The human neuroendocrine thyrotropin-releasing hormone receptor promoter is activated by the haematopoietic transcription factor c-Myb

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Thyrotropin-releasing hormone (TRH) receptor (TRHR) is a G-protein-coupled receptor playing a crucial role in the anterior pituitary where it controls the synthesis and secretion of thyroid-stimulating hormone and prolactin. Its widespread presence not only in the central nervous system, but also in peripheral tissues, including thymus, indicates other important, but unknown, functions. One hypothesis is that the neuropeptide TRH could play a role in the immune system. We report here that the human *TRHR* promoter contains 11 putative response elements for the haematopoietic transcription factor c-Myb and is highly Myb-responsive in transfection assays. Analysis of Myb binding to putative response elements revealed one preferred binding site in intron 1 of the receptor gene. Transfection studies of promoter deletions confirmed that this high-affinity element is necessary for efficient Myb-dependent transactivation of reporter plasmids in CV-1 cells. The Myb-dependent activation of the *TRHR* promoter was strongly suppressed by expression of a dominant negative Myb–Engrailed fusion. In line with these observations, reverse transcriptase PCR analysis of rat tissues showed that the *TRHR* gene is expressed both in thymocytes and bone marrow. Furthermore, specific, high-affinity TRH agonist binding to cell-surface receptors was demonstrated in thymocytes and a haematopoietic cell line. Our findings imply a novel functional link between the neuroendocrine and the immune systems at the level of promoter regulation.

Key words: c-Myb binding site, gene regulation, promoter analysis, thymus, thyrotropin-releasing hormone (TRH), thyrotropin-releasing hormone receptor (TRHR).

INTRODUCTION

Thyrotropin-releasing hormone (TRH) is a tripeptide (pGlu-His-Pro-NH_2; where pGlu stands for pyroglutamic acid) that is released from the hypothalamus and transported via the portal vascular system to the anterior pituitary where it stimulates the release of thyroid-stimulating hormone (TSH) and prolactin from the anterior pituitary [1,2]. In addition to its classical function as a releasing hormone, the distribution of TRH and its receptor indicates supplementary roles. TRH and TRH receptor (TRHR) are found distributed throughout the central and peripheral nervous systems as well as in other tissues [3–6] including thymus [6] and small intestine epithelial cells [7], consistent with its proposed role in the immune system.

cDNAs encoding the highly conserved TRHR have been cloned from mouse, rat and hamster cell lines [8–10], from human brain [11] and from avian and bovine tissues [12,13]. This receptor is now also designated TRHR1, to be distinguished from TRHR2, a recently discovered subtype of the rat TRH receptor, 51 % identical to TRHR1 but with a distinct mRNA tissue distribution [14,15].

We have reported previously the cloning of the gene encoding the human TRHR [16]. The gene encompasses 35 kb and the open reading frame was found to be interrupted by a large intron of 31 kb with an additional 0.5 kb intron in the 5′-untranslated region. Sequencing of 2.5 kb of promoter region DNA allowed the identification of elements that may contribute to the regulation of the *TRHR* gene expression. One transcription start site was mapped to position −885 upstream of the putative translation start site [17]. Nonetheless, cell-type-specific promoter activity was found within a fragment smaller than this (− 770 to + 1), implying not only multiple transcription start sites, but also that important regulatory elements may be present in the first intron of the gene [16]. This intron 1 included two potential recognition elements for the pituitary transcription factor Pit-1, both of which were found to bind a protein factor in extracts from rat pituitary tumour cells [16]. These Pit-1 sites are likely to be important determinants for the cell-type-specific expression of the receptor gene in the anterior pituitary. In addition, two glucocorticoid response elements were identified and found to be necessary for transcriptional regulation of the *TRHR* gene by glucocorticoids [18].

The analysis of the *TRHR* promoter also revealed several potential binding sites for transcription factors that normally exert their functions during proliferation and differentiation of haematopoietic cells. Cells of the immune system have been found to contain receptors for neuroendocrine hormones and can also be considered a source of pituitary and hypothalamic peptides (reviewed in [7,19–21]). For TRH signalling in particular, it was reported that a proper T-cell-dependent immune response is critically dependent on the early activation of TRH and prolactin, suggesting an important relationship between the immune and endocrine systems [22]. To our knowledge, the role of shared transcriptional control in the circuitry between the immune system

Abbreviations used: TRH, thyrotropin-releasing hormone; TRHR, TRH receptor; MeTRH, methylated TRH; TSH, thyroid-stimulating hormone; DBD, DNA-binding domain; MRE, Myb-responsive element; tk, thymidine kinase; EMSA, electrophoretic mobility-shift assay; RT-PCR, reverse transcriptase PCR.

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and the neuroendocrine system has so far not been explored. The present study addresses whether the *TRHR* promoter really contains functional elements for a haematopoietic transcription factor, focusing on putative response elements for c-Myb.

The c-Myb transcription factor regulates the proliferation, differentiation and apoptosis of immature lymphoid and myeloid cells [23]. In addition to its well-studied role in myeloid cells, c-Myb also plays a central role in T-cell development and function [24]. While resting T-cells express very low levels of c-Myb protein, T-cell activation leads to induction of c-Myb expression [25]. c-Myb is also expressed at high levels in immature thymocytes and may be a regulator of T-cell differentiation [26]. Transgenic mice with dominant interfering Myb alleles were found to have partially blocked thymopoiesis and proliferation of mature T-cells was inhibited [27]. Parts of this effect may be related to the regulation of the *bcl-2* gene by c-Myb [28]. A T-cell-specific transcriptional enhancer of the human T-cell receptor δ gene also contains a highly critical binding site for c-Myb that is essential for enhancer activity [29]. Similarly, in the locus control region ('LCR') in the human adenosine deaminase (*ADA*) gene first intron, c-Myb acts as an organizer of thymocyte-specific gene expression [30]. The retroviral v-Myb was also found to be oncogenic in T-cells [31]. Finally, a study of homozygous null *c-Myb/Rag1* chimaeric mice concluded that c-Myb plays an important role at multiple stages of haematopoiesis, and is essential at a specific stage early in T-cell development just before the oligopotent thymocytes mature into the definitive T-cell precursors [24].

In the present work we have addressed the role of the haematopoietic transcription factor c-Myb in the activation of the *TRHR* promoter. Analysis of 11 putative Myb-responsive elements (MREs) in the human *TRHR* promoter pinned down a single MRE in the first intron that plays a particularly important role in the activation of the receptor gene. The *TRHR* promoter was found to be Myb-responsive and fragments from intron 1 conferred Myb-responsiveness on a heterologous thymidine kinase (*tk*) promoter. Expression analysis in rat demonstrated the presence of TRHR mRNA in thymocytes, bone marrow and in the rat natural-killer cell line RNK-16. This expression was shown to be functional as evidenced by TRH binding to surface receptors on thymocytes and RNK-16 cells. Altogether, these data suggest a novel link at the transcriptional level between the neuroendocrine and haematopoietic systems.

EXPERIMENTAL

Cell culture and reagents

CV-1, COS-1, HeLa, $GH_1 2C_1$ and GH_4C_1 cells were cultured in Dulbecco's modified Eagle's medium with 10 % fetal calf serum. CV-1, COS-1 and HeLa cells were supplemented with 20 mM glutamine, penicillin and streptomycin. RNK-16 cells were cultured in RPMI 1640 medium with 10 % fetal calf serum, penicillin and streptomycin. Cells were grown under 5 % CO_2 in a humidified atmosphere. All culture media, sera and other additions were from Life Technologies.

