Functions of PIT1 in GATA2-dependent transactivation of the thyrotropin β promoter

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Abstract

Thyrotropin (TSH) is a heterodimer consisting of α and β chains, and the β chain (TSHβ) is specific to TSH. The coexistence of two transcription factors, PIT1 and GATA2, is known to be essential for TSHβ expression. Using kidney-derived CV1 cells, we investigated the role of PIT1 in the expression of Tshb gene. GATA2 Zinc finger domain, which is known to recognize GATA-responsive elements (GATA-REs), is essential for cooperation by PIT1. Transactivation of TSHβ promoter requires PIT1-binding site upstream to GATA-REs (PIT1-US), and the spacing between PIT1-US and GATA-REs strictly determines the cooperation between PIT1 and GATA2. Moreover, truncation of the sequence downstream to GATA-REs enabled GATA2 to transactivate the TSHβ promoter without PIT1. The deleted region (nt −82 to −52) designated as a suppressor region (SR) was considered to inhibit transactivation by GATA2. The cooperation of PIT1 with GATA2 was not conventional synergism but rather counteracted SR-induced suppression (derepression). The minimal sequence for SR was mapped to the 9 bp sequence downstream to GATA-REs. Electrophoretic mobility shift assay suggested that some nuclear factor exists in CV1 cells, which binds with SR and this interaction was blocked by recombinant PIT1. Our study indicates that major activator for the TSHβ promoter is GATA2 and that PIT1 protects the function of GATA2 from the inhibition by SR-binding protein.

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Introduction

Thyrotropin (TSH) is a heterodimer consisting of α and β chains, and the β chain (TSHβ) is specific for TSH while the α chain (α subunit of glycoprotein hormone, αGSU) is common to LH, FSH, and chorionic gonadotropin (Zhu et al. 2007). The DNA sequence between nt −271 and −80 in the mouse TSHβ promoter, which corresponds to sequence nt −269 to −78 in the human gene, was reported to be sufficient for maximal promoter activity in thyrotroph (Wood et al. 1990). In this region, a functional PIT1-binding site (PIT1-US) is located upstream of two GATA-responsive elements (GATA-REs; Haugen et al. 1996, Gordon et al. 1997, 2002, Dasen et al. 1999). Although TSHβ expression is specific to thyrotrophs in vivo, co-expression of PIT1 with GATA2 is able to activate TSHβ promoters even in kidney-derived CV1 cells (Gordon et al. 1997, Dasen et al. 1999, Nakano et al. 2004, Matsushita et al. 2007).

PIT1 is a pituitary-specific transcription factor expressed in somatotrophs, lactotrophs, and thyrotrophs, and has pivotal roles in the transcriptional activation of genes for GH, prolactin (PRL), TSHβ subunit, and PIT1 itself (Andersen & Rosenfeld 1994). PIT1 possesses a transactivation domain in the N-terminal region, while the central POU-specific domain (POUS) and C-terminal POU homeodomain (POUH) are important for the DNA recognition (Andersen & Rosenfeld 1994). At least 16 different mutations of the Pit1 gene have been reported in patients with combined pituitary hormone deficiency (CPHD), who show deficiencies in the production of TSH, GH, and PRL (Cohen & Radovick 2002, Kishimoto et al. 2002). Although the functional features of these mutant PIT1s have been investigated regarding GH and PRL promoters (Cohen & Radovick 2002), few studies have been reported in the context of TSHβ expression.

GATA2 is a subtype of GATA family transcription factors and is expressed in thyrotrophs and gonadotrophs in the pituitary (Dasen et al. 1999). GATA2 is known to regulate the transcription of target genes via GATA-RE, the consensus sequence of which is 5′-(A/T)GATA(A/G)-3′. GATA1, 2 and 3 are mainly expressed in the hematopoietic lineage, while GATA4, 5 and 6 are found in visceral organs including the heart,
blood vessels, adrenal glands, gonads, and the intestinal tract (Viger et al. 2008).
A functional GATA-RE was also identified in the zGSU promoter (Steger et al. 1994).
In the central portion of GATA families, there are two zinc fingers, of which the C-terminal finger recognizes GATA-RE (Ko & Engel 1993, Merika & Orkin 1993).
The zinc finger domain of GATA2 (GATA2-Zf) has high homology with that of GATA1 and 3, while the amino acid sequences of N- and C-terminal regions show diversity (Viger et al. 2008).

Dasen et al. (1999) found that the expression of PIT1 converted gonadotrophs to thyrotrophs. They proposed that, when the promoter has binding sites for both PIT1 and GATA2, these two transcription factors exhibit synergistic activation. In solution, however, the POUH domain of PIT1 directly interacts with GATA2-Zf, resulting in the reduction of GATA2-induced transactivation of promoters containing GATA-REs only (Dasen et al. 1999). They showed that, in thyrotrophs, the existence of PIT1 represses GATA2-induced transactivation of the Tshb gene, which possesses only GATA-RE, while co-expression of PIT1 and GATA2 enhances Tshb gene transcription, the promoter of which contains the binding sites for these two factors.

