Thyroid-hormone-dependent negative regulation of thyrotropin β gene by thyroid hormone receptors: study with a new experimental system using CV1 cells

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The molecular mechanism involved in the liganded thyroid hormone receptor suppression of the TSHβ (thyroid-stimulating hormone β, or thyrotropin β) gene transcription is undetermined. One of the main reasons is the limitation of useful cell lines for the experiments. We have developed an assay system using non-pituitary CV1 cells and studied the negative regulation of the TSHβ gene. In CV1 cells, the TSHβ–CAT (chloramphenicol acetyltransferase) reporter was stimulated by Pit1 and GATA2 and suppressed by T_3 (3,3′,5-tri-iodothyronine)-bound thyroid hormone receptor. The suppression was dependent on the amounts of T_3 and the receptor. Unliganded receptor did not stimulate TSHβ activity, suggesting that the receptor itself is not an activator. Analyses using various receptor mutants revealed that the intact DNA-binding domain is crucial to the TSHβ gene suppression. Co-activators and co-repressors are not necessarily essential, but are required for the full suppression of the TSHβ gene. Among the three receptor isoforms, β2 exhibited the strongest inhibition and its protein level was the most predominant in a thyrotroph cell line, TαT1, in Western blotting. The dominant-negative effects of various receptor mutants measured on the TSHβ–CAT reporter were not simple mirror images of those in the positive regulation under physiological T_3 concentration.

Key words: GATA2, negative-feedback loop, Pit1, thyroid hormone, thyroid hormone receptor, thyroid-stimulating hormone β gene (TSHβ gene).

INTRODUCTION

In the past 15 years, the mechanism of thyroid hormone receptor (TR) function in the positive transactivation of target genes has been studied extensively [1]. TR heterodimerizes with retinoid X receptor (RXR) and binds to a positive T_3 (3,3′,5-tri-iodothyronine)-responsive element (pTRE) on the promoter region of the genes. The typical pTRE is referred to as DR4 (direct repeat 4) in which two half sites (AGGTCA) are aligned in the same direction and separated by a space of four random nucleotides. In the absence of T_3, TR/RXR recruits co-repressors, such as NCoR (nuclear receptor co-repressor) and SMRT (silencing mediator for retinoid and thyroid hormone receptor), and associates with Sin3 and histone deacetylase (HDAC), inducing the suppression of target genes [2]. In the presence of T_3, TR/RXR dissociates from the co-repressor complex and recruits co-activators including p160 family proteins, CBP [CREB (cAMP-response-element-binding protein)-binding protein]/p300, PCAF (p300/CBP-associated factor) and others. The histone acetyltransferase activity of the co-activator complex relaxes the chromatin structure, resulting in transactivation.

Contrary to the positive regulation, little is known about the mechanism of T_3-dependent negative regulation of the genes. Negative regulation of target genes by T_3/TR is very important in terms of thyroid hormone homoeostasis. In fact, Feng et al. [3] have shown that approx. 50 % of T_3-dependent genes in the liver are suppressed. Among the negatively regulated genes, TSH (thryoid-stimulating hormone, or thyrotropin) genes, which consist of an α-subunit (TSHα) and a TSH-specific β-subunit (TSHβ), are most important in the hypothalamus–pituitary–thyroid axis. It is clear that TR, especially TRβ, plays a critical role in the regulation of TSH, since patients with resistance to thyroid hormone (RTH) who possess mutations in the TRβ gene show impairment of TSH regulation [4].

Despite its importance, suppression of target genes by T_3/TR has not been investigated extensively. There are several difficulties in the study, and one of the main reasons has been the limitation of useful cell lines for the experiments. To study the positive regulation, the TR and reporter plasmid containing pTRE are introduced into cells such as CV1, and a T_3-induced increase in the transcriptional activity of the reporter gene can be easily observed. To study the negative regulation, however, the basal transcription activity should be high enough to detect the suppression. Unfortunately, it is very difficult to introduce the TSHβ gene into the non-pituitary cells commonly used for transfection experiments. Exceptionally, the transcriptional activity of the TSH gene promoter can be observed in some culture cells, such as GH3, JEG3 and TSA201, and there have been several reports analysing the negative regulation of TSHα and β using these cells [5–8]. Such culture cells do not themselves express the TSH gene, and we

Abbreviations used: CAT, chloramphenicol acetyltransferase; CBP, cAMP-response-element-binding protein-binding protein; CoR, co-repressor; DBD, DNA-binding domain; DMEM, Dulbecco's modified Eagle's medium; DR4, direct repeat 4; ER, oestrogen receptor; FCS, foetal calf serum; HDAC, histone deacetylase; Luc, luciferase; NCoR, nuclear receptor co-repressor; PPAR, peroxisome-proliferator-activated receptor; pTRE, positive T_3-response element; RAR, retinoic acid receptor; RTH, resistance to thyroid hormone; RXR, retinoid X receptor; SMRT, silencing mediator for retinoid and thyroid hormone receptor; SRC-1, steroid receptor co-activator-1; T_3, 3,3′,5-tri-iodothyronine; TR, thyroid hormone receptor; TRH, thyroid-stimulating-hormone-releasing hormone; TSH, thyroid-stimulating hormone (thyrotropin); VDR, vitamin D_3 receptor.

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do not know how transfected TSHα and β genes can be activated in these non-thyrotrophic cells. The fact that the mechanism of the TSH gene activation in these cells is completely unknown hinders the study of the T_3/TR-induced suppression of the TSHβ promoter activity.

The ideal cell line for the TSH study should have the following properties: (i) a reporter gene containing a TSH gene promoter is easily and efficiently expressed, and the basal activity of the expressed TSH in cells is sufficiently high; (ii) the cells do not contain endogenous nuclear receptors; and (iii) the cells are easily maintained under conventional culture conditions. In the present study, we established a new experimental system for the negative regulation of the TSHβ gene by T_3/TR using CV1 cells, the most frequently used cells in the study of T_3-dependent positive regulation. With this assay system, we provide data showing that unliganded TR itself may not be an activator of the TSHβ gene. The intact DNA-binding domain (DBD) of TRβ is crucial to the T_3/TR-dependent TSHβ gene suppression. Co-activators and co-repressors are not necessarily essential, but are required for the full suppression of the TSHβ gene. Among the three TR isoforms, β2 exhibited the most potent inhibitory function, and its protein level was predominant in a thyrotroph cell line, TαT1, in Western blotting. The dominant-negative effects of various TRβ mutants measured on the TSHβ–CAT (chloramphenicol acetyltransferase) reporter were not simple mirror images of those on the reporters for positive regulation under physiological T_3 concentration.