Effector and reporter plasmids

The mammalian expression plasmids encoding full-length murine c-Myb (pEQP2-CMV-c-Myb [32]) and truncated murine c-Myb [residues 1–360; pEQP2-CMV-c-Myb(1–360)] were kind gifts from Dr B. Lüscher, Institut für Biochemie, Klinikum der RWTH, Aachen, Germany. The dominant-negative effector pSCDMS/MEnT encodes the murine DNA-binding domain (DBD) fused to the *Drosophila Engrailed* transcription repressor domain [27] and was a kind gift from Dr K. Weston, CRC Centre for Cell and Molecular Biology, Chester Beatty Laboratories, London, U.K. The luciferase reporter constructs pGL2/tk-3xGG and pGL2/tk-3xTT have been described in [33]. The reporter plasmids pGL2/tk-MREcA, pGL2/tk-MREcB and pGL2/tk-3xTR10 were constructed by inserting one of the following fragments into pGL2/tk: a PCR product spanning the −2160 to −2047 region or the −404 to −145 region of the human *TRHR* promoter, or a duplex oligonucleotide with the sequence 5′-GCATTATAACCGTCACTAACCGTCACTAACCGTCACT-TAGCGCGAGCT-3′, where the repeated MRE-TR10s (MRE-TR10 is one of the putative response elements for c-Myb; see the Results section for details) are underlined. The reporter plasmids with upstream deletions (designated pGL2b-TRHR-*n*) were constructed by inserting in pGL2basic (Promega) PCR products spanning the region from *n* to +1 (initiator ATG position) from the *TRHR* promoter (where *n* indicates the deletion endpoints, −2530, −2250, −2000, −1750, −1500 and −1250). Reporter plasmids with internal deletions were all derived from pGL2b-TRHR-1250 using a common strategy to introduce deletions in intron 1. The plasmid was digested with *Sac*II (−819) and *Bcl*I (−139) and the excised fragment replaced with a PCR fragment spanning the region from *Sac*II to an engineered *Bcl*I site introduced by PCR in the positions −162, −251, −351 and −449, giving rise to the plasmids ΔI-A, ΔI-B, ΔI-C and ΔI-D respectively. Mutagenesis of MRE-TR10 was performed by using the QuikChange site-directed mutagenesis kit (Stratagene) on a *Sac*II–*Xho*I subfragment of pGL2b-TRHR-1250. All constructs generated by PCR were verified by sequencing.

Expression of c-Myb proteins

Whole-cell extracts of Myb-transfected COS-1 cells were prepared as described previously [32,33]. The minimal DBD of human c-Myb, R_2R_3 (residues 89–104), designated c-Myb-DBD, was expressed in *Escherichia coli* and purified as described in [34].

Electrophoretic mobility-shift assay (EMSA)

DNA binding was monitored by EMSA. The *mim-1* A site oligonucleotide was labelled and purified as described in [35]. Unlabelled double-stranded oligonucleotides for competition were similarly annealed, filled in and PAGE-purified. Binding reactions with recombinant c-Myb or whole-cell extracts of Myb-transfected COS-1 cells were performed as described previously [33]. Typically 1–3 μl of COS-1 cell extract (10 μg of protein) or 20 fmol of purified recombinant c-Myb R_2R_3 was used per binding reaction. 5′ γ-^{32}P-labelled DNA oligonucleotide probe (10 fmol) was added and the binding mixture (total volume 20 μl) was incubated for 10 min at 25 °C before electrophoresis.

Transactivation assays in mammalian cells

CV-1 cells were transfected with Fugene (Roche Molecular Biochemicals) and HeLa cells using Lipofectamine (Life Technologies) as recommended by the manufacturers. Cells were seeded the day before at (1–2) × 10^5 cells/plate in 35 mm plates. Cells were transfected with the indicated amounts of effector and reporter plasmids in combinations indicated in the Figures. Cells were lysed and scraped into 250 μl of reporter lysis buffer (Promega). All lysates were analysed for luciferase activity using

either a standard or a dual-luciferase assay kit as described by the manufacturer (Promega). Comparable Myb expression in transfected cells was verified by Western analysis.

Isolation of cells from the bone marrow, thymus, spleen and lymph nodes

Rats, strain PVG, age 7–10 weeks, were killed using CO_2 gas. Cells and tissues were immediately dissected out and placed in sterile ice-cold PBS, pH 7.4. The tissues were disrupted and homogenized by cutting and squeezing through a nylon membrane (cell strainer) and centrifuged. The pellet was washed twice in ice-cold PBS and immediately lysed in a guanidinium thiocyanate solution consisting of 5 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5 % sodium larcosyl sulphate and 0.7 % β-mercaptoethanol. For isolation of natural killer cells from the spleen the procedure of [36] was followed.

RNA isolation and reverse transcriptase PCR (RT-PCR) analysis

Total RNA was prepared from different tissues and cell lines [37]. Total RNA (3 μg) was reverse transcribed using Moloney murine leukaemia virus reverse transcriptase (Promega). In the PCR reactions, 20 % of the cDNA served as template in 50 μl of 1 × Vent buffer in the presence of 0.1 mM of each dNTP and 0.2 μM of each primer. The primers were rTRHR-Forward (5′-TTACCGGAATTCATATGGAGAATGAAACCGT-3′) and rTRHR-Reverse (5′-GCGCATGGATCCTATCATATTTTCTCC-TGTTTGGCAG-3′). RNA samples from GH_4C_1 and GH_12C_1 cells were used as positive and negative controls respectively. Vent DNA polymerase (0.6 units; New England Biolabs) was added after a hot start of 5 min at 94 °C. The PCR program proceeded as follows: 1 min at 94 °C, 1 min at 60 °C and 2 min at 70 °C for 34 cycles, and finally 10 min at 70 °C.

TRH-binding assay

The assessment of the binding of tritium-labelled methylated TRH ([^{3}H]MeTRH) was performed according to established methods [38].

RESULTS

The *TRHR1* promoter contains 11 potential Myb-responsive elements arranged in two clusters

Previous reports have suggested that the TRHR is expressed not only in the anterior pituitary, but also in rat peripheral tissues including thymus [6] and small intestine epithelial cells [7], indicating a thus-far unknown role in the immune system. We were intrigued by the finding of response elements for the haematopoietic transcription factor c-Myb in the *TRHR* promoter. When a region of 2.5 kb in the *TRHR* gene upstream of the translation start site was sequenced, we identified 11 putative response elements for c-Myb, all fitting the consensus sequence YAACNG (Figure 1). Three sites were clustered in a 100 bp region far upstream (−2161 to −2059) and four were clustered in a 250 bp region in the first intron (−397 to −155; Figure 1, top panel). In addition, four other potential sites were found scattered throughout the sequenced region. We named the two clustered regions MRE-cluster A and MRE-cluster B and designated the putative sites MRE-TR1 to MRE-TR11, as listed in Figure 1 (bottom panel). From the theoretical scores for all the potential sites (estimated using a matrix procedure [39]), we predicted that MRE-cluster A contains two strong (MRE-TR2 and MRE-TR3) and one weak (MRE-TR1) MREs, whereas MRE-cluster B contains three reasonably strong sites (MRE-TR7, -TR9 and -TR10) and one weak one (MRE-TR8). The analysis of scores furthermore predicted that the four scattered MREs are either of modest strength (MRE-TR4, -TR5 and -TR6) or very weak (MRE-TR11).

