The TRH-R in human endocrine disease: structure, function and characterisation

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# Contents

Declaration ................. vi
Acknowledgements ....... vii
Abstract ................. viii
List of Figures ............. xi
List of Tables and Appendices xiv
Abbreviations ............. xv

1 Introduction

1.1 Physiological background .......................... 1
1.2 Aim of the study ................................. 1
1.3 Importance of the study .......................... 2
1.4 Structure of the thesis ........................... 2

2 Literature review

2.1 The hypothalamic-pituitary axis ................. 5
  2.1.1 The hypothalamus ............................ 5
  2.1.2 Hypothalamic hormones ....................... 6
  2.1.3 The pituitary gland .......................... 7
  2.2 Thyrotrophin-releasing hormone (TRH) ........ 10
   2.2.1 Structure of TRH ............................ 11
   2.2.2 Synthesis and metabolism of TRH .......... 11
   2.2.3 Localisation of TRH to the hypothalamus . 12
   2.2.4 TRH and the hypothalamic-pituitary-thyroid axis . 12
   2.2.5 Feedback regulation of hypothalamic TRH .. 14
   2.2.6 TRH and regulation of prolactin release .... 15
   2.2.7 TRH in extra-pituitary locations .......... 16
  2.3 G-protein-coupled receptors (GPCRs) .......... 17
   2.3.1 Structure and function of GPCRs .......... 17
   2.3.2 Disulphide bridge formation ............... 18
   2.3.3 GPCR glycosylation ......................... 19
   2.3.4 GPCR palmitoylation ....................... 19
   2.3.5 GPCR phosphorylation ..................... 19
   2.3.6 GPCR-induced G-protein activation ....... 22
  2.4 G-proteins .................................. 22
  2.5 Effectors and second messenger systems ....... 27
   2.5.1 The phosphatidylinositol phosphate (PI) pathway .... 27
   2.5.2 The adenyl cyclase (AC) pathway .......... 28
  2.6 G-proteins, GPCRs and disease ................. 30
   2.6.1 G-proteins, signal transduction and disease ... 30
   2.6.2 GPCR dysfunction and disease ............. 31
2.7 The pituitary TRH receptor (TRH-R) 34
  2.7.1 Isolation and characterisation of TRH-R cDNAs 34
  2.7.2 The TRH-R in extra-hypothalamic tissues 36
  2.7.3 Control of TRH-R expression 36
  2.7.4 Upstream and downstream regulatory elements of the TRH-R gene 37
2.8 The thyroid gland 38
  2.8.1 Structure and function 38
  2.8.2 Functions of the thyroid hormones 40
  2.8.3 Hypothyroidism 41
  2.8.4 Central hypothyroidism: an example of specific type of hypothyroidism 43
  2.8.5 Hyperthyroidism 47
2.9 Pituitary tumours 48
2.10 Concluding remarks 50

3 Materials and methods 52
  3.1 Introduction 52
  3.2 The polymerase chain reaction (PCR) 52
  3.3 Detection of single-strand conformational polymorphisms in PCR products (PCR-SSCP) 54
  3.4 TA cloning 55
  3.5 Automated fluorescent DNA cycle sequencing 56
  3.6 Horizontal agarose electrophoresis of DNA fragments 57
  3.7 DNA Purification 58
    3.7.1 Plasmid recovery 58
    3.7.2 Extraction of high molecular weight genomic DNA 58
    3.7.3 Recovery of DNA from an agarose gel 59
    3.7.4 Phenol:chloroform DNA extraction 59
  3.8 Salt and ethanol precipitation of DNA 60
  3.9 Spectrophotometric DNA quantitation 60
  3.10 Restriction endonuclease digestion of DNA 60
  3.11 DNA ligation 61
  3.12 Transformation of plasmid DNA into E. coli 62
    3.12.1 Chemically competent E. coli 62
    3.12.2 Electrocompetent E. coli 62
  3.13 Site-directed mutagenesis 64
    3.13.1 General methodology 64
    3.13.2 Eukaryotic expression vectors 64
    3.13.3 Helper phage 66
    3.13.4 Preparation of uracil-containing template 66
    3.13.5 Single strand DNA preparation 66
    3.13.6 Oligonucleotide design 67
    3.13.7 Transformation of the cccDNA for plasmid recovery 67
  3.14 Tissue culture 67
  3.15 Transfection of eukaryotic cells 68
3.15.1 Transient transfection
3.15.2 Stable Transfections
3.16 Total inositol phosphate (IP) production assay
  3.16.1 Methodology
  3.16.2 Expression of results
3.17 Calcium imaging
  3.17.1 Intracellular calcium measurements
  3.17.2 Stimulation of cells with TRH and [Ca^{2+}]_{i} imaging
  3.17.3 Data analysis and presentation
3.18 Radioligand receptor binding assay
  3.18.1 Cell membrane preparation
  3.18.2 Radioligand receptor binding assay (dose displacement)
  3.18.3 Determination of protein concentration of membrane preparations
  3.18.4 Data analysis
3.19 In-Vitro translation
3.20 Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE)
3.21 Rapid amplification of 5' cDNA ends (5' RACE)
3.22 Quantitation of promoter activity
  3.22.1 Luciferase assay
  3.22.2 β-galactosidase assay
3.23 Statistical analysis of experimental results

4 Structure of the TRH-R in human pituitary adenomas
  4.1 Introduction
  4.2 Materials and methods
    4.2.1 Patients
    4.2.2 Oligonucleotide PCR primer design and synthesis
    4.2.3 PCR-SSCP analysis of TRH-R mutants generated by site-directed mutagenesis
    4.2.4 PCR-SSCP analysis of TRH-R from genomic DNA samples
    4.2.5 Cloning of PCR fragments and DNA sequencing
  4.3 Results
  4.5 Discussion

5 Naturally occurring inactivating mutations of the human TRH-R
  5.1 Introduction
  5.2 Materials and methods
    5.2.1 Patient 1
    5.2.2 Patient 2
    5.2.3 Screening TRH-R genes for mutations
### Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.2.4</td>
<td>Construction of wild-type and mutant TRH-R cDNA expression vectors</td>
<td>99</td>
</tr>
<tr>
<td>5.2.5</td>
<td>Functional studies of mutant TRH-Rs in a transient expression system</td>
<td>100</td>
</tr>
<tr>
<td>5.2.6</td>
<td><em>In vitro</em> translation</td>
<td>100</td>
</tr>
<tr>
<td>5.3</td>
<td>Results</td>
<td>101</td>
</tr>
<tr>
<td>5.3.1</td>
<td>Tests of Thyroid Function (Patient 2)</td>
<td>101</td>
</tr>
<tr>
<td>5.3.2</td>
<td>DNA sequence analysis</td>
<td>104</td>
</tr>
<tr>
<td>5.3.3</td>
<td>Functional Studies</td>
<td>107</td>
</tr>
<tr>
<td>5.4</td>
<td>Discussion</td>
<td>112</td>
</tr>
<tr>
<td>6</td>
<td>Site-directed mutagenesis and expression studies of the hTRH-R</td>
<td>115</td>
</tr>
<tr>
<td>6.1</td>
<td>Introduction</td>
<td>115</td>
</tr>
<tr>
<td>6.2</td>
<td>Materials and methods</td>
<td>118</td>
</tr>
<tr>
<td>6.3</td>
<td>Results</td>
<td>120</td>
</tr>
<tr>
<td>6.3.1</td>
<td>Thr265 mutants compared to WT hTRH-R</td>
<td>120</td>
</tr>
<tr>
<td>6.3.2</td>
<td>Glu122 mutants compared to WT hTRH-R</td>
<td>123</td>
</tr>
<tr>
<td>6.3.3</td>
<td>Comparison of rat and human TRH-R expression</td>
<td>125</td>
</tr>
<tr>
<td>6.3.4</td>
<td>Calcium imaging of rat and human TRH-R stable cell lines</td>
<td>127</td>
</tr>
<tr>
<td>6.4</td>
<td>Discussion</td>
<td>130</td>
</tr>
<tr>
<td>7</td>
<td>Isolation and characterisation of the 5'-flanking region of the mTRH-R gene</td>
<td>135</td>
</tr>
<tr>
<td>7.1</td>
<td>Introduction</td>
<td>135</td>
</tr>
<tr>
<td>7.2</td>
<td>Materials and methods</td>
<td>136</td>
</tr>
<tr>
<td>7.2.1</td>
<td>Isolation of the upstream 5'-flanking region of the mTRH-R gene</td>
<td>136</td>
</tr>
<tr>
<td>7.2.2</td>
<td>5′ RACE analysis</td>
<td>136</td>
</tr>
<tr>
<td>7.2.3</td>
<td>Construction of pGL-3 constructs for functional study of promoter activity</td>
<td>138</td>
</tr>
<tr>
<td>7.2.4</td>
<td>Transient transfections</td>
<td>140</td>
</tr>
<tr>
<td>7.3</td>
<td>Results</td>
<td>141</td>
</tr>
<tr>
<td>7.3.1</td>
<td>Sequencing of the 5'-flanking region of the mTRH-R gene.</td>
<td>141</td>
</tr>
<tr>
<td>7.3.2</td>
<td>5′ RACE analysis of mouse pituitary RNA</td>
<td>146</td>
</tr>
<tr>
<td>7.3.3</td>
<td>Expression of -1045TRH-R-luc in pituitary and non-pituitary cells</td>
<td>148</td>
</tr>
<tr>
<td>7.4</td>
<td>Discussion</td>
<td>149</td>
</tr>
<tr>
<td>8</td>
<td>Summary of results and concluding remarks</td>
<td>155</td>
</tr>
<tr>
<td>8.1</td>
<td>Concluding remarks</td>
<td>155</td>
</tr>
<tr>
<td>8.2</td>
<td>Directions for future study</td>
<td>156</td>
</tr>
</tbody>
</table>
Appendix I 158
Appendix II 160
Appendix III 161
Bibliography ......................................................... 163
Declaration

The experiments described in this thesis were the unaided work of the author unless otherwise stated and acknowledged. No part of this work has been accepted for any other degree, nor is any part of it being submitted concurrently in candidature for another degree. All experiments were carried out at the Medical Research Council Reproductive Biology Unit in Edinburgh, except where otherwise indicated.

Elena Faccenda

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Finally, I would like to dedicate this thesis to my parents, by way of thanks, and in appreciation of their unconditional support and encouragement, and without whom my further education would have been severely curtailed.

This thesis is a product of the support, help and friendship of all the people mentioned. Thank you all.
Abstract

Thyrotrophin releasing hormone (TRH) is a small neuropeptide of three amino acids originally isolated from hypothalamic extracts as a stimulator of thyrotrophin (TSH) release from the anterior pituitary gland. TRH also has an auxiliary role in the pituitary of modulating prolactin (PRL) secretion from lactotroph cells. However, since its initial identification TRH has been shown to be widely distributed throughout the central and peripheral nervous system where it is thought to act as a neurotransmitter/neuromodulator, as well as in extraneural tissues where its action is less well understood.

TRH stimulates hormone secretion via specific high affinity binding sites on target anterior pituitary cells. The human homologue of the pituitary TRH receptor (hTRH-R) has been cloned and shares a high degree of sequence homology with TRH-Rs previously cloned from lower species including mouse and rat. The TRH-R is a member of the superfamily of G-protein coupled receptors (GPCRs), and communicates to the cell interior through the phospholipase C (PLC) second messenger system.

The overall aims of this project were to determine whether naturally occurring TRH-R mutations exist in relevant human clinical conditions, and if so, to investigate their effects on receptor function in vitro. The molecular mechanisms of TRH action in wild type and mutant TRH-Rs would be investigated using the techniques of site-directed mutagenesis and expression of TRH-Rs in mammalian cell lines. The role of specifically chosen sites within regions of the TRH-R believed to be important for intracellular signalling would be studied simultaneously by the same methods.

Pituitary tumours secreting either TSH, PRL or GH may respond to TRH by enhanced, blunted or paradoxical hormone release. Non-functioning pituitary adenomas may also show abnormal responses to TRH. Little is understood of the cellular and molecular mechanisms regulating inappropriate hormone release or abnormal growth of these tumours. However, activating and inactivating mutations in a number of GPCRs have been implicated in a variety of human diseases. Mutations in the TRH-R may therefore be involved in the aetiology of pituitary adenoma formation and/or activity. A group of pituitary adenomas collected at surgery, comprising 11 TSH-secreting, 15 GH-secreting and 15 PRL-secreting adenomas as well as 5 non-functioning tumours were screened for mutations in the TRH-R gene.
Abstract

DNA extracted from these adenomas was screened for somatic TRH-R mutations by polymerase chain reaction-single stranded conformational polymorphism analysis (PCR-SSCP). No TRH-R mutations were identified in any of the tumour groups, so it appears that the TRH-R is not of significant causative importance in aberrant TRH responses or tumourigenesis of anterior pituitary cells.

Blood lymphocyte DNA was also obtained from two unrelated patients suffering from isolated central hypothyroidism, a rare disease characterised by low circulating thyroid hormone levels in the presence of normal plasma TSH levels and a lack of TRH-inducible TSH or PRL secretion. The DNA obtained from these patients was screened for germline TRH-R mutations. The first of these patients (Patient 1) retained normal TRH-R structure suggesting that his hypothyroidism must have another aetiology (e.g. abnormalities in downstream TRH-R signalling mechanisms). However, Patient 2 was found to have inherited a mutant TRH-R allele from each of his unaffected parents. The maternally-derived mutation involved the substitution of one nucleotide (C49-to-T) which altered the codon for amino acid 17 (Arg-CGA) such that it became a premature stop codon (TGA), thereby severely truncating the TRH-R polypeptide. The mutation inherited from the father affected the third transmembrane region. Three amino acids were deleted (Ser115, Ile116 and Thr117) and another was substituted (Ala118-to-Thr). It is presumed that this mutant will be translated into a full length receptor (except for 3 amino acids). When the mutated TRH-R constructs were transfected into mammalian expression systems no TRH binding or function was measurable, explaining this patient's unresponsiveness to TRH administration. The incidence of this novel cause of central hypothyroidism remains to be ascertained. This is the first report of a mutation in a PLC-linked GPCR and for that reason alone it is an important discovery. In addition, this patient appears to represent a naturally occurring TRH-R knock-out, a condition which due to the postulated various and widespread actions of TRH was presumed to be a lethal genetic condition.

In vitro mutagenesis of the hTRH-R was also carried out in combination with PCR-SSCP analysis to further understanding of the underlying causes of pituitary tumours and other TRH-related pituitary conditions. Specific amino acids within the receptor were altered by mutating the receptor cDNA. The function of the mutant receptors was then measured in terms of ligand binding and second messenger activation. Substitution of Glu122 (in the conserved (E)DRY motif in the second intracellular loop) to Leu (E122L) resulted in complete ablation of TRH binding, and subsequent inositol phosphate accumulation. In contrast, substitution of this same residue with Asp (E122D) caused no change in either radioligand binding or second messenger...
Abstract

Substitution of Thr265 (a potential phosphorylation site at the interface of transmembrane region VI/intracellular loop 3) by any of the three residues Ser, Ala or Asn (T265S, T265A and T265N respectively) resulted in mutant hTRH-Rs which exhibited a three to six-fold increase in receptor expression at the cell surface compared to wild type, and an increased affinity for ligand.

In order to address the issue of whether the inactivating TRH-R mutations from Patient 2 indeed represent a non-lethal TRH-R knockout the final aim of the project was to determine those regions of the upstream, or 5'-flanking, region of the mouse TRH-R (mTRH-R) responsible for transcriptional initiation and control. The mTRH-R 5'-flanking region was analysed by cloning regions of various lengths into a luciferase (luc) reporter vector and measuring resultant luc activity. The transcription start site appears to lie within a region 300-350 bases upstream of the ATG translational start site. Expression of the region -1045 to -182, (relative to the ATG translational start site) in pituitary GH3 cells results in a 3.85 fold increase in luc activity over no promoter controls. No increase in luc activity was seen with expression of this same fragment in COS-1 cells (non-pituitary). By characterising the DNA elements responsible for initiating and controlling TRH-R transcription it will be possible to create a DNA construct and to produce a transgenic mouse lineage with no TRH-R expression, a true TRH-R knock-out. Such an animal model will allow more detailed analysis of the functions (if there are any) of TRH during embryogenesis and subsequent postnatal development, both in the brain and in extraneural locations.

The results of the work described in this thesis indicate that the possession of inactivated TRH-Rs results in central hypothyroidism in one out of two randomly selected patients. It remains to be determined how prevalent such mutations may be in patients with this rare disorder and indeed in other congruous diseases. Furthermore, TRH-R inactivation was achieved by substitution of a single amino acid in a different region of the hTRH-R (intracellular loop 2) suggesting that additional naturally occurring inactivating mutations may be identified in additional patients as yet unidentified. The effects of TRH-R knock-out will be studied in a transgenic mouse model and comparison with the apparent human equivalent identified here will give further insight into the functional roles of TRH and its receptor both during embryogenesis and in later stages of development. By uncovering how TRH and its receptor interact and the responses triggered by ligand binding, it may be possible to fully understand the mechanisms underlying the effects of TRH and to improve TRH treatments currently in use in the clinic.
## List of Figures

| Figure 2.1  | The hypothalamus | 6 |
| Figure 2.2  | Structure of the pituitary gland | 8 |
| Figure 2.3  | Hormones released by the anterior pituitary gland | 10 |
| Figure 2.4  | Structure of TRH | 11 |
| Figure 2.5  | The hypothalamo-pituitary-thyroid (HPT) axis | 13 |
| Figure 2.6  | Schematic representation of GPCR structure | 21 |
| Figure 2.7  | The GTPase cycle. | 26 |
| Figure 2.8  | The G_q and G_\text{G1} effector (following page) | 28 |
| Figure 2.9  | Two-dimensional representation of the primary sequence of the hTRH-R. | 35 |
| Figure 2.10 | Structure of thyroid tissue | 41 |
| Figure 3.1  | The hTRH-R/pcDNA-3 construct and pcDNA-3 | 65 |
| Figure 3.2  | Cartoon representation of Promega's Coupled Rabbit Reticulocyte in vitro Translation System. | 76 |
| Figure 3.3  | Overview of 5' RACE | 78 |
| Figure 4.1  | Responses of thyrotrophs, lactotrophs and somatotrophs of the anterior pituitary gland to TRH. | 82 |
| Figure 4.2  | PRL, TSH and GH responses to exogenous TRH in normal subjects and in patients with pituitary adenomas/acromegaly. | 83 |
| Figure 4.3  | Regions of the hTRH-R amplified for PCR-SSCP analysis | 88 |
| Figure 4.4  | PCR-SSCP analysis of rat TRH-R wild-type and mutant sequences (following page) | 90 |
**List of figures**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 4.5</td>
<td>Representative examples of PCR-SSCP analysis of pituitary adenoma DNA samples</td>
<td>92</td>
</tr>
<tr>
<td>Figure 5.1</td>
<td>TRH-induced responses of TSH and PRL of all Patient 2 family members</td>
<td>103</td>
</tr>
<tr>
<td>Figure 5.2</td>
<td>PCR-SSCP analysis of the TRH-R from Patient 1</td>
<td>104</td>
</tr>
<tr>
<td>Figure 5.3</td>
<td>Inheritance pattern of mutant alleles</td>
<td>105</td>
</tr>
<tr>
<td>Figure 5.4</td>
<td>Location of the inactivating hTRH-R mutations identified from Patient 2</td>
<td>106</td>
</tr>
<tr>
<td>Figure 5.5</td>
<td>Radioligand displacement assay results</td>
<td>107</td>
</tr>
<tr>
<td>Figure 5.6</td>
<td>Total inositol phosphate production</td>
<td>109</td>
</tr>
<tr>
<td>Figure 5.7</td>
<td>In vitro translation of WT, M-STOP and F-TM3 receptor cDNAs</td>
<td>110</td>
</tr>
<tr>
<td>Figure 6.1</td>
<td>Primary structure of the hTRH-R</td>
<td>117</td>
</tr>
<tr>
<td>Figure 6.2</td>
<td>Functional determinations of Thr265 mutations of the hTRH-R (following page)</td>
<td>121</td>
</tr>
<tr>
<td>Figure 6.3</td>
<td>Functional analysis of Glu122 mutants of the hTRH-R (following page)</td>
<td>123</td>
</tr>
<tr>
<td>Figure 6.4</td>
<td>Comparison of total TRH-induced inositol phosphate production in COS-1 cells transiently transfected with WT and start-stop versions of the rat and human TRH-Rs</td>
<td>126</td>
</tr>
<tr>
<td>Figure 6.5</td>
<td>Calcium imaging of 293-h10 and 293-E2 stable cell lines</td>
<td>129</td>
</tr>
<tr>
<td>Figure 6.6</td>
<td>Flanking regions of the rat and human TRH-R translation initiation codons</td>
<td>133</td>
</tr>
<tr>
<td>Figure 7.1</td>
<td>The mTRH-R 5' flanking promoter containing region</td>
<td>138</td>
</tr>
<tr>
<td>Figure 7.2</td>
<td>Nucleotide sequence of the mTRH-R gene 5'-flanking region</td>
<td>142</td>
</tr>
<tr>
<td>Figure 7.3</td>
<td>5' RACE analysis of the mTRH-R 5'-flanking region (following page)</td>
<td>146</td>
</tr>
<tr>
<td>Figure 7.4</td>
<td>Expression of -1045TRH-R-luc in pituitary and non-pituitary cells</td>
<td>148</td>
</tr>
</tbody>
</table>
Figure 7.5  Alignment of the nucleotide sequences of the mouse and human TRH-R upstream regions (following page) 152
List of Tables and Appendices

Table 2.1........ Mammalian α-subunits previously identified 25
Table 2.2........ G-protein mutations in human tumours. 31
Table 2.3........ Some of the GPCRs in which in vivo mutations cause disease (following page). 32
Table 2.4........ Summary of diagnostic test results in hypothyroidism 42
Table 2.5........ Differential diagnosis of central hypothyroidism. 45
Table 3.1........ Outline of a basic PCR temperature cycling protocol 53
Table 3.2........ Some of the plasmid vectors and their E. coli host strains used 63
Table 4.1........ Details of the 50 patient tumours used in this study (following page) 85
Table 4.2........ Primers used to PCR amplify the coding region of the hTRH-R 87
Table 5.1........ Hormone levels of Patient 2 and his family 101
Table 6.1........ Oligonucleotides used to generate site-specific mutant hTRH-Rs (following page) 117
Table 6.2........ Functional measurements of each TRH-R construct. 126
Table 7.1........ Sequences of the primers used to amplify segments of the mTRH-R promoter region. 139
Table 7.2........ Putative transcription factor binding sites of the mTRH-R 5'-flanking region (following page) 143
Table 7.3........ Positions of putative splice sites 145
Appendix I.................................................. 158
Appendix II.................................................. 160
Appendix III.................................................. 161
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>αα</td>
<td>amino acid</td>
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<tr>
<td>Ala (A)</td>
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<td>Amp</td>
<td>ampicillin</td>
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<td>AMP</td>
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</tr>
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</tr>
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<td>dopamine D2 receptor</td>
</tr>
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</tr>
<tr>
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<td>el</td>
<td>extracellular loop</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>$G_\alpha$</td>
<td>heterotrimeric G-protein $\alpha$-subunit</td>
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<tr>
<td>$G_\beta$</td>
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<tr>
<td>$G_\gamma$</td>
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<td>growth hormone</td>
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<tr>
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<td>GH-secreting pituitary adenoma</td>
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<td>kilobase</td>
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<td>kilodalton</td>
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<td>luteinising hormone receptor</td>
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<td>phosphate buffered saline</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>-----------</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>polyethylene glycol</td>
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<td>phospholipase C</td>
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<td>prolactin</td>
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<td>prolactinoma</td>
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<td>pertussis toxin</td>
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<td>ribonucleic acid</td>
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<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<td>sodium dodecyl sulphate</td>
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<td>tri-iodothyronine</td>
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<td>thyroxine</td>
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<tr>
<td>TE</td>
<td>tris, EDTA</td>
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<tr>
<td>TBE</td>
<td>tris, borate, EDTA</td>
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<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetra-methylene diamine</td>
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<td>Tet</td>
<td>tetracycline</td>
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<td>Thr (T)</td>
<td>threonine</td>
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<tr>
<td>TM</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>TRH</td>
<td>thyrotrophin-releasing hormone</td>
</tr>
<tr>
<td>h/r/m-TRH-R</td>
<td>(human, rat, mouse) TRH receptor</td>
</tr>
<tr>
<td>Trp (W)</td>
<td>tryptophan</td>
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<tr>
<td>TSH</td>
<td>thyrotrophin (thryroid stimulating hormone)</td>
</tr>
<tr>
<td>TSH-oma</td>
<td>TSH-secreting pituitary adenoma</td>
</tr>
<tr>
<td>Tyr (Y)</td>
<td>tyrosine</td>
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<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>UV</td>
<td>ultra-violet</td>
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<tr>
<td>Val (V)</td>
<td>valine</td>
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<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactoside</td>
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Chapter 1
Introduction

1.1 Physiological background

Thyrotrophin-releasing hormone was the first of the trophic neurohormones to be isolated and purified from the hypothalamus (Schally et al, 1966). The role of TRH in control of the thyrotroph and thyroid function is well established, although this is only one of its diverse biological functions. In addition to additional effects on the anterior pituitary gland, TRH is also widely distributed in extrahypothalamic brain regions, in the spinal cord, and in several peripheral tissues, suggesting additional functions of TRH in these areas. The recognised effects of TRH in neural tissues suggest a role as a neurotransmitter/neuromodulator of central nervous system (CNS) activity. The function(s) of TRH in the gastrointestinal and reproductive tracts, and in the retina are poorly understood, but such wide spread distribution of this hormone suggests that it may be a hormone involved in a complex set of interactions with other hormones and transmitters which regulate normal bodily functions. Phylogenetic data indicates that TRH exists in species which do not possess a pituitary gland. This suggests that the putative paracrine role of TRH in extra-neural tissues may have pre-existed evolution of the pituitary gland and that TRH has subsequently been recruited by this organ.

1.2 Aim of the study

The primary aim of this PhD study was to determine the role of the TRH-R in human endocrine disorders by screening TRH-R genes from various patient and tissue sources for somatic or germline mutations altering receptor function. Structure-function relationships in the TRH-R were in addition, sought by functional assessment of mutant TRH-Rs generated by site-specific mutagenesis of amino acids within the receptor structure highlighted as playing a potential role in receptor function.
1.3 Importance of the study

Since its identification TRH has been used in clinical practice to treat a wide variety of disorders, including the treatment of patients with mild disturbance of consciousness (due to head injury or subarachnoid haemorrhage) (Manaka et al, 1977; Sano et al, 1979), and foetal respiratory distress syndrome (Shennan and Peek, 1995). The effects of TRH therapy in depression, based on its CNS stimulatory effects, remains equivocal (Kastin et al, 1972; Prange et al, 1972). TRH has some clinical disadvantages, including a short duration of action (due to its short half life resulting from endopeptidase activity), its weak CNS effects relative to its TSH-releasing activity, and its hypertensive effects. Elucidation of the structure and function relationships of TRH and the TRH-R may facilitate the synthesis of TRH analogues with increased resistance to metabolism, more specific activity as applicable to the proposed treatment and with fewer or less marked side-effects.

Many human diseases have been linked to mutations in GPCRs and in light of this the TRH-R may be implicated in certain conditions characterised by abnormal HPT axis activity and may also be of significance in the discordant and paradoxical responses of pituitary adenoma cells to TRH.

The hypothalamo-pituitary-thyroid axis bears strong relevance to reproductive endocrinology. In addition to the well established prolactin-releasing effects of TRH, it is known that the anterior pituitary gland becomes more sensitive to TRH at pro-oestrus (Brandi et al, 1990; de Lean et al, 1977a) and under the influence of oestrogen (de Lean et al, 1977b), which is probably a result of an increased number of TRH-Rs on mammotrophic cells in response to oestrogen (Gershengorn et al, 1979). Therefore it appears that there is some degree of interplay between the HPT and hypothalamo-pituitary-gonadal (HPG) axes.

1.4 Structure of the thesis

The thesis begins in Chapter 2 with an extensive review of the current literature concerning the TRH-R as an example of a GPCR, the function of TRH and the TRH-R in the wider context of the HPT axis, disturbances of the HPT axis and the role of GPCR mutations in pathogenesis.
Chapter 3 describes in detail the experimental protocols used throughout the following experimental chapters (Chapters 4 to 7) from basic molecular biology methods to more specific descriptions of methods for expression in eukaryotic cells, receptor function assays and promoter/transcription site isolation and characterisation.

Pituitary adenomas arise from the clonal expansion of a single transformed progenitor cell, although the factors involved in oncogenic transformation in these neoplasms is undetermined. Pituitary adenomas often show discordant responses to TRH, suggesting that there may be a TRH-R abnormality in association with abnormal cell proliferation and secretion. Chapter 4 describes the screening of 50 pituitary adenomas for somatic mutations in the TRH-R genes contained in DNA extracted from patient adenoma tissue samples. A rapid screening method was used in which fragments of the TRH-R coding region were analysed for single-strand conformational polymorphisms. The majority of the work constituting this study was performed by the author in the laboratory of Dr Shlomo Melmed (Division of Endocrinology and Metabolism, Cedars-Sinai Research Institute, UCLA, Los Angeles, California, USA) during a four month working visit.

Chapter 5 describes the analysis of the TRH-R genes of two patients with central hypothyroidism, a condition characterised by low plasma total and free T4 concentrations in the presence of normal TSH values. This is a rare disorder which may arise from either hypothalamic or pituitary lesions. Inactivating mutations in the TRH-R were sought as an explanation for the absent TRH-induced TSH and PRL responses observed in these patients. It should be pointed out that this study was performed in collaboration with two other investigators. Patient 1 DNA was obtained from Dr David Repaske (Division of Endocrinology, Children's Medical Center, Cincinnati, Ohio, USA) and was analysed by PCR-SSCP by the author. Patient 2 mutant TRH-R constructs were obtained from Dr Robert Collu (Hopital Sainte Justine, Universite de Montreal, Montreal, Canada) who asked the author to perform the experiments necessary for functional characterisation of these mutant TRH-Rs as his own laboratory was finding this very difficult to achieve. In vitro translation experiments were designed and performed by the author.

Site-directed mutagenesis is a powerful tool used by molecular biologists to help to identify specific molecular interactions involved in GPCR structure and function. In Chapter 6 the hTRH-R was subject to such mutation at two sites within the cytoplasmic domains of the receptor. Glu122 and Thr265 were identified as having potential roles in receptor structure and/or function and were subsequently mutated.
The function of the mutant receptors was analysed by measuring radioligand binding and TRH-induced G-protein activation of the phospholipase C second messenger pathway.

In order to study the effects of TRH-R knockout it was decided to begin the process of establishing a transgenic mouse line. In order to execute this plan it was first essential to characterise the 5'-flanking region of the mouse (m)TRH-R gene. Clone 9, a 6.5kbp fragment of the mTRH-R gene had been isolated by a previous PhD student working with the author's supervisor (Duthie et al., 1993b) and in the same article 400bp of the upstream sequence (with respect to the ATG translation initiation codon) was published. Further sequence analysis and all of the characterisation experiments were performed as an integral part of this thesis and this work is described in Chapter 7.

Chapter 8 summarises and concludes the work performed to establish the set of related studies constituting this thesis and outlines future experimental research.
Chapter 2
Literature Review

2.1 The hypothalamic-pituitary axis

The idea that hypothalamic neurones might regulate normal pituitary function by secreting specific chemical messengers into the blood flowing through the portal vessels and thence into the adenohypophysis was first outlined by Harris almost 50 years ago in a monograph describing the hypothalamic control of anterior pituitary hormones (Harris, 1948). The hypothalamus is now known to be the central regulator of adenohypophyseal hormone release, forming the terminal links between the neuronal circuitry of the brain and the endocrine cells of the anterior pituitary (Yen, 1991). The basic structural organisation of the hypothalamus is shown in Figure 2.1.

2.1.1 The hypothalamus

The hypothalamus is a poorly defined and primitive structure located at the base of the vertebrate forebrain. In order to serve its neuroendocrine function the hypothalamus is both highly vascularised and strongly innervated. Afferent and efferent fibres connect the hypothalamus with other regions of the brain including the forebrain, limbic system, visual cortex, thalamus and brain stem. The hypothalamus is composed of various bundles of cells which secrete hormones either into the portal blood system for transport to the adenohypophysis, or directly into the general circulation after storage in the hypothalamus. On reaching target cells located in the anterior pituitary the hypothalamic hormones, also known as releasing or inhibiting hormones because of the nature of their actions, act to regulate the synthesis and secretion of various trophic hormones. In turn these trophic hormones regulate gonadal, thyroid and adrenal function, in addition to lactation, bodily growth and somatic development.
2.1.2 Hypothalamic hormones

To date five classical hypophysiotrophic peptide hormones, thyrotrophin-releasing hormone (TRH) (Guillemin, 1978) gonadotrophin-releasing hormone (GnRH) (Burgus et al, 1970); (Matsuo et al, 1971) somatostatin or somatotroph-releasing inhibitory hormone (SRIH) (Brazeau et al, 1973) corticotrophin-releasing hormone (CRH) (Vale et al, 1981) and growth hormone-releasing hormone (GHRH) (Speiss et al, 1983) have been isolated, and their role in the regulation of anterior pituitary hormone synthesis and secretion extensively studied. Other factors, e.g., neurotransmitters and neuropeptides, shown to influence anterior pituitary hormone release include catecholamines (dopamine (DA), adrenaline and noradrenaline), acetylcholine, gunminobutyric acid (GABA), opioid peptides and vasoactive intestinal peptide (VIP). The precise action of many of these factors is unknown but the roles of
dopamine and VIP in the regulation of prolactin (PRL) secretion have been more precisely established (Abe et al, 1985; Jonathan, 1985)

Many of the hypothalamic-releasing/inhibiting hormones have also been localised outside the hypothalamic-pituitary axis in tissues including the brain, spinal cord, autonomic nervous system, gastrointestinal, respiratory and reproductive tracts (Yen, 1991). A potential role for these peptides in cellular communication prior to the development of the neuronal or endocrine systems has been postulated (Kreiger, 1983).

2.1.3 The pituitary gland

Figure 2.2 shows a diagrammatic representation of the anatomy of the pituitary gland. The pituitary gland has two parts: the neurohypophysis (median eminence, infundibular stem and neural lobe) is of neural origin whilst the adenohypophysis (anterior pituitary and intermediate lobe) is of ectodermal origin, developing from the embryonic pharyngeal evagination known as Rathke's pouch. The main part of the neurohypophysis, the neural lobe, is connected to the median eminence by the infundibular stem. Adenohypophyseal tissue (pars tuberalis) surrounds the median eminence and infundibular stem creating the pituitary stalk. The adenohypophysis consists of three main regions: the pars distalis (ventral portion commonly termed anterior lobe), pars intermedia (adjacent to neural lobe; rudimentary in humans) and the pars tuberalis.
Figure 2.2  Structure of the pituitary gland
Diagram summarising the structure of the pituitary gland and its vascular network.

