

Novel splicing variants of the human thyrotropin receptor encode truncated polypeptides without a membrane-spanning domain

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The thyrotropin receptor is of fundamental importance to normal thyroid function and is considered to be the predominant antigen affected by the autoantibodies of Graves' autoimmune hyperthyroidism. The identification of the epitopes on the receptor to which the autoantibodies bind or the mechanism by which the autoantibodies arise remain to be established. In this report we have analysed in detail the *in vivo* transcription of the human TSH receptor gene (hTSH-R), demonstrating the presence of numerous novel TSH receptor transcripts. Northern blot analysis of mRNA from human thyroid tissue using a radiolabelled cDNA probe specific for the extracellular domain of the hTSH-R revealed the presence of small polyadenylated mRNAs, in addition to the full-length hTSH-R mRNA. A PCR strategy devised to clone transcripts with 3' polyadenylation and 5' hTSH-R specific sequences was used to clone five different hTSH-R transcripts (hTSH-R. ST1 to ST5; 250bp–1.7 kb) from human thyroid tissue. Sequence analysis demonstrated that the small transcripts arose by alternative splicing of the hTSH-R mRNA. The transcripts were associated with polysomes and were demonstrated in human thyroid tissue from patients suffering from Graves' disease, sporadic goiter as well as in healthy lobes of thyroid tissue. *In situ* hybridization demonstrated that two of the alternative transcripts adopted a tissue distribution pattern identical to that of the full-length hTSH-R transcript. The two major truncated transcripts ST4 and ST5 contained unique sequences at the 3' end of the mRNAs and thus potentially represent the molecular origin of soluble TSH receptor variants which have been postulated on numerous occasions.

Keywords: TSH receptor; alternative splicing; goiter and Graves' disease

Introduction

Pathological dysfunction associated with human thyroid stimulating hormone (hTSH) and its receptor (hTSH-R) is most profound in Graves' autoimmune hyperthyroidism; a condition of hyperthyroxemia and thyroid hyperplasia with low or undetectable levels of circulating TSH. The hyperstimulation of the thyroid in Graves' disease has been attributed to an autoimmune reaction, resulting in the production of a spec-

trum of autoantibodies some of which interact directly with the hTSH-R and induce chronic endocrine activity (Smith *et al.*, 1988). A temporal variation in the populations of these autoantibodies, which may either block or stimulate receptor activity, is offered as an explanation for the diversity of clinical symptoms and sequelae observed in Graves' disease. Recent data from this laboratory have shown the presence of 'soluble' TSH binding molecules in human thyroid tissue of Graves' patients (Hunt *et al.*, 1992a,b; Willey *et al.*, 1993). Indeed a number of recent studies have demonstrated that the extracellular portion of the hTSH-R is able to bind TSH (Shi *et al.*, 1993a). Such activities have been demonstrated either by transfection studies or with recombinant protein produced in insect cells (Gattadahalli *et al.*, 1994). In this current report a total of five alternatively-spliced variants of the hTSH-R are described, providing a possible molecular origin for these 'soluble' TSH binding entities. One of the five variants has been previously described in detail by Graves *et al.* (1992) and Takeshita *et al.* (1992). The relevance of such alternatively spliced molecules to thyroid activity, in general, and Graves' hyperthyroidism, in particular, is discussed.

Results

Human thyroid expresses several small hTSH-R transcripts

Total RNA was isolated from thyroid tissue removed by surgery from patients either suffering from Graves' disease, sporadic goiter or from non-pathological thyroid tissue. Total RNA samples were separated by electrophoresis and blotted as described in Materials and methods. The Northern blots were hybridized with a cDNA probe corresponding to the 5' portion of the extracellular domain of the hTSH-R and the results are shown in Figure 1.

In all RNA samples analysed, a transcript corresponding to the full-length hTSH-R (4.3 kb) was demonstrable. Indeed a transcript of approximately 4.2 kb can also be seen which represents a full length mRNA utilizing an alternative transcription start site as previously described (Gross *et al.*, 1991). In addition, two smaller transcripts of approximately 1.2 and 1.7 kb were also seen. These two transcripts were evident in all of the thyroid samples and upon first appraisal, at roughly equivalent levels to the full-length hTSH-R transcript. The small transcripts failed to hybridize with a probe originating from the membrane-

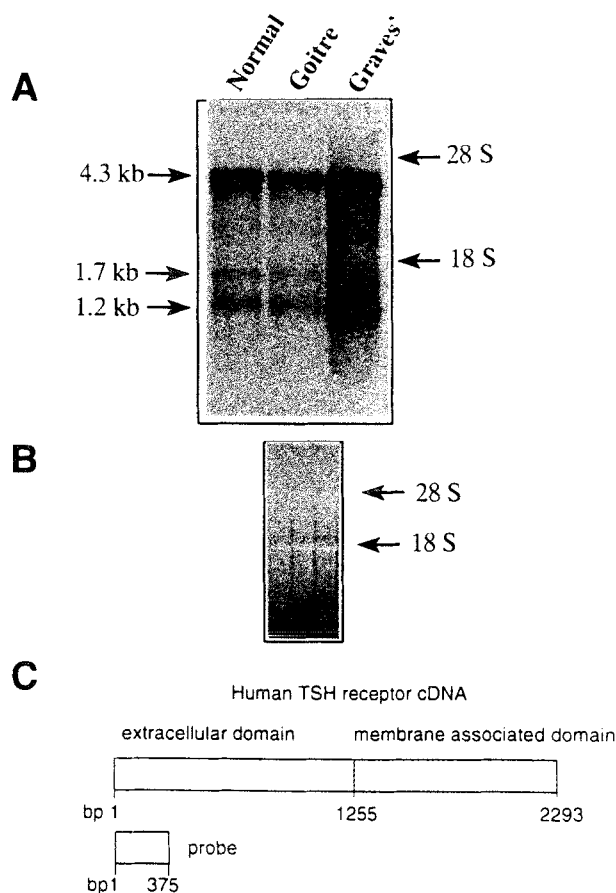


Figure 1 Expression of the hTSH-R in pathological and normal human thyroid. (A) 10 μ g samples of total RNA isolated from the thyroid of Graves, goitre or non-pathological origin were separated by denaturing agarose gel electrophoresis and blotted onto nylon nitrocellulose membranes as in Materials and methods. The membrane was hybridized under conditions with a radiolabelled probe corresponding to bp 1–375 of the extracellular domain of the hTSH-R. The signal was developed after a 24 hour exposure at -70°C with one intensifying screen. (B) RNA loading control of the ethidium bromide stained gel after electrophoresis over night at a constant 40V. Staining of the prominent 28S and 18S ribosomal bands demonstrate the loading and integrity of the RNA samples. (C) Structural map of the hTSH-R demonstrating the domains present within the coding region of the hTSH-R cDNA. The probe used in this experiment originates from the 5' end of the extracellular domain (bp 1–375).

spanning region of the hTSH-R (as previously described Graves *et al.*, 1992), suggesting that they arose by alternative processing and contained only sequences from the extracellular domain of the receptor. Detailed density scanning of the autoradiographs demonstrated that, although the absolute level of all transcripts varied for the different individuals and thyroid pathologies, the ratio of expression for the full-length receptor transcript and the 1.2 kb and 1.7 kb variants was always 4:2:1. The constancy of this ratio would suggest that the processing of all of the hTSH-R variants was under stringent control and did not differ in the two thyroid pathologies investigated here and in normal tissue.