However, it should be noted that GATA2 alone is able to activate the GATA-REs derived from various genes including CD34 (Tisuzuki et al. 2000), interleukin-5 (Miaw et al. 2000), T cell receptor δ (Smith et al. 1995) or artificial GATA-RE (Dasen et al. 1999). These findings raise the possibility that the GATA2 function may be suppressed by an unknown mechanism in the Tshb gene, which GATA2 alone cannot transactivate. If some inhibitory factor may be involved in this suppression, it is unlikely to be a thyrothroph-specific one, because co-expression of PIT1 is required for GATA2-induced activation of the Tshb gene in non-pituitary cells, including CV1 (Gordon et al. 1997, Nakano et al. 2004), 293T (Nakano et al. 2004), and Hela cells (data not shown). The fact that PIT1 enables GATA2 to stimulate the TSHβ promoter led to the idea that inhibition may be relieved by PIT1. In this study, we studied the molecular mechanism of how PIT1 cooperates with GATA2 to transactivate the Tshb gene.

Materials and methods

Plasmid constructions

The TSHβ–CAT reporter gene in which the human TSHβ promoter (nt −128/+37) was fused to the CAT gene was described previously (Sasaki et al. 1999, Nakano et al. 2004, Matsushita et al. 2007). The expression plasmids for mouse GATA2 (pcDNA3-mGATA2), human PIT1 (pCB6+-hPIT1), and rat TRβ2 (pCMX-rTRβ2) have been described elsewhere (Nakano et al. 2004). Using TSHβ–CAT as the PCR template, PCR fragments 1 and 2 were amplified with an upstream primer (hTSHβL-U1: 5’-accgcatagcgggtgatgtcg-3’) and downstream primer hTSHβ-DPIT1-2 (5’-gctgtggtgacccaaactaagcttgctttatcttttgattata-ctttggtcattttataactgctttctatctgaaaa-3’) or hTSHβ-DGATA2-2 (5’-gctgtggtgacccaaactaagcttgctttatcttttgattataactgctttctatctgaaaa-3’) respectively. The sequence between EcoRI and BstEII sites in TSHβ–CAT was substituted with PCR-fragment 1 or 2 to generate TSHβ–M1-CAT or TSHβ–M2-CAT respectively. Both the sequences between nt −82/−80 (TGA) and nt −55/−53 (GAA) in TSHβ–CAT were mutated to GAT to create two EcoRV sites (GATATC) using a site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The mutated plasmid was digested with EcoRV and self-ligated to generate TSHβ–M3-CAT. IV-M2, IV-M3, M4, and M5 were created by a standard subcloning technique. M6-11, TSHβ-mP, 2G, 4G, and 3T-CAT were also created using site-directed mutagenesis kit. The sequence between suppressor region (SR) and TATA box (nt −64/−26) in TSHβ–CAT was amplified using PCR with primers (5’-ggggGATACTaagcagcaagatacgagg-3’ and 5’-ggggGA TATaagcagcaagatacgagg-3’) of the Tshb sequence between EcoRI and BstEII sites in TSHβ-M3-CAT to be generated in TSHβ-Rpl-CAT. To generate the deletion constructs of GATA2, the GATA2-cDNA region in pcDNA3-mGATA2 was amplified by PCR with primer mGATA2U (5’-ccccgctctagctcactctctcgtcagctctgccattgaa-3’) and mGATA2D2 (5’-gcctgccgtagctactctcattgcccgttggtgacc-3’) for GATA2-NZ, with primer mGATA2U2 (accggagtcaagctgtgcggtcagctgtgcgtgactctccgtgga-3’) and mGATA2D2 (5’-taactcctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctc
using the calcium phosphate technique (Sasaki et al. 1995). Cells at a density of 10^6 cells/plate were transfected with 4·0 μg wild-type or the mutant THB–CAT reporter gene, β-galactosidase expression vector pCH111 (1·8 μg; Nakano et al. 2004), human PIT1 expression vector pCB6^-hPIT1 (0·4 μg), mouse GATA2 expression vector pcDNA3-mGATA2 (0·2 μg), and pCMX empty vector as carrier DNA (to make 7·2 μg DNA per dish). After the cells were exposed to calcium phosphate/DNA precipitates for 20 h, the medium was replaced with fresh DMEM containing 5% FCS depleted of thyroid hormones (Samuels et al. 1979). After incubation for an additional 24 h, cells were harvested and CAT activity was measured as described previously (Sasaki et al. 1995, Nakano et al. 2004). CAT activity was normalized for transfection efficiency determined by the β-galactosidase assay. In each CAT reporter assay, we also performed transfection with CMV-CAT (10 ng/dish), and took the magnitude of CAT activity as 100% to standardize the activities of THB–CAT (Nakano et al. 2004).