The posterior pituitary is composed of magnocellular neurosecretory nerve endings. Oxytocin and vasopressin with their corresponding neurophysin molecules are synthesised in the supraoptic nucleus (SON) and paraventricular nucleus (PVN; Figure 2.1), packaged into granules and transported through the nerve axons, via the pituitary stalk, to the posterior pituitary. When stimulated, the neurosecretory cells fire action potentials and exocytose hormones directly into the capillary vessels of the inferior hypophyseal artery. Cholinergic and noradrenergic fibres synapse with the SON and PVN and have been shown to stimulate hormone release, while inhibitory regulation occurs by dopaminergic and opioidergic pathways. Purification of oxytocin and
vasopressin (du Vigneud *et al*, 1954) revealed that both of these hormones have a nonapeptide structure requiring a disulphide bond between cysteine residues (at positions 1 and 6) for biological activity. Oxytocin is released in response to peripheral stimuli from cervical stretch receptors or from suckling stimulus at the breast. Oxytocin action in the cervix promotes foetal expulsion during labour, whereas in the mammary gland it causes milk release. The main physiological stimulus for the release of vasopressin is provided by alterations in body fluid balance, an increase in osmotic pressure being counterbalanced by the action of vasopressin on water reabsorption from the kidneys.

In contrast, the anterior pituitary is composed of a number of different endocrine cell types. The mature anterior pituitary gland consists principally of five major main endocrine cell types: somatotrophs (c. 50% of anterior pituitary cells), lactotrophs (c. 15%), gonadotrophs (c. 10%), thyrotrophs (c. 5%) and corticotrophs (15-20%) (Figure 2.3). Hypothalamic-releasing/inhibiting hormones bind to specific receptors located on the surface of anterior pituitary cells which facilitate coupling to a second messenger cascade system, resulting in alterations in effector system activity, and ultimately hormone exocytosis.
The five major cell types of the anterior pituitary gland are depicted in relation to the adenohypophyseal trophic hormones which each cell type produces. The effects of the trophic hormones on their downstream target tissues is shown.

2.2 Thyrotrophin-releasing hormone (TRH)

Like the other hypothalamic releasing/inhibiting factors TRH was initially isolated from pituitary extracts (Boler et al, 1969; Guillemin, 1978), its existence having been proposed by Greer as early as 1951 (Greer, 1951). Once isolated TRH was the first hypothalamic releasing factor to be chemically synthesised and successfully administered to humans which was able to disrupt the thyroid axis (Guillemin et al, 1971; Schally et al, 1984). TRH is released directly into the pituitary portal circulation and has effects on two distinct target cell populations. The primary effect of TRH is to stimulate the release of TSH from thyrotroph cells. TRH has the ancillary action of
stimulating the release of PRL from lactotroph cells, although the physiological significance of this action remains uncertain.

2.2.1 Structure of TRH

TRH was first isolated from porcine hypothalamus as a TSH releasing factor (Schally et al, 1966). The characterisation of porcine and ovine TRH (Folkers et al, 1984; Burgus et al, 1970) showed this compound to be a weakly basic, cyclic tripeptide, pyroglutamyl-histidyl-prolineamide (Figure 2.4). At this time it was demonstrated that the cyclicised glutamic acid residue and an intact amide were essential for ligand activity.

![Figure 2.4 Structure of TRH](Image)

The chemical structure of TRH [pyroGlu-His-Pro-NH₂] (from Folkers et al., 1969)

2.2.2 Synthesis and metabolism of TRH

TRH is synthesised in vivo as part of a larger precursor molecule, or pro-hormone that undergoes extensive post-translational processing to produce the mature peptide (Jackson and Wu, 1985; Nilnii et al, 1993; Richter et al, 1984). Interestingly the prepro-TRH (amino acids 178-199) molecule has been suggested as an inhibitor of ACTH secretion, that is as a corticotrophin release-inhibiting factor (CRIF) (Redei et al, 1995a; Redei et al, 1995b), although more recent work disputes these findings
(Nicholson and Orth, 1996). Several TRH-like peptides have recently been characterised, some of which may be produced by alternative post-translational processing of the TRH pro-hormone. Others appear to be the products of distinct genes (Bilek et al, 1992; Fuse et al, 1990).

TRH has a short half-life in both tissues and serum, being rapidly degraded into TRH-free acid (diamino-TRH), histidyl-proline-diketopiperazine (His-Pro-DKP) and constituent amino acids (Bower et al, 1978; Jackson et al, 1979; Jikihara et al, 1993). His-Pro-DKP is proposed to be active in the rat hypothalamus as a dopamine-uptake blocker (Jikihara et al, 1993; Peters et al, 1985).

2.2.3 Localisation of TRH to the hypothalamus

The highest concentration of TRH is localised in the paraventricular nucleus (PVN) of the hypothalamus which is the classic thyrotrophic area of the hypothalamus (Jackson and Reichlin, 1974; Lechan and Jackson, 1982). TRH can be detected in the nerve fibres of the posterior pituitary, with a concentration second only to the hypothalamus. Ablation of the PVN in the rat depletes hypothalamic TRH by 70% which results in hypothyroidism. In addition, the same lesion reduces posterior pituitary TRH suggesting that the TRH located in both the PVN and the posterior pituitary is of hypothalamic origin (Jackson and Reichlin, 1977). TRH may be synthesised in the pituitary and may act on local target cells as a paracrine regulator of anterior pituitary hormone secretion (Childs et al, 1978).

2.2.4 TRH and the hypothalamic-pituitary-thyroid (HPT) axis

TRH release has not been extensively investigated but may be under CNS derived adrenergic control and is likely to be pulsatile in nature. Pituitary regulation of TSH secretion is primarily under the dual control of hypothalamic TRH and thyroid hormones. TRH activates the phosphatidylinositol second messenger system on binding to TRH receptors (TRH-Rs) on thyrotrophs and to a lesser extent on mammotrophs. The TRH-R and its effects on the phosphatidylinositol second messenger system will be discussed more fully in sections 2.5 and 2.3.3 respectively. TSH secreted in response to TRH in the pituitary acts on the thyroid gland to stimulate the synthesis and release of the thyroid hormones $T_3$ and $T_4$. Figure 2.5 shows in schematic form the mechanisms of control in the hypothalamic-pituitary-thyroid axis.
Figure 2.5 The hypothalamo-pituitary-thyroid (HPT) axis

Diagrammatic representation of the neuroendocrine regulation of TSH secretion. The rate of TSH secretion is modulated by the positive TRH signal from the pituitary gland and by two negative feedback signals; one from the brain (somatostatin) and another from the circulating thyroid hormones T₃ (triiodothyronine) and T₄ (thyroxine). T₃ is the more potent of the thyroid hormones although both exert effects at the pituitary and hypothalamic levels. Specific enzymes (5′ mono-deiodinases) present in these two tissues remove an iodine moiety from T₄, generating T₃. Lines with arrows indicate positive effects; lines with bars indicate inhibitory control.

TSH is a member of the family of heterodimeric glycoproteins which includes FSH, LH and hCG. All of these family members share a common α-subunit. The β-subunit however, is distinct, and it is this part of the molecule which confers the specific biological activity of each hormone. TSH binds to cell surface receptors on thyroid follicular cells and promotes adenylyl cyclase activity and hence increases
intracellular levels of the second messenger cyclic AMP (cAMP). Cyclic AMP is able to mimic the intracellular effects of TSH even in its absence.

In order to maintain homeostatic balance TSH secretion cannot be permitted to go unchecked. In common with most other endocrine systems a set of positive and negative control mechanisms interact to maintain normal hormone levels. The ability of the thyrotroph to respond to TRH with increased TSH release is controlled by the feedback inhibition of thyroid hormones. Both T$_4$ and T$_3$ are capable of inhibiting TSH secretion; however, T$_3$ formed by the removal of an iodine residue from T$_4$ in the pituitary appears to be more important as a mediator of feedback inhibition than is circulating T$_3$. The effects of thyroid hormones on TRH secretion are not known. Studies in which exogenous TRH is administered to both normal subjects and to patients with thyroid deficiency or thyroid hormone excess, indicate that feedback at the pituitary level is sufficient to explain the regulation of TSH secretion. The TSH response to administered TRH is enhanced in patients with thyroid hormone deficiency compared to normal subjects. In contrast, thyroid hormone excess diminishes or abolishes the TSH response to TRH. The release of TSH is not absolutely dependent on TRH since low residual secretion of TSH remains even after TRH secretion has stopped. Elimination of the source of TSH by removal of the pituitary gland reduces thyroid function to a greater extent than does hypothalamic trauma.

In common with other pituitary hormones, TSH appears to be secreted in discrete pulses (Samuels et al, 1990), approximately nine per 24 hours in normal subjects. There also appears to be circadian rhythmicity of TSH secretion, with a nocturnal increase in pulse amplitude.

**2.2.5 Feedback regulation of hypothalamic TRH**

Thyroid hormones may be involved in feedback at the hypothalamic level. Administration of T$_3$ to the hypothalamus of monkeys inhibited TSH secretion by the pituitary (Belchetz et al, 1978), apparently by inhibiting the release of TRH and/or regulating its breakdown (Jackson et al, 1979). However, the thyroid hormones may be acting instead to stimulate the release of neurotransmitters, including dopamine and somatostatin, known to be involved at both TRH and TSH levels of control (Berelowitz et al, 1980; Chen and Ramirez, 1981; Montoya et al, 1979; Smythe et al, 1982).

Other substances of hypothalamic origin have been found to play some role in TSH secretion. Somatostatin from the hypothalamus may tonically inhibit TSH secretion,
since injection of anti-somatostatin antiserum into normal animals increases TSH levels. Dopamine antagonists cause elevation of serum TSH in normal patients suggesting that dopamine (a neurotransmitter) may also have a role as a tonic inhibitor. TSH release is inhibited by dopamine acting directly on thyrotrophs (Besses et al., 1975) and by somatostatin, the distinctive inhibitor of GH (Maeda and Frohman, 1980), and it is stimulated by noradrenaline (Montoya et al., 1979). Excess glucocorticoids also lead to a partial inhibition of TSH secretion (Re et al., 1976).

Oestrogens stimulate TSH release apparently by increasing the number of TRH-Rs on thyrotrophs (de Lean et al., 1977b). Lastly, TSH may be able to inhibit hypothalamic TSH release via a 'short-loop' feedback (Roti et al., 1978).

2.2.6 TRH and regulation of prolactin release

The synthesis and release of prolactin is under complex plurifactorial control, including the interaction of prolactin-inhibiting factors (PIFs; dopamine for example) and prolactin-releasing factors (PRFs), including TRH.

The major regulatory role of the hypothalamus is inhibitory with respect to prolactin secretion. It is now known that the withdrawal of dopamine results in the release of prolactin from the anterior pituitary. This is brought about by the dissociation of dopamine from its lactotroph receptors (dopamine type 2 receptors, D2-R) and the subsequent release of adenylyl cyclase inhibition which results in activation of the cAMP pathway. This process may potentiate PRF action (de la Escalera and Weiner, 1992). PIFs other than dopamine, such as α-melanocyte-stimulating hormone and possibly GABA may act in a more direct but less potent manner than dopamine (Yen, 1991).

TRH probably acts as a modulator of PRL secretion rather than as a true PRF. Initial evidence suggesting a stimulatory role for TRH in PRL secretion has been contradicted by more recent findings, including the observed inability of anti-TRH antiserum to prevent PRL release in ewes (Fraser and McNeilly, 1982) or rats (Harris et al., 1978), and the fact that circadian rhythms of PRL and TSH are dissociated in humans (Harris et al., 1978). Vasoactive intestinal peptide (VIP) from the hypothalamus stimulates PRL release in an autocrine manner (Nagy et al., 1988).
2.2.7 TRH in extra-pituitary locations

Although TRH plays the central regulatory role in the hypothalamic-pituitary-thyroid axis (Yamada and Wilber, 1990) it is also present in many extra-hypothalamic loci, including the mammalian central nervous system, retina, testis, pancreas and gut. Most of the early studies of TRH distribution ensued from the production of TRH-specific antibodies which could be used for immunoassay and immunohistochemistry. At early stages of mammalian development relatively high concentrations of TRH can be localised in the embryonic pancreas (Martino et al, 1978) and gastro-intestinal tract where it may be involved in the regulation of gastro-intestinal secretion and motility (Morley et al, 1979a). These neonatal hormone levels decline to low levels in the adult (Kawano et al, 1983; Morley et al, 1979b). A large literature exists concerning the possible extra-pituitary roles of TRH as a neurotransmitter and/or neuromodulator in the central nervous system (CNS) (O'Leary and O'Connor, 1995; Sharif, 1985)). TRH is present in the preoptic area of the brain where it is postulated to be involved in thermoregulation and alertness, in the hippocampus where it may have a role in controlling seizure thresholds, in the brain stem where it may modulate the autonomic nervous system (ANS; respiration, gut motility, gastric acid secretion, heart rate and blood pressure for example), and in the spinal cord where it may have a neurotrophic effect on α motoneurones. The adult rat testis has been identified as a source of hypothalamic neuropeptides including TRH. In the adult rat testis prepro-TRH mRNA was 13% of that found in the hypothalamus, and was especially evident in Leydig cell extracts (Feng et al, 1993). In the same study prepro-TRH mRNA and TRH were measured during rat testicular development. The concentrations of TRH (measured by radioimmunoassay), paralleled the observed changes in prepro-TRH mRNA, suggesting that TRH may be acting as a novel paracrine or autocrine regulator of testicular function and perhaps also of development. Wilber et al. (1996) describe the expression of the TRH gene in testis and also in the heart. TRH appears to be regulated by environmental lighting in the retina (Schaeffer et al, 1977).

The authenticity of the findings described in many of the original reports of the localisation of TRH has recently been questioned in light of the identification of TRH-like peptides which may have cross-reacted with the antibodies previously presumed to be TRH-specific (Fuse et al, 1990; Gkonos et al, 1993).

Respiratory distress syndrome (RDS) and its complications is one of the main causes of perinatal morbidity and mortality in premature babies. Prevention of RDS currently depends on β-adrenoceptor agonist therapy and on medications which accelerate
functional development of the antenatal lung, and optimisation of neonatal care, including the use of exogenous surfactants. The use of corticosteroids during the antenatal period has been well studied and is known to result in a 50% reduction in the risk of RDS before 34 weeks gestation. Prenatal morbidity and mortality are decreased without adverse effects. TRH, in conjunction with corticosteroids, is effective in promoting lung maturation and preventing RDS and its long-term complications (Zauli et al, 1996) and appears to be a promising antenatal therapy. In a comparison of antepartum combined TRH-betamethasone treatment of women at risk of preterm delivery, the incidence of RDS was reduced from 52% in the betamethasone treatment only group to 31% in the combination therapy group. The incidence of severe RDS was reduced from 42% to 20% in the same groups (Knight et al, 1994). This treatment gives better results than corticosteroids alone, without unfavourable neonatal consequences. In vitro studies of rat and rabbit lung suggest that the action of TRH on the lung may be mediated in part by one of the numerous, non-hormonal pathways known to be stimulated by TRH, particularly the ANS (Liggins, 1995). A study of TRH-cortisol treated foetal sheep indicates that the hormones function synergistically to increase connective tissue maturation in the lung, which is associated with increases in collagen and elastin content, and lung distensibility and stability (Campos et al, 1992). Rats with congenital diaphragmatic hernia (CDH) have been shown to benefit from TRH-dexamethasone (Suen et al, 1994), which supports the potential clinical use of pharmacological therapies to treat human foetuses with prenatally diagnosed CDH. At present the optimum dose, frequency and method of hormone administration have not been established. It has been suggested that TRH therapy should not be used in women with pre-eclampsia as it significantly raises blood pressure in this group and should definitely not be used in women at high risk of preterm delivery as this treatment is associated with unacceptable maternal and perinatal risks (Crowther et al, 1995). In light of conflicting evidence, it is clear that further trials are needed to confirm the beneficial effects, if any exist, of combined TRH-corticosteroid antenatal treatment. Moreover, its use in growth retarded foetuses and its long-term effects have not been addressed.

It is unlikely that TRH exerts its effects through the promotion of surfactant synthesis and secretion, as Yokoyama et al. (Yokoyama et al, 1995) found that there was no difference in the surfactant mRNA levels or in disaturated phosphatidylcholine (DSPC) levels in control, dexamethasone or TRH and dexamethasone treated rats. If TRH-corticosteroid therapy does indeed accelerate lung maturation, the mechanism by which this action is mediated remains to be determined.
2.3 G-protein-coupled receptors (GPCRs)

2.3.1 Structure and function of GPCRs

The proteins which are assigned to the superfamily of GPCRs share common structural, as well as functional properties. GPCRs are integral membrane proteins which couple primary messengers (external stimuli) to changes in cellular metabolism by stimulating the exchange of bound GDP for GTP on associated G (guanine-nucleotide-binding) protein α-subunits. Extracellular signals as diverse as hormones, growth factors, neurotransmitters, photons and odourants cause second messenger responses by acting via the increasing multitude of GPCR superfamily members.

There is as yet no definitive 3-dimensional model of GPCR structure. As these molecules exist only within the context of a cell membrane, X-ray crystallographic studies have been fraught with technical difficulties. However, based on physical studies of the evolutionarily-related proteins bacteriorhodopsin (Bonatti et al, 1989) and rhodopsin (Omary and Trowbridge, 1981) and in the light of X-ray crystallographic and molecular modelling work originating from the Cambridge Laboratory for Molecular Biology (Baldwin, 1993; Henderson et al, 1990) extrapolations have been made which allow computer modelling of members of the GPCR family. When combined with other methods such as site-directed mutagenesis and expression studies of chimeric receptors, the hypotheses made from computer models of receptor structure can be tested and the model refined accordingly. The amino tail portion of GPCRs is extracellular and the carboxy tail is intracellular as shown by protease digestion studies and immunological mapping (Dohlman et al, 1987a; Wang et al, 1989). The greatest degree of sequence homology between different GPCRs exists in the transmembrane (TM) domains.

The main structural feature shared by GPCRs is based on hydropathicity analysis of the amino acids forming the receptor peptide. The protein is believed to span the plasma membrane seven times (hence the supplementary terminology, seven transmembrane or heptahelical receptors), with the transmembrane domains (TM I-TM VII) adopting α-helical formation. The TMs generally contain between 20 and 25 amino acids and based on structural similarities with the extensively studied protein bacteriorhodopsin (Henderson et al, 1990), are believed to be oriented to form a ligand binding pocket (Findlay and Eliopoulos, 1990). The helices are conjoined by intra- and extracellular domains for which less structural information is available. Figure 2.6
below, shows a cartoon example of a general GPCR showing only its primary structure.

It is thought that ligand binding properties of the GPCRs reside principally within the pore created by the TM bundle (Grand, 1989). The reason for conservation of TM region sequences are unknown, although, within a given receptor family conserved amino acids with key roles in agonist binding have been identified. For example, a conserved TM II aspartate and two conserved cysteines in TM V of the adrenoceptor family interact with the catecholamine nitrogen and the hydroxyl groups of the chatechol ring respectively (Strader et al, 1989). In the TRH-R it has been suggested that two hydrogen bonds form on ligand binding. The hydroxyl group of the pyroGlu of TRH interacts with tyrosine 106 in TM III (Perlman et al, 1994a) and additionally the N-H of pyroGlu associates with the C=O of Asn 110 in TM III (Perlman et al, 1994b).

2.3.2 Disulphide bridge formation

Disulphide bridge formation is proposed between two conserved cysteine residues of the extracellular loops (el 1 and el 2, Figure 2.6). The purpose of these bonds is apparently to stabilise the 3-dimensional conformation of the receptor to ensure function, as shown by two independent studies of disulphide bond formation between extracellular cysteines of the TRH-R (Cook et al, 1996; Perlman et al, 1995). Each of these studies show that by both site-directed mutagenesis (thereby removing the cysteine residues) and by the addition of strong reducing agents to receptor assays (which disrupt the disulphide bonds) ligand binding is subsequently perturbed. These results indicate that the cysteine residues do indeed form a bond which maintains the receptor in a conformation required for high affinity ligand binding. In addition to maintaining the receptor in its binding conformation the extracellular domains may be involved in assisting ligand insertion into the binding pocket as has been shown for some small peptides (Fong et al, 1993) and glycoprotein hormones (Reichert et al, 1991).

GPCRs also share the potential to be posttranslationally modified, for example by glycosylation, phosphorylation or myristoylation.
2.3.3 GPCR glycosylation

Asparagine residues lying within glycosylation consensus sequences (Asn-X-Ser/Thr, X = any amino acid), especially in the amino tail, may be modified. Glycosylation is responsible for the discrepancy observed between the predicted molecular weights of GPCRs and that which is experimentally determined. Deglycosylation of the β2-adrenoreceptor reduces its molecular weight by approximately 16 kDa (Benovic et al., 1987). Glycosylation is not believed to be of major importance in ligand binding but may play a role in determining correct cellular distribution of receptors. Of note is the observation that removal of a consensus glycosylation site by mutation of Thr 17 of human rhodopsin is responsible for causing retinitis pigmentosa (Sung et al., 1991).

2.3.4 GPCR palmitoylation

Palmitoylation, thioester-linked palmitic acid addition, occurs at intracellular cysteine residues located in the carboxy tails of the β2-adrenoreceptor and rhodopsin and appears to create a fourth intracellular loop by intercalation of the palmitate into the lipid bilayer (O'Dowd et al., 1989; Ovchinnikov et al., 1988). Removal by mutation of the appropriate cysteine in the β2-adrenoreceptor negates Gs coupling indicating a role for its posttranslational modification in receptor coupling to G-proteins (O'Dowd et al., 1989). This palmitoylation might represent another common structural feature of GPCRs as other superfamily members include cysteine residues in similar positions and may therefore be similarly modified.

2.3.5 GPCR phosphorylation

Serine and threonine residues in il 3 and in the carboxy tail may act as sites for regulatory phosphorylations by second messenger-dependent or receptor-specific protein kinases (Lefkowitz et al., 1993) and may play a role in receptor desensitisation. Consensus sites for phosphorylation by protein kinases A and C (PKA and PKC) are Arg/Lys-Arg/Lys-X-Ser/Thr and Ser/Thr-X-Arg/Lys respectively. Phosphorylation of photo-excited rhodopsin leads to multiple intracellular phosphorylations and a concomitant conformational change. The phosphorylated rhodopsin then becomes a target for arrestin binding which prevents activation of transducin (Hargrave and McDowell, 1992). β-adrenergic receptor kinase (β-ARK) and β-arrestin are believed to perform the same function at the β2-adrenoreceptor and perhaps for other adenylyl cyclase-linked receptors. Other receptor kinases, including PKA and PKC may provide alternative desensitisation pathways for other receptors.
Sites of potential posttranslational modifications
- N-linked glycosylation
- Phosphorylation
- Cysteine residue-disulphide bond formation

Figure 2.6  Schematic representation of GPCR structure

G-protein coupled receptors have a hypothetical structure based on the hydrophobic nature of stretches of amino acid residues which suggests that these will be buried in the plasma membrane (I-VII). The transmembrane helices are connected by a series of extracellular loops (el 1-3) and intracellular loops (il 1-3). The carboxy tail is intracellular and the amino tail is to the outside of the cell. GPCRs may be modified by the posttranslational addition of various types of molecules at sites as indicated by differently coloured circles. Disulphide bonds in the extracellular portion of the receptor maintain the 3-dimensional conformation required for ligand recognition and binding.
2.3.6 GPCR-induced G-protein activation

Studies of the β2-adrenoceptor have highlighted two regions of GPCRs with potential functions in G-protein-coupling (Ostrowski et al, 1992). These sites occur at the N- and C-regions of il 3, but il 2 is also involved in the overall 3-dimensional receptor structure which facilitates receptor-G-protein association.

Prior to ligand binding GPCRs exist in an inactive 3-dimensional conformation which shields or otherwise constrains the regions of the peptide involved in G-protein-coupling. An allosteric transition is believed to be elicited by agonist binding, thereby overriding the constraining function of the prevailing molecular determinants of structure and allowing the receptors to relax into the active conformation at which point G-protein-coupling can take place.

2.4 G-proteins

G-proteins form a subgroup of the superfamily of GTP-binding G-proteins that includes the ras and ras-like proteins. Despite the diversity within the G-protein subfamily, just as with the GPCRs, its members share common structural and functional properties. G-proteins are heterotrimers consisting of α-, β- and γ-subunits. The majority of the sequence homology with other members of the GTP-binding G-protein superfamily resides within the α-subunit which is also the location of both specific high affinity guanine nucleotide binding and intrinsic GTP-ase activity. Certain regions of all α-subunits are highly conserved probably due to the common functions subserved by these regions of this subunit, GTP binding and hydrolysis for example, and have therefore been under evolutionary pressure to maintain these essential features. The less well conserved regions are presumably those areas responsible for subunit-specific functions such as receptor and effector coupling. The β- and γ-subunits form a tightly coupled dimer but otherwise the particular functions of the dimer are less well understood in comparison to what is known of the α-subunit. G-proteins couple many kinds of cell surface receptors to intracellular effector enzymes or ion channels. Each cell contains a variety of GPCRs, G-proteins and effectors. Whether a cellular response to an external signal is diffused through several pathways or is focused to a single pathway is determined by the specificity of G-protein interactions with both GPCRs and effectors, making the G-
protein a critical control point in signal transduction. Some of the G-proteins which have been identified in mammalian species are presented in Table 2.1.

G-proteins are located at the cytoplasmic face of the cell membrane. Although the primary amino acid sequence of heterotrimer subunits does not indicate regions of hydrophobicity, co- and post-translational modifications are believed to increase protein lipid-solubility which facilitates anchorage to the membrane. Many of the \( \alpha \)-subunits are known to undergo N-terminal fatty acylation (at glycine residue position 2) by addition of myristic acid. Substitution of this glycine for alanine within both \( \alpha_{11} \) and \( \alpha_0 \) results in unpalmitoylated peptides which are found predominantly in the cytosol (Jones et al., 1990; Mumby et al., 1990). However, \( \alpha_s \) does not myristoylate (Buss et al., 1987), and both \( \alpha_{11} \) and \( \alpha_q \) lack a consensus myristoylation signal (MGXXXS; X= any amino acid: (Gutowski et al., 1991; Pang and Sternweis, 1990; Wu et al., 1992), yet all three are membrane associated. Therefore, some other mechanism of membrane anchorage must exist. There have been suggestions that cysteine residues in the N-terminal domain may be palmitoylated (Parenti et al., 1993). A role for the C-terminal region of \( \alpha_s \) and other \( \alpha \)-subunits with similar C-terminal consensus motifs has been proposed. \( \beta \gamma \)-dimers can only be isolated from membrane preparations by detergent extraction, although, like the \( \alpha \)-subunits, their amino acid sequences do not predict regions of high hydrophobicity. As established for p21\textsuperscript{ras}, which shares the isoprenylation motif (CAAX; A= aliphatic residue) with \( \gamma \)-subunits, \( \gamma \) undergoes a post-translational modification at a C-terminal cysteine, which increases protein hydrophobicity. Both farnesyl (C-15) and geranylgeranyl (C-20) isoprenyl chains have been identified on different \( \gamma \)-subunits. Mutation of the crucial cysteine residue within the consensus motif results in \( \gamma \)-subunits which are both functionally inactive and localised to soluble fractions (Fukada et al., 1991; Katz et al., 1992; Ohguro et al., 1991; Simonds et al., 1991; Spiegel et al., 1991) underscoring the role of this type of protein modification in \( \gamma \)-subunit structure and function. It can be seen then, that \( \alpha \)-subunits may be membrane anchored either independently or by association with an anchored \( \beta \gamma \)-dimer.

G-proteins transduce information across the cell membrane by coupling diverse receptors to intracellular effector systems. G-protein association with receptors activated by extracellular signals enables tightly bound GDP to be released from the \( \alpha \)-subunit and replacement by GTP. GTP binding results in a conformational change of the \( \alpha \)-subunit which leads to G-protein activation and dissociation from the \( \beta \gamma \)-dimer. Both the activated \( \alpha \)-subunit and the \( \beta \gamma \)-dimer have been shown to be important regulators of intracellular effectors. G-protein activation is terminated by
dephosphorylation of GTP to GDP by the intrinsic GTP-ase activity of the α-subunit. Subsequently, the α-subunit regains both its inactive conformation and its high affinity for the βγ-dimer. Formation of the heterotrimer is essential for high affinity coupling between the G-protein and the receptor. Figure 2.7 shows the cycle of G-protein activation/inactivation.
<table>
<thead>
<tr>
<th>$\alpha$-Expression</th>
<th>Effector</th>
<th>Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>$G_s$ ubiquitous</td>
<td>↑ adenylyl cyclase, Ca$^{2+}$ channel</td>
<td>βAR, TSH, glucagon, others</td>
</tr>
<tr>
<td>$G_{olf}$ olfactory</td>
<td>↑ adenylyl cyclase</td>
<td>Odorant</td>
</tr>
<tr>
<td>$G_i$ rod photoreceptors</td>
<td>↑ cGMP-phosphodiesterase</td>
<td>Rhodopsin</td>
</tr>
<tr>
<td>$G_{i2}$ cone photoreceptors</td>
<td>↑ cGMP-phosphodiesterase</td>
<td>Cone opsin</td>
</tr>
<tr>
<td>$G_{i1}$ neural &gt; other tissues</td>
<td>↓ adenylyl cyclase, ↑ K$^+$ channel</td>
<td>$\alpha_2$AR, M$_2$, others</td>
</tr>
<tr>
<td>$G_{i2}$ ubiquitous</td>
<td>↓ adenylyl cyclase, ↑ K$^+$ channel</td>
<td>&quot;</td>
</tr>
<tr>
<td>$G_{i3}$ other tissues &gt; neural</td>
<td>↓ adenylyl cyclase, ↑ K$^+$ channel</td>
<td>&quot;</td>
</tr>
<tr>
<td>$G_o$ neural, endocrine</td>
<td>↓ Ca$^{2+}$ channel</td>
<td>&quot;</td>
</tr>
<tr>
<td>$G_7$ neural, platelets</td>
<td></td>
<td>M$_2$, others</td>
</tr>
<tr>
<td>$G_q$ ubiquitous</td>
<td>↑ phospholipase C-β</td>
<td>M$_2$, $\alpha_1$AR, TRH, NT, GnRH</td>
</tr>
<tr>
<td>$G_{i1}$ ubiquitous</td>
<td>↑ phospholipase C-β</td>
<td>&quot;</td>
</tr>
<tr>
<td>$G_{i4}$ liver, lung, kidney</td>
<td>↑ phospholipase C-β</td>
<td>?</td>
</tr>
<tr>
<td>$G_{i5/1}$ blood cells</td>
<td>↑ phospholipase C-β</td>
<td>?</td>
</tr>
</tbody>
</table>

Table 2.1 Mammalian $\alpha$-subunits previously identified

Some of the G-proteins which have been identified. The proteins shown are divided into their family groups ($G_s$, $G_i$, $G_q$, and $G_{i2}$) and the effectors which they activate are shown as is their tissue distribution. Where known the receptors to which $\alpha$-subunits couple are shown. Information in this table is adapted from Neer (1995)
Several crucial roles have been assigned to the G-protein $\beta\gamma$-dimers. These dimers may be important for presenting $\alpha$-subunits in the correct orientation for receptor activation, facilitating guanine nucleotide exchange and targeting certain kinases to enhance agonist-induced receptor phosphorylation and desensitisation (Cerione et al, 1986; Fung and Nash, 1983; Phillips et al, 1992; Pitcher et al, 1992). $\beta\gamma$-dimers may also be direct mediators of signal transduction. Both purified and recombinantly expressed $\beta\gamma$-dimers have been shown to be capable of activating phospholipase C.
(PLC) to cause phosphatidylinositol 4,5-bisphosphate breakdown. PLC-β2 and PLC-β3 appear to be particularly sensitive to activation by G-protein βγ while PLC-γ1 and PLC-δ1 are unresponsive (Camps et al, 1992; Carozzi et al, 1993; Katz et al, 1992; Park et al, 1993). It is possible that βγ derived from G_1 or G_0, rather than α-subunit, may be responsible for the pertussis-toxin-sensitive activation of phosphoinositide hydrolysis identified in some cell types.

Bacterial toxins are known to interrupt G-protein activity by interfering with either GDP/GTP exchange (pertussis toxin effect on α_i and α_0 subunits) or by inhibiting GTP-ase activity (cholera toxin-modification of αs resulting in persistent GTP activation).

2.5 Effectors and second messenger systems

G-protein linked effector and second messenger systems are structurally and functionally more diverse than their upstream signalling partners. Effector systems include retinal cyclic GMP phosphodiesterases (GMP-PDE), ion channels (e.g. K^+ and Ca^{2+}) and several phospholipase and adenylyl cyclase subtypes.

2.5.1 The phosphatidylinositol phosphate (PI) pathway

Phosphoinositide-specific phospholipase C (PLC) plays an important role in mediating the cellular actions of many hormones, neurotransmitters and some growth factors. Agonist-dependent activation of PLC, via the G_q proteins, results in the hydrolysis of the membrane phospholipid, phosphatidylinositol 1,4-bisphosphate (PIP_2) by the β1 and β2 PLC isoforms (Park et al, 1992; Taylor, 1991). This process results in the production of two major second messengers, inositol 1, 4, 5-triphosphate (IP_3) and diacylglycerol (DAG) (Limor et al, 1989) (Figure 2.8). IP_3 production results in the release of calcium ions (Ca^{2+}) from intracellular Ca^{2+} stores via binding and activation of IP_3 receptors/Ca^{2+} channels located on the surface of Ca^{2+}-containing vesicles. DAG-induced protein kinase C (PKC) activation stimulates the phospholipase A2 and phospholipase D2 pathways which result in the production of arachidonic acid (which is further metabolised to prostaglandins and leukotrienes) and DAG respectively. This secondary production of DAG prolongs PKC activation.
2.5.2 The adenyl cyclase (AC) pathway

Cyclic AMP (cAMP) is the second messenger associated with the AC pathway and is a ubiquitous molecule of critical importance, regulating a variety of cellular processes including gene transcription, mitogenesis, metabolism, smooth muscle contractile state and ion channel activity. Cyclic AMP is capable of regulating many different biochemical pathways. GPCR-induced activation of AC catalyses the conversion of ATP to cAMP. Cyclic AMP production occurs more rapidly than the intrinsic GTPase activity of the activated G-protein α-subunit is able to switch off the system, resulting in an amplification of the original signal. Cyclic AMP exerts its effects on the intracellular environment through the cAMP-dependent protein kinase A (PKA) (Levitski, 1988). This AC activity is inhibited by G_i proteins. In addition, AC may be regulated by calcium/calmodulin (Tang and Gilman, 1992) and βγ-subunits (Gilman, 1987) as shown in Figure 2.8.