The screening of >30 thyroid samples from normal subjects and those with Graves' disease or sporadic goitre have substantiated these findings (data not shown).

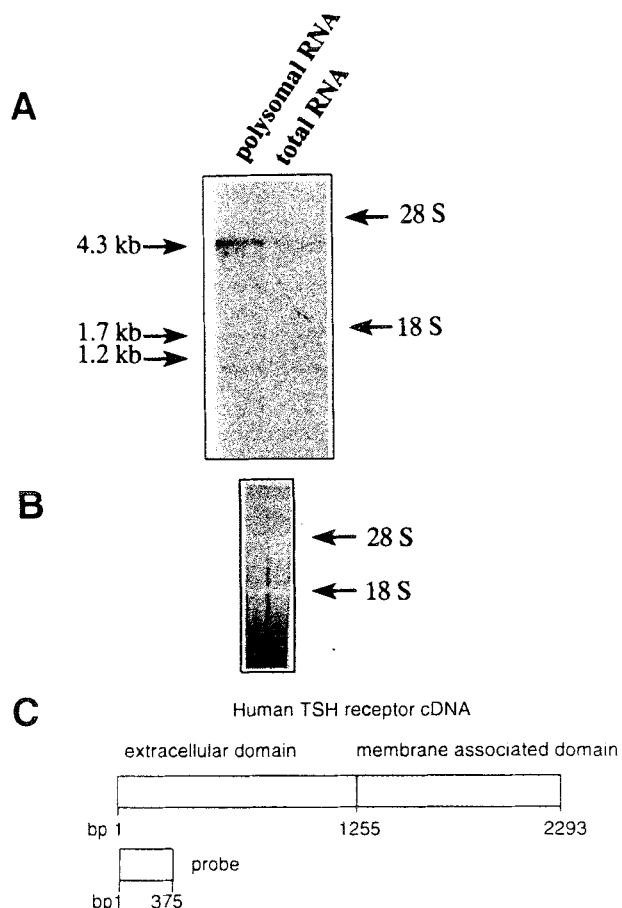


Figure 2 The small hTSH-R transcripts are associated with polysomes. (A) 5 μ g of polysomal or 10 μ g of total RNA isolated from the same Graves' thyroid tissue were separated electrophoretically and subsequently blotted onto a nylon nitrocellulose membrane. The membrane was hybridized under stringent conditions with a radiolabelled probe corresponding to the 5' end of the extracellular domain as in Figure 1. The filter was exposed for 24 h at -70°C with an intensifying screen. (B) Ethidium bromide stained gel after completion of the electrophoretic separation, demonstrating the loading and integrity of the two RNA samples with respect to the 18S and 28S ribosomal RNA species. Considering that only 5 μ g of polysomal RNA was loaded in contrast to 10 μ g total RNA then the hybridization signal observed for all transcripts in the polysomal fraction is enriched. (C) Structural map of the hTSH-R demonstrating the domains present within the coding region of the receptor cDNA. The probe used in this experiment originates from the 5' end of the extracellular domain (bp 1–375).

Small hTSH-R transcripts are associated with polysomes

The transcription of the full-length hTSH-R for cellular export requires association of the mRNA with polysomes a prerequisite which we also wished to demonstrate for small hTSH-R transcripts. Total and polysomal RNA was isolated from the thyroid of a Graves' patient and analysed by Northern blotting (Figure 2). Both of the two prominent small transcripts, as well as the full-length receptor mRNA, was seen when one compared the hybridization signal for the polysomal RNA with the signal for the total RNA preparation.

The co-association of the small and full length hTSH-R mRNA with the polysomal RNA fraction would suggest that they were being actively transcribed, and theoretically processed by the same translation machinery. Such molecules lacking a trans-membrane-spanning region could potentially encode

molecules which would behave like the 'soluble' hTSH-receptors which we have described previously (Hunt *et al.*, 1992a,b; Willey *et al.*, 1993). One must presume however that the extracellular domain of the receptor protein is able to interact/bind with its' cognate hormone. This has indeed been postulated and partially demonstrated by a number of workers (Hunt *et al.*, 1992; Shi *et al.*, 1993; Gattadahalli *et al.*, 1994). Results from this laboratory have recently shown that

such 'soluble' TSH binding proteins are indeed present in crude human thyroid tissue homogenates (Willey *et al.*, 1993). Indeed a construct coding for amino acids 1–418 of the hTSH-R (the entire extracellular domain) produced a protein, after transfection of HeLa cells, which is secreted from the cell and is able to interact with TSH in solution, albeit with a low affinity (Hunt *et al.*, 1992a; Shi *et al.*, 1993a). This has now been also demonstrated in more detail by a number of groups (Huang *et al.*, 1993; Seetharamaiah *et al.*, 1993). To further clarify the origin and coding capacity of these small transcripts a PCR cloning strategy was developed.