**Electrophoretic mobility shift assay**

Oligonucleotides for PG-probe (wild-type THB sense; 5'-cagatgaattttcaatggtgctttcaaatagaagaa-3' and antisense; 5'-ttctttagagagctttgcttttaagaa3'–5'), 2G sense; 5'-cagatgaattttcaatggtgctttcaaatagaagaa-3' and antisense; 5'-cagatgaattttcaatggtgctttcaaatagaagaa-3'), and probe-SR (sense; 5'-agtgaatcactgtgtgaaagcatttt-3' and antisense; 5'-acacctgatgtgattattttctgaa-5').**Fig. 5A** were labeled with γ[32P]-ATP using T4 polynucleotide kinase (Toyobo, Tokyo, Japan). CV1 cells were transfected with PIT1 or GATA2 expression plasmids (5 μg per 1 cm dish). They were harvested and lysed in lysis buffer containing 125 mM Tris–HCl (pH 7·6) and 0·5% Triton X-100.
Recombinant full-length human PIT1 (r-PIT1) was generated by baculovirus system according to the manufacturer’s protocol. The -32P-labeled probes and nuclear extracts from transfected CV1 cells were incubated for 30 min on ice in 20 mM binding buffer containing 10 mM Tris–HCl (pH 7.6), 50 mM KCl, 0.05 mM EDTA, 2.5 mM MgCl2, 8.5% glycerol, 1 mM dithiothreitol, 0.5 mg/ml poly (dI-dC), 0.1% TritonX-100, and 1 mg/ml nonfat dry milk. Electrophoretic mobility shift assay (EMSA) for the SR region was also prepared using nuclear extracts with non-transfected CV1 cells. A 25- to 50-fold molar excess of cold oligonucleotides for 2G, 4G (sense 5'-cagtatgaattttcaatggggggagatgcttttcagataagaaa-3' and antisense 5'-tttcttatctgaaaagcatctcccctcccattgaaaattcatactg-3'), 6G (sense 5'-cagtatgaattttcaatggggggagatgcttttcagataagaaa-3' and antisense 5'-tttcttatctgaaaagcatctcccctcccattgaaaattcatactg-3'), 8G (sense 5'-cagtaaattttcaatggggggagatgcttttcagataagaaa-3' and antisense 5'-tttcttatctgaaaagcatctcccctcccattgaaaattcatactg-3') and double-stranded probe-SR were used as competitors in binding reactions. DNA–protein complexes were resolved by electrophoresis on a 5% polyacrylamide gel at 100 V for 80 min at room temperature. For the supershift assay, antibodies against PIT1 (Santa Cruz, CA, USA, SC442) and GATA2 (Santa Cruz, CA, USA, SC-267) were added.

The gel was dried and labeled bands were visualized using the BAS-1000 autoradiography system (Fuji Film, Tokyo, Japan).

**Statistical analysis**

Each CAT reporter assay was performed in duplicate more than three times, and each result is expressed as the mean ± s.d. Statistical significance was examined by ANOVA and Fisher’s protected least significant difference test using StatView 4.0 software (Abacus Concepts, Berkeley, CA, USA). *P < 0.05 was considered significant.

**Results and discussion**

**DNA binding of PIT1 at PIT1-US is necessary to cooperate with GATA2**

Figure 1A shows the structure of the human TSHβ promoter encompassing nt −128/+37 fused to a CAT reporter gene (TSHβ–CAT; Sasaki et al. 1999, Nakano et al. 2004, Matsushita et al. 2007). Consistent with the previous reports (Gordon et al. 1997, Dasen et al. 1999, Nakano et al. 2004), transactivation of the TSHβ...
Cooperation of PIT1 with GATA2 in the Tshb gene

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Cooperation of PIT1 with GATA2 in the TSHβ promoter is independent of the nucleotide spacing between their binding sites

