2, page 43)
SRIF Immunohistochemistry

Of the 30 control nontumorous pituitaries, all of which were formalin-fixed and paraffin-embedded, SRIF immunoreactivity could not be demonstrated in a single instance despite application of vigorous antigen retrieval methods [179]. Similarly, in all of 20 formalin-fixed and paraffin-embedded, somatotroph adenomas that were studied by immunohistochemistry, all of which expressed SRIF mRNA transcripts by ISH and/or Northern analysis, immunoreactive SRIF protein could not be demonstrated.

SRIF mRNA signal intensity and Ki-67 labeling index

In comparing SRIF mRNA signal intensity with Ki-67 labeling index (tumor growth fraction), a strong, highly significant, and negative linear correlation was observed ($r = -0.64; 95\% \text{ CI}:-0.74, -0.50, p <0.001$) (Figure 3.7).

![Figure 3.7](scatterplotΜ.png)  
**Figure 3.7** Scatterplot analysis showing a significant inverse correlation between the SRIF mRNA signal intensity and the Ki-67 derived tumor growth fraction ($r = -0.64; p <0.001$). Dotted lines represent the 95% confidence interval around the expected mean SRIF mRNA signal intensity for any given Ki-67 LI.

SRIF mRNA signal intensity and tumor size/invasion status

All tumors were graded according to size, invasion status, and radiological appearance according to the Hardy classification [63]. Of grades 0-I, II, III, IV, the primary study group was represented by 9, 42, 31, and 18 tumors, respectively. The mean SRIF mRNA signal intensity of microadenomas (ie. tumors $< 1.0 \text{ cm in diameter, Grades 0-I}$) was significantly higher than that
of larger tumors (ie. macroadenomas, Grades II-IV) (28.18 ± 2.42 versus 17.28 ± 1.45, respectively; ANOVA, F-ratio = 7.28, posthoc linear orthogonal contrast, t-statistic=2.03, p< 0.01). The mean SRIF mRNA signal intensity among noninvasive adenomas (Grades 0-II) was significantly higher than that of invasive adenomas (Figure 3.8).

Relationships between SRIF mRNA signal intensity and GH secretion

In exploring the relationship between SRIF transcript accumulation and GH secretion, two separate analyses were performed: (i) At a cellular level, the distribution of SRIF mRNA transcripts were compared with the distribution of GH immunoreactivity on the same tissue section; and (ii) At a clinical level, the SRIF mRNA signal intensity in the tumor was correlated with serum GH level in the patient.

(i) Combined in-situ hybridization for SRIF mRNA and immunohistochemistry for GH

In the 30 cases wherein in ISH for SRIF mRNA transcripts was combined with immunohistochemistry for GH on the same tissue section, 2 different patterns were seen. In the first, and more common of the two, both SRIF transcripts and immunoreactivity for GH were diffusely present in all tumor cells, revealing a diffuse co-localization of SRIF message and immunoreactive GH (Figure 3.9A). Less frequently, a second pattern was observed wherein an inverse relationship was distinctly evident between the cellular accumulation of SRIF transcripts and the
presence of GH immunoreactivity (Figure 3.9B). In such cases, tumors appeared to be comprised of two distinct cell populations; one population exhibited intense signal for SRIF but was immunonegative for GH, whereas a second population of cells was immunopositive for GH but contained no message for SRIF. This inverse relationship was especially obvious among mammosomatotroph adenomas, but was also seen in some cases of sparsely and densely granulated GH cell adenomas.

Figure 3.9 Cellular relationship between the distributions of SRIF mRNA and immunoreactive GH in two somatotroph adenomas. The more common pattern (A) shows a diffuse co-localization of the SRIF hybridization signal with the cellular content of immunoreactive GH. Less often (B) an inverse relationship was evident; cells expressing SRIF message were devoid of GH. (Hematoxylin, GH counterstain, original magnification x400).

(ii) Correlation between SRIF mRNA signal intensity and preoperative serum GH levels

In all patients, multiple basal determinations of the preoperative serum GH level had been made, the mean of which was used for this analysis. In correlating the mean preoperative blood GH level and tumoral SRIF mRNA signal intensity, a weak but significant negative linear correlation was observed ($r = -0.31$; 95%CI: -0.48, -0.12); $P<0.001$. (Figure 3.10).

Figure 3.10 Scatterplot analysis showing a weak, but statistically significant negative linear correlation between the serum GH level and the tumoral SRIF mRNA signal intensity ($r=-0.31$, $P<0.001$). Although highly significant statistically, the magnitude of the relationship is too small to be of practical or clinical importance.
SRIF mRNA signal intensity and surgical outcome

As described in chapter 2, response to surgical therapy was considered a dichotomous outcome variable defined solely on the basis of postoperative suppressibility of the serum GH level to less than 2 ng/ml during an oral glucose tolerance test. Based on this criterion, remission was achieved in 43 of 100 patients. Although nearly all of the remaining 57 patients experienced substantial declines in basal GH levels as well as symptomatic improvement, their failure to suppress below the established threshold placed them in the "no remission" category. When stratified on the basis of postoperative remission status, the distribution of SRIF mRNA signal intensities among tumors in which remission was achieved differed significantly from those in which it had not. First, the mean SRIF signal intensity of tumors amenable to remission was higher than those not achieving remission (26.7 ± 1.9 versus 11.9 ± 1.5, respectively; two sample t-test for independent samples, t-statistic = 6.26, 95%CI for difference (10.1, 19.4); p<0.001). Moreover, the distribution of individual SRIF signal intensities in each group was clearly separable, with that of the remission group being shifted rightward (Figure 3.11).

To delineate more precisely the relationship between SRIF mRNA transcript accumulation and endocrinologic outcome, a univariate logistic regression model of remission likelihood was fitted as is represented as follows:

\[
\text{Logit} \left( \frac{p_{\text{remission}}}{1 - p_{\text{remission}}} \right) = -2.1313 + 0.0978(\text{SRIF})
\]

\[
p_{\text{remission}} = \frac{1}{1 + e^{-[-2.1313 + 0.0978(\text{SRIF})]}}
\]

(Equation 3.1)
As illustrated in Figure 3.12, SRIF transcript accumulation was favourably associated with remission likelihood; increases in tumoral SRIF mRNA signal intensity were associated with a higher probability of postoperative remission. For example, an increment in SRIF signal intensity of 10 silver grains/cell was associated with more than a two fold increase in the odds favoring postoperative remission (odds ratio = 2.66, 95% CI: 1.83, 4.12).

To validate the adequacy with which a logistic model actually represented our data, two standard statistical criteria of goodness-of-fit were performed evaluated. First, a Hosmer-Lemeshow statistic was calculated ($X^2 = 7.378$, 8df, $p=0.50$); its lack of significance legitimized our acceptance of adequate model fit. Second, the area under the receiver operator characteristic (ROC) curve was high ($c=0.81$), indicating both good fit and satisfactory predictive accuracy for this model.

**Outcome prediction in a secondary test population**

In evaluating tumor samples from the secondary population, the investigators were blinded to all information except the pathologic subtype of the tumor. In situ hybridization for SRIF mRNA was performed and the signal intensity quantified as described. Based only on the SRIF mRNA signal intensity, the derived outcome model (Equation 3.1) was applied and the probability of a surgical remission was determined for each case. A threshold or "cut-off" probability for remission of 0.5 was selected. Accordingly, when the model predicted a remission probability of greater than 0.5, the case was designated as a predicted remission; alternatively, values of 0.5 or less were designated as predicted failures. Predicted results were compared to actual results using a contingency table analysis (Table 3.1). Although the model correctly predicted actual surgical outcome in 21 of 30 cases (70%), correctly predicting 14 of 19 successful outcomes.
(79%) and 7 of 11 unsuccessful outcomes (94%), the model's performance fell short of statistical significance (continuity corrected $X^2 = 2.638, p = 0.10$).

**Table 3.1**

<table>
<thead>
<tr>
<th>PREDICTED REMISSION STATUS (Equation 3.1)</th>
<th>ACTUAL REMISSION STATUS</th>
<th>MAYO CLINIC POPULATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Remission</td>
<td>Remission</td>
<td>14</td>
</tr>
<tr>
<td>No remission</td>
<td>No remission</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>11</td>
</tr>
</tbody>
</table>

**DISCUSSION**

A neuropeptide with wide anatomic distribution, SRIF has primarily been regarded for its physiologic role in the regulation of adenohypophyseal, pancreatic, neuronal, and smooth muscle functions [148, 151]. Given that its dominant physiologic effect in most target tissues is inhibitory, there has been increasing interest in the role of SRIF in both the pathogenesis and therapy of neoplastic disease [155]. Insofar as the pituitary somatotroph was the first recognized substrate for SRIF action and remains the prototypical target tissue subject to SRIF regulation, alterations of SRIF activity might be expected to be of particular pathophysiologic relevance in the setting of somatotroph adenomas. Indeed, several lines of evidence point to SRIF as a potential negative autocrine/paracrine regulator in somatotroph adenomas. First, SRIF is a peptide which, in virtually all tissues, acts locally, near its site of synthesis. Second, our data and that of others indicates that somatotroph adenomas are a biosynthetic site for SRIF [98, 100, 101, 132, 133]. Third, secretion of SRIF by somatotroph adenomas has been documented in vitro [124]. Fourth, somatotroph adenomas almost invariably express multiple SRIF receptors (SSTR1, SSTR2, SSTR5, and less frequently SSTR3) [39b, 58, 59, 118, 163b]; only rarely are these tumors wholly SSTR negative. In addition, SRIF receptor expression in human somatotroph adenomas has been documented in vivo using SSTR scintigraphy with radionuclide labeled SRIF analogs [39a, 86]. Finally, when expressed by somatotroph adenomas, SSTRs retain their functionality, as evidenced by the facts that almost 90% of somatotroph...
(79%) and 7 of 11 unsuccessful outcomes (94%), the model's performance fell short of statistical significance (continuity corrected $X^2 = 2.638, p = 0.10$).

### Table 3.1

<table>
<thead>
<tr>
<th>PREDICTED REMISSION STATUS (Equation 3.1)</th>
<th>ACTUAL REMISSION STATUS</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Remission</td>
<td>No remission</td>
</tr>
<tr>
<td>Remission</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>No remission</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>11</td>
</tr>
</tbody>
</table>

**DISCUSSION**

A neuropeptide with wide anatomic distribution, SRIF has primarily been regarded for its physiologic role in the regulation of adenohypophyseal, pancreatic, neuronal, and smooth muscle functions [148, 151]. Given that its dominant physiologic effect in most target tissues is inhibitory, there has been increasing interest in the role of SRIF in both the pathogenesis and therapy of neoplastic disease [155]. Insofar as the pituitary somatotroph was the first recognized substrate for SRIF action and remains the prototypical target tissue subject to SRIF regulation, alterations of SRIF activity might be expected to be of particular pathophysiologic relevance in the setting of somatotroph adenomas. Indeed, several lines of evidence point to SRIF as a potential negative autocrine/paracrine regulator in somatotroph adenomas. First, SRIF is a peptide which, in virtually all tissues, acts locally, near its site of synthesis. Second, our data and that of others indicates that somatotroph adenomas are a biosynthetic site for SRIF [98, 100, 101, 132, 133]. Third, secretion of SRIF by somatotroph adenomas has been documented in vitro [124]. Fourth, somatotroph adenomas almost invariably express multiple SRIF receptors (SSTR1, SSTR2, SSTR5, and less frequently SSTR3) [39b, 58, 59, 118, 163b]; only rarely are these tumors wholly SSTR negative. In addition, SRIF receptor expression in human somatotroph adenomas has been documented in vivo using SSTR scintigraphy with radionuclide labeled SRIF analogs [39a, 86]. Finally, when expressed by somatotroph adenomas, SSTRs retain their functionality, as evidenced by the facts that almost 90% of somatotroph
adenomas treated with SRIF analogs will respond with some degree of secretory inhibition [86, 87, 160]. Collectively, these data indicate that somatotroph adenomas, being that they are both a local source of SRIF synthesis and a responsive target for SRIF action, may be subject to SRIF mediated autocrine/paracrine regulation. Until now, however, the biologic, clinical, and prognostic relevance of such putative activity had not been systematically explored.

In this report, involving a consecutive series of 100 morphologically well classified and clinically well detailed somatotroph adenomas, we have demonstrated that SRIF mRNA transcript accumulation is a prognostically informative event/marker for somatotroph adenomas. Whereas SRIF transcripts were either absent or present at low levels in the normal pituitary, transcript accumulation was demonstrable in 76% of somatotroph adenomas. Overall, the mean level of expression among adenomas was more than four fold higher than that seen in the normal gland. These data are in keeping with the in vitro findings of Joubert et al., wherein in vitro release of SRIF by somatotroph adenomas was significantly higher than that released by the normal gland [76]. Of particular importance, however, was that fact that accumulation of SRIF mRNA transcripts was not a random phenomenon among somatotroph adenomas. Instead, the level of SRIF message was associated with a number of clinically relevant differences in tumor biology and behavior. Specifically, the SRIF mRNA signal intensity was: (i) inversely correlated with the Ki-67 labeling index (tumor growth fraction); (ii) was significantly higher in microadenomas as compared to macroadenomas; (iii) was three fold higher among noninvasive adenomas as compared to invasive adenomas; (iv) was inversely correlated with GH secretory activity; and (v) was favourably associated with the likelihood of postoperative remission. Accordingly, a reciprocal relationship exists between SRIF mRNA transcript accumulation and oncologic behavior (ie. size, proliferative activity, invasiveness), secretory activity, and response to surgical therapy. The potential mechanisms by which SRIF may mediate these effects are each discussed separately.

Precisely how SRIF inhibits tumor growth is uncertain, however, a variety of potent effector responses have been invoked as underlying the SRIF antiproliferative effect [155]. First, SRIF appears to have a number of direct inhibitory effects which are readily reflected at multiple sites in the cell cycle. In rodent pituitary tumor cell lines, SRIF has been shown to: (i) induce a G0/G1 cell cycle block and reduce progression of cells into S-phase [32]; (ii) induce a block just prox-
imal to the G1/S restriction point by inhibiting early response gene induction and AP-1 binding [198]; and (iii) induce apoptotic cell death in G2 [173]. In addition, SRIF is a potent stimulator of phosphoprotein phosphatases, suggesting that dephosphorylation of transcription factors may also account for some of SRIF's direct cell cycle effects [155, 199]. Aside from its direct cell cycle effects, SRIF is known to have a number of indirect growth inhibitory effects. For example, SRIF has been shown to suppress epidermal growth factor (EGF) mediated cell proliferation [144, 155]. This may be of some relevance in the present context, as EGF-receptor overexpression has been associated with postoperative recurrence in some somatotroph adenomas [97]. Finally, preliminary evidence indicates that SRIF and its analogs may adversely affect tumor microvasculature and inhibit vascular cell proliferation [7, 35, 81, 153-155, 209]. For example, SRIF has been shown to increase vasomotor tone and induce hypoxic damage in some tissues, while inhibiting angiogenesis in others [155]. Parenthetically, no association between histological evidence of tumor necrosis or hypoxic injury and SRIF mRNA signal intensity was observed in the present series. Clearly, SRIF exerts a wide range of antiproliferative effects, all of which are believed to be differentially coupled to the various SRIF receptors subtypes. In the case of somatotroph adenomas, it is not known which of the aforementioned mechanisms are operative, although multiple mechanisms are likely. The antiproliferative effects of SRIF and its analogs are explored further in chapter 5.