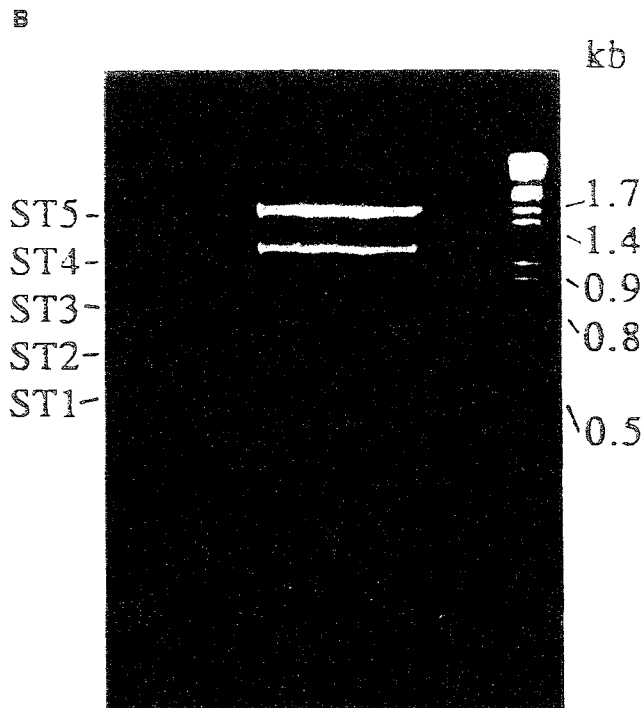
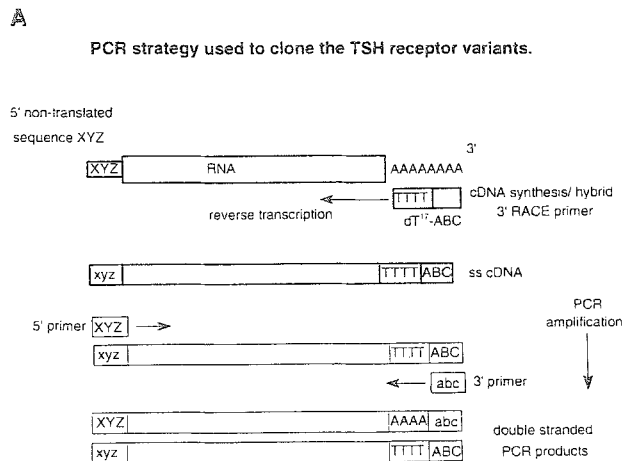


Figure 3 PCR amplification of the alternatively spliced hTSH-R transcripts. The PCR amplification strategy (A) employed a hot start touch down program in combination with asymmetric amplification due to the different primer lengths and melting temperatures. This methodology was semi-quantitative in amplifying the target molecules. The TTTTABC sequence represents the cDNA synthesis oligonucleotide primer and is a hybrid oligo dT 3' RACE primer. The abc oligonucleotide is complementary to the sequence to the ABC hybrid oligonucleotide and was thus utilized as a specific 3' PCR primer. XYZ represents the sequence in the 5' non-translated region used for the 5' PCR oligonucleotide primer and xyz represents the complementary sequence. (B) Preparative agarose gel electrophoresis of the amplified products. The five amplified hTSH-R variants are designated ST1 to ST5 according to their size (from 275 bp to 1.7 kb).

PCR cloning of the small hTSH-R transcripts

From the information that the small hTSH-R transcripts contained a substantial 5' portion of the hTSH-R gene and that they were transcribed and associated with polysomes, a PCR cloning strategy was devised to clone these molecules in order to define their exon usage (Figure 3A). The presumption was made that the small transcripts used a similar transcription start site as the full-length receptor and, therefore, an oligonucleotide located 30 bp upstream of the ATG start-site was synthesized. This sequence was used as the 5' PCR primer. Previous results had shown that the 3' ends of the small transcripts were polyadenylated (Hunt *et al.*, 1992a). Based upon these facts the poly-somal RNA prepared for the previous experiment was reverse transcribed using an oligo dT-hybrid primer as described in Materials and methods. An aliquot of the single stranded cDNA product was amplified using a 'hot-start, touch-down' PCR protocol with a two minute extension incubation in order to amplify the longer 1.7 kb transcript. The results of this analysis are shown in Figure 3B. In the ethidium bromide-stained gel, five prominent bands were visualized and were labelled hTSH-R.ST1 to ST5. The variants ranged in size from 270 bp to 1.7 kb, whereby the 1.2 and 1.7 kb variants were the most prominent in both the preceding Northern blot analysis and the PCR amplification. These five bands were excised and cloned into the PCRII cloning vector as described in Materials and methods. Sequence analysis of a large number of independent clones (>10) from three individual thyroid tissues (representing the different pathologies examined above) corresponding to the transcripts ST1-ST5, revealed that all of the clones were polyadenylated at their 3' ends and had identical 5' non-translated sequences (corresponding to the 5' PCR primer), the correct ATG start-site and leader sequences as the full length hTSH-R cDNA sequence. Thus, based upon the sequence data all five of the transcripts ST1 to ST5 represented alternatively spliced hTSH-R variants.

Exon usage in the hTSH-R splice variants

The elucidated sequences allowed the exon origins of the different molecules to be established (Figure 4).

ST1 (270 bp) represented a variant in which the first exon was transcribed and then at the exon 1/intron 1 boundary a splicing event occurred which introduced a novel sequence at the 3' end of the transcript. The novel 30 bp addition maintained a correct reading frame with a TGA stop signal and a non-translated

region in which a standard polyadenylation signal (AATAAA) was found. This novel 3' sequence did not originate from the known hTSH-R gene coding and intron/exon boundary sequences (those cloned and sequenced in this laboratory or published elsewhere) and, presumably, was derived from one of the intron sequences of the hTSH-R gene.

The hTSH-R.ST2 variant (546 bp) contained the first three exons in its sequence, however intron 3 was

not spliced out. The newly incorporated sequence from intron 3 terminated at the exon 3/intron 3 boundary with an in frame TAA stop codon after the usage of one codon, and a polyadenylation tail approximately 20 bp 3' of a polyadenylation consensus sequence.

hTSH-R.ST3 variant (637 bp) was identical to ST2 except that a different polyadenylation signal (85 bp downstream of that used in ST2) was utilized, resulting in a cDNA approximately 100 bp longer than the ST2 clone.

The ST4 hTSH-R variant (1.2 kb; 1102 bp) was readily seen in Northern blot analysis of both total and polysomal RNA. It contained the first eight exons of the hTSH-R sequence before a splicing event added a unique 3' end to the molecule. The origin of the sequence 3' to the exon 8/intron 8 boundary was not evident, as this sequence was not found in the full-length hTSH-R cDNA nor in the published partial genomic sequence. The novel splicing event introduced a 340 bp in-frame sequence with a TAA stop codon after 72 bp which, according to its' coding capacity would provide a unique 22 amino acid sequence to the