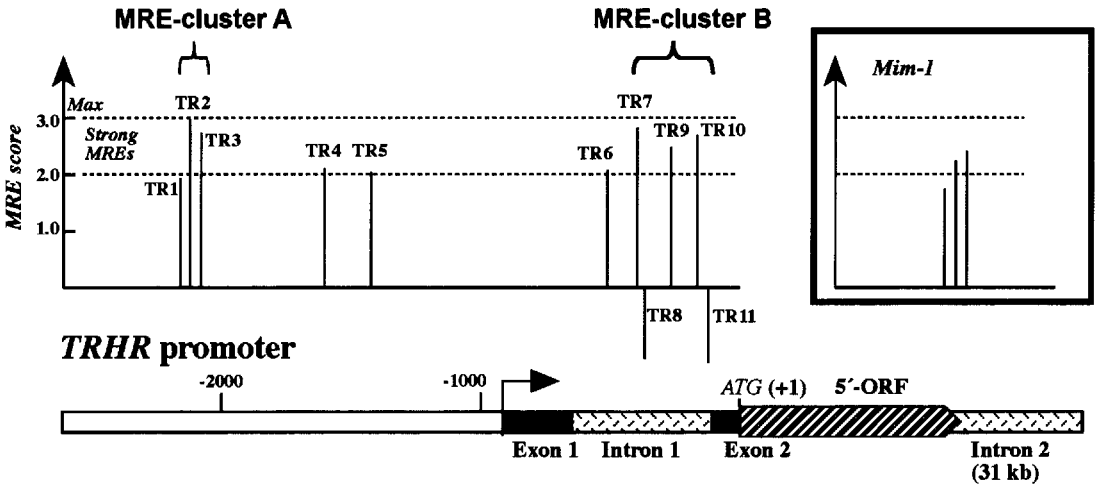
EMSA of putative MREs

To assess which of the putative MREs were in fact recognized by c-Myb, oligonucleotides corresponding to the elements with the highest score ($\geqslant$ 2.40) were labelled and used as binding probes for recombinant c-Myb-DBD. The MRE-TR1 oligonucleotide was also labelled as a representative of a weaker site (score 1.90). A reasonable correlation between theoretical scores and efficiency of binding was observed (Figure 2, left-hand panel). While the MRE-TR1 probe gave barely detectable binding, strong binding was observed for MRE-TR2, MRE-TR3, MRE-TR7 and MRE-TR10. Somewhat weaker binding was observed for MRE-TR9, as expected (score 2.40). The binding data indirectly supports our assumption that MREs with a score lower than 2.4 (MRE-TR4, -TR5, -TR6 and -TR11) are not major binding sites although they may contribute to Myb-responsiveness in combination with stronger sites. The most efficiently bound probes appeared to be MRE-TR2 (score 2.99) and MRE-TR10 (score 2.66).

To verify that the sites bound by recombinant c-Myb-DBD were also recognized by Myb proteins expressed in mammalian cells, we analysed the labelled MRE oligonucleotides for binding using extracts from Myb-transfected cells as protein source. COS-1 cells were transfected by an expression plasmid pEQP2-CMV-c-Myb(1–360) and whole-cell extracts were used as a source for Myb proteins [32]. Each probe was tested in the absence and presence of an excess of unlabelled Myb-specific oligonucleotide to verify that the retarded complexes showed specific binding. In this system, essentially the same pattern of binding affinities was seen as with the recombinant protein, but the selectivity seemed to be more stringent than with recombinant proteins (Figure 2, right-hand panel). Thus the best MREs (MRE-TR2, MRE-TR3, MRE-TR7 and MRE-TR10) all gave rise to retarded bands, but now MRE-TR10 was clearly superior to the other MREs with respect to binding affinity. In fact, the complex formed on MRE-TR10 was as strong as that obtained with the positive control probe from the *mim-1* gene (Figure 2, right-hand panel, compare lanes 11 and 13). The increased stringency also resulted in a rather weak binding to MRE-TR7 and no detectable binding to MRE-TR9. We conclude that among the 11 putative Myb-binding elements in the *TRHR1* promoter, one site (MRE-TR10) was clearly a preferred binding site for c-Myb.

Regions of the human *TRHR1* promoter harbouring MRE-TR10 mediate Myb-dependent transactivation

To test whether the regions harbouring the best MRE sites also conferred Myb-dependent transactivation, two luciferase-reporter plasmids were constructed harbouring either of the MRE–cluster A or MRE-cluster B regions upstream of the tk basal promoter. These reporters were co-transfected with two different c-Myb effector plasmids into Myb-negative CV-1 cells. One effector encoded full-length c-Myb, the other encoded a chimaera of the minimal DBD of human c-Myb (R_2R_3) fused to the strong transactivation domain of herpes simplex virus VP16. As shown in Figure 3 (top panel), the cluster A gave weak Myb-dependent transactivation (4.3-fold), only slightly above the Myb response of



MRE	Position	Score	oligo	Sequence	Region
MRE-TR1	MRE [-2161/-2153]	1.90	+	TAACTGGTG	MRE cluster A
MRE-TR2	MRE [-2128/-2104]	2.99	+	TAACTGACA	MRE cluster A
MRE-TR3	MRE [-2083/-2059]	2.72	+	CAACTGAAT	MRE cluster A
MRE-TR4	MRE [-1605/-1597]	2.10		TAACAGAAT	
MRE-TR5	MRE [-1424/-1416]	2.03		TAACAGACC	
MRE-TR6	MRE [-513/-505]	2.09		CAACAGCTA	
MRE-TR7	MRE [-397/-389]	2.79	+	TAACTGAAA	MRE cluster B
MRE-TR8	MRE [-369/-361]	-1.74		CAACTGGGC	MRE cluster B
MRE-TR9	MRE [-266/-258]	2.40	+	TAACTGTTT	MRE cluster B
MRE-TR10	MRE [-163/-155]	2.66	+	TAACCGTCA	MRE cluster B
MRE-TR11	MRE [-124/-116]	-1.93		TAACAGTGT	

Figure 1 Putative c-Myb-binding sites in the human *TRHR* promoter

The human *TRHR* gene sequence upstream of the ATG initiation codon was analysed for the presence of putative MREs using matrix analysis [39]. Top panel: the scores calculated for each putative MRE (designated TR1–TR11) are plotted along the *TRHR* gene sequence to illustrate the locations and clustering of the MRE elements. For comparison, a similar plot for the Myb-responsive *mim-1* gene [50] is included (box). Bottom panel: list of putative MRE sequences (TR1–TR11) in the human *TRHR* gene promoter including matrix scores [39]. The oligo column shows which elements were used to probe for binding to c-Myb.