MATERIALS AND METHODS

Plasmid constructions

The TSHβ($-128/+37$)–CAT reporter gene was constructed by fusing the human TSHβ promoter (-128 to $+37$) to the CAT gene [9]. Using partial digestion of TSHβ–CAT with *Eco*RI, the human genomic DNA fragment (-1193 to -129) was ligated into one of two *Eco*RI sites upstream of the TSHβ promoter region (-128 to $+37$) to generate TSHβ($-1193/+37$)–CAT. The cDNA encoding mouse GATA2 was subcloned into the *Hin*dIII/*Xba*I site in pcDNA3 vector (Invitrogen, CA, U.S.A.). The expression vector pCMX containing wild-type human TRα1, human TRβ1 and rat TRβ2 were used. The human TRβ1-deletion mutants (pCI-C1, pCI-C2) [9], mutant TRβ1 in the zinc fingers [C127S, C145G and C164S (single letter amino acid codes, e.g. C127S is a $Cys^{127} \rightarrow$ Ser mutation)] [10] and in the CoR (co-repressor) box {AHT (mutations at positions 228, 229 and 233 in human TRβ1 to glycine, glycine and alanine respectively, as described in [2], according to the numbering system of Sakurai et al. [10a]) and P214R} [2,11] were previously described. The expression vectors pCMX containing the mutants TRβ1-F451X, E449X, K443E, R338W, G345R, F451I and F451L, all identified from patients with RTH, were described elsewhere [12–16]. Mutant TRβ1s (I280K, V284K, I302R and C309K) [17] and rat TRβ2-F504X and E502X, which have identical amino acid mutations with mutant TRβ1-F451X and E449X respectively, were artificially constructed by site-directed mutagenesis (Stratagene, La Jolla, CA, U.S.A.) and confirmed by sequencing.

Cell culture

CV1 and HEK-293T cells were grown in monolayer culture at 37 °C under CO_2/air (1:19) in Dulbecco's modified Eagle's medium (DMEM) containing 10 % (v/v) foetal calf serum (FCS), penicillin G (100 units/ml) and streptomycin (100 μg/ml). TαT1 cells, a thyrotroph cell line from the mouse pituitary, were seeded on Matrigel-coated plates (Becton Dickinson Labware, Bedford, MA, U.S.A.). The cells were maintained under the same conditions as CV1 and HEK-293T cells.

Transient transfection of TSHβ–CAT in CV1 and HEK-293T cells

CV1 and HEK-293T cells were trypsinized and plated in 60-mm diameter dishes for 24 h before the transient transfection using the calcium phosphate technique. The cells at a density of 10^6 cells/plate were transfected with 0.4 μg of wild-type and/or mutant TR expression vector along with 4.0 μg of the TSHβ–CAT reporter gene, 2.2 μg of β-galactosidase expression vector pCH111 (a modified version of pCH110; Pharmacia LKB Biotechnology, Piscataway, NJ, U.S.A.), 0.2 μg of human Pit1 expression vector pCB^{6+}-hPit1, 0.4 μg of mouse GATA2 expression vector pCDNA3-mGATA2 and pCMX empty vector as carrier DNA (a total of 7.2 μg of DNA per dish). After the cells were exposed to the calcium phosphate/DNA precipitates for 20 h, the medium was replaced with fresh DMEM containing 5 % (v/v) FCS depleted of thyroid hormones [18] or the medium was supplemented with 1 μM T_3. After incubation for an additional 24 h, the cells were harvested and the CAT activity was measured as described previously [19]. The transfection efficiency was normalized by the β-galactosidase assay. In each CAT reporter assay, we performed the transfection with CMV (cytomegalovirus)–CAT (10 or 25 ng/dish), the magnitude of which was adjusted to a value of 100 to standardize the activities of TSHβ–CAT.

Antibodies and immunoblotting

The anti-FLAG antibody (M2; Sigma, St. Louis, MO, U.S.A.) was used to detect expression of the FLAG-tagged wild-type TRβ1 (pCMX-FLAG-hTRβ1), pCI-C1 and pCI-C2. The expression of wild-type TRβ1, and AHT, P214R, C309K, E457A, RT338W, G345R, K443E, C446X, E449X, F451X, F451I and F451L mutants were confirmed with the antibody against the C-terminal amino acid sequence of TRβ1 (MA1-215; Affinity Bioreagents, Golden, CO, U.S.A.). The antibody against the common C-terminal 40 amino acids among TRβ1, TRβ2 and TRα1, which was raised previously in our laboratory and referred to as 4BII [20], was utilized to confirm the comparable expression of pCMX-hTRβ1, pCMX-rTRβ2 and pCMX-hTRα1 in CV1 cells. To study the expression of TR isoforms in TαT1 cell, a Western blot was performed with specific antibodies against TRβ1 (J51; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), TRβ2 (06-540; Upstate Biotechnology, Lake Placid, NY, U.S.A.) and TRα1 (PA1-211A; Affinity Bioreagents). Each antibody was diluted with 0.5 % (w/v) non-fat dried milk in PBS. To confirm the expression of plasmids for wild-type and mutant TRs in CV1 cells, 3 μg/6 cm diameter dish of the expression plasmids were transfected and the whole-cell extracts were subjected to Western blotting with specific antibodies. Nuclear extracts from the TαT1 cells were prepared as described previously [9]. Briefly, the cells were suspended in one packed cell volume of buffer A (10 μM Hepes, pH 7.9, 1.5 μM $MgCl_2$ and 10 μM KCl) and sheared by passage through a 25 G needle five times on ice. They were centrifuged at 18 000 g for 10 min and resuspended in buffer B [20 μM Hepes, pH 7.9, 25 % (v/v) glycerol, 420 μM NaCl, 1.5 μM $MgCl_2$ and 0.2 μM EDTA] for 30 min at 4 °C. The samples were dialysed against buffer C [20 μM Hepes, pH 7.9, 20 % (v/v) glycerol, 42 μM $(NH_4)_2SO_4$, 0.5 μM dithiothreitol and 0.2 μM EDTA] at 4 °C for 2 h, and stored at -80 °C. The protein concentration was determined by the method of Bradford (Bio-Rad, Hercules, CA, U.S.A.). As controls, we used whole-cell