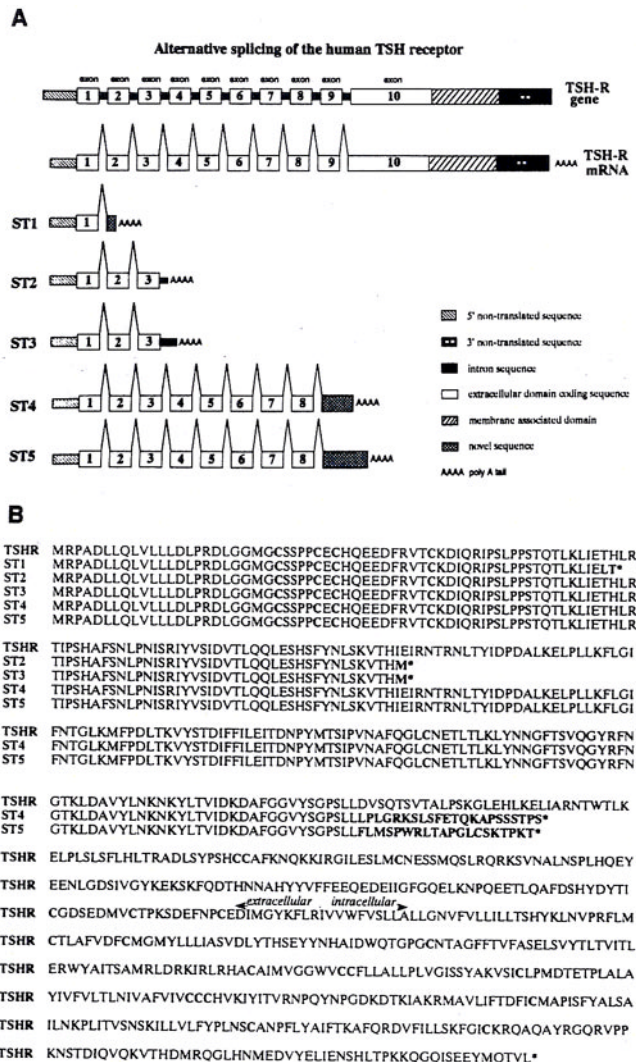


Figure 4 Alternative splicing of the human hTSH-R gene. (A) This figure depicts the gene organization and coding capacity of the hTSH-R gene (Gross *et al.*, 1992). The full length hTSH-R mRNA (4.3 kb) can be divided into two portions, the 3' non-translated region and the coding domain, which can be further divided into the extracellular and the transmembrane/cytosolic portion. At the gene level the extracellular portion of the receptor protein is encoded in the first complete 9 exons and part of exon 10. The whole of the transmembrane spanning region and cytosolic tail are coded for in the remainder of exon 10. Alternative splicing of the hTSH-R results in the synthesis of five variant transcripts which are all devoid of the membrane anchoring portion of the receptor. ST1-ST5 all have the same 5' non translated region as the hTSH-R RNA and are all polyadenylated. The detailed composition of ST1 to ST5 is depicted in the figure and described in detail in the corresponding text. (B) Potential coding capacity of the translated DNA sequences of ST1 to ST5. Novel carboxyl terminal sequences are depicted in bold print and termination codons are shown as a bold asterisk. The **Cysteine** (C) residues are highlighted in bold and underlined print to orientate the protein sequence with respect to the full length membrane associated hTSH-R protein sequence.

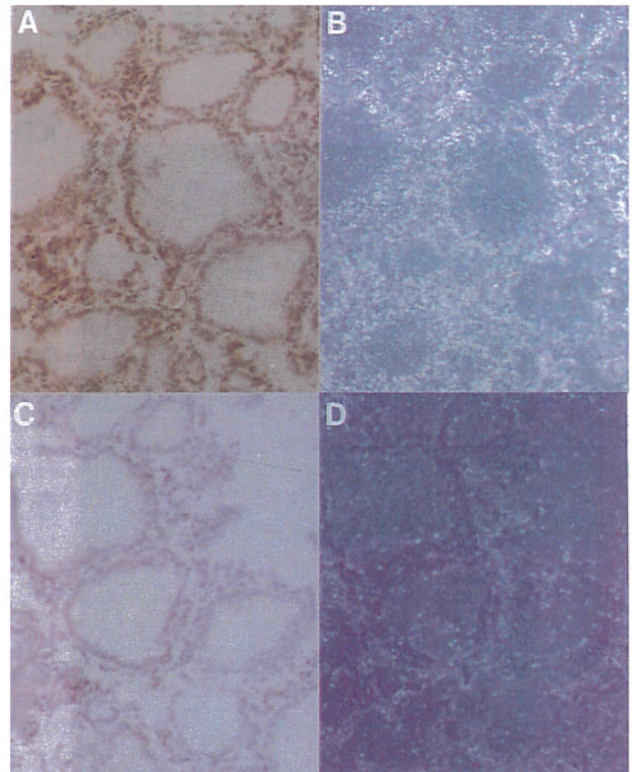


Figure 5 *In situ* hybridization of human thyroid sections with sense and antisense probes corresponding to probe 1. 10 μ m cryostat sections of human thyroid from a Graves' patient were prepared and hybridized with sense and antisense RNA probes corresponding to the 5' portion of the full length hTSH-R cDNA as described in Materials and methods. This probe was used in both Figures 1 and 2 and thus will hybridize with all of the hTSH-R variant mRNAs. (A) Bright field illumination showing the thyroid section histology and (B) dark field illuminations of sections hybridized with the same antisense probe. Silver grains are localized to the epithelial cells of the thyroid follicle. The strong signal is a total representation of all of the hTSH-R transcripts found within these cells. (C) and (D) represent the negative control after hybridization with the sense probe. No specific signals are seen localized to any particular cell type. In the (A, B, C and D) the sections were photographed with a 20 \times optic and the exposure time was 17 days.

carboxyl end of the molecule. Approximately 172 bp downstream of the stop codon was a consensus polyadenylation signal and subsequently a polyadenylation tail.

