A Thyrotrope-specific Variant of Pit-1 Transactivates the Thyropitin β Promoter*

(Received for publication, March 10, 1993, and in revised form, June 15, 1993)

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Thyropitin (TSH) is a subunit of TSH, the expression of which is limited to the thyrotrope cells of the anterior pituitary gland. Tissue-specific expression of the mouse TSHβ gene is conferred by sequences between -270 and -80 of the 5'-flanking region. We have investigated tissue-specific expression of the TSHβ promoter in two thyrotrope-derived cell types: 1) TtT-97 thyrotropic tumors, which express the endogenous TSHβ gene, and 2) an α-TSH cell line, which was generated from a thyrotropic tumor that has lost the ability to express the TSHβ gene. The pituitary-specific transcription factor Pit-1 is present in thyrotropes and interacts with three cis-acting elements in the functionally important region of the TSHβ promoter. Pit-1 protein is present in TtT-97 tumor cells but is absent from α-TSH cells. Reintroduction of Pit-1 into α-TSH cells by transient transfection does not restore TSHβ promoter activity. We have identified an alternate spliced variant of Pit-1, called Pit-1T, the mRNA and protein expression of which is limited to thyrotrope-derived cells. Pit-1T contains a 14-amino acid insert in the transactivation domain due to an alternate 3' splice acceptor site. Transiently transfected Pit-1T increases TSHβ promoter activity in TtT-97 thyrotropic tumor cells, whereas additional Pit-1 has no effect. The α-TSH cell line, which lacks all Pit-1 proteins, requires both isoforms in order to stimulate TSHβ promoter activity. These data suggest that Pit-1T is a thyrotrope-specific splice variant of Pit-1 that is required for TSHβ promoter stimulation; furthermore, both Pit-1 and Pit-1T are required for TSHβ promoter activity in thyrotrope cells.

Thyropitin (TSH) is a glycoprotein hormone that is produced only by thyrotrope cells of the anterior pituitary gland (1). TSH contains two dissimilar, noncovalently associated α and β subunits. While the α subunit is shared among the other glycoprotein hormones, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and choric gonadotropin (CG), the TSHβ subunit is immunologically and functionally unique, and its expression is restricted to thyrotropes. It is now well established that gene expression is affected by **trans-acting factors that bind directly to cis-acting elements on promoter regions of specific genes, but the molecular mechanisms that dictate cell-specific expression of the TSHβ gene remain elusive (2).**

The sequence of the mouse TSHβ gene and its 5'-flanking region have been reported previously (3). Transfection experiments in thyrotrope cells with both homologous and heterologous promoters have shown that cell-specific activity of the mouse TSHβ promoter is localized between -270 and -80 of the 5'-flanking region (4, 5). DNase I protection studies using nuclear protein extracts from a TSHβ-expressing mouse thyrotropic tumor, TtT-97, have identified four cis-acting elements in this region designated D1 (-253 to -222), D2 (-196 to -176), P1 (-132 to -100), and P2 (-86 to -64) (5). These regions are not protected by nuclear extracts from L-cell fibroblasts or a thyrotrope-derived α-TSH cell line that has lost the ability to express the TSHβ gene. These data suggest that specific trans-acting factors present only in thyrotropes that bind to this promoter region may play a significant role in tissue-specific expression of TSHβ.

Pit-1 is a well characterized pituitary-specific transcription factor (6, 7). It is a 31-33-kDa protein that is a member of the POU-homeodomain family (8). Pit-1 mRNA transcripts are detectable in all cell types of the anterior pituitary but expression of Pit-1 protein is limited to thyrotropes, sommatotropes, and lactotropes (7). Pit-1 has been shown to be required for efficient transcription of the growth hormone (GH) and prolactin genes (9, 10) as well as autoregulation of its own expression (11, 12). The role of Pit-1 in TSHβ gene expression is not clear. It is well documented in Snell and Jackson dwarf mice as well as several human case reports that a deletion or mutation of the Pit-1 genomic locus results in deficiency of TSH, GH, and prolactin as well as pituitary hypoplasia (13-16), suggesting that Pit-1 is necessary for normal development of these pituitary cell types. Recent studies have identified thyropitin-releasing hormone (TRH) and cAMP responsive elements within the TSHβ gene promoter that interact with Pit-1 (17-19). Although the sequence of these elements are not exact Pit-1 consensus binding sites as defined by the GH (TATNCAT) and prolactin promoters, they mediate a 4-10-fold stimulation of TSHβ promoter activity in the presence of transacted Pit-1 with forskolin, phorbol esters, or TRH in non-thyrotrope cells. However Pit-1 alone does not significantly activate TSHβ promoter activity in the presence of transacted Pit-1 with forskolin, phorbol esters, or TRH in non-thyrotrope cells. Therefore Pit-1 may play a role in TRH-mediated TSH production, but its role in cell-specific expression remains undefined.