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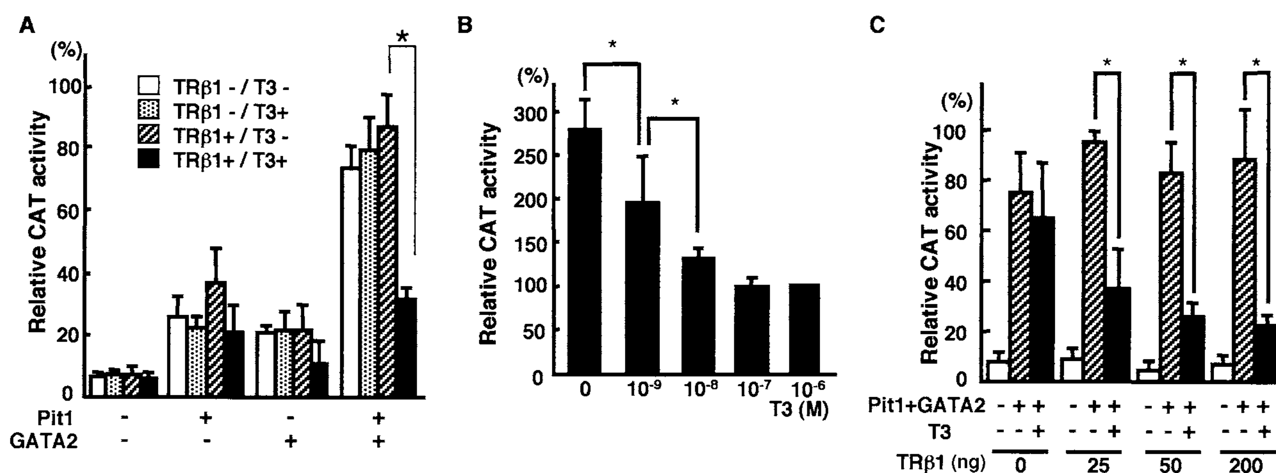

Figure 1 T_3-dependent negative regulation of the TSHβ promoter (− 128/ + 37) by TRβ1 in CV1 cells

(**A**) TSHβ(− 128/ + 37)–CAT plasmid (4.0 μg) was transfected into CV1 cells with or without Pit1 (0.2 μg), GATA2 (0.4 μg) and TRβ1 (0.4 μg) plasmids. After incubation for 24 h with or without 1 μM T_3, the cells were harvested and the CAT activity was measured, with normalizing transfection efficiency by β-galactosidase activity. As an interassay control, 10 ng of CMV (cytomegalovirus)–CAT was transfected, the magnitude of which was adjusted to a value of 100. The experiment was repeated several times and the results are means ± S.D. $*P < 0.05$. (**B**) Dose dependency of T_3. TSHβ(− 128/ + 37)–CAT, Pit1, GATA2 and TRβ1 were expressed in CV1 cells and 0–1 μM of T_3 was supplemented. The CAT activity at each T_3 concentration was expressed relative to that at 1 μM T_3, which was adjusted to a value of 100. The results are means ± S.D. for three separate experiments. $*P < 0.05$. (**C**) Dose-dependency of the amount of TRβ1 expressed. pCMX-hTRβ1 (25–200 ng) was transfected in CV1 cells under the same conditions as described in **A**. CMV–CAT (10 ng) was used as the interassay control and the expression level of CMV–CAT was adjusted to a value of 100. The TSHβ(− 128/ + 37)–CAT activities without Pit1 or GATA2 (open bars), with Pit1 and GATA2 in the absence of T_3 (shaded bars), and with Pit1 and GATA2 in the presence of 0.1 μM T_3 (solid bars) are indicated. The results are means ± S.D. for four independent experiments. $*P < 0.05$.

extracts of CV1 cells transfected with pCMX plasmid expressing wild-type human TRα1 [21], TRβ1 and rat TRβ2.

Equal amounts (100 μg) of nuclear extract were separated by SDS/PAGE (10 % gel) and transferred on to the Immobilon-P membrane (Millipore, Bedford, MA, U.S.A.). The membrane was incubated overnight at 4 °C in blocking solution [10 % (w/v) non-fat dried milk in PBS]. After the membrane was washed in PBS with 0.05 % (v/v) Tween 20 three times for 10 min, it was incubated for 1 h at room temperature (25 °C) with the TR-isoform-specific antibodies described above. After incubation with horseradish-peroxidase-linked donkey anti-mouse or anti-rabbit immunoglobulin (Amersham Biosciences, Little Chalfont, Bucks., U.K.) diluted 1:500 for 1 h at room temperature, the antibody–protein complexes were visualized using an ECL® (enhanced chemiluminescence) detection reagent (Amersham Biosciences).

Statistics

Each experiment was performed in duplicate more than three different times and each result is expressed as the mean ± S.D. The statistical significance was determined by ANOVA and Fisher's Protected Least Significant Difference (PLSD) test using StatView 4.0 software (Abacus Concepts, Berkeley, CA, U.S.A.). $P < 0.05$ was considered significant.

RESULTS

T_3-dependent suppression of the TSHβ gene by TR in CV1 cells

We have established an assay system to study the negative regulation of the TSHβ gene using CV1 cells. When simply transfected into CV1 cells, the TSHβ(− 128/ + 37)–CAT reporter gene was very low in its activity. Although the expression of Pit1 alone or GATA2 alone did not highly stimulate the activity, co-expression of both transcription factors dramatically enhanced the TSHβ promoter activity. The expression of TRβ1 alone did not stimulate the TSHβ gene activity when T_3 was absent, suggesting that TRβ1 itself was not an activator of the TSHβ gene (Figure 1A). This result was confirmed independently by two different researchers repeating the experiments several times. The transfection method, either calcium phosphate or lipofection, did not affect the result (results not shown). A supplement of T_3 decreased the TSHβ (− 128/ + 37)–CAT activity dose-dependently, and 1 nM T_3 suppressed it significantly, at approx. 25 % of the basal activity without T_3 (Figure 1B). T_3-induced suppression of the TSHβ gene was dependent on the amount of TRβ1 (Figure 1C). Without Pit1 and GATA2, even very large amounts of unliganded TRβ1 did not activate the TSHβ gene.

Nuclear receptor specificity in TSHβ gene regulation

The negative regulation of TSH is specific to T_3 *in vivo*. Since our assay system contains no endogenous nuclear receptor, we examined the effects of other receptors including vitamin D_3 receptor (VDR), RXRβ, oestrogen receptor (ER) α, retinoic acid receptor (RAR) α and peroxisome-proliferator-activated receptor (PPAR) γ2 on the TSHβ(− 128/ + 37)–CAT activity to see whether or not suppression of the TSHβ–CAT reporter gene was specific to T_3/TR. In the presence of cognate ligand, no significant suppression was detected by any other nuclear receptors, except for ERα, which decreased the CAT activity slightly, but significantly (Figure 2).