To explore the physical interaction of PIT1 and GATA2 in the TSHβ gene, we performed a gel shift assay with 32P-labeled probe-PG, which encompasses PIT1-US and GATA-REs (Fig. 3A). We detected both PIT1 (Fig. 3B, left panel) and GATA2 bindings (right panel), the signals of which were abolished or supershifted by the addition of antibody against PIT1 and GATA2 respectively. When nuclear extracts containing PIT1 and GATA2 were mixed, we observed an additional band, which was supposed to be a PIT1/GATA2 complex (Fig. 3C, left panel). Similar results were reported previously (Dasen et al. 1999, Gordon et al. 2002). To address whether DNA binding of these two proteins may be mutually dependent, we examined the effect of nucleotide insertion between PIT1-US and GATA-REs (Fig. 3A). In earlier studies, a consensus sequence for the PIT1-binding site was postulated to be TATNCAT (Andersen & Rosenfeld 1994); however, analysis of the X-ray crystal structure of the PIT1–DNA complex on GH and PRL promoters (Scully et al. 2000) revealed that the POU5 domain recognizes four nucleotides (ATTC), while the POU1 domain binds with two nucleotides (AT), and that the spacing between ATTC and AT is 4 bp in the PRL promoter (Fig. 3D, left panel), and 6 bp in the GH promoter (right panel). In the TSHβ promoter, we inspected the antisense strand of the 5' flanking region of GATA-REs (Fig. 3A), and speculated that PIT1-US may have a similar configuration with 5 bp spacing between the nucleotides recognized by the POU5 and POU1 domains. We generated an artificial oligonucleotide (2G) that had a 2 bp insertion in the putative boundary (Fig. 3A) and performed the gel shift assay. As shown in Fig. 3C, we found that PIT1 and GATA2 bound radiolabeled 2G (right panel) as well as wild-type probe-PG (left panel). The fact that a 2 bp insertion, i.e., a 72° rotation of the DNA double helix, had no influence on the pattern of DNA binding of PIT1 and GATA2 suggested that DNA binding of PIT1 and GATA2 might be physically independent. To confirm this, we performed a competition assay using artificial oligonucleotides that had 2–8 bp spacing between PIT1-US and the upstream GATA-RE (Fig. 3A). As shown in Fig. 3E, a 25-fold molar excess of these oligonucleotides efficiently competed against 32P-labeled wild-type probe for PIT1 and GATA2 binding, indicating that at least 2–8 bp spacing does not affect the DNA binding of PIT1 and GATA2. Although protein–protein interaction between PIT1 and GATA2 has been reported in the promoter that has GATA-RE only (Dasen et al. 1999, Gordon et al. 2002), formation of the PIT1/GATA2 complex on probe-PG was not affected by the increasing amount of PIT1.

DNA binding of PIT1 and GATA2 in the TSHβ promoter is independent of the nucleotide spacing between their binding sites

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Thus, it is unlikely that the direct interaction between PIT1 and GATA2 is required for their DNA recognition in PG probe.

The cooperative function of PIT1 is strictly determined by the positions of PIT1-US and GATA-REs

In the promoter of the erythropoietin receptor gene, GATA1 and SP-1 are predicted to bind to the same surface of the double helix of DNA (Zon et al. 1991, Merika & Orkin 1995), and DNA bindings of these transcription factors are physically independent; however, Merika & Orkin (1995) reported that additional 5 bp insertion in the spacing between GATA-RE and SP-1-binding sites resulted in fourfold reduction in the transactivation of this gene. The cooperation between PIT1 and ETS family transcription factor in the PRL promoter is also determined by the spacing between these two transcription factors (Duval et al. 2003). To examine the effect of the spacing between PIT1-US and GATA-REs on the transactivation of the Tshb gene, we generated TSHb-2G-CAT and TSHb-4G-CAT, which have an additional 2 or 4 bp insertion of guanines between PIT1-US and GATA-REs respectively (Fig. 4A). The positions of guanine insertions were identical to those of oligonucleotides, 2G and 4G, used in the gel shift assay (Fig. 3A). As shown in Fig. 4B, potentiation by PIT1 was increasingly attenuated in TSHb-2G- and 4G-CAT. This observation indicates that increased spacing interferes with the function of PIT1. Therefore, cooperation between PIT1 and GATA2 was strictly determined by the distance between PIT1-US and GATA-REs. Gordon et al. (2002) reported that PIT1

(Fig. 3F). Thus, it is unlikely that the direct interaction between PIT1 and GATA2 is required for their DNA recognition in PG probe.

