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Biochemistry

Thyrotropin-releasing hormone regulation of human *TSHB* expression: Role of a pituitary-specific transcription factor (Pit-1/GHF-1) and potential interaction with a thyroid hormone-inhibitory element

(cis-acting element/thyrotroph/DNA-binding assay/trans-acting factor)

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ABSTRACT **Regulation of human thyrotropin β subunit gene (*TSHB*) expression by thyrotropin-releasing hormone (TRH) was examined in a clonal rat pituitary-cell line (GH_3). Transient expression studies were done with various 5′-flanking DNA sequences of *TSHB* coupled to reporter gene chloramphenicol acetyltransferase. Deletion analysis defined two discrete regions (−128 to −92 base pairs and −28 to +8 base pairs) that each mediated an ≈2-fold TRH induction. The upstream site contains a DNA sequence with close homology to the DNA-binding site for a pituitary-specific transcriptional factor Pit-1/GHF-1. DNase I footprinting analysis of mouse thyrotropic tumor extract as well as DNA-transfection studies using an expression vector containing an N-terminal deletion of Pit-1/GHF-1 cDNA suggest that Pit-1/GHF-1 or a closely related protein in the thyrotroph mediates TRH responsiveness of this gene. In addition, the downstream site overlaps with the recently characterized thyroid hormone-inhibitory element of *TSHB*. In fact, deletion of DNA sequences important in thyroid hormone-receptor binding (c-*erbAB*/c-*ERBA2*) from +3 to +8 base pairs, significantly reduced (30%) TRH responsiveness. The location of a TRH-stimulatory element near a thyroid hormone-inhibitory element may allow for fine control of *TSHB* expression *in vivo*.**

Thyroid-stimulating hormone like other pituitary glycoprotein hormones contains two dissimilar noncovalently linked subunits, α and β. The major negative regulator of the synthesis of both subunits is thyroid hormone. Shupnik *et al.* (1) first demonstrated that thyroid hormone acted predominately at a transcriptional level to regulate expression of these subunit genes. Recently, we and others have shown that thyroid hormone-inhibitory elements are located near the transcriptional start site of the common *TSHA* and *TSHB* (α and β subunit genes, respectively) genes (2–6), suggesting that the mechanism for inhibition may involve a displacement of or interaction with other transcriptional proteins. Moreover, our laboratory and others (7, 8) have reported that a thyroid hormone-inhibitory element of *TSHB* has at least two thyroid hormone receptor-binding sites (c-*ERBA*), and both appear necessary for thyroid hormone inhibition (8).

In contrast, much less is known about the effect of the major positive regulator of TSH synthesis, thyrotropin-releasing hormone (TRH). It is known that TRH increases the transcriptional rate of both TSH subunit genes 3- to 5-fold in pituitary cells from hypothyroid animals (9) and that the increase in steady-state common TSHA and TSHB mRNAs in response to TRH treatment appears to depend on the thyroidal state (9, 10). Moreover, Carr *et al.* (11) have shown that TRH stimulates expression of the rat *Tshb* gene in a transient transfection assay, but a clear localization of the cis-acting element(s) that mediates this effect was not found. Taken together, these studies suggest that thyroid hormone and TRH may not act independently to regulate expression of β subunit.

In the current study, DNA-transfection studies and DNA-binding assays were used to localize discrete cis-acting elements that mediate TRH stimulation of *TSHB* and to begin to determine the trans-acting factors that mediate this response. Experiments were done in GH_3 cells, a rat pituitary cell line producing growth hormone (GH) and prolactin (12) and containing functional TRH receptors (11, 13, 14).

METHODS

Plasmid Construction. Construction of pTKCAT, p−1200/+8, p−128/+37, and p−128/+8hTSHβCAT has been described (5). An internal deletion was constructed between −613 and −199 bp of p−1200/+8hTSHβCAT using *Sph* I (pΔSShTSHβCAT). Other deletion constructs were obtained using PCR. The 5′ primers were synthesized with *Eco*RI or *Kpn* I restriction sites, and 3′ primers were synthesized with *Hin*dIII sites. The −128/+8 construct was used as a template. PCR products were ligated to the *Hin*dIII site of the chloramphenicol acetyltransferase (CAT) coding sequence, and the product was inserted into the *Eco*RI/*Kpn* I and *Bam*HI sites of pUC18/19 vectors. pTKCAT constructs containing regions of the *TSHB* 5′-flanking region were obtained by inserting PCR fragments, containing *Kpn* I and *Bam*HI ends, upstream of the thymidine kinase (TK) promoter in pTKCAT.