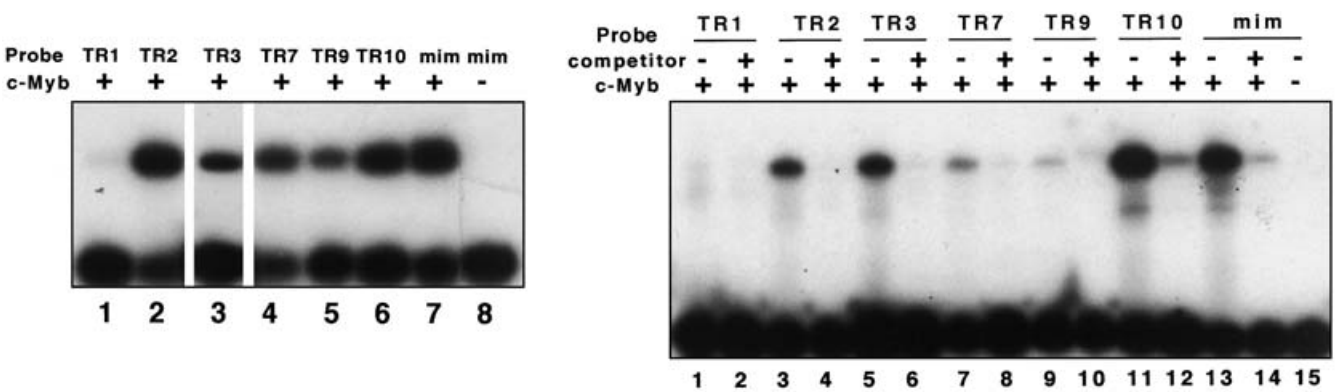


Figure 2 DNA binding of c-Myb to putative MREs from the *TRHR* gene promoter

Six different duplex oligonucleotides representing putative Myb-recognition elements from the *TRHR* gene promoter were used as probes for binding to c-Myb. The sequence of each probe is listed in Figure 1 (bottom panel). Left-hand panel: DNA binding of purified recombinant c-Myb-DBD proteins. In each lane 20 fmol of recombinant protein was incubated with 10 fmol of the indicated probe at 25 °C for 10 min before analysis by the EMSA as described in the Experimental section. The probes analysed were MRE-TR1 (lane 1), MRE-TR2 (lane 2), MRE-TR3 (lane 3), MRE-TR7 (lane 4), MRE-TR9 (lane 5), MRE-TR10 (lane 6) and *mim-1* A reference probe (lanes 7 and 8). All lanes had added recombinant c-Myb except lane 8, which shows the migration of probe only. Right-hand panel: DNA binding of whole-cell extracts from transfected CV-1 cells expressing mouse c-Myb(1–360). In each lane 2 μl of standardized COS-1 cell lysate was used as a source of c-Myb. Each probe was analysed in the absence (−) and presence (+) of a 50-fold excess of unlabelled *mim-1* probe. The probes analysed were MRE-TR1 (lanes 1 and 2), MRE-TR2 (lanes 3 and 4), MRE-TR3 (lanes 5 and 6), MRE-TR7 (lanes 7 and 8), MRE-TR9 (lanes 9 and 10), MRE-TR10 (lanes 11 and 12) and *mim-1* A reference probe (lanes 13–15). Lane 15 shows the migration of probe only.

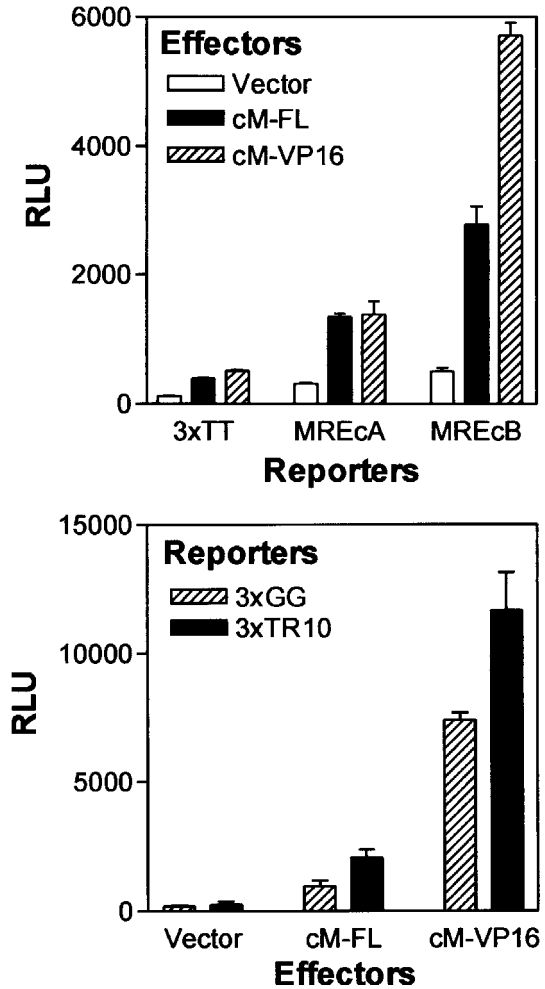


Figure 3 MRE clusters from the *TRHR* promoter mediate Myb-dependent transactivation

CV-1 cells were co-transfected with the indicated combinations of Myb effector and MRE reporter plasmids. The effectors used expressed either full-length c-Myb (black columns, cM-FL; effector, pEQP2-CMV-c-Myb) or the c-Myb DBD fused to the VP16 transactivation domain (hatched columns, cM-VP16; effector, pCIneo-R_2R_3VP16). As a negative control we used empty expression vector (white columns, Vector; effector, pCIneo). Top panel: the luciferase reporters analysed in this panel contained either the cluster A or B region of the *TRHR* promoter (reporters pGL2/tk-MREcA or pGL2tk/MREcB respectively, see text for details). As negative control the reporter pGL2/tk-3xTT was used [33]. Bottom panel: two reporters with multimerized MREs were compared. pGL2/tk-3xTR10 (black columns) contained three tandem copies of the TR10 element and pGL2/tk-3xGG (hatched columns) contained three tandem copies of the strong *mim*-1 A site (positive control [33]). In both panels results are expressed in relative luciferase units (RLU). In all transfections CV-1 cells were transfected with 0.2 μg of effector and 1.0 μg of reporter plasmid.

the negative control reporter plasmid (3.2-fold) containing three copies of a very weak MRE. The MRE-cluster B conferred a significantly higher Myb-dependent transactivation, in particular with the R_2R_3 VP16 construct (5.5- and 11.4-fold respectively). Thus the cluster in intron 1 encompassing the MRE-TR10 element conferred a clear Myb-dependent transactivation on the reporter.

To test the role of the MRE-TR10 element, a reporter plasmid was constructed harbouring only the MRE-TR10 element (in three copies). When this reporter was co-transfected with a c-Myb effector plasmid into CV-1 cells, strong Myb-dependent transactivation was measured demonstrating that this element alone was sufficient to mediate strong Myb-dependent promoter activation (Figure 3, bottom panel). When compared with the reference reporter pGL2/tk-3xGG (with the strong *mim-1* A site in three copies), the pGL2/tk-3xTR10 reporter gave apparently as high or even higher transactivation.

The *TRHR1* promoter is Myb-responsive

To test whether the *TRHR1* promoter itself was Myb-responsive, we constructed a TRHR1–luciferase chimaera with the region from −1250 to +1 (initiator ATG position) from the *TRHR1* promoter fused to the luciferase reporter gene. When this reporter was co-transfected with the two c-Myb effector plasmids in CV-1 cells, strong Myb-dependent activation was observed for both effectors (results not shown, but see Figure 4, top-right panel, below). This shows that not only does this promoter contain Myb-responsive elements active in the context of a heterologous basal promoter (*tk*), but the *TRHR1* promoter is itself highly Myb-responsive.

Supporting evidence was obtained by examining activation of the *TRHR1* promoter in transfected HeLa cells in the presence or absence of a dominant-negative Myb–Engrailed fusion. Again introduction of a Myb-effector plasmid caused activation of the *TRHR1* promoter, whereas co-expressing the dominant-negative Myb abolished this activation almost completely (Figure 4, top-left panel). The dominant-negative construct alone also reduced the background level observed in this cell line, most likely by binding and repression through the many MREs present in the promoter.