Figure 3 PIT1 binding with PIT1-US is physically independent from GATA2 binding. (A) Schematic representation of probe-PG that has PIT1-US and two GATA-REs (sense and antisense strands). Inspection of the antisense strand of the TSHβ promoter suggests 5 bp spacing between DNA contacts made by the POUS and POUi domains. Two to eight guanines were inserted between PIT1-US and upstream GATA-RE to create 2G–8G. Open arrowhead indicates the insertion point of three thymines in TSHβ-3T-CAT (Fig. 4A). (B) EMSA using radiolabeled PG-probe with nuclear extract from CV1 cells transfected with PIT1 (left panel) and GATA2 (right panel). Binding signals were abolished or supershifted by the addition of antibody against PIT1 or GATA2 respectively. PIT1- and GATA2-binding signal is indicated by an arrow. Ab, antibody against PIT1 (left panel) or GATA2 (right panel); PG, cold PG-probe; NS, non-specific oligonucleotide competitor; free, free radiolabeled PG-probe. (C) EMSA using 32P-probe-PG (left panel) and 32P-2G (right panel). PIT1–GATA2 complexes (PIT1/GATA2) on the radiolabeled PG-probe and 2G were detected. PG, cold probe-PG; NS, non-specific oligonucleotide competitor. (D) Reported PIT1-binding sites in PRL (left panel) and GH (right panel) promoters. Spacing between POUS-binding nucleotides (ATTTC) and POUH-binding nucleotides (AT) is 4 bp in the PRL promoter (left panel) and 6 bp in the GH promoter (right panel). (E) Binding of PIT1 and GATA2 effectively competed with a 25-fold molar excess of cold 2G, 4G, 6G, and 8G as well as cold PG-probe (PG). (F) PIT1–GATA2 complex (PIT1/GATA2) on probe-PG was not affected by the increasing amount of PIT1. Constant amount of nuclear extract (0.4 μl) containing GATA2 was mixed with increasing amounts of PIT1 (0–0.4 μl) extracted from transfected CV1 cells and was incubated with 32P-labeled probe-PG.
may also recognize upstream GATA-RE in the TSHβ gene and suggested that this composite DNA element may play a role in the cooperation between PIT1 and GATA2. To test this, we generated a mutant reporter gene, TSHβ-3T-CAT, which had an insertion of three thymidines between two GATA-REs (Fig. 4A). Although upstream GATA-RE was intact in this construct, the cooperation with PIT1 was also reduced (Fig. 4A). Similar but modest effects were observed when one or two thymidines were inserted (data not shown). Given that DNA bindings by these transcription factors does not require the direct protein–protein interaction between PIT1 and GATA2 (Fig. 3), there is a possibility that proper alignment of PIT1-US and GATA-REs on TSHβ promoter is necessary to recruit coactivators including CBP. Following findings support this hypothesis. First, functional cooperation between CBP and PIT1 is impaired in a mutant PIT1, P24L (Fig. 1D), which has a defect in the association with CBP in the PRL promoter (Kishimoto et al. 2002). Second, CBP is also associated with Zn-finger domains of GATA1 and 2, which mediates the cooperation with PIT1 (Fig. 2; Boyes et al. 1998, Grass et al. 2003, Hayakawa et al. 2004). Third, transactivation of TSHβ promoter by PIT1 and GATA2 was abolished by the expression of adenovirus E1A (data not shown), which is known to interfere with the function of CBP (Kishimoto et al. 2002).

Sequence downstream to GATA-REs has a suppressive effect on the GATA2-induced transactivation of the TSHβ promoter

The above results indicate the functional importance of the nucleotide positions of PIT1-US and GATA-REs in the TSHβ promoter. However, these findings do not fully explain why this promoter requires coexistence of PIT1 while GATA-REs in other promoters can be activated by GATA2 alone (Smith et al. 1995, Dasen et al. 1999, Miaw et al. 2000, Tsuzuki et al. 2000). We speculated the existence of a molecular mechanism that represses GATA2 function. Dasen et al. (1999) demonstrated that PIT1 was required for the transactivation of the short promoter sequence encompassing nt −145/−62 of the mouse Tshb gene (corresponding to human nt −143/−60) in CV1 cells. Except for PIT1-US and GATA-REs, this sequence contains only 30 bp between GATA-REs and TATA box (nt −89/−60 in human TSHβ gene), and this 30 bp sequence is conserved among humans, mice, and rats (Wondisford et al. 1988). We generated deletion constructs, TSHβ-M1, 2, and 3-CAT (Fig. 5A). As shown in Fig. 5B, TSHβ-M1-CAT, in which the sequence between GATA-REs and TATA box was completely deleted, exhibited much stronger transactivation by PIT1 and GATA2 than that of the wild-type TSHβ–CAT. This result suggested that the deleted sequence...
Figure 5 TSHβ promoter lacking for SR can be activated by GATA2 alone. (A) Schematic representation of TSHβ–CAT and its deletion constructs. In these mutant reporters, the transcription start site remains intact. The sequence of SR (−82/−52) is indicated above wild-type TSHβ–CAT. (B) Deletion of SR enhanced the transactivation by PIT1 and GATA2. TSHβ–CAT plasmid or its deletion constructs (4.0 µg) were transfected into CV1 cells together with expression plasmids for PIT1 (0.4 µg) and GATA2 (0.4 µg). The magnitude of CAT activity of CMV–CAT (10 ng/dish) was taken as 100. The results are shown as the mean ± s.d. from at least three experiments. *P<0.05 versus without PIT1 or GATA2. #P<0.05 versus TSHβ–CAT (wild type) in the presence of PIT1 and GATA2. (C) In the absence of PIT1, GATA2 was able to transactivate the constructs that lacked SR (TSHβ–M1 and M3–CAT) as well as (GATA-RE)2-tk–CAT. Four micrograms of reporter plasmid were transfected into CV1 cells together with or without expression plasmids of GATA2 (0.4 µg). All experiments were performed without PIT1. The magnitude of CAT activity of CMV–CAT (10 ng/dish) was taken as 100. The results are shown as the mean ± s.d. of four experiments. *P<0.001. (D) Schematic representations of TSHβ–hRLuc, -mP–hRLuc, and -mP-M1–hRLuc. The promoter of the Tshβ gene encompassing nt −128 to +37 was fused to a modified renilla luciferase (hRLuc) reporter gene. (E) Using lipofection, GATA2 expression plasmid was co-transfected into the pituitary somatotroph cell line GH3 together with TSHβ–hRLuc, TSHβ–mP–hRLuc, or TSHβ–mP-M1–hRLuc. The magnitude of hRLuc activity for phRLuc-control vector (Promega Corp., 100 ng/dish) was taken as 100. The results are shown as mean ± s.d. from four experiments. *P<0.05.