Our data confirm an inverse relationship between the level of SRIF message and tumor invasiveness. The phenomenon had been previously raised by Levy et al in their study of pro-SRIF precursor protein expression in somatotroph adenomas. In that report, pro-SRIF levels were higher among noninvasive adenomas as compared to invasive tumors, although the difference failed to achieve statistical significance [100]. Recently, Alvaro et al., reported point mutations of the protein kinase C gene as being present among invasive pituitary adenomas [1, 2]. In addition to its role in regulating genes related to various components of the extracellular matrix, PKC has also been shown to phosphorylate the cAMP response element (CRE) binding protein (CREB) [122]. A CRE is situated upstream to the SRIF gene promoter [121]. Phosphorylation of CREB by PKC has been shown to induce the formation of CREB dimers, which exhibit a 10 fold higher affinity for the CRE than the monomeric form. Theoretically, a deactivating mutation of the PKC gene among invasive tumors, as proposed by Alvaro et al., might be expected to be associated with a reduction in CREB phosphorylation and a reduction in CREB dimer forms,
resulting in reduced activation of the CRE within the SRIF gene and thus, reduced SRIF gene transcription. Such a mechanism would link some aspects of tumor invasion with secondary repression of SRIF transcription, explaining the differences we observed in the SRIF mRNA signal intensity between invasive and noninvasive tumors.

Whereas strong relationships were observed between SRIF mRNA transcript accumulation and the oncologic behavior, the relationship between the former and the endocrine activity of somatotroph adenomas was less definitive. In some instances, particularly involving densely granulated and mammosomatotroph adenomas, a reciprocal relationship between SRIF mRNA signal intensity and immunoreactive GH content could be resolved at the level of an individual tumor cell. Cells expressing high levels of SRIF message tended to be immunonegative for GH, whereas neighboring tumor cells having low levels of SRIF message tended to express an abundance of immunoreactive GH. This relationship, although consistent with an autocrine inhibitory role for SRIF, was not uniformly present. At the clinical level, a negative linear correlation was observed between the the tumoral SRIF mRNA signal intensity and the patients preoperative blood GH level. Although statistically significant, it was not an especially strong relationship. In part, this relates to the episodic pattern of GH secretion both in normal subjects and in acromegalic patients, in whom wide fluctuations between peak and trough GH levels are typically encountered [195]. Correspondingly, among the patients in this series, in each of whom multiple GH determinations had been made, considerable variability was observed between one random GH level and the next. In attempting to minimize this effect, the mean of multiple random GH determinations was used for comparison. A 24 hr-integrated blood GH level, although not available in this series, may have provided a more precise means of gaging this relationship.

Proliferative activity, tumor size, invasion status, and preoperative GH levels are interrelated clinicopathologic parameters that reflect different aspects of the intrinsic aggressiveness of somatotroph adenomas. In any given tumor, each has some bearing on the responsiveness to surgical intervention and prognosis [186, 190]. This was demonstrated in our study population from the univariate analysis performed in Chapter 2. To more precisely delineate the prognostic relevance of SRIF mRNA transcript accumulation, postoperative endocrine remission was chosen as a final endpoint for comparison. This is an especially relevant endpoint because it essentially distinguishes those tumors whose biological characteristics lend themselves to complete removal
from those whose size and invasiveness preclude complete excision. This assumes, of course, that the surgical procedure was performed by an experienced neurosurgeon, as was true in this series. Therefore in this context, postoperative remission status can also be viewed as a reflection of tumor aggressiveness. In our analysis of surgical outcome, the degree of SRIF mRNA transcript accumulation among tumors amenable to postoperative remission was more than two-fold higher than those in which surgery failed to provide endocrine cure. This relationship was more precisely defined by our logistic regression outcome model wherein a unit increase in SRIF mRNA signal of 10 silver grains per cell was associated with an increase in the odds favoring remission by more than 2.5 fold. Finally, the generalizability of our outcome model in predicting postoperative remission was evaluated in a second population of acromegalic patients. Based only on the tumoral SRIF mRNA signal intensity and the application of our outcome model (Equation 1), the model correctly predicted 70% of surgical outcomes in the secondary (Mayo Clinic) population of acromegalic patients. The association between predicted and actual outcomes, although falling just short of statistical significance ($p = 0.10$), was still of conceptual importance. Particularly in the context of the aforementioned clinicopathologic analyses wherein accumulation of SRIF mRNA transcripts was indicative of a favorable clinical phenotype, the same trend was reflected in the performance of our outcome model. It should also be pointed out that the statistical significance of these data were evaluated with a conservative statistical procedure (continuity corrected chi-square rather than standard chi square).

Our correlations between SRIF and tumor behavior have been based exclusively on the levels of SRIF message. Our attempts to demonstrated immunoreactive SRIF protein on formalin fixed paraffin-embedded sections were unsuccessful, despite the application of vigorous antigen retrieval techniques. Using a different fixative, Li et al could demonstrate SRIF immunoreactive cell populations in paraffin-embedded somatotroph adenomas [101]. With Western blotting, however, we demonstrated a protein band with migrational characteristics identical to that of mature SRIF-28 protein in all of 6 somatotroph adenomas studied and in the single autopsy control pituitary. This indicates that technical limitations rather than a failure of protein translation account for our inability to detect SRIF immunohistochemically. Given that only low level protein expression is required for autocrine or paracrine activity, it is likely that the levels of SRIF present were below immunohistochemically detectable threshold. In contrast to the report of Levy et al., in which only the pro-SRIF precursor protein was demonstrable by Western blotting
in somatotroph adenomas [100], we identified only a single band (3-5 kDa) which co-migrated with SRIF-28, corresponding to mature SRIF; the pro-SRIF precursor, having a molecular mass of 10 kDa, was not detected in any instance. Furthermore, even when we repeated the experiment using protocol modifications specifically designed to resolve small proteins of 10 kDa or less, only a single protein band corresponding to SRIF-28 was detectable.

In this report, we have comprehensively drawn upon morphologic, molecular, cell kinetic, and clinical data to provide evidence that SRIF mRNA transcript accumulation is a prognostically relevant event/marker with respect to the clinical behavior of somatotroph adenomas. Accumulation of SRIF transcripts was shown to be a marker of clinically and prognostically favorable behavior, as reflected by its inverse relationship to tumor size, proliferative activity, invasiveness, GH levels, and its positive association with postoperative remission. The converse was also found to be true, with absent or low level transcript accumulation being a marker of clinically aggressive behavior. While it has been previously recognized that the neoplastic somatotroph is both a source of SRIF synthesis and a target for SRIF action, and thus, may itself be subject to SRIF-mediated autocrine/paracrine regulation, this is the first demonstration of the clinical and prognostic correlates that might accompany putative activity of this type. What remains unknown, however, are the mechanisms responsible for SRIF overexpression in these tumors. One possibility is that SRIF mRNA accumulation may represent an adaptive response of the neoplastic somatotroph to chronic GH excess, analogous to the feedback effects that occur in the hypothalamus. Physiologically, elevations in GH levels stimulate hypothalamic SRIF release and SRIF gene transcription. This constitutes an important and well established short feedback loop that enables GH to regulate its own secretion. Were neoplastic somatotrophs subject to similar feedback responses, one might speculate those tumors capable of responding with increases in SRIF gene transcription, translation, and secretion would have a more indolent phenotype, whereas those incapable of a negative feedback response would be inherently more aggressive. From this point of view, tumors capable of generating such negative feedback responsive might, on some level, be considered ‘better differentiated’. Such a paradigm would be compatible with the findings observed herein.

An alternative explanation is that alterations in tumoral SRIF transcriptional and secretory activity are themselves primary events and/or causal determinants of somatotroph adenoma behavior.
In all likelihood, this is probably an oversimplification. Complicating the issue is the fact that numerous hormones, neuropeptides, growth factors, cytokines, and intracellular mediators are now recognized for their direct and indirect influences on SRIF transcriptional and/or secretory activity (reviewed in [134]). Moreover, situated between the typical TATA and CAAT promoter regions of the SRIF gene is a cAMP response element [121-123, 202], a transcriptional enhancer sequence which is itself subject to tissue-specific regulation by various signaling cascades that converge on the adenylate cyclase second messenger system; several such pathways are operative in the somatotroph. Accordingly, the primary events responsible for behavioural differences in these lesions may be considerably upstream to and only indirectly related to alterations observed in SRIF transcript levels. As discussed previously, mutations of the PKC gene and their effect on pituitary tumor invasion might be mediated by such a mechanism. Even the GHRH mediated pathway, although often regarded as distinct and opposite in effect to the SRIF pathway, may also project on to the latter. Moudiennine et al., in a series of in vitro experiments, demonstrated that the GHRH released from neoplastic somatotrophs can repress, presumably by autocrine and/or paracrine mechanisms, the somatotrophs pro-SRIF synthesis and SRIF release [124]. This indicates that the direct stimulatory effects of GHRH on somatotroph proliferation may be further potentiated by inhibition of SRIF activity. As illustrated by these examples, the regulation of the neoplastic somatotroph is clearly complex, involving multiple independent and inter-related regulatory elements and pathways, the net effect of which will ultimately determine the clinical behavior of these lesions. Still, such secondary alterations in SRIF activity may represent an important pathway by which at least some of the effects of primary upstream events may ultimately be mediated.
Chapter 4: GHRH and SRIF mRNAs and somatotroph adenoma behavior: Multivariate modelling

Summary

To better understand the relationship between the accumulation of GHRH and SRIF mRNAs and tumor behavior, multivariate methods were applied to data obtained in the previous two chapters. First, tumors were grouped according to their level of GHRH and SRIF mRNA signal intensities. This identified three phenotypically distinct groups: (i) an aggressive group (Group 1), defined by high levels of GHRH and low levels of SRIF mRNAs, and characterized by the highest tumor growth fractions, high preoperative GH levels, the highest frequency of gross invasion, and the lowest rate of postoperative remission; (ii) a clinically favorable group (Group 4), defined by low levels of GHRH and high levels of SRIF mRNAs, and characterized by the lowest tumor growth fractions, low preoperative GH levels, the lowest frequency of gross invasion, and the highest rate of postoperative remission; and (iii) an intermediate group defined by GRHR and SRIF mRNA levels that were both high or low (Groups 2,3), and characterized by a behavior intermediate to the aggressive and clinically favorable groups. Next, a series of multivariate models, based on the GHRH and SRIF mRNA signal intensities, were fitted and tested in the secondary (Mayo Clinic) population of somatotroph tumors. These analyses demonstrated that both tumor cell proliferation and responsiveness to surgical resection could be regarded as a function of the levels of GHRH and SRIF message within the tumor. Finally, a significant reciprocal relationship between the levels of GHRH and SRIF message was demonstrated. Collectively, these data indicate that accumulation of GHRH and SRIF mRNA transcripts are not random phenomena. Instead, their presence is reliably and reproducibly associated with a number of clinical and prognostically relevant differences in tumor behavior.
INTRODUCTION
Clinically relevant differences in the behavior of somatotroph adenomas are accompanied by measurable differences in the accumulation of GHRH and SRIF mRNA transcripts. Whereas accumulation of the GHRH message is associated with a number of clinicopathologic features indicative of aggressive behavior, the opposite is true for SRIF, whose mRNA accumulation defines a more clinically indolent phenotype. As presented in the preceding chapters, these conclusions are based upon a series of univariate analyses wherein the clinicopathologic correlates of mRNA levels of each hypophysiotropic hormone were considered separately. Although this univariate approach, in proposing a GHRH-mediated stimulatory loop in parallel with a SRIF-mediated inhibitory loop is both statistically valid and conceptually simple, it probably underestimates the complexity of the hypophysiotropic hormone regulatory circuitry in the neoplastic somatotroph and, in turn, its relationship to neoplastic progression. For example, under both physiological and experimental conditions, a clear synergy can be demonstrated between the secretory and transcriptional activities of hypothalamic GHRH and SRIF [15, 151]. Indeed, there is evidence that GHRH and SRIF may each have regulatory influence upon the other. For example, Mouhieddine et al have shown that GHRH can suppress SRIF secretion for somatotroph adenomas in vitro [124]. Alternatively, data also exists to support a repression of GHRH by SRIF [16, 39, 136, 211]. Clearly interactions do exist between these two regulatory elements, indicating that each regulatory cascade does not operate in isolation of the other. Moreover, since cAMP, PKA, and CREB serve as intermediate messengers for both GHRH and SRIF pathways, a convergence and mechanistic link may exist between the two (reviewed in chapter 1). Collectively, these data suggest that both in health and in disease, an interaction likely exists between GHRH and SRIF regulatory pathways. If present, such interactions would escape detection by a simple univariate approach.

To more comprehensively explore the relationship between GHRH and SRIF mRNAs to each other, as well as their simultaneous and combined effects† on several aspects of tumor behavior, this chapter uses multivariate methods to integrate data presented in preceding chapters. This will allow the relationships between GHRH and SRIF mRNAs and tumor behavior to be more precisely characterized and allow for the derivation of a multivariate model of postoperative outcome. Theoretically, such a model should have improved predictive accuracy over either of the

†Usage of the term “effect” in this chapter refers to a statistical or mathematical association. Unless otherwise stated, it does not imply, nor for that matter, does it preclude causality.
two previously fitted univariate models.

MATERIALS AND METHODS

Study variables and approach to data analysis
In this chapter, various statistical procedures are applied to data sets obtained in previous chapters. For each of the somatotroph adenomas in the primary study population, the following variables were considered: GHRH mRNA signal intensity, SRIF mRNA signal intensity, Ki-67 derived tumor growth fraction, mean preoperative GH level level, Hardy radiologic grade (ie. invasion status), and presence or absence of postoperative remission (defined on the basis of suppressibility of GH levels to less than 2 ng/ml on an oral glucose tolerance test)

Three methods of data analysis were used in this chapter. In the first, tumors were simply clustered on the basis of like GHRH and SRIF mRNA profiles and then groups were subjected to various categorical data analysis procedures. In the second approach, conventional multivariate methods of data analysis and modelling were used. Finally, the relationship between GHRH and SRIF mRNA signal intensities, themselves, were compared using simple bivariate analysis.

RESULTS

PART 1: Grouping and categorical data analysis

As a first step in understanding the collective relationship between GHRH and SRIF transcript accumulation and tumor behavior, the primary study population was partitioned on the basis of similar GHRH and SRIF mRNA profiles. Non hierarchical clustering was used to group tumors into four mutually exclusive clusters on the basis of the median GHRH and SRIF mRNA signal intensities of primary study population. Specifically, each tumor was classified as being either below or above the median (ie. 50th percentile) mRNA signal intensity for each hypophysiotropic hormone mRNA. For each of GHRH and SRIF mRNAs, an mRNA signal intensity greater than or equal to the median for the overall study population was designated as
“high’, whereas values below the median were given a designation of “low”. Accordingly, this two-way design permitted reduction of the primary study population into four recognizable tumor groups:

- **Group 1** (high GHRH, low SRIF)
- **Group 2** (high GHRH, high SRIF)
- **Group 3** (low GHRH, low SRIF)
- **Group 4** (low GHRH, high SRIF)

Pertinent clinicopathologic characteristics of each group are presented in Table 4.1. As in previous chapters, there were four main parameters of interest in each group: i) Ki-67 derived tumor growth fraction; (ii) preoperative GH level; (iii) frequency of gross invasion; and (iv) rate of postoperative remission. Difference in continuous variables (ie. Ki-67 LI and GH levels) between groups were evaluated using ANOVA, whereas categorical variables (ie. invasion and remission status) were evaluated with a chi-squared analysis.