Three recent reports have described a new isoform of Pit-1 in somatotrope cells, called Pit-1β/Pit-1a/GHF-2 (22-24). This isoform appears to be a splice variant of Pit-1 gene transcripts, which contains an additional 26 amino acids in the transactivation domain. The Pit-1β mRNA has approximately 14% the abundance of Pit-1 in GH3 rat pituitary tumor cells, and the Pit-1β protein is less than 3% as abundant as Pit-1 (22). When
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MATERIALS AND METHODS

Experimental animals—All TtT-97 mice used in these studies were treated in accordance with the National Institutes of Health guidelines on animal use and care. All protocols were reviewed and approved by the University of Colorado Health Science Center Committee on Use and Care of Animals.

Reverse transcription-PCR to identify Pit-IT—Total RNA (10 μg) from mouse thyrotropic tumor (TtT-97), α-TSH, GH3, GH4, and mouse kidney cells was reverse-transcribed using avian myeloblastosis virus reverse transcriptase and 500 ng of an antisense oligonucleotide (EOA) complementary to the 3' end of Pit-I and encompassing the TAA stop codon (underlined) (5'-CGAAGCTTTTATCCGCTGTCAT-3'). A region of the 5'-lFAUT origin activation encoding exons 1-3 was amplified by polymerase chain reaction (PCR) with a sense strand oligonucleotide (E1S) beginning at the Pit-1 translation start site (untranslated) (5'-GGATCTAGGAGGGATATACTTATGAGTTAAGCCATGGTCGGTTC-3') containing a BamHI cloning site and the E3A oligonucleotide described above. PCR was carried out with 500 ng of each primer and 2.5 units of Taq polymerase (Boehringer Mannheim) for 35 cycles (each cycle 94°C for 1 min, 59°C for 1 min, and 72°C for 90 s).

An aliquot of each PCR product was separated on a 1.5% agarose gel, transferred overnight to a 0.45-μm nylon membrane (Micron Separations, Inc.), and cross-linked for 5 min with UV irradiation at 300 nm. The filter was probed with a 32P-labeled antisense Pit-1 oligonucleotide (5'-CCGGATCCATGAGTTGCCAATCTTTCACCTCGG-3') of specific activity 1.7 TIBS (Taq polymerase (Boehringer Mannheim) for 35 cycles (each cycle 94°C for 1 min, 59.5°C for 1 min, and 72°C for 90 s). Washes were carried out with TBS-0.02%T and the filter was incubated for 1 h at 25°C. Washes were carried out with TBS-0.05%T and the filter was incubated for 1 h at 25°C with 10 ml of a 1:100 dilution of purified TtT-97 antibody. The filter was then washed with TBS-0.05%T and exposed to X-ray film for 7 h.

Isolation of full-length Pit-IT Clones from TtT-97 Cells—In order to obtain full-length coding regions of the Pit-1 isoforms for transient transfection studies, coding cDNA fragments were obtained by reverse transcription following PCR with oligonucleotides spanning the translation start and termination sites of Pit-1. TtT-97 total RNA (10 μg) was reverse-transcribed with a HindIII-containing exon 6 oligonucleotide (E6A) complementary to the 3' end of Pit-1 and encompassing the 14-bp region immediately upstream of E6A. Filters were hybridized with the probe overnight at 68°C. The appropriate bands were excised from the X-ray film and sequenced by the chain termination reaction. Cells were grown in 800 ml of Luria-Bertani (LB) medium with 50 μg/ml ampicillin at 37°C as an optical density of 1.1 at 600 nm and protein production was induced with a final concentration of 1 mM isopropylthio-β-D-galactoside for 2 h. Cells were pelleted and resuspended in 100 ml of buffer (20 mM Tris-HCl, 0.5 M NaCl, 0.1% SDS, and 1 mM EDTA) and adjusted to 2.5 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride. Cells were sonicated in 0.5-ml aliquots for 10 s on ice, pooled, and centrifuged at 25,000 rpm for 30 min at 0°C, and the supernatant was collected and adjusted to 10% glycerol and stored at -70°C. The fusion proteins were cleaved with 0.5 units of thrombin (Sigma) in 50 μl Tris-HCl, pH 8, 150 μM NaCl, and 2.5 mM CaCl₂ for 30 min at 25°C. Proteins were separated by electrophoresis on a denaturing 10% SDS-polyacrylamide gel and electrotransferred to nitrocellulose overnight at 10 mA with a Hoeffer Transphor apparatus at 4°C. The nitrocellulose was blocked for nonspecific binding with 5% nonfat milk in 20 mM Tris, pH 7.5, 157 mM NaCl, (0.2% Tween 20 (TBS-0.2%T) for 1 h at 25°C. Washes were carried out with TBS-0.05%T and the filter was incubated for 1 h at 25°C with 10 ml of a 1:100 dilution of purified TtT-97 antibody. The filter was then washed with TBS-0.05%T and incubated for 1 h at room temperature with a horseradish peroxidase-conjugated goat anti-rabbit IgG antisera (Life Technologies, Inc.) at 1:5000 dilution in TBS-0.2%T containing 1% nonfat milk. The filter was washed with TBS-0.05%T and exposed to radiographic film for 30 min.