Figure 2.8 The G_q and G_s/G_i effector (following page)

In this schematic representation of effector systems the G_q effector system is shown in the upper section and the G_s/G_i system is shown below. Three different agonists (A, shown in different colours) are shown to be activating the GPCRs (R). The agonist-bound Rs cause the dissociation of G-proteins. At the top, G_q mediates the activation of phospholipase-β1 (PLC-β1) to hydrolyse phosphatidylinositol 4,5-bisphosphate (PIP2). This degradation causes formation of the second messengers inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) which elevate intracellular Ca^{2+} and activate protein kinase C (PKC) respectively. The βγ-dimer may also stimulate PLC-β, in particular the -β2 and -β3 isoforms. In the lower section interaction of free α_s and the adenyl cyclase (AC) catalytic region results in increased cytosolic cyclic AMP in response to agonist binding (A(s)). Some AC subtypes may also be stimulated by βγ-dimers from other G-proteins or by calmodulin in the presence of increased intracellular Ca^{2+}. The mechanism by which G_i inhibits AC is not yet clear, but may involve α_i and βγ.
Chapter 2

Literature Review

PI system

AC system

GDP  GTP  GDP  GTP  GDP  GTP  GDP

Ca^{2+}  Ca^{2+} - calmodulin

cytosol

cyclic AMP

ATP
2.6 G-proteins, GPCRs and disease

Many and varied extracellular signals reach the interior of the cell as second messengers through the hormone-GPCR-G-protein complex which acts as a signal transducer to modulate a number of key signalling enzymes (adenylyl cyclase and phospholipases for example) or cellular effectors such as ion channels. Defects which alter the function of the first messenger (hormone, neurotransmitter, autocrine or paracrine substance) or either of the transducing elements (GPCR or G-protein) can lead to defective signalling and, ultimately, to disease. The clinical manifestations of hereditary or acquired defects of all of these signalling components have been described. The existence of diverse families of GPCRs, G-proteins and effectors means that a large number of signalling pathways can be mediated through a relatively small number of signalling components. The complexity of the interactions involved in cellular signalling allows many opportunities for small or large scale dysregulation of the normally finely tuned signalling network.

2.6.1 G-proteins, signal transduction and disease

Mutations in the genes encoding G-protein polypeptides have been identified as the primary lesion leading to the development of several clinical pathophysiologies.

One of the lines of research which led to the identification of G-proteins as a component of hormonal cAMP stimulation was investigation into the role of *Vibrio cholerae* toxin (cholera toxin, CTX) in the diarrhoeal disease cholera. It was discovered that CTX, which was already known to increase cAMP levels in cells, covalently modified a 45 kDa, GTP-dependent, polypeptide coupling factor. It transpired that the diarrhoea produced by *V. cholerae* is the result of efflux of Cl⁻ and water from intestinal epithelial cells in response to elevated levels of cAMP caused by inappropriate activation of Gs. This happens because the G-protein's GTPase activity is reduced by CTX-induced modification, locking the G-protein in its active, GTP-bound state. Gₛ was eventually purified from the reconstitution of rabbit liver GTP regulatory protein fractions into the S49 cyc⁻ cell line (Bourne et al, 1975) which does not produce endogenous Gₛ due to a gene mutation predicted to be located in the promoter region of the Gₛ gene. Pertussis toxin (PTX) also causes disease by modifying several members of the Gₛ subunit G-protein subfamily. PTX effectively uncouples the G-protein from the receptor (Figure 2.7), resulting in a disinhibition (= stimulation) of adenylyl cyclase activity.
More recently $G_\alpha$ mutations have been identified in human tumours (Table 2.2), the most common of which are the $G_{s\alpha}$, or $gsp$ oncogenic mutations which cause constitutive activity of adenylyl cyclase. Originally discovered in a subset of GH-producing pituitary adenomas, $gsp$ mutations have also been found in thyroid tumours. Gln 227 is equivalent to Gln 61 of p21ras and mutation of this site leads to a reduction in GTPase activity and a promotion of oncogenic activity. The oncogenicity of these mutations arises from the stimulatory effects that intracellular cAMP has on cell growth, proliferation and transformation.

<table>
<thead>
<tr>
<th>G-protein modified</th>
<th>Position and identity of alteration</th>
<th>Tissue</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$G_{s\alpha}$</td>
<td>R201C</td>
<td>Pituitary</td>
<td>Landis et al. (1989)</td>
</tr>
<tr>
<td>$G_{s\alpha}$</td>
<td>R201H</td>
<td>Pituitary</td>
<td>Landis et al. (1989)</td>
</tr>
<tr>
<td>$G_{s\alpha}$</td>
<td>Q227R</td>
<td>Pituitary</td>
<td>Landis et al. (1989)</td>
</tr>
<tr>
<td>$G_{s\alpha}$</td>
<td>Q227L</td>
<td>Pituitary</td>
<td>Vallar (1990)</td>
</tr>
<tr>
<td>$G_{s\alpha}$</td>
<td>Q227C</td>
<td>Thyroid</td>
<td>Lyons et al. (1990)</td>
</tr>
<tr>
<td>Gi2\alpha</td>
<td>R179C</td>
<td>Adrenal</td>
<td>Lyons et al. (1990)</td>
</tr>
<tr>
<td>Gi2\alpha</td>
<td>R179C</td>
<td>Ovary</td>
<td>Lyons et al. (1990)</td>
</tr>
</tbody>
</table>

Table 2.2  G-protein mutations in human tumours.
Amino acid substitutions use the one letter amino acid nomenclature, for example, R179C indicates the substitution of Arg at position 179 with Cys.

With the recent identification of G-proteins in novel systems or in those where their function remain undetermined, it is clear that the potential for implicating alterations in the level of expression or function of these proteins with disease states is vast.

2.6.2  GPCR dysfunction and disease

Alterations in the primary, secondary or tertiary structure of GPCRs could lead to various functional aberrations, as could defects of receptor processing, intracellular transport, G-protein coupling, recycling or phosphorylation. The clinical manifestations of any receptor defect will depend on its severity and the tissue distribution of the defective receptor. Mutation of a receptor of wide tissue distribution
and of critical function could be lethal, perhaps in utero, whereas a defect in a more specifically expressed and functional receptor might have a more distinctive pattern of clinical symptoms. An increasing number of GPCRs have been found to be involved in the pathogenic mechanisms of disease, both in vivo and in vitro.

Table 2.3  
Some of the GPCRs in which in vivo mutations cause disease  
(following page)

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhodopsin</td>
<td>autosomal dominant retinitis pigmentosa</td>
<td>1-9</td>
</tr>
<tr>
<td>Rhodopsin</td>
<td>autosomal recessive retinitis pigmentosa</td>
<td>10</td>
</tr>
<tr>
<td>Blue opsin</td>
<td>tritanopia colour blindness</td>
<td>9, 11, 12</td>
</tr>
<tr>
<td>Vasopressin V2</td>
<td>X-linked nephrogenic diabetes insipidus</td>
<td>13-16</td>
</tr>
<tr>
<td>ACTH</td>
<td>familial glucocorticoid resistance</td>
<td>17</td>
</tr>
<tr>
<td>MSH</td>
<td>pigmentation phenotypes (mouse)</td>
<td>18</td>
</tr>
<tr>
<td>LH</td>
<td>male precocious puberty</td>
<td>19</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid adenoma, Grave's disease, hypothyroidism</td>
<td>20-22</td>
</tr>
<tr>
<td>human CaR</td>
<td>familial hypocalciuric hypercalcemia, neonatal severe hyperparathyroidism</td>
<td>23</td>
</tr>
<tr>
<td>GHRH</td>
<td>little mouse phenotype</td>
<td>24</td>
</tr>
<tr>
<td>β2-adrenergic</td>
<td>steroid dependent asthma</td>
<td>25</td>
</tr>
<tr>
<td>β1-adrenergic</td>
<td>idiopathic dilated cardiomyopathy</td>
<td>26</td>
</tr>
</tbody>
</table>
2.7 The pituitary TRH receptor (TRH-R)

2.7.1 Isolation and characterisation of TRH-R cDNAs

The TRH-R cDNA was originally isolated from mouse by expression cloning of a cDNA library constructed from mouse TSH-producing tumours (Straub et al., 1990) at a time when there was no sequence data available on which to base DNA probe design. The mouse TRH-R (mTRH-R) was recognised to be a member of the GPCR superfamily of receptors. Subsequently, the TRH-R has been cloned from several species including rat (de la Pena et al., 1992a; Sellar et al., 1993) and human (Duthie et al., 1993a; Hinuma et al., 1994; Hirata et al., 1994; Yamada et al., 1993) There is very high sequence homology between all three of these receptors at both the nucleic acid and protein levels. At the extreme 3' end of the carboxy-tail there is a point of sequence divergence which results in each species of receptor having a different tail. All isoforms share amino acid 392 as this common point of sequence divergence. This site marks the position of an intron/exon boundary in the mouse gene, beyond which, only one more amino acid is encoded on exon 3 before the stop codon (Duthie et al., 1993b). The hTRH-R encodes five amino acids beyond position 392 and the rat (long form, 412 amino acids) receptor encodes 19 amino acids beyond this site. The short form of the rTRH-R (387 amino acids; de la Pena et al., 1992b) also has position 392 as a 3' exon/intron junction. All isoforms of the TRH-R are functional. The role of the variant carboxy-tails is not known. As the carboxy-tail of other GPCRs is implicated in G-protein coupling and perhaps in desensitisation and internalisation it is possible that the alternative sequences could engender receptor stability or cell specific variations in function. However, it is equally feasible that the divergent sequences identified represent nothing more than functionless sequence polymorphisms.

The hTRH-R gene has been mapped to chromosome 8q23 (Morrison et al., 1994). The hTRH-R is encoded on two exons, with the intervening intron site located at Val 264 of the receptor's amino acid sequence (Figure 2.8).
Figure 2.9 Two-dimensional representation of the primary sequence of the hTRH-R.

The hTRH-R is a member of the GPCR superfamily and as such displays the classical features of this group of receptors; namely, the seven membrane spanning α-helices linked by intra- and extra-cellular loops, an extracellular amino tail, an intracellular carboxy tail and consensus sites of post-translational modification. Amino acids are shown in the single letter code. The intron/exon junction at Val 264 is indicated by the white circle in il 3, the consensus ERY sequence at the amino end of il 2 is also shown, as is the location of Met 75 in TM I (the significance of which will be discussed in Chapter 5).
2.7.2 The TRH-R in extra-hypothalamic tissues

The TRH-R receptor, similar or identical to that of the pituitary isoform has been detected by Northern blot analysis in the hypothalamus, cerebrum, cerebellum, brain stem, spinal cord, and eye (Satoh et al, 1993a). In another study the TRH-R has been identified in the rat testis (Satoh et al, 1994), with the transcript exhibiting approximately 10% of the anterior pituitary expression level, and the receptor exhibiting binding (to 3Me-His2-TRH) and antagonism (by chlordiazepoxide) comparable to the cloned rTRH-R. The role of the TRH-R in such tissues is not currently known.

2.7.3 Control of TRH-R expression

The responsiveness of cells to external signal molecules can be modulated by the down-regulation of the number of cell surface receptors, either by the action of the receptor ligand (homologous down-regulation), or by the action of other extracellular substances which interact with other receptors (heterologous down-regulation). Several steps in the turnover of receptors have been shown to be involved in down-regulation (Collins et al, 1991; Hadcock and Malbon, 1991). The processes involved in receptor down-regulation have been shown to include both pretranslational processes such as inhibition of receptor synthesis as a result of decreased receptor mRNA levels (Burnstein et al, 1990; Hadcock and Malbon, 1988), and posttranslational processing, such as internalisation and subsequent intracellular receptor degradation (Chen et al, 1990; Valiquette et al, 1990). Studies of regulation of mRNA levels include those which investigate actual mRNA synthesis (i.e. gene transcription) and those which investigate alterations in mRNA stability and degradation in response to transduced signals. The regulation of mRNA stability has been linked to GPCR expression by several groups of investigators (Akamizu et al, 1990; Hadcock et al, 1989; Saji et al, 1991).

Down-regulation of the number of TRH-Rs on anterior pituitary cells has been well documented (Hinkle, 1989) and leads to diminished TRH responsiveness. The TRH-R has been shown to undergo both homologous down-regulation (Gershengorn, 1978; Hinkle and Tashjian, 1975) and heterologous down-regulation in response to molecules such as the thyroid hormones (Gershengorn, 1978; Perrone and Hinkle, 1978), epidermal growth factor (Hinkle et al, 1991) and VIP (Fujimoto et al, 1991; Imai and Gershengorn, 1985). It is believed that the homologous down-regulation observed in the TRH-R is the result of decreases in receptor mRNA rather than the
result of rapid receptor internalisation, since the half-time of TRH-induced TRH-R
down-regulation is approximately 12 hours (Oron et al, 1987). More recent studies
using GH3 cells which express endogenous TRH-Rs, show that TRH-R mRNA is
similarly reduced by both TRH and PMA (a PKC activator) and this reduction can be
blocked by H-7 (an inhibitor of protein kinases; Gershengorn, 1993). An increase in
intracellular calcium levels does not decrease TRH-R mRNA on its own, which taken
together with the effects of PKC activator/inhibitor data suggests that TRH-R down-
regulation by TRH is mediated by a PKC-linked mechanism. VIP, which stimulates
hormone secretion from anterior pituitary cells via the PKA system, is also able to
decrease the level of TRH-R mRNA in GH3 cells. This process is mimicked by
dorskolin (which directly activates adenylyl cyclase to increase intracellular cAMP
levels), cholera toxin and a cAMP phosphodiesterase inhibitor, which when taken
together suggests that VIP down-regulation of TRH-R mRNA is mediated by cAMP.
When cells are treated with the cAMP increasing agents (VIP or forskolin) in addition
to the PKC increasing agents (TRH or PMA) the decrease in TRH-R mRNA is greater
than observed by stimulation of one intracellular system alone. These results indicate
that TRH and VIP down-regulate TRH-R mRNA by activating PKC and PKA
respectively, and that these affects are additive. In cell lines expressing exogenous
TRH-Rs under the control of the strong CMV promoter (Gershengorn, 1993), TRH-R
mRNA was likewise decreased by TRH, suggesting that the rate of transcription was
not likely to be responsible for the decrease in receptor mRNA, particularly in view of
the finding that TRH actually stimulated the rate of TRH-R transcription rather than
reducing it. However this is probably a non-specific increase as the TRH-R construct
used in these transfection studies did not carry any significant portion of upstream
gene regulatory elements upon which transcription factors could have any effect (Yan
et al, 1991). It was subsequently discovered that TRH caused an increase in the rate
of TRH-R mRNA turnover, decreasing the mRNA half-life from 3 hours in untreated
cells to 45 minutes in TRH treated cells.

2.7.4 Upstream and downstream regulatory elements of the
TRH-R gene

Truncation of the 3' untranslated region (UTR) of the mouse TRH-R such that 143
bases inclusive of the poly (A)+ tail is deleted, results in mRNA that is not down-
regulated or has a decreased half-life as is found with the full-length mRNA. This is
despite the fact that in general plasmid vector transcripts expressed in a transfected cell
will be polyadenylated by the presence of the bovine growth hormone polyadenylation
signal which is part of the vector. Therefore, there appears to be a sequence within the
3' end of the TRH-R mRNA that is essential for TRH-induced regulation of TRH-R mRNA which is not the poly (A)+ tail. The decrease in TRH-R mRNA in transfected cells could therefore the result of decreased mRNA stability, possibly mediated by the activity of an RNase which has been shown to degrade the mRNA in TRH stimulated GH3 cells (Narayanan et al, 1992). A similar mechanism may cause the same decrease in TRH-induced TRH-R mRNA turnover in thyrotroph cells. No similar effects on TRH-R mRNA stability/degradation have been shown in non-pituitary cells or in AtT20 adrenocorticotroph cells (Gershengorn et al, 1994).

The rate of TRH-R transcription was not considered as important in the extensive study reported above, however the 5' UTR of the mouse TRH-R gene should not be ignored. After all, many other gene promoter regions contain transcription factor binding sites which mediate effects required for cellular regulation. This is of particular significance to the TRH-R as the rate of transcription has been shown to be increased by dexamethasone (Yang and Tashjian, 1993) and oestradiol (Kimura et al, 1994). The effects of dexamethasone and oestradiol are not inhibited by cyclohexamide (a protein synthesis inhibitor), suggesting that the protein(s) mediating transcriptional regulation are present in unstimulated cells. Glucocorticoid and/or steroid response elements may be present in the sequence of the TRH-R promoter region.

2.8 The thyroid gland

2.8.1 Structure and function

The thyroid gland is located just below the larynx with its lateral lobes, which are connected by a mass of tissue called the isthmus, lying to the left and right of the trachea. When present, the pyramidal lobe extends upwards from the isthmus. The thyroid gland is able to deliver high levels of hormones into the bloodstream in a short period of time due to its rich blood supply.

Histologically the thyroid gland is composed of spherical sacs, or follicles, encompassed by an epithelial layer. The colloid lumen is lined with follicular cells of the epithelium, whereas the outer surface is adjacent to parafollicular, or C cells (Figure 2.10) and capillaries located within the connective tissue of the gland. The epithelial cells vary in shape in relation to their level of secretory activity. When
inactive, they are squamous or cuboidal in shape, but attain a more columnar appearance when actively secreting hormones. The follicular cells manufacture the thyroid hormones (T₃ and T₄). As mentioned previously, although T₄ is the major secretory component, T₃ is three to four times more potent, with conversion of T₄ to T₃ taking place in several peripheral tissues including the liver and lungs. Despite these factors it appears that both hormones are functionally similar. The parafollicular cells produce calcitonin.

One of the thyroid's unique features is its ability to store hormones and release them in a steady flow over an extended period of time. Essential to the synthesis of the thyroid hormones is the uptake of dietary iodide ions. Inorganic iodide from the blood is concentrated in the follicles by active transport across the cell membrane and rapid translocation to the colloid lumen. Under normal conditions the follicular cells contain a forty times higher concentration of iodide than is present in the blood, with this ratio increasing up to 300 times during periods of maximal activity. Peroxidase within the follicular cells oxidises the iodide to iodine which is then chemically bound to tyrosine residues to form mono- and di-iodotyrosines (inactive metabolites MIT and DIT respectively). Thyroglobulin (TGB), a large glycoprotein produced by the follicular cells and secreted into the lumen binds MIT and DIT. The TGB-bound MIT and DIT are then coupled by further enzymatic activity to form TGB-bound thyroid hormones. The TGB-thyroid hormone complex is stored within the follicular lumen where it constitutes the thyroid colloid. Droplets of colloid are pinocytosed by the follicular cells in advance of hormone secretion. Within these small endocytotic vesicles colloid is proteolysed by lysosomes resulting in uncoupling of the thyroid hormones. Thyroid hormones secreted into the blood associate with plasma proteins, predominantly thyroxine-binding globulin (TBG), with pre-albumin and to a lesser extent albumin. Very little free circulating thyroid hormones are detected in blood samples although it is this level which determines the individual's metabolic state and thyroid status. TBG binding capacity is altered in many conditions. Pregnancy, myxoedema, use of oral contraceptives, oestrogen levels, and genetic TBG excess cause increased TBG capacity. Malnutrition, severe illness, thyrotoxicosis and drugs such as anabolic steroids and phenytoin cause decreased TBG capacity. Hence the T₄:TBG ratio or the free thyroxine index (FTI) were often used to correct the effects of these differences in TBG capacity. However, with the advent of more sensitive free T₃ and T₄ assays these latter parameters are now used routinely in clinical practice.
2.8.2 Functions of the thyroid hormones

The thyroid hormones have three principal effects on the body: 1) regulation of organic metabolism and energy balance, 2) regulation of growth and development and 3) regulation of nervous system activity. Since the overall effect of the thyroid hormones is to increase catabolism, they increase basic metabolic rate generating the energy required to increase the rates of lipid and carbohydrate catabolism and protein synthesis. The body produces heat during this energy production, a phenomenon termed the calorigenic effect.

The thyroid hormones help to regulate tissue growth and development, especially in children. In concert with hGH, body and nervous tissue growth is accelerated. Deficiency of the thyroid hormones during foetal development can result in fewer and smaller neurones, defective myelination of axons, and mental retardation (cretinism). During early years of life, thyroid hormone deficiency leads to small stature and poor development of certain organs such as the brain and reproductive structures.

The thyroid hormones increase reactivity of the nervous system, resulting in increased heart rate, blood pressure, gastrointestinal motility and nervousness.

Figure 2.5 illustrates the control mechanisms involved in thyroid hormone secretion. Essentially, chemical sensors in the hypothalamus detect changes in the levels of circulating thyroid hormone. If this level drops below normal, the hypothalamus responds by secreting TRH into the portal system. TRH stimulates the thyrotroph cells of the anterior pituitary gland to secrete TSH, and hence thyroid hormones are secreted by the thyroid gland until the blood levels are normalised. Conditions which increase the body’s energy requirements also trigger this negative feedback system and increase thyroid hormone secretion (a cold environment, hypoglycaemia, high altitude and pregnancy, for example).

The thyroid gland is regulated by the co-ordinate action of the hypothalamus and pituitary (Figure 2.5). Therefore, thyroid dysfunction frequently occurs in subjects with lesions of these organs.
Figure 2.10 Structure of thyroid tissue
Left; photomicrograph of thyroid follicles. Right; diagram showing the arrangement of follicular structures, highlighting the cuboidal cells of the epithelium which secrete the factors which together form the thyroid colloid, into the interior lumen of the follicle.

2.8.3 Hypothyroidism

The most common cause of hypothyroidism and goitre world-wide is iodine deficiency. Where iodine levels are not deficient, autoimmune disease is the most common cause of hypothyroidism, followed by hypothyroidism as a result of thyroid resection or radio-iodine treatment of thyrotoxicosis. Hypothyroidism can be the result of failure of the thyroid gland itself (primary hypothyroidism), can be due to failure of the pituitary gland (secondary hypothyroidism), or can be the result of
hypothalamic failure (tertiary hypothyroidism) (Table 2.4). Primary hypothyroidism is the most common form of the disease and can be divided into congenital and acquired types of disease. The congenital forms of hypothyroidism can result from inherited conditions of lack of thyroid function, ectopic thyroid conditions or disruptions of thyroid hormone synthesis. The acquired forms include those arising from iodine deficiency, autoimmunity, thyroid tissue removal (by partial thyroidectomy or radio-iodine treatment) or iodine excess. Primary hypothyroidism is ten times more common in females than males. Secondary hypothyroidism can be caused by pituitary tumours or other destructive lesions, both of which can interrupt pituitary signalling to the thyroid gland. Hypothalamic lesions and isolated TRH deficiency are causes of tertiary hypothyroidism.

<table>
<thead>
<tr>
<th>Disease</th>
<th>T₄</th>
<th>basal TSH</th>
<th>TRH response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary hypothyroidism</td>
<td>low</td>
<td>raised</td>
<td>exaggerated</td>
</tr>
<tr>
<td>Secondary hypothyroidism</td>
<td>low</td>
<td>low or normal</td>
<td>absent</td>
</tr>
<tr>
<td>(pituitary)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tertiary hypothyroidism</td>
<td>low</td>
<td>low or normal</td>
<td>normal or delayed</td>
</tr>
<tr>
<td>(hypothalamic)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>non-thyroid illness</td>
<td>low</td>
<td>normal</td>
<td>normal</td>
</tr>
</tbody>
</table>

Table 2.4 Summary of diagnostic test results in hypothyroidism

Cretinism, caused by insufficient foetal thyroid hormone exposure is an example of primary congenital hypothyroidism. The disease is characterised by several outstanding clinical symptoms: dwarfism, mental retardation, low body temperature and heart rate, and general lethargy, for example. Dwarfism is caused by the failure of the skeleton to grow and mature. hGH on its own is not sufficient for normal development. Mental retardation occurs as a result of poor neuronal development as discussed in Section 2.8.2. Cretins also show retarded sexual development and a yellowish skin colour. The affected infant attains characteristic facial features due to the formation of fat pads under the skin and the development of a large protruding
tongue. Carbohydrates are stored rather than metabolised in this state of general metabolic underactivity. Congenital hypothyroidism is not widely recognisable clinically at birth. Measurement of TSH five days postnatally may reveal a persistent elevation of this hormone reflecting hypothyroidism due to primary thyroid failure. However, it will not detect the rare case of hypothyroidism secondary to pituitary-hypothalamic disease (central hypothyroidism, which will be discussed at length in a following section). If congenital hypothyroidism is diagnosed early, the symptoms can be somewhat alleviated by thyroid hormone therapy.

Severe hypothyroidism in the adult, myxoedema, is most often the end result of autoimmune destruction of the thyroid gland (Hashimoto's thyroiditis), although subclinical hypothyroidism can occur in which low levels of thyroid antibodies in the circulation are associated with moderately elevated TSH, but normal T4. Approximately 50% of patient with subclinical hypothyroidism progress to overt hypothyroidism. For reasons identical to the infantile syndrome, the adult sufferer of hypothyroidism exhibits decreased heart rate, low body temperature, sensitivity to cold, muscular weakness, general lethargy, a tendency to gain weight easily along with a characteristic puffiness of the face and thick, dry flaky skin. Since neurological maturation has not been affected, the patient with myxoedema does not suffer from mental retardation. However, in some cases neuronal reactivity may be dulled resulting in a loss of mental alertness. Symptoms are alleviated by administration of thyroid hormones. Circulating thyroid antibodies are more common in women than in men at all ages but are most common in postmenopausal women.

Hypothyroidism is treated with replacement thyroxine therapy which should be maintained for life, with regular follow-through testing.

2.8.4 Central hypothyroidism: an example of specific type of hypothyroidism

Central hypothyroidism (and hyperthyroidism) is far less common than primary thyroid disease. More than 95% of patients with hypothyroidism, have disease of the thyroid gland. The remaining group who have normal thyroid associated with reduced TSH secretion, normally as a result of either hypothalamic or pituitary disease, have central hypothyroidism. Such hypothyroidism occurs most often in association with GH-deficiency. Isolated central hypothyroidism is considered rare. Any lesion that leads to insufficient TRH or TSH production, or interrupts anatomic connections between the hypothalamus and pituitary can cause central hypothyroidism. The
recognition of central hypothyroidism as a *bona fide* condition has implications which are important in both the diagnosis and subsequent therapeutic regime used with individual patients. Failure to recognise and treat central hypothyroidism can result in poor growth and less than optimal state of health (Manasco *et al*, 1994; Samuels and Ridgway, 1992).

By definition patients with central hypothyroidism have low circulating- and free-T₄ levels. T₃ levels are often low, but are less predictable than T₄ levels. TSH levels are generally lower in patients with pituitary lesions, compared with those with hypothalamic lesions, who have been found to have normal or even elevated TSH levels (Patel and Burger, 1973). Hypothyroidism in the presence of normal or elevated TSH levels in central hypothyroidism may be a result of decreased TSH bioactivity. Direct data from some patients with central hypothyroidism suggests that their TSH has reduced activity in human thyroid membrane radioligand binding and adenylyl cyclase activating assays, compared to the TSH from patients with primary hypothyroidism (Beck-Peccoz *et al*, 1985; Faglia *et al*, 1983). In addition, lectin chromatography of the sera from patients with central hypothyroidism has shown their TSH to be differentially glycosylated in comparison to TSH from euthyroid or primary hypothyroid patients (Miura *et al*, 1989). The bioactivity of TSH is impaired in this pattern of glycosylation, which has effects on subunit combination and hormone clearance (Magner, 1990), although another study by the same investigator found no strict correlation between glycosylation patterns and bioactivity (Magner *et al*, 1992). However, animal studies of central hypothyroidism and studies of the effects of exogenous TRH on TSH glycosylation in humans (Magner, 1990) suggests that the changes observed in central hypothyroidism may be due to TRH deficiency and indeed, one group of investigators found that TSH bioactivity in central hypothyroidism was enhanced (and even pushed into the normal range) by exogenous TRH, either by acute injection or chronic oral administration (Beck-Peccoz *et al*, 1985). T₄ also increased or was normalised in this and another study (Faglia *et al*, 1983), suggesting that at least in some patients, TRH deficiency is implicated in the production of inactive TSH. Horimoto *et al*. (1995) deduced that TSH bioactivity was reduced in patients with central hypothyroidism (as determined by a reduction in the ratio of the T₃ increment to mean TSH increment after TRH administration, as compared to normal). However, even when using their novel serum TSH purification method they were unable detect any TSH bioactivity reduction *in vitro*. Therefore, at this time it is not possible to draw concrete conclusions as to the existence or function
of differential TSH glycosylation in central hypothyroidism, and the role that TRH deficiency might play in this process.

Cases of central hypothyroidism can be classified based on the pathophysiological cause of the condition. These include:

- destructive hypothalamic hypothyroidism; caused by mass or infiltrative lesions (see pituitary hypothyroidism below), congenital defects, degenerative diseases, traumatic injury or iatrogenic effects
- idiopathic hypothalamic hypothyroidism
- tumorous pituitary hypothyroidism; mass lesions e.g. pituitary adenomas, craniopharyngiomas, metastatic cancer, cysts and abscesses
- atrophic pituitary hypothyroidism; infiltrative lesions e.g. tuberculosis and toxoplasmosis, pituitary atrophy
- idiopathic pituitary hypothyroidism

Each of these subgroups can be identified by differential diagnosis (Table 2.5).

<table>
<thead>
<tr>
<th></th>
<th>Destructive Hypothalamic</th>
<th>Idiopathic Hypothalamic</th>
<th>Tumorous Pituitary</th>
<th>Atrophic Pituitary</th>
<th>Idiopathic Pituitary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes insipidus</td>
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<td>N</td>
<td>rare</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Neurologic defects</td>
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<td>N</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
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<td>N</td>
<td>none/moderate</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Hypoprolactinaemia</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>Anterior hypopituitarism</td>
<td></td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>TSH/PRL response to TRH</td>
<td></td>
<td>Y/Y</td>
<td>Y/Y</td>
<td>Y/Y</td>
<td>rare/N</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N/Y</td>
</tr>
</tbody>
</table>

Table 2.5  Differential diagnosis of central hypothyroidism.
Causes of central hypothyroidism can be diagnosed by combination of the symptoms and signs observed as indicated. Annotations: Y= yes, N= no. Adapted from Emerson (1985).
A less specific subdivision is that which categorises the causes of central hypothyroidism into one group resulting from TSH deficiency (pituitary based) and a second group which arises from TRH deficiency (hypothalamus based).

When the symptoms presented by a patient with central hypothyroidism cannot be assigned to any of the above categories a diagnosis of idiopathic central hypothyroidism is made. Idiopathic TRH deficiency is diagnosed in patients who retain intact TSH responses to exogenous TRH, perhaps in association with isolated TRH- or pluri-hypothalamic releasing hormone deficiencies, but in the absence of anatomical abnormalities. The aetiology of idiopathic TRH deficiency remains unknown, although a few cases have been found to be transient, with spontaneous resolution of hypothyroidism (Gharib and Abboud, 1987).

Idiopathic TSH deficiency is diagnosed when a patient with central hypothyroidism has low, or undetectable TSH levels, no TSH or PRL response to TRH, no evidence of other pituitary hormone deficiencies, and no abnormalities on radiological investigation. Idiopathic TSH deficiency may have several plausible aetiologies one of which is the mutation of the TSH-β gene resulting in inactive, or no TSH.

Genetic analysis of five Japanese kindreds with isolated TSH deficiency has indicated that the disease is inherited in an autosomal recessive pattern (Hayashizaki et al, 1990). All of the affected individuals have severe growth failure and mental retardation, accompanied by a failure to secrete active TSH in response to TRH administration, despite the expected increase in free α-subunit. In three of the kindreds, a single nucleotide substitution in the second exon of the TSH β-subunit was discovered. This mutation resulted in an amino acid substitution (within exon 2, previously established as a region critical for αβ-dimer formation) which has been proposed to cause a deleterious structural change in the β-subunit which prevents formation of biologically active TSH. In a similar study of two related Greek families with three children affected by congenital TSH deficiency, a different exon 2 mutation was found. In this case the mutation resulted in a premature stop codon and the gene therefore encoded a truncated β-subunit (Dacou-Voutetakis et al, 1990).

Acquired central hypothyroidism probably accounts for less than 5% of all cases of hypothyroidism which makes this condition much rarer than primary hypothyroidism. Pituitary tumours (functioning and non-functioning) are the most common cause of acquired central hypothyroidism in adults. TSH deficiency usually occurs in association with other pituitary hormone deficiencies which may arise due to the
existence of a macroadenoma and very rarely of a microadenoma (Abboud, 1995), but may also result from ablative surgery, radiotherapy or other organic causes.

Patients with primary hypothyroidism show an increased TSH pulse amplitude and a blunting of the normal nocturnal TSH surge. Patients with central hypothyroidism also exhibit atypical pulsatile TSH secretion. It has been shown that the normal nocturnal TSH surge is absent or blunted in central hypothyroidism, giving rise to a sensitive and specific test for identification of this condition (Rose et al, 1990). Isolated central hypothyroidism has been identified in children previously diagnosed with idiopathic short stature using this more accurate method (Rose, 1995). It is interesting to note that a blunted nocturnal TSH surge has been identified in patients suffering from seasonal defective disorder (SAD; (Coiro et al, 1994)). Although SAD patients experienced psychiatric depression in the winter the blunted TSH secretory pattern was consistent throughout the seasons. Normal subjects showed a significant nocturnal TSH surge in the tests at all times. The authors of this report suggest that patients with SAD exhibit symptoms of mild central hypothyroidism, i.e. low TSH response to TRH in the presence of normal serum thyroid hormone levels and an absent nocturnal TSH surge. Patients with SAD may be suffering from a TRH-linked defect, perhaps located in the retina, since TRH has previously been detected in this tissue (Schaeffer et al, 1977), where the hormone appears to be regulated by environmental lighting, although the outcome of TRH activity in the retina is presently unknown.

In three reports (Costom et al, 1971; Miyai, 1985; Miyai et al, 1988) patients with central hypothyroidism had undetectable basal and TRH-stimulated TSH levels, suggesting that these rare patients had thyrotroph insensitivity due to a receptor or post-receptor defect or primary thyrotroph disease interfering with the synthesis and secretion of TSH.

2.8.5 Hyperthyroidism

Hyperthyroidism is much less common than hypothyroidism although its symptoms are those which are more often brought to mind when discussing thyroid disease.

Grave's disease (also more common in women than in men) is an example of a hyperthyroid state caused by autoimmune destruction of thyroid tissue. One of the primary symptoms is a vast enlargement of the thyroid tissue resulting from excessive lymphocytic infiltration of the gland, the end result of which is goitre formation.
Additional causes of hyperthyroidism include toxic multinodular goitre and toxic adenoma formation. More rare causes are de Quervain's thyroiditis, 'silent' thyroiditis and neonatal hyperthyroidism caused by transplacental passage of thyroid stimulating antibodies into the foetal circulation.