The pSVL-c-erbAβ expression vector was constructed by inserting a human c-ERBA2 cDNA (15) containing *Xba* I and *Bam*HI ends into the multiple-cloning site of pSVL (Pharmacia LKB). The pCMVPit-1 expression vector (from L. Staudt, National Institutes of Health) contains the cytomegalovirus promoter (CMV) upstream and the rabbit β-globin

Abbreviations: Pit-1/GHF-1, pituitary transcriptional factor 1; TRH, thyrotropin-releasing hormone; TSH, human thyrotropin; CAT, chloramphenicol acetyltransferase; T_3, triiodothyronine; CMV, cytomegalovirus promoter; TK, thymidine kinase; GH, growth hormone; rGH, rat growth hormone; ABCD assay, avidin–biotin complex DNA-binding assay; MTT, mouse thyrotropic tumor; Y, pyrimidine; W, deoxyadenosine or thymidine; M, deoxycytidine or deoxyadenosine.

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intron and polyadenylylation site downstream of Pit-1/GHF-1. pCMV was constructed by removing the Pit-1/GHF-1 coding region with *Xba* I and *Bam*HI and religating blunt ends of the vector. pCMVΔPit-1 was constructed by inserting a truncated version of the Pit-1/GHF-1 coding region lacking DNA sequences encoding amino acids 2–45. All constructs were confirmed by multiple restriction-enzyme analysis and/or DNA sequencing.

Transfection and CAT Assays. GH_3 cells were cultured and transfected using described methods (16). Twenty-five micrograms of the phTSHβCAT constructs and 5 μg of a pTKGH construct were cotransfected per 100-mm plate. The morning after transfection, cell cultures were washed with serum-free medium and shocked for 2 min with 20% glycerol in Hepes saline (pH 7.5). Thereafter cell cultures were incubated in serum-free Dulbecco's modified essential medium/10 μM $ZnSO_4$/100 μM $MgSO_4$/4 mM L-glutamine/insulin at 10 μg/ml/transferrin at 5 μg/ml/selenite at 5 ng/ml/1% bovine serum albumin. CAT activity in the cell lysate was usually determined after 24-hr treatment, as described (5). The concentration of human GH was determined in the medium, and CAT activity was corrected for transfection efficiency.

DNA-Binding Assays. The avidin–biotin complex DNA-binding (ABCD) assay was used to localize human c-*ERBA* binding in the human and rat β subunit gene by using described methods (5). Each DNA fragment contained identical 5′ overhangs (10 base overhangs on each end), which, when repaired by *Taq* polymerase and biotin 11-dUTP, incorporated 11 biotin residues.

DNase I footprinting was performed using a *TSHB* DNA fragment from −199 (*Sph* I) to +79 (*Afl* II) bp, labeled at the *Afl* II site by using *Taq* polymerase, dTTP, and [α-^{32}P]ATP. Nuclear extract was prepared from mouse thyrotropic tumor (MTT) by using the method of Dignam *et al.* (17). DNA-binding reactions were done in 50 μl of 50 mM KCl/20% (vol/vol) glycerol/20 mM Hepes, pH 7.9/1 mM dithiothreitol/0.1% Nonidet P-40/10 nM triiodothyronine (T_3) containing 4 μg of poly(dI-dC) for 30 min at 25°C. DNase I (0.6 μg) partially digested the radiolabeled template (2 min at 25°C), and the reaction was terminated with 100 μl of 50 mM EDTA/1% SDS containing 20 μg of yeast tRNA. The digested products were extracted with phenol and chloroform, precipitated with ethanol, and resolved on an 8% denaturing polyacrylamide gel. One hundred-fold molar excess (2nM) of a rat specific or mutated DNA fragment of a high-affinity Pit-1/GHF-1-binding site (−99 to −60 bp), as described (18), was used in some binding experiments

RESULTS

Localization of TRH-Responsive Regions. Initial experiments were done with three different hTSHβCAT constructs in GH_3 cells (Fig. 1). The p−1200/+8hTSHβCAT and pΔSShTSHβCAT constructs were stimulated 2.0- to 2.2-fold by TRH (10 nM), but the p−128/+8hTSHβCAT construct displayed the most pronounced induction (4-fold). The concentration–response of CAT stimulation by TRH was studied over 10^{-10}–10^{-7} M TRH; the concentration necessary for half-maximal stimulation was ≈2 nM (data not shown) and was identical to that for half-maximal stimulation of prolactin secretion in these cells (13).