<table>
<thead>
<tr>
<th></th>
<th>GHRH High SRIF Low Group 1</th>
<th>GHRH High SRIF High Group 2</th>
<th>GHRH Low SRIF Low Group 3</th>
<th>GHRH Low SRIF High Group 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N</strong></td>
<td>38</td>
<td>12</td>
<td>12</td>
<td>38</td>
</tr>
<tr>
<td><strong>Mean GHRH mRNA SI</strong> (silver grains / cell)</td>
<td>31.6 ± 1.8</td>
<td>25.3 ± 2.2</td>
<td>8.2 ± 1.4</td>
<td>5.6 ± 0.9</td>
</tr>
<tr>
<td><strong>Mean SRIF mRNA SI</strong> (silver grains / cell)</td>
<td>5.6 ± 0.8</td>
<td>26.0 ± 2.5</td>
<td>9.6 ± 1.7</td>
<td>31.2 ± 1.4</td>
</tr>
<tr>
<td><strong>Mean Ki-67 growth fraction (%)</strong></td>
<td>5.3 ± 0.3</td>
<td>3.4 ± 0.7</td>
<td>3.3 ± 0.5</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td><strong>Mean preoperative blood GH level (ng/ml)</strong></td>
<td>55.9 ± 6.1</td>
<td>50.5 ± 17.2</td>
<td>25.7 ± 4.5</td>
<td>26.8 ± 3.0</td>
</tr>
<tr>
<td>rate of invasion (%)</td>
<td>71.1</td>
<td>42.6</td>
<td>50.0</td>
<td>28.9</td>
</tr>
<tr>
<td>rate of remission (%)</td>
<td>13.2</td>
<td>33.3</td>
<td>50.0</td>
<td>73.7</td>
</tr>
</tbody>
</table>

*Table 4.1* Clinicopathologic characteristics of tumors clustered on the basis of median GHRH and SRIF mRNA levels. The median GHRH and SRIF mRNA signal intensities for the primary study population were 16.9 and 16.1 silver grains / cell, respectively. Individual tumors were then clustered into one of 4 groups, depending on whether the GHRH and SRIF mRNA signal intensities were above or below their respective median value.
(i) **Ki-67 derived tumor growth fraction between groups**

Significant differences were identified in the mean Ki-67 LI between groups (one way ANOVA, F-ratio = 26.38; P <0.001, Figure 4.1A). Pairwise comparisons between groups (Figure 4.1A,B) showed: (i) the Ki-67 LI to be highest in Group 1, differing significantly from that of all other groups (P<0.05, Bonferroni correction); (ii) the Ki-67 LI to be lowest in Group 4, differing significantly from that of all other groups (P<0.05, Bonferroni correction); and (iii) the Ki-67 LIs of Group 2 and Group 3 did not differ significantly from each other, although their means were intermediate to, and significantly different from, those of Group 1 and Group 4.

Based on this grouped data, the statistical effect of GHRH and SRIF message on the Ki-67 LI was summarised with a two-way, fixed effects, crossed design ANOVA (Figure 4.1C). This analysis confirmed that GHRH and SRIF transcript level, whether high or low, each accounted significantly, and independently, for the variation observed in the tumor growth fraction. Although GHRH and SRIF mRNAs are each highly significant contributors to the variation in the Ki-67 LI, the residual sum of squares is large, indicating that factors other than the levels of GHRH and SRIF message must also contribute to the variability observed in the tumor growth fraction.

*(text continued on page 86)*
A. Boxplot analysis showing distribution of Ki-67 labeling indices in tumors grouped on the basis of GHRH and SRIF mRNA signal intensities. For each group, the 10th, 25th, 50th, 75th, and 90th percentiles of the Ki67-LI distribution are depicted in box and whiskers format. One-way ANOVA confirms that significant differences in Ki-67 LI exist between groups (F-ratio = 26.38; 3, 96 df; p < 0.001).

Group 1: GHRH mRNA > median & SRIF mRNA < median (n = 38)
Group 2: GHRH mRNA > median & SRIF mRNA > median (n = 12)
Group 3: GHRH mRNA < median & SRIF mRNA < median (n = 12)
Group 4: GHRH mRNA < median & SRIF mRNA > median (n = 38)

B. Matrix of Bonferroni corrected p-values showing significant differences in the mean Ki-67 LI between groups. The mean Ki-67 LI of group 1 is significantly higher than that of all other groups whereas that of group 4 is significantly lower than that of all others. No difference in Ki-67 LI was identified between groups 2 and 3.

<table>
<thead>
<tr>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>0.0102</td>
<td>0.0084</td>
</tr>
<tr>
<td>Group 2</td>
<td>0.9615</td>
<td>0.0246</td>
</tr>
<tr>
<td>Group 3</td>
<td></td>
<td>0.0312</td>
</tr>
</tbody>
</table>

C. Results of a factorial, fixed effects, crossed design ANOVA confirming the significant effect of GHRH and SRIF status, whether high or low, on the Ki-67 LI.

Figure 4.1 Evaluation of Ki-67 LI in tumors grouped on the basis of median GHRH and SRIF-mRNA signal intensities using A. Boxplot analysis and one-way ANOVA; B. Pairwise comparisons; and C. Factorial ANOVA.
(ii) Preoperative GH level between groups

Significant differences were identified in the mean preoperative GH level between groups (one way ANOVA, F-ratio = 6.02; P < 0.001, Figure 4.2A). Pairwise comparisons between groups revealed the mean GH level to be highest in Group 1, differing significantly from that of Group 3 and Group 4 (P < 0.05, Bonferroni correction) (Figure 4.2A, B). Based on these grouped data, the statistical effect of GHRH and SRIF message on the preoperative GH level was summarised with a two-way, fixed effects, crossed design ANOVA (Figure 4.2C). This analysis confirmed that the GHRH mRNA level, whether high or low, accounted significantly for the variation observed in the preoperative GH level, whereas the SRIF mRNA did not. In addition, the residual sum of squares was large, indicating that factors other than GHRH mRNAs must also contribute to the variability observed in the preoperative serum GH levels.

**Figure 4.2** Evaluation of differences in preoperative GH levels of tumors grouped on the basis of median GHRH and SRIF-mRNA signal intensities using A. Boxplot analysis and one-way ANOVA; B. Pairwise comparisons; and C. Factorial ANOVA.
(iii) Frequency of tumor invasion between groups

The frequency of tumor invasion was highest in Group 1 (71%), lowest in Group 4 (29%), and intermediate in Group 2 (43%) and Group 3 (50%). A significant association between the tumor group and invasiveness was present ($\chi^2 = 13.77$, 3 df, $P < 0.004$ (Table 4.2)).

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-invasive</td>
<td>11</td>
<td>7</td>
<td>6</td>
<td>27</td>
<td>51</td>
</tr>
<tr>
<td>Invasive</td>
<td>(71%)</td>
<td>(42%)</td>
<td>(50%)</td>
<td>(29%)</td>
<td>49</td>
</tr>
<tr>
<td>Totals</td>
<td>38</td>
<td>12</td>
<td>12</td>
<td>38</td>
<td>100</td>
</tr>
</tbody>
</table>

LEGEND:
- Group 1: GHRH mRNA ≥ median & SRIF mRNA < median (n = 38)
- Group 2: GHRH mRNA ≥ median & SRIF mRNA ≥ median (n = 12)
- Group 3: GHRH mRNA < median & SRIF mRNA < median (n = 12)
- Group 4: GHRH mRNA < median & SRIF mRNA ≥ median (n = 38)

(iv) Rate of postoperative remission between groups

The rate of postoperative remission was lowest in Group 1 (13%), highest in Group 4 (74%), and intermediate in Group 2 (33%) and Group 3 (50%). A significant association between tumor group and postoperative remission rate was present ($\chi^2 = 29.10$, 3 df, $P < 0.001$) (Table 4.3).

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>No remission</td>
<td>33</td>
<td>8</td>
<td>6</td>
<td>10</td>
<td>57</td>
</tr>
<tr>
<td>Remission</td>
<td>(13%)</td>
<td>(33%)</td>
<td>(50%)</td>
<td>6 (74%)</td>
<td>43</td>
</tr>
<tr>
<td>Totals</td>
<td>38</td>
<td>12</td>
<td>12</td>
<td>38</td>
<td>100</td>
</tr>
</tbody>
</table>

LEGEND:
- Group 1: GHRH mRNA ≥ median & SRIF mRNA < median (n = 38)
- Group 2: GHRH mRNA ≥ median & SRIF mRNA ≥ median (n = 12)
- Group 3: GHRH mRNA < median & SRIF mRNA < median (n = 12)
- Group 4: GHRH mRNA < median & SRIF mRNA ≥ median (n = 38)
Part II: Multivariate modelling

The preceding approach to data analysis involved comparing clinicopathologic correlates between tumors that had been clustered into groups on the basis of like hypophysiotropic mRNA profiles. In this section, a more conventional approach to multivariate data analyses was used, one that evaluates the relationships between GHRH and SRIF mRNAs and tumor behavior in a parametric and more quantitative fashion. Specifically, the approach involved the development of a series of multivariate models to more precisely quantify, characterize, and ultimately, to better predict the relationships between the variability in hypophysiotropic hormone mRNAs and the variability in three important clinicopathologic variables: i) Ki-67 LI; ii) serum GH levels; and iii) surgical outcome. For each of these variables, a multivariate model was fitted, based on the GHRH and SRIF mRNAs. The validity and generalizability of each model was then tested in a secondary population of acromegalic patients (Mayo Clinic cohort).

(i) Model for tumor growth fraction

To quantify the relationship between GHRH and SRIF mRNAs and tumor growth fractions, a multiple regression model of Ki-67 LI was fitted (Figure 4.3A). The fitted model was as follows:

\[ \text{Ki-67 LI} = 2.925 + 0.083 \times \text{(GHRH mRNA)} - 0.055 \times \text{(SRIF mRNA)} \]  
(Equation 4.1)

As reflected in several pertinent indices (Figure 4.3A), this regression model was significant (F-ratio = 61.75, P < 0.001, R² = 0.56). Both GHRH and SRIF mRNA signal intensities were highly significant predictors of Ki-67 LI \((\text{GHRH: t-statistic} = 5.84; \ P < 0.001; \ 95\% \ CI: \ 0.054, 0.111); \ \text{(SRIF: t-statistic} = -3.70; \ P < 0.001; \ 95\% \ CI: -0.085, -0.026))\}. There are two noteworthy features of this model. First, the positive and negative coefficients for GHRH and SRIF, respectively, indicate their corresponding effects on the growth fraction. Second, the absolute value of the regression coefficient is greater for GHRH than for SRIF, indicating that an incremental change in GHRH mRNA signal intensity is associated with a larger change in the Ki-67 LI than is a corresponding change in the SRIF mRNA signal intensity.

As regression diagnostic procedures, several standard scatter plot analyses were constructed to
**Fitted Model:**

\[ \text{Ki-67 LI} = 2.925 + 0.083 \times \text{[GHRH mRNA]} - 0.055 \times \text{[SRIF]} \]  

**Equation 4.1**

<table>
<thead>
<tr>
<th>Case</th>
<th>GHRH mRNA</th>
<th>SRIF mRNA</th>
<th>Ki-67 LI predicted (%)</th>
<th>Ki-67 LI actual (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>15.97</td>
<td>31.16</td>
<td>2.54</td>
<td>1.85</td>
</tr>
<tr>
<td>M2</td>
<td>8.67</td>
<td>19.40</td>
<td>2.58</td>
<td>1.99</td>
</tr>
<tr>
<td>M3</td>
<td>11.11</td>
<td>28.15</td>
<td>2.30</td>
<td>1.15</td>
</tr>
<tr>
<td>M4</td>
<td>32.12</td>
<td>26.80</td>
<td>4.12</td>
<td>5.13</td>
</tr>
<tr>
<td>M5</td>
<td>3.80</td>
<td>29.28</td>
<td>1.63</td>
<td>1.11</td>
</tr>
<tr>
<td>M6</td>
<td>17.79</td>
<td>23.51</td>
<td>3.11</td>
<td>0.50</td>
</tr>
<tr>
<td>M7</td>
<td>31.96</td>
<td>28.60</td>
<td>1.71</td>
<td>1.52</td>
</tr>
<tr>
<td>M8</td>
<td>4.87</td>
<td>30.20</td>
<td>3.50</td>
<td>1.68</td>
</tr>
<tr>
<td>M9</td>
<td>18.87</td>
<td>12.60</td>
<td>3.80</td>
<td>2.48</td>
</tr>
<tr>
<td>M10</td>
<td>12.14</td>
<td>7.00</td>
<td>3.82</td>
<td>2.97</td>
</tr>
<tr>
<td>M11</td>
<td>6.60</td>
<td>21.14</td>
<td>2.31</td>
<td>3.14</td>
</tr>
<tr>
<td>M12</td>
<td>14.21</td>
<td>28.81</td>
<td>4.52</td>
<td>8.7</td>
</tr>
<tr>
<td>M13</td>
<td>9.78</td>
<td>18.14</td>
<td>2.74</td>
<td>1.87</td>
</tr>
<tr>
<td>M14</td>
<td>10.65</td>
<td>22.00</td>
<td>2.60</td>
<td>1.15</td>
</tr>
<tr>
<td>M15</td>
<td>16.68</td>
<td>4.40</td>
<td>4.07</td>
<td>3.96</td>
</tr>
<tr>
<td>M16</td>
<td>33.14</td>
<td>22.60</td>
<td>4.43</td>
<td>3.45</td>
</tr>
<tr>
<td>M17</td>
<td>14.45</td>
<td>18.80</td>
<td>3.09</td>
<td>1.98</td>
</tr>
<tr>
<td>M18</td>
<td>5.50</td>
<td>33.16</td>
<td>1.56</td>
<td>2.69</td>
</tr>
<tr>
<td>M19</td>
<td>4.10</td>
<td>22.12</td>
<td>2.05</td>
<td>1.12</td>
</tr>
<tr>
<td>M20</td>
<td>7.50</td>
<td>34.25</td>
<td>1.66</td>
<td>0.23</td>
</tr>
<tr>
<td>M21</td>
<td>2.15</td>
<td>26.14</td>
<td>2.69</td>
<td>0.50</td>
</tr>
<tr>
<td>M22</td>
<td>22.21</td>
<td>42.50</td>
<td>2.41</td>
<td>1.98</td>
</tr>
<tr>
<td>M23</td>
<td>25.54</td>
<td>25.50</td>
<td>3.64</td>
<td>1.14</td>
</tr>
<tr>
<td>M24</td>
<td>9.78</td>
<td>10.00</td>
<td>3.19</td>
<td>1.99</td>
</tr>
<tr>
<td>M25</td>
<td>14.45</td>
<td>19.90</td>
<td>3.03</td>
<td>0.89</td>
</tr>
<tr>
<td>M26</td>
<td>36.41</td>
<td>26.60</td>
<td>4.65</td>
<td>4.99</td>
</tr>
<tr>
<td>M27</td>
<td>7.20</td>
<td>23.64</td>
<td>2.22</td>
<td>1.24</td>
</tr>
<tr>
<td>M28</td>
<td>18.84</td>
<td>10.00</td>
<td>3.94</td>
<td>0.00</td>
</tr>
<tr>
<td>M29</td>
<td>10.45</td>
<td>4.19</td>
<td>3.56</td>
<td>1.54</td>
</tr>
<tr>
<td>M30</td>
<td>4.33</td>
<td>0.00</td>
<td>3.28</td>
<td>4.68</td>
</tr>
</tbody>
</table>

† mRNA signal intensity (mean silver grains/cell)

\[ \text{Ki-67 LI} = 2.925 + 0.083 \times \text{[GHRH mRNA]} - 0.055 \times \text{[SRIF]} \]

**Figure 4.3.** Fitting a multivariate model for prediction of Ki-67 LI from the primary study population (A); and model testing in the secondary (Mayo Clinic) population (B&C).

Evaluate the adequacy with this linear model actually represented our data. These included scatter plots of residual Ki-67 LI versus: (i) fitted values; (ii) GHRH mRNA values; and (iii) SRIF mRNA values. In each instance, the scatter plot revealed only a random distribution of residual values; this confirmed that the assumption of linearity underlying this model was not violated.