In Grave's disease thyroid stimulating antibodies activate adenylyl cyclase by binding to the surface TSH receptors on thyroid cells in much the same manner as TSH. The TSH receptors are thereby switched on, promoting the secretion of excess levels of thyroid hormones. In addition to goitre, thyroiditis is characterised by other overt physical symptoms many of which are associated with periorbital swelling and include exophthalmos and lid retraction. More occasionally localised myxoedema and vitiligo are evident. The metabolic rate as could be expected is abnormally high, resulting in symptoms of increased pulse, high blood pressure, high body temperature, heat intolerance, flushed skin, irritability and possibly muscle tremors caused by increased responsiveness of the nervous system.

Hyperthyroidism is usually treated by antithyroid drugs (e.g. carbimazole which is metabolised to its active counterpart, methimazole), surgical removal of excess tissue or radio-iodine treatment.

2.9 Pituitary tumours

Pituitary tumours account for approximately 10% of intracranial tumours. These tumours are classified into two broad groups; those which are associated with hormone secretion are termed functioning, whereas those showing no overt signs of hormone output are classified as non-functioning. Hormonal stimulation of hypothalamic source seems an unlikely primary cause of adenoma formation, although these signals may have a secondary effect on adenoma growth and secretion. Pituitary tumours are generally benign neoplasms which in the majority of cases are detected only as a result of mass effects from tumour expansion, such as visual field defects, hypopituitarism (due to compression of the pituitary stalk and blockade of the delivery of hypothalamic hormones to the pituitary or compression of other secretory cells of the normal gland) or hyperprolactinaemia (if dopamine delivery to the pituitary is blocked by compression of the portal system). Pituitary tumours have been shown to arise from the clonal expansion of a single, transformed progenitor cell (Herman et al, 1990) but little is known of the mechanisms which underlie oncogenic transformation.
in most pituitary adenomas. However, the gsp mutations (Section 2.4.1) have been shown to be associated with the transformation of a subset of GH-secreting pituitary adenomas and thyroid tumours.

Adenomas of the anterior pituitary gland may arise from any of the major cell types making up this tissue. The hormone produced by the resulting lesions therefore depends on the nature of the progenitor cell.

**Lactotroph cell adenomas** or prolactinomas are one of the most common anterior pituitary tumours, accounting for approximately 60% of all identified pituitary tumours.

**Somatotroph cell adenomas** which account for approximately 15% of pituitary tumours are usually associated with gigantism (prior to epiphyseal fusion) or acromegaly (after epiphyseal fusion). Indeed, over 98% of patients with acromegaly have pituitary adenoma, 30% of whom also have hyperprolactinaemia due to the incidence of tumours of mammotroph origin.

**Corticotroph adenomas** are the cause of Cushing's disease. These tumours tend to be very small (microadenomas) lesions located in the centre of the pituitary gland.

**Gonadotroph cell adenomas** were until relatively recently believed to be rare with an incidence of less than 1%. Recently, it has been shown that a large proportion of non-functioning pituitary adenomas are of gonadotroph origin, indeed taking this finding into account makes gonadotroph adenomas one of the largest groups of pituitary tumours, accounting for 40 to 50% of all macroadenomas. Symptoms associated with hormonal hypersecretion are the least common clinical presentation of gonadotrophinoma. They usually come to recognition as a result of neurological effects caused by growth and expansion of the tumour. Therefore, gonadotroph adenomas are generally recognised only once they have attained macroadenoma proportions. The reasons for the failure to recognise gonadotroph adenomas are two fold; firstly, they secrete inefficiently, and secondly, the hormones which they secrete (the gonadotrophins and their subunits) do not produce recognisable clinical symptoms. In comparison with a prolactinoma of equivalent size, which can produce a 100-1000-fold increase in normal serum prolactin levels, the gonadotroph adenoma increases serum FSH to up to 10-fold and often levels are not elevated above the normal range at all (Snyder, 1995). FSH is most commonly the secretory product of gonadotrophinomas. LH hyper-secretion is less frequently observed. Additionally, the secretion of intact gonadotrophins is rare. The hormone output usually includes a
combination of intact FSH, FSH- and LH-β subunits and some α-subunit (Snyder, 1995), with the proportions of each secretory product being inconsistent amongst adenomas. Hormonal output by these tumours appears to depend to a certain extent on endogenous GnRH, since treatment with Nal-Glu-GnRH (a GnRH antagonist) reduces elevated FSH levels in patients with gonadotroph adenomas to normal (Daneshdoost et al, 1990). Patients with gonadotrophinomas often show a paradoxical rise in gonadotrophins and/or their subunits in response to exogenous TRH (Daneshdoost et al, 1993; Daneshdoost et al, 1991; Katznelson et al, 1992). The incidence of truly non-functioning adenomas is approximately 20%.

**The thyrotroph cell tumour**, or thyrotrophinoma, is the most infrequently encountered pituitary adenoma. Approximately 30% of patients with thyrotroph adenomas show a rapid rise in serum TSH in response to TRH administration (Bevan et al, 1989). The remainder show no response to TRH challenge. TRH binding sites have been detected on the cells of some thyrotrophinomas (Chanson et al, 1988) but not on others (Filetti et al, 1982). Patients with TSH-secreting adenomas often present late, with large and usually invasive tumours.

### 2.10 Concluding remarks

The HPT axis has been extensively studied by many researchers using the tools of the pharmacologist, the physiologist and most recently the molecular biologist. The identification of many of the molecules involved in control of the HPT axis, and in particular the cloning of cell surface receptors has allowed a hitherto unprecedented level of investigation. The cloning of the TRH-R has allowed studies of the molecular and cellular aspects of this receptor, including elucidation of its structure, function, and regulation. However, there still exist many aspects of TRH-R structure and activity that remain to be elucidated. Many questions as to the mechanisms of receptor activation, ligand binding, desensitisation as well as to the mechanisms and roles of down-regulation and the possible existence of TRH-R isoforms possibly binding the TRH-like peptides in extra-hypothalamic tissues, still exist. Additionally, in view of the already large number of GPCRs that have been linked with disease, is there a place for the TRH-R on this list? With the continual advancement of the molecular and biological tools which are available, and in conjunction with high power computer
modelling techniques, the pace of progress into the understanding of the TRH/TRH-R system is not likely to slow.
Chapter 3
Materials and methods

3.1 Introduction

The methods detailed in this chapter are general tools of molecular biology but are discussed in relation to their use specifically as applied to the study of GPCRs. The experimental chapters contain the detailed information pertaining to individual experiments.

A detailed list of buffers and solutions used is included in Appendix I. Appendix II details the suppliers of consumables and equipment used in the experiments.

3.2 The polymerase chain reaction (PCR)

The polymerase chain reaction which originated in the mid 1980s (Mullis and Faloona, 1987) is used to enzymatically amplify a stretch of DNA between two regions of known sequence. Two amplimers, or PCR primers (short single strand oligonucleotides), are synthesised to complement sequences which lie on each of the complementary strands of the DNA and which flank the segment to be amplified. These amplimers are added to a reaction mix which contains a molar excess of the amplimers and the four deoxynucleoside triphosphates (dNTPs), buffer, a thermostable DNA polymerase, and a source of template DNA. This mixture is processed through a series of heating and cooling cycles which perform the following functions;

a) Denaturation by heating to form the single strands of target DNA to which the amplimers can anneal.

b) Annealing which allows the amplimer to bind to its complementary region on the single-stranded DNA target generated in step a.

c) Extension by the DNA polymerase which recognises and binds only to the double-stranded DNA formed in step c.
Chapter 3  Materials and Methods

The products of one round of amplification become the template for the next, resulting in an exponential increase in the number of amplified molecules with each PCR cycle. The major product of a PCR reaction is a fragment of DNA delineated by the 5' ends of the amplimers and whose length is the distance between the amplimers.

Table 3.1 shows a general PCR temperature cycling protocol

<table>
<thead>
<tr>
<th>Step</th>
<th>Number of cycles</th>
<th>Temperature (°C)</th>
<th>Time (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>denaturation</td>
<td>1</td>
<td>95</td>
<td>120</td>
</tr>
<tr>
<td>denaturation</td>
<td></td>
<td>95</td>
<td>45</td>
</tr>
<tr>
<td>annealing</td>
<td>20-30</td>
<td>50-60; depending on primers</td>
<td>45</td>
</tr>
<tr>
<td>extension</td>
<td>1</td>
<td>72</td>
<td>30+; depending on length of product</td>
</tr>
<tr>
<td>extension</td>
<td></td>
<td>72</td>
<td>300</td>
</tr>
</tbody>
</table>

Table 3.1 Outline of a basic PCR temperature cycling protocol

After the initial denaturation cycle, 20-30 cycles of amplification are performed (shaded section) with the annealing temperature determined by the average melting temperature of the primers used.

A PCR reaction necessarily includes the following components: however, the final volume of the reaction can be scaled to complement specific experimental requirements

**Reaction components**

- 5' amplimer, 0.5 μl (20μM)
- 3' amplimer, 0.5 μl (20μM)
- dNTPs, 1.0μl (10mM)
- Magnesium chloride, 5-10μl (25mM)
- 10x reaction buffer, 5μl
- DNA polymerase, 0.5μl (5U/μl)
- template DNA, 1μl (10-50ng)
- dH2O, to 49μl

The product of the PCR is analysed for length and integrity by agarose gel electrophoresis (see section 3.6)
3.3 Detection of single-strand conformational polymorphisms in PCR products (PCR-SSCP)

PCR-SSCP is a method which is used to screen DNA sequences (usually of genomic DNA or mRNA origin) for the presence of mutations or polymorphisms. Mutations are detected as a shift in the migration pattern of the single strands of DNA PCR amplified from the DNA under analysis. This method is based upon the hypothesis that single-stranded DNA adopts a secondary structure which is determined by its primary structure (or nucleotide sequence). If even a single nucleotide in a given strand of DNA is altered then it is proposed that the secondary conformation of that DNA will be different and may be detected as a shift in electrophoretic mobility under non-denaturing conditions.

The first step in this method of analysis is PCR which is performed as in section 3.2. A PCR product of 200-250 bp achieves the most accurate results. A larger PCR product can be incubated with appropriate restriction endonuclease/s in order to generate smaller fragments for PCR-SSCP analysis.

A final PCR reaction volume of 10µl is used which requires the relative reduction of all reaction components. Either pfu (Boehringer Mannheim) or ULTMA (Perkin Elmer) DNA polymerase is utilised as these enzymes have proof-reading activity and therefore exhibit higher fidelity (i.e. incorporate fewer erroneous nucleotides) than Taq DNA polymerase. 32P-α-dCTP (Amersham) is added to the reaction in order that the product can be detected by autoradiography.

2µl of the PCR product is heat denatured (5 minutes at 95°C) in 18µl of SSCP sample buffer (Appendix I). 10-20µl of the denatured PCR product is then applied to the well of a vertical non-denaturing 10% polyacrylamide/0.5x TBE gel and is electrophoresed at 30 Watts overnight in 0.5x TBE electrophoresis buffer.

The gel may require to be run with external water-cooling or in a temperature controlled cold-room to prevent over-heating which would destroy the secondary structure of the single-stranded DNA as it migrates through the gel matrix. If the gel is run slowly at a low power setting it may be possible to electrophorese at ambient temperature. To enhance the resolution achieved by PCR-SSCP analysis a 5-10% final volume of glycerol may be added to the polyacrylamide gel mixture but this may necessitate a reduction in the electrophoresis rate or require cooling of the electrophoresis set-up. Combinations of conditions must be tested in order to optimise
this method for the specific PCR product and to enhance the possibility of detecting all of the nucleotide substitutions which may be present.

Following electrophoresis the gel is transferred to filter paper, covered with cling-film and exposed to photographic film for 4-16 hours at -70°C. On development of the film discrete bands are visualised as a record of the migration patterns of the individual single strands of the PCR DNA. If the sample PCR is electrophoresed with PCR from a known wild-type template source the presence of a single nucleotide base difference may be visible as a shift in the migration pattern as compared to wild-type. However, SSCP analysis cannot detect all possible base changes since this screening method only detects those which result in a conformational change.

3.4. TA cloning

This method allows the rapid and effective ligation of PCR-generated DNA fragments into plasmid vectors which allow the propagation of the amplified DNA in E. coli for post-PCR applications.

TA cloning is used extensively by molecular biologists. In the studies detailed in the following experimental chapters, this method was used to clone PCR products for several purposes. The products of the PCR step of PCR-SSCP analysis (unlabelled of course) were cloned to check that no mutations were missed by this screening method. In addition several PCR fragments of different origins (the mTRH-R 5' UTR and genomic DNA extracted from pituitary adenomas for example) were cloned prior to sequencing and/or subsequent subcloning into vectors with desired functions.

TA cloning utilises the fact that non-proof-reading DNA polymerases such as Taq add single deoxyadenosine triphosphate residues to the 3' end of the PCR fragment in a template-independent fashion. The construction of a linearised plasmid vector with single deoxythymosine triphosphate overhangs allows annealing of the complementary As and Ts, with the plasmid/PCR fragment junction being covalently linked by the use of a DNA ligase enzyme. The circular construct thus generated can be transformed into an appropriate strain of E. coli which permits amplification of the PCR fragment in its plasmid carrier. The PCR fragment-containing plasmid can be extracted from bacterial cell lysate by various methods. The TA vectors contain the lacZ gene which when intact enables E. coli expressing this gene to metabolise X-gal which results in a
blue coloration of arising colonies. When a PCR product is successfully inserted the reading frame of lacZ may be disrupted and therefore colonies arising from vector + insert plasmids are unable to metabolise X-gal and remain white. This is known as blue/white screening and is a useful way of identifying positive colonies.

3.5 Automated fluorescent DNA cycle sequencing

Cycle sequencing is a rapid and convenient method for performing enzymatic extension reactions for DNA sequencing. The strategy of chain termination by the incorporation of radiolabelled deoxynucleoside triphosphates (dNTPs) in the presence of unlabelled dideoxynucleoside triphosphates (ddNTPs) which was originally described by Sanger et al. (1977) has recently been combined with fluorescent technology.

The chemistries of the original radioisotopic method and the more recently developed non-radioisotopic system are identical. Each method generates a population of labelled oligonucleotides that begin from a fixed point and end randomly at a fixed residue. Every base in the DNA has an equal chance of being the variable terminus which results in a population of oligonucleotides which differ in length by one base. The population of fragments are resolved by electrophoresis through a denaturing polyacrylamide gel.

The Sanger method requires using four separate reaction tubes, each with only one of the four nucleosides in the form of a radiolabelled ddNTP. Each of these four reactions are electrophoresed in individual lanes of the resolving gel, with the sequence determined by the pattern of bands observed on an autoradiogram of the resulting gel.

However, the modern chemistry utilises the incorporation of ddATP, ddGTP, ddCTP and ddTTP conjugated to distinctive fluorophores which can all be added in a single reaction tube. During the cycle sequencing reaction, the oligonucleotide chains synthesised are terminated by a fluorescently labelled ddNTP. When electrophoresed through a denaturing polyacrylamide gel these oligonucleotides are separated by size. The automated DNA sequencing apparatus uses a laser source to excite the fluorophores as they migrate past a detection point. Computer software subsequently constructs a screen image of the gel with coloured bands representing each nucleotide
base of the DNA chain. The actual DNA sequence is then automatically computed and can be further analysed as required.

All DNA sequence analysis and manipulation was performed using the programmes Gene Jockey and most recently, Gene Jockey II (Phil Taylor, Cambridge Biosoft).

### 3.6 Horizontal agarose electrophoresis of DNA fragments

DNA may be size-separated by electrophoresis through an agarose gel matrix. The percentage of agarose added to the gel mixture determines the size separation which can be achieved and therefore the resolving power of the gel. For most general purpose electrophoresis a 1% agarose (Boehringer Mannheim) gel was made using 0.5x TBE buffer (Appendix I), as this gives high resolution of DNA fragments between 500 bp and 7 kb.

The agarose was melted in 0.5x TBE buffer, and when hand hot, 0.3μg/ml ethidium bromide was added. The gel was poured into a casting tray and allowed to solidify. A 10% volume of agarose gel loading dye (Appendix I) was added to the DNA samples (50ng to 1μg DNA) which was then diluted to the final loading volume in distilled water. Once set, the gel was inserted into its electrophoresis tank and was submerged in electrophoresis buffer (0.5x TBE). As DNA carries an overall negative charge the samples are placed at the negative terminal of the electrophoresis tank and migrate towards the positive terminal. Electrophoresis was carried out at a constant voltage of 5-10V/cm until the dye front reached the bottom of the gel. When electrophoresis was complete the gel was placed on a UV transilluminator (Vilber Lourmat) and photographed to provide a permanent record. The DNA appeared as bright, luminous bands. Size markers (Hind III/Eco R1 and pGEM, both Promega) were run alongside the sample DNA so that its size (in base pairs) could be estimated.
3.7 DNA purification

3.7.1 Plasmid recovery

The plasmid to be amplified was transformed into E. coli cells by either a heat-shock method or by electroporation as described in section 3.12.

Cells from a resulting single bacterial colony were used to inoculate 10ml of LB broth (Appendix I) containing appropriate antibiotics (Table 3.2 indicates the bacterial strains used for different vectors used throughout the experiments and indicates the selective antibiotics used in their culture). The E.Coli culture was grown overnight with shaking at 37°C and the DNA extracted using a Promega Wizard™ Mini-prep DNA purification system as follows: Bacterial cells were pelleted by centrifugation at 12000 rpm for 5 minutes, resuspended, lysed and neutralised with 200μl of resuspension, lysis and neutralisation solutions respectively. Following further centrifugation at 12000 rpm for 5 minutes, the cleared supernatant was removed and added to 0.5ml of Mini-prep DNA purification resin in a clean 2ml Eppendorf tube. The DNA/resin mixture was subsequently filtered through Miniprep columns using a vacuum manifold. Finally, the columns were washed with ethanol, dried, and the DNA eluted with 50μl of TE buffer (Appendix I) or autoclaved water by centrifugation at 12000 rpm for 1 minute. Approximately 30μg of plasmid DNA was obtained from a 10ml E.Coli bacterial culture. Larger scale plasmid DNA preparations were carried out using a Promega Wizard™ Maxi-prep kit. Using this procedure, 0.5ml of a 10ml E. Coli starter culture was used to inoculate 300ml of antibiotic-containing LB broth and the DNA extracted using a similar but scaled up version of the Mini-prep procedure described above. Approximately 500-1000μg of DNA was obtained using this method.

The extracted plasmid DNA was analysed by restriction digest and agarose gel electrophoresis to check both its integrity and its identity.

3.7.2 Extraction of high molecular weight genomic DNA

The medium from which the DNA was to be extracted was homogenised in DNA extraction buffer (150mM NaCl, 10mM Tris.HCl (pH 7.5), 10mM EDTA, 0.4% SDS). Proteinase K was added to the homogenate to a final concentration of 5μg/ml. The samples were then incubated at 55°C overnight. DNA was isolated by phenol:chloroform extraction followed by ethanol precipitation and pelleting by
centrifugation at 10000 rpm for 10 minutes. The air-dried pellet was resuspended in autoclaved, distilled water or TE buffer and quantified by UV spectrophotometry at 260nm. To confirm that DNA had not degraded during tissue storage and extraction procedures, aliquots of the DNA were electrophoresed in 0.7% agarose gels containing 0.5x TBE as described above.

### 3.7.3 Recovery of DNA from an agarose gel

It is often necessary to extract DNA of a certain size from a heterogeneous mixture. One method of achieving this is to electrophorese the mixture of DNA fragments through a low melting point agarose (Sea Plaque agarose, Flowgen) gel. This method is similar to the protocol described above except that the 1% low melting point agarose gels were set and run at 4°C. In addition, to prevent any damage to the DNA, ethidium bromide staining was performed after completion of electrophoresis. The gel was then viewed under UV light, the position of the required fragment(s) ascertained and the correct DNA bands excised with a scalpel blade for further purification. DNA recovery was achieved using a Qiagen gel purification kit. In essence this procedure involves melting the DNA-containing gel slice in extraction buffer and then binding the DNA to a matrix in a dedicated centrifuge spin column. The liquid portion of the mixture is spun out of the column whilst the DNA is retained, bound to the matrix. The DNA is then eluted by adding Tris-Cl buffer (10mM, pH 8.0).

### 3.7.4 Phenol:chloroform DNA extraction

Phenol:chloroform extraction separates nucleic acids from proteins, and is a widely used technique for the removal/inactivation of enzymes or for the purification of DNA from agarose gels. An equal volume of TE-saturated phenol was added to the DNA containing sample and the sample vortexed for 1 minute followed by centrifugation at 10000 rpm for 1 minute. The upper aqueous phase was removed into a clean 1.5ml Eppendorf tube and the vortexing and centrifugation procedure repeated, first with an equal volume of phenol:chloroform and finally with an equal volume of chloroform. The DNA was extracted from the chloroform phase using a salt/ethanol precipitation method (see section 3.8 below).
3.8 Salt and ethanol precipitation of DNA

Nucleic acids may be concentrated by precipitation with ethanol, in the presence of monovalent cations. Ammonium acetate (10M, pH 5.0) or sodium acetate (3M, pH 5.0) was added to a final concentration of 2.5M or 0.3M respectively, together with 2 volumes of 100% ethanol. The samples were precipitated at 4°C/on ice for 30 minutes and centrifuged at 12000 rpm for 30 minutes at 4°C. The supernatant was then removed, the DNA pellet washed in 70% ethanol and resuspended in TE buffer/water.

3.9 Spectrophotometric DNA quantitation

The concentration of a 1:100 dilution of a DNA sample was determined by measuring the optical density (OD) at 260nm and 280nm in a Kontron Analytical spectrophotometer. An OD$_{260}$ reading equivalent of one corresponds to a solution containing 50μg/ml of double stranded DNA, 40μg/ml of either single stranded DNA or RNA or 20μg/ml of single stranded oligonucleotides. Depending on the relative base composition of the DNA an OD 260:280 ratio of between 1.5 and 2.3 indicates good DNA sample purity.

3.10 Restriction endonuclease digestion of DNA

Restriction endonucleases are enzymes found naturally in bacterial cells. These enzymes are highly specific in their activity, recognising short sequences (generally palindromic in nature) in double-stranded DNA. On binding to their individual recognition site the endonucleases cleave the DNA. Restriction endonucleases are used extensively in molecular biology and are useful for several protocols. If the sequence of a length of DNA is known, the restriction sites it contains can be identified. On incubation with a given restriction endonuclease, fragments of expected lengths are generated confirming the identity of the DNA. This is especially useful in confirmation of the identity of plasmid DNA extracted from *E. coli* host strains. Restriction endonucleases are also used in the transfer of stretches of DNA between plasmid vectors, a method known as subcloning.

1-5μg of purified DNA was digested with 10 units of restriction enzyme (sourced from several suppliers), in 1x enzyme buffer (supplied with the enzyme in 10x
solution) at the optimum digestion temperature (generally 37°C) for 1 hour, and the digestion products subsequently analysed by agarose gel electrophoresis.

3.11 DNA ligation

The cut ends of restriction endonuclease-treated DNA fragments can be rejoined with the use of a DNA ligase enzyme. Bacteriophage T4 DNA ligase is most commonly used as it gives efficient ligation of both blunt and sticky ended DNA. The ligase enzyme regenerates the phosphodiester bond between the 3' terminal phosphate group on one strand of the DNA fragments and the 5' hydroxyl group of the other DNA fragment by transferring the phosphate group from ATP present in the ligation reaction buffer.

It is often advantageous to dephosphorylate a linearised (cut with a restriction endonuclease so that it is no longer circular) plasmid vector. If the plasmid into which another stretch of DNA is to be ligated is dephosphorylated, the ligase only forms a bond in one strand of the DNA at each end of the ligation. This results in unligated strands containing "nicks". This enhances transformation as nicked DNA is more effectively taken up and replicated by E. coli than is fully ligated DNA. Dephosphorylation also inhibits recircularisation of the plasmid DNA during ligation so more of the desired product is formed.

Ligation may be enhanced by the addition of condensing agents such as polyethylene glycol or hexaminecobalt chloride. These agents condense the DNA into aggregates which increases molecular proximity and subsequently increases the rate of the ligation reaction.

The molar ratio of vector to insert DNA is of great importance for efficient ligation. The insert DNA must be at least equimolar to the vector, but should preferentially be in a 3 to 5-fold molar excess, particularly if a less efficient blunt end ligation is being performed.

As with restriction endonuclease digestion of DNA, ligation requires the incubation of the cut DNA fragments with the ligase enzyme of choice in a buffer which is usually supplied with the enzyme. Optimal reaction conditions are dictated by the enzyme being used.
3.12 Transformation of plasmid DNA into *E. coli*

Plasmid vectors are circular stretches of DNA which carry various pieces of genetic information; antibiotic resistance, mating factors, replication origins, promoter sequences and enzymatic capability for example. It is possible to ligate a chosen length of DNA, a GPCR cDNA for example, into a plasmid vector for further analysis. The plasmid vector construct must be transformed into appropriate *E. coli* strains so that large quantities of the desired DNA can be extracted from the resulting bacterial cultures.

3.12.1 Chemically competent *E. coli*

*E. coli* cells are grown in 300ml of the appropriate medium until the cells reach mid log phase (at an optical density of 0.5-0.6 at 600nm). Making sure that all solutions are ice cold and that all processes are performed at 4°C, the cells are pelleted at 4000rpm for 10 min and are then resuspended in 10ml of 0.1M CaCl₂. This cell suspension is centrifuged as described previously, resuspended in 10ml of the 0.1M CaCl₂ and dispensed into 200μl aliquots which can be stored at -70°C until use.

To introduce exogenous plasmid DNA into chemically competent cells the following protocol is followed. The cells are incubated with the DNA to be transformed on ice for 30 minutes. The *E. coli* are then shocked at 42°C for 30-45 seconds which provokes a transient permeability in the cell wall of the bacteria allowing the DNA to pass into the cells. The cells are then placed on ice for 2 minutes during which time the cell wall reintegrates. Finally the *E. coli* are incubated at 37°C for 60 minutes in SOC medium (Appendix 1). This allows the selective antibiotic resistance carried in the sequence of the plasmid vector to be expressed prior to the cells being plated onto LB agar (Appendix 1) containing selective antibiotic/s. The LB agar plates are incubated in an inverted position at 37°C overnight. Post-transformation, isolated single colonies are selected and grown in liquid culture for the extraction of plasmid DNA as previously described.

3.12.2 Electrocompetent *E. coli*

*E. coli* cells are prepared from a liquid culture such that they are suspended in a medium with very low electrical conductance properties. This involves washing the cells pelleted from a 300ml log phase bacterial culture twice with 20ml of ice cold sterile water, and then once with 2ml of ice cold sterile 10% glycerol solution. The
resulting cell pellet is resuspended in a small volume (approximately 800μl) of the 10% glycerol and 50μl aliquots stored at -70°C for up to six months.

The DNA to be transformed is mixed with the bacterial cells which are placed between the metal contacts of a 2mm electroporation cuvette (Flowgen). A high voltage pulse (2.5kV) is then discharged through the E. coli/DNA mixture using a Gene Pulser II apparatus (Bio-Rad). The electric shock permeabilises the bacterial cell wall allowing DNA to diffuse into the cell interior where it is replicated alongside the bacterial chromosomal DNA (episomal replication). The cells are incubated for 1 hour in SOC buffer to allow antibiotic resistance to develop and then the bacteria are spread onto LB agar + antibiotics plates. Following overnight incubation, isolated colonies are selected for liquid culture and DNA isolation.

The amount of DNA isolated by both of the above methods depends on the inherent copy number of the plasmid vector and the volume of bacterial culture extracted.

<table>
<thead>
<tr>
<th>Plasmid vector</th>
<th>E. coli strain</th>
<th>Selective antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcR2.1 (TA vector, Invitrogen)</td>
<td>INVα F'</td>
<td>50μg/ml Amp or Kan</td>
</tr>
<tr>
<td>pT7-blue (TA vector, R&amp;D)</td>
<td>NovaBlue</td>
<td>50μg/ml Amp or Kan</td>
</tr>
<tr>
<td>pcDNA-3</td>
<td>TOP10 F'</td>
<td>50μg/ml Amp</td>
</tr>
<tr>
<td>pGL3-basic</td>
<td>JM109</td>
<td>50μg/ml Amp</td>
</tr>
<tr>
<td>pcDNA-3</td>
<td>CJ236 (dut/ung mutant)</td>
<td>50μg/ml Amp</td>
</tr>
</tbody>
</table>

Table 3.2 Some of the plasmid vectors and their E. coli host strains used
The TA vectors can be selected on either Amp or Kan which allows for the destruction of any plasmid template which may be carried over from the PCR reaction. For example, if the PCR template is in an Amp resistant plasmid, the TA clones should be selected on Kan, and vice versa. CJ236 is the dut/ung mutant strain which produces DNA containing uracils which is required for the site-directed mutagenesis method described in Section 3.13 below.
3.13 Site-directed mutagenesis

3.13.1 General methodology

DNA mutations were introduced into the TRH-R using a method adapted from Kunkel (1985) and performed using a Muta-GeneTM phagemid in vitro mutagenesis kit (Bio-Rad). The coding DNA of interest was cloned into an eukaryotic expression vector and the DNA replicated within a dut/ung double mutant E. Coli bacterium. The dut mutation inactivates dUTPase whereas the ung mutation inactivates the enzyme uracil-N-glycosylase. Together these mutations result in the incorporation of uracils into the DNA. This 'parental' DNA single-strand was then used as a template to anneal an oligonucleotide containing the desired mutation. A complementary, non-uracil containing DNA strand, identical except that it carried the mutation, was subsequently synthesised. The double stranded DNA construct was transformed into cells containing an efficient uracil-N-glycosylase enzyme system which resulted in the inactivation of the parental DNA strand, thus permitting only the DNA strand carrying the mutation to replicate.

3.13.2 Eukaryotic expression vectors

The plasmid pcDNA-3 (Invitrogen) is a 5.4 kb vector derived from pRc/CMV (Figure 3.1a). The vector contains a CMV promoter and enhancer, SV40 origin for transient episomal replication of cells expressing the SV40 large T antigen, and a bovine growth hormone polyadenylation signal. It also contains an f1 origin for the rescue of single stranded DNA, T7 and SP6 promoters for the production of sense/antisense RNA transcripts, and a ColE1 (from pUC 19) high copy number origin. The β-lactamase gene confers Amp resistance in the E.Coli strain TOP 10F'. In addition, this vector has the advantage of a neomycin resistance marker, expressed from the SV40 early promoter, for the selection of stable transformants in the presence of G418 (a neomycin analogue). A recombinant eukaryotic expression vector for the human TRH-R was constructed by subcloning the hTRH-R cDNA into the Eco RI restriction site of pcDNA-3 (Invitrogen, Figure 3.1b). The receptor cDNA was inserted downstream of the sequence of the human CMV promoter which permits the membrane expression of the receptor protein in the chosen mammalian host cells.
Figure 3.1 The hTRH-R/pcDNA-3 construct and pcDNA-3
Schematic representation of pcDNA-3 highlighting some of the control elements of this vector (a) and the hTRH-R/pcDNA-3 expression construct (b) assembled by ligating the receptor cDNA into the Eco RI site of the pcDNA-3 polylinker (or MCS).
Single strand DNA was produced from wild-type hTRH-R/pcDNA-3 transformed into CJ236 E. coli. This uracil-containing single strand DNA was subsequently used to generate mutant receptor cDNA sequences as described in the method below. Mutant hTRH-Rs were transfected into mammalian cells for expression and subsequent functional studies.

3.13.3 Helper phage

M13KO7 (Invitrogen) is a single-stranded interference resistance helper phage carrying the mutant gene II. Phage infection of plasmids containing either an f1 or M13 origin (for the rescue of single stranded DNA) results in the preferential replication, packaging and extrusion of single strand plasmid DNA. M13KO7 had a titre of 2.6x10^10 plaque forming units (pfu)/ml and was added at a multiplicity of infection of 20:1 (phage:cells) to a cell culture containing 1x10^8 colony forming units (cfu)/ml. Kan was used to select for cells infected with helper phage.

3.13.4 Preparation of uracil-containing template

CJ236 cells were streaked onto LB agar plates containing 30μg/ml chloramphenicol (Chl) and single colonies selected for further use. Individual colonies were subsequently grown in an appropriate antibiotic environment and electrocompetent cells prepared. Electrocompetent CJ236 cells (50μl) were electroporated (Section 3.12.2). The transformed cells were plated onto LB agar plates, containing Chl and Amp, incubated overnight at 37°C and single colonies picked. The DNA plasmids were then extracted and the presence of inserts checked by Eco R1 restriction digests.

3.13.5 Single-strand DNA preparation

Selected CJ236/pcDNA-3 colonies were grown in 2xYT broth, containing appropriate concentrations of Chl, Amp until an OD reading (at 600 nm) of 0.3 was reached. M13KO7 helper phage was added, the incubation continued for 1 hour prior to the addition of Kan (70μg/ml) and then continued overnight. The E. Coli cells were pelleted by centrifugation and the supernatant treated with 10μg/ml RNase and 10μg/ml DNase at 37°C for 10 minutes. One quarter of the total volume of 3.5M ammonium acetate/20% polyethylene glycol was added, the solution precipitated on ice for 10 minutes and the DNA pelleted by centrifugation at 12000 rpm for 20 minutes. The DNA was resuspended in TE buffer and purified using a phenol/chloroform...
extraction method. The single-strand uracil-containing DNA was then used as a template to anneal mutagenic oligonucleotides.

3.13.6 Oligonucleotide design

Mutagenic oligonucleotides, complementary to the receptor DNA except containing internal base mismatches, were designed to the targeted area of the receptor and synthesised on an Applied Biosystems PCR Mate synthesiser. The oligonucleotides were deprotected in 1ml of ammonia solution at 55°C overnight, purified by salt/ethanol precipitation, and subsequently phosphorylated using T4 polynucleotide kinase. These phosphorylated oligonucleotides were then annealed to a uracil containing template in a 25:1 primer to template ratio. A complementary strand of DNA was synthesised using T7 DNA polymerase and the ends of this de novo DNA strand ligated together with T4 DNA ligase generating covalently closed circular DNA (cccDNA) which should carry the desired mutation(s).

3.13.7 Transformation of the cccDNA for plasmid recovery

1 or 2 µl of the cccDNA was electroporated into TOP10F E. coli (Section 3.12.2) and resulting colonies grown in liquid culture for plasmid purification (Section 3.7.1). The plasmid DNA was sequenced to confirm the presence of the desired mutation prior to transfection experiments. In general, 40-70% of colonies selected would contain the expected mutation.