Deletional Analysis of the Region from −128 to +37 bp of *TSHB*. TRH stimulated the p−128/+8hTSHβCAT construct 3.8-fold, and a 5′ deletion to −28 bp (p−28/+8hTSHβCAT) resulted in 2.2-fold induction (Fig. 1). This induction was significantly higher than the 1.2-fold effect of the agent on pTKCAT activity. To test whether inclusion of the first exon of *TSHB*, which contains a thyroid hormone-inhibitory element, affected TRH induction without any T_3 treatment, we

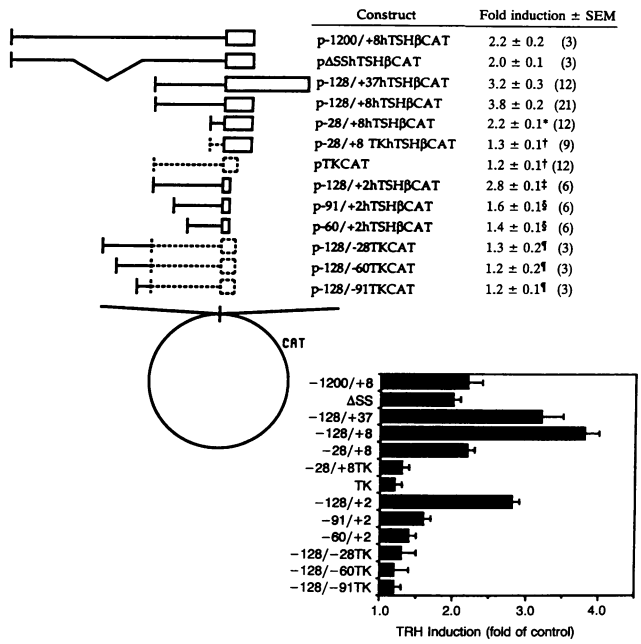

FIG. 1. Analysis of 5′ deletions in *TSHB* in GH_3 cells to isolate TRH-responsive regions. GH_3 cells were transfected with the indicated constructs (—, *TSHB* sequences; – –, TK sequences) and, after glycerol shocking, exposed to serum-free medium containing either no TRH or 10 nM TRH. CAT activity of TRH-treated cells is expressed as -fold of that seen in respective untreated cells. Values in parentheses are numbers of individual transfections. *Unpaired *t* test: $P < 0.01$, −128/+8 versus −28/+8, †unpaired *t* test: $P < 0.01$, −28/+8 versus indicated construct, ‡unpaired *t* test: $P < 0.05$, −128/+8 versus −128/+2, §unpaired *t* test: $P < 0.01$, −128/+2 versus indicated construct, and ¶unpaired *t* test: not significant when compared with pTKCAT.

compared the TRH effect on expression of the p−128/+8hTSHβCAT and p−128/+37hTSHβCAT constructs. Inclusion of bases +9 to +37 did not seem to alter TRH induction; in fact, induction was slightly lower in the construct containing a complete thyroid hormone-inhibitory element (Fig. 1).

To localize the TRH response to more discrete regions, another set of plasmids was constructed ranging from −128 bp, −91 bp, and −60 bp to only +2 bp of the first exon. When compared with the activity seen from p−128/+8hTSHβCAT, TRH-stimulated CAT activity dropped from 3.8- to 2.8-fold in the p−128/+2hTSHβCAT construct (Fig. 1). Two successive deletions, each of ≈30 bp, the p−91/+2hTSHβCAT and p−60/+2hTSHβCAT constructs, resulted in a drastic loss of induction. Thus, the region from +3 to +8 bp and from −128 to −92 bp seemed essential for a full stimulatory effect by TRH.