As an additional assessment of adequate model fit and more importantly, as the most direct means of testing the generalizability of our conclusions to other population of somatotroph
adenomas, the model was tested in the secondary (Mayo Clinic) population of somatotroph adenomas. Based on the GHRH and SRIF mRNA signal intensities of the tumors in the Mayo Clinic cohort (as determined in Chapters 2 and 3), equation 4.1 was used to predict the expected Ki-67 growth fraction of each of the 30 tumors in the Mayo Clinic population (Figure 4.3B). Predicted values were compared to actual Ki-67 LI using a scatterplot analysis (Figure 4.3C). A significant correlation was observed between predicted and actual values (r = 0.51, R² = 0.259, P = 0.004, 95% CI: 0.182, 0.734).

(ii) Model for GH level

To delineate the relationship between GHRH and SRIF mRNAs and GH secretory activity, a multiple regression model of Ki-67 LI was fitted (Figure 4.4A). The fitted model was as follows:

\[ \text{GH} = 11.135 + 1.458 \text{(GHRH mRNA)} + 0.161 \text{(SRIF mRNA)} \]  
\[ \text{(Equation 4.2)} \]

As reflected in several pertinent indices (Figure 4.4A) this regression model was significant (F-ratio = 21.82, P < 0.001, R² = 0.30), however, only GHRH was a significant predictor of the GH level \{(GHRH: t-statistic = 5.48; P < 0.001; 95% CI: 0.930, 1.986); (SRIF: t-statistic = 0.571; P =0.57; 95% CI: -0.398, 0.719)\}.

Based on their GHRH and SRIF mRNA signal intensities, Equation 4.2 was used to predict the expected preoperative GH level of each of the 30 tumors in the Mayo Clinic population (Figure 4.4B). Predicted values were compared to actual GH levels using a scatterplot analysis (Figure 4.4C). Model performance was weak, as the correlation between predicted and actual values was poor ( r = 0.190, P = 0.317, 95% CI: -0.183, 0.515).
A. Multiple regression model for predicting the GH level, fitted from the primary (i.e. Toronto) study population. The overall model is significant ($F$-ratio = 21.82, $p < 0.001$, $R^2 = 0.30$), however only GHRH mRNA signal intensity is a significant predictor of GH.

B. (left). Table of GHRH and SRIF mRNA signal intensities, and predicted and actual preoperative GH level in the secondary (Mayo Clinic) population. The predicted GH level for each case, calculated from the fitted model (Equation 4.2) is shown, as is the actual GH level.

C. (below) Scatterplot analysis of predicted versus actual preoperative GH level for the secondary (Mayo Clinic) population ($n = 30$). Although the model fitted from the primary (Toronto) population was significant, the lack of any linear correlation between predicted and actual values in the Mayo Clinic population indicates a failure of the model to be generalized to secondary populations of acromegaly associated pituitary tumours. ($r = 0.19, R^2 = 0.036, P = 0.32$).

---

**Figure 4.4** Fitting a multivariate model for predicting preoperative GH from the primary study population (A) and model testing in the secondary (Mayo Clinic) population (B&C).
(iii) Model for postoperative outcome (remission)

To evaluate the relationship between GHRH and SRIF mRNAs and postoperative remission status, a multiple logistic regression model was fitted to determine the degree of precision with which both of the former could predict the latter. The model was as follows:

\[
\text{Logit} \left( \frac{p_{\text{remission}}}{1 - p_{\text{remission}}} \right) = 0.1439 - 0.0910 \cdot (\text{GHRHmRNA}) + 0.0527 \cdot (\text{SRIFmRNA})
\]

\[
p_{\text{remission}} = \frac{1}{1 + e^{-\left[0.1439 - 0.0910 \cdot (\text{GHRHmRNA}) + 0.0527 \cdot (\text{SRIFmRNA})\right]}}
\]

The overall model was significant, as judged by several conventional indices. Akaike's informational criterion (AIC) and Schwartz's Baysean criterion (SC), each an adjustment of the -2 Log Likelihood score, both showed a reduction in their absolute value, indicating that inclusion of GHRH and SRIF mRNAs as explanatory variables provided a better model than one containing an intercept alone (Figure 4.5). The -2 Log likelihood statistic and the maximum likelihood score statistic, both of which have a chi-square distribution under the null hypothesis, were highly significant (P < 0.0001).

In the model, both GHRH and SRIF mRNAs were significant predictors of postoperative remission (GHRH, P < 0.002; SRIF, P < 0.025, Wald chi-squared statistic). In spite of the significance of the overall model its constituent variables, it was still necessary to validate the adequacy with which this logistic model represented our data. For this purpose, two standard goodness-of-fit criteria were evaluated. First, a Hosner-Lemeshow statistic was calculated (\(X^2 = 8.42, 8\text{df}, P=0.394\)); its lack of significance legitimized our acceptance of adequate model fit. Second, the area under the receiver-operator characteristic curve was high (c=0.859), indicating both good fit and high predictive accuracy for this model.
To evaluate the generalizability and predictive accuracy of our outcome model, it was tested in the secondary (Mayo Clinic) cohort. Based on their GHRH and SRIF mRNA signal intensities, Equation 4.3 was used to predict the expected postoperative remission status of each of the 30 tumors in the Mayo Clinic population. A cutoff probability of surgical remission of 0.5 was selected. When the model generated a remission probability of greater than 0.5, the tumor was classified as a predicted remission; alternatively remission probabilities of 0.5 or less were designated as predicted failures (Figure 4.6A). The predicted remission status was compared to the actual remission status using a chi-squared analysis (Figure 4.6B). The model correctly predicted the actual surgical outcome in 24 of 30 (80%) of cases (continuity adjusted $X^2 = 9.19$, 1 df, $P = 0.002$). Correctly predicting 14 of 19 successes and 10 of 11 failures, the model performed with a sensitivity and specificity of 74% and 91%, respectively.

![Figure 4.6 Outcome prediction in the Mayo Clinic population. A. In each of the 30 cases of the Mayo Clinic population, a predicted remission probability was determined by Equation 4.3. This was compared to the actual remission status. Discordant results are shown in bold. B. Contingency table analysis showing a significant association between predicted and actual outcomes (continuity adjusted $X^2 = 9.19$, 1 df, $P = 0.002$).](image-url)
(iv) Comparative analysis of other possible outcome models

As it was our objective to specifically evaluate the relationship between GHRH and SRIF mRNAs and postoperative outcome, the outcome model that was selected and tested (Equation 4.3) was deliberately fitted to include only GHRH and SRIF mRNAs as predictors. A number of other clinicopathologic predictors of postoperative outcome also exist. As demonstrated in Chapter 2, patient age, tumor pathology, Hardy grade, preoperative GH level, Ki-67 LI, were all significant univariate predictors of postoperative outcome in our primary study population. When all these univariate predictors are considered, including all levels of the categorical variables (ie. 6 pathologic subtypes and 4 Hardy grades), as well as GHRH and SRIF mRNA signal intensities, at least 157 meaningful outcome models could be fitted (APPENDIX 1). To determine how well our model compared to others, a comparative analysis of all possible outcome models was performed. A program was written to generate all possible logistic models using SAS system language/code. The significance of each model was evaluated on the basis of maximum likelihood score statistic, which has an asymptotic chi-square distribution under the null hypothesis; the higher the value of this statistic, the more significant the model. Each model and its corresponding maximum likelihood score statistic are presented in APPENDIX 1. The score statistic values ranged from 0.118 in the ‘worst’ univariate model, to 42.88 in the ‘best’ multivariate, fully saturated model that contained 13 predictor variables. The model containing only GHRH and SRIF mRNA signal intensities as predictors (Equation 4.3), had a score statistic of 37.15. This implies that our selected model contained 87% (37.15/42.88) of the predictive information present in the fully saturated model. Given that GHRH and SRIF mRNA signal intensities were the most significant predictors of postoperative outcome overall, the inclusion of any and all additional predictors in any possible model could result in an absolute maximum improvement of only 13%.

Part III. Relationship between GHRH and SRIF mRNA signal intensities

In the preceding analyses, GHRH and SRIF mRNA signal intensities were each regarded as independent variables as their relationship to various clinicopathologic parameters was sought. In this analysis, however, the potential relationship between the levels of GHRH message and SRIF message is explored in each of the 100 tumors of the primary study population. To evalu-
ate for any linear association between the two, a scatterplot analysis was performed comparing GHRH and SRIF mRNA signal intensities. As shown in Figure 4-7, a highly significant and inverse linear correlation exists between the level of these transcripts in somatotroph adenomas ($r = -0.60; p < 0.01; 95\% \text{ CI}: -0.73, -0.49$).

**DISCUSSION**

The data presented in this chapter provide further evidence that the accumulation of GHRH and SRIF mRNA transcripts within somatotroph adenomas are not random events. Indeed, each of the three sets of analyses presented herein indicate that accumulation of these transcripts is associated with a number of biologically relevant consequences. The first set of analyses has clearly shown that somatotroph adenomas can be divided into several clinically and prognostically relevant groups based upon the pattern with which these transcripts accumulate. The second set of analysis further quantified these relationships with the development and testing of various statistical models which help to explain several important clinicopathologic aspects of somatotroph adenomas in the context of the levels of GHRH and SRIF message expressed by these tumors. The third set of analysis highlight the presence of a reciprocal relationship between the levels of GHRH and SRIF transcripts in somatotroph adenomas, arguing again for a nonrandom pattern of transcript accumulation in these tumors. Each of these analysis are discussed separately.

*Characterization of tumors grouped on the basis of GHRH and SRIF mRNA profiles*

This analysis indicates that the GHRH and SRIF mRNA levels can define, significantly, three phenotypically distinct groups, each having a different clinicopathologic profile. The first is an aggressive group (Group 1), defined by high levels of GHRH and low levels of SRIF mRNAs,
and characterized by the highest tumor growth fractions, high preoperative GH levels, the highest frequency of gross invasion, and the lowest rate of postoperative remission. The second is a clinically favorable group (Group 4), defined by low levels of GHRH and high levels of SRIF mRNAs, and characterized by the lowest tumor growth fractions, low preoperative GH levels, the lowest frequency of gross invasion, and the highest rate of postoperative remission. Between these two extremes is a third subset of tumors (Groups 2 and 3) whose defining feature are levels of GHRH and SRIF mRNAs that are both either high or low. The proliferative activity, preoperative GH levels, frequency of invasion, and response to surgery of this group was intermediate to those of the aggressive and clinically favorable groups.

A second important observation evident from this analysis is that the effects of GHRH and SRIF mRNA on tumor behavior appear to be both independent and additive, insofar as the transcript level of both have some bearing on the ultimate clinical phenotype of the tumor. These data provide additional evidence for the existence of a putative GHRH mediated stimulatory circuit in parallel with a SRIF mediated inhibitory circuit, each of which appear to have competing regulatory effects on the neoplastic somatotroph. That the previously recognized association between high levels of GHRH message and adverse clinical behavior (Chapter 2) could be potentiated by low levels of SRIF message, certainly supports the possibility of such competing circuitry, as does the finding that previously recognized associations between high levels of SRIF message and favorable clinical behavior (Chapter 3) could be potentiated by low levels of GHRH message.

**Multivariate modelling**

Whereas the previous analysis of grouped data convincingly correlated differences in GHRH and SRIF mRNA profiles with differences in tumor behavior, there were two limitations inherent to this approach. First, the strategy was qualitative insofar as the groups were defined on the informative, but nonetheless arbitrary basis of whether the level of GHRH and SRIF transcripts were either high or low (ie. above or below the 50th percentiles). Second, and more importantly, that approach did not lend itself to efficient testing in a secondary population of somatotroph adenomas, the successful validation of which would be essential to providing conclusions that would be generalizable to other populations of acromegalic patients. With the application of
multivariate modelling methods, these limitations were overcome.

Based on the levels of GHRH and SRIF mRNAs in the 100 tumors of the primary study population, three models were fitted and subsequently tested in the secondary (Mayo Clinic) population.

Model for tumor growth fraction (Ki-67):
The first model quantified the relationship between GHRH and SRIF transcript levels and tumor growth fractions, providing a means by which the former could predict the latter. Validated by its significant performance in predicting the Ki-67 labeling index of tumors in the secondary (Mayo Clinic) population, the model permits, without invoking causality, the conclusion that the proliferative activity of any given somatotroph adenoma is a function of levels of GHRH and SRIF message present in that tumor. Furthermore, the model indicates that increases in GHRH message are associated with increases in proliferative activity whereas the opposite is true of SRIF message, conclusions raised in chapters 2 and 3, but now generalizable to other populations of somatotroph adenomas. In spite of the significance of this model and its performance, it is important to emphasize that the coefficient of multiple determination associated with this model is 0.56, indicating that only 56% of the variability in Ki-67 LI can be explained by variability in the levels of GHRH and SRIF mRNAs, indicating that factors other than GHRH and SRIF mRNA account for almost half of the variability observed in the Ki-67 LI.

Model for secretory activity (preoperative GH):
A model delineating the relationship between the tumoral level of GHRH and SRIF mRNAs and the preoperative serum GH level was fitted for the primary study population. Despite the statistical significance of this model overall, it performed very poorly when tested in the secondary population. There was minimal linear correlation between predicted and actual GH values, indicating that the secretory activity of the tumor could not be reproducibly represented as a linear function of GHRH and SRIF mRNA levels as structured in our model. There are at least two possible explanations for this outcome. The first is that the tumoral content of GHRH and SRIF transcripts have no bearing on the secretory dynamics of the tumor. Given that these two hypophysiotrophic hormones are the most important regulators of GH secretion in the normal somatotroph, together with the fact that the neoplastic somatotroph also retains responsiveness
to these hormones, it seems improbable that the levels of GHRH and SRIF message should negligibly impact on GH secretion. Were this the case, the fitting of a statistically significant model with satisfactory goodness-of-fit would not have been possible from our primary study population; this was not the case. A more likely explanation stems from the fact that somatotroph adenomas are well known to exhibit great variability in preoperative serum GH levels from one measurement to another during any 24 hour period. It has been this variability that has led to measurements of 24-hour integrated GH levels rather than a single random GH level as the more accurate means of directly determining GH levels. In each of the 100 cases in the primary study population, the mean of multiple preoperative GH levels was used; these data were used to fit the model. In the secondary population, only a single random GH level was available for most patients. Accordingly, it may be the imprecision of a single random GH determination and a limitation of data collection rather than a failure of model performance which accounted for the poor correlation between predicted and actual values.

Model for postoperative outcome

The development of a multivariate model of postoperative outcome, including both GHRH and SRIF mRNA signal intensities as independent variables resulted in a model with improved predictive accuracy as compared to the respective univariate models developed in chapters 2 and 3. The combined model correctly predicted the postoperative remission status in 80% of the patients in the Mayo Clinic cohort, as compared with the 73% accuracy of the GHRH outcome model (Equation 2.1, Chapter 2) and the 70% accuracy of the SRIF outcome model (Equation 3.1, Chapter 3). These data indicate that the likelihood of postoperative remission of somatotroph adenomas, assuming the procedure is performed by an experienced surgeon, can be considered a function of both the GHRH and SRIF mRNA content in the tumor. As indicated by the coefficients in the logistic function (Equation 4.3), increasing levels of GHRH mRNA are associated with a reduced likelihood of postoperative remission, whereas the opposite is true for SRIF mRNA. Since surgical responsiveness is essentially a reflection of the intrinsic aggressiveness of the tumor which either permits or precludes surgical removal, and since GHRH and SRIF message levels can reliably and reproducibly predict surgical responsiveness, it follows that these hypophysiotrophic hormone mRNAs are markers of tumor aggressiveness. Again, these findings neither imply, nor for that matter preclude a causal relationship; the design of these experiments simply does not permit an assessment of causality.
Whereas our objective was to specifically evaluate the relationship between GHRH and SRIF mRNAs and postoperative remission, it is recognized, both from our data and from the literature, that a number of clinicopathologic factors have bearing on the postoperative outcome and remission likelihood. For example, in the univariate analysis presented in chapter 2, patient age, tumor morphology, tumor invasiveness (Hardy grade), tumor Ki-67 LI, and preoperative GH levels were all significant predictors of postoperative outcome in our primary study population. Accordingly, it was of some theoretical interest to compare our selected outcome model to every other outcome model that could possibly be fitted from our data. Although our selected model was not the ‘best’ model overall, it was the best two variable model possible, and given that it contained 87% of the predictive power of the fully saturated 13 variable model, it was among the most efficient and parsimonious models possible. This underscores the relative strength of GHRH and SRIF transcript levels as markers of surgical responsiveness, and in turn, markers of tumor aggressiveness.