The importance of the DBD of TRβ1

The functions of the receptor domains in terms of the negative regulation of the TSHβ gene were examined using various mutant TRβ1s which possessed functional disruption in each domain. The mutant C1, which lacked the N-terminal A/B domain of TRβ1, showed T_3-dependent negative regulation, whereas mutant C2, which lacked the DBD, failed to suppress the TSHβ(− 128/ + 37)–CAT (Figure 3A). As expected, a truncated TR of the

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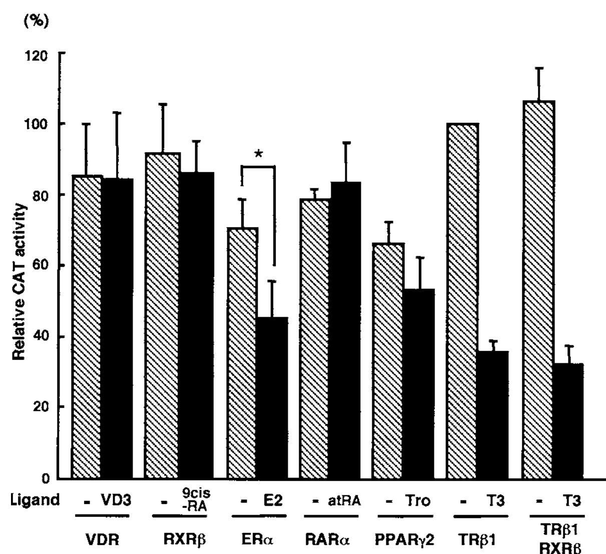

Figure 2 Receptor specificity in the negative regulation of the TSHβ gene promoter

The expression plasmids for VDR, RXRβ, ERα, RARα and PPARγ2 were co-transfected into CV1 cells in the absence (shaded bars) or presence (solid bars) of 1 μM 1α25(OH)$_2$ vitamin D$_3$ (VD3), 9-*cis* retinoic acid (9-*cis* RA), oestradiol (E2), all-*trans* retinoic acid (atRA) and troglitazone (Tro), respectively. The CAT activity of the cells expressed with TRβ1 in the absence of T$_3$ is represented as 100 and other activities are expressed as relative values. The results are means $\pm$ S.D. for four experiments. $^*P < 0.05$.

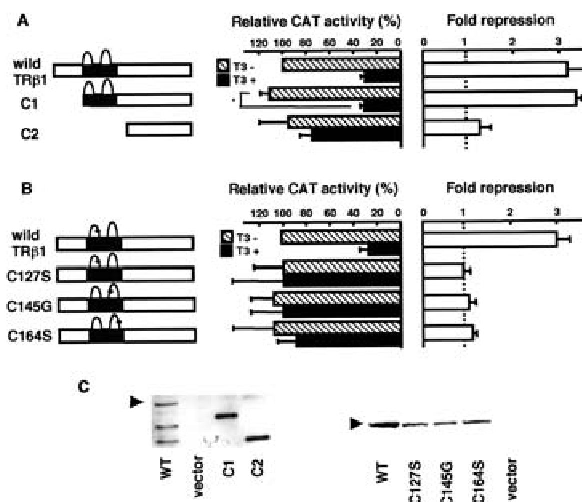

Figure 3 Regulation of TSHβ promoter by mutant TRβ1s possessing deletions in the A/B or A/B + C domains or mutations in the zinc fingers

(**A**) The effect of the deletion in the A/B domain (C1) or A/B + C domains (C2). (**B**) The effects of mutant TRβ1s which have point mutations in the zinc-finger motifs in the DBD. The CAT activity of the cells expressed with wild-type TRβ1 in the absence of T$_3$ is represented as 100 and other activities are expressed as relative values. The fold repression was calculated from the CAT activity without T$_3$ (shaded bar) divided by that with T$_3$ (solid bar). The results are means $\pm$ S.D. for four separate experiments. $^*P < 0.05$. (**C**) Expression of wild-type or mutant TRβ1s in CV1 cells. Whole-cell extracts of CV1 cells transfected with equal amounts of expression plasmids for wild-type and mutant TRβ1s were analysed by Western blotting with anti-FLAG antibody (left panel) and the antibody against the C-terminal amino acid sequence of TRβ1 (right panel). The position of wild-type TRβ1 is indicated by the arrowhead.

ligand-binding domain did not exhibit the suppression, since it lost T$_3$ binding (results not shown). To investigate the DBD function further, we examined three different TRβ1s possessing

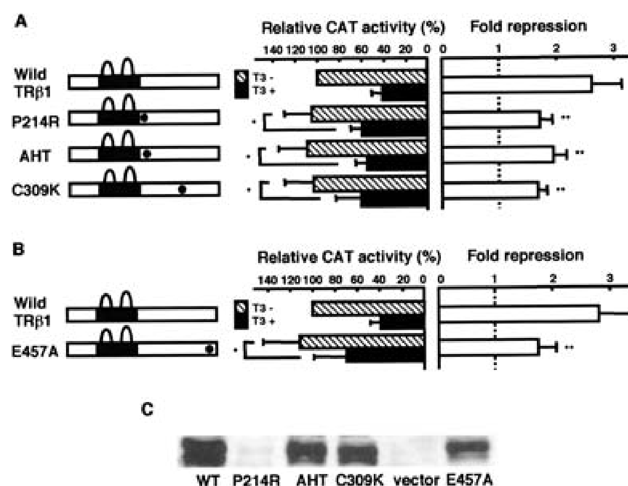

Figure 4 Effects of the mutant TRβ1s, which have impaired co-repressor-binding (A) or co-activator-binding ability (B), on T$_3$-dependent suppression of TSHβ($-$128/+37)–CAT reporter gene

The CAT activity of the cells expressed with wild-type TRβ1 in the absence of T$_3$ was represented as 100 and other activities were expressed as relative values. The fold repression was calculated from the CAT activity without T$_3$ (shaded bar) divided by that with T$_3$ (solid bar). The results are means $\pm$ S.D. for four to seven separate experiments. $^*P < 0.05$. (**C**) Western blot analysis of mutant TRβ1s expressed in CV1 cells performed under the same conditions as in Figure 3(C).

point mutations in the zinc fingers. C127S, C145G and C164S mutants all abrogated the negative regulation, indicating that an intact DBD is essential (Figure 3B). There was no remarkable difference in the expression level among mutant TRβ1s (Figure 3C).