Dissection of the *TRHR* promoter: a prominent role for the TR10 element

In an effort to map the Myb-responsive regions in the *TRHR1* promoter, we constructed luciferase reporters driven by successive upstream deletions of the *TRHR1* promoter and tested these in co-transfection experiments. Very similar Myb-responsiveness was observed with deletion endpoints at −2530, −2250, −2000, −1750, −1500 and −1250 (Figure 4, top-right panel). The only consistent difference between these reporters was an increased basal promoter activity in the longest construct (−2530) compared with the rest. It seems that neither the MRE-cluster A (−2161 to −2059) nor the dispersed MREs in the upstream region make major contributions to the overall Myb-responsiveness of the *TRHR1* promoter, consistent with the modest effect of MRE-cluster A observed in the enhancer analysis (Figure 3, top panel).

Having found stronger activating effects of MRE-cluster B (Figure 3, top panel) and evidence for an important role for the TR10 element (Figures 2, right-hand panel, and 3, bottom panel), we constructed successive internal deletions in intron 1 as well as a promoter construct where TR10 had been mutated as illustrated in Figure 4 (bottom panel). When these reporters were analysed in co-transfection experiments, a major decrease in Myb response was observed even with the smallest intron deletion (ΔI-A) where the TR10 element was removed. A very similar fall was observed when the TR10 element was mutated (Figure 4, bottom panel). Larger intron deletions slightly only reduced the Myb response further, supporting a prominent role for the TR10 element in mediating Myb-responsiveness of the *TRHR1* promoter. It is noteworthy that none of the constructs totally eliminated the Myb response, suggesting that some low-level residual responsiveness could be due to indirect effects or the presence of Myb-responsive elements outside the region of analysis. The slight activation of the 3xTT control construct (Figure 3, top panel) is consistent with this.

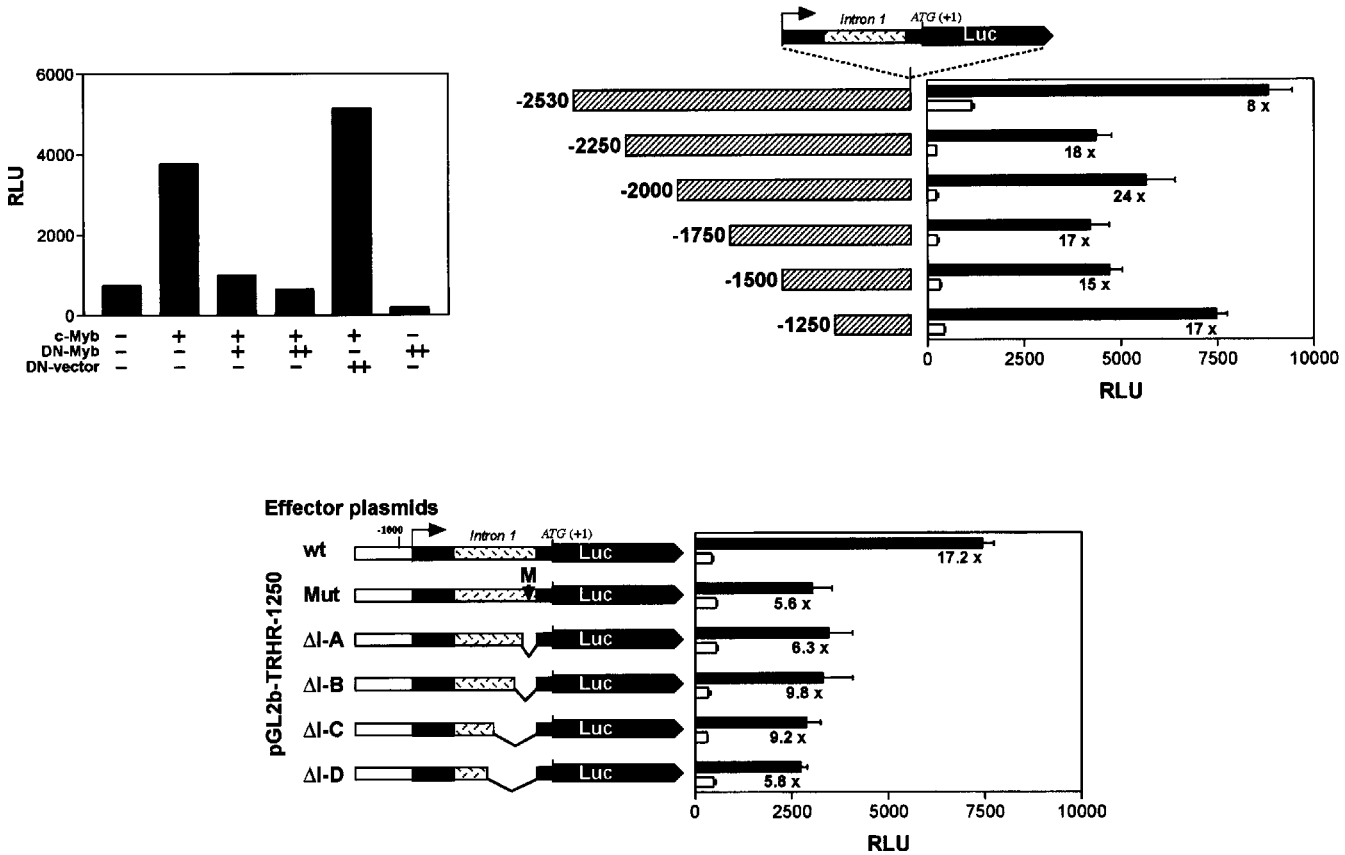


Figure 4 The *TRHR* promoter is Myb-responsive

Top-left panel: HeLa cells were co-transfected with 0.5 μg of the TRHR reporter pGL2b-TRHR-1250 and the indicated combinations of Myb effectors. These expressed either full-length c-Myb [effector, pCIneo-ccM-FLK (0.17 μg)] [33], or a dominant-negative Myb (DN-Myb; effector, pSCDMS/MenT) [27]. DN-vector indicates use of the empty expression vector pSCDMS, which is the backbone of the dominant-negative construct. Different inputs of pSCDMS/MEnT and pSCDMS are indicated by + (1 μg) or ++ (2 μg). Top-right panel: CV-1 cells were co-transfected with the reporter plasmid pGL2b-TRHR-n (1.0 μg) containing the basal *TRHR* promoter including the transcription start site and intron 1, where *n* indicates the upstream deletion endpoints: −2530, −2250, −2000, −1750, −1500 and −1250. The effector used was full-length c-Myb (0.2 μg of pEQP2-CMV-c-Myb). Bottom panel: CV-1 cells were co-transfected with the indicated reporter plasmids containing deletions in intron 1 derived from pGL2b-TRHR-1250 as described in the Experimental section. The effector used was as in the top-right panel. In all transfections 1.0 μg of reporter and 0.2 μg of effector plasmid were used. Numbers on the bars indicate fold induction (the ratio RLU with Myb/RLU without Myb). In all panels results are expressed in relative luciferase units (RLU). Luc, luciferase; wt, wild type; Mut, mutated.