A reciprocal relationship between GHRH and SRIF mRNAs

That a significant reciprocal relationship should exist between the levels of GHRH and SRIF mRNAs in somatotrophs adenomas is an intriguing finding. Insofar as correlation analysis measures association and not causation, it is not possible to discern from this experiment alone, the mechanism of this finding. Assuming this is a biologically significant finding, there are at least three theoretical explanations for its occurrence: i) GHRH may repress SRIF either directly or indirectly ii) SRIF may repress GHRH either directly or indirectly, or iii) some other factor may simultaneously repress and stimulate GHRH and SRIF, respectively, or vise versa. Evidence exists in support of all three possibilities.

The possibility that GHRH may repress SRIF is supported by the finding of Moudieddine et al, who demonstrated that human somatotroph adenomas exposed to exogenous GHRH in vitro respond with an inhibition of SRIF release [124]. These authors suggested that GHRH exerted this inhibitory effect at the level of SRIF gene transcription, although levels of SRIF mRNA were not specifically studied.

The possibility that SRIF may repress GHRH is supported by several lines of evidence [16, 39,
In a recent report of an acromegalic patient harboring a GHRH secreting bronchial carcinoid tumor, treatment with the long-acting SRIF analog resulted in a 70% reduction in the serum GHRH levels [39]. In another report involving one acromegalic patient with a GHRH secreting pancreatic islet cell tumor and a second acromegalic patient harboring a GHRH secreting bronchial carcinoid tumor, the ability of SRIF to repress GHRH secretion was demonstrated both in vitro and in vivo [16]. The authors also demonstrated that these tumors possessed functional SRIF receptors which were negatively coupled to adenylate cyclase, of the types known to be present on somatotroph adenomas. In a mouse hypothalamic perfusion system model, Giraldi and Frohman were able to deplete hypothalamic SRIF content by incubation with cysteamine and anti-SRIF serum [136]. The response was an increase in hypothalamic GHRH secretion. Whereas most reports indicate an inhibitory effect of SRIF on GHRH secretion, this has not been uniform finding. There have been isolated accounts wherein exogenously delivered SRIF or its analogs has resulted in a paradoxical increase in GHRH secretion under various experimental conditions [136]. A substantive explanation for these discordant findings has not been forthcoming.

A final possibility is that the reciprocal relationship observed between GHRH and SRIF mRNA levels is unrelated to a direct effect of one hypophysiotrophic hormone on the other, but is instead the result of some other factor. For example, in a rat model of chronic GH hypersecretion, reciprocal changes were observed in GHRH mRNA and SRIF mRNA in hypothalamic nuclei [15]. Whereas GHRH mRNA were reduced by approximately 50%, SRIF mRNA levels increased by approximately 80%. The authors concluded that these reciprocal effects were the direct result of negative feedback effects of GH excess, although they could not exclude a secondary effect of SRIF induced repression of GHRH gene transcription. Contrary to that paper, we cannot invoke simple feedback effects of chronic GH excess as being responsible for the reciprocal changes we observed; our data indicates that tumors having higher levels of GHRH message also tended to have higher GH levels.

Although a satisfactory explanation for the inverse relationship observed between GHRH and SRIF mRNAs is not provided by our data nor can it be conclusively gleaned from existing literature, the weight of evidence does suggests that GHRH may, to some extent, be under regulatory control of SRIF. This issue is revisited in Chapter 6.
Chapter 5: Antiproliferative effect of a somatostatin analog on somatotroph adenomas

Summary

The extent to which somatostatin analogs suppress the growth of somatotroph adenomas is poorly understood. We sought to determine and quantify the in vivo effects of octreotide on the cell cycle kinetics of growth hormone producing pituitary adenomas. Pituitary tumor tissues were obtained from 32 acromegalic patients, all of whom participated in a multicenter randomized controlled trial studying the clinical efficacy of the somatostatin analog, octreotide. These included 16 tumors from the patient group randomized to 4 months of octreotide therapy prior to surgical resection, and 16 tumors from the group randomized to surgical resection alone. All tumors had been fully characterized on the basis of their immunophenotypic profile and their ultrastructural morphology. Included were 16 densely and 16 sparsely granulated somatotroph adenomas. In each case, immunostaining for the cell cycle specific nuclear antigen Ki-67 was performed using the MIB-1 antibody. The staining reaction was manually quantified and a tumor growth fraction was derived in each case. The mean growth fraction of tumors exposed to octreotide was suppressed by 83% as compared to untreated controls (0.011 ± 0.004% versus 0.065 ± 0.016%, respectively, P=0.0068). The association between octreotide treatment and lower tumor growth fractions was statistically independent of tumor subtype, being evident among both sparsely and densely granulated somatotroph adenomas. Octreotide exerts a significant antineoplastic effect on somatotroph adenomas, one readily reflected at the level of the cell cycle. This antiproliferative response provides insight into a number of clinicopathologic issues surrounding octreotide therapy of these neoplasms.
INTRODUCTION

Even when transformed to the neoplastic phenotype, growth hormone (GH) producing cells of the pituitary do not entirely escape physiologic regulatory controls, particularly those mediated by the inhibitory hypothalamic hormone, somatostatin (somatotropin-releasing inhibiting factor or SRIF) [88]. This observation has now found clinical application in the form of somatostatin analogs, the most recent of available therapies for acromegaly associated pituitary tumors [87, 160]. Whereas the use of these agents to exploit the inhibitory effects of native SRIF has both theoretical and practical appeal, their precise mechanisms of action against somatotroph tumors have yet to be fully understood, particularly with regard to suppression of tumor growth. Although it is known that somatostatin analogs substantially reduce serum GH levels in the majority of acromegalic patients [44, 87, 128, 160, 204], it remains unclear why so few patients respond with a significant reduction in tumor size [86]. Even more fundamental is the unresolved question of whether, and to what extent, SRIF analogs exert any significant antiproliferative effect on such tumors. Morphologic analyses of somatotroph adenomas that have undergone preoperative treatment with SRIF-analogs have provided few mechanistic insights into the problem, as no consistent morphologic alteration has been demonstrable [6, 41, 55]. Thus, in the context of somatotroph adenomas, the precise subcellular targets of these agents is not known.

Recent experimental evidence implicates the cell cycle as a potentially important target of activity for SRIF and its analogs. Octreotide, the most widely used of the SRIF analogs, has been shown to inhibit cell cycle progression in rodent pituitary tumor cell lines [32], however, it is unknown whether this agent can cause a comparable and clinically relevant in vivo cell cycle blockade in human somatotroph adenomas. In this study, the question of whether octreotide exerts an inhibitory effect on the cell cycle of somatotroph adenomas is addressed in the context of a multicenter randomized trial.
MATERIALS AND METHODS

Tumor samples and treatment protocol

Of 86 acromegalic patients who participated in a multicenter North American and European randomized controlled trial of octreotide therapy, sufficient tumor tissue for this morphologic component of the study was available in 32 cases. Details of the study protocol have been outlined in previous publications relating to this trial [41, 42]. Briefly, the inclusion criteria for trial included: (i) serum GH levels greater than 2μg/L throughout a 2 hour glucose tolerance test; and (ii) a pituitary tumor mass greater than 10 mm (ie. macroadenoma) on computed tomography or magnetic resonance imaging. Patients having undergone prior surgery or radiation therapy were excluded, as were patients having received bromocriptine therapy one month prior to enrolment and those patients whose acromegaly was related to ectopic growth hormone-releasing hormone (GHRH) hypersecretion. Patients were randomized into one of two study arms: (i) immediate transsphenoidal surgery (n = 43) or (ii) transsphenoidal surgery after 4 months of octreotide therapy (n = 43). Of those randomized to the latter group, treatment was initiated with octreotide acetate (Sandostatin, Sandoz Pharmaceuticals, Basel, Switzerland), 50μgm being delivered subcutaneously every 8 hours for the first week and thereafter, 100 μgm every 8 hours for the remainder of the 4 month study period. All specimens had been routinely processed, fixed in 4% buffered formalin, embedded in paraffin, and classified on the basis of their histology, immunohistochemical profile, and ultrastructure. Due to limited availability of adequate amounts of suitably preserved tissue, this study necessarily focused on only the most common somatotroph adenoma subtypes, the densely and sparsely granulated variants. Of the 43 tumors in the octreotide treated group, adequate tissue for this study was available in 16 cases, including 8 densely and 8 sparsely granulated variants. Similarly, of the 43 tumors in the surgery only group, tissue was available in 16 cases, again including 8 densely and 8 sparsely granulated variants. At this level, the only exclusion criteria were: (i) poor morphologic preservation of the tissue; and (ii) the absence of an adenoma in the surgical specimen. Patient demographics were comparable in both groups. Among the octreotide treated group, there were 9 women and 7 men with a mean age of 47.6 years (range: 25-71 years). Representing the surgery only group were 9 men and 7 women, having a mean age of 43.5 years (range: 21-69). Case summaries are presented in Table 5.1.
Ki-67 immunostaining

In all 32 specimens, Ki-67 derived tumor growth fractions were determined using the monoclonal antibody MIB-1 (AMAC Inc. Westbrook, ME). This antibody recognizes an epitope encoded by a 1002 base pair fragment of the Ki-67 complimentary deoxyribonucleic acid (cDNA) [31]. The Ki-67 antigen is a nuclear protein of unknown function which, as a result of its selective and transient expression in G1 to M phases of the cell cycle, reliably discriminates proliferating from quiescent cells [56, 179]. The effectiveness of this strategy in determining pituitary tumor growth fractions was recently described [187]. Briefly, 5μm thick formalin fixed, paraffin embedded sections were mounted onto glass slides and dried until ready for use. Using the avidin-biotin-peroxidase complex method of Hsu et al [72], Ki-67 immunostaining was performed in a batch fashion. Microwave antigen retrieval was employed as previously described [179, 187]. Using a 1:50 dilution, sections were incubated overnight at 4° C in the MIB-1 monoclonal antibody. Antigen-antibody complexes were detected with the chromogen 3,3'-diaminobenzidine. Slides were lightly counterstained with methyl green.

Determination of tumor growth fractions

In each case, a tumor growth fraction was determined, being represented as the percentage ratio of Ki-67 immunopositive nuclei to total nuclei. Cell counts were undertaken at high power (400X), being performed by a single technologist (JL) who was blinded to the pathological subtype and the treatment group of the tumor. In all specimens, every neoplastic cell within the full sectional area of the specimen was enumerated. Depending on the size and cellularity of the specimen, the number of cells counted per case ranged from approximately 9500 to 21000 cells. Vascular endothelial cells and nontumorous adenohypophyseal cells were excluded. Dividing the total number of immunopositive nuclei by the total number of nuclei evaluated, a growth fraction was determined in each case, and expressed as a percent.

Statistical analysis

Three statistical procedures were used to evaluate these data. First, in comparing the mean growth fractions of tumors in the two treatment groups, a two-sample t-test for independent samples was performed. Second, to evaluate the statistical effect and/or interaction between tumor pathology and treatment group on the tumor growth fraction, a balanced two-way, fixed-effects, analysis of variance (ANOVA) was performed. Third, to evaluate any statistical effects between
tumor growth fraction and tumor pathology, tumor immunohistochemical profile (monohormonal, bihormonal, trihormonal, etc.), sex of the patient, and treatment group, while controlling for patient age, an analysis of covariance (ANCOVA) was performed. For all statistical analyses, two-tailed probability values less than 0.05 were designated as significant. All means are reported as mean ± standard error of the mean (SEM). Statistical analyses were performed using SAS system software version 6.10 (SAS Institute, Cary, NC.).

RESULTS
The individual growth fractions of all pituitary adenomas were uniformly low, ranging from 0 to 0.185%. Among the 16 adenomas which did not receive preoperative octreotide, the mean growth fraction was 0.065 ± 0.016%. By comparison, the mean growth fraction among octreotide-treated adenomas was significantly lower (0.011 ± 0.004%, two sample t-test for independent samples, t-statistic = 3.08, 30 degrees of freedom, p = 0.0068). Overall, the mean growth fraction of octreotide treated adenomas was suppressed by 83%, as compared to the control group treated by surgery alone (Figure 5.1).
The association between octreotide treatment and lower growth fractions appeared to be independent of tumor subtype. In both densely and sparsely granulated variants, preoperative octreotide treatment was associated with lower growth fractions, although the difference was slightly more marked in the densely granulated tumors (Figure 5.2). To define more precisely this relationship between tumor subtype, presence or absence of octreotide treatment, and tumor growth fractions, a two-way ANOVA was performed. In this analysis, only the presence or absence of octreotide treatment had any statistical effect on tumor growth fraction (F-ratio = 8.93, P = 0.006). In particular, there was neither a primary effect of tumor subtype (P = 0.744), nor a significant interaction effect of tumor subtype and treatment status on the tumor growth fraction (P = 0.697). This indicated that the association of octreotide therapy with lower tumor growth fractions was independent of the densely or sparsely granulated pathologic subtype.

In evaluating the data with an ANCOVA model, the significance of the octreotide effect on tumor growth fraction was again demonstrable, however, no significant effect of patient sex (P=0.539), patient age (P = 0.679), or the number of hormonal immunoreactivities present in the tumor (P=0.193) was evident.

**DISCUSSION**

Whereas surgical resection has been, and continues to be, the treatment of choice for acromegaly associated pituitary tumors, there has been increasing recognition that curative resections are not routinely achieved, even in the most experienced neurosurgical hands. Especially today, when definitions of “cure” for this disease have necessarily been subject to increasingly stringent bio-
chemical criteria, reported rates of curative resections seldom exceed 50-60% [38, 48].

Moreover, in comparison to other hormone-secreting pituitary adenomas, a disproportionately large number of somatotroph adenomas are already invasive macroadenomas at presentation, a fact which further undermines the success of surgical management [186]. These considerations emphasize the fact that a sizable proportion of acromegalic patients are in need of adjuvant therapy if significant reductions in the morbidity and premature mortality accompanying GH hypersecretion are to be realized.

In response to the foregoing, SRIF-analogs have evolved as an effective form of medical therapy for acromegaly associated pituitary tumors, especially those in which surgical resection fails to reverse GH hypersecretion. These agents exploit the physiologic inhibition normally exercised by hypothalamic SRIF, the principal negative regulator of pituitary somatotrophs. Pharmacologically more stable and of higher potency than native SRIF, SRIF-analogs such as octreotide have proved effective in reducing serum GH levels and in providing prompt symptomatic relief from a number of acromegaly associated signs and symptoms. These include headache, arthralgias, hyperhidrosis, sleep apnea, and cardiac dysfunction [44, 87, 128, 160, 204]. Whereas these are fairly consistent responses that are observed in the majority of treated patients, the effect of octreotide in reducing tumor size is far less predictable. Indeed, only 20-40% of all treated patients will experience any significant reductions in tumor size, and although these may occasionally be sufficient to effect subjective visual improvement in some patients with chiasmal compression, seldom can tumor shrinkage be regarded as dramatic [86]. These clinical observations aside, a paucity of quantitative data exists regarding the the antiproliferative activity of octreotide in these tumors, and no data has been presented with regard to its effect on their cell cycle kinetics.