The functions of co-repressors and co-activators

To study whether the co-repressors participate in the T$_3$/TR-dependent suppression of the TSHβ gene, we examined the functions of the mutant TRs, which had impaired co-repressor binding. Two CoR-box mutants, P214R and AHT, the properties of which have been extensively studied [2,22], significantly suppressed the TSHβ($-$128/+37)–CAT activity (Figure 4A). Recently, Marimuthu et al. [17] identified TR surfaces that interact with NCoR, reporting that a CoR box is not important. Among the mutant TRs, we confirmed that C309K possessed normal T$_3$-binding activity and did not interact with NCoR or SMRT in the absence of T$_3$. It exhibited no silencing activity, nor dominant-negative function against wild-type TR on pTRE (results not shown). C309K significantly decreased the TSHβ–CAT activity similarly to P214R and AHT (Figure 4A). The strength of suppression by P214R, AHT and C309K was diminished slightly compared with that by wild-type TRβ1. The data suggested that co-repressors are not critical, but may be required for full suppression by T$_3$/TR.

Another mutant TRβ1, E457A, has been known not to bind co-activators, despite its normal T$_3$-binding ability [23]. As shown in Figure 4B, E457A could also decrease the TSHβ($-$128/+37)–CAT activity. The extent of the decrease was, however, significantly smaller by E457A than by wild-type TRβ1 (36.6 $\pm$ 15.8 % compared with 59.3 $\pm$ 13.4 %, $P < 0.05$). This indicates that a co-activator is not indispensable, but is again required for T$_3$/TR-induced full suppression of the TSHβ gene. When we checked the protein levels of TRs by Western blotting, we found unexpectedly that the expression of P214R was low, while that of

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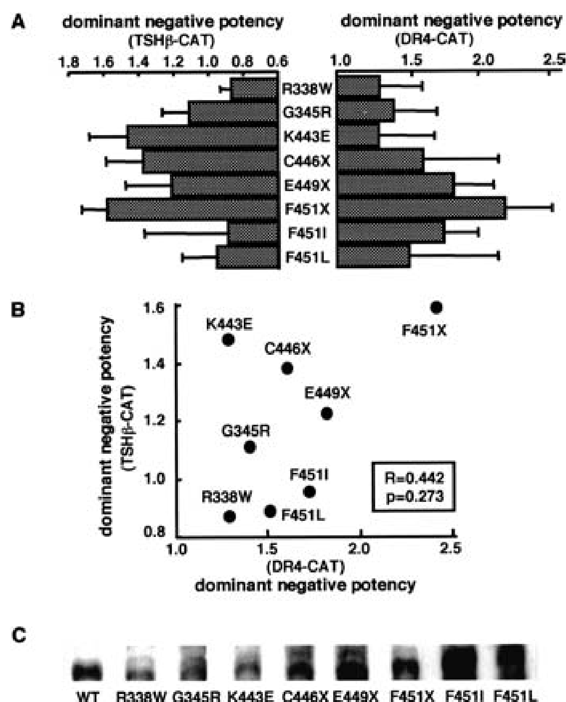

Figure 5 Dominant-negative potency of mutant TRβ1s on the positive and negative TREs

(**A**) Equal amounts (0.4 μg) of expression vectors for wild-type and various mutant TRβ1s were transfected into CV1 cells, and transcriptional activities were measured using DR4–CAT (right) and TSHβ(− 128/ + 37)–CAT reporter gene (left) in the presence of 1 nM T_3. Dominant-negative potency was calculated from the following ratio: CAT activities of wild-type TRβ1 only/CAT activities of wild-type + mutant TRβ1 on DR4–CAT and CAT activities of wild-type + mutant TRβ1/CAT activities of wild-type TRβ1 only on TSHβ–CAT. Results are means ± S.D. for three to six experiments. (**B**) Correlation of the dominant-negative potency between DR4–CAT and TSHβ(− 128/ + 37)–CAT. There was no significant correlation at 1 nM T_3. (**C**) Protein expression of mutant TRβ1s in CV1 cells was analysed under the same conditions as in Figure 3(C).

AHT, C309K and E457A was comparable. The CV1 cells that expressed P214R exhibited transcriptional activity as high as wild-type TR when T_3 was supplemented (results not shown). It is unlikely that the expression level of P214R was very low; rather the antibody against TR that was used might have failed to recognize the conformation of P214R.

Dominant-negative effect of various mutant TRβ1s identified from patients with RTH on TSHβ(− 128/ + 37)–CAT

Various mutant TRβ1s identified from patients with RTH express inhibitory effects on the wild-type TRβ1 functions. For example, co-transfection of equimolar amounts of mutant TRβ1s impaired the T_3-dependent transactivation of wild-type TRβ1 on pTREs, such as DR4, as shown in our previous study [24]. Our present assay system has enabled us to examine the negative regulation of the TSHβ promoter by T_3/TR as well as the positive regulation on pTRE using the same CV1 cells under similar assay conditions. We measured the transcriptional activities on DR4–CAT and TSHβ(− 128/ + 37)–CAT after transfection with an equal amount of wild-type and various mutant TRs. The CAT activity of each mutant TRβ1 was assayed at the physiological T_3 concentration of 1 nM and expressed relative to that of wild-type TRβ1. The dominant-negative potency was calculated from CAT activities of wild-type + mutant TRs and wild-type TR only (Figure 5A). When the dominant-negative potencies were compared between the positive and negative TREs, a correlation was not obtained, suggesting that the negative regulation by T_3/TR might not be a simple mirror image of positive regulation (Figure 5B). The expression levels of wild-type and mutant TRβ1 were confirmed (Figure 5C).

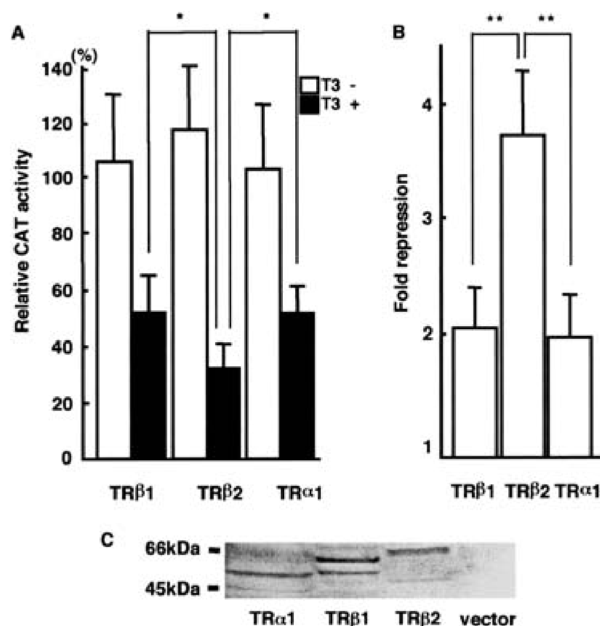

Figure 6 T_3-dependent inhibition of the TSHβ–CAT reporter gene activity by TR isoforms

(**A**) Expression vectors of TRβ1, TRβ2 or TRα1 were transfected into CV1 cells, and the transcriptional activity of the TSHβ(− 128/ + 37)–CAT gene was assayed in the presence (closed bars) or absence (open bars) of 1 μM T_3. Results are means ± S.D. for four experiments. (**B**) The fold repression is indicated as the ratio between the CAT activity in the absence and that in the presence of T_3. (**C**) Western blot analysis was performed using the anti-TR antibody, 4BII, raised against the C-terminal 40 amino acids common to TRα1, β1 and β2.