TRH Induction Could Not Be Transferred to a Heterologous Promoter. We next evaluated whether DNA sequences from +3 to +8 bp of the first exon were sufficient to mediate a TRH response. A hybrid plasmid was constructed with −28 to −1 bp of the TK promoter upstream of the first 8 bp of the *TSHB* first exon (p−28/+8TKhTSHβCAT). TRH treatment of cell cultures transfected with this construct resulted in 1.3-fold stimulation (Fig. 1), comparable to that seen with TKCAT-containing construct (1.2-fold). These data suggest that, in addition to sequences between +3 and +8 bp, sequences from −28 to −1 bp must also be important for TRH induction. Moreover, we were unable to transfer a TRH-stimulatory effect to the TK promoter using the more upstream region (Fig. 1, p−128/−28, −128/−60, and −128/−91TKCAT).

Potential Interaction Between TRH-Stimulatory and Thyroid Hormone-Inhibitory Elements. We next addressed

whether an interaction between the thyroid hormone-inhibitory element located between +3 and +37 bp by functional assays (5) and an adjacent TRH-stimulatory element might occur. Therefore, we studied the effect of TRH on the p−128/+8hTSHβCAT construct (which contains only part of the thyroid hormone-inhibitory element), the p−128/+37hTSHβ construct (which contains the complete element), and TKCAT with and without T_3 (10 nM). T_3 treatment of *TSHB* (hTSHβ) constructs inhibited basal CAT activity by only 10–20%, whereas CAT activity from pTK-CAT increased ≈10% (Table 1, experiment I). When stimulated with TRH, on the other hand, a significant inhibition by T_3 (30%) was seen with the p−128/+37hTSHβCAT construct (310% versus 220%), but T_3 inhibited the p−128/+8hTSHβCAT construct only 10% (340% versus 300%).

Interestingly, when the experiment was repeated with cotransfection of 5 μg of pSVL-c-erbAβ, TRH-stimulated CAT was only 230% of basal activity in the p−128/+37hTSHβCAT construct, either with or without T_3 (Table 1, experiment II). In contrast, the p−128/+8hTSHβCAT construct displayed a similar pattern of stimulation to TRH whether pSVL-c-erbAβ was cotransfected (experiment II) or not cotransfected (experiment I). This effect, however, was not from a change in basal expression because basal expression was similar in both constructs whether pSVL-c-erbAβ was or was not cotransfected (data not shown).

Localization of Thyroid Hormone Receptor (c-*ERBA2*)-Binding Sites Near the Transcriptional Start Site of *TSHB*. The ABCD assay was used to localize c-*ERBA2*-binding sites in the rat *Tshb* and human *TSHB* genes. Fig. 2 illustrates the avidity of various DNA fragments of the human *TSHB* and rat *Tshb* genes for ^{35}S-labeled c-ERBA2 protein. A segment of the long terminal repeat of the adenovirus 5 was used as a negative control, and a 5′-flanking region of the rat GH gene (rGH, −188 to −160 bp) was used as a positive control. Both the rGH fragment and a DNA fragment containing −12 to +43 bp of *TSHB* bound significantly more c-ERBA2 protein than the negative control fragment adenovirus 5. Within the region from −12 to +43 bp of *TSHB* are two regions, from −12 to +19 bp and +18 to +43 bp, that appear to bind the c-*ERBA2* receptor with different avidities. The 3′ site bound approximately the same amount of c-ERBA2 protein as the rGH fragment but bound 8-fold less protein than the more-5′ site. Equivalent regions of rat *Tshb* (numbering differs due to a 10-bp difference in transcriptional start sites) bound with a similar pattern to the c-ERBA2 protein.

Table 1. Potential interaction between cis-acting elements mediating stimulatory TRH and inhibitory thyroid hormone responses in *TSHB*

Construct	CAT activity, % basal		
	T_3	TRH	T_3 + TRH
Exp. I			
p−128/+37hTSHβCAT	90 ± 10	310 ± 20*	220 ± 10
p−128/+8hTSHβCAT	80 ± 10	340 ± 20†	300 ± 30
pTKCAT	110 ± 10	140 ± 10†	150 ± 10
Exp. II			
p−128/+37hTSHβCAT		230 ± 10†‡	230 ± 10
p−128/+8hTSHβCAT		320 ± 50†§	280 ± 20