3.14 Tissue culture

Tissue culture reagents were purchased from Gibco-BRL. COS-1 cells (Monkey kidney, SV40 transformed fibroblasts), HEK-293 cells (human embryonic kidney epithelial cells), GH3 and GH4C1 cells (mouse GH/PRL secreting cells of lactotroph origin) and AtT20 cells (mouse corticotroph tumour cell line) were obtained from the American Type Culture Collection. Alpha-T3-1 cells (immortalised anterior pituitary cells of gonadotroph lineage) were a gift from Dr. Pamela Mellon (The Salk Institute, La Jolla, CA, USA). Cells (except GH4C1 cells) were routinely maintained and passaged in complete Dulbecco's modified Eagle's medium (complete DMEM, see Appendix I) and incubated at 37°C in a humidified atmosphere of 5% (v/v) CO2 in air. GH4C1 cells were maintained in Ham's F10 medium supplemented with 10% horse
serum and antibiotics/glutamine as for complete DMEM. Incubation of GH4C1 cells was identical to that of the other cell lines used.

3.15 Transfection of eukaryotic cells

All transfections whether transient or stable were performed using a liposome-mediated transfection protocol (Transfectam™, Promega). The lipid molecules in the transfection reagent form liposome structures or micelles which carry a positive charge. This positive charge interacts with the overall negative charge of the DNA resulting in the lipid coating of the DNA which can then be intercalated into the lipid bilayer of the cell membrane allowing the DNA to enter the cell. On reaching the nucleus the plasmid DNA is episomally transcribed into RNA and then translated into the protein of interest.

The specific cell lines transfected, the density at which they were plated out, the amount of DNA transfected, and the culture medium used, are all detailed in the relevant chapters.

3.15.1 Transient transfection

Transient transfections were performed according to manufacturers instructions.

Briefly, on the afternoon prior to transfection the cells to be treated were trypsinsised, harvested, counted and plated out at the desired density in 60mm tissue culture grade petri dishes in complete growth medium. On the morning of transfection the cells were washed twice with serum-free medium and 0.5ml replaced into the dishes whilst the DNA and Transfectam™ were mixed. The appropriate amount of DNA (usually 5μg/60mm dish) was mixed with 0.75ml serum-free medium. In a separate tube 10μl Transfectam™/60mm dish and 0.75ml of serum-free medium were mixed together by vortexing. 0.75ml aliquots of diluted DNA and diluted Transfectam™ were mixed together and the resulting 1.5ml of DNA/liposome were added dropwise to the cells in the petri dishes. Cells were incubated at 37°C in 5% CO₂ in a humidified incubator for 6 hours after which time the DNA/liposome-containing medium was removed and replaced with complete culture medium.

Using this method, the DNA is not incorporated into the genome of the host cells and therefore daughter cells generated by cellular division do not contain the receptor DNA
or express the receptor protein. Functional assays were performed 48-72 hours post-transfection.

3.15.2 Stable transfections

HEK-293 cells were generally used for stable transfections. To generate a clonally derived stably expressing cell line, the protocol as described above was followed except that the transfected DNA was restriction digested (linearised) at a site in the multiple cloning site of the vector downstream of the receptor sequence. 48 hours post-transfection the cells were trypsinised from the 60mm petri dish and seeded into a 125cm² tissue culture flask. On reaching approximately 50% confluency the cells were seeded into a 175cm² tissue culture flask and allowed to grow to 60% confluency. At this point the Geneticin™ analogue, G418 (Sigma), was added to the culture medium at a final concentration of 800μg/ml. Over the next 10-14 days those cells not expressing the resistance gene died, leaving isolated colonies which had expanded from a single G418-resistant progenitor cell. The culture flask was cut open and the colonies were trypsinised in cloning rings and removed to the chambers of a 24 well tissue culture plate. The cells were maintained in G418-containing complete culture medium and were seeded into larger volume vessels as required, until the cell lines had been expanded to provide adequate numbers of cells for receptor function assays.

3.16 Total inositol phosphate (IP) production assay

3.16.1 Methodology

24 hours post transient transfection cells were trypsinised and transferred to 24-well tissue culture plates (2x 60mm plates per 24 wells). The cells were labelled to isotopic equilibrium with 1 μCi/ml myo-³[H]-inositol (Amersham) in inositol-free DMEM (GIBCO-BRL).

48 hours later this medium was removed and the cells washed once with 250μl of Buffer A (Appendix I). A further 500μl Buffer A containing LiCl (10mM) was replaced into the wells and the plates incubated at 37°C for 30 minutes. 55μl of ligand agonist of increasing concentrations (0-10⁻⁵M) was added into the wells in triplicate and the plates returned to the incubator for a further 45 minutes.
The drug-containing medium was aspirated and the reaction stopped by adding 500µl of PCA stop solution (0.5M PCA/5mM EDTA), followed by the addition of 50µl phytic acid (1.8mg/ml) and incubation at 4°C for 10 minutes. The total reaction mixture was removed to numbered test tubes and neutralised with 0.5mM potassium hydroxide/60mM HEPES containing Universal pH indicator, producing a 'mid-green' colour.

The neutralised solutions were transferred to a second set of numbered test tubes containing 500µl of Dowex anion exchange resin (Analytical grade anion AG® 1-X8 resin, 100-200 mesh, Bio-Rad), vortexted briefly, allowed to settle and the supernatant aspirated. Inositol, glyceroinositol phosphates and total inositol phosphates were eluted from the resin by washing sequentially with 1ml dH2O, 1ml 60mM ammonium formate/5mM sodium tetraborate and 1ml 1M ammonium formate/0.1M formic acid respectively. 800µl aliquots of the total IP-containing fractions were individually transferred to scintillation vials and 4ml Optiphase HP (Pharmacia) liquid scintillant was added. Radioactivity was measured in an LKB Rackbeta liquid scintillation counter.

Total cell radioactivity was measured to compensate for interwell variations in cell numbers. Wells were washed twice with 250µl PCA/EDTA stop solution and the cells solubilised with 500µl 0.1M NaOH solution. Each well was neutralised with the required volume of 0.66% acetic acid. The resultant solution was transferred to individual scintillation vials and radioactivity measured as above.

3.16.2 Expression of results

Results are expressed as dpm/10^5 Total Counts (Tc) with assay points representing the mean of triplicate samples. Assays were carried out on at least three independent occasions unless otherwise stated. The ED50 was estimated by fitting a sigmoid curve of the dose response data using the program SigmaPlot on Macintosh computers.
3.17 Calcium imaging

3.17.1 Intracellular calcium measurements

Before intracellular calcium ([Ca²⁺]ᵢ) measurements could be determined, cells first had to be loaded with the fluorescent calcium dye fura-2 AM. Trypsinised and dispersed cells were plated on to sterile glass coverslips. After 2 days attached cells were washed (X2) with Buffer A (Appendix I). Cells were loaded with fura-2 AM ester (4μM final concentration in Buffer A) for 30min at 37°C in a 5% CO₂ humidified incubator. Unincorporated dye was removed by washing (X3) with Buffer A. Coverslips were then transferred to the heated stage (37°C) of an inverted epifluorescence microscope (see below).

3.17.2 Stimulation of cells with TRH and [Ca²⁺]ᵢ imaging

Dynamic video imaging was carried out using the MagiCal hardware and Tardis software provided by Joyce Loebl Ltd. A Nikon Diaphot microscope with a X40 quartz oil immersion objective, operated in epifluorescence mode, was used to image the cells.

Fluorescent images were obtained by exposing cells to 340nm and 380nm light, alternated under computer control, at a speed of approximately 0.6Hz. The images, viewed at wavelengths of 510nm (with a 40nm wide band filter), were focused on to the face of an intensified charge-coupled device camera (Photonic Sciences, UK) interfaced in turn, to an analogue hardware averager and 8-bit analogue-to-digital converter, both operating at video frame rate. Typically, eight images were averaged at each wavelength. A similar number were collected for background images which were subsequently subtracted on a pixel-by-pixel basis from the image samples. These images were held in dynamic random access memory for subsequent processing and analysis.

Fluorescence excitation shifts occur when fura-2 binds calcium, i.e. the excitation efficiency increases at 340nm and 380nm. Ratios of values obtained at 340/380nm represent changes in [Ca²⁺]ᵢ. The 340/380nm ratio was calculated from averaged video frames on a pixel-by-pixel basis, and was proportional to the [Ca²⁺]ᵢ. Calibration was performed on cells loaded with dye which had been made permeable
to extracellular Ca\textsuperscript{2+} using 1μM ionomycin. Exposure of cells to solutions containing either 10mM Ca\textsuperscript{2+} or 10mM EGTA provided an estimate of the minimal and maximal fluorescence at 340nm and 380nm. A dissociation constant of 225nM for fura-2 and Ca\textsuperscript{2+} at 37°C was used. Possible errors in [Ca\textsuperscript{2+}]\textsubscript{i} determination were estimated to be 5% from frame to frame, and were measured by collecting data from known standards of Ca\textsuperscript{2+}/EGTA buffers, containing 50-100μM fura-2 free acid. Within a single frame, the maximal pixel-to-pixel standard error (spatial variation) was less than 3% in concentration for an average of 4 frames, and this fell to under 2% for an average of 8 frames.

### 3.17.3 Data analysis and presentation

Software-based image analysis using MagiCal allowed quantitation of ionised Ca\textsuperscript{2+} in whole cells versus time. This was accomplished by the use of a light pen accessed into dynamic memory to define a pixel data-set on a given image frame and the software then automatically constructed a graphical presentation of Ca\textsuperscript{2+} concentration with time. ASCI files of quantitative data could also be derived, and these were used to derive plots of [Ca\textsuperscript{2+}]\textsubscript{i} versus time for either single cells, or the averages of several cells.

### 3.18 Radioligand receptor binding assay

#### 3.18.1 Cell membrane preparation

48-72 hours post transient transfection the 60mm dishes of monolayer cells were washed twice with phosphate buffered saline (PBS, Sigma) and the cells harvested by scraping in a further 2ml of PBS. Whole cells were pelleted by centrifuging at 2500 rpm for 5 minutes at 4°C. The cell pellets were resuspended and lysed in 4ml of hypotonic ice-cold 1x radioligand binding assay (RBA) buffer (Appendix I) for 10 minutes. The disrupted cells were homogenised with coarse and then fine pestles in a glass homogeniser. Homogenates were transferred to 2ml Eppendorf tubes and centrifuged at 11000rpm for 30 minutes at 49°C. The supernatant was aspirated and the membrane preparations stored at -70°C until use.
3.18.2 Radioligand receptor binding assay (dose displacement)

Freshly made or stored membrane pellets were kept on ice and diluted in the required volume of 1x RBA buffer. An even suspension was obtained by aspirating the membranes through a 19 gauge needle. TRH binding assays were performed using the tritiated TRH agonist [³H](3-Me-His2)TRH (NEN-Du Pont). The radiolabel was diluted to 2000 counts/µl in 1x RBA buffer and 50µl added to each one of a set of glass tubes (approximately 10⁵ counts/tube). Unlabelled peptide (3-Me-His2)TRH (50µl, Peninsula) was added at varying concentrations (0-10⁻¹⁰M) in triplicate. Finally, 400µl of the diluted membrane preparation was added to each tube and the reaction allowed to reach equilibrium over 2 hours at 4°C. Maximal binding (B₀) was measured when no cold peptide was added, non-specific binding (NSB) measured by adding a saturating dose (10⁻⁶M) of [3-Me-His2]TRH and blank values from tubes with no membranes.

Membranes were filtered through GF/B filter papers (Whatman) soaked in 1x RBA buffer containing 0.5% polyethylenimine (Sigma) using a Brandel cell harvester (SEMAT). The filters were washed quickly 3 times with 1x RBA buffer to remove free ligand, leaving receptor-ligand complexes bound to the filter paper discs. Filter paper discs were transferred to individual scintillation vials to which 4ml of Ecoscint (National Diagnostics) liquid scintillation fluid was added. Radioactivity was measured as previously described.

3.18.3 Determination of protein concentration of membrane preparations

The protein concentration of the membrane preparations was determined colorimetrically using Bio-Rad assay reagent in a 96 well microtitre plate format. 50µl protein standard (bovine serum albumin, BSA) or test sample, 100µl 0.2M NaOH, 150µl dH₂O and 50µl assay reagent were added to each well and the results measured using a Labsystems plate reader set at an absorbance wavelength of 620nm. Results were analysed using the computer programme Assay Zap (Phil Taylor, Cambridge Biosoft) run on Apple Macintosh computers. Protein concentration was corrected for intersample variations allowing the estimation of the concentration of receptors/µg of total protein (Bₘₐₓ).
3.18.4 Data analysis

Receptor dissociation constant (Kd) and receptor number (Bmax) were calculated from the results arising from Scatchard analysis or Cheng and Prusoff analysis (Cheng and Prusoff, 1973) of assay data using SigmaPlot. When the same ligand is used as the hot and cold competitors in a displacement experiment an adaptation of the Cheng and Prusoff equation (DeBlasi et al, 1989) can be used to calculate receptor number. Cheng and Prusoff analysis involves the calculation of Kd from measurements of the concentration of unlabelled ligand required to displace half the specific binding (ED50 values). This analysis is only appropriate when the same compound is used as both the radioligand and competitor. The proof of this equation is shown below:

If \( IC_{50} = K_c \left(1 + \frac{L}{K_h}\right) \) where \( K_c = K_d \) of cold ligand
then \( IC_{50} = K_d \left(1+\frac{L}{K_h}\right) \)
\( = K_d + \frac{(L.K_d)}{K_d} \)
\( = K_d + L \)

and hence, \( K_d = IC_{50} - L \)

If \( B_{\text{max}} = B_0 \left(K_d + L\right)/L \), substituting \( K_d = IC_{50} - L \)
then \( B_{\text{max}} = B_0 \left(IC_{50} - L + L\right)/L \)
and \( B_{\text{max}} = \frac{B_0 \cdot IC_{50}}{L} \)

Alternatively Scatchard analysis may be used which is a method based on the calculation of the amount of radioactivity bound (B) to cell membranes versus the amount of free radioactivity (F). A Scatchard plot involves the plotting of B/F versus B. A linear relationship indicates a single affinity binding site with a Kd value calculated from the slope of this line and a Bmax value from the point at which the line intercepts the x-axis.

Assays were carried out on a minimum of three independent occasions, unless otherwise specified, with individual assay points representing the mean of triplicate samples.
3.19 **In-Vitro translation**

*In-vitro* translation is a method whereby the polypeptide encoded by any stretch of cDNA can be produced in a cell-free system. Using the Coupled Rabbit Reticulocyte System (Promega) both the transcription and translation reactions can be performed in a single reaction tube. The only requirements with regards to the DNA sequence are 1) that it is cloned downstream of an RNA polymerase promoter sequence (T7, SP6 or T3 for example), 2) that the DNA is linearised downstream of the sequence of interest and 3) that the sequence contains an ATG translation initiation signal. The reaction is set up to include the RNA polymerase of choice, purified rabbit reticulocyte preparation, reaction buffer, amino acids (minus methionine) and radio-labelled methionine ($^{35}$S-Met, Amersham). Reactions are incubated at $30^\circ$C for 90 minutes and then an aliquot is loaded onto a SDS-PAGE gel and analysed to ascertain the relative molecular weight of the protein product. The SDS-PAGE gel is dried and exposed to photographic film (at $-70^\circ$C) with dark bands indicating the position of $^{35}$S-Met-labelled reaction products. Figure 3.2 shows a schematic representation of the *in-vitro* translation method.
Plasmid vector containing the DNA sequence encoding a protein of interest is first linearised by restriction digest and is then included in the reaction mix which transcribes RNA initiated from the RNA polymerase promoter and subsequently translates this RNA into protein. The inclusion of radio-labelled methionine in the amino acid (αα) mixture allows autoradiographic detection of the protein products following SDS-PAGE analysis.
3.20 Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE)

This method allows the estimation of the molecular weight of proteins as they are electrophoresed in a denatured state in which structure and native charge bear no influence. The proteins are denatured using a reducing agent such as β-mercaptoethanol, and the chaotropic detergent SDS which essentially forms a negatively charged 'cage' around the protein. This results in an electrophoretic migration pattern which is influenced only by the molecular weight of the protein. Therefore, the size of proteins can be estimated by co-electrophoresis with proteins of pre-determined sizes. SDS-PAGE can be used in the first stage of Western blot analysis or simply as a method of analysis of protein products. The PAGE gel can be fixed and stained by several different methods, or radio-labelled proteins can be used to produce an autoradiographic record of migration patterns.

3.21 Rapid amplification of 5' cDNA ends (5' RACE)

Obtaining a full-length cDNA is of critical importance for structural and expression studies. For analysis of promoter regions of genes it is essential to ascertain the exact site of transcriptional initiation. This requires detecting the 5' (or upstream) sequence of RNA which is required for efficient transcription. 5' RACE allows the amplification of unknown sequences at the 5' end of the messenger RNA (mRNA). The methodology of 5' RACE is outlined in Figure 3.3, and is based on the protocol of the Boehringer Mannheim 5'3' RACE Kit.
Figure 3.3 Overview of 5’ RACE

First strand cDNA is synthesised from total or poly(A)+ RNA using a sequence specific primer, SP 1, using AMV reverse transcriptase and dNTP mix. The first strand cDNA is purified from unincorporated nucleotides and primers, and a homopolymeric-adenosine tail is added to its 3' end using the terminal transferase enzyme. A-tailed cDNA is then PCR amplified using a second sequence specific primer, SP 2, and the oligo dT-anchor primer. The use of a second primer increases the specificity of the reaction products, eliminating amplification of products arising from mispriming. The obtained DNA is further amplified by a second PCR using a nested specific primer, SP 3 and the PCR anchor primer. The 5’ RACE products can be TA cloned for subsequent sequencing and characterisation.

The size of the PCR band produced by 5’ RACE should correspond to the length of the sequence which exists in the mature RNA transcript, giving the exact position of the transcriptional start site.
3.22 Quantitation of promoter activity

In order to ascertain the regions of a promoter which have intrinsic stimulatory/inhibitory affects on the levels of transcription within cells, methods using reporter genes have been developed. The principle underlying all of these methods is the same. Essentially, the sequence of interest is cloned in front of a reporter gene within a plasmid vector which, unlike other mammalian expression vectors, has no intrinsic promoter activity. The resulting plasmid construct is transfected and expressed in host cells. The cells will only transcribe RNA from the reporter gene if the cloned sequence contains transcription factor binding sites. The level of transcription initiated by the experimental sequence is extrapolated from the level of reporter activity by assay of reporter gene products.

One of the most sensitive and simple methods utilises the gene for luciferase as the reporter. Promoter activity is measured as a function of the light emitted from transfected cell extracts on addition of luciferin, the enzyme substrate.

3.22.1 Luciferase assay

The DNA sequences to be tested were cloned into the pGL3-Basic vector (Promega) upstream of the luciferase gene. Cells were transiently transfected as previously described, using 3μg of DNA/60mm dish. 48 hours post-transfection cells were lysed for 15 minutes in 400μl of 1x Reporter Lysis Buffer (Promega) and scraped from the plates. The cell debris was pelleted by centrifugation in 1.5ml Eppendorf tubes. 20μl of the supernatant was added to 100μl of luciferase assay reagent (Promega) and the light emission measured in a luminometer with an integration time of 60 seconds.

3.22.2 β-galactosidase assay

β-gal was used to correct for transfection efficiency when co-transfected with luciferase vector constructs. Transfection with pSVβ-gal, a vector which contains the β-gal gene under the control of the SV40 promoter, allows for correction of inter-plate variations in cell numbers. 3μg of pSVβ-gal was co-transfected with the pGL3 constructs in the promoter experiments. The luciferase cell lysis samples (50μl) were used to assess β-gal activity which is measured by adding 50μl of β-gal assay reagent in a 96 well plate format (Promega) and measuring absorption at 540nm (OD540). Luciferase activity was subsequently expressed as a function of β-galactosidase levels.
3.23 Statistical analysis of experimental results

Where necessary, data was analysed for statistical significance using Student’s t test in the statistics programme Statview on an Apple Macintosh computer. Results were deemed significant when \( p < 0.05 \).

The following chapters describe and discuss the results obtained using the methodologies which have been described in this chapter. These techniques were applied to the study of the hTRH-R in endocrine disease and in site-directed mutagenesis experiments, and to the characterisation of the 5'-flanking and untranslated region (UTR) of the mTRH-R gene.
Chapter 4

Structure of the TRH-R in human pituitary adenomas

4.1 Introduction

Under physiological conditions hypothalamic thyrotrophin-releasing hormone (TRH) stimulates the release of thyrotrophin (TSH) and prolactin (PRL) from pituitary thyrotrophs and lactotrophs respectively (Kaplan et al, 1972). TRH exerts its effects by binding to specific high-affinity receptors located on the surface membrane of target cells. After the initial isolation of the murine cDNA for the TRH-R (Straub et al, 1990), the human TRH-R has recently been cloned (Duthie et al, 1993a; Hinuma et al, 1994; Yamada et al, 1993), confirming it to be a G-protein coupled receptor (GPCR), and its gene mapped to chromosome 8q23 of the human genome (Morrison et al, 1994). The human TRH-R protein is encoded by a gene which has two exons, separated by a single intron longer than 1kb, which is found within the region of the DNA sequence encoding the third cytoplasmic loop (Hinuma et al, 1994). The TRH-R couples principally to the inositol phosphate second messenger pathway, and the regulatory G-proteins associated with TRH-R signal transduction have been identified as Gq and G11 (Aragay et al, 1992; Hsieh and Martin, 1992)

TRH binding sites have previously been detected by classical radioligand-binding techniques on PRL- and GH-secreting adenomas (Le Dafniet et al, 1985a; Le Dafniet et al, 1985b) and non-functioning tumours (Le Dafniet et al, 1987). In approximately half of patients with acromegaly TRH induces the release of GH (Irie and Tsutsima, 1972). This discordant GH response to TRH does not occur in healthy normal individuals. Over 50% of patients with an apparently non-functional tumour secrete LHβ subunit in response to TRH (Daneshdoost et al, 1990). PRL responses to TRH administration in patients with prolactinomas can vary (Snyder et al, 1974; Yovos et al, 1981). Administration of TRH to patients with TSH-secreting adenomas causes brisk TSH release in about one third of patients, the remainder are unresponsive (Bevan et al, 1989). Therefore, a variety of abnormal hormone responses to TRH have been described in patients with pituitary adenomas. However, controversy
surrounds the existence of TRH binding sites in TSH-secreting adenoma cells. Radioligand-binding techniques could not detect TRH-Rs from the cells of two TSH-secreting tumours which were shown to be unresponsive to TRH (Chanson et al, 1988). Other reports however, describe a normal response to TRH by cultured thyrotroph adenoma cells (Filetti et al, 1982), implying normal TRH-R function. Figure 4.1 shows a schematic representation of the pathways involved in TRH-induced responses from cells of the anterior pituitary gland. Figure 4.2 illustrates representative responses to TRH in normal subjects and in patients with pituitary adenomas.

Figure 4.1 Responses of thyrotrophs, lactotrophs and somatotrophs of the anterior pituitary gland to TRH.

Thyrotrophs and lactotrophs respond to TRH under normal conditions whereas TRH induces paradoxical GH secretion from somatotrophs in acromegalic states.
Figure 4.2  PRL, TSH and GH responses to exogenous TRH in normal subjects and in patients with pituitary adenomas/acromegaly.

(a) Patients with prolactinoma generally show no response to TRH. (b) Patients with GH-secreting adenomas and resulting acromegaly exhibit a paradoxical TSH response after TRH challenge. (c) Patients with TSH-secreting adenomas produce varying responses ranging from complete lack of TRH-induced stimulation of TSH secretion to exaggerated TSH response.
Despite the high incidence of pituitary tumours, the molecular mechanisms underlying both cellular transformation and clonal expansion (Herman et al, 1990) in the majority of these adenomas are poorly understood. Mutagenesis studies of various GPCRs have shown that single amino acid substitutions and deletions can alter ligand binding and signal transduction. Recent studies have identified mutations in human GPCR genes as the causative factors in several diseases (Hirata et al, 1994; Pollack et al, 1994). These mutations invariably lead to either constitutive receptor activation or receptor inactivation. No naturally occurring mutations have yet been ascribed to the TRH-R, although an artificial mouse TRH-R construct with a truncated carboxy terminal tail has resulted in a TRH-R which is constitutively active in both Xenopus oocytes and AtT20 mammalian cells (Matus-Leibovitch et al, 1995).

The ability of TRH to stimulate the secretion of PRL and TSH from different pituitary cell types under normal physiological conditions makes the pituitary TRH-R a candidate for potential GPCR mutations associated with disease states. Thus, oncogenic mutations of this receptor might be important in the aetiology of prolactinomas and the less common TSH-secreting tumours. Additionally, the paradoxical stimulatory effect of TRH on GH secretion in acromegaly and glycoprotein subunits in non-functioning adenomas, makes the TRH-R a candidate for involvement in the pathogenesis of these tumours. A mutant TRH-R could cause excessive hormone output from dysregulated cells in vivo and may therefore be involved in the unrestrained hormone secretion exhibited by certain types of pituitary tumours.

Functional pituitary tumours expressing TSH, PRL or GH may respond to TRH by either enhanced, blunted or paradoxical hormone release. Little however is understood of the mechanisms regulating hormone release in these tumours. To assess whether the TRH-R is involved in aberrant hormone secretion, the entire coding region of the TRH-R gene from pituitary adenoma tissues was screened for somatic mutations. In this study the objective was to determine the TRH-R structure in TSH-, PRL- and GH-secreting adenomas as well as in non-functioning pituitary adenomas. Tissue from fifty patients with pituitary adenomas (11 TSH-secreting, 15 PRL-secreting, 17 GH-secreting, and 7 non-functioning tumours) was screened using SSCP analysis of PCR amplified TRH-R gene fragments from extracted genomic DNA. The PCR products from tumour DNA were analysed in parallel with DNA derived from matching lymphocytes.
4.2 Materials and methods

4.2.1 Patients

Pituitary adenoma tissue samples were obtained at surgery from 50 patients with pituitary adenoma (17 acromegaly, 15 prolactinoma, 11 TSH-secreting and 7 non-functioning adenoma) along with blood samples to provide lymphocyte DNA as control sequence. Pituitary adenoma tissue samples were freshly obtained from 50 patients at surgery and subsequently stored at -70°C. In 45 of the 50 cases, patient’s peripheral blood samples (20ml) were collected in ethylenediaminetetraacetic acid (EDTA)-coated tubes for subsequent lymphocyte separation. Genomic DNA was extracted from adenoma (5-20mg) and lymphocyte samples (1.5ml) as described in Section 3.7.2

All 50 tumours were diagnosed by standard clinical, imaging, immunocytochemical and biochemical criteria. Details for the tumours used in this study are shown in Table 4.1.

Table 4.1 Details of the 50 patient tumours used in this study (following page)

AC 1-17 - GH-secreting adenomas, TSH 1-11 - TSH-secreting adenomas, PRL 1-15 - prolactinomas, NF 1-7 - non-functioning adenomas (NFPA). Immunohistochemical staining was scored on a relative scale; ++++, ++ and + representing high, medium and low levels of positive staining respectively; n.d. - not done.
<table>
<thead>
<tr>
<th>Tumour No</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Immunohistochemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC1</td>
<td>F</td>
<td>Acromegaly</td>
<td>GH+</td>
</tr>
<tr>
<td>AC2</td>
<td>F</td>
<td>Acromegaly</td>
<td>GH+/PRL+</td>
</tr>
<tr>
<td>AC3</td>
<td>F</td>
<td>Acromegaly</td>
<td>GH+</td>
</tr>
<tr>
<td>AC4</td>
<td>M</td>
<td>Acromegaly</td>
<td>GH+</td>
</tr>
<tr>
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<td>F</td>
<td>Acromegaly</td>
<td>GH+</td>
</tr>
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<td>F</td>
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<td>GH+</td>
</tr>
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</tr>
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<td>F</td>
<td>Acromegaly</td>
<td>GH+</td>
</tr>
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</tr>
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<td>Acromegaly</td>
<td>GH+++</td>
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<td>PRL-oma</td>
<td>PRL+</td>
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<td>PRL-oma</td>
<td>PRL+</td>
</tr>
<tr>
<td>PRL3</td>
<td>F</td>
<td>PRL-oma</td>
<td>PRL+++</td>
</tr>
<tr>
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<td>F</td>
<td>PRL-oma</td>
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</tr>
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</tr>
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</tr>
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<td>PRL-oma</td>
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</tr>
<tr>
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<td>F</td>
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</tr>
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</tr>
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</tr>
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<td>F</td>
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<td>PRL+</td>
</tr>
<tr>
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<td>&gt;50% PRL+/&gt;50% GH</td>
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<td>F</td>
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<td>TSH+/+GH+</td>
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</tr>
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</tr>
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<td>NF1</td>
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<tr>
<td>NF5</td>
<td>F</td>
<td>NFPA</td>
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</tr>
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<td>nd</td>
</tr>
<tr>
<td>NF7</td>
<td>M</td>
<td>NFPA</td>
<td>nd</td>
</tr>
</tbody>
</table>
4.2.2 Oligonucleotide PCR primer design and synthesis

The primers for PCR reactions were synthesised on a PCR MATE Model 391 DNA Synthesiser (Applied Biosystems). Primer sequences (Table 4.2) were derived from published receptor sequences (Duthie et al, 1993a; Hinuma et al, 1994), and were used to amplify exon sequence from the flanking intron where necessary. The regions of the hTRH-R amplified by each primer pair are represented in the schematic diagram of the receptor gene structure shown in Figure 4.3.

<table>
<thead>
<tr>
<th>Primer pairs</th>
<th>Sequence (5'-3')</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>TAGCTTCAGCGGACCTGAAGATGGCTCTGCATTACCTACCTCCAG</td>
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</tr>
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</tr>
<tr>
<td>5</td>
<td>TCCCCAGAAATTCCCGTGAGCGRATGGAAAGCGGACTGAG</td>
<td>241</td>
</tr>
</tbody>
</table>

Table 4.2 Primers used to PCR amplify the coding region of the hTRH-R

Sequence of primer pairs 1-5 are given in the 5' to 3' orientation, those shown in upper case anneal to the hTRH-R cDNA sequence (Duthie et al, 1993a). Primer sequences shown in lower case anneal to intronic sequence (Matre, unpublished data). The lengths of the PCR bands produced by each of the primer pairs is given in the right-most column.
Figure 4.3 Regions of the hTRH-R amplified for PCR-SSCP analysis

Exons 1 and 2 and the single coding region intron of the hTRH-R are shown in relation to the deduced transmembrane regions (I-VII) of the receptor structure. The sections of the hTRH-R gene amplified using the primer pairs given in Table 4.2 are indicated by the numbered bars below the exon/intron representation. The hTRH-R is encoded by two exons separated by a single intron (believed to be >1000bp) in il 3 at amino acid position Val 264 (Figure 2.8).

4.2.3 PCR-SSCP analysis of TRH-R mutants generated by site-directed mutagenesis

Mutations were generated in the rat TRH-R cDNA by the method of Kunkel (1985). In one mutant (SSR260AAW), three bases were substituted from T to G, T to G and A to T, and in two others single bases were substituted (R262W, A to C; K263N, G to C). The region of the receptor containing the mutations was amplified with TRH-R specific primers (upstream primer anneals at position 546-568, 5’GATCTCCAGGAATTACTACTCAC3’; downstream primer anneals at position 851-876, 5’CTCTAGTGGTTGCTAACTCATTCTC3’; results in a 330bp PCR fragment) and analysed by PCR-SSCP alongside wild type PCR products similarly amplified. PCR-SSCP analysis of the cloned wild-type and mutant rat TRH-Rs was performed to confirm the ability of this method to detect the subtle mobility shifts caused by single base alterations in PCR fragments of a size comparable to those analysed from patient-derived genomic DNA.
4.2.4 PCR-SSCP analysis of TRH-R from genomic DNA samples

PCR amplification was performed with 50 ng of genomic DNA in a final volume of 10 μl containing 2 pmol of each 5' and 3' primer, 1 μl Taq buffer (GIBCO-BRL), 0.1 μl of Taq DNA polymerase (GIBCO-BRL), 0.2 μl of 10 mM dNTPs, MgCl₂ to concentration specified by primers, and 0.1 μl α-[^32P]-dCTP (Amersham). PCR amplification was performed using an Omigene Thermal Cycler (Hybaid) applying the following parameters: an initial denaturation step at 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 52-60°C for 45 s, 72°C for 45 s, and then a final extension cycle at 72°C for 5 min.

1 μl of the labelled PCR product was diluted in 19 μl of SSCP sample buffer (Appendix I), heated for 3 min at 94°C and rapidly cooled on ice. 3-5 μl of the denatured sample was analysed by electrophoresis on 5% neutral polyacrylamide gels containing 10% glycerol and 0.5x TBE. Gels were electrophoresed in 0.5x TBE at 250V overnight at room temperature and then exposed to X-ray film (Kodak X-OMAT AR) at -70°C for 12-24 hours.

4.2.5 Cloning of PCR fragments and DNA sequencing

To confirm the results obtained by PCR-SSCP analysis, genomic DNA was PCR amplified and subjected to DNA sequencing. In brief, unlabelled PCR reactions (50 μl) were run using the same ratios of components as for PCR-SSCP above. The amplified product was then cloned into the pT7-Blue vector (Novagen) following the manufacturer's protocol. Plasmid-containing clones were identified and grown up for DNA isolation using Wizard™ miniprep purification system (Promega) according to the manufacturer's instructions. Plasmid DNA was then cycle sequenced using fluorescent dideoxy terminator chemistry as described in section 3.5. Sequence analysis was performed using GeneJockey II DNA analysis software (Biosoft).
4.3 Results

Table 4.2 shows the oligonucleotides used in this study. These sequences were derived from the structure of the hTRH-R gene depicted in Figure 4.3. The hTRH-R gene comprises at least two exons with the coding region of the receptor interrupted by a single intron in the third cytoplasmic loop. Typical donor and acceptor consensus sequences flank the splice site which is found in the codon for valine 264 at base pair 790 (Hinuma et al, 1994; Matre et al, 1993). PCR primer pairs 1 to 5 (Table 4.2) were used to amplify the entire coding region of the hTRH-R. These primer pairs PCR-amplified successfully from all genomic DNA samples, as shown by the resolution of a single PCR band of the correct size on 1% agarose gel electrophoresis (not shown).

Figure 4.4 shows an autoradiograph of PCR-SSCP analysis of wild type TRH-R and three mutant TRH-Rs generated by site-directed mutagenesis. The 330bp band amplified from the plasmids containing the cDNA for the triple mutant (SSR260AAW) and both of the single mutants (R262W and K263N) were detected by this method, visualised as an electrophoretic shift in position of the bands when compared to wild type sequence. This control experiment confirms that PCR-SSCP analysis is sufficiently sensitive to detect single base alterations in PCR fragments at the maximum end of the size range examined in this study.