ST5 (1654 bp) represented the 1.7 kb transcript and was shown to be very similar to **ST4** in its general

composition. As in **ST4** the first eight exons were present and a novel splicing event also occurred at the exon 8/intron 8 boundary. However, the introduced in-frame sequence was unique to **ST5**, adding 880 bp to the 3' end and a polyadenylated tail 80 bp downstream from a consensus polyadenylation signal. The

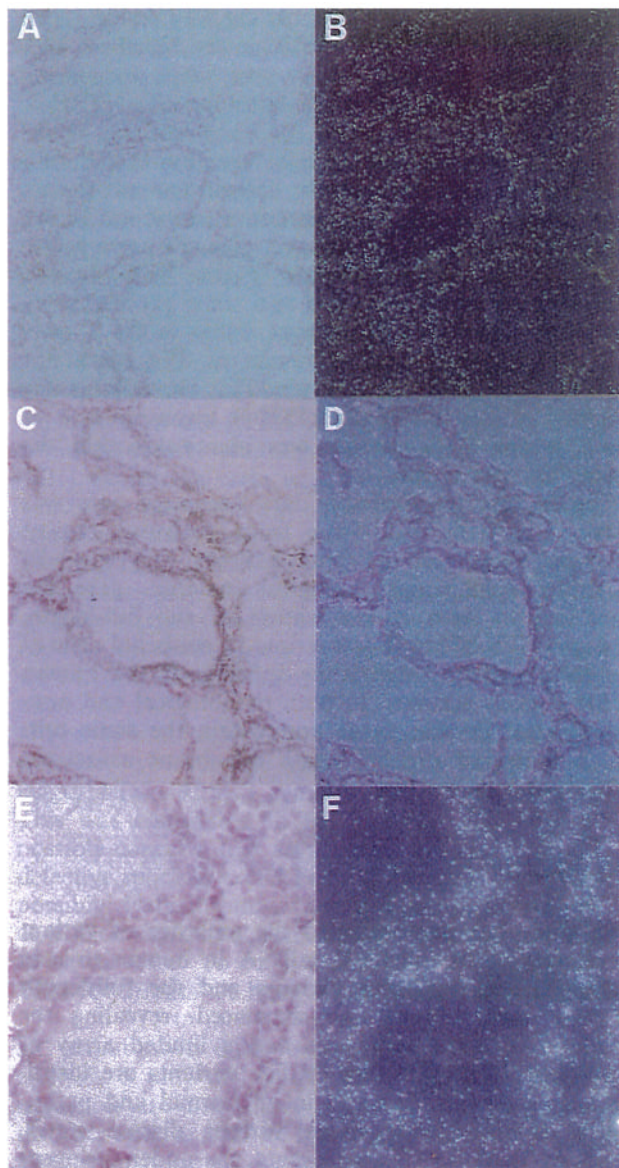


Figure 6 *In situ* hybridization of human thyroid sections using full length hTSH-R specific sense and antisense probes. 10 μ m cryostat sections of human thyroid from a Graves' patient were prepared and hybridized with sense and antisense RNA probes corresponding to the 3' nontranslated portion of the hTSH-R as described in Materials and methods. This fragment hybridizes specifically and only with the full length hTSH-R mRNA as this 3' non-translated sequence is only found in the 4.3 kb mRNA species and does not cross hybridize with any related molecules (LH/hCG or FSH receptor). (A) Bright field illumination demonstrating the histology of the thyroid section, and (B) dark field illumination of sections hybridized with the antisense probe. Silver grains are associated only with the epithelial cells of the thyroid follicle. (C) and (D) represent the negative control after hybridization with the sense probe demonstrating no specific hybridization with any thyroid structures. In figures A, B, C and D magnifications were 20 \times and the exposure time was 17 days. In E and F a 40 \times optic was used and the exposure time was 17 days.

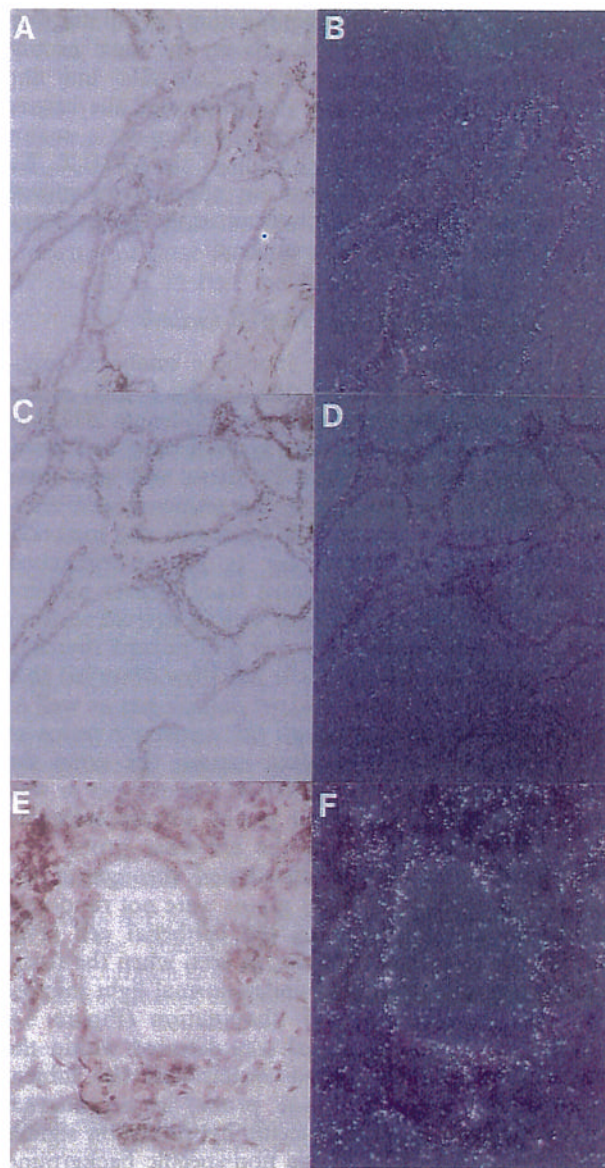


Figure 7 *In situ* hybridization of human thyroid sections using ST4 specific sense and antisense probes. 10 μ m cryostat sections of human thyroid from a Graves' patient were prepared and hybridized with sense and antisense RNA probes corresponding to the unique region of the cDNA for **ST4**. This probe corresponds to the sequence immediately 3' to the end of exon 8 and is not found in either the full length of any other variant TSH-R mRNA as denoted by a data base computer search. (A) Bright field illumination and (B) dark field illumination of sections hybridized with the antisense probe. Silver grains (less intense than for the full length TSH-R mRNA) are only associated with the epithelial cells of the thyroid follicles. (C) and (D) represent the negative control after hybridization with the sense probe with no apparent specific signals being associated with any of the thyroid structures. All magnifications were 20 \times and exposure time was 26 days. (E) and (F) are at 40 \times magnification for the antisense probe after 20 days exposure. The longer exposure times were necessary for as seen in the Northern blot experiment in Figure 1 then the relative levels of ST4 (1.2 kb mRNA variant) are less than those of the full length TSH-R mRNA.

in-frame sequence following exon 8 would potentially add 20 amino acids before termination with a TGA stop codon. The rest of the 3' non-translated sequence was again unique and had no homology to any of the known cDNA or genomic sequences of the hTSH-R gene. The coding capacity of the cloned and sequenced variants is shown in Figure 4B, based upon the translation of the nucleotide sequence.