TRHR1 is expressed in haematopoietic tissues and cell lines

To investigate whether *TRHR1* was expressed in haematopoietic cells, total RNA was isolated from a selection of haematopoietic tissues and cell lines and analysed for presence of *TRHR1*-related sequences by RT-PCR and Southern blotting. As shown in Figure 5 (upper panel) a clear *TRHR1*-specific amplification product was detected using RNA from thymocytes. The amplicon was of the same order of intensity as obtained with the positive control RNA isolated from the receptor-positive pituitary adenoma cell line GH_4C_1. A haematopoietic cell line included in the experiment (the RNK-16 cell line) also showed high levels of expression. When subjected to Southern analysis, clear hybridization signals verified the TRHR1 specificity of the amplicons (Figure 5, lower panel). In addition, it revealed detectable expression of TRHR1 in both bone marrow and natural killer cells. On the other hand, no expression could be detected in GH_12C_1 cells (negative control), Peyer plaques, splenocytes or lymph node samples, even after prolonged exposure. This shows that TRHR1 is expressed in specific haematopoietic cells and tissues.

To verify that the detected mRNA expression led to the expression of functional receptors on the cell surface, we analysed the capacity of TRHR1-positive cells to bind specifically the TRH analogue [^{3}H]MeTRH. As expected from the RT-PCR profile, both thymocytes and RNK-16 cells showed specific binding of the agonist, as seen by competition with unlabelled TRH displacing in the naonmolar range (Figure 6). This confirms that active TRHR1 is present on these cells. The haematopoietic cells had several-fold lower binding capacity, reflecting a much lower number of receptors compared with GH_4C_1 cells. No binding was detected to splenocytes or lymphocytes (Figure 6) or to the receptor-negative pituitary cell line GH_12C_1 (results not shown). We conclude that thymocytes as well as the cell line RNK-16 express a single class of high-affinity binding sites for TRH.

DISCUSSION

We have in the present work shown that TRHR1 is expressed in important haematopoietic tissues such as thymus and bone marrow and provided evidence that the promoter of the human receptor is activated by the haematopoietic transcription factor c-Myb. Both findings point to a novel link at the transcriptional level between the neuroendocrine system and the immune system.

Different experimental approaches were used to examine the hypothesis that c-Myb is an activator of human *TRHR1*. The multiplicity of binding sites for c-Myb, 11 within a region

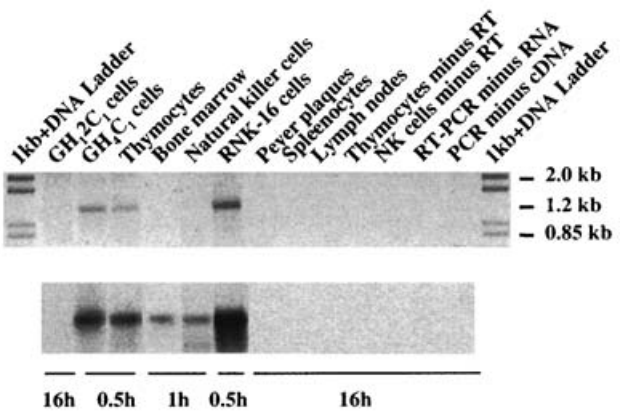


Figure 5 RT-PCR analysis of TRHR mRNA expression in a selection of haematopoietic tissues and cell lines

Rat total RNA was extracted from different tissues and cell lines and expression of rat TRHR analysed by RT-PCR performed as described in the Experimental section. The upper panel shows the agarose gel analysis of the PCR products, while the lower panel shows the autoradiogram of the blotted gel hybridized to a rat TRHR-specific probe. In all lanes 40 % of the final PCR reaction was loaded on the gel, except for the positive control GH_4C_1 sample where only 10 % was loaded. The PCR product was of the expected size (1.2 kb). For the Southern blot different exposure times were used as indicated (h). The receptor-negative GH_12C_1 cell line and the other tissues together with negative controls did not generate signals even after 16 h of exposure. NK, natural killer.

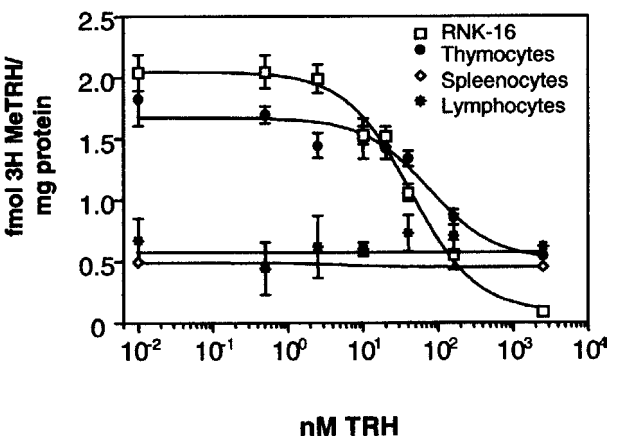


Figure 6 TRH agonist binding to thymocytes and natural killer RNK-16 cells

Equilibrium binding of [^{3}H]MeTRH to different cell types was performed as described previously [38]. The respective K_d values and maximal specific binding to TRHR were: in thymocytes, 75.5 ± 1.5 nM and 1.2 ± 0.1 fmol/mg of protein; in RNK-16 cells, 43.0 ± 1.2 nM and 2.0 ± 0.1 fmol/mg of protein. The corresponding values for the anterior pituitary GH_4C_1 TRHR (results not shown) were: 25.2 ± 1.1 nM and 115.0 ± 3.5 fmol/mg of protein. No detectable binding was observed with splenocytes or lymphocytes or with the receptor-negative control GH_12C_1 cells (the latter is not shown). The results are means ± S.E.M. from two independent experiments each carried out in triplicate.

of 2 kb, is in itself one argument. Genome-wide analysis of transcription-factor-binding sites in *Caenorhabditis elegans* has revealed that multiplicity is a highly discriminating criterion for the biological relevance of response elements [40]. Secondly, several of the putative MREs were found to bind recombinant c-Myb. In addition, extracts from c-Myb-expressing cells gave specific complexes with several of the putative MREs, with TR10 as a particularly strong binding element. A DNA fragment from intron 1 harbouring the TR10 element conferred Myb-responsiveness on a heterologous *tk* basal promoter. The same observation was made using an isolated multimerized TR10 element. Furthermore, TRHR1's own basal promoter was found to be highly Myb-responsive in transient transfection experiments, and the activation was cancelled out by a dominant-negative Myb construct and strongly reduced upon deletion or mutation of the TR10 element.

Intron 1 seems to be an important regulatory region controlling TRHR expression. We have previously mapped two binding sites for the anterior-pituitary-specific transcription factor Pit-1 in intron 1 [16]. These most probably control tissue-specific expression of the receptor in the anterior pituitary. The strongest of these sites, Pit-1B, is located only 40 bp downstream of the strong TR10 MRE. We have previously also reported the identification of a response element for the glucocorticoid receptor in intron 1 (proximal GRE) [18]. Other predicted candidate sites are present, but remain to be investigated. Intron 1 thus seems to harbour multiple response elements probably controlling in a co-ordinated fashion both tissue-specific expression and responsiveness of the promoter.

Our expression analysis in rat tissues and cell lines showed that TRHR1 is indeed expressed in major haematopoietic tissues like thymus and bone marrow. These results confirm and extend the findings of Fukusumi et al. [6], which showed by RT-PCR and Northern blot analysis that TRHR1 mRNA was expressed at relatively high levels in several peripheral tissues including thymus. The expression of TRHR1 in thymus was also confirmed by Montagne et al. [41]. Their finding in thymus of low levels of both TRH mRNA (corresponding to about 6 % of the level in the hypothalamus) and TRH peptide suggested that TRH may act locally in the thymus. Additional evidence comes from studies using a peptide-derived anti-TRHR1 antibody, also supporting the presence of TRHR1 in lymphoid tissues [42]. An important confirmation is also our present demonstration of TRH agonist binding to cell-surface receptors present on thymocytes and RNK-16 cells. Although the level of receptors expressed in these cells seems to be several-fold lower than what has been reported in the specialized pituitary adenoma cell line GH_4C_1, it could still be a level sufficiently high to play a functional role in thymocytes.