Effects of T_3 (10 nM) and/or TRH (10 nM) on expression of p−128/+37hTSHβCAT, p−128/+8hTSHβCAT, and pTKCAT were tested in GH_3 cells, cultured in serum-free conditions, without (experiment I) or with (experiment II) a cotransfected c-*ERBA2* expression plasmid. CAT activity is expressed as percentage of untreated cell activity. Values are the mean ± SEM of 3–10 individual transfections. *Unpaired *t* test: $P < 0.01$, TRH versus T_3 + TRH; †unpaired *t* test: not significant, TRH versus T_3 + TRH; ‡unpaired *t* test: $P < 0.05$, TRH with c-*ERBA2* versus without; and §unpaired *t* test: not significant, TRH with c-*ERBA2* versus without c-*ERBA2*.

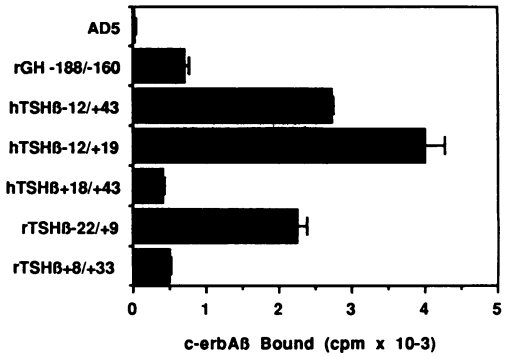

FIG. 2. ABCD assay using ^{35}S-labeled c-ERBA2 protein and various regions of the human *TSHB* and rat *Tshb* genes. Biotinylated DNA fragments (1 pM) from the adenovirus 5 (AD5), rGH, human *TSHB* (hTSHβ), and rat *Tshb* (rTSHβ) genes and 1.5 × 10^4 cpm of ^{35}S-labeled c-ERBA2 (c-erbAβ) protein were used. Results are expressed as mean ± SEM.

To localize further *TSHB* sequences that bind the thyroid hormone receptor, DNase I footprinting analysis was done with *in vitro*-synthesized c-ERBA2 protein. This translation extract yielded a specific footprint from +4 to +16 bp (Fig. 3, lanes 4 and 5), as compared to no added extract or unprogrammed translation extract (Fig. 3, lane 1 and lanes 2

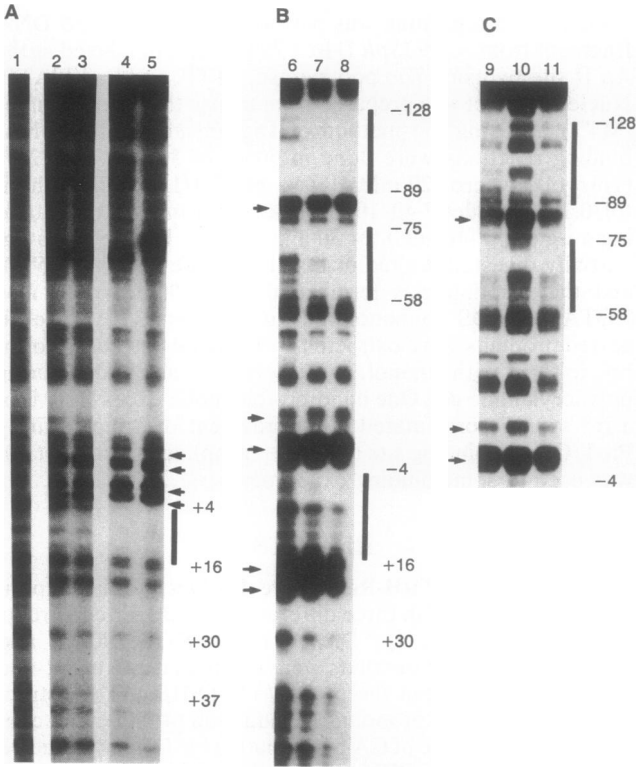

FIG. 3. DNase I footprint of a *TSHB* DNA fragment (−199 to +79 bp). DNase I footprinting was done with various protein extracts. Arrowheads, DNase I hypersensitivity sites; bars, footprinted regions. (*A*) Lanes: 1, probe only; 2 and 3, 4 μl and 8 μl of unprogrammed *in vitro* translation extract, respectively; 4 and 5, 4 μl and 8 μl of *in vitro* translation extract programmed with c-ERBA2 mRNA. (*B*) Lanes: 6–8, 15, 30, and 60 μg of MTT extract, respectively. (*C*) Lane: 9, 30 μg of MTT extract without specific competitor DNA; 10, 30 μg of MTT extract and 100-fold molar excess of rGH Pit-1/GHF-1 competitor DNA fragment (see text); 11, 30 μg of MTT extract and 100-fold molar excess of rGH mutated-Pit-1/GHF-1 competitor-DNA fragment (see text).

and 3, respectively). This footprint corresponds to the more 5′-binding site that displayed higher avidity in the ABCD assay. A DNase I footprint corresponding to the more 3′-binding site in the ABCD assay may be present between +30 and +43 bp. An equivalent DNase I footprinting analysis was obtained without any T_3 (data not shown).

The DNase I footprinting analysis was repeated with nuclear extract from MTT, a TSH-secreting mouse tumor propagated in hypothyroid mice. Increased amounts of this extract (Fig. 3, lanes 6–8) yielded a footprint from −4 to +16 bp of *TSHB*. Hypersensitivity sites were found surrounding this footprint, which extended more 5′ than the footprint generated by c-*ERBA2* itself (Fig. 3, lanes 4 and 5). A weaker footprint between +30 and +43 bp and corresponding to the lower-avidity site in the ABCD assay was also identified.

Pit-1/GHF-1 or a Closely Related Protein Binds to the *TSHB* 5′-Flanking Region. MTT nuclear extract also yielded two strong DNase I footprints at −128 to −89 bp and −75 to −58 bp of *TSHB*, separated by a DNase I hypersensitivity site (Fig. 3). Because these regions contain DNA sequences homologous to Pit-1/GHF-1 DNA-binding site (see *Discussion*), we next tested whether a specific or a mutated Pit/GHF-1 DNA-binding site from the rGH gene (−99 to −60 bp) would compete for these DNase I footprints. One hundred-fold molar excess of the specific (lane 10) Pit-1/GHF-1 eliminated the footprints at −128 to −89 and −75 to −58 bp; 100-fold molar excess of mutated Pit-1/GHF-1 (lane 11) did not have this effect.

Cotransfection of an Expression Vector Containing a Truncated Version of Pit-1/GHF-1 Blocks TRH Induction of *TSHB*. Because functional domains for TRH induction in *TSHB* contain binding sites for Pit-1/GHF-1 or a closely related protein, we tested whether a truncated version of Pit-1/GHF-1 protein, which lacks most of the N-terminal transactivation domain (19, 20) but contains an intact DNA-binding domain, might function as a competitive antagonist and block TRH induction. Fig. 4 shows that increased amounts of a cotransfected pCMV expression vector containing this Pit-1/GHF-1 deletion mutant (pCMVΔPit-1) specifically blocked TRH induction of *TSHB* (p−128/+8hTSHβCAT), whereas the expression vector alone (pCMV) did not significantly alter TRH induction of the control (pTKCAT) or *TSHB* (p−128/+8hTSHβCAT) constructs.

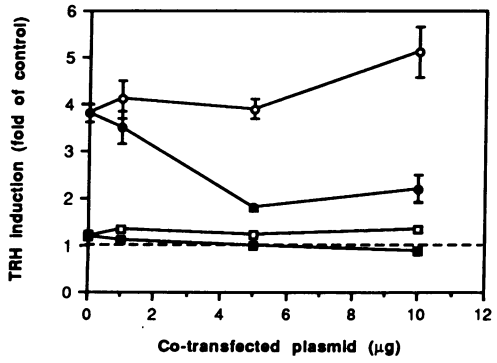

FIG. 4. Cotransfection of an expression vector containing a deletion mutant of Pit-1/GHF-1 in GH_3 cells. TRH induction (-fold of control) of pTKCAT (TK) or p−128/+8hTSHβCAT (TSHB) without cotransfection or after cotransfection of increased amounts of either expression vector alone, pCMV (CMV), or pCMV containing an N-terminal deletion mutant of Pit-1/GHF-1, pCMVΔPit-1 (CMVΔPIT). □, TK, CMV; ■, TK, CMVΔPIT; ○, TSHB, CMV; ●, TSHB, CMVΔPIT. Results are expressed as mean ± SEM of triplicate determinations.

DISCUSSION

Deletion analysis defined two regions, each ≈30 bp long, that independently mediated a 2- to 2.5-fold increase in *TSHB* expression in GH_3 cells. When tested together in the p−128/+8hTSHβCAT construct, the stimulatory effect was additive (4-fold). Data regarding magnitude, concentration-dependency, and time course of induction of the *TSHB* promoter by TRH agree well with *in vitro* analysis of mRNA levels, transcriptional activity, and DNA transfection in rat pituitary cells (9–11, 21). However, Carr *et al.* (11) and Shupnik *et al.* (21) have localized TRH-responsive regions more upstream in rat *Tshb*. The reason for this discrepancy is unclear at present.

Expression of the prolactin and GH genes is regulated in a cell-specific manner by the transcription factor Pit-1/GHF-1 (22, 23). Day *et al.* (14) have shown that TRH-responsive regions in the rat prolactin gene contain Pit-1/GHF-1 consensus sequences that bind Pit-1/GHF-1. Because recent data suggest that approximately half of the thyrotrophs in the pituitary express Pit-1/GHF-1 (24), this factor might also be important in regulating *TSHB* and *Tshb*. A consensus Pit-1/GHF-1 DNA-binding site was defined (YYWNWNAWWTATNCAT) in the prolactin and GH genes (22, 23). Within −128 to −28 bp of the *TSHB* 5′-flanking DNA are three regions with high homology to this sequence (Table 2). The most-5′ of these elements (−119 to −104 bp) has the highest homology to the core sequence of the Pit-1/GHF-1 binding sequence (8 of 10). Fig. 3 shows that nuclear extract from MTT, a pure population of thyrotrophs, generates two DNase I footprints at −128 to −89 bp and −75 to −58 bp, which correspond closely to the homologous regions noted in Table 2. In addition, 100-fold molar excess of a specific competitor of the Pit-1/GHF-1 DNA-binding site (Fig. 3, lane 10) but not a mutated version of the same site (Fig. 3, lane 11) eliminated the footprints at −128 to −89 bp and −75 to −58 bp. Analogously, DNA fragments from rat *Tshb* were effective competitors for Pit-1/GHF-1 binding to the prolactin distal enhancer (23). These data indicate that Pit-1/GHF-1, or a closely related protein in the thyrotroph, binds to this region in the *TSHB* gene.

Therefore, because deletion from −128 to −91 bp caused the most pronounced loss of TRH induction and because Pit-1/GHF-1 or a closely related protein in the thyrotroph binds to this region, we directly tested whether an antagonist of Pit-1/GHF-1 might abolish TRH induction of *TSHB*. A CMV expression vector containing an N-terminal deletion mutant of Pit-1/GHF-1 (amino acids 2–45 deleted) specifically blocked TRH induction of *TSHB* in GH_3 cells. This mutant lacks most of the N-terminal region, which is rich in hydroxylated amino acids, and is defective in transcriptional activation; however, this mutant was shown to bind normally to the Pit-1/GHF-1 DNA-recognition site (19). Presumably, the mutant acted as a competitive antagonist in GH_3 cells, which are rich in Pit-1/GHF-1, to block TRH induction. These data suggest that Pit-1/GHF-1, or a closely related

Table 2. Possible Pit-1/GHF-1 recognition sequences in the 5′-flanking region of *TSHB*

	Sequence	Match
Consensus	YYWNWNAWWTATNCAT	
Core	AWWTATNCAT	
Boundary		
−104/−119	TCTATTGAAAATTCAT	14/16
−89/−104	CTTATCTGAAAAGCAT	12/16
−58/−73	GTTTATACAATTGCAT	12/16

The *TSHB* from −128 to +8 bp was screened for homologies to the consensus binding sequence of Pit-1/GHF-1 as characterized by Nelson *et al.* (23).

Table 3. Comparison of human, rat, and mouse thyrotropic β-subunit genes and rGH gene

H	−1	T	GGG	T	C	ACC	AC	A	GCA	TCTGCTCACCAA	TG	C	AAAG		TAAG	+37
R	−11	T	GGG	T	C	ATC	AC	A	GCA	TTAACTCGCCAG	TG	C	AAAG		TAAG	+27
M	−11	T	GGG	T	C	ATC	AC	A	GCA	GTAACTCACTCA	TG	C	AAAG		TAAG	+27
GH	−179	A	GGG	A	C	GTG	AC	C	GCA	−165 −194	TG	G	AAAG	G	TAAG	−183

Human (H), rat (R), and mouse (M) genes are aligned with two 5′-flanking regions of rGH (GH) thyroid hormone-response element. Boxed nucleotides are identical among the four genes.

protein in the thyrotroph, is the trans-acting factor responsible for TRH stimulation of *TSHB* expression.

A second region from the TATA box (beginning at −28 bp) to +8 bp of the first exon, which still confers an ≈2.2-fold induction by TRH, might be important for structural interaction between a trans-acting factor responsible for stimulatory regulation by TRH and inhibitory regulation by thyroid hormone. The thyroid hormone receptor, c-*ERBA2*, binds to two regions of the *TSHB* first exon, as determined by the ABCD assay; each region shares homology with DNA sequences from the rGH thyroid hormone-response element (see Table 3). The more 5′ site appears to have a higher avidity for c-*ERBA2* (see Fig. 2) and overlaps with a TRH-response element described in this study (−28 to +8 bp). In addition, both the 5′ and 3′ sites contain a DNA sequence (+1 GGGTCA +6, and +31 AAGTAA +36) homologous to a consensus thyroid hormone receptor-binding site proposed by Brent *et al.* (25) from studies in rGH gene, AGGTMA. Interestingly, when bases from +3 to +8 bp of *TSHB* were deleted in our study, TRH-stimulated CAT activity was significantly reduced by ≈30% (see Fig. 1).

Thus the effect of thyroid hormone in modulating TRH-stimulated expression of *TSHB* was evaluated by using either the p−128/+8hTSHβCAT or p−128/+37hTSHβCAT construct. Unlike a previous report by Carr *et al.* (4) in this cell line, we observed only a 10–20% decrease in basal activity of *TSHB* in response to T_3 treatment. The reason for the discrepancies in these results is unclear but may be due to the particular constructs, transfection method, and/or GH_3 clonal line we used. On the other hand, T_3 treatment significantly reduced TRH-stimulated CAT activity from the p−128/+37hTSHβCAT construct (30%); and this reduction was greater than that obtained with a construct containing only part of the thyroid hormone-inhibitory element, p−128/+8hTSHβCAT (10%). Although reduction in TRH stimulation of *TSHB* expression by T_3 was only 30%, this reduction by T_3 was consistently seen and was of the same magnitude as that seen *in vivo* (26). Moreover, cotransfection of a human c-*ERBA2* expression (pSVL-c-erbAβ) plasmid resulted in a significant T_3-independent reduction in TRH-stimulated CAT activity from the p−128/+37hTSHβCAT construct but did not alter the pattern of TRH-stimulated expression, with and without T_3, from the p−128/+8hTSHβCAT construct. These data suggest that the thyroid hormone receptor may interact with a nuclear factor responsible for TRH stimulation via overlapping cis-acting elements. In support of this hypothesis, a DNase I footprint from −4 to +16 bp was noted with the MTT extract, which encompasses the c-*ERBA2* footprint from +4 to +16 but also extends more 5′ to −4 bp.

A confounding variable in these studies is the fact that thyroid hormone has been shown to decrease the number of TRH receptors in GH_3 cells (27). Alternatively, TRH was shown to decrease nuclear thyroid hormone receptor number in GH_4 cells (28). Thus, the reduction in TRH-stimulated CAT activity by T_3 could be due to a reduction in TRH action from a decrease in TRH receptor number or T_3 receptor number. However, the differential effects of T_3 on the p−128/+8hTSHβCAT and p−128/+37hTSHβCAT constructs with and without transfected c-*ERBA2*, as well as the reduction in TRH-stimulated CAT activity after deletion of part of the thyroid hormone inhibitory element (+3 to +8 bp) make these explanations less likely.

In conclusion, treatment with TRH induces CAT activity of a construct containing −128 to +8 bp of *TSHB*. Two regions, each of ≈30 bp, contributed to this effect. The upstream region is located between −128 and −92 bp and binds Pit-1/GHF-1 or a closely related protein in the thyrotroph. The downstream region is located between −28 and +8 bp. This element might be important for a structural interaction between the stimulatory regulation of *TSHB* expression by the hypothalamic mediator TRH and its inhibitory regulation by thyroid hormones.

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