Comparison of suppressive efficiency of the three TR isoforms on the TSHβ promoter

The functional properties of three active TR isoforms, TRβ1, TRβ2 and TRα1, were compared in the context of suppression of the TSHβ promoter (Figure 6). In the presence of T_3, the TSHβ(− 128/ + 37)–CAT activity was inhibited approx. 50 % by TRα1 and TRβ1, and 70 % by TRβ2 compared with the basal activity with empty vector. All three TR isoforms are functional and TRβ2 exhibits the strongest inhibition among them. The protein expression was confirmed using antibody against the C-terminal 40-amino-acid sequence common to the three isoforms [20]. Collectively, TRβ2 exhibited the strongest inhibition among them (Figure 6).

Protein expression of three TR isoforms in the pituitary thyrotroph cell line, TαT1

The transfection study in CV1 cells showed the predominant suppressive efficiency of TRβ2. Thus it is desirable to examine the protein expression of the three TR isoforms in pituitary thyrotrophs. Since it is difficult to isolate sufficient amounts of thyrotrophs, we studied the TR protein level using a thyrotroph cell line, TαT1. As positive controls of the Western blot analysis, the whole-cell extracts of CV1 cells transfected with expression

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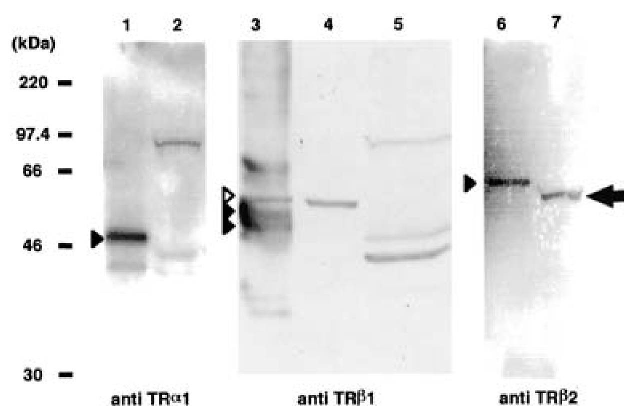

Figure 7 Protein expression of TRα1, β1 and β2 in mouse thyrotroph TαT1 cells

Nuclear extracts (100 μg) from TαT1 cells (lanes 2, 5 and 7) and whole-cell extracts from CV1 cells transfected with empty plasmid (lane 4) or plasmid for human TRα1 (lane 1), β1 (lane 3) or rat β2 (lane 6) were resolved by SDS/PAGE (10 % gel). The blot was probed with isoform-specific antibody against TRα1 (lanes 1 and 2), TRβ1 (lanes 3–5) and TRβ2 (lanes 6 and 7), followed by chemiluminescent detection. The protein standards are shown on the left (molecular mass in kDa). The position of wild-type TR isoforms and endogenous TRβ2 in TαT1 cells are indicated as solid arrowheads and an arrow respectively. The open arrowhead indicates the non-specific protein in CV1 cells.

vector encoding wild-type human TRα1 and TRβ1, and rat TRβ2 were used. As shown in Figure 7, the antibodies against each TR isoform detected the cognate receptors expressed in CV1 cells with similar intensity, suggesting that the recognition ability of each antibody is comparable. In TαT1 nuclear extracts, only the protein band of TRβ2 was detected with anti-TRβ2 antibody, the molecular mass of which was identical with that calculated from the reported amino acid sequence of mouse TRβ2 (54 kDa), slightly smaller than that of rat TRβ2 (58 kDa). No signals of TRα1 and TRβ1 were observed, indicating that at least TRβ2 predominantly exists in this thyrotroph cell line.

Mutant TRβ2 exhibited dominant-negative potency

Since TRβ2 exhibited the most potent inhibitory activity in TSHβ–CAT, and since TRβ2 is clearly present in thyrotroph TαT1 cells, we determined whether or not mutant TRβ2s showed dominant-negative effects similar to the mutant TRβ1s. We constructed the mutants TRβ2-F504X and E502X possessing C-terminal truncations identical with those of TRβ1-F451X and E449X respectively. They did not mediate T_3-dependent negative regulation because of the deletion of the T_3-binding activity (Figure 8A), and exhibited similar dominant-negative potencies against wild-type TRβ2, as well as TRβ1 (Figure 8B). No significant difference was observed between the inhibitory effects of mutant TRβ1s and mutant TRβ2s.

TSHβ gene suppression by T_3/TRβ2 using different culture cells or a reporter gene containing a longer promoter region

We confirmed T_3-dependent suppression of the TSHβ gene by TRβ2 using different culture cells or a reporter gene containing a longer human TSHβ promoter. HEK-293T cells, which have no endogenous nuclear receptors, exhibited the enhanced TSHβ ($-128/+37$)–CAT activity with expression of Pit1 and GATA2. TRβ2 significantly decreased the CAT activity by administration of 1 μM T_3 (Figure 9A). The TSHβ($-1193/+37$)–CAT reporter

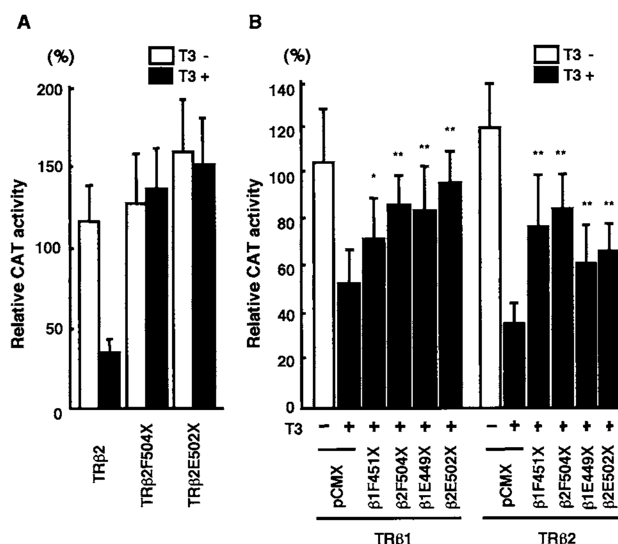

Figure 8 Functional properties of the mutant TRβ2-F504X and E502X which have the C-terminal truncations identical with those of TRβ1-F451X and E449X respectively