Pit-1 antisera—kind gift of Dr. R. Maurer was used at a 1:10,000 dilution to test the bacterially derived Pit-1 and Pit-1T proteins.

Gene Transfer Studies of Pit-1 Isoforms—A plasmid containing sequences from -392 to +40 of the mTSHβ promoter fused to luciferase was constructed as previously described (29). Pit-1 and Pit-1T expression plasmids were generated by insertion of the full-length coding regions into a pCMV5-galactosidase vector (Clontech) from which the

Quantitation of Pit-1 IT mRNA in TtT-97 Cells by RNase Protection Analysis—Transcripts containing the unique Pit-IT splice in TtT-97 thyrotropes were quantitated by an RNase protection strategy employing hybridization of dephosphorylated RNA to terminal transferase (TdT)-cDNA of Pit-1 to exon 3 PCR-derived Pit-IT sequence in pGEM3zf+. Linearization with EcoRI at position 45 of the mPIT-1 sequence and synthesis for 1 h at 39°C using [γ-32P]UTP (800 Ci/m mole) and SP6 RNA polymerase as described (25) in a 155-bp antisense RNA which is complementary to Pit-1T RNA at 14-15 bp sequence at the 3' end. After digestion with BumHI and HindIII, the agarose gel-purified fragments were size-fractionated by electrophoresis on an 8% denaturing polyacrylamide sequencing gel containing 8 M urea. The dried gel was exposed to radiographic film at -70°C for 17 days with two enhancing screens. Fragments generated by a HpaII digest of pRE922 DNA were used as size standards. Following visualization by autoradiography, the area of the dried gel containing the Pit-1 isoform bands was exposed to a PhosphorImager screen (Molecular Dynamics, Inc.) for 5 days. The radioactivity emitted from the appropriate bands was quantified by integration analysis using ImageQuant software (Molecular Dynamics, Inc.).
β-galactosidase coding region was removed. The BamHI to HindIII PCR-generated coding sequence of each Pit-1 isoform and a NotI fragment containing the CMV promoter were blunted by reverse transcription and subsequently ligated. Pit-1- and Pit-1T-positive clones were identified by colony blot hybridization with a 32P-nick-translated Pit-1 cDNA and subsequently ligated. Pit-1- and Pit-1T-positive clones were selected by colony hybridization with a BamHI to Taq1 (1-200 nucleotides) fragment derived from the PCR product of mouse Pit-1 into the Pit-1T full-length coding cDNA in pGEM3zf+ (Promega) between the BamHI and Taq1 sites. The Pit-1β PCR product was generated by RT-PCR as previously described using the E1S (BamHI site) and E3A (HindIII site), followed by subcloning into pGEM3zf+ and sequence verification. Correct orientation and ligation borders of the full-length Pit-1β were verified by sequence analysis. The BamHI to HindIII full-length Pit-1p cDNA was then excised and ligated into the pCMV vector as described (29).

Transient transfection assays were performed as previously described (29). Briefly, 20 μg of TSHβ-luciferase DNA, 10 μg of each pCMV Pit-1 isoform DNA, and 1 μg of a pCMV β-galactosidase DNA as an internal control for transfection efficiency were transfected by electroporation into 3 million n-TSH cells or 5-10 million dispersed TtT-97 tumor cells in a total of 140 μl of 50 μm Tris, pH 8, 10 μm EDTA. An Rous sarcoma virus promoter-luciferase plasmid was transfected in parallel to correct for inter-assay variation. Cells were incubated at 37 °C for 48 h prior to harvest, subjected to freeze-thaw extraction, and assayed for both luciferase and β-galactosidase activity. Transfections were performed in duplicate and constructs with the appropriate Pit-1 isoform in the reverse orientation were used as negative controls. Luciferase activity was measured in a Monolight 2010 luminometer (Analytical Luminescence Laboratory) from duplicates of the freeze-thaw lysates while β-galactosidase activity and expression relative to 1 million Rous sarcoma virus-luciferase units. Number of independent tumor or cell preparations and transfections are designated by n values.

**RESULTS**

**Identification and Isolation of Pit-1 Isoforms in Thyrotrope Cells**—10 μg of total RNA was reverse-transcribed with an exon 3 oligonucleotide (E3A), and the cDNA coding for the activation domain of Pit-1 (31) was amplified by PCR with an exon 1 oligonucleotide (E1S) and E3A. Southern blot hybridization with a 32P-labeled exon 3 oligomer upstream of E3A revealed differently sized PCR products derived from TtT-97, α-TSH, GH3, and mouse pituitary RNA (Fig. 1). TtT-97, α-TSH, and hypothyroid mouse pituitary RNA yielded two distinct products (Fig. 1, lanes 1 and 2). The smaller, more intense band was consistent with Pit-1; however, the upper band was larger than those found in the other cell lines, and its size was consistent with the previously described Pit-1β (381 nucleotides) (22-24). These fragments were isolated, subcloned, and sequenced. Three different activation domain sequences were identified