In this prospective controlled study, we have demonstrated that octreotide therapy is associated with an 83% suppression in the mean growth fraction of somatotroph adenomas, as compared with surgical controls. Although the magnitude of the response appeared slightly greater among densely granulated tumors, the overall octreotide effect was essentially independent of tumor subtype. Accordingly, it can be concluded that therapeutic doses of octreotide exert a definite antimitotic effect in GH cell adenomas, one readily reflected in the fraction of cycling cells. As elaborated below, this finding validates, in the context of a controlled clinical trial, a number of
octreotide induced cell cycle effects that were previously only demonstrable in-vitro, using rodent pituitary tumor cell lines [32, 173, 198, 199]. Of some interest is the fact that all somatotroph adenomas in this study, whether octreotide treated or control, had very low growth fractions (range: 0 - 0.185%). In fact, the growth fraction of the tumors in this trial were more than one order of magnitude less than those in either the 100 tumors in the primary study population or in the 30 tumors of the secondary (Mayo Clinic population). The reason for this marked difference is unclear. In part, this may reflect uncontrollable differences in tissue processing and fixation that almost invariably accompany multicenter trials of this type. In spite of the small magnitude and narrow range of growth fractions exhibited by these tumors overall, it is both interesting and important that so large an effect of octreotide treatment could still be discerned, reflecting again upon the potent antiproliferative effect exercised by this agent.

To better appreciate the antiproliferative effects of octreotide, and to put the findings of the present study in perspective with prior experimental data, several basic aspects of the cell cycle merit emphasis. As illustrated in Figure 5.3, the cell cycle, representing the highly regulated cyclic sequence of cellular maturation and division, is divided into four well known functional phases: G1, S, G2, and M phases. Terminally differentiated or otherwise quiescent cells that no longer participate in the cell cycle, either temporarily or permanently, are designated as being in the G0 phase. In progressing through the cell cycle ((G0)->G1->S->G2->M), a series of restriction points are encountered, each representing the

![Figure 5.3 Cell cycle specific effects of octreotide and/or SRIF. The Ki-67 antigen is expressed in G1-M phases of the cell cycle, and therefore discriminates proliferating cells from quiescent, non-cycling (G0) cells. In this report, octreotide was associated with an 83% suppression in the proportion of cells in G1-M phases of the cell cycle. The precise site(s) within the G1-M interval where octreotide exerts its inhibitory effect in human somatotroph adenomas is not known. Previous studies in rodent pituitary tumor cell lines have shown 3 potential sites of octreotide induced cell cycle blockade. These include: (i) blocking the entry of nonproliferating (G0) cells into the cell cycle; (ii) repression of early response gene induction and inhibition of AP-1 binding, events crucial to passage through the G1/S restriction point; and (iii) induction of apoptotic cell death in G2.](image-url)
point of irreversible transit from one phase of the cell cycle to the next. Passage through these restriction points is contingent upon the appropriate and highly specific complexation of a series of regulatory proteins, including the cyclins, cyclin-dependent kinases, inhibitors of cyclin-dependent kinases, and various transcription factors and gene products [143]. The Ki-67 labeling strategy used herein specifically identifies cells in G1, S, G2, and M phases of the cell cycle, but not the nonproliferating (G0) cells. Accordingly, it permits a reliable estimation of that proportion of the cellular population that is actively proliferating. Our finding of a lower Ki-67 labeling index in association with octreotide treatment indicates a reduction in the proportion of cells in G1 to M phases of the cell cycle, but does not indicate specifically where in this interval octreotide exerts its antiproliferative effect. In this regard, prior studies involving rodent pituitary tumor cell lines have indicated at least 3 potential sites of octreotide induced cell cycle blockade. In the GH3 pituitary tumor cell line, octreotide was shown to induce a cytostatic block at the G0/G1 interface, indicating that it inhibits entry of quiescent cells into the pool of cycling cells [32]. In another study involving the same cell line, a second level of blockade was demonstrated just proximal to the G1/S restriction point. In that study, octreotide was shown to inhibit both the expression of the early response gene c-fos, as well as DNA binding of the heterodimeric transcription factor complex (AP-1) [198]. The latter event, which occurs in late G1, is essential to activation of the multimeric S-phase promoting factor which, in turn, leads the cell to irreversible entry into S-phase. Finally, in the AtT-20 mouse pituitary tumor cell line, octreotide has been shown to induce apoptotic cell death in G2 [173], again effectively reducing the pool of cycling cells. Any and all of these mechanisms may account for the reduction in tumor growth fraction that we observed in association with octreotide treatment.

Aside from its direct cell cycle effects, octreotide is also believed to have at least two indirect effects which may further contribute to its inhibition of tumor growth. First, SRIF and its analogs appear to suppress growth factor mediated tumor growth, specifically that mediated by the epidermal growth factor (EGF)-transforming growth factor-a system [144, 155]. In the case of somatotroph adenomas, this mechanism may be of some relevance, particularly in light of a recent link between overexpression of EGF-R transcripts with aggressive behavior and recurrence in some somatotroph adenomas [97]. A second indirect effect of SRIF and its analogs relates to their effects on peritumoral vasculature. It is known that SRIF receptor density is markedly increased in peritumoral blood vessels, and that ligand activation induces vasocon-
striction [154]. Ostensibly, SRIF analogs may increase the tone of the tumor microvasculature, inducing a chronic hypoxic response which, although insufficient to induce necrosis routinely, may be sufficient to attenuate tumor growth.

From the foregoing, it is clear that octreotide exhibits a wide range of direct and indirect antiproliferative effects, all of which ultimately converge on the cell cycle. In this regard, blockade of cell cycle progression can be regarded as a "final common pathway" of octreotide action, however, the precise upstream events and post-receptor effector mechanisms that actually mediate this inhibition remain poorly understood. Complicating the issue is the existence of multiple SRIF receptors (SSTRs), five distinct subtypes having been cloned to date, with each exhibiting functional differences in their post-receptor coupling [58, 59, 70, 87, 118]. Since somatotroph adenomas ordinarily express several functionally distinct SRIF receptors, it is likely that multiple effector mechanisms are responsible for mediation of octreotide's inhibitory effect on tumor growth [58, 59, 118]. Among other receptor subtypes, somatotroph adenomas commonly co-express somatostatin receptor subtypes 2 and 5 (SSTR2, SSTR5), both of which bind octreotide with high affinity [118]. Although activation of either will inhibit cell proliferation, they do so by different effector mechanisms; activation of SSTR2 stimulates a tyrosine phosphatase, whereas activation of sstr5 simulates the inositol phospholipid/calcium pathway [29]. Future characterization of post receptor pathways for each SRIF receptor subtype will be of importance in fully understanding the mechanisms underlying octreotide's antiproliferative effects.

Whatever the precise mechanism, intermediary events, and second messenger systems involved, the present data do indicate that the cell cycle is ultimately a target of octreotide action in somatotroph adenomas. That being the case, these data also lend insight into several clinico-pathologic aspects of these lesions and their responsiveness to this agent. First, in contrast to dopamine agonist therapy of prolactin-producing pituitary adenomas wherein a reduction in tumor size may be evident within hours or days of initiating treatment, octreotide induced tumor shrinkage, if it occurs at all, frequently requires weeks of continuous therapy. That dopamine agonists induce rapid and morphologically obvious reductions in cytoplasmic volume [197], whereas octreotide acts primarily by suppressing cell proliferation without concomitant reductions in cytoplasmic volume and organelles helps explain differences in both the magnitude and the temporal course of their respective therapeutic responses. Thus, it would appear that octreotide therapy should be regarded as a means of suppressing and stabilizing ongoing tumor growth rather than a means of shrinking the size of established tumors. With regard to the
minority of tumors which do exhibit significant size reduction in response to octreotide [6]. It is important to acknowledge that at any given time, the size of a tumor is determined by a dynamic balance between cell proliferation and cell loss. Our finding that octreotide is associated with reduced cell proliferation would suggest that, in such volumetrically responsive tumors, this equilibrium is shifted in favor of cell loss, thus accounting for the eventual reduction in tumor size. Alternatively, given the aforementioned possibility of octreotide-induced apoptosis, reductions in tumor size may be a function of both decreased proliferation and increased cell loss, although this possibility has yet to be explored specifically. To the extent they can be recognized histologically, morphologic features of apoptosis are not evident among octreotide treated pituitary tumors. Finally, the present data are also consistent with several previous reports concerning the effects of octreotide on the morphology of somatotroph adenomas [6, 41, 55]. Although, occasional tumors subject to octreotide treatment exhibit increased lysosomal activity, alterations in cytoplasmic hormone granularity, interstitial fibrosis, and subtle changes in cell and nuclear size, no consistent morphologic alterations have been found. Indeed, many tumors have shown no change whatsoever. Since the observed suppression of cell proliferation would not be expected to induce conspicuous alterations in tumor morphology, our findings are in keeping with the seemingly nonspecific morphology previously described in octreotide treated adenomas.

Most studies to date have emphasized the benefits of octreotide from an endocrinologic point of view, however, this report demonstrates, unequivocally and for the first time, that octreotide also exerts a significant antiproliferative effect in somatotroph adenomas. Many questions remain, particularly with regard to which of the various SRIF receptors and signal transduction pathways actually mediate the antiproliferative effect we observed. Future studies into these areas will allow for better strategic design and development of more functionally specific SRIF analogs, ones capable of fully exploiting the antiproliferative effects of this promising class of antitumor agents.

The author acknowledge the following clinical investigators for their contribution of patients to this study:

A. Barkan (Ann Arbor, USA), A. Beckers (Liege, Belgium), P.E. Belchetz (Leeds, England), W. F. Chandler (Ann Arbor, USA), M. Buchfelder (Erlangen, Germany), P.J. Derome (Suresnes, France), M. Dupuy (Suresnes, France), R.P. Dullart (Groningen, Netherlands), C. Edwards (Edinburgh, UK), R. Fahibusch (Erlangen, Germany), M. Giovannelli (Milano, Italy), J. Habas (New York, USA), J. Hardy (Montreal, Canada), A.G. Harris (Los Angeles, USA), D. Killinger (Toronto, Canada), A. Klibaner (Boston, USA), S.W.J. Lamberts (Rotterdam, Netherlands), M. Losa (Milano, Italy), S. Melmed (Los Angeles, USA), G. Pernin (Lyon, France), K. Post (New York, USA), O. Muller (Munich, Germany) G. Sassolas (Lyon, France), R. Singh (Rotterdam, Netherlands), O. Serri (Montreal, Canada), H. Smyth (Toronto, Canada), A. Verenaert (Liege, Belgium), C.A. ter Weeme (Groningen, Netherlands), M. Weiss (Los Angeles, USA), N. T. Zervas (Boston, USA).
Chapter 6: Effects of SRIF analog therapy on GHRH and SRIF mRNAs

Summary

When studied by in situ hybridization, somatotroph adenomas exposed to 4 months of presurgical octreotide therapy (n = 10) were found to have a significant (37%) suppression in their mean GHRH mRNA signal intensity as compared to control adenomas (p = 0.01). Correspondingly, in none of 4 octreotide-treated tumors evaluated by Western analysis was any GHRH protein detectable. No differences were observed in the levels of SRIF mRNA between octreotide-treated adenomas and controls. In view of the previously described association between GHRH mRNA transcript accumulation and proliferative activity (Chapter 2), and the fact that octreotide therapy is associated with an 84% suppression in the Ki-67 tumor growth fraction (Chapter 5), the current observation that SRIF analogs may downregulate endogenous GHRH is of particular relevance, suggesting that perhaps some of the antiproliferative effects of SRIF analogs in somatotroph adenomas may be mediated and/or potentiated by GHRH inhibition. Such a mechanism has never before been ascribed to the activity of SRIF analogs in pituitary tumors.
INTRODUCTION

Whereas we have demonstrated that SRIF analogs exert a significant antiproliferative effect on somatotroph adenomas, the precise mechanisms responsible for this effect are unknown. As discussed in the previous chapter, SRIF and its analogs are known to be coupled to a variety of potential effector systems, the direct and indirect effects of which may converge to inhibit cell cycle progression. One general effector system of particular interest in this context concerns the ability of SRIF and its analogs to suppress growth factor mediated tumor growth. For example, in mouse models of gastric cancer transplants and chondrosarcoma transplants, SRIF has been shown to downregulated EGF and IGF-1 mediated tumor growth, respectively [144, 152]. In the preceding chapters we have shown that accumulation of GHRH mRNA transcripts is associated with higher rates of tumor proliferation whereas the opposite is true for SRIF mRNA transcript accumulation. Moreover, we have shown that there is a reciprocal relationship between the levels of GHRH and SRIF message in somatotroph adenomas. One of the mechanisms proposed for this relationship was that SRIF may inhibit GHRH activity at the transcriptional level or beyond. In this chapter we evaluate the simple hypothesis that the SRIF analog, octreotide, can inhibit GHRH expression at both the mRNA and protein levels. Validation of such would not only provide one specific mechanism for the antiproliferative effects of SRIF and its analogs on somatotroph adenomas, but it would provide additional support for the functionality of GHRH mediated autocrine and/or paracrine loop in somatotroph adenomas.

MATERIALS AND METHODS

Of the 32 samples of pituitary tumor tissue obtained from patients who participated in a multicenter North American and European randomized controlled trial (Chapter 5), there were 20 cases wherein sufficient tumor tissue was available for this component of the study. This include 10 tumors from patients randomized to immediate transsphenoidal resection and 10 tumors from patients randomized to 4 months of therapy with the SRIF analog, octreotide acetate (Sandostatin) followed by transsphenoidal resection. Of those randomized to the latter group, octreotide therapy consisted of 50 μgm administered subcutaneously every 8 hours for the first week and 100 μgm every 8 hours for the rest of the 4 month period of the study. Details of the trial protocol and tissue processing was presented in Chapter 5.
All tissues available from the randomized trail had been formalin fixed and embedded in paraffin; no fresh tissue was available. Accordingly, fresh-frozen tissue for western analysis had to be obtained from other sources. Fragments from 4 somatotroph adenomas pretreated with octreotide were obtained. Two were obtained from the Toronto Hospital Brain Tumor Bank (courtesy of Dr. A. Guha) and two were obtained from the University of Virginia, Charlottesville, VA (courtesy of Dr. E. Laws). All patients were treated with a standard of dose of 100 μgm every 8 hours. The duration of therapy varied from 3 weeks to 4 months, and all patients were being treated at the time of surgery. As controls, protein extracts from tumors not treated with octreotide were used.

**In situ hybridization**

In each of the 20 cases, in situ hybridization for GHRH and SRIF mRNAs and quantification by manual densitometry were performed according to protocols outlined in Chapters 2 and 3.

**Western analysis**

In the 4 tumor samples for which fresh frozen tissue was available, protein extraction, SDS PAGE and immunodetection for GHRH was performed as outlined in Chapter 2.

**RESULTS**

Accumulation of GHRH mRNA transcripts could be identified by in situ hybridization in 19 of the 20 cases. The mean GHRH mRNA signal intensity of tumors in the octreotide treated group was significantly lower than those in the untreated group (9.02 ± 1.23 versus 14.3 ± 1.37; P = 0.01, Figure 6.1). Differences between the two groups were not qualitatively obvious when evaluat-
ing individual cases; differences were apparent only after densitometric quantification and formal statistical comparisons.