(**A**) The effects on the TSHβ promoter ($-128/+37$). Expression vectors for wild-type or mutant TRβ2s were transfected into CV1 cells and transcriptional activities were measured in the absence (open bars) and presence (closed bars) of 1 μM T_3. The CAT activities are indicated as percentages compared with the activities in which empty vector was co-transfected. Result are means $\pm$ S.D. for four experiments. (**B**) The dominant-negative effects by mutant TRβ1s or TRβ2s. Expression vectors of mutant TRβ1s or TRβ2s were co-transfected into CV1 cells together with equimolar wild-type TRβ1 or TRβ2. Transcriptional activities were measured in the absence or presence of 1 μM T_3. The CAT activities are indicated as percentages compared with the activities in which empty vector was co-transfected. Results are means $\pm$ S.D. for four experiments. $^*P < 0.05$, $^{**}P < 0.01$.

gene activity in CV1 cells also showed augmentation by Pit1 and GATA2, and a significant decrease by 1 μM T_3. These indicate that TSHβ gene suppression by T_3/TR in our assay system does not relate to the cell lines used or small fragment of the promoter used.

DISCUSSION

In the present study, we tried to establish a suitable new assay system to examine the T_3/TR-dependent transcriptional repression of the TSHβ gene promoter, considering the following two points: (i) using a conventional culture cell line and (ii) using a CAT, not a luciferase, reporter system. The CV1 cell line is derived from monkey kidney cells and is one of the most frequently used cells for transfection experiments of nuclear receptors because of its good transfection efficiency, ease of handling and lack of endogenous nuclear receptors. In fact, numerous studies have been performed on T_3/TR-dependent transcriptional regulation of a reporter gene containing pTRE using CV1 cells. Unfortunately, no basal activity of the TSHβ gene is usually obtained when simply introduced into these cells. Pit1 and GATA2 have been reported to be important for TSHβ expression [25] and to be determinants of the differentiation from immature pituitary cells to thyrotrophs [26]. We confirmed that the expression of these pituitary-specific transcriptional factors in CV1 cells enhanced the TSHβ promoter activity. The activity was sufficiently high, and when TR was co-expressed, it decreased the TSHβ promoter activity in a T_3-dependent manner. The fold suppression was dose-dependent of the ligand concentration and the receptor amount. In addition,

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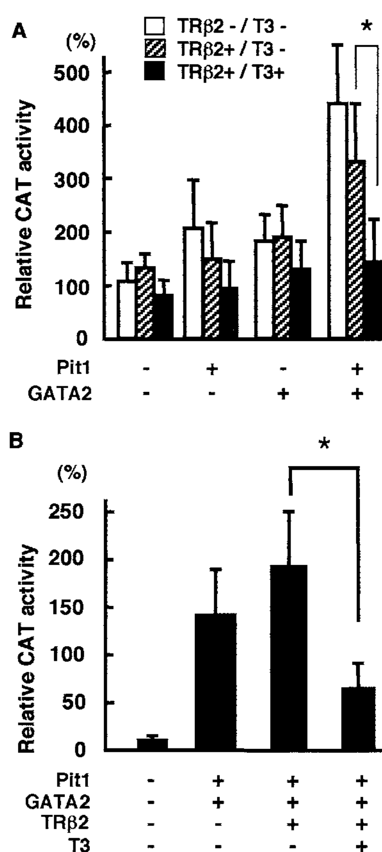

Figure 9 T_3-dependent suppression of the TSHβ gene by TRβ2

(**A**) TSHβ ($-128/+37$)–CAT plasmid (4.0 μg) was transfected into HEK-293T cells with or without Pit1 (0.2 μg), GATA2 (0.4 μg) and TRβ2 (0.4 μg) plasmids. The experimental procedure was the same as in Figure 1. (**B**) The CAT reporter gene containing TSHβ ($-1193/+37$) promoter was introduced into CV1 cells. Results are means $\pm$ S.D. for four experiments. $^*P < 0.05$.

RARα, VDR, PPARγ2 and RXRβ, nuclear receptors belonging to the same subgroup of TR, did not affect the transcriptional activity, supporting the ligand/receptor specificity in feedback regulation of TSHβ expression and the validity of this assay system (Figure 2).

The second point we considered, using a CAT, not a luciferase, reporter system, is important, although it has often been overlooked. A luciferase reporter gene itself was found to mediate the T_3-dependent decrease in the activity in CV1 [27], JEG3 and other cells [28]. There have been many investigations of the T_3-dependent negative regulation of the TSH gene using a luciferase reporter system [5,6,8,22,29,30]. It may sometimes evoke confusing results of T_3/TR-dependent decrease in the reporter activity caused by the TSH promoter or by the luciferase gene itself.

The important finding in this study is that unliganded TR itself does not enhance the basal transcriptional activity of the TSHβ promoter. No basal TSHβ promoter activity was obtained without co-expression of activator(s) in our assay system and simple introduction of TR did not activate the TSHβ gene. Pit1 and GATA2 were absolutely required to enhance it. Furthermore, co-expression of TR did not stimulate the Pit1/GATA2-driven TSHβ–CAT promoter further. This was confirmed with two different cell line cultures, and with two different shorter and longer TSHβ promoters. Clinically, one may easily misinterpret that unliganded TR itself is an activator of the TSHα and TSHβ genes, since thyroid hormone deficiency enhances TSH production *in vivo*. If this is the case, the absence of TR should cause a decrease in the transcriptional activity of the TSH gene. Knockout mice targeting TRβ alone [31], or both TRα and TRβ [32], did not support this. The serum TSH level and TSHβ mRNA in the pituitary remarkably increased in these mice. Weiss et al. [33] examined precisely TRβ1-knockout mice and concluded that the absence of TRβ does not impair the up-regulation of TSH induced by T_3 deprivation. Collectively, these data, including our findings in the present study, may support the notion that it may not be unliganded TR itself, but another factor that stimulates the TSH gene transcription.

When we studied the functions of each domain of TR in terms of the negative regulation of TSHβ, we found that the intact C domain (DBD) was critical to express the suppression. Mutant TRβ1-C2 with deletion of the A/B and C domains, but not C1 with the A/B deletion, failed to express the inhibition. Three different zinc-finger mutants also demonstrated the absolute necessity of an intact DBD. This may raise a possibility that TR binding to some specific DNA region on the promoter is essential. Immediately downstream of the transcriptional start site in the TSHβ promoter, there is a short sequence, GGGTCA, which is similar to a single half site of pTRE. Several reports have suggested that this sequence might mediate the T_3-dependent negative regulation [34–36]. We are currently examining the significance of this region using various mutated TSHβ reporter genes in our assay system. An alternative possibility is that the DNA-binding activity of TR is not necessarily crucial, but the receptor conformation with an intact DBD is important. In addition to DNA binding, DBD has some other important functions. For example, we reported previously that DBD of TR interacted with HDAC2 in a T_3-dependent manner on the TSHβ promoter [9]. Destruction of DBD may change the conformational structure of the receptor and hinder the recruitment of necessary factor(s) for negative regulation of the TSHβ promoter, resulting in failure of transcriptional repression.