It was evident from both the Northern blot analysis and the subsequent PCR cloning that, of all the five hTSH-R transcripts described above, the most prominent mRNA variants were the 1.2 kb ST4 and the 1.7 kb ST5. These molecules also possessed the largest potential coding capacity, corresponding to a major portion of the extracellular domain of the hTSH-R. To determine the cellular origin of the alternatively spliced hTSH-R transcripts, *in situ* hybridization studies using thyroid tissue sections were utilized.

In situ hybridization of the hTSH-R variants

The 5' probe used for the Northern analyses, originated from the extracellular domain (bp 1–375) of the hTSH-R, and hybridized with the full-length hTSH-R and the ST4 and ST5 variants in both total and poly-somal RNA (Figures 1 and 2). Sense and anti-sense RNA probes corresponding to this sequence were transcribed *in vitro*, as described in Materials and methods, and were used as probes for *in situ* hybridization studies in thyroid tissue isolated from Graves patients (although identical results were seen in thyroid sections from other pathologies, ie. goiter and normal thyroid). Graves tissue was chosen for it has been observed that in comparison with other thyroid pathologies as well as normal thyroid, then although the respective ratios of all of the different transcripts remain the same the overall expression of the hTSH-R variants *per se* was generally elevated. The silver grains corresponding to the hybridization signals of the antisense probe with the mRNA were equally distributed throughout the tissue (Figure 5B). The silver grains were not randomly distributed but restricted to the epithelial cell layer forming the individual follicles, as seen when the tissue morphology was compared under normal light microscopy and dark-background illumination (Figure 5A and B). No significant signal was detected within the connective tissue, the blood vessels and the lumen. Hybridization with the sense probe, as an internal negative control, gave no specific hybridization signals and provided an index of the non-specific background signal (Figure 5C and D). Since the probe was capable of detecting ST4, ST5 and the full-length receptor without distinction, then the widely distributed hybridization signal would reflect the presence of all three of the transcripts in the epithelial cells of the follicle.

Full-length receptor transcripts (4.3 kb) were visualized by using a specific probe derived from the 3' non-translated region of the 4.3 kb hTSH-cDNA. Hybridization of thyroid sections with an antisense RNA transcribed from this cDNA fragment is shown in Figure 6B. As had been observed for the antisense 5' probe, the silver grains were concentrated in the epithelial cell layer (as seen in the higher magnifications, Figure 6 E and F) associated with the thyroid follicle and were equally expressed throughout the tissue.

Again no significant signal was seen with the sense probe (Figure 6 C and D).

In situ hybridization using the sense and anti-sense probes derived from the novel portion of the ST4 cDNA (described in Materials and methods), gave a similar signal as for the extracellular domain receptor probe and also the specific full length hTSH-R probe with respect to the tissue distribution and cellular location. However the intensity of the signal was much reduced due to the lower levels of the ST4 transcript in these cells, as also readily visible in the Northern blot analysis. This was also readily seen when comparing the 5' probe, which is able to hybridize to all TSH-R variants, to the full length (4.3 kb) specific probe (Figure 5B and 6B). Silver grains were associated with the epithelial cells forming the follicle and no signals were associated with the connective tissue or lumen (Figure 7B and F). Hybridization with the sense probe gave only background signals (Figure 7D). *In situ* hybridization using sense and anti sense probes corresponding to the unique sequence found in the 3' portion of ST5 gave identical results as ST4 (data not shown). These data demonstrated that the cellular distribution of the full-length hTSH-R transcript and of the ST4 and ST5 variants was identical within the human thyroid tissue.

The *in situ* hybridization data presented here was obtained with thyroid tissue from a Graves patient although tissue isolated from patients with sporadic goiter or even non-pathological material, gave the same results, with co-localization of the full-length, ST4 and ST5 hTSH-R transcripts in epithelial cells of the follicle. Thus, alternative splicing of the human hTSH-R gene occurred in both pathological and non-pathological thyroid tissue and within the same cells which processed the full-length membrane associated hTSH receptor mRNA.

Discussion

This study has demonstrated the presence of variant transcripts of the hTSH-R mRNA in human thyroid tissue. The five splicing variants and the full-length hTSH-R were cloned and sequenced, revealing the adoption of novel sequences and a limited array of exon usage. The fact that all five variants are identifiable in similar ratios and in all normal and pathological thyroid tissue, from various disease states, indicates that the splicing events are under stringent control and not purely random. More recently, the presence of ST4 has been confirmed by workers in the USA and in Japan (Graves *et al.*, 1992; Takeshita *et al.*, 1992). Although the small transcripts were also seen (in the same molar ratios) in non-pathological thyroid material and so are not specific to Graves' disease, this does not detract from their potential that their translation products may act as an immunogen and thus may be related to the early onset of Graves' disease when anti-TSH-R antibodies begin to become detectable. Although the physiological relevance of the alternatively spliced hTSH-R molecules has yet to be definitively proven, their presence in the human thyroid would lend weight to our findings, and those of Murakami *et al.* (1992), of a 'soluble' TSH-binding entity in thyroid tissue and in Graves' sera. These hTSH-R

mRNA variants also potentially code for the largest portion of the TSH receptor, covering most of the extracellular domain, and thus were conceived as being possible 'soluble' receptor candidates as we and other groups have previously speculated (Hunt *et al.*, 1992; Huang *et al.*, 1993; Shi *et al.*, 1993; Willey *et al.*, 1993). Transfection studies of HeLa and COS cells are at present in progress to test whether ST1-ST5 translation products are capable of interacting with the hormone TSH, or are capable of influencing the TSH/hTSH-R interaction in co-transfection experiments with the full length receptor. Furthermore recombinant ST4 protein has been synthesized and purified from bacteria and has been used to immunize rabbits. The resulting antiserum is at present being used to probe for the presence of the ST4 protein in both human thyroid tissue and in Graves' serum. This recombinant protein is also being used to screen Graves' serum for the presence of autoantibodies directed against the soluble receptor species.

An interesting aspect regarding expression of the hTSH-R variants has recently been demonstrated by Shi *et al.* (1993a) in which in a study to examine the expression of the hTSH-R in thyroid tumor tissues it could be clearly seen that in some samples that the 4:2:1 expression ratio was disturbed. That the tissues which demonstrated this altered ratio appeared to correlate with neoplasia of poor prognosis remains to be clarified in a larger detailed study. Interestingly, we have previously shown that none of these hTSH-R splicing variants were present in canine, bovine or rodent thyroids and, therefore, these transcripts appear, like Graves' disease, to be a peculiarly human phenomenon.