(nt −81/−29) possesses an inhibitory function. While the transactivation of TSHβ–M2–CAT by PIT1 and GATA2 was comparable with that of TSHβ–CAT, potent transactivation was again observed in TSHβ–M3–CAT, which lacks nt −82/−52 (Fig. 5B). As the deleted sequence in TSHβ–M3–CAT was thought to have a repressive effect on the TSHβ promoter driven by PIT1 and GATA2, we designated the sequence (nt −82/−52) as a SR (Fig. 5A). Interestingly, we found that both TSHβ–M1–CAT and TSHβ–M3–CAT, but not the wild-type TSHβ–CAT, were also strongly activated by GATA2 in the absence of PIT1 (Fig. 5C). Although GATA2 modestly transactivated TSHβ–M2–CAT, the increase was not statistically significant (data not shown). We wanted to confirm PIT1-independent transactivation by GATA2 in the pituitary somatotroph cell line, GH3. Because the transfection efficiency of this cell line was very low, we generated TSHβ–hRLuc, in which the TSHβ promoter (nt −128/+37) was fused to modified renilla luciferase (hRLuc) gene (Fig. 5D), and transfected into GH3 cells with or without GATA2 expression plasmid (Fig. 5E). Because GH3 cell expresses endogenous PIT1 (Gordon et al. 1997), transfection of GATA2 only was able to activate TSHβ–hRLuc. To exclude the effect of endogenous PIT1, we generated TSHβ–mP–hRLuc, of which PIT1-US was mutated (Fig. 5D), and found that GATA2 alone did not activate this construct. However, truncation of SR from TSHβ–mP–hRLuc (TSHβ–mP-M1–hRLuc) enabled GATA2 alone to stimulate transcription again (Fig. 5E). These observations suggest that SR has a suppressive effect on GATA2 activity.
Suppression by SR is independent of the distance from the TATA box or its orientation and PIT1 counteracts this inhibition (derepression)

We created a reporter construct, TSH\textsuperscript{b}-Rpl-CAT (Fig. 6A), in which SR was replaced with the 39 bp sequence between SR and TATA box (nt $-64/-26$). This construct was activated by GATA2 alone as well as TSH\textsuperscript{b}-M3-CAT, while the activation of wild-type TSH\textsuperscript{b}-CAT by GATA2 alone was minimal (Fig. 6B). We also tested IV-CAT, IV-M1-CAT, and IV-M3-CAT, in which the sequence between GATA-REs and TATA box (nt $-123/-29$) was ligated in the inverted orientation (Fig. 6C, left panel), and found that IV-M1-CAT and IV-M3-CAT were transactivated by GATA2 alone ($\sim 12$ fold) without PIT1 (Fig. 6C, right panel). These results indicate that the suppressive effect by SR was independent of the distance from the TATA box or its orientation. To examine whether PIT1 relieves the SR-mediated inhibition, we compared the dose-dependent effects of PIT1 on wild-type TSH\textsuperscript{b}-CAT and TSH\textsuperscript{b}-M3-CAT activity (Fig. 6D). Increasing amounts of PIT1 enhanced the activity of wild-type TSH\textsuperscript{b}-CAT, while the stimulation of TSH\textsuperscript{b}-M3-CAT by GATA2 was independent of PIT1 (Fig. 6D). This was confirmed in the experimental condition where GATA2 expression plasmid was reduced to a submaximal amount of 0.2 µg/dish. These findings suggest that the major transcriptional activator for the Tsh\textsuperscript{b} gene is GATA2, and that the cooperation between PIT1 and GATA2 in the regulation of the TSH\textsuperscript{b} promoter is not a conventional synergism, but rather derepression of the SR-induced suppression against GATA2-mediated transactivation.