Accumulation of SRIF mRNA transcripts were identified by ISH in all 20 of the somatotroph adenomas tested. Although the mean SRIF mRNA signal intensity of tumors in the octreotide treated group was lower than those in the untreated group, the difference was not significant (12.74 ± 1.73 versus 10.34 ± 1.13; two sample t-test for independent samples, t-statistic = 1.13, 18 df. P = 0.27; Figure 6.1)

By western analysis, GHRH protein was not demonstrable in any of the 4 tumors that had undergone preoperative octreotide therapy (Figure 6.2). In contrast, and as reported in Chapter 2, GHRH protein was demonstrable by Western analysis in 9 of 10 tumors that had not been treated with octreotide.

DISCUSSION

These data demonstrate that exposure to an SRIF analog is associated with a reduction of endogenous production of GHRH in somatotroph adenomas. The phenomenon was evident at both transcriptional and translational levels and, taken together with the data presented in chapter 5, was commensurate with an 84% suppression of tumor cell proliferation. We could not demonstrate any change in the levels of SRIF mRNA with octreotide therapy, despite the expectation that endogenous SRIF might be suppressed due to negative feedback effects.

As described in Chapter 5, the antiproliferative effect of SRIF and its analogs are thought to be
mediated by a diverse range of effector systems. Whereas some of these involve direct blockade of cell cycle progression, others, such as inhibition of growth factor mediated tumor growth, are indirect. In the case of somatotroph adenomas, the precise mechanisms underlying the antiproliferative effect of SRIF and its analogs are unknown. In view of the previously described association between GHRH mRNA transcript accumulation and proliferative activity (Chapter 2), the current finding that SRIF analogs may downregulate endogenous GHRH is of particular relevance, suggesting that perhaps some of the antiproliferative effects of SRIF analogs in somatotroph adenomas may be mediated by GHRH inhibition.

From a conceptual standpoint, these data lend additional support to the presence and functionality of GHRH mediated autocrine/paracrine stimulatory loop within somatotroph adenomas. Until now, our assertion that such a loop should exist was based primarily on the demonstration that the anatomic components necessary for such a circuit are present in somatotroph adenomas. Specifically, we demonstrated that GHRH is produced by tumor cells and that these same cells also express mRNA for the GHRH receptor (GHRH-R). Moreover it is known that somatotroph adenomas retain responsiveness to exogenously administered GHRH, confirming functionality of the GHRH-R. Our assertion was strengthened further by the demonstration of significant clinicopathologic correlations between GHRH mRNA levels and tumor behavior, associations which could only be explained by the presence of a GHRH mediated autocrine/paracrine stimulatory loop. The current demonstration that inhibition of endogenous GHRH in the neoplastic somatotroph is accompanied by an antiproliferative response provides further functional support for the existence and functionality of a GHRH-mediated stimulatory loop, as well as the biological effects that may accompany its interruption.

That a SRIF analog should downregulate endogenous GHRH and induce an antiproliferative response provides further support for SRIF as a negative regulator in the neoplastic somatotroph. It also establishes a functional link between GHRH and SRIF mediated pathways in this tumor system. Having previously demonstrated that the neoplastic somatotroph is an endogenous source of SRIF, we now show that it is also a responsive target for SRIF action as reflected by reductions in tumor cell proliferation and GHRH expression. Accordingly, the case for SRIF as a negative autocrine/paracrine regulator is strengthened. The concept that the neoplastic somatotroph, like the normal somatotroph, should be subject to dual regulation by SRIF and
GHRH has been an underlying theme throughout this thesis. Both SRIF and GHRH cascades converge on the adenylate cyclase second messenger system. Activation of the former, via its coupling to an inhibitory G-protein leads to reduction in cAMP whereas activation of the latter, through its coupling to a stimulatory G-protein leads to an increase in cAMP. Since the level of cAMP determines the cellular response (ie either stimulation or inhibition), these two pathways had been regarded as competing circuits, wherein the relative balance between the two ultimately defined the behavior of the cell. The current observation, indicating that one regulatory element may repress the activity of the other elevates the complexity of the paradigm, arguing against the simplicity of two parallel pathways of regulatory control. Instead, our observation suggests a more dynamic concept of regulation in the somatotroph wherein a clear interaction exists between one regulatory pathway and the other. In doing so, the current observation also helps to explain the reciprocal relationship observed between the levels of GHRH and SRIF mRNAs in somatotroph adenomas (Chapter 4).

Whereas this is the first indication of SRIF analog mediated downregulation of GHRH in human somatotroph adenomas, the phenomenon is supported by several lines of evidence in other tumor systems and experimental models [16, 39, 136, 211]. In a recent report of an acromegalic patient harboring a GHRH secreting bronchial carcinoid tumor, treatment with the long-acting SRIF analog resulted in a 70% reduction in the serum GHRH levels [39]. In another report involving one acromegalic patient with a GHRH secreting pancreatic islet cell tumor and a second acromegalic patient harboring a GHRH secreting bronchial carcinoid tumor, the ability of SRIF to repress GHRH secretion was demonstrated both in vitro and in vivo [16]. The authors also demonstrated that these tumors possessed functional SRIF receptors which were negatively coupled to adenylate cyclase, specifically the types known to be present on somatotroph adenomas. In a mouse hypothalamic perfusion system model, Giraldi and Frohman were able to deplete hypothalamic SRIF content by incubation with cysteamine and anti-SRIF serum [136]. The response was an increase in hypothalamic GHRH secretion. Whereas most reports indicate an inhibitory effect of SRIF on GHRH secretion, this has not been a uniform finding. There have been isolated accounts wherein exogenously delivered SRIF or its analogs has resulted in a paradoxical increase in GHRH secretion under various experimental conditions [11, 136]. The significance of these discordant findings is uncertain.
The precise mechanism by which SRIF can downregulate GHRH is unknown. Whether this downregulation involves direct repression GHRH gene transcription and translation, or whether it is secondary to some other intermediate factor(s) inducible by SRIF is unknown. Because virtually nothing is known of GHRH gene regulation in health or in disease, even speculation about mechanism is difficult in this area.

Whereas our conclusions from these data are that the antiproliferative effects of SRIF are mediated by both a direct inhibition of cell cycle progression and a further potentiation of the effect due to downregulation of GHRH, an alternative interpretation does exist. Specifically, there is the possibility that the antiproliferative effects are entirely due to direct inhibition of cell cycle progression or some other mechanism, and the changes in GHRH represent a secondary epiphenomenon without biologic consequence. Although this interpretation of the data cannot be dismissed, we believe it to be unlikely. In view of the facts that GHRH is (i) the most important positive regulator of the normal somatotroph; (ii) a potent mitogen and inducer of early response genes; and (iii) that its mRNA levels strongly correlate with proliferative activity, our interpretation that its downregulation is a biologically relevant event that may contribute to the antiproliferative effect of SRIF is certainly far more compelling than is the alternative.

**Therapeutic implications**

In chapter 2, we demonstrated that accumulation of GHRH mRNA transcripts is a marker of an aggressive clinical phenotype. As evidenced by the significant associations between the levels of GHRH message and proliferative activity, invasiveness, and preoperative GH levels, GHRH transcripts were seen to preferentially accumulate among aggressive tumors not subject to surgical cure. Those data, together with the grouping analysis presented in Chapter 4 indicate that high levels of GHRH message can, in essence, be equated with a prognostically unfavorable state. Now, we show that a downregulated GHRH state represents a therapeutically advantageous state, at least from the standpoint of the proliferative activity of the tumor. It follows, therefore, that achievement of a downregulated GHRH state may represent a reasonable therapeutic strategy in the pharmacologic therapy of somatotroph adenomas. Since GHRH antagonists would represent the most direct means of achieving this goal, they may hold some therapeutic potential in the pharmacologic management of somatotroph adenomas. The addition of a
GHRH antagonist to the existing regimen of SRIF analogs would permit maximal therapeutic exploitation of the regulatory mechanisms operative in the somatotroph, leading to a maximally downregulated GHRH state in the presence of maximal somatostatinergic tone. Although GHRH antagonists have not yet been tried in the treatment of somatotroph adenomas, our data certainly provide theoretical justification for their use. The principle has, however, been recently applied in a patient with acromegaly due to a GHRH secreting bronchial carcinoid tumor; administration of a competitive GHRH antagonist led to suppression of GHRH levels and, correspondingly, blood GH levels [75].
Chapter 7 Conclusions and Relevance

The objective of this thesis was to comprehensively evaluate the biologic, clinicopathologic, and prognostic significance of GHRH and SRIF mRNAs in human somatotroph adenomas. The data presented herein indicates that the majority of somatotroph adenomas have, at some point in their evolution, assumed the capacity to transcribe and translate GHRH and SRIF genes. More importantly, accumulation of GHRH and SRIF mRNA transcripts were not random phenomena. To the contrary, GHRH and SRIF transcript levels were quantifiably associated with a number of clinically and prognostically relevant differences in tumor behavior. These are summarized below:

1. The accumulation of GHRH transcripts was an adverse prognostic event, one that defined a more aggressive clinical phenotype. Increasing levels of GHRH message were associated with higher GH secretory activity, a higher tumor proliferation index, a greater frequency of invasive growth, and a reduced likelihood of postoperative remission.

2. The accumulation of SRIF transcripts was a favorable prognostic event, one that defined a more indolent clinical phenotype. Increasing levels of SRIF message were associated with lower GH secretory activity, lower tumor proliferation index, a reduced frequency of invasive growth, and an increased likelihood of postoperative remission.

3. The statistical effects of GHRH and SRIF transcript levels on tumor behavior were additive. Tumors expressing high levels of GHRH and low levels of SRIF mRNAs were clearly the most aggressive, whereas those expressing high levels of SRIF and low levels of GHRH mRNAs were distinctly more benign.

4. Since surgical responsiveness is the most important prognostic outcome variable, one that essentially reflects whether the inherent aggressiveness of the tumor permitted or precluded complete surgical removal by an experienced surgeon, it was selected as the primary outcome variable to be modelled. In univariate analysis, the GHRH mRNA signal intensity was the single most powerful predictor of outcome, eclipsing any and all other conventional clinicopatho-
logic outcome predictors. Surgical outcome could also be modelled as a significant logistic function of the levels of GHRH and SRIF mRNAs. Both these models performed significantly when applied to a second population of somatotroph adenomas. This validation of these models indicated that our findings were generalizable to other populations of somatotroph tumors.

5. A significant reciprocal relationship appeared to exist between the levels of GHRH and SRIF mRNAs in somatotroph tumors. Presurgical treatment with the SRIF analog, octreotide, was associated with significant reductions in tumor proliferation and GHRH mRNA levels and, in the small number of cases studied by Western analysis, an absence of GHRH protein expression. Collectively, these data suggest that the antiproliferative effects of SRIF analogs may be potentiated by a secondary repression of GHRH transcription and translation.

These data support the hypothesis that accumulation of GHRH and SRIF mRNAs are prognostically informative events in somatotroph adenomas. These conclusions neither imply nor, for that matter, preclude a causal relationship between hypophysiotropic hormone mRNAs and tumor behavior; the design of our experiments does not permit an assessment of causality.

RELEVANCE

The data presented herein has theoretical, clinical, and therapeutic relevance; each is discussed separately.

Theoretical relevance

From a theoretical standpoint, these data necessitate a reevaluation of the role of hypothalamic hypophysiotropic hormones in pituitary tumorigenesis. Our data indicate that the expression of hypophysiotropic hormones in pituitary adenomas is a nonrandom, clinically relevant, and prognostically informative phenomenon. Furthermore, it is a phenomenon whose most logical explanation invokes a presence and functionality of GHRH and SRIF mediated autocrine/paracrine regulatory loops within somatotroph adenomas. We have conclusively demonstrated that the neoplastic somatotroph is a source of GHRH and SRIF synthesis. As reviewed in chapter 1, evidence from the literature clearly indicates that the neoplastic somatotroph is also a responsive tar-
get for GHRH and SRIF action. Accordingly, there is little doubt that the neoplastic somatotroph is adequately configured to engage in autocrine/paracrine activity of this type. Proceeding with the assumption that the clinicopathologic correlates observed between hypophysiotropic hormone mRNAs and tumor behavior are the end result of an autocrine/paracrine process, a new paradigm for a hypophysiotropic component to pituitary tumorigenesis can be envisaged. In that pituitary tumor development is a multistep process which begins with a tumor initiation phase that is maintained thereafter by a growth promotion phase, it is in the latter phase that locally produced hypophysiotropic hormones would be most likely to exert their trophic effects. In this regard, and as suggested by our data, hypophysiotropic hormones may represent important determinants of neoplastic progression in the pituitary. Thus the net effect of local stimulatory and inhibitory activity would ultimately define the behavioural phenotype. Furthermore, recent observations indicate that GHRH may serve as an autocrine stimulator in several other human tumors [78a, 78b], suggesting a much broader role of this growth factor in human neoplasia.

Contrary to conventional concepts of pituitary tumorigenesis which have considered the contemporary ‘pituitary hypothesis’ and the traditional ‘hypothalamic hypothesis’ as being mutually exclusive theories, we propose that the two can be effectively merged without violating the underlying essence of either. What emerges is a ‘unified theory’ of pituitary tumor development that not only highlights the merits of existing theories, but also addresses the problems of biological behavior and neoplastic progression, issues not readily reconciled by previous hypothesis.

Clinical relevance
A secondary, but nonetheless important, aim of this thesis was to develop a prognostically informative strategy whereby the clinical behavior of somatotroph adenomas could be predicted. Although we have conclusively shown that GHRH and SRIF are important prognostic markers for predicting the likelihood of immediate postoperative remission, the durability of the prediction and the long term significance of these findings in terms of tumor recurrence and disease free survival still need to be determined. A practical limitation of our approach, one that will preclude its adoption as a routine diagnostic test, is that all clinicopathologic and prognostic correlates have been based on mRNA levels obtained by in situ hybridization. This method is tech-
nically laborious, requires vigorous control procedures, and is not available routine use in most hospital diagnostic labs. Since we only had ready access to formalin-fixed, paraffin-embedded tissues, this method of analysis was a necessity for this work. An important next step to bring these data to a level of routine diagnostic applicability would involve determining whether the clinicopathologic correlates described herein are also valid at a protein/immunohistochemical level. In this regard, the development of more reliable antisera for GHRH and SRIF and the development of more quantifiable immunohistochemical methods for their detection would be a necessity.

**Therapeutic relevance**

Perhaps the most important aspect of these data lie in their potential therapeutic application. Proceeding again, with the assumption that local autocrine/paracrine regulatory circuits are responsible for the correlations observed between GHRH and SRIF transcript levels and tumor behavior, a menu of potential therapeutic options emerge. Our data indicate that a downregulated GHRH state represents an oncologically favorable situation. Accordingly, rationale would now exist for the use of GHRH antagonists in somatotroph adenomas. A number of such agents have been developed [213], and one has been successfully used in an acromegalic with a GHRH-producing carcinoid tumor [75]. To our knowledge, these agents have not been used in somatotroph adenomas so far. A second therapeutic option involves the use of somatostatinergic agents. Octreotide, the prototypical SRIF analog, has been used for more than a decade in this regard. Although this agent is an effective adjuvant, it does not reduce GH levels to the desired endpoint in almost half of treated patients [203], nor does it consistently reduce tumor size. Accordingly, other somatostatinergic agents, perhaps those with different receptor affinities and post receptor coupling, may prove more effective. Our data would suggest that an upregulated SRIF state would be an oncologically favorable situation that may forestall neoplastic progression in somatotroph adenomas. Accordingly additional rationale now exists for pharmacologic augmentation of somatostatinergic tone in these lesions. A final therapeutic avenue would involve pharmacologic targeting of various effectors and/or signaling pathways downstream to the GHRH and SRIF receptors.
APPENDIX 1: Model selection for multivariate analysis

Appendix to ‘RESULTS/ (iv) Comparative analysis of other possible outcome models’ section, Chapter 4, page 94

This appendix contains a comparative analysis of each of 157 possible models that could be used to predict postoperative outcome. The significance of each model was evaluated on the basis of maximum likelihood score statistic, which has an asymptotic chi-square distribution under the null hypothesis; the higher the value of this statistic, the more significant the model. Each model, its size (i.e. the number of predictive variables contained in the model), and its corresponding maximum likelihood score statistic are listed below. The model containing only GHRH and SRIF mRNA signal intensity as predictors had a score statistic of 37.149. The fully saturated model having 13 predictors had a score statistic of 42.879 (shown on final page of appendix). This implies that the model containing only GHRH and SRIF maintained 87% (37.149/42.879) of the predictive power of the fully saturated, 13-variable model.