The *in situ* analysis was conceived to answer the question whether the cells that transcribe the full length hTSH-R also synthesize the variant mRNAs. This was considered to be important, for one could possibly conceive that an aberrant splicing event (i.e. a defective splicing) could possibly occur in cells that are possibly dysfunctioning (due to damage or possibly a de-differentiated function). However this appeared not to be the case. To further analyse the splicing of the TSH-R variants we have assigned the novel 3' ends of ST4 and ST5 to intron 8 of the hTSH-R gene (Dietz *et al.*, unpublished data) by genomic cloning and PCR analysis. It may well be of interest to see whether these sequences are also found in similar intronic sequences in other species and are for some reason not used, for as previously stated the equivalent TSH-R variants are not observed in many other species.

Interestingly, the FSH receptor splicing variants cloned so far (from man and sheep) also represent molecules originating from exon 1–8 as shown for the hTSH-R (Gromoll *et al.*, 1992; Haseena *et al.*, 1993) variants ST4 and ST5. The translation of such a molecule could explain the presence of buffer-soluble FSH binding proteins found in bovine testis extracts (Dias and Reichert, 1982). One could envisage that such 'soluble' receptor molecules could be playing a role in hormone clearance, as has been demonstrated for a number of other hormones and growth factor molecules (Leung *et al.*, 1987; Taga *et al.*, 1989; Engelman *et al.*, 1990; Goodwin *et al.*, 1990; Waldmann, 1991). A well studied model, that of the growth hormone binding proteins (GHBP), demonstrates two mechanisms

whereby soluble receptors can be generated. In this system human and rabbit GHBP arise from proteolytic cleavage of the membrane associated receptor, whereas in mouse and rat alternative splicing accounts for the GHBP variants (Mathews, 1991; Baumann, 1994). This appears to be in contrast to the TSH-R in the human.

Further, alternatively spliced variants coding for the extracellular portion of a receptor have been demonstrated for the closely related LH and FSH receptors, in both man and other species, although their translation and physiological significance remain to be elucidated (Loosfelt *et al.*, 1989; Gromoll *et al.*, 1992; Haseena *et al.*, 1993). On the one side this substantiates our findings here, that members of this family of receptors (glycoprotein hormones) are alternatively spliced, but again as for many aspects of the biochemistry of these receptors the hTSH-R appears to be the exception, in that the alternative splicing is human specific, as is of course Graves' disease.

Materials and methods

General analytical reagents and other materials were from Sigma (Deisenhofen, Germany) and Calbiochem (Bad Soden, Germany).

Tissue samples and preparation

Human thyroid tissues were obtained from patients with either Graves' disease or sporadic goiter (in accordance with the local ethics committee guidelines). Normal human thyroid was obtained from an accident victim after official consent. The Graves' patients had undergone a combined Carbimazole, L-thyroxine treatment prior to operative therapy. Goiter patients were treated with either Iodine or L-thyroxine or both depending upon the gravity of the symptoms.

RNA isolation and Northern blot hybridization

RNA from freshly isolated human thyroid tissue was extracted by the guanidinium isothiocyanate/cesium chloride method (Chirgwin *et al.*, 1979). Polysomal RNA from human thyroid was isolated essentially as described (Melcher, 1987). For Northern blot analysis, RNA was denatured and fractionated on 1.3% agarose/2.2 M formaldehyde gels, transferred to nylon membranes (Hybond-N; Amersham, Braunschweig, Germany) hybridized and washed (at 65°C) in a simplified hybridization buffer containing 5% SDS (Virca *et al.*, 1990). A restriction fragment of the extracellular domain (bp 1–375) was prepared from hTSH-R encoding cDNA clones and labelled using [α^{32} P]dCTP and T7 DNA polymerase (Stratagene, Heidelberg, Germany) after the method of Feinberg and Vogelstein, 1983).

Polymerase chain reaction (PCR) analysis

Polysomal RNA (5 μ g) from human thyroid tissue was reverse transcribed using Superscript (Moloney Murine Leukaemia Virus reverse transcriptase, Gibco-BRL, Eggenstein) primed with 20 pmol of a 35-mer antisense oligonucleotide (5'-GACTCGAGTCGA-CATCGAT^{17-3'}). The RNA was hydrolyzed with 0.3 M NaOH/30 mM EDTA for 15 min at 55°C. The single stranded cDNA products were then neutralized with 1 M Tris-HCl pH 8.0 before being precipitated and redissolved in 10 μ l TE_{Seq} (10 mM Tris-HCl, pH 8.3, 0.1 mM EDTA). Single stranded cDNA aliquots were amplified using a 'hot start touch down' protocol (Don *et al.*, 1991; Chou *et al.*

al., 1992). This methodology adds specificity to the normal PCR reaction by amplifying templates annealed at near optimal melting temperatures. 1 µl of cDNA was mixed with 25 pM (1 µl) of the respective 3' primer (5'-GACTCGAGTC-GACATCG-3') and 5' primer (5'-CGGAGGATGGAGAA-ATAGCCCCGAGTC-3'). The remainder of the reaction, containing 200 µM deoxyribonucleotide triphosphates and Super Taq PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM MgCl₂, 1 mg/ml BSA) and 2.5 U of Super Taq polymerase (ITC, Heidelberg) was overlaid with 50 µl of mineral oil in a final volume of 47 µl and was heated to 80°C for 5 min (hot start) in a Hybaid thermocycler (Teddington, England). The 3 µl mixture containing the cDNA and primers was added, and the following 'touch down' program was run using a denaturation temperature of 95°C for 1 min, an initial annealing temperature of 65°C for 1 min and an extension temperature of 72°C for 2 min. Additional two cycle amplifications through five annealing temperatures of 62, 59, 56, 54 and 52°C were performed followed by a final 20 cycles at 50°C.

Cloning of PCR products

PCR products were separated by agarose gel electrophoresis (low melting) in TAE buffer. The specific bands were excised, eluted and purified using a QIAEX purification system (Qiagen, Hilden). The fragments were cloned into the PCR II

vector using a TA cloning kit (Invitrogen, California) and subsequently transformed into competent XL1-blue bacteria (Stratagene, Heidelberg). Inserts were sequenced by the dideoxy method and T7 DNA polymerase using a commercially available sequencing kit (Pharmacia, Uppsalla) in conjunction with exoIII deletion (Stratagene, Heidelberg). Sequences were compiled and analysed with the DNASTar software package (DNASTar, Madison, Wisconsin).