Nine bp sequence in SR is essential for the repression of GATA2-induced transactivation

As shown in Figs 1A and 7A, SR contains a PIT1-binding site-like sequence (PIT1-like; Steinfeld et al. 1992) and a TATA box-like sequence (TATA-like; Wondisford et al. 2009) (2009). 225–237

Figure 6 The suppressive effect by SR was independent of its orientation or the distance from the TATA box and is counteracted by PIT1 (derepression). (A) Schematic representations of TSH\textsuperscript{b}–CAT, M3-CAT, and Rpl-CAT. In TSH\textsuperscript{b}–Rpl-CAT, SR was replaced with the 39 bp sequence between SR and TATA box (nt $-64/-26$) in inverted orientation. The original nucleotide positions (nt $-64$ and $-26$) are indicated above TSH\textsuperscript{b}–CAT and in parentheses below TSH\textsuperscript{b}–Rpl-CAT. (B) TSH\textsuperscript{b}–M3-CAT and Rpl-CAT, but not wild-type TSH\textsuperscript{b}–CAT, were transactivated by GATA2 alone in a dose-dependent fashion. Four micrograms of reporter plasmid were transfected into CV1 cells together with or without expression plasmids of GATA2 (0.4 µg). All experiments were performed without PIT1. (C) Schematic representation of IV, IV-M1-, and IV-M3-CAT (left panel). The sequence from nt $-123$ to $-29$ in TSH\textsuperscript{b}–IV, TSH\textsuperscript{b}–M1-, and TSH\textsuperscript{b}–M3-CAT was digested with restriction enzymes and ligated in inverted orientation (left panel). The original nucleotide positions are indicated in parentheses. CAT activity with GATA2 was divided by that without GATA2 to calculate fold activation (right panel). (D) The cooperative function of PIT1 with GATA2 is not conventional synergism but prevention of the suppression by SR (derepression). Transactivation of TSH\textsuperscript{b}–M3-CAT by GATA2 (0.4 µg) was independent of the dose of the PIT1 gene, and TSH\textsuperscript{b}–M3-CAT could be stimulated even by a submaximal amount of GATA2 (0.2 µg). *$P<0.05$ versus without GATA2.

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Figure 7 Nine bp sequence in SR is important for the inhibition of GATA2-induced transactivation of TSHβ promoter. (A) Schematic representation of TSHβ–CAT and its deletion constructs (M4 and 5). The PIT1-binding site-like sequence and TATA box-like sequence are indicated as PIT1-like and TATA-like respectively. In M4 and M5, the sequences between nt −63/−29 and −72/−29 were deleted from TSHβ–CAT respectively. (B) M10 exhibits transactivation by GATA2 alone. Four micrograms of reporter plasmid (M6–M11) were transfected into CV1 cells together with or without plasmids of GATA2 (0.4 µg). Except for wild-type promoter (wt), experiments were performed without PIT1 expression plasmid. CAT activity with GATA2 was divided by that without GATA2 to calculate fold activation. *P<0.001. #P<0.001 versus wild-type TSHβ–CAT. (C) Schematic representation of TSHβ–CAT and its mutants (M6–M11). Mutated sequences are underlined. Sequences similar to the PIT1-binding site (PIT1-like), AP-1 site (AP-1 like), and TATA box (TATA like) are indicated above the wild-type sequence (Wt). Nucleotide positions for nt −82, −72, −63, and −52 are indicated.

Figure 8 PIT1 blocks the recognition of AP-1 like sequence by SR-binding protein (SRBP). (A) Schematic representation of SR-probe (nt −84/−65) and mutated oligo-DNAs (mut1–6). (B) EMSA using radiolabeled SR-probe with nuclear extract from non-transfected CV1 cells (CV1-NE). Single specific binding (arrow) was detected (lane 2) and a mutation in AP-1 like sequence (mut2) failed to compete with this binding (lane 6). SRBP, SR-binding protein; comp, competitor; NS, non-specific competitor; free, free radiolabeled SR-probe (arrowhead). (C) EMSA using radiolabeled probe-SR and recombinant full-length PIT1 (r-PIT1). Single specific binding (open arrowhead) was detected. Comp, competitor. (D) r-PIT1 binding (open arrowhead) was competed by wild-type SR and mut6 but not by mut2–5. (E) SRBP binding (arrow) was competed by increasing amount of r-PIT1 (open arrowhead).
To evaluate the function of these sequences, we generated two deletion constructs, M4 and M5, that lack nt K63/K29 and nt K72/K29 respectively (Fig. 7A). As shown in Fig. 7B, we found a modest activation of M4 by GATA2 alone, but no activation of M5. Since GATA2 alone could not activate M5 that contains nt K82/K72 (Fig. 7A and B), we speculated that this short sequence might mediate the inhibitory effect on GATA2-dependent transcription. Among the series of mutations in SR (Fig. 7C), M9 and M10 were strongly activated by GATA2 alone, while M6, M7, and M8 were not (Fig. 7B). In M11, GATA2-induced transactivation was again attenuated. These results suggested that the 9 bp sequence mutated in M10 is critical for the suppressive effect on GATA2-induced transactivation. Although GATA2 modestly stimulated M4 (Fig. 7B), disruption of the TATA-like sequence (M6) did not cause transactivation by GATA2 (Fig. 7B and C).