What could the biological function of TRH be in a peripheral tissue like thymus? One possible role is growth control since TRH has been reported to increase thymocyte cell proliferation in rats [43]. Similarly, Lesnikov et al. [44] made the interesting observation that a stereotactic electrolytic lesion of the anterior hypothalamic area in mice produced a rapid involution of the thymus and a reduction of lymphocytes in the peripheral blood, an effect that was prevented by postoperational administration of TRH or melatonin and which seemed to reflect a direct activity of TRH on thymic targets or binding sites on lymphocytes.

Another possibility is that TRH could act as a local hormone in one of the neuroendocrine circuits within the thymus and thus play an immunomodulatory role during an active immune response. A large body of work has established that intricate interactions of both a stimulatory and inhibitory nature exist between the neuroendocrine and immune systems (reviewed in [19–21,45]). Thymus is one central tissue in this respect [45]. Supporting evidence for a role of TRH in this network is the finding that a proper T-cell-dependent immune response requires the early activation of TRH and prolactin [22]. T-cell-dependent antigens elicited a rapid increase of hypothalamic TRH and TRHR1 mRNAs, while specific antibody production could be inhibited by intracerebro-ventricular injection of antisense TRH oligonucleotides. There is also evidence that TRH and TSH act as local hormones in the lymphoepithelial communication in the intestine, where the expression pattern of receptors for TRH and TSH suggested a hormone-mediated link between the

lymphoid and non-haematopoietic components of the intestine [46]. Consistent with this, mice with a mutant TSH receptor (*hyt*/*hyt* mice) were found to have a selectively impaired intestinal T-cell repertoire [46]. It would have been interesting to study whether the high expression of c-Myb in the intestine [47,48] also plays a role in controlling expression of TRHR in this organ. Another attractive line of investigation would have been to study links between mutations in the gene for the TRHR and impaired immune function. Mutations in TRHR causing reduced or absent receptor activity were found in a patient with isolated central hypothyroidism, but defects in immune function were not investigated [49].

In conclusion, our study provides evidence for a novel functional link between the neuroendocrine and the immune systems operating at the level of promoter regulation. The neuroendocrine receptor TRHR1 seems to be transcriptionally regulated by the haematopoietic transcription factor c-Myb. This link is also reflected in TRHR1 being functionally expressed in haematopoietic tissues and cell lines.

We thank Tor Lea for critical reading of the manuscript. We are grateful to B. Lüscher and K. Weston for kind gifts of plasmids. This work was supported by The Norwegian Research Council, The Norwegian Cancer Society, the Novo Nordic Foundation and the Anders Jahres Foundation.