The legend used to code each of the variables is presented in the table below. Each of the 157 models is presented on the pages that follow.

Legend: for variable names in model

GRH GHRH mRNA signal intensity (silver grains / cell)
SRIF SRIF mRNA signal intensity (silver grains/cell)
KI Ki-67 labeling index (%) 
GH mean preoperative GH level (ng/ml)
PATH1 Acidophil stem cell adenoma 
PATH2 Densely granulated GH cell adenoma
PATH3 Mammosomatotroph adenoma
PATH4 Mixed GH-PRL cell adenoma
PATH5 Sparsely granulated GH cell adenoma
PATH6 Other plurihormonal GH cell adenoma
HARD1 Hardy grades 0-1
HARD2 Hardy grade 2
HARD3 Hardy grade 3
HARD4 Hardy grade 4
AGE Patient age
The SAS System
The LOGISTIC Procedure

Data Set: WORK.MESIS
Response Variable: REMISS
Response Levels: 2
Number of Observations: 100
Link Function: Logit

Regression Models Selected by Score Criterion

<table>
<thead>
<tr>
<th>Score</th>
<th>Size</th>
<th>Value</th>
<th>Variables Included in Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31.735</td>
<td>GRH</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>28.571</td>
<td>SRIF</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>24.689</td>
<td>KI</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7.940</td>
<td>GH</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.211</td>
<td>HARD3</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5.910</td>
<td>HARD1</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4.155</td>
<td>AGE</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.930</td>
<td>PATH2</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.116</td>
<td>PATH3</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.698</td>
<td>PATH4</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.466</td>
<td>PATH5</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.115</td>
<td>HARD2</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.118</td>
<td>PATH1</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Score</th>
<th>Size</th>
<th>Value</th>
<th>Variables Included in Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>37.149</td>
<td>GRH SRIF</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>34.336</td>
<td>GRH PATH5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>33.979</td>
<td>GRH PATH2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>33.701</td>
<td>GRH KI</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>33.092</td>
<td>GRH PATH3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>32.939</td>
<td>GRH HARD3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>32.853</td>
<td>AGE GRH</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>32.695</td>
<td>SRIF KI</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>32.669</td>
<td>GRH HARD1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>31.994</td>
<td>GRH HARD2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>31.918</td>
<td>GRH PATH1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>31.873</td>
<td>GRH GH</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>31.742</td>
<td>GRH PATH4</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Score</th>
<th>Size</th>
<th>Value</th>
<th>Variables Included in Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>39.297</td>
<td>GRH SRIF PATH5</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>39.077</td>
<td>GRH SRIF PATH2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>37.866</td>
<td>AGE GRH SRIF</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>37.727</td>
<td>GRH SRIF HARD3</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>37.667</td>
<td>GRH SRIF PATH3</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>37.538</td>
<td>GRH SRIF KI</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>37.535</td>
<td>GRH SRIF HARD1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>37.279</td>
<td>GRH SRIF PATH4</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>37.205</td>
<td>GRH SRIF GH</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>37.181</td>
<td>GRH SRIF HARD2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>37.155</td>
<td>GRH SRIF PATH1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>36.353</td>
<td>GRH PATH2 PATH1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>36.288</td>
<td>GRH KI PATH2</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Score</th>
<th>Size</th>
<th>Value</th>
<th>Variables Included in Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>40.250</td>
<td>GRH SRIF PATH2 PATH3 PATH</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>40.229</td>
<td>GRH SRIF PATH2 PATH5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>39.858</td>
<td>GRH SRIF PATH2 PATH4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>39.799</td>
<td>GRH SRIF HARD3 PATH5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>39.752</td>
<td>AGE GRH SRIF PATH5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>39.661</td>
<td>GRH SRIF KI PATH2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>39.605</td>
<td>GRH SRIF HARD1 PATH5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>39.545</td>
<td>AGE GRH SRIF PATH2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>39.486</td>
<td>GRH SRIF PATH3 PATH5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>39.485</td>
<td>GRH SRIF KI PATH5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>39.355</td>
<td>GRH SRIF PATH1 PATH5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>39.334</td>
<td>GRH SRIF PATH4 PATH5</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Score</th>
<th>Size</th>
<th>Value</th>
<th>Variables Included in Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>41.816</td>
<td>GRH SRIF PATH2 PATH3 PATH4</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>40.998</td>
<td>AGE GRH SRIF PATH2 PATH3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>40.861</td>
<td>GRH SRIF PATH2 PATH3 PATH5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>40.659</td>
<td>GRH SRIF HARD3 PATH2 PATH5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>40.624</td>
<td>GRH SRIF HARD3 PATH2 PATH3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>40.580</td>
<td>AGE GRH SRIF PATH2 PATH5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>40.572</td>
<td>GRH SRIF KI PATH2 PATH5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>40.418</td>
<td>GRH SRIF HARD3 PATH2 PATH4</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>40.375</td>
<td>GRH SRIF HARD1 PATH2 PATH5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>40.362</td>
<td>GRH SRIF PATH2 PATH4 PATH5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>40.285</td>
<td>GRH SRIF HARD2 PATH2 PATH3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>40.275</td>
<td>GRH SRIF HARD1 PATH2 PATH3</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Score</th>
<th>Size</th>
<th>Value</th>
<th>Variables Included in Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>42.307</td>
<td>GRH SRIF HARD3 PATH2 PATH3 PATH4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>42.196</td>
<td>AGE GRH SRIF PATH2 PATH3 PATH4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>41.965</td>
<td>GRH SRIF PATH1 PATH2 PATH3 PATH4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>41.907</td>
<td>GRH SRIF HARD1 PATH2 PATH3 PATH4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>41.882</td>
<td>GRH SRIF KI PATH2 PATH3 PATH4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>41.838</td>
<td>GRH SRIF HARD2 PATH2 PATH3 PATH4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>41.838</td>
<td>GRH SRIF GH PATH2 PATH3 PATH4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>41.827</td>
<td>GRH SRIF PATH2 PATH3 PATH4 PATH5</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>41.439</td>
<td>AGE GRH SRIF PATH2 PATH3 PATH5</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>41.266</td>
<td>AGE GRH SRIF HARD3 PATH2 PATH3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>41.236</td>
<td>GRH SRIF HARD3 PATH2 PATH3 PATH5</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>41.212</td>
<td>AGE GRH SRIF KI PATH2 PATH3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>41.084</td>
<td>GRH SRIF KI PATH2 PATH3 PATH5</td>
<td></td>
</tr>
</tbody>
</table>
42.587 AGE GRH SRIF HARD3 PATH2 PATH3 PATH4
42.404 GRH SRIF HARD3 PATH1 PATH2 PATH3 PATH4
42.402 GRH SRIF HARD3 GH PATH2 PATH3 PATH4
42.347 GRH SRIF KI HARD3 PATH2 PATH3 PATH4
42.337 GRH SRIF HARD2 HARD3 PATH2 PATH3 PATH4
42.331 AGE GRH SRIF PATH1 PATH2 PATH3 PATH4
42.329 GRH SRIF HARD3 PATH2 PATH3 PATH4 PATH5
42.312 GRH SRIF HARD1 HARD3 PATH2 PATH3 PATH4
42.254 AGE GRH SRIF HARD1 PATH2 PATH3 PATH4
42.241 AGE GRH SRIF KI PATH2 PATH3 PATH4
42.229 AGE GRH SRIF HARD2 PATH2 PATH3 PATH4
42.207 AGE GRH SRIF PATH2 PATH3 PATH4 PATH5
42.199 AGE GRH SRIF GH PATH2 PATH3 PATH4

42.680 AGE GRH SRIF HARD3 PATH1 PATH2 PATH3 PATH4
42.631 AGE GRH SRIF HARD3 GH PATH2 PATH3 PATH4
42.615 AGE GRH SRIF KI HARD3 PATH2 PATH3 PATH4
42.608 AGE GRH SRIF HARD3 PATH2 PATH3 PATH4 PATH5
42.599 AGE GRH SRIF HARD2 HARD3 PATH2 PATH3 PATH4
42.589 AGE GRH SRIF HARD1 HARD3 PATH2 PATH3 PATH4
42.518 GRH SRIF HARD3 GH PATH1 PATH2 PATH3 PATH4
42.505 GRH SRIF HARD3 PATH1 PATH2 PATH3 PATH4 PATH5
42.460 GRH SRIF KI HARD3 GH PATH2 PATH3 PATH4
42.449 GRH SRIF KI HARD3 PATH1 PATH2 PATH3 PATH4
42.444 GRH SRIF HARD2 HARD1 PATH1 PATH2 PATH3 PATH4
42.442 GRH SRIF HARD2 HARD3 GH PATH2 PATH3 PATH4
42.419 AGE GRH SRIF PATH1 PATH2 PATH3 PATH4 PATH5

42.776 AGE GRH SRIF HARD3 PATH1 PATH2 PATH3 PATH4 PATH5
42.737 AGE GRH SRIF HARD3 GH PATH1 PATH2 PATH3 PATH4
42.712 AGE GRH SRIF KI HARD3 PATH1 PATH2 PATH3 PATH4
42.698 AGE GRH SRIF HARD2 HARD3 PATH1 PATH2 PATH3 PATH4
42.683 AGE GRH SRIF HARD1 HARD3 PATH1 PATH2 PATH3 PATH4
42.671 AGE GRH SRIF KI HARD3 GH PATH2 PATH3 PATH4
42.649 AGE GRH SRIF HARD2 HARD3 GH PATH2 PATH3 PATH4
42.647 AGE GRH SRIF HARD3 GH PATH2 PATH3 PATH4 PATH5
42.639 AGE GRH SRIF HARD1 HARD3 GH PATH2 PATH3 PATH4
42.633 AGE GRH SRIF KI HARD3 PATH2 PATH3 PATH4 PATH5
42.622 AGE GRH SRIF KI HARD2 HARD3 PATH2 PATH3 PATH4
42.619 AGE GRH SRIF HARD2 HARD3 PATH2 PATH3 PATH4 PATH5
42.615 AGE GRH SRIF KI HARD1 HARD3 PATH2 PATH3 PATH4

42.826 AGE GRH SRIF HARD3 GH PATH1 PATH2 PATH3 PATH4 PATH5
42.800 AGE GRH SRIF KI HARD3 PATH1 PATH2 PATH3 PATH4 PATH5
42.792 AGE GRH SRIF HARD2 HARD3 PATH1 PATH2 PATH3 PATH4 PATH5
42.784 AGE GRH SRIF KI HARD3 GH PATH1 PATH2 PATH3 PATH4
42.779 AGE GRH SRIF HARD1 HARD3 PATH1 PATH2 PATH3 PATH4 PATH5
42.763 AGE GRH SRIF HARD2 HARD3 GH PATH1 PATH2 PATH3 PATH4
42.748 AGE GRH SRIF HARD1 HARD3 GH PATH1 PATH2 PATH3 PATH4
42.722 AGE GRH SRIF KI HARD2 HARD3 PATH1 PATH2 PATH3 PATH4
42.712 AGE GRH SRIF KI HARD1 HARD3 PATH1 PATH2 PATH3 PATH4
42.703 AGE GRH SRIF HARD1 HARD2 HARD3 PATH1 PATH2 PATH3 PATH4
42.683 AGE GRH SRIF KI HARD3 GH PATH2 PATH3 PATH4 PATH5
42.681 AGE GRH SRIF KI HARD2 HARD3 GH PATH2 PATH3 PATH4
42.674 AGE GRH SRIF KI HARD1 HARD3 GH PATH2 PATH3 PATH4

42.863 AGE GRH SRIF KI HARD3 GH PATH1 PATH2 PATH3 PATH4 PATH5
42.849 AGE GRH SRIF HARD2 HARD3 GH PATH1 PATH2 PATH3 PATH4 PATH5
42.835 AGE GRH SRIF HARD1 HARD3 GH PATH1 PATH2 PATH3 PATH4 PATH5
42.810 AGE GRH SRIF KI HARD2 HARD3 PATH1 PATH2 PATH3 PATH4 PATH5
42.801 AGE GRH SRIF KI HARD1 HARD3 PATH1 PATH2 PATH3 PATH4 PATH5
42.799 AGE GRH SRIF KI HARD2 HARD3 GH PATH1 PATH2 PATH3 PATH4
42.796 AGE GRH SRIF HARD1 HARD2 HARD3 PATH1 PATH2 PATH3 PATH4 PATH5
42.789 AGE GRH SRIF KI HARD1 HARD3 GH PATH1 PATH2 PATH3 PATH4
42.764 AGE GRH SRIF HARD1 HARD2 HARD3 GH PATH1 PATH2 PATH3 PATH4
42.729 AGE GRH SRIF KI HARD1 HARD2 HARD3 PATH1 PATH2 PATH3 PATH4
42.695 GRH SRIF KI HARD2 HARD3 GH PATH1 PATH2 PATH3 PATH4 PATH5
42.692 AGE GRH SRIF KI HARD2 HARD3 GH PATH2 PATH3 PATH4 PATH5
42.685 AGE GRH SRIF KI HARD1 HARD3 GH PATH2 PATH3 PATH4 PATH5

42.878 AGE GRH SRIF KI HARD2 HARD3 GH PATH1 PATH2 PATH3 PATH4 PATH5
42.868 AGE GRH SRIF KI HARD1 HARD3 GH PATH1 PATH2 PATH3 PATH4 PATH5
42.850 AGE GRH SRIF HARD1 HARD2 HARD3 GH PATH1 PATH2 PATH3 PATH4 PATH5
42.816 AGE GRH SRIF KI HARD1 HARD2 HARD3 PATH1 PATH2 PATH3 PATH4 PATH5
42.801 AGE GRH SRIF KI HARD1 HARD2 HARD3 GH PATH1 PATH2 PATH3 PATH4
42.697 GRH SRIF KI HARD1 HARD2 HARD3 GH PATH1 PATH2 PATH3 PATH4 PATH5
42.693 AGE GRH SRIF KI HARD1 HARD2 HARD3 GH PATH2 PATH3 PATH4 PATH5
42.661 AGE GRH SRIF KI HARD1 HARD2 GH PATH1 PATH2 PATH3 PATH4 PATH5
41.934 AGE GRH SRIF KI HARD1 HARD2 HARD3 GH PATH1 PATH2 PATH3 PATH5
41.333 AGE GRH SRIF KI HARD1 HARD2 HARD3 GH PATH1 PATH2 PATH4 PATH5
40.720 AGE GRH SRIF KI HARD1 HARD2 HARD3 GH PATH1 PATH3 PATH4 PATH5
39.979 AGE GRH KI HARD1 HARD2 HARD3 GH PATH1 PATH2 PATH3 PATH4 PATH5
39.252 AGE SRIF KI HARD1 HARD2 HARD3 GH PATH1 PATH2 PATH3 PATH4 PATH5

42.879 AGE GRH SRIF KI HARD1 HARD2 HARD3 GH PATH1 PATH2 PATH3 PATH4 PATH5
REFERENCES


163. Schonbrunn A. Somatostatin action in pituitary cells involves two independent transduction mechanisms. Metabolism 39:96-100 (suppl 1), 1990.