In situ hybridization

Surgically removed human thyroid was frozen immediately in liquid nitrogen and stored at -80°C until use. 10 µm frozen sections were cut, and prepared for *in situ* hybridization essentially as described (Ivell *et al.*, 1990). For hybridization ³⁵S-labelled RNA probes were prepared from cDNA fragments coding for the extracellular domain of the hTSH-R (XbaI-SstI fragment cloned in pBS, Stratagene, Heidelberg; bp 1-375). This fragment hybridizes with all of the hTSH-R variants. Fragments corresponding to the unique 3' non-translated end of the full length hTSH-R (HincII-XbaI; bp 2299-2546; in pBS) the variant hTSH-R.ST4 (HindIII-Sall; bp 684-1040; in pBS) were utilized as specific templates. Anti-sense and sense probes were labelled by incorporation of ³⁵S ribo CTP using T3 or T7 polymerase, following standard procedures (Wahl *et al.*, 1987).

References

- Buamann, G. (1994). *J. Endo.*, **141**, 1-6.
- Chirgwin, J., Przybyla, A., MacDonald, R. & Rutter, W. (1979). *Biochemistry*, **18**, 5294-5299.
- Chou, Q., Russell, M., Birch, D., Raymond, J. & Bloch, W. (1992). *Nucleic Acids Research*, **20**, 1717-1723.
- Dias, J.A. & Reichert, L.E. (1982). *J. Biol. Chem.*, **257**, 613-620.
- Don, R.H., Cox, P.T., Wainwright, B.J., Baker, K. & Matlick, J.S. (1991). *Nucleic Acids Research*, **19**, 4008.
- Engelmann, H., Holtmann, H., Brakebush, C., Avni, Y.S., Sarov, I., Nophr, Y., Hadas, E., Leitner, O. & Wallach, D. (1990). *J. Biol. Chem.*, **265**, 14497-14504.
- Feinberg, A.P. & Vogelstein, B. (1983). *Anal. Biochem.*, **132**, 6-13.
- Gattadahalli, S., Seetharamaiah, S., Kurosky, A., Desai, R.K., Dallas, J.S. & Prabhakar, B.S. (1994). *Endocrinology*, **134**, 549-554.
- Goodwin, R.G., Friend, D., Ziegler, S.F., Jerzy, R., Falk, B.A., Gimpel, S., Cosman, D., Dower, S.K., March, C.J., Namen, A. & Park, L.S. (1990). *Cell*, **60**, 941-951.
- Graves, P.N., Tomer, Y. & Davies, T.F. (1992). *Biochem. Biophys. Res. Commun.*, **187**, 1135-1143.
- Gromoll, J., Gudermann, T. & Nieschlag, E. (1992). *Biochem. Biophys. Res. Commun.*, **188**, 1077-1083.
- Gross, B., Misrahi, M., Sar, S. & Milgrom, E. (1991). *Biochem. Biophys. Res. Commun.*, **177**, 679-687.
- Haseena, K., Yarney, T.A. & Sairam, M.R. (1993). *Biochem. Biophys. Res. Commun.*, **190**, 888-892.
- Huang, G.C., Page, M.J., Nicholson, L.B., Collison, K.S., McGregor, A.M. & Banga, J.P. (1993). *J. Mol. Endo.*, **10**, 127-142.
- Hunt, N., Willey, K.P., Northemann, W. & Leidenberger, F. (1992a). *J. Endocrinol. Invest.*, **15**, (suppl 2) 2.
- Hunt, N., Willey, K.P., Jähner, D., Ivell, R., Castel, M.A. & Leidenberger, F. (1992b). *Exp. Clin. Endocrinol.*, **4-5**, 22-28.
- Ivell, R., Furuya, K., Nollmeyer, D., Ungefroren, H. & Chiwakata, C. (1990). *Hormonal communicating events in the testis*. Sero symposia publications. Volume 70. Raven Press: New York.
- Leung, D.W., Spencer, S.A., Cachianes, G., Hammonds, R.G., Collins, C., Henzel, W.J., Barnard, R., Waters, M.J. & Wood, W.I. (1987). *Nature*, **330**, 537-543.
- Loosfelt, H., Misrahi, M., Atger, M., Salesse, R., Vu Hai-Luu Thi, M.T., Jolivet, A., Guiochon-Mantel, A., Sar, S., Jallai, B., Garnier, J. & Milgrom, E. (1989). *Science*, **245**, 525-528.
- Mathews, L.S. (1991). *TEM*, **2**, 176-180.
- Melcher, B.M. (1987). *Guide to molecular cloning techniques*. Berger, S.L. and Kimmel, A.R. (eds). Methods in Enzymology. Volume 152. Academic press: New York. pp. 241-248.
- Murakami, M., Miyashita, K., Yamada, M., Iriuchijima, T. & Mori, M. (1992). *Biochem. Biophys. Res. Commun.*, **186**, 1074-1080.
- Seetharamaiah, G.G., Desai, R.K., Dallas, J.S., Tahara, K., Kohn, L.D. & Prabhakar, B.S. (1993). *Autoimmunity*, **14**, 315-320.
- Shi, Y., Minjing, Z., Parhar, R.S. & Farid, N.R. (1993a). *Thyroid*, **3**, 129-133.
- Shi, Y., Minjing, Z. & Farid, N.R. (1993b). *Clinical Endocrinology*, **39**, 269-274.
- Smith, B.R., McLachlan, S.M. & Furmaniak, J. (1988). *Endo. Rev.*, **9**, 106-121.
- Taga, T., Hibi, M., Hirata, Y., Yamasaki, K., Yasukawa, K., Matsuda, T., Hirano, T. & Kishimoto, T. (1989). *Cell*, **58**, 573-581.
- Takeshita, A., Nagayama, Y., Fujiyama, K., Yokoyama, N., Namba, H., Yamashita, S., Izumi, M. & Nagataki, S. (1992). *Biochem. Biophys. Res. Commun.*, **188**, 1214-1219.
- Virca, G.D., Northemann, W., Shiels, B.R., Widera, G. & Broome, S. (1990). *Biotechniques*, **8**, 370-371.
- Wahl, G.M., Meinkoth, J.L. & Kimmel, A.R. (1987). *Guide to molecular cloning techniques*. Berger, S.L. & Kimmel, A.R. (eds). Methods in Enzymology, Volume 152. Academic press: New York. pp. 572-581.
- Waldmann, T.A. (1991). *J. Biol. Chem.*, **266**, 2681-2684.
- Willey, K.P., Hunt, N., Abend, N., Northemann, W., Ivell, R. & Leidenberger, F. (1993). *J. of Endo.*, **139**, 317-328.