REFERENCES

1 O'Leary, R. and O'Connor, B. (1995) Thyrotropin-releasing hormone. J. Neurochem. **65**, 953–963
2 Gershengorn, M. C. and Osman, R. (1996) Molecular and cellular biology of thyrotropin-releasing hormone receptors. Physiol. Rev. **76**, 175–191
3 Zabavnik, J., Arbuthnott, G. and Eidne, K. A. (1993) Distribution of thyrotrophin-releasing hormone receptor messenger RNA in rat pituitary and brain. Neuroscience **53**, 877–887
4 Calza, L., Giardino, L., Ceccatelli, S., Zanni, M., Elde, R. and Hokfelt, T. (1992) Distribution of thyrotropin-releasing hormone receptor messenger RNA in the rat brain: an *in situ* hybridization study. Neuroscience **51**, 891–909
5 Satoh, T., Feng, P., Kim, U. J. and Wilber, J. F. (1993) Identification of thyrotropin-releasing hormone receptor messenger RNA in the rat central nervous system and eye. Brain Res. Mol. Brain Res. **19**, 175–178
6 Fukusumi, S., Ogi, K., Onda, H. and Hinuma, S. (1995) Distribution of thyrotropin-releasing hormone receptor mRNA in rat peripheral tissues. Regul. Pept. **57**, 115–121
7 Wang, J., Whetsell, M. and Klein, J. R. (1997) Local hormone networks and intestinal T cell homeostasis. Science **275**, 1937–1939
8 Straub, R. E., Frech, G. C., Joho, R. H. and Gershengorn, M. C. (1990) Expression cloning of a cDNA encoding the mouse pituitary thyrotropin-releasing hormone receptor. Proc. Natl. Acad. Sci. U.S.A. **87**, 9514–9518
9 Zhao, D., Yang, J., Jones, K. E., Gerald, C., Suzuki, Y., Hogan, P. G., Chin, W. W. and Tashjian, Jr, A. H. (1992) Molecular cloning of a complementary deoxyribonucleic acid encoding the thyrotropin-releasing hormone receptor and regulation of its messenger ribonucleic acid in rat GH cells. Endocrinology **130**, 3529–3536
10 Sellar, R. E., Taylor, P. L., Lamb, R. F., Zabavnik, J., Anderson, L. and Eidne, K. A. (1993) Functional expression and molecular characterization of the thyrotrophin-releasing hormone receptor from the rat anterior pituitary gland. J. Mol. Endocrinol. **10**, 199–206
11 Matre, V., Karlsen, H. E., Wright, M. S., Lundell, I., Fjeldheim, A. K., Gabrielsen, O. S., Larhammar, D. and Gautvik, K. M. (1993) Molecular cloning of a functional human thyrotropin-releasing hormone receptor. Biochem. Biophys. Res. Commun. **195**, 179–185
12 Sun, Y. M., Millar, R. P., Ho, H., Gershengorn, M. C. and Illing, N. (1998) Cloning and characterization of the chicken thyrotropin-releasing hormone receptor. Endocrinology **139**, 3390–3398
13 Takata, M., Shimada, Y., Ikeda, A. and Sekikawa, K. (1998) Molecular cloning of bovine thyrotropin-releasing hormone receptor gene. J. Vet. Med. Sci. **60**, 123–127
14 O'Dowd, B. F., Lee, D. K., Huang, W., Nguyen, T., Cheng, R., Liu, Y., Wang, B., Gershengorn, M. C. and George, S. R. (2000) TRH-R2 exhibits similar binding and acute signaling but distinct regulation and anatomic distribution compared with TRH-R1. Mol. Endocrinol. **14**, 183–193
15 Cao, J., O'Donnell, D., Vu, H., Payza, K., Pou, C., Godbout, C., Jakob, A., Pelletier, M., Lembo, P., Ahmad, S. and Walker, P. (1998) Cloning and characterization of a cDNA encoding a novel subtype of rat thyrotropin-releasing hormone receptor. J. Biol. Chem. **273**, 32281–32287
16 Matre, V., Høvring, P. I., Ørstavik, S., Frengen, E., Rian, E., Velickovic, Z., Murray-McIntosh, R. P. and Gautvik. K. M. (1999) The structural and functional organization of the gene encoding the human thyrotropin releasing hormone receptor. J. Neurochem. **72**, 40–50
17 Iwasaki, T., Yamada, M., Satoh, T., Konaka, S., Ren, Y., Hashimoto, K., Kohga, H., Kato, Y. and Mori, M. (1996) Genomic organization and promoter function of the human thyrotropin-releasing hormone receptor gene. J. Biol. Chem. **271**, 22183–22188
18 Høvring, P. I., Matre, V., Fjeldheim, A. K., Loseth, O. P. and Gautvik, K. M. (1999) Transcription of the human thyrotropin-releasing hormone receptor gene – analysis of basal promoter elements and glucocorticoid response elements. Biochem. Biophys. Res. Commun. **257**, 829–834
19 Roszman, T. L. and Brooks, W. H. (1997) Interactive signaling pathways of the neuroendocrine-immune network. Chem. Immunol. **69**, 203–222
20 Kruger, T. E. (1996) Immunomodulation of peripheral lymphocytes by hormones of the hypothalamus-pituitary-thyroid axis. Adv. Neuroimmunol. **6**, 387–395
21 Wang, J. (1996) Dynamic regulation of intestinal immunity by hormones of the hypothalamus-pituitary-thyroid axis. Adv. Neuroimmunol. **6**, 407–417
22 Perez Castro, C., Penalva, R., Paez Pereda, M., Renner, U., Reul, J. M., Stalla, G. K., Holsboer, F. and Arzt, E. (1999) Early activation of thyrotropin-releasing-hormone and prolactin plays a critical role during a T cell-dependent immune response. Endocrinology **140**, 690–697
23 Oh, I. H. and Reddy, E. P. (1999) The myb gene family in cell growth, differentiation and apoptosis. Oncogene **18**, 3017–3033
24 Allen, III, R. D., Bender, T. P. and Siu, G. (1999) c-Myb is essential for early T cell development. Genes Dev. **13**, 1073–1078
25 Phan, S. C., Feeley, B., Withers, D. and Boxer, L. M. (1996) Identification of an inducible regulator of c-myb expression during T-cell activation. Mol. Cell. Biol. **16**, 2387–2393
26 Weston, K. (1998) Myb proteins in life, death and differentiation. Curr. Opin. Genet. Dev. **8**, 76–81
27 Badiani, P., Corbella, P., Kioussis, D., Marvel, J. and Weston, K. (1994) Dominant interfering alleles define a role for c-Myb in T-cell development. Genes Dev. **8**, 770–782
28 Taylor, D., Badiani, P. and Weston, K. (1996) A dominant interfering Myb mutant causes apoptosis in T cells. Genes Dev. **10**, 2732–2744
29 Hernandez-Munain, C. and Krangel, M. S. (1995) c-Myb and core-binding factor/PEBP2 display functional synergy but bind independently to adjacent sites in the T-cell receptor delta enhancer. Mol. Cell. Biol. **15**, 3090–3099
30 Ess, K. C., Whitaker, T. L., Cost, G. J., Witte, D. P., Hutton, J. J. and Aronow, B. J. (1995) A central role for a single c-Myb binding site in a thymic locus control region. Mol. Cell. Biol. **15**, 5707–5715
31 Badiani, P. A., Kioussis, D., Swirsky, D. M., Lampert, I. A. and Weston, K. (1996) T-cell lymphomas in v-Myb transgenic mice. Oncogene **13**, 2205–2212
32 Oelgeschläger, M., Krieg, J., Lüscher-Firzlaff, J. M. and Lüscher, B. (1995) Casein kinase II phosphorylation site mutations in c-Myb affect DNA binding and transcriptional cooperativity with NF-M. Mol. Cell. Biol. **15**, 5966–5974
33 Andersson, K. B., Berge, T., Matre, V. and Gabrielsen, O. S. (1999) Sequence selectivity of c-Myb *in vivo*. Resolution of a DNA target specificity paradox. J. Biol. Chem. **274**, 21986–21994
34 Gabrielsen, O. S., Sentenac, A. and Fromageot, P. (1991) Specific DNA binding by c-Myb: evidence for a double helix-turn-helix-related motif. Science **253**, 1140–1143
35 Ording, E., Bergholtz, S., Brendeford, E. M., Jamin, N. and Gabrielsen, O. S. (1996) Flexibility in the second half-site sequence recognised by the c-Myb R2 domain – *in vitro* and *in vivo* analysis. Oncogene **13**, 1043–1051
36 Naper, C., Vaage, J. T., Lambracht, D., Lovik, G., Butcher, G. W., Wonigeit, K. and Rolstad, B. (1995) Alloreactive natural killer cells in the rat: complex genetics of major histocompatibility complex control. Eur. J. Immunol. **25**, 1249–1256
37 Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**, 5294–5299
38 Gautvik, K. M. and Lystad, E. (1981) Demonstration of a heterogenous population of binding sites for thyroliberin in prolactin-producing tumour cells and their possible functional significance. Eur. J. Biochem. **116**, 235–242
39 Deng, Q. L., Ishii, S. and Sarai, A. (1996) Binding site analysis of c-Myb: screening of potential binding sites by using the mutation matrix derived from systematic binding affinity measurements. Nucleic Acids Res. **24**, 766–774

40 Clarke, N. D. and Berg, J. M. (1998) Zinc fingers in *Caenorhabditis elegans*: finding families and probing pathways. Science **282**, 2018–2022
41 Montagne, J. J., Ladram, A., Nicolas, P. and Bulant, M. (1999) Cloning of thyrotropin-releasing hormone precursor and receptor in rat thymus, adrenal gland, and testis. Endocrinology **140**, 1054–1059
42 Mellado, M., Fernandez-Agullo, T., Rodriguez-Frade, J. M., San Frutos, M. G., de la Pena, P., Martinez, A. C. and Montoya, E. (1999) Expression analysis of the thyrotropin-releasing hormone receptor (TRHR) in the immune system using agonist anti-TRHR monoclonal antibodies. FEBS Lett. **451**, 308–314
43 Pawlikowski, M., Zerek-Melen, G. and Winczyk, K. (1992) Thyroliberin (TRH) increases thymus cell proliferation in rats. Neuropeptides **23**, 199–202
44 Lesnikov, V. A., Korneva, E. A., Dall'ara, A. and Pierpaoli, W. (1992) The involvement of pineal gland and melatonin in immunity and aging: II. Thyrotropin-releasing hormone and melatonin forestall involution and promote reconstitution of the thymus in anterior hypothalamic area (AHA)-lesioned mice. Int. J. Neurosci. **62**, 141–153
45 Moll, U. M. (1997) Functional histology of the neuroendocrine thymus. Microsc. Res. Tech. **38**, 300–310
46 Wang, J. and Klein, J. R. (1994) Thymus-neuroendocrine interactions in extrathymic T cell development. Science **265**, 1860–1862
47 Thompson, M. A. and Ramsay, R. G. (1995) Myb: an old oncoprotein with new roles. Bioessays **17**, 341–350
48 Thompson, M. A., Flegg, R., Westin, E. H. and Ramsay, R. G. (1997) Microsatellite deletions in the c-myb transcriptional attenuator region associated with over-expression in colon tumour cell lines. Oncogene **14**, 1715–1723
49 Collu, R., Tang, J., Castagne, J., Lagace, G., Masson, N., Huot, C., Deal, C., Delvin, E., Faccenda, E., Eidne, K. A. and Van Vliet, G. (1997) A novel mechanism for isolated central hypothyroidism: inactivating mutations in the thyrotropin-releasing hormone receptor gene. J. Clin. Endocrinol. Metab. **82**, 1561–1565
50 Ness, S. A., Marknell, A. and Graf, T. (1989) The v-myb oncogene product binds to and activates the promyelocyte-specific mim-1 gene. Cell **59**, 1115–1125

Received 6 January 2003/3 March 2003; accepted 10 March 2003
Published as BJ Immediate Publication 10 March 2003, DOI 10.1042/BJ20030057