Figure 4.4 PCR-SSCP analysis of rat TRH-R wild-type and mutant sequences

 Autoradiograph of SSCP-PCR analysis of cDNA from wild type rTRH-Rs and rTRH-Rs with point mutations introduced by site-directed mutagenesis. Lanes 1 and 2 are wild-type sequence, lane 3 is triple mutant SSR260AAW and lanes 4 and 5 are two different mutants with single base changes, R262W and K263N, respectively. For base changes refer to Materials and Methods (this Chapter).
As pituitary tumours are generally clonal in origin, disease related mutations would be expected to be confined to the tumour tissue. PCR-SSCP analysis of the entire hTRH-R coding region derived from the tumour DNA samples from 50 patients exhibited no electrophoretic shifts as shown in the representative examples illustrated in Figure 4.5. In 45 of the 50 patients, respective matched lymphocyte DNA samples were available. This lymphocyte DNA served as normal controls and exhibited identical migration patterns to the tumour DNA samples. This indicates that the TRH-R is structurally normal in TSH-, PRL- and GH-secreting pituitary adenomas, and in non-functional pituitary tumours.
Figure 4.5 Representative examples of PCR-SSCP analysis of pituitary adenoma DNA samples

Representative examples of PCR-SSCP analysis of tumour (T) and matching lymphocyte (L) DNA derived from separate patients. TSH, GH, PRL and NFPA represent TSH-secreting, GH-secreting, PRL-secreting and non-functioning pituitary adenomas respectively. The numbers in parentheses indicate the set of primers used for PCR (see Table 4.2).

The PCR-SSCP results were confirmed by sequencing of the PCR fragments amplified from 10 randomly selected tumours. Alignment of these DNA sequences with the wild type human TRH-R sequence showed that there were no nucleotide substitutions in any of the samples amplified from patient DNA (data not shown). Sequencing of these DNA fragments confirmed the absence of a second intron in the coding region which had been postulated to exist at nucleotide position 1177 in the carboxy tail of the human TRH-R (Matre et al, 1993)
4.5 Discussion

Compelling evidence suggests that pituitary adenomas arise from intrinsic pituitary cell defects caused by spontaneous or induced somatic mutations (Faglia, 1993; Melmed, 1994) with the resulting neoplastic mass consisting of a body of clonally derived cells (Herman et al, 1990). TRH has known stimulatory effects on lactotrophs, thyrotrophs and under certain circumstances somatotrophs. In patients with pituitary adenomas which secrete excessive amounts of either PRL, TSH or GH, a somatic mutation of the TRH-R could be implicated in the pathogenesis of the disease. In this study of 50 different human pituitary tumours, the coding region of the TRH-R was found to be unaltered, indicating that the TRH-R protein is likely to retain its normal structure in the majority of these tumours. One possible explanation for the lack of observed mutations could be that the PCR-SSCP method is not sufficiently sensitive. However, the sensitivity of the PCR-SSCP analysis used in control experiments with known TRH-R mutants was sufficient to detect single point mutations. Furthermore, DNA sequencing confirmed the results obtained by PCR-SSCP. Another possibility is that a defect might exist in non-coding portions of the receptor gene i.e. in the promoter region, or in other regulatory intronic sequences, which have not yet been analysed. Mutations in intronic sequence have been reported in isolated cases of hereditary diseases (Cooper, 1992; Naylor et al, 1992) but have not yet been associated with endocrine tumour formation. Increasing evidence does indicate though that most disease-causing mutations associated with GPCRs occur in the coding region or at the splice sites of receptor genes.

Several pathogenetic activating mutations in human GPCRs have been reported (Hirata et al, 1994; Pollack et al, 1994; Rao et al, 1994). Activating mutations frequently appear in the carboxy terminal region of il 3 of the GPCRs, an area that is believed to be important for G-protein coupling. This is coincidentally the location of the single intron in the coding region of the human TRH-R. This region of the TRH-R therefore becomes increasingly important as a candidate site for receptor mutations. In the present study it was therefore essential that the primers used to PCR the TRH-R splice junction should amplify from within the intron so that none of the receptor sequence be masked by primer sequence. The splice sites at both ends of this intron were therefore extensively analysed in all tumour types, but were found to be unaltered.

In the presence of an apparently normal TRH-R, another possible explanation for aberrant pituitary cell function is a post-receptor signal-transducing defect. Mutation(s) could occur in the G-protein (Gq/G11 in the case of the TRH-R; Aragay et
al., 1992; Hsieh and Martin, 1992) or other participants in the second messenger signalling pathway. Somatic G-protein gene mutations have so far only been identified in GH-secreting adenomas of somatotroph origin. There have been several reports of activating mutations in the α-subunit of the adenyl cyclase linked G-protein, Gs. Known as gsp mutations, these are believed to occur in approximately 40% of GH-secreting pituitary adenomas (Landis et al., 1989; Spada et al., 1994). A study by Dong and colleagues (1996) found no evidence of mutations in Gaq, Gai or Gas from nine TSH-secreting tumours. This recent study also confirms the lack of TRH-R mutations in TSH-secreting adenomas as described here. It is also possible that chronic desensitisation and down-regulation of the TRH-R and/or its associated G-proteins could be responsible for the lack of TRH responsiveness exhibited by some TSH- and PRL-secreting tumours. Anderson et al. (1995) have shown that the human TRH-R can be desensitised by TRH stimulation and in addition it has been shown that the levels of Gq/G11 are downregulated in the presence of TRH (Kim et al., 1994) both of which may be partly responsible for the reduced or absent response to TRH exhibited by certain pituitary adenomas.

Despite the presence of high affinity binding sites for TRH, non-functioning pituitary tumours show no TSH or PRL immunoreactivity. However, most of these tumours have a few LH/FSH/α-subunit positive cells and may respond to TRH (Daneshdoost et al., 1990; Katzenelson et al., 1993). These findings suggest that the TRH-R is not stimulating the same intracellular response in non-functioning pituitary tumour cells as it does in thyrotrophs. This could perhaps be the result of a TRH-R mutation allowing the receptor to couple to an inappropriate intracellular signalling system. In this study genomic DNA extracted from a series of non-secreting pituitary adenomas and analysed by PCR-SSCP analysis did not show any abnormalities in the TRH-R from non-functioning pituitary adenomas.

The dopamine type 2 (D2) receptor inhibits pituitary hormone secretion so it is feasible that a loss of function mutation of this receptor could release pituitary tumour cells from tonic inhibition resulting in a secretory pattern similar to that expected of an activating mutation of a stimulatory GPCR. However, it has been shown that the D2 receptor retains normal structure in a range of pituitary adenomas including those secreting PRL, TSH and GH (Friedman et al., 1994).

In conclusion, the TRH-R examined in a large number of human pituitary tumours was found to possess an intact DNA coding sequence, indicating that this receptor is not likely involved in the pathogenic transformation of anterior pituitary cells. Future
studies to elucidate the role of possible defects in pituitary tumour signal transduction pathways should probably be directed both at proteins involved in post-receptor events as well as at the regulatory elements of these proteins.
Chapter 5

Naturally occurring inactivating mutations of the human TRH-R

5.1 Introduction

Hypothyroidism is the result of functional failure at one of several points on the hypothalamo-pituitary-thyroid axis (Fig 2.5). Deficiency of the thyroid gland itself (primary hypothyroidism) is more common than both pituitary failure (secondary hypothyroidism) and hypothalamic failure (tertiary hypothyroidism). Central hypothyroidism is a very rare disorder, occurring in approximately 0.005% of the general population (Martino et al, 1996) and resembles secondary hypothyroidism in that there is no TSH response to exogenous TRH. However, central hypothyroidism can be due either to pituitary diseases, leading to TSH deficiency, or to hypothalamic diseases, leading to TRH deficiency (Emerson, 1985). Such problems result in characteristic symptoms of insufficient bioactive TSH secretion and resultant insufficiency of thyroid hormones. Central hypothyroidism is rarely isolated, being most usually associated with other pituitary hormone abnormalities. Deficiencies causing central hypothyroidism are known to result from tumours or infiltrative diseases of the hypothalamo-pituitary area or to pituitary atrophy (Emerson, 1985). In a few kindreds with isolated central hypothyroidism, inactivating mutations in the TSHβ-subunit gene have been described (Dacou-Voutetakis et al, 1990; Hayashizaki et al, 1990). Inactivating mutations of pituitary receptors for hypothalamic hormones also are a plausible cause of hypopituitarism. Indeed, such mutations have recently been reported for the growth hormone-releasing hormone receptor (Wajnrajch et al, 1996). It is possible therefore that inactivating mutation(s) of the TRH-R which would result in blunted TSH (and PRL) response to TRH challenge may be involved in the aetiology of this disease in some cases. To determine the role of the TRH-R in central hypothyroidism we analysed the TRH-R gene from two patients with conditions diagnosed as having isolated central hypothyroidism.
5.2 Materials and methods

5.2.1 Patient 1

Patient 1, the first of two similarly affected male siblings of two unaffected parents, was a 37 week gestation 7lb 9oz baby of an unremarkable pregnancy, but suffered from prolonged post-partum indirect hyperbilirubinaemia. Newborn screening revealed an abnormally low T4 level of 30nmol/litre which was still low in a repeat test (36nmol/litre). At this time TSH was measured at 14 mU/l with a T3-radioligand uptake (T3RU) of 35% (normal range 30-40%) but showed no other signs of marked hypothyroidism. A TRH stimulation test revealed absent TSH and PRL responses, whilst FSH and LH were within normal limits. A cranial CT scan did not reveal any abnormalities. Thyroid hormone replacement (thyroxine) therapy (50 μg/day increasing to 62.5 μg/day) was initiated 8 days post-partum. T4 therapy rapidly corrected hormone levels but at age 2 years thyroxine therapy was discontinued which resulted in rapid fall in T4, TSH and T3RU values. Therapy was re-established and maintained for a further 5 years after which time the mother withdrew thyroxine. Within 2 months T4 had dropped to 60nmol/litre, TSH was 6.9 mU/l, and T3 was 192nmol/litre (normal 145-370nmol/litre). Thyroxine therapy was again restarted at 100 μg/day. When assessed at 10 years of age and whilst still taking thyroxine, Patient 1’s T4 level was normal at 104nmol/litre. At age 11 years (June 1995) Patient 1 is clinically in early puberty with 5 cubic centimetre testes and Tanner II pubic hair, suggesting no problem with gonadotrophin secretion. Until 10.5 years of age Patient 1’s growth rate was within the 75 percentile, but at 12.5 years had dropped into the 50 percentile. His weight has always been at or above, and is currently within the 95 percentile. Patient 1 is doing well at school. Clinically there has been a long term problem with constipation but Patient 1 does not suffer from any other chronic complaints.

A younger sibling of Patient 1 also suffered from prolonged hyperbilirubinaemia after birth and when tested was found to have low T4 (73nmol/litre), inappropriately low TSH (2.65 mU/l) and normal T3RU (31.2%) levels. At 24 days old thyroxine therapy was initiated at 37.5 μg/day. Ten days later a TRH stimulation test failed to elicit any TSH or PRL response. Magnetic resonance imaging of the head revealed no abnormalities. FSH, LH and testosterone levels were normal (15 mU/ml, 9.1 mU/ml and 127 ng/ml respectively). This sibling is a large child with length within the 75-90 percentile, but with a very high weight of 20 kg and a large head circumference of
53.5 cm at 26 months of age. Development of speech and walking has been slow in this infant, the latter most likely attributable to the obesity. Most recent thyroid tests (whilst on 50μg/day thyroxine) show T₄ at 152nmoles/litre and a free T₄ of 0.03nmoles/litre, both of which are at the high end of the normal range. Subsequent assessment may indicate that the daily thyroxine dose be reduced in order to normalise thyroid hormone levels.

5.2.2 Patient 2

Patient 2, the second of three sons born to nonconsanguineous Caucasian parents, was referred for evaluation of short stature at 8.9 years of age. His clinical history was unremarkable except for poor school performance. On examination, his height was 115.2 cm (-2.6 SD), his weight was 23.0 kg (-0.4 SD), and his heart rate was 64 beats per minute without others signs of hypothyroidism. The bone age was 4 years (-4.1 SD). Plasma thyroxine (T₄) was 52 nmoles per liter (normal, 60-150 nmoles/litre) and TSH was 1.3 mU/l (normal, 0.1-5.0; Table 5.1). Peak plasma growth hormone response to clonidine and levodopa were 31.8 and 15.6 (iɡ per liter, respectively (normal, >8). Baseline plasma prolactin was 6.2 μg per liter (normal, <15) and intravenous TRH failed to induce a rise in either TSH or prolactin levels. On a computerised tomographic scan, the pituitary volume was normal at 175 cubic mm (Huot et al, 1989); on films of the abdomen, taken after the scan, a stippled right femoral epiphysis was noted. A diagnosis of central hypothyroidism of unknown cause was made. The patient was started on T₄ at a daily dose of 50μg. Normalisation of plasma T₄ levels (Table 5.1) was associated with an increase in heart rate (to 100 beats per minute) and a slight and transient increase in height velocity (from 4.8 to 7.3 cm per year). At 10.9 years, height velocity decreased again to 2.7 cm per year and the dose of T₄ was increased to 75 μg per day. At 12.3 years, after one month of T₄ withdrawal, plasma T₄ was 43nmoles/litre and TSH was 2.2 mU/l; intravenous TRH failed again to release TSH and prolactin (Figure 5.1). At 11.9 years, his overall intellectual quotient (IQ) was evaluated with the Wechsler Intelligence Scale for Children - Revised (Wechsler, 1974) at 92 (Verbal 79, Performance 109). The testicular volume started to increase at 12.5 years, with normal pubertal progression thereafter. The two brothers and the parents had no signs or symptoms of hypothyroidism. The overall IQ of the elder brother at age 16 years was 76 (Verbal 74, Performance 81), and that of the younger brother, at age 11 years, was 89 (Verbal 86, Performance 93). After obtaining the consent of the parents and
the assent of the brothers, basal thyroid function studies and a TRH test were performed and gave normal results (Figure 5.3).

Plasma T₄, triiodothyronine (T₃) and TSH were measured on the AutoDelfia by time-resolved immunofluorometric assays (Wallac). Thyroxine binding globulin (TBG) and prolactin were measured by radioimmunoassays. The free T₄ index was calculated as the ratio of plasma T₄ to TBG times 100. The TRH test was performed as previously reported (Collu, 1977; Collu et al, 1977) using 7 µg per kilogram (maximum 200 µg) of Relefact™.

### 5.2.3 Screening TRH-R genes for mutations

Genomic DNA was isolated from the peripheral blood of both patients as described in section 3.7.2.

Patient 1 DNA was PCR-amplified and analysed by SSCP analysis as described in Chapter 4.

For Patient 2 the entire coding region of the TRH-R was amplified by the polymerase chain reaction and sequenced directly. Mutations were identified by sequence alignment with wild type hTRH-R cDNA. New restriction sites created by each of the two mutations was used to confirm the presence of the mutations and to identify family members as carriers of the mutated alleles. PCR was performed under the same conditions as previously described (Tang et al, 1995), except for the addition of α³²P-dATP. After amplification of genomic DNA of each family member, the PCR products were digested with the appropriate enzymes and electrophoresed on a polyacrylamide gel. The gel was dried and exposed overnight to an X-ray film. The appearance of additional digested fragments indicated the presence of a mutation in one of the two alleles.

### 5.2.4 Construction of wild-type and mutant TRH-R cDNA expression vectors

Mutant TRH-Rs (maternally-derived, designated M-STOP and paternally-derived, designated F-TM3) were constructed by removing Bgl II/Hind III fragments of the mutant hTRH-R clones and ligating them into similarly digested wild-type hTRH-R/pcDNA-3 expression construct. The entire sequence of the cloned receptors was
verified and found to be identical to that of the published hTRH-R (Duthie et al, 1993a; Yamada et al, 1993) except for the expected mutations in the mutant clones.

5.2.5 Functional studies of mutant TRH-Rs in a transient expression system

HEK-293 and COS-1 cells were maintained as described in Section 3.14. Monolayer cultures of either HEK-293 or COS-1 cells (1.2X10^6 cells per 60mm dish) were transiently transfected with either the wild-type or the mutant human TRH-R cDNAs (5μg per 60mm dish) using Transfectam™ (Promega) as described in Section 3.15.1.

Radioligand binding assays were performed with cell membranes as described in Chapter 3. Sigmoid displacement curves were generated using SigmaPlot. All values are means of triplicate determinations and the experiments were performed on at least three independent occasions.

Total inositol phosphate accumulation assays were performed and results analysed as described in Chapter 3.

5.2.6 In vitro translation

Receptor cDNA samples (10μg) were linearised by digestion with Xba I, followed by heat inactivation of the restriction endonuclease at 85°C for 15 min. 2μg of the linearised DNA was added to the translation reaction as described in Section 3.19 and was transcribed by the addition of T7 RNA polymerase which transcribes from the T7 promoter site of pcDNA-3 and through the receptor coding sequence (Figure 3.1). Following incubation 5μl of the translation reaction was added to 20μl of SDS sample buffer (Appendix I) and run on a 12.5% SDS-PAGE gel at 100V overnight. The resulting gel was treated as described in Section 3.19 and an autoradiographic image of the gel produced using X-Omat AR film (Kodak) for 2-5 days at -70°C.
5.3 Results

5.3.1 Tests of thyroid function (Patient 2)

The results of thyroid function tests of all Patient 2 family members are shown in Table 5.1. Patient 2 was the only family member to have abnormally low plasma T₄ and free T₄ index values, but normal TSH levels when evaluated at age 8.9 years. Retrospective verification of T₄ and TSH values obtained at neonatal screening of two days of age revealed that these anomalies were present since birth: T₄ was 64 nmoles per liter at neonatal screening (normal, >88 nmoles/litre), but the baby was not recalled because his TSH concentration was 13 mU per liter (normal, <50). Treatment with T₄ at 8.9 years of age resulted in normalisation of plasma T₄ and free T₄ index values; these values returned to abnormally low levels one month after interruption of the therapy. The intravenous administration of TRH failed to induce any rise in plasma TSH and prolactin levels when the patient was tested after one month of T₄ withdrawal at 12.3 years of age (Figure 5.1). Similar results had been obtained before initiating T₄ treatment at 8.9 years of age, and also at 10.3 years of age one month after stopping T₄. Plasma TSH and prolactin levels rose after TRH administration in all the other family members (Figure 5.1).

Table 5.1 Hormone levels of patient 2 and his family (following page)

Hormone levels for Patient 2 were measured before, during and after treatment. As comparison his parents' and brothers' hormone levels were measured. The normal ranges for the measurements are included for reference.
### Naturally occurring inactivating mutations of the hTRH-R

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>T4 therapy</th>
<th>T4 (nmol/litre)</th>
<th>T3 (nmol/litre)</th>
<th>Free T4 index</th>
<th>TSH (mU/l)</th>
</tr>
</thead>
</table>
| Patient 2 = brother 2

- 8.9  None  52  1.8  9.1  1.3
- 9.1  50μg/day  121  3.2  17.1  0.3
- 11.6  75μg/day  116  2.4  16  <0.1
- 12.3  None for 1 month  43  1.2  6.7  2.2

- Brother 1

- 15  None  96  1.8  _  0.9

- Brother 3

- 10  None  96  2.0  _  0.9

- Mother

- 37  None  133  2.3  _  0.4

- Father

- 46  None  96  1.5  _  1.9

- Normal Range

- 60-150  0.9-2.9  15-23  0.1-0.5
Figure 5.1 TRH-induced responses of TSH and PRL of all Patient 2 family members

Plasma TSH and PRL measurements after TRH injection at time zero minutes. All family members except the affected son (Patient 2) exhibited normal TSH and PRL responses after administration of 200μg TRH i.v. Patient 2= brother 2
5.3.2 DNA sequence analysis

Patient 1 DNA was found to carry normal TRH-R sequence suggesting that this receptor is not involved in the aetiology of this case of central hypothyroidism. Figure 5.2 shows the PCR-SSCP results from Patient 1.

![PCR-SSCP analysis of the TRH-R from Patient 1](image)

There were no apparent mobility shifts in the TRH-R fragments amplified from the DNA of Patient 1 (P) as compared to those amplified from the DNA from an unaffected individual (WT). The numbers below the figures indicate the primer pairs used to amplify the individual segments of the hTRH-R (Table 4.2).

In contrast Patient 2 was found to be a compound heterozygote, having inherited a different mutated allele from each of the parents. Several germline mutations were found in the patient's TRH-R-encoding DNA. Sequencing of 10 clones indicated that the mutations were in different alleles. In one allele, a cytosine-to-thymosine (C to T) mutation at nucleotide position 49 resulted in arginine (CGA) being substituted by a premature stop codon (TGA) at amino acid position 17. In the other allele, a deletion
of nine nucleotides from position 343 to 351, plus a mutation of guanine-to-adenine (G to A) at position 352 were found. The deletion resulted in the loss of three amino acid residues (Ser115, Ile116, Thr117) at the cytoplasmic end of the third transmembrane domain of the receptor. The mutation at position 352 resulted in the replacement of alanine (GCC) by threonine (ACC) at amino acid position 118 (Figure 5.4). The presence of different mutations in each of the patient's alleles indicates that he is a compound heterozygote. The mutation at position 349 generated a new restriction site for the enzyme Nla III while the deletion plus mutation at positions 343-352 created a new restriction site for the enzyme Rsa I. Family studies applying restriction site enzymatic digestion to the respective parts of the gene traced each of the mutant alleles to either one of the parents and provided evidence that the elder brother of the proband had also inherited the mother's mutant allele (Figure 5.3). The heterozygous state of each parent and of the elder brother was confirmed by the presence of one normal allele. These results indicate that the patient and his elder brother had inherited germline mutations. The location of the mutations in the predicted topographical structure of the hTRH-R is shown in Figure 5.4. Mutation M-STOP (maternal) was located within the extracellular amino tail and mutation F-TM3 (paternal) was located in TM III (Figure 5.4). No other mutations were found by direct sequencing of the remainder of Patient 2's TRH-R DNA.

![Figure 5.3 Inheritance pattern of mutated alleles](image)

Patient 2 (2*), one of 3 sons, inherited one defective allele from each parent. One son carried a WT allele from his father and a mutant allele from his mother (1) and the third had inherited two wild type (WT) alleles (3). Only possession of two defective genes results in symptomatic manifestation.
Chapter 5  Naturally occurring inactivating mutations of the hTRH-R

Extracellular

Figure 5.4  Location of the inactivating hTRH-R mutations identified from Patient 2

Amino acids are shown in the one letter code. The putative locations of the seven transmembrane helix bundles are indicated as is the predicted disulphide-bridge between el 1 and el 3. The location of the mutations identified are highlighted; M-STOP, maternal mutation; F-TM3, paternal mutation.
5.3.3 Functional studies

The biological activity of the receptor constructs was examined by evaluating binding of labelled TRH analogue and stimulation by TRH of total inositol phosphate accumulation in transfected cells. Mutated TRH-R cDNAs were transfected into HEK-293 cells for binding assays and into COS-1 cells for total inositol phosphate accumulation assays. Radioligand displacement curves with (3Me-His2)-TRH and membranes prepared from HEK-293 cells expressing wild-type and mutant TRH-Rs M-STOP and F-TM3 are represented in Figure 5.5. The wild-type receptor exhibited high affinity binding with an affinity constant (Kd) of approximately 1.6 nM (± 0.09 SEM), similar to that previously reported (Duthie et al, 1993a). In contrast, the maternally-derived mutant (M-STOP) showed no measurable binding, while the paternally-derived mutant (F-TM3) showed a very small amount of binding. Untransfected HEK-293 cells did not demonstrate any binding (results not shown).
Chapter 5 Naturally occurring inactivating mutations of the hTRH-R

Figure 5.5 Radioligand displacement assay results
Representative curves of displacement of $^3$H-[3Me-His2]-TRH binding by [3Me-His2]-TRH in membrane preparations from HEK-293 cells transiently expressing wild type and mutant hTRH-Rs (maternally-derived mutant M-STOP and paternally-derived mutant F-TM3). Data points represent the mean (± SEM) of triplicate samples. Assays were performed on at least three independent occasions.

In accord with the binding assay results mutated receptors were as expected unable to activate total inositol phosphate accumulation when transfected into COS-1 cells, indicating that both of the mutated forms of the TRH-R are functionally inactive (Figure 5.6). The EC$_{50}$ value of total inositol phosphate stimulation by TRH for COS-1 cells expressing the wild-type receptor was 2.4 nM (± 0.12 SEM). Sham transfected COS-1 cells demonstrated no inositol phosphate response at any of the TRH doses used.
Chapter 5 Naturally occurring inactivating mutations of the hTRH-R

Figure 5.6 Total inositol phosphate production

TRH-induced total inositol phosphate accumulation in COS-1 cells transiently expressing wild type and mutant hTRH-Rs (maternally-derived mutant M-STOP and paternally-derived mutant F-TM3). Sham transfected COS-1 cells were used as a negative control. Data points represent the mean (± SD) of duplicate samples. Assays were performed on at least three independent occasions.

In vitro translation was performed to ascertain the possibility of translation of a truncated hTRH-R from the second methionine in the receptor sequence, Met75 (in TM 1), even from the DNA encoding M-STOP (Figure 5.4). Figure 5.7 shows an autoradiograph of an in vitro translation experiment. In Figure 5.7 the protein products appear as dark bands on the gel. The predicted molecular weight of the unglycosylated, full length hTRH-R is 45kDa, which is reduced to 40kDa if translated from Met75 instead of the initiation codon for Met 1. The 40kDa band is visible in all three lanes but the full length hTRH-R only appears in the lanes representing translation products from cDNAs without a premature stop codon (namely WT and F-TM3). The smaller molecular weight band is the only specific protein translated from the M-STOP construct. There were no translation products in the reaction which was
performed with no added DNA (not shown), indicating the specificity of the production of the bands which are visible.

---

**Figure 5.7**  *In-vitro* translation of WT, M-STOP and F-TM3 receptor cDNAs (following page)

Protein products arising from *in vitro* translation of WT hTRH-R (WT lane), M-STOP and F-TM3 DNA appear as dark bands on the autoradiograph. The 45kDa full length product is only produced from WT and F-TM3 cDNAs, whereas the shorter 40kDa band translated from Met 75 is visible in all three lanes.
Naturally occurring inactivating mutations of the hTRH-R
Chapter 5  Naturally occurring inactivating mutations of the hTRH-R

5.4 Discussion

The TRH-R is a member of the large family of G protein-coupled, seven transmembrane domain receptors (Straub et al, 1990). The hTRH-R has recently been cloned and its structure and functionality have been found to be similar to that of the rat and mouse receptors (Duthie et al, 1993a; Hinuma et al, 1994; Yamada et al, 1993). Receptor-mediated stimulation of the synthesis and release of TSH and PRL is exerted through activation of the inositol phosphate-calcium-protein kinase C signal transduction pathway (Gershengorn, 1989). The third transmembrane helix is an essential constituent of the TRH-R binding pocket (Perlman et al, 1994b). In this study of two patients with outwardly similar clinical symptoms, one (Patient 2) was found to confirm the hypothesis that inactivating mutation of the TRH-R could be responsible for human disease whereas Patient 1 carried apparently normal TRH-R genes (at least within the coding region). Although Patient 1's TRH-R genes were normal, Patient 2 was found to be a compound heterozygote, having inherited a different mutation in the TRH-R gene from each parent. The presence of a stop codon at amino acid position 17 in the mutated maternal allele (M-STOP) presumably results in a truncated protein, missing all seven transmembrane domains. On the other hand, deletion of three amino acids and substitution of alanine by threonine in the mutated paternal allele (F-TM3) may result in alteration of the tertiary structure of the third transmembrane helix, so disrupting normal receptor function. Both mutated receptors, when expressed in eukaryotic cells, resulted in severely reduced or absent TRH binding and TRH-induced inositol phosphate accumulation confirming the role of the mutant TRH-Rs in the pathophysiology of this patient's central hypothyroidism. The results suggest that the mutant TRH-Rs have essentially absent biological activity which explains the observed failure of this patient to elicit a rise in either TSH or PRL following the administration of TRH. The mutations are recessive and therefore, both mutations must be present for symptoms to be observed, as both parents had normal thyroid function, as did the sibling who also carried the mutated maternal allele. This case provides an opportunity to study the effect of isolated TRH insensitivity in the brain and elsewhere in the absence of compounding effects arising from deficiencies of other hypothalamic or pituitary hormones which often accompany central hypothyroidism. It would be interesting to assess both the glycosylation status of the TSH present in Patient 2's serum, and the profile of any TSH secretion with regards to the presence/absence of the nocturnal TSH surge observed in euthyroid subjects. However, the ethics of performing such studies, which are not clinically indicated, particularly in this patient who is at present only 15 years old, must be considered.
Although naturally occurring activating or inactivating mutations of G protein-coupled receptors linked to other intracellular signalling systems have been reported (Pearce and Trump, 1995) to the author's knowledge this is the first description of naturally occurring inactivating mutations of a GPCR linked to the PLC second messenger pathway. This is also only the second report of a defect in a pituitary gland receptor. In addition, identification of the receptor mutations from Patient 2 expands the range of molecular defects within the thyroid axis, which already includes defects in TSHβ, the TSH-R, thyroid hormone synthesis and thyroid hormone insensitivity, to include defects in the TRH-R. Central hypothyroidism, defined as low plasma total and free T4 concentrations in the presence of normal TSH values, is rare. It may result from either hypothalamic or pituitary lesions and usually occurs in association with other pituitary hormone deficiencies (Emerson, 1985). The clinical manifestations of central hypothyroidism are usually mild, especially when it is isolated (Martino et al, 1996). Indeed, in Patient 2, the only presenting symptoms were short stature with markedly delayed bone maturation, which shows the exquisite sensitivity of these markers of hypothyroidism in children (Rose, 1995). Substitution therapy with T4 did increase height velocity and there was a catch-up in bone maturation; however, the growth acceleration during treatment was modest and temporary. Furthermore, although the patient had cognitive deficiencies, his two unaffected brothers had similar learning difficulties and low IQs. Extrapituitary expression of the TRH-R has been reported, suggesting that TRH may have non-endocrine functions (Collu et al, 1980). The reasons as to why the TRH-R mutations do not appear to cause dysfunction in any of the extrahypothalamic systems believed to be influenced by TRH (Section 2.2.7) remain unclear at this time. This patient appears to represent a naturally occurring TRH-R knock-out, the result of which was previously predicted to be lethal in utero as a consequence of the possible widespread functions of TRH outside of the pituitary gland. If TRH is indeed involved in foetal development it would appear that the almost negligible binding of mutant F-TM3, whilst insufficient to maintain mature thyroid function, may be adequate to permit normal foetal development. In addition the in vitro translation experiment seems to indicate that low levels of a truncated receptor may be translated from the alternative Met75 in TM I. Whether this peptide would express as a functional receptor is unknown. Interestingly, it has recently been shown that a TRH-R missing the entire amino tail binds ligand with wild type characteristics (Han and Tashjian, 1995). It is therefore conceivable that an amino terminally truncated receptor could be responsible for the non-lethality of this apparent TRH-R knockout, providing enough receptor activity to allow almost normal TRH function. The prevalence and phenotypic spectrum of TRH-R mutations in isolated central
hypothyroidism as well as in other related diseases remain to be established. In light of the recent description of the hTRH-R promoter region (Iwasaki et al, 1996) it would be informative to screen this region of the receptor in Patient 1 for mutations which may interfere with either the initiation or control of transcription as non-expression of the TRH-R could explain the lack of responsiveness to TRH in this patient.

In order to investigate the effects of TRH-R knock-out it would be useful to establish a transgenic mouse line with targeted receptor ablation. In preparation for this project it will be necessary to identify and characterise the promoter elements of the mTRH-R gene. The approaches and experimental design of such an undertaking are described in Chapter 7 of this thesis.
Chapter 6

Site-directed mutagenesis and expression studies of the hTRH-R

6.1 Introduction

The intracellular domains of GPCRs contain regions which on ligand binding are believed to undergo conformational changes permitting G-protein coupling, and which may be phosphorylated conferring receptor regulation. Unlike the transmembrane domains of GPCRs, there is no model of 3-dimensional structure for the cytoplasmic regions, so predictions concerning structure-function relationships are more difficult to make. The most useful tool is comparison of different GPCRs in an attempt to identify conserved amino acids as these are likely to be of functional significance. Based on such comparisons it is possible to carry out site-specific mutagenesis of any given amino acid or group of amino acids of a GPCR and to examine the effects of mutation(s) on in vitro receptor function. The results of such experiments allow the extrapolation of predicted relationships between specific amino acids and receptor function. Several regions of the internal, or intracellular domains of GPCRs have been identified as being involved in coupling to G-proteins, receptor phosphorylation, regulation and internalisation.

A conserved region at the junction between TM III and il 2, known as the AspArgTyr or DRY motif, has been linked with mediation of receptor activation. Several GPCRs have a conserved substitution of Glu at the Asp position of the DRY motif. Spectral studies of rhodopsin show that movements in this region of the molecule occur upon photoactivation (Farahbakhsh et al, 1995). Indeed Glu134, of the DRY motif of rhodopsin appears to undergo proton uptake immediately upon retinal isomerisation (Arnis et al, 1994). Substitution of Glu134 for Gln results in low level constitutive activity and enhanced light-dependent responses (Arnis et al, 1994; Cohen et al, 1993). Scheer and co-workers (1996) suggest that protonation of the equivalent residue of the $\alpha_{1B}$-adrenergic receptor, Asp142, alters the equilibrium between the inactive and active receptor states and replacement of this residue with Ala leads to high constitutive receptor activity. In addition, this study finds that disruption of the molecular interactions between Arg143 and nearby residues displaces Arg143 from its
'polar pocket' thereby activating the photoreceptor. Both the Asp (or Glu) and the Arg of this conserved motif appear to be fundamental for mediating receptor activation. The TRH-R has Glu instead of Asp at this intracellular location.

Site-directed mutagenesis studies of several GPCRs have indicated that coupling to specific G-proteins seems to be predominantly mediated by sequences within il 3, in particular, those regions at the membrane interface. Mutation of Ala293 (in il 3) of the $\alpha_1B$-AR, which lies 3 amino acids from the cell membrane, to any other amino acid, results in agonist-independent, or constitutive, receptor activity (Kjelsberg et al, 1992).

In this study the functional roles of two specific amino acids of the hTRH-R were studied by measuring ligand binding and effector activation in cells transfected with mutant hTRH-Rs. Two specific residues were chosen for study:

1. Thr265 (in il 3) was highlighted for two reasons. Firstly, the analogous Thr residue (Thr373) in the $\alpha_2$-adrenergic receptor when mutated results in constitutive activity (Ren et al, 1993) and secondly, Thr265 is a potential phosphorylation site which may be involved in receptor activation/desensitisation.

2. Glu122 was studied as it is part of the DRY motif which has previously been implicated in GPCR function.
Chapter 6  Site-directed mutagenesis and expression studies of the hTRH-R

Figure 6.1  Primary structure of the hTRH-R

This diagram shows the primary sequence of the hTRH-R and the proposed arrangement of the seven transmembrane helices and the extra- and intracellular domains. Amino acids are shown in the single letter code. The mutated residues are highlighted in blue (Glu122) and magenta (Thr265), and the substitutions are indicated by the letters at the ends of the arrows.
6.2 Materials and methods

Site-directed mutagenesis was carried out as described in Section 3.13.

Oligonucleotides were synthesised using a PCR MATE Model 391 DNA Synthesiser (Applied Biosystems) based on the wild type sequence of the hTRH-R. Single nucleotide base changes resulted in amino acid substitutions generating the receptor constructs outlined in Table 6.1. The sequences of the oligonucleotides used to generate these mutant TRH-Rs are also shown in Table 6.1.

Thr265 was mutated to one of three different amino acids; Ser (conservative change which retains the hydroxyl side chain), Ala (moderate change) and Asn (large change of side chain charge). Glu122 was substituted for one of two amino acids; Asp (reverts receptor to DRY consensus sequence) or Leu (large alteration in side chain).

Receptor expression in mammalian cells, radioligand binding assays and total inositol phosphate accumulation assays, were performed as described in Chapter 3.

Table 6.1 Oligonucleotides used to generate site-specific mutant hTRH-Rs

| Mutant hTRH-R constructs generated by site-directed mutagenesis using the oligonucleotides described to alter specific amino acids within the receptor peptide. Thr265 lies at the membrane proximal region of the carboxy end of the third intracellular loop (the oligonucleotide spans from 801 to 825). Glu122 is located at the amino end of the second intracellular loop and is found within the DRY motif (the oligonucleotide spans from 372 to 396). As the single-strand DNA isolated from pcDNA-3 is synthesised in the forward orientation, the sequences shown were inverted for oligonucleotide synthesis to allow annealing of the oligonucleotide primer to the single-strand DNA. |
Chapter 6  Site-directed mutagenesis and expression studies of the hTRH-R  119

<table>
<thead>
<tr>
<th>Construct</th>
<th>Oligonucleotide Sequence</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>80\textsubscript{1}GGAAGCAGGTCA\textsubscript{ACC}AAAATGCTGGC\textsubscript{825}</td>
<td>none</td>
</tr>
<tr>
<td>T265A</td>
<td>GGAAGCAGGTG\textsubscript{CC}AAGATGCTGGC</td>
<td>Thr265 to Ala</td>
</tr>
<tr>
<td>T265S</td>
<td>GGAAGCAGGTCT\textsubscript{CCA}AGATGCTGGC</td>
<td>Thr265 to Ser</td>
</tr>
<tr>
<td>T265N</td>
<td>GGAAGCAGGTCA\textsubscript{TCA}AGATGCTGGC</td>
<td>Thr265 to Asn</td>
</tr>
<tr>
<td>Wild Type</td>
<td>37\textsubscript{2}CCTT\textsubscript{AT}AGAGGTACATAGC\textsubscript{396}</td>
<td>none</td>
</tr>
<tr>
<td>E122D</td>
<td>CCTT\textsubscript{AT}AGAGGTACATAGC</td>
<td>Glu122 to Asp</td>
</tr>
<tr>
<td>E122L</td>
<td>CCTT\textsubscript{AT}AGAGGTACATAGC</td>
<td>Glu122 to Leu</td>
</tr>
</tbody>
</table>

The rTRH-R was used as a positive control in the transfection experiments. Throughout the expression experiments it became obvious that the hTRH-R expressed at a much lower level in comparison with the rTRH-R. In order to carry out experiments comparing expression levels, different TRH-R constructs were generated. The coding regions (i.e. from ATG start codon to AGT stop codon) of the rat and human TRH-Rs were PCR amplified, ligated into the TA vector pcR2.1, and then subcloned into the Eco RI site of the pcDNA-3 polylinker. These 'start-stop' (s-s) clones were expressed in COS-1 cells for comparison of function with the WT cDNA clones which were expressed with intact 5' and 3' UTRs.

Additionally, a stable cell line was established in which the hTRH-R was expressed in HEK-293 cells as described in Section 3.15.2. Resulting stable cell lines were assayed for TRH-induced changes in the concentration of intracellular calcium ([Ca\textsuperscript{2+}]\textsubscript{i}) using calcium imaging (Section 3.17). The newly established hTRH-R-expressing cell line was compared with the 293-E2 cell line which is a stable line expressing the rTRH-R (established by Dr Jelka Zabavnik; (Kim et al, 1994)).
6.3 Results

6.3.1 Thr265 mutants compared to WT hTRH-R

Substitution of Thr265 with either one of the three amino acids chosen here (Ala, Thr or Asn) results in increased hTRH-R expression levels. Radioligand binding measurements were carried out using the same amount of membranes of WT and mutant expressing cells, however the level of binding was approximately three to six times greater in the mutants. The $B_{\text{max}}$ value was 0.1 pmol/mg protein for the WT hTRH-R and was significantly increased to 0.66 (p<0.05, n=3), 0.34 (p<0.02, n=3) and 0.32 (p<0.02, n=3) pmol/mg protein for mutants T265A, T265S and T265N respectively (Table 6.2). These three mutant TRH-Rs had increased affinity for ligand as compared to that of the WT hTRH-R. The dissociation constant (Kd) was 5.43 nM (± 0.6 SEM) for the WT hTRH-R and 1.38 (± 0.15 SEM), 2.17 (± 0.05 SEM), and 1.98 nM (± 0.07 SEM) for mutant receptors T265A, T265S and T265N respectively (Figure 6.2a and Table 6.2). The reduced Kd values for the mutant hTRH-Rs were significantly lower than the Kd of the WT receptor with p<0.02 from 3 separate experiments. Similarly, in the IP assays, although basal levels of second messenger turnover were not significantly altered by these mutant receptors, maximum stimulation was higher compared to WT (Figure 6.2b). The EC$_{50}$ of total inositol phosphate stimulation was reduced from 4.0 nM (± 0.45 SEM) for the WT hTRH-R to 1.0 nM (± 0.02 SEM) for each of the Thr265 mutant receptors (significant with p<0.05, n=3), indicating that these receptors can be activated at a lower ligand concentration compared to wild type hTRH-R. COS-1 cells alone showed no measurable level of ligand binding or inositol phosphate stimulation.
Thr265 was substituted with one of three different amino acids (Ala, Ser or Asn) producing the constructs T265A, T265S and T265N. Each of these mutant receptors was transiently expressed in COS-1 cells alongside WT hTRH-R. Cells from a single transfection were used for both radioligand displacement assays and total inositol phosphate accumulation assays. a) Scatchard displacement plots (which plot bound ligand versus bound/free ligand) were derived from a competition radioligand binding displacement experiment using $[^3]$H(3Me-His2-TRH) tracer. Data points are the mean of triplicate samples ±SEM, and are representative of at least three independent experiments. b) shows the TRH-induced total inositol phosphate production in cells from the same transfection as used in the binding assay above. Results are shown as the mean of triplicate determinations ±SEM.
Chapter 6  Site-directed mutagenesis and expression studies of the hTRH-R

(a)  

(b)
6.3.2 Glu122 mutants compared to WT hTRH-R

The mutant receptor carrying the Glu122 to Asp substitution (E122D) exhibited a reduced EC50 value (0.5nM ± 0.03) as compared to TRH-induced total IP stimulation by the WT TRH-R (4.0nM ± 0.45 SEM; p<0.05, n=3). Mutant E122D also showed a reduced dissociation constant (calculated using the adapted Cheng and Prusoff equation described in Section 3.18.4) compared to the WT hTRH-R (WT hTRH-R Kd=5.43nM compared to 0.46nM for mutant E122D; p<0.05, n=3), indicating a relative increase in ligand affinity for mutant E122D. Substitution of Glu122 with Leu (E122L) totally abolished binding and was therefore unable to stimulate IP accumulation (Figure 6.3). Radioligand binding and IP assay results for Glu122 mutants are summarised in Table 6.2.

Figure 6.3 Functional analysis of Glu122 mutants of the hTRH-R (following page)

a) shows the results of a radioligand displacement binding experiment using [3H](3Me-His2-TRH) and membranes prepared from COS-1 cells transiently expressing WT and mutant hTRH-Rs (E122D and E122L). The data points represent the mean of triplicate samples and the graph is representative of at least three separate experiments. b) shows the total inositol phosphate accumulation induced by varying concentrations of TRH in COS-1 cells from the same transfection as those used for the radioligand binding assay above.
Chapter 6  Site-directed mutagenesis and expression studies of the hTRH-R  124

(a)  

[3H] 3Me-His2-TRH bound (dpm)  

\[ \text{[3H] 3Me-His2-TRH] log (M)} \]  

- WT hTRH-R  
- E122D  
- E122L

(b)  

Total inositol phosphate production (dpm/10^5 dpm Tc)  

- WT hTRH-R  
- E122D  
- E122L  
- COS-1 cells

\[ \text{[TRH] log (M)} \]  

10000  
9000  
8000  
7000  
6000  
5000  
4000  
3000  
2000  
1000  
0  
C  
10^{-11}  
10^{-10}  
10^{-9}  
10^{-8}  
10^{-7}  
10^{-6}  
10^{-5}
6.3.3 Comparison of rat and human TRH-R expression

In order to establish an hypothesis regarding the difference in expression levels between the rat and human TRH-Rs, several receptor constructs were generated. Essentially, the receptors were expressed either from full length constructs for WT determinations, that is with 5' and 3' untranslated regions (UTR) intact, or were expressed from clones without any UTR (designated 'start-stop' or s-s clones). The WT TRH-Rs-s forms of the rat and human TRH-Rs expressed at lower levels than did their respective full length clones with untranslated regions intact as measured by accumulation of TRH-induced total inositol phosphate. However, rTRH-Rs-s still expressed much more highly than both the intact and s-s hTRH-R cDNAs. In fact, there was a convincing gradation of IP stimulation with intact rTRH-R producing the highest IP response, with rTRH-Rs-s next, the intact hTRH-R next, and the hTRH-Rs-s having the lowest IP stimulatory effect of all four constructs (Figure 6.4).
Figure 6.4  Comparison of total TRH-induced inositol phosphate production in COS-1 cells transiently transfected with WT and start-stop versions of the rat and human TRH-Rs

COS-1 cells transiently expressing the full length WT cDNAs for the rTRH-R and hTRH-R containing intact 3' and 5' UTRs were assayed alongside cells expressing the 'start-stop' (s-s) forms of the receptor constructs. The results are the mean of samples performed in triplicate (±SEM). The upper inset graph shows the full length and s-s hTRH-Rs separately to indicate that these constructs when expressed in COS-1 cells retain TRH-induced dose dependent total inositol phosphate production.

Table 6.2 summarises the functional parameters of each of the mutant TRH-R constructs discussed above.
<table>
<thead>
<tr>
<th>Construct</th>
<th>Kd ± SEM (nM)</th>
<th>Bmax ± SEM (pmol/mg protein)</th>
<th>EC50 IP production (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT hTRH-R</td>
<td>5.43 ± 0.6</td>
<td>0.1 ± 0.05</td>
<td>4.0 ± 0.45</td>
</tr>
<tr>
<td>T265A</td>
<td>1.38 ± 0.15</td>
<td>0.66 ± 0.02</td>
<td>1.0 ± 0.02</td>
</tr>
<tr>
<td>T265S</td>
<td>2.17 ± 0.05</td>
<td>0.34 ± 0.05</td>
<td>1.0 ± 0.01</td>
</tr>
<tr>
<td>T265N</td>
<td>1.98 ± 0.07</td>
<td>0.32 ± 0.05</td>
<td>1.0 ± 0.01</td>
</tr>
<tr>
<td>E122L</td>
<td>no binding</td>
<td>not detected</td>
<td>no stimulation</td>
</tr>
<tr>
<td>E122D</td>
<td>0.46 ± 0.03</td>
<td>0.30 ± 0.03\textsuperscript{a}</td>
<td>0.5 ± 0.03</td>
</tr>
<tr>
<td>WT hTRH-R s-s</td>
<td>n.d.</td>
<td>n.d.</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>WT rTRH-R</td>
<td>2.73 ± 0.68\textsuperscript{§}</td>
<td>n.d.</td>
<td>0.6 ± 0.04</td>
</tr>
<tr>
<td>WT rTRH-R s-s</td>
<td>n.d.</td>
<td>n.d.</td>
<td>3.0 ± 0.2</td>
</tr>
</tbody>
</table>

Table 6.2  Functional measurements of each TRH-R construct.

K_d values were determined either from Scatchard analysis or from the Cheng and Prusoff equation (DeBlasi et al, 1989). § previously determined (Cook et al, 1996). \textsuperscript{a} was calculated using Cheng and Prusoff analysis (see Section 3.18.4). n.d. = not done.

### 6.3.4 Calcium imaging of rat and human TRH-R stable cell lines

Single cells expressing the rat and hTRH-Rs were assessed using the technique of calcium imaging which allows visualisation of ligand-induced changes in intracellular calcium ([Ca^{2+}]_i) as it is released from intracellular stores. Figure 6.5 shows traces of changes in [Ca^{2+}]_i levels in human (293-h10, 6a) and rat (293-E2, 6b) TRH-R stable cell lines. TRH (1\mu M) induces a rapid increase in [Ca^{2+}]_i levels. A second pulse of TRH has no effect on [Ca^{2+}]_i. Bradykinin (BK) acts through a specific receptor (BK-R) to mobilise intracellular calcium. The BK-R is endogenously expressed in kidney derived HEK-293 cells (Van Zoelen et al, 1994) and was used in comparison with the TRH-R to assess the characteristics of homologous versus heterologous desensitisation of the two receptors. However, BK was a poor stimulator of calcium changes in these cells, and the spikes observed following addition of BK were often
small. High-dose TRH desensitised both subsequent Ca\(^{2+}\) responses to TRH and BK, whereas pre-treatment with BK desensitised only the BK-induced Ca\(^{2+}\) response. The mobilisation of intracellular Ca\(^{2+}\) may negatively control PLC activity (Stauderman and Pruss, 1990), and therefore, the lack of BK-induced Ca\(^{2+}\) response following TRH pretreatment may be attributable to this negative feedback control at the level of PLC activity, especially in view of the high level of intracellular Ca\(^{2+}\) mobilised in the rat or human TRH-R stable cell lines. Although the hTRH-R has shown lower expression levels as compared to the rTRH-R in the transient expression experiments, the level of TRH-induced Ca\(^{2+}\) responses were not statistically different between the stable cell lines 293-h10 and 293-E2 (p<0.04, n=6).

These experiments were carried out at early passage stages of clone 293-h10 (passage 4-6). Unfortunately, by passage 8-10 hTRH-R expression was lost, despite maintenance of the cell line in Geneticin\(^{TM}\) containing medium. All clones which were isolated and characterised showed similar loss of TRH-R function whilst surviving in the selective growth medium.
Figure 6.5  Calcium imaging of 293-h10 and 293-E2 stable cell lines
Stably expressing cells were plated onto glass coverslips and were imaged as described in Section 3.17. TRH (1μM) and BK were added to the incubation buffer at the times indicated by the arrows. a) represents the results obtained from 293-h10 cells and b) represents the results from 293-E2 cells. The results are the average of at least six individual cells from one field of imaged cells.
6.4 Discussion

On the basis of previous studies of the β-adrenergic receptor it was hypothesised that mutation of Thr265 of the hTRH-R would result in constitutive activity of the receptor. The $B_{\text{max}}$ values showed that all three of the Thr265 mutants expressed at a much higher level than the WT receptor (Table 6.2), between three and six times greater than WT in fact. The basal levels of IP turnover were not increased as is often characteristic of constitutively active receptors, although these mutants reached half maximum stimulation at a lower TRH concentration than the wild type, indicating that the EC$_{50}$ was reduced. The EC$_{50}$ for IP stimulation in the Thr265 mutant TRH-Rs was reduced 5-fold in comparison to the WT hTRH-R. The binding affinity of each of the Thr265 mutants was increased in comparison to the wild type binding affinity, which is a phenomenon also observed in other cases of constitutively active GPCRs (Lefkowitz et al, 1993). The result of increased affinity in the presence of increased ability to stimulate phosphoinositide hydrolysis is probably a result of the increased number of mutant receptors expressed at the cell membrane, rather than being due to ligand-independent, or constitutive activation. Why such mutations result in increased expression is not known, however this is an interesting finding deserving further investigation.

Substitution of Glu122 for Asp resulted in a mutant TRH-R with increased binding affinity, and reduced EC$_{50}$ of total IP stimulation as compared to the wild type hTRH-R. This alteration in apparent receptor function may be due either to a change in the expression level of the mutant receptor or to altered receptor function. Substitution of Glu by Asp is a relatively conservative change and probably allows the receptor to retain near normal structure thereby not altering function to any great extent. In contrast substituting this same Glu residue with Leu produces a mutant receptor which shows no ligand binding or agonist-induced IP accumulation. It is often possible to detect low level cell surface expression of GPCRs by increasing the concentration of ligand used in IP stimulation assays into the μM range. If there are any receptors present they should bind ligand and elicit a measurable IP response. Indeed, in a study of the role of disulphide bond formation between cysteine residues of the rTRH-R it was found that a mutant receptor which was not stimulated by this IP 'push' method also showed no expression by Western blotting of epitope-tagged mutant receptor, whereas other non-binding mutants responded to high level TRH doses and were demonstrable in the cell membrane (Cook et al, 1996). It is unlikely therefore, that mutant E122L is expressed at the cell membrane as no IP production was
measurable even at very high (100µM) TRH concentrations. Construction of an epitope-tagged version of this mutant receptor would enable the cell surface expression, or lack thereof, to be measured. Personal communication with Professor G Milligan (Department of Pharmacology, University of Glasgow, UK) suggested that the E122L substitution would result in constitutive receptor activity, in analogy to a D to L substitution in another GPCR with a conserved DRY motif. The loss of function of mutant E122L whilst unexpected is not totally inexplicable. The change from Glu to Leu is a relatively drastic one, with Leu being very much more hydrophobic than Glu. However, this type of substitution is not without precedent as substitution of a single residue in the α2-adrenergic receptor with any other amino acid resulted in constitutively active receptors and there was no apparent correlation between the change in side group and the degree of ligand independent activity (Ren et al, 1993). Substitution of Glu122 for Leu could potentially alter the 3-dimensional structure of the receptor in this region, thereby destroying either normal membrane insertion or receptor functionality.

The combined results of the site-directed mutagenesis of the hTRH-R highlight both the sensitivity of certain regions of GPCRs to mutation and the effects that the choice of substitution can have. Although Thr265 was substituted with a very dissimilar amino acid, Asn, the effects were indistinguishable to those caused by only slight alteration in the amino acid (i.e. T265S), assuming that the hydroxyl group is involved in phosphorylation of this residue of the receptor. This site of the receptor is therefore sensitive to any change. As this region may be involved in the processes of either desensitisation and/or receptor internalisation this is not a surprising finding as the alterations occurring during these processes (e.g. phosphorylation) are highly specific and are also likely to be relatively subtle. Future studies would include investigation of the pattern of desensitisation of this mutant, again using an epitope-tagged construct so that receptor endocytosis (including agonist-induced receptor internalisation and receptor recycling) could be monitored.

Our experience of hTRH-R expression studies indicated that this receptor has a variable expression level which is consistently lower than that seen with the rTRH-R. Other groups have experienced difficulty in expressing their hTRH-R clones (personal communication with Dr R Collu). Using the rTRH-R as a positive control for confirmation of receptor expression throughout the experiments highlighted this phenomenon. When comparing receptor sequences at the DNA level, the major difference between the rat and human TRH-R clones is in the amount of untranslated region (UTR), with the rTRH-R clone (Sellar et al, 1993) having substantially more
UTR sequence than the hTRH-R clone (Duthie et al, 1993a) and changes in the 3’ region encoding the carboxy tails of these related receptors. The hTRH-R cDNA clone has only 19 bp of 5’ UTR, whereas the rat has 340 bp of 5’ UTR. At the 3’ end of the human clone there are 130 bp of UTR and the rat has 1800 bp UTR. The rat 3’ UTR contains a polyA+ tail, whereas in the hTRH-R clone this RNA stability region is absent from the 3’ UTR. The absence of a polyA+ tail should not affect expression levels as the expression vector pcDNA-3 encodes the bovine polyadenylation signal and therefore adds this sequence to transcripts synthesised from the expression vector. It has been suggested that there are often AU rich elements (AREs) within the 3’ UTR which confer RNA instability. The mRNA molecules containing the AREs are believed to be targeted for rapid and selective degradation, in particular, systematic removal of the polyA+ tail. The rTRH-R 3’ UTR contains single AUUUA sequences at 1747-1751 and 1974-1978 and a double AUUUAUUUA between bases 2613 and 2621. The latter likely represents a more significant ARE as these regions are usually composed of multiple copies of the sequence. The hTRH-R 3’ UTR does not contain any ARE consensus sequences. This would suggest that if anything the rTRH-R mRNA should be less stable than that of the hTRH-R, and would therefore be expected to express less efficiently. In reality the opposite is true. It would therefore appear that there is some intrinsic element of the actual coding region which may regulate expression, as even when expressed without any UTR sequence the rTRH-Rs still expresses to a much higher level than the human isoform of the TRH-R. However, the difference may not lie at the RNA level, the rat and human TRH-R peptides may not be processed and targeted to the cell membrane equivalently. Future studies lie in the direction of assessing expression of epitope-tagged WT and mutant hTRH-Rs as a function of receptor mRNA.

The loss of hTRH-R expression in the HEK-293 stable cells lines was very unexpected, especially as the rat TRH-R (293-E2) cell line has maintained expression over a period of almost five years. Personal communication with Dr S Seal fon indicates that similar difficulties have been experienced in expression of the human gonadotrophin-releasing hormone receptor (hGnRH-R). No reason for this phenomenon has yet been determined. Different approaches have been taken to improve levels of expression of several GPCRs. Davidson et al. (1996) have shown that incorporation of an additional glycosylation site in the hGnRH-R increases cell surface receptor expression 1.8-fold. However, the expression levels reported, even with the addition of an extra glycosylation site are still low and do not reach the levels of GnRH-R expression attained in the rat GnRH-R (293-A2, established by Dr JVF
Cook) stable cell line. Another group has inserted the hGnRH-R into an expression vector containing the sequence for an internal ribosomal entry site (IRES) which has led to the successful generation of an over-expressing hGnRH-R stable cell line (Beckers et al, 1995). In addition it has been reported that there exists a consensus sequence flanking the AUG translation initiation codon which creates an optimal site of initiation for the 40S ribosomal subunit on the mRNA (Kozak, 1984; Kozak, 1981). The original consensus sequence, known as a Kozak initiation sequence, takes the nucleotide form CC(A/G)CCAUGG (AUG initiation codon shown in bold type), but it has subsequently been shown that the optimal sequence for initiation by eukaryotic ribosomes is ACCAUGG (Kozak, 1986). Neither the rat nor the human TRH receptors have this consensus sequence flanking their initiation codons, but both contain an A at position -3 and a G at position +4 which have been shown to be important nucleotides for ribosomal translation initiation (Kozak, 1981) (Figure 6.6). Creation of TRH-Rs containing a Kozak initiation sequence or the addition of extra glycosylation sites, or use of an IRES expression vector might improve hTRH-R expression.

![Figure 6.6 Flanking regions of the rat and human TRH-R translation initiation codons](image)

There is no Kozak consensus sequence in either the rat or the human TRH-R cDNA sequences, although the -3 and +4 positions have the A and G nucleotides implicated by Kozak in efficient eukaryotic ribosomal function (Kozak, 1981).

Although removal of the 3' and 5' UTRs of the rat and human TRH-Rs reduces levels of receptor expression, some intrinsic sequence remains within the coding region of the receptors which appears to mediate expression, since the rTRH-R retains its superior expression in the absence of UTR sequences. The other significant difference between the rat, mouse and human TRH-Rs exists in the carboxy tail region where
there exists a point of sequence divergence (at amino acid 392), with each species having a different receptor carboxy tail which exhibits no comparative sequence homology. The rTRH-R has the longest tail, 19 amino acids after position 392, the human has 5 amino acids after this same position and the mouse has the shortest tail with only one amino acid following 392. The carboxy tail of GPCRs has been implicated in receptor desensitisation (Hausdorff et al, 1991) and receptor internalisation (Nussenzveig et al, 1993), and may also be involved in directing receptor peptides to the cell membrane, although expression of a mTRH-R with a truncated carboxy tail is still targeted to the cell membrane (Yeaman et al, 1996).

There are other more subtle amino acid differences between the rat and human TRH-Rs which may create the molecular signals which direct receptor expression to the cell surface. There are more than ten sites at which the hTRH-R has unique amino acids as compared to the rat and mouse isoforms. Whether these differences are functionally significant has not been determined to date.

In conclusion, the residues targeted by sequence analogy to the results obtained from other mutant GPCRs do not produce the same results in the hTRH-R, indicating that the function of amino acid residues is dictated by the context in which that residue exists, and not only the characteristics of that particular amino acid. The increased expression of mutant hTRH-Rs was an unexpected but interesting finding, the cause(s) of which remain(s) to be determined. The reasons for decreased hTRH-R expression level compared to the rTRH-R also remain elusive. In order to study the hTRH-R further it will be necessary to increase and stabilise its expression, either by introducing enhancing elements such as a Kozak sequence, or by using an IRES expression vector. Stable hTRH-R cell lines would be highly useful for the study of this receptor in pituitary and in extra-pituitary cells in the absence of available human tissue samples or appropriate animal models, especially for screening novel drugs. The murine TRH-R has been successfully expressed in a variety of cell types using recombinant, replication-defective adenovirus vectors (Falck-Pedersen et al, 1994; Wolff et al, 1996; Yeaman et al, 1996). This method of gene transfer allows more accurate manipulation of the number of expressed receptors (as compared to plasmid vector transfection) as this parameter is directly proportional to the multiplicity of viral particle infection. It should also be possible to co-infect with different adenovirus constructs in order to express multiple peptides in the same cell, such as other GPCRs or components of intracellular effector systems not normally produced by the chosen cell type.
Chapter 7
Isolation and characterisation of the 5'-flanking region of the mTRH-R gene

7.1 Introduction

Thyrotrophin-releasing hormone (TRH) plays an important role in the control of the hypothalamic-pituitary-thyroid axis. It stimulates both synthesis and release of TSH from the thyrotrophs of the anterior pituitary. In addition TRH causes increased synthesis and release of prolactin from the lactotrophs, although the physiological significance of this is unclear. TRH has also been implicated in both affective disorders and motor neurone diseases (Munsat et al, 1989; Nemeroff and Evans, 1989). TRH exerts its actions by binding to specific high affinity GPCRs on its target cells. Binding studies have shown that the TRH-R is expressed in a tissue specific manner. High affinity binding sites have been demonstrated in specific cells of the anterior pituitary, i.e. lactotrophs and thyrotrophs (Hinkle, 1989), various regions of the brain, in particular the amygdala, and in the anterior horn cells of the spinal cord (Satoh et al, 1993b).

The mouse TRH-R (mTRH-R) was cloned from a TiT97 mouse thyrotrophic tumour cDNA library (Straub et al, 1990), and this was quickly followed by the isolation of TRH-R cDNAs from rat (de la Pena et al, 1992a; Sellar et al, 1993; Zhao et al, 1992) and human (Duthie et al, 1993a) tissues. These cDNAs have been used to further investigate the expression and regulation of the TRH-R, in particular to demonstrate a more detailed pattern of expression of the TRH-R in the brain (Zabavnik et al, 1993) and spinal cord, and in other tissues (Satoh et al, 1993b).

In pituitary cells, both homologous and heterologous hormonal regulation of TRH-R concentration, as measured by [3H]-TRH binding, has been demonstrated (de Lean et al, 1977b; Gershengorn, 1978; Gershengorn et al, 1979; Hinkle and Tashjian, 1975; Perrone and Hinkle, 1978). The ability to modulate gene expression in response to extracellular signals is a basic property of all living cells. The mechanisms whereby specific genes are expressed in a temporal or tissue-specific manner or are activated in response to extracellular inducers have been studied extensively by molecular
biologists. It has been discovered that cis-acting DNA sequences are required for eukaryotic gene regulation. The DNA sequences involved in transcriptional control are short stretches of nucleotides or modular recognition elements which interact with specific transcription factors (trans-acting elements). Positive and negative regulatory elements which function in a cell-specific manner or in response to extracellular inducers have been identified. In addition, pre-existing transcription factors may be covalently or allosterically modified presenting a further level at which gene regulation may be modified.

Additional studies have shown that the TRH-R is regulated in pituitary cells at the pre-translational level by oestrogens (Kimura et al, 1994), glucocorticoids (Yang and Tashijian, 1993) and thyroid hormones (Yamada et al, 1992), and that for the steroid hormones, this regulation occurs at the level of transcription (Kimura et al, 1994; Yang and Tashijian, 1993). The mechanism by which TRH decreases TRH-R mRNA levels appears to be mainly post-transcriptional (Fujimoto et al, 1992; Narayanan et al, 1992), though there is some evidence of transcriptional regulation (Yang and Tashijian, 1993). Both TRH itself and thyroid hormones down-regulate TRH-R number in pituitary cells (Gershengorn, 1978), whereas oestrogens and glucocorticoids increase receptor number (Gershengorn et al, 1979; Yang and Tashijian, 1993).

In order to study the factors involved in the tissue specific expression and transcriptional regulation of the mTRH-R gene, a 6.5kb genomic fragment containing sequences upstream of the translation initiation codon has been isolated and sequenced. This has been the preliminary step in identification of the sequences necessary for expression and regulation of the TRH-R gene in pituitary cells. In addition to the identification of the elements of the TRH-R promoter which regulate receptor expression, the information regarding the TRH-R gene structure is essential for designing transgenic and receptor knock-out animal studies. The technique of homologous recombination in embryonic stem cells can be used to generate a mouse lacking the TRH-R gene. This mutant mouse model will enable the determination of the exact role that TRH input via the TRH-R plays in the control of the hypothalamo-pituitary-thyroid axis and also its role in PRL secretion and lactation. TRH and its receptor are also found in many extra-pituitary locations including the pancreas, the prostate, the testis and the CNS, in particular the amygdala and spinal cord. TRH has been shown to have effects on cognition, motor function and recovery after spinal injury in both experimental models and in clinical trials. The TRH-R deficient mice will advance our understanding of the role TRH plays in the development and function
of the CNS. The effects of this knockout mutation will also provide information on the role of TRH in other extra-pituitary locations as mentioned above.

7.2 Materials and methods

7.2.1 Isolation of the upstream 5'-flanking region of the mTRH-R gene

A mouse genomic library constructed in EMBL3 (Clontech) was screened with a \[^{32}\text{P}\]-dCTP labelled 2.5 kb fragment of the rTRH-R cDNA (Sellar \textit{et al}, 1993). Hybridisation was performed at 42°C in hybridisation buffer containing 50% formamide according to standard procedures. Five positive clones were isolated from a total of 6 x 10^5 screened (Duthie \textit{et al}, 1993b). Phage DNA from isolated clones was purified and digested with restriction endonucleases. Southern blots of digested genomic DNA clones were probed with \[^{32}\text{P}\]\label{label} labelled oligonucleotides corresponding to different regions of the mouse TRH receptor. Clone 9, hybridising to an oligonucleotide representing nucleotides-155 to -138 of the mTRH-R sequence was approximately 19kb. This clone was digested with BglII and a 6.5kb fragment, also hybridising to a probe in the same region (-156 to -181) was subcloned into the Bam H1 site of pBluescript II (Stratagene). Sequencing was carried out on as described in Section 3.5.

7.2.2 5' RACE analysis

Normal mouse pituitary RNA was used as template for 5' RACE as described in Section 3.21. As described, three gene specific primers (SP1, SP2 and SP3) were designed to anneal regions within the 5'-flanking region of the mTRH-R gene (Duthie \textit{et al}, 1993b). Primer sequences were as follows: SP1 was oligo 382 (Table 7.1) which anneals at a position spanning the translation start site (-22 to +27), SP2 was 5'GACAGAAAGGTGAAGGCTG3' (-68 to -50) and SP3 was 5'CAGGCAGCGTGACAGAGTG3' (-119 to -101). The SP1 primer was used to transcribe the first strand cDNA from the RNA template and SP2 and SP3 were used as nested primers to increase amplification and specificity of the PCR reactions. The resulting PCR products were separated on a 10-20% polyacrylamide precast minigel (Bio-Rad) and stained with ethidium bromide following electrophoresis.
7.2.3 Construction of pGL-3 constructs for functional study of promoter activity

Figure 7.1 shows the relative positions of PCR primers used to amplify different length fragments of the genomic mTRH-R gene upstream region. Table 7.1 gives the exact sequences of the PCR primers and the sizes of the PCR bands amplified.

**Figure 7.1 The mTRH-R 5' flanking promoter containing region**

The 5' flanking region of the mTRH-R gene is numbered with 1 at the A of the ATG translation start site. The genomic DNA sequences amplified and subcloned into the luc reporter vector are shown by the double-ended arrows at the bottom of the figure (not to scale). Primer PX2 (annealing at -182 to -157) was used as an anchor primer with primers 450, 750 and 1000. These 5' primers anneal to the sequence at -609 to -640, -902 to -619 and -1154 to -1177 respectively. Primers 382 (-22 to +27) and 2000 (-2050 to -2030) resulted in production of a large PCR fragment (2700 bp) as it included the intronic sequence (645 bp intron at position -95/-96) which was shortened by restriction digest with the enzymes Kpn I and Xho I to just 1045 bp. All four of the fragments generated were inserted into the cloning site of the promotorless luc reporter vector pGL3. The position of the most likely CAATT and TATAA sequences are shown. The DNR spans a total of 74 bp from -396 to -470.
Chapter 7  Isolation and characterisation of the mTRH-R 5'-flanking region

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence (5'-3')</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PX2 &lt;</td>
<td>CCAATCCTGCAGAAGAAATCTTGTCC</td>
<td></td>
</tr>
<tr>
<td>450 &gt;</td>
<td>ATTTCAAGCACGTCACGGCACAAGAGTTTCTG</td>
<td>452</td>
</tr>
<tr>
<td>750 &gt;</td>
<td>CCCCTCTGACTCCTGACC</td>
<td>763</td>
</tr>
<tr>
<td>1000 &gt;</td>
<td>GGACTGCAGTCTCTTTGGGCTAG</td>
<td>997</td>
</tr>
<tr>
<td>382/SP1 &lt;</td>
<td>GGAGATATGATACTGTCAGTGAATG</td>
<td></td>
</tr>
<tr>
<td>2000 &gt;</td>
<td>CAGTGATGGCCGACTAGACC</td>
<td>2700</td>
</tr>
</tbody>
</table>

Table 7.1  Sequences of the primers used to amplify segments of the mTRH-R promoter region.

The sequences are shown in the 5'-3' direction. < indicates priming at the 3' end of the sequence and > indicates the 5' primers. PX2 was used as the 3' primer in separate PCR reactions with 450, 750 and 1000. Primer 382/SP1 was used with primer 2000 in another PCR reaction. The sizes of the PCR bands produced using these primers are given in bp in the last column of the table and are illustrated in Figure 7.1 above.

PX2 was used as an anchor primer with primers 450, 750 and 1000 giving 3 different PCR bands whose sizes (in bp) are shown in the last column of Table 7.1. The PCR products were TA cloned and then subcloned into the Kpn I/Xho I sites of the poly linker of the pGL3 luciferase (luc) reporter vector. Primers 382/SP1 and 2000 were used together to generate a PCR band of 2.0kb which contained an endogenous Kpn I site at -1045 of the promoter sequence. This large PCR product (as shown in Figure 7.1) was restriction digested from its TA clone with Kpn I (which cuts within the promoter sequence) and Xho I (cutting within the multiple cloning site of the TA vector sequence). The resulting fragment (1045bp) was subcloned into pGL3-basic as described above. The luc expression constructs were sequenced to confirm the presence and orientation of the promoter fragments. The resulting constructs were now referred to as -450TRH-R-luc, -750TRH-R-luc, -1000TRH-R-luc and -1045TRH-R-luc.
7.2.4 Transient transfections

5μg of each TRH-R-luc construct was transfected into GH3, GH4C1 and COS-1 cells, along with 2μg of pSVβ-gal construct, used to control for transfection efficiency. 5μg of the pGL3-basic and pGL3-control luciferase vectors, again along with the pSVβ-gal vector were transfected into replicate dishes as negative and positive controls respectively. The cells were transfected in 60mm tissue culture plates using 5μl Transfectam™/well or were electroporated as described in Chapter 3. Cells were harvested 48 hours after transfection into 400μl of Reporter Lysis Buffer (Promega) and luciferase activity of the cell extracts determined using the Luciferase Assay System (Promega). β-gal activity was measured using the β-galactosidase Assay System (Promega). Total proteins were determined using the BioRad protein assay method. Relative luciferase activity was determined (light units/β-gal/mg protein) and results for each construct were compared using the Students t-test (*p<0.05).
Chapter 7  Isolation and characterisation of the mTRH-R 5'-flanking region

7.3  Results

7.3.1  Sequencing of the 5'-flanking region of the mTRH-R gene.

Figure 7.2 shows the nucleotide sequence of the first 2251 nucleotides of the mTRH-R gene upstream from the ATG translation initiation codon. Sequence analysis reveals a long dinucleotide repeat (DNR), d(TG)$_{16}$d(AG)$_{21}$ upstream of position -396 and an intron at -95/-96 with respect to the initiation codon as previously described (Duthie et al., 1993b). There are consensus CAATT and TATAA motifs at -780/-775 and -756/-752 respectively, at -1562/-1557 and -1468/-1464 respectively and a TATAA motif at -2186/-2182 (highlighted in Figure 7.2). However, none of these sites are within an appropriate distance from the position of the possible transcriptional start site around -348 as indicated by alignment of the human$^1$ and mouse 5'-flanking sequences (Figure 7.5). There are other putative consensus motif sequences which may be the binding sites for several regulatory elements/transcription factors (Figure 7.2 and summary in Table 7.2).

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$^1$A paper by Iwasaki et al describing the promoter of the hTRH-R has recently been published (during the course of this work) (Iwasaki et al., 1996).
Figure 7.2  Nucleotide sequence of the mTRH-R gene 5'-flanking region
Sequences of interest as summarised in table 7.2 are highlighted above. Consensus sites are underlined and labelled accordingly. Only one of the E-box (Yoon and Chikaraishi, 1992) sites is shown (indicated by an asterisk in Table 7.2) and none of the PEA-3 (Wasylyk et al., 1990) sites are shown as they are too numerous. Only the perfect match Sp-1 (Schmidt et al., 1989) site is shown. The positions of CAAATT and TATAAA boxes are indicated in bold and bold/underlined text respectively and the location of the 3' splice site intron junction is shown as bold GG at position -95/-96. The numbering is relative to the ATG translation initiation site in which the adenosine is numbered +1.
Table 7.2  Putative transcription factor binding sites of the mTRH-R 5'-flanking region (following page)

Sequence analysis of approximately 2kb of the mTRH-R 5'-flanking region sequenced reveals the locations of putative cis-active binding sites for several regulatory trans-acting factors, including Pit-1 (Kapiloff et al., 1991), AP-1 (Angel et al., 1987), AP-2 (Imagawa et al., 1987), AP-3 (Mitchell et al., 1987), GRE (glucocorticoid response element; (Forman and Samuels, 1990)), ER (oestrogen receptor binding site; (de Verneuil and Metzger, 1990; Forman and Samuels, 1990)), E-box (Yoon and Chikaraishi, 1992), PEA-3 (Wasylyk et al., 1990), TRE (thyroid hormone response element) (Forman and Samuels, 1990; Forrest et al., 1991) and Sp1 (Schmidt et al., 1989). * indicates a site where the central NN nucleotides are GG in the indicated binding location, a motif which often appears in this cis-active site.
<table>
<thead>
<tr>
<th>Site</th>
<th>Consensus sequence (5'-3')</th>
<th>Position</th>
<th>Match</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pit-1</td>
<td>(A/T)TAT(C/T)CAT</td>
<td>-1998/-1991</td>
<td>7/8</td>
</tr>
<tr>
<td>AP-1</td>
<td>TGA(G/G)T(C/A)A</td>
<td>-2110/-2104</td>
<td>6/7</td>
</tr>
<tr>
<td>AP-2</td>
<td>CCC(A/C)N(G/C)3</td>
<td>-2251/-2244</td>
<td>7/8</td>
</tr>
<tr>
<td>AP-3</td>
<td>TGTGG(A/T)3</td>
<td>-1452/-1445</td>
<td>7/8</td>
</tr>
<tr>
<td>Sp-1</td>
<td>(G/T)(G/A)GGC(G/T)(G/A)(G/A)(G/T)</td>
<td>-1324/-13167</td>
<td>9/9</td>
</tr>
<tr>
<td>GRE</td>
<td>AGAACAN3TGTTCT</td>
<td>-2083/-2069</td>
<td>11/15</td>
</tr>
<tr>
<td>ER</td>
<td>AGGTCAN3TCACCT</td>
<td>-569/-555</td>
<td>12/15</td>
</tr>
<tr>
<td>E-Box</td>
<td>CANNTG</td>
<td>-2121/-2116</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-1920/-1915</td>
<td>6/6</td>
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<td>-1569/-1564</td>
<td>6/6</td>
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<td>-1244/-1239*</td>
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<td>-1230/-1225</td>
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<td>-1004/-999</td>
<td>6/6</td>
</tr>
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<td></td>
<td></td>
<td>-488/-483</td>
<td>6/6</td>
</tr>
<tr>
<td>PEA-3</td>
<td>AGGAA(G/A)</td>
<td>-1835/-1830</td>
<td>5/6</td>
</tr>
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<td>-1747/-1742</td>
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<td>-95/-90</td>
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<td>-66/-61</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>-51/-65</td>
<td>5/6</td>
</tr>
<tr>
<td>TRE</td>
<td>(AGGTCA)2</td>
<td>-1408/-1397</td>
<td>8/12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-1229/-1218</td>
<td>8/12</td>
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<tr>
<td></td>
<td></td>
<td>-1063/-1052</td>
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<td></td>
<td></td>
<td>-806/-795</td>
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<td></td>
<td></td>
<td>-599/-588</td>
<td>8/12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-502/-491</td>
<td>8/12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-61/-49</td>
<td>8/12</td>
</tr>
</tbody>
</table>
It is not known how much of the sequenced 5'-flanking region appears in the mRNA for the mTRH-R. It is feasible that some of this sequence may be spliced out of the primary transcript prior to nuclear export to the ribosomes for translation. Analysis of the sequence of the mTRH-R 5'-flanking region reveals several sites which adhere to the consensus sequences for 5' splice donor and 3' splice acceptor sites (Senapathy et al, 1990). The positions and sequences of these sites are given in Table 7.3 below.

<table>
<thead>
<tr>
<th>Consensus sequence</th>
<th>5' donor splice site/position</th>
<th>3' acceptor splice site/position</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAGGTAAGT</td>
<td>YYYYYYYYYYNCAGR</td>
<td></td>
</tr>
<tr>
<td>CTGGTTAGT (-2089/-2081)</td>
<td>CTTTTGCCTTACAGA (-1067/-1593)</td>
<td></td>
</tr>
<tr>
<td>AAATGTAAGG (-1064/-1056)</td>
<td>CCTTTTCACTGTAAGG (-1335/-1321)</td>
<td></td>
</tr>
<tr>
<td>AAGGTACCT (-1051/-1043)</td>
<td>GAATTTCTTCTGCAGG (-185/-172)</td>
<td></td>
</tr>
<tr>
<td>AAAGTAAAT (-487/-479)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAGGGATGT (-268/-260)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTGCCTAAGT (-221/-213)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7.3 Positions of putative splice sites
Sequence analysis of the mTRH-R 5'-flanking region reveals several potential splice donor/acceptor sites. The consensus sequences for these sites are given in the top row. The sequences located at the given positions are shown with mismatches shown in bold type. Y= C or T, R= A or G, M= A or C and N= any nucleotide.
7.3.2 5' RACE analysis of mouse pituitary RNA

Figure 7.3 shows a photographic representation of the PCR bands produced by 5' RACE analysis. The upper panel shows the products of the first PCR reaction using SP2 and the d(T) PCR primer supplied by the kit manufacturer, Boehringer Mannheim. This reaction produced a band of approximately 270bp as compared to the size standards. The 234bp band was produced by the kit control to indicate that the reverse transcriptase step had produced amplifiable cDNA. The lower panel shows that the 270bp band was reduced to approximately 220bp following nested PCR with SP3 (which anneals to the 5'-flanking sequence 50bp upstream of SP2) and the PCR anchor primer (again supplied as a kit component). SP3 anneals at a position 101bp upstream from the ATG transcription start site so this would give a position of -321 for the transcription start site. However, the d(T) PCR primer adds 39bp onto the length of the PCR product, so subtracting this from the initial position of -321 results in a possible transcription start site at -282.

Figure 7.3 5' RACE analysis of the mTRH-R 5'-flanking region (following page)

The upper panel shows the results of PCR amplification of reverse transcriptase derived cDNA from both the control RNA supplied in the 5'/3' RACE kit (Boehringer Mannheim) and mouse total pituitary RNA. The control sample cDNA was amplified in the same reaction tube as the sample cDNA and gave a band of 234bp as expected (when compared to the DNA size markers electrophoresed in the lane labelled pGem markers). The sample cDNA gave a band of 270bp when amplified with the d(T) PCR primer (supplied with the kit) and SP2. This sample band was reduced to 220bp on a second amplification with the PCR anchor primer (supplied with kit) and SP3. The control amplification again gave the expected sized band (157bp) as shown in the lower panel. Negative control samples were performed at each step. In the upper panel (first PCR) RNA was added to the PCR reaction components to discount the possibility of DNA contamination of the RNA samples (labelled 'no RT'). In the second PCR reaction all components except first round PCR DNA was added (labelled '-ve control') to eliminate the possibility of DNA contamination of reaction components.
Chapter 7  Isolation and characterisation of the mTRH-R 5'-flanking region

approx 270 → 234

no RT  mouse pit RNA  pGem markers

approx 220 → 157

mouse pit RNA -ve control pGem markers
7.3.3 Expression of -1045TRH-R-luc in pituitary and non-pituitary cells

-1045TRH-R-luc induced a 3.85-fold increase in luciferase activity in GH3 cells compared to the pGL3 vector alone, and when this same construct was transfected into COS-1 cells, which are derived from monkey kidney and do not normally express the TRH-R, no increase in luciferase activity was seen (Figure 7.4). These findings indicate that this fragment of the 5'-flanking region contains sequences necessary for expression of the TRH-R gene specifically in pituitary cells.

![Graph showing luciferase activity](image)

**Figure 7.4** Expression of -1045TRH-R-luc in pituitary and non-pituitary cells

GH3 and COS-1 cells were co-transfected with -1045TRH-R-luc and pSVβ-gal. Luciferase and β-gal levels were measured 48 hours later using the appropriate assay system (Promega). Luc activity was corrected for β-gal activity (to control for transfection efficiency) and total protein content (to control for cell number). pGL3 (without insert/promoter) transfected cells were used as a negative control and the pGL3 control vector (in which luciferase transcription is controlled by the SV40 promoter) was used as a positive control. Assay points are the mean of triplicate samples (± SEM, n=3). Significance was determined when p<0.05 and is indicated by asterisks.
3.4 Discussion

The mTRH-R cDNA (Straub et al, 1990) contains 265bp of true 5' UTR which indicates that the mRNA must be greater or equal in length to this sequence. The rTRH-R cDNA (Sellar et al, 1993) has 340bp of 5' UTR indicating that this is contained in the mature mRNA and that the transcription start site must be further 5' of this location. Alignment of the mouse and rat 5' UTR regions reveals a high level of sequence homology. Analysis of the sequence of the mTRH-R 5'-flanking region reveals two perfect CAATT and TATAA pairs at positions -780 and -756, and -1562 and -1458, along with a further TATAA motif at -2186 (shown in Figure 7.2). Although the CAATT/TATAA pairs are spaced at appropriate distances from each other, none of these are at the correct distance from the translation initiation start site. The TATA box is usually centred at -28 relative to the transcription initiation site and the CAATT box is most often found between -50 and -129 (Bucher and Tifonov, 1986). The absence of typical TATAA and CAATT consensus sequences is characteristic of other G-protein-coupled receptors such as those for GnRH (Albarracin et al, 1994), TSH (Ikuyama et al, 1992), LH (Huhtaniemi et al, 1992; Tsai-Morris et al, 1993) and FSH (Heckert et al, 1992) as well as the melatonin 1a receptor (Roca et al, 1996), and instead possibly use other elements such as the GATA motif. The GPCR genes may therefore be regulated by a common yet alternate mechanism. Initial results using normal mouse pituitary total RNA in 5' RACE analysis suggests the existence of a transcription start site in the region of -282 with respect to the translation initiation codon. The precise position of this site requires the cloning and sequencing of the PCR product which implicates this site. The analogous upstream region of the hTRH-R has been recently cloned (Iwasaki et al, 1996), and alignment of this sequence with the equivalent mouse sequence shows a remarkable level of homology (Figure 7.5). The transcription initiation site of the human sequence is at the adenosine base at -344 (marked by an asterisk in Figure 7.5), directly adjacent to the 3' end of the DNR which appears in similar positions in both species. Alignment of the analogous human sequence indicates that the transcription start sites for both of these TRH-R genes are likely to be in close proximity, around -344 which is the transcription start site for the hTRH-R mRNA. The analogous position is -348 in the mTRH-R gene so it is therefore feasible that the transcriptional initiation site of both of these genes will be found to lie at homologous positions with respect to the ATG translation start site as this area of both promoter regions is almost identical. The nucleotide homology continues beyond -650, to at least -800. It may be possible to determine whether the mTRH-R mRNA extends beyond the -282 position.
by performing RT-PCR using an upstream primer based on the sequence alignment shown in Figure 7.5. Choosing sequence spanning -348 to synthesise a PCR primer should allow detection of this longer RNA transcript if it exists in cDNA reverse transcribed from mouse pituitary RNA extracts. However, the presence of the transcription site in such close proximity to the DNR may interfere with PCR based techniques as such a sequence is both difficult to amplify and PCR primers cannot be made to this type of repeat element as they will form primer-dimer complexes.

Table 7.2 outlines the putative transcription factor consensus binding sites identified in the mTRH-R gene upstream region. TRH-R expression in the pituitary, in lactotrophs and thyrotrophs (Hinkle, 1989), is restricted to cell types that are known to express and translate the pituitary specific transcription factor, Pit-1. Therefore, it might be expected that the regulatory region of the TRH-R gene would contain binding sites for Pit-1, as is the case for the growth hormone and prolactin genes. Because TRH-R mRNA has been identified in PRL- and GH-producing pituitary tumours (Kaji et al, 1995), Pit-1 may be involved in the regulation of anterior pituitary TRH-Rs through activation of the TRH-R gene. Sequence analysis indicates the existence of a possible Pit-1 binding site at -1998/-1991 on the coding strand of the TRH-R promoter region. There are several consensus binding site matches for the transcription factor Sp-1 (-1324/-1316, -1742/-1734, -1865/-1857 and -1853/-1845) which is thought to be involved in modulating the activity of essential genes. There are putative binding sites for the activator protein factors AP-1 (-2110/-2104), AP-2 (-2251/-2244) and AP-3 (-1452/-1445) and these may be involved in the down-regulation of TRH-R mRNA levels by phorbol esters (Fujimoto et al, 1991). A consensus motif known as the E-box (an enhancer box recognition site for helix-loop-helix regulatory proteins) may also be involved in the regulatory effects of phorbol esters upon TRH-R transcription. There are approximately twenty putative PEA-3 binding sites, which may be targets for transcriptional control via signal transduction-linked mechanisms (see Table 7.2). TRH-R transcription may be regulated by oestrogen to a certain extent (Kimura et al, 1994), and might be mediated via the putative oestrogen receptor binding site located at -569/-555.

Initial transfection studies suggest that -1045TRH-R-luc is sufficient to promote luciferase activity in a pituitary cell specific manner. Given that these experiments were performed in rat GH3 cells, this indicates that cross-species transcriptional control is possible at least in vitro. In addition, the hTRH-R promoter was also active in rat GH4C1 cells. It is noteworthy that the untranslated region of the rTRH-R cDNA is also highly homologous to the mouse and human 5'-flanking regions. It
would be interesting to investigate the cellular specificity of TRH-R expression, to ascertain whether pituitary cells of non-thyrotrophic origin (α-T3-1 gonadotroph cells or AtT20 corticotroph cells, for example) will express this gene. There is evidence which indicates that the AtT20 cell line (an adrenocorticotroph tumour cell line) expresses cell surface TRH-Rs (Gershengorn et al, 1980), and may therefore possess the machinery required for transcriptional initiation of this receptor gene.

Several different approaches are available to determine whether any of these putative sites are involved in the control of TRH-R transcription. One method involves mutation or deletion of consensus sites followed by measurement of the resulting effects on promoter-induced luciferase activity as previously described for the -1045TRH-R-luc construct. Luciferase activity would be expected to be lost when regulatory sites in the RNA are mutated/deleted and can therefore no longer bind trans-activating protein factors. Alternately, antisense oligonucleotides which bind to the RNA and therefore block protein-RNA interactions could be tested in similar protocols. Blocking regulatory sites in this way would result in a loss of luciferase expression. It is possible to determine the presence of nuclear proteins which bind to DNA molecules using a method which detects DNA-protein interactions as a function of electrophoretic gel-shifts. In essence, this assay involves the radio-labelling of an oligonucleotide probe which mimics a consensus transcription factor binding site, and mixing of this probe with nuclear proteins extracted from tissues or cell lines which are known to express the gene of interest. A DNA-protein complex will form if any transcription factors are present which recognise and bind to the probe. When electrophoresed through a polyacrylamide gel alongside the native probe the DNA-protein complex should be retarded in its migration and should produce a higher band on the resulting autoradiographic image. This is known as a gel shift. Specific antibodies to many transcription factors are available and if added to an appropriate DNA-protein complex and electrophoresed alongside the samples described above should result in a 'super shift', or further migratory retardation, confirming the identity of the protein which has bound the oligonucleotide probe.

The DNR element (d(TG)_{16}.d(AG)_{21}) of the 5' flanking sequence may be involved in modulation of gene replication, transcription and recombination. It has been suggested that an alternating purine-pyrimidine sequence such as the TG repeat may allow the DNA to assume its lowest energy state, Z-DNA (Rich et al, 1984). Z-DNA adopts a left-handed helical conformation as opposed to the more characteristic B-DNA which forms right-handed helices. Z-DNA is often found in controlling regions of genes (Schroth et al, 1992) where supercoiling of the DNA permits switching between B-
and Z-DNA which is perhaps responsible for the activity of such regions as enhancers or promoters for example (Liu and Wang, 1987). The rat prolactin gene 5' UTR (Naylor and Clark, 1990) and the rat ceruloplasmin gene 5' UTR (Fleming and Gitlin, 1992)) both contain similar DNR sequences which have been shown to exert negative effects on gene transcription. The gene encoding the substance P receptor, a GPCR, encodes a d(GT)\(_n\) sequence in its 5' UTR and a d(CT)\(_m\).d(CA)\(_n\) sequence in its 3' UTR, although the function of these regions is not known. Expression of the comparable region from the mTRH-R gene in a reporter vector may allow determination of the function of this region in the control of gene transcription. Alternately, the DNR may be spliced out of the mature mRNA as splice donor and acceptor consensus sequences have been identified in the 5'-flanking region of the mTRH-R gene, and the DNR may therefore play no significant role in transcriptional regulation of this receptor.

Figure 7.5  Alignment of the nucleotide sequences of the mouse and human upstream regions (following page)

The first 650 bp of the 5'-flanking region of the mouse and human TRH-R genes are aligned to show areas of sequence homology (indicated by dots between the lines of nucleotides), excluding the intronic sequences which were removed at the positions of the arrows (between -96/-95 in the mouse and -89/-88 in the human). The sequences are numbered with +1 being the adenosine of the translation initiation ATG/Met codon (shown in shaded box). The upper lines show the mouse sequence and the lower lines represent the equivalent region of the human sequence. The position of the exact transcription start site for the human TRH-R promoter is marked by an asterisk (adenosine at -344).
Chapter 7  Isolation and characterisation of the mTRH-R 5'-flanking region

-650 -640 -630 -620 -610 -600 -590 -580

**Mouse**
In conclusion, we have isolated a 6.5kb genomic fragment of the mTRH-R gene, of which almost 2.3kb of the 5'-flanking region (upstream of the translation initiation codon) has been sequenced. Several putative transcription factor binding sites for factors known to affect TRH-R transcription can be identified in the sequence although many of these are not perfect matches to the consensus sequences. However, the contribution of these sites to control of TRH-R expression should not be discounted until further studies have been performed. Resection or mutation of the promoter region should provide more accurate information as to the role of regulatory protein binding sites in both the functional and tissue-specific control of TRH-R transcription. Although the transcription start site for the mTRH-R has not been conclusively identified it is possible that the site identified at -282 is one of multiple transcription start sites. Several other GPCR genes have been found to be transcribed from multiple positions within their promoters (Roca et al, 1996). It is note-worthy that no appropriately located TATA or CAATT boxes are to be found in the mTRH-R gene as this is appearing as a common feature of GPCRs, suggesting that these proteins share an as yet unidentified, but common mechanism of transcriptional initiation. However, given that the exact location of the transcriptional start site of the mTRH-R gene remains elusive, and that the existence and use of possible intron splice sites (shown in Table 7.3) remain to be determined, these identified sites may yet be found to be involved in transcriptional activation. However, none of the putative splice donor/acceptor pairs identified here would bring the CAATT and TATAA pairs closer to the site potential transcriptional initiation site at -282. Precise characterisation of the mTRH-R gene promoter region is essential if it is to be utilised in gene targeting and transgenic studies.
Chapter 8
Summary of results and concluding remarks

8.1 Concluding remarks

Cells are constantly bombarded by a multitude of external signals. Many of these signals bind to cell surface receptors to initiate a flow of information to the cell interior, rather than entering the cell directly. A group of membrane receptors which, despite sharing structural and sequence homologies, transmit signals as diverse as hormones, neurotransmitters, biogenic amines, odourants and light (see (Dohlman et al., 1991) for review) are coupled to a group of guanine-nucleotide binding proteins (G-proteins). These G-protein coupled receptors (GPCRs) regulate a variety of effector enzymes and ion channels. The cellular response to any given signal depends on the second messenger system affected by activation of the receptor concerned. The role of GPCRs in disease is now well established for many human conditions, as is the role of abnormally functioning G-proteins. The main aim of the work carried out for this thesis was to determine whether the TRH-R may also be a causative factor in human endocrine disease, including pituitary adenomas and central hypothyroidism.

The hTRH-R was found to retain normal structure in a sample of 50 various human pituitary adenoma tissues suggesting that TRH-R dysfunction is not responsible for the discordant and/or paradoxical effects of TRH in these patients. However, in an additional study, one patient (out of two studied) was found to carry two separate mutations in his genes for the TRH-R (M-STOP, which has a premature stop codon in the extracellular amino tail and F-TM3 which has a deletion and substitution in TM III). Both of these mutations result in inactive receptors with no measurable TRH binding. Whether this patient represents a TRH-R knockout remains to be determined, as there may be some residual receptor activity from the mutant F-TM3. Additionally, in vitro translation experiments show that there is a low level of translation from Met75, the next ATG in the reading frame of the hTRH-R cDNA. It is not known whether this second translation initiation site will be utilised in vivo, and whether it would bind ligand restoring TRH-induced cellular activity.
Site-directed mutational analysis of two amino acid residues in regions of GPCRs which are important for structure/function was performed with the aim of determining the significance of these residues in TRH-R function. Mutation of Thr265 resulted in mutant receptors which had characteristics of constitutive activity, although there was no significant increase in basal second messenger activity under the conditions used in these experiments.

The mTRH-R 5'-flanking region was isolated and sequenced in preparation for the generation of a transgenic mouse line. The location of the transcriptional initiation site was investigated using 5' RACE analysis and was found to reside potentially at a location 282bp upstream of the translational start site (ATG). It is interesting to note that the GnRH receptor RNA transcript may be initiated from one of several positions within the 5'-flanking region of this receptor (personal communication with Dr S Kakar, Department of Physiology and Biophysics, University of Alabama, USA). Sequence analysis of the 5'-flanking region of the mTRH-R identifies several putative sequences homologous to the consensus sites for transcription factor binding.

8.2 Directions for future study

With respect to mutational analysis of the hTRH-R the recent publication of the sequence of the human promoter region will allow analysis of this region for naturally occurring mutations which would alter TRH-R expression. These might arise either by mutating out the transcription start site or mutating sites crucial for the binding of transcription regulating proteins. The TRH-Rs of pituitary adenomas (especially in view of the fact that a study of two TSH-secreting adenomas showed that they had no measurable TRH binding) and of Patient 1 (see Chapter 5 in which Patient 1 was found to have a normal TRH-R coding region despite being unresponsive to TRH challenge) should be screened. Stable cell lines expressing the inactivating mutations identified in Patient 2 (Chapter 5) should be established to determine whether there is low level binding from either mutation and also whether a truncated receptor translated from Met75 is detectable in the cell membrane, either by radioligand binding or immunodetection of an epitope-tagged receptor construct. In order to help identify a cause for the poor expression of the hTRH-R as compared to the rTRH-R (Chapter 6) stable cell lines should be established in order to maximise the reproducibility of the experiments, as transient expression of the hTRH-R in particular can give quite variable results on occasion. Stable cell lines provide a source of cells which produce
large numbers of receptors and which therefore increase the ability to detect experimentally-induced alterations in receptor function for example. It would therefore be advantageous to establish cell lines stably expressing the potentially constitutively active T265 mutations of the hTRH-R (Chapter 6) which would allow more accurate determination of the effects of these mutations on receptor function. Such cell lines would be especially useful for the detection of changes in ligand-independent basal IP turnover. If these mutants do indeed prove to be active in the absence of ligand the effects of such receptors on cell morphology could be determined. Overactive receptors may enhance mitogenesis and tumorigenicity in cell cultures as described for the α1B-adrenergic receptor (Allen et al, 1991) which suggests that GPCRs may be involved in cellular transformation and tumour formation. Constitutively active GPCRs are also useful in the screening of compounds for inverse agonist activity (Barker et al, 1994), that is for the ability of a substance to switch off the conformationally active (in the absence of stimulating ligand) form of the receptor. A further experiment which would identify increased basal IP turnover would be to transfect cells with increasing amounts of receptor (WT or mutant) and then plot basal IP levels against B\text{max}. Increasing the dose of DNA transfected will increase the number of expressed receptors. If the mutant receptor is constitutively active to even a small degree this should become detectable as the receptor number increases. In comparison the WT receptor should show no significantly increased ligand-independent IP accumulation. Stable cell lines will be established using an IRES vector as described in Chapter 6.

The TRH-R promoter region can be further analysed by performing a 'footprint' assay which detects regions of DNA protected by binding of added DNA binding proteins. A ribonuclease protection assay will be carried out to identify any additional transcription initiation sites in addition to that found in the experiments carried in the Chapter 7 study. Mutation/deletion of the putative promoter region will allow determination of the effects of protein binding sites on the level of transcription and in fact whether substances such as oestradiol, glucocorticoids and phorbol esters are acting either directly or indirectly to alter TRH-R mRNA levels. In order to establish a transgenic mouse model of a TRH-R gene knockout, the exact location of the transcription start site must be known so that this portion of the gene can be deleted by homologous recombination and so produce a gene which cannot transcribe TRH-R RNA. It will be very interesting to investigate the effects of absent TRH-induced effects on foetal development and whether this would be a lethal genetic condition or not.
## Appendix I

Composition of buffers and stock solutions used in experimental procedures

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<th>Buffer</th>
<th>Component</th>
<th>Concentration (pH)</th>
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<tr>
<td>SSCP loading dye</td>
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<tr>
<td></td>
<td>xylene cyanol</td>
<td>0.05%</td>
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<tr>
<td>SDS-PAGE sample buffer</td>
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<tr>
<td>(1x)</td>
<td>SDS</td>
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<td></td>
<td>Tris.Cl (pH 6.8)</td>
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<td></td>
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<tr>
<td></td>
<td>glycerol</td>
<td>10%</td>
</tr>
<tr>
<td>10x tris-glycine electrophoresis buffer</td>
<td>Tris base</td>
<td>250mM</td>
</tr>
<tr>
<td></td>
<td>glycine</td>
<td>2.5M</td>
</tr>
<tr>
<td></td>
<td>SDS</td>
<td>1%</td>
</tr>
<tr>
<td>6x sample buffer (agarose gels)</td>
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</tr>
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<tr>
<td>10x TBE</td>
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<tr>
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<td>Boric acid</td>
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<tr>
<td></td>
<td>EDTA</td>
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<tr>
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<td></td>
<td>MgCl₂</td>
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<td>Buffer A (IP Assay)</td>
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<td>LB agar</td>
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<tr>
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<td>bacto yeast extract</td>
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<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>KCl</td>
<td>2.5mM</td>
</tr>
<tr>
<td></td>
<td>MgSO₄</td>
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<tr>
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<td>glucose</td>
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<th>Concentration (pH)</th>
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<td>Complete DMEM</td>
<td>L-glutamine</td>
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</tr>
<tr>
<td></td>
<td>penicillin</td>
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<tr>
<td></td>
<td>streptomycin</td>
<td>100μg/ml</td>
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<tr>
<td></td>
<td>HIFCS</td>
<td>10%</td>
</tr>
<tr>
<td>10x Trypsin-EDTA</td>
<td>trypsin</td>
<td>0.5%</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>2%</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>sodium acetate</td>
<td>2M (5.0)</td>
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Appendix II

Suppliers of consumables and equipment used

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<th>Supplier</th>
<th>Address</th>
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<tr>
<td>Ambion (AMS Biotechnology, UK, Ltd)</td>
<td>Oxon, UK</td>
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<tr>
<td>Amersham</td>
<td>Chester-le-Street, UK</td>
</tr>
<tr>
<td>Applied Biosystems</td>
<td>Warrington, UK</td>
</tr>
<tr>
<td>BIO-RAD</td>
<td>Hemel Hempsted, UK</td>
</tr>
<tr>
<td>BIO-101 (Stratech Scientific Ltd)</td>
<td>Luton, UK</td>
</tr>
<tr>
<td>Boehringer Mannheim</td>
<td>Lewes, UK</td>
</tr>
<tr>
<td>Cambridge Biosoft</td>
<td>Cambridge, UK</td>
</tr>
<tr>
<td>Costar</td>
<td>Cambridge, UK</td>
</tr>
<tr>
<td>CP Laboratories</td>
<td>Bishop's Stortford, UK</td>
</tr>
<tr>
<td>Falcon</td>
<td>Cowley, UK</td>
</tr>
<tr>
<td>Flowgen</td>
<td>High Wycombe, UK</td>
</tr>
<tr>
<td>GIBCO-BRL</td>
<td>Paisley, UK</td>
</tr>
<tr>
<td>Hoechst-Roussel Canada Inc</td>
<td>Montréal, Québec, Canada</td>
</tr>
<tr>
<td>Hybaid</td>
<td>Teddington, UK</td>
</tr>
<tr>
<td>Invitrogen</td>
<td>NV leek, the Netherlands</td>
</tr>
<tr>
<td>Kodak</td>
<td>Cambridge, UK</td>
</tr>
<tr>
<td>Kontron Instruments</td>
<td>Cumbernauld, UK</td>
</tr>
<tr>
<td>MBI Fermentas (IGi Immunogen Intnl, Sunderland, UK</td>
<td></td>
</tr>
<tr>
<td>DuPont UK, Ltd</td>
<td>Stevenage, UK</td>
</tr>
<tr>
<td>National Diagnostics</td>
<td>Atlanta, GA, USA</td>
</tr>
<tr>
<td>New England Biolabs</td>
<td>Hitchin, UK</td>
</tr>
<tr>
<td>Novagen</td>
<td>Madison, WI., USA</td>
</tr>
<tr>
<td>Peninsula</td>
<td>Belmont, CA, USA</td>
</tr>
<tr>
<td>Perkin Elmer Cetus</td>
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</tr>
<tr>
<td>Promega</td>
<td>Madison, WI, USA</td>
</tr>
<tr>
<td>Qiagen Ltd</td>
<td>Dorking, UK</td>
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<td>R&amp;D Systems Europe, Ltd</td>
<td>Abingdon, UK</td>
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<td>St. Albans, UK</td>
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<td>Sigma</td>
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<tr>
<td>Stratagene Ltd</td>
<td>Cambridge, UK</td>
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<tr>
<td>Vilber Lourmat</td>
<td>Cedex 2, France</td>
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<tr>
<td>Whatman LabSales Ltd</td>
<td>Maidstone, UK</td>
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</tbody>
</table>
Appendix III

Publications

Arising from the work carried out for this thesis:


Additional publications:


Attendance at scientific meetings:

1. **Faccenda E, Melmed S and Eidne K A.** Human PRL-, GH- and TSH-secreting pituitary adenomas harbor structurally intact dopamine type 2 and thyrotropin releasing hormone receptors. 77th Annual Meeting of the Endocrine Society, June 14-17, 1995: Washington DC, USA.


Bibliography