A database search suggests that the 9 bp sequence (TGAATCAA between nt −82/−74) contains a sequence that resembles the AP-1 site. Indeed, a very similar sequence (TGAATCA) was reported as a potent AP-1 site in the plasminogen activator inhibitor type 2 gene (Cousin et al. 1991). However, the overexpression of Jun and Fos in CV1 cells did not affect the activity of TSHβ–CAT (data not shown). Delhase et al. (1996) reported that a homologous sequence (ATGAATCA) in the human PIT1 gene promoter is recognized by not only Jun/Fos in Hela cells but also PIT1 in GH3 cells. However, as shown in Figs 5C and 7B, SR inhibits GATA2-induced transactivation without PIT1. Thus, it is unlikely that the function of SR may be mediated by PIT1, although PIT1 associates with nuclear receptor corepressor (NCoR; Xu et al. 1998, Scully et al. 2000). Recently, Kim et al. (2007) reported that a LIM homeodomain transcription factor, LHX2, may trans-activate the TSHβ promoter through the recognition of the DNA sequence that includes the 9 bp element (nt −82/−74). In our experimental condition, however, the expression plasmid for porcine LHX2 (Susa et al. 2006) did not enhance TSHβ–CAT and TSHβ–hRluc in CV1 cells and GH3 cells (data not shown).

**Minimal sequence of SR is recognized by nuclear factor in CV1 cells**

We performed an EMSA using radiolabeled probe-SR (Fig. 8A) and nuclear extract from non-transfected CV1 cells. As shown in Fig. 8B, we detected a single retarded band (lane 2). This band was thought to be specific because a large excess of cold SR-probe (lane 3), but not unrelated oligonucleotide (NS, lane 4), abolished the signal. Competition assay with a series of mutant oligo DNAs (Fig. 8A) revealed that a mutant, mut2,
failed to compete with radiolabeled probe-SR (Fig. 8B, lane 6). The positions of substituted nucleotides in mut2 (GAA to CGG) corresponded to mutations in M9 and M10 (Fig. 7C), which permitted transactivation by GATA2 alone (Fig. 7B). An attempt to identify the SR-binding protein (SRBP) in CV1 cell is currently underway in our laboratory.

**PIT1 blocks the SR-mediated repression by the competition with SRBP**

Although PIT1-like sequence is inactive for the activation of Tshb gene (Fig. 1E), it was reported that the recombinant PIT1 recognizes this sequence (Haugen et al. 1996). We carried out EMSA with radiolabeled SR-probe and recombinant PIT1 (r-PIT1) generated from baculovirus system. As shown in Fig. 8C, the binding of r-PIT1 was detected (lanes 1–4) and this signal was efficiently competed by cold SR (lane 5). Competition assay revealed that not only mut2 but also mut3–5 can compete with wild-type SR (Fig. 8D, lanes 5–8), suggesting that PIT1-binding site includes sequence recognized by SRBP. As shown in Fig. 8E, SRBP was replaced by the increasing amounts of r-PIT1. These results suggest that PIT1 interferes with the SRBP binding. We tested the cooperation between PIT1 and GATA2 using M7 contrast, which possesses the mutation in PIT1-like sequence (Fig. 9A). As shown in Fig. 9B, cooperation by PIT1 was impaired in M7 but not in wild-type TSHβ–CAT or M6 construct. These results indicate that PIT1 blocks the SR-mediated repression by the competition with SRBP.

Our hypothesis is summarized in Fig. 10. The major transcriptional activator for TSHβ promoter is GATA2, while PIT1 creates the environment for the transactivation by GATA2. This observation is reminiscent of the role of PIT1 in the *Prl* gene regulation. First, the association of CBP by PIT1 is important for the activation of the PRL and TSHβ promoters (Kishimoto et al. 2002; Fig. 1C and D). Secondly, the synergy of PIT1 with ETS family transcription factor including ETS1 is known to be necessary for the full activation of the *Prl* gene (Gutierrez-Hartmann et al. 2007). Finally, Duval et al. (2003) reported that this synergy does not require the physical interaction of PIT1 with ETS1 on DNA but is influenced by the spacing between the binding sites for these transcription factors (8 bp in the wild-type promoter). It should be noted, however, that the cooperation of PIT1 with GATA2 is not conventional synergism but rather derepression against SRBP on SR (Figs 6, 7 and 8). Current findings may explain why Tshb gene is exclusively expressed in the thyrotroph where both PIT1 and GATA2 coexist.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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