The contribution of co-repressors such as NCoR and SMRT to the T_3/TR-dependent TSHβ suppression is considered to be small. The two CoR-box mutants, P214R and AHT, could suppress the TSHβ($-128/+37$)–CAT activity. Recently, Marimuthu et al. [17] have identified residues of TR that are critical for NCoR binding by testing more than 100 separate mutations of human TRβ. Since the CoR box was not important for NCoR-binding according to their report, we generated four TR mutants of the receptor surfaces for NCoR binding (results not shown). All of them showed impaired NCoR binding, but three of them also lost T_3-binding ability and were unsuitable for the study of T_3-dependent negative regulation. C309K had T_3-binding ability and significantly decreased the TSHβ($-128/+37$)–CAT activity similarly to P214R and AHT. The fact that two CoR-box mutants and also one TR mutant in the 'co-repressor-binding surface' could exhibit the T_3-dependent suppression of TSHβ($-128/+37$)–CAT activity suggests that co-repressors are not critical.

Tagami et al. [8] proposed a two-step model in negative regulation of the TSH promoter by T_3/TR: (i) unliganded TR, which does not bind to the TSH gene, recruits co-repressors, such as SMRT and NCoR, and withdraws HDAC from the basal promoter to cause activation; (ii) T_3 binding to TR dissociates the co-repressors/HDACs, thereby causing T_3-dependent repression of the TSH gene. In their study, the DBD was not important, and mutation in the CoR-box region was fatal. The results of our present study were very different from theirs and did not support their model. As described, unliganded TR itself did not stimulate the basal transcriptional activity. The DBD of TR is crucial to the TR-mediated suppression. Additionally, RAR that can recruit

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the co-repressors similarly to TR did not affect the basal activity in our experiment. According to their model, liganded RAR should also decrease the TSHβ($-128/+37$)–CAT activity. Furthermore, our study showed that co-repressors are not necessarily critical. The reason for the discrepancy between their data and ours is unknown, but two points described previously should be noted. First, TSA201 and JEG3 cells used in their study exceptionally exhibit some degree of the basal transcriptional activity of TSHα and β promoter when introduced, and the mechanism of the activation in these cells is uncertain. It may be conceivable that co-expressed TR interacts with some endogenous factors necessary for the TSH-gene activation, causing augmentation of the basal transcriptional activity in these cells. Secondly, they used the luciferase reporter gene in their assay system, which is known to show a T_3-dependent inhibition of the activity.

E457A, which does not bind co-activators despite its normal T_3-binding ability, significantly decreased the TSHβ($-128/+37$)–CAT activity, but not to the extent of wild-type TRβ1. This indicates that co-activators such as SRC-1 (steroid receptor co-activator-1) are not indispensable, but are required to express the full suppression of the TSHβ gene by T_3/TR. The result seems to agree well with a study of SRC-1-knockout mice, which showed partial resistance to T_3 in TSH suppression [37]. The TSHβ mRNA levels in SRC-$1^{+/+}$ and SRC-$1^{-/-}$ mice were comparably high after treatment with low iodine/propylthiouracil, and administration of T_3 resulted in a marked decrease in TSHβ mRNA in SRC-$1^{+/+}$ mice, but blunted the reduction in SRC-$1^{-/-}$ mice significantly. The findings that the T_3-mediated suppression of the TSHβ promoter was attenuated in CV1 cells expressed with E457A and also SRC-1-knockout mice indicate that co-activator SRC-1 partially participates in T_3/TR-dependent transcriptional suppression of the TSHβ gene.

Although all three functional TR isoforms showed the suppression of TSHβ promoter, TRβ2 exhibited the strongest inhibition among them. TRα1 and β1 are expressed ubiquitously in the tissues [38–40], whereas TRβ2 is expressed specifically in the anterior pituitary and hypothalamus [41,42]. A pituitary somatolactotroph cell line GH3 expresses both TRβ1 and β2 equally, but TRβ2 is more abundant than TRβ1 in the rat pituitary gland [41]. Childs et al. [43] demonstrated the expression of the message and protein of TRβ2 in rat thyrotrophs by *in situ* hybridization and immunohistochemical staining. In the present study, we also demonstrated that at least TRβ2 protein exists in a thyrotroph cell line, TαT1, by immunoblotting. In functional experiments, Langlois et al. [44] reported that TRβ2 showed greater T_3-dependent repression than TRα1 and TRβ1 in the negative regulation of the TRH (TSH-releasing hormone) gene. Transgenic mice with targeted disruption of the TRβ2 gene demonstrated elevated basal T_3 and T_4 (thyroxine) concentrations associated with inappropriate TSH production, suggesting that TRβ2 plays an important role in the hypothalamus–pituitary–thyroid axis [45]. These data and ours indicated that TRβ2 has pivotal roles in the negative regulation of TSH and TRH.

Our present assay system enabled us to compare the dominant-negative potency of mutant TRs on the positive and negative target-gene promoters under the same conditions using the same cells. When eight different mutants identified from patients with RTH were studied, no correlation was observed between dominant-negative potency on DR4–CAT and TSHβ–CAT at the physiological concentration of 1 nM T_3 (Figure 5B). Although the interpretation of the data should be cautious, this may suggest that the dominant-negative effect of mutant TRβs on the negative regulation is not the simple mirror image of that on the positive regulation, as previously reported [23].

The mechanism of how TR inhibits the transcriptional activity of the TSHβ promoter in a T_3-dependent fashion remains to be investigated. Pit1 and GATA2, which activate the TSHβ gene, have been known to interact with nuclear receptors. Palomino et al. [46] found a possible interaction between TR and Pit1. The GATA family proteins have been reported to be associated with ER [47], glucocorticoid receptor [48] and steroidogenic factor 1 [49]. We currently postulate a model that liganded TR associated with HDAC2 on the TSHβ gene promoter may interact directly with Pit1 and/or GATA2, resulting in the inactivation of their functions as activators. In this sense, it is interesting that liganded ER, which associates with GATA2, did suppress the TSHβ–CAT activity slightly, but significantly in our study (Figure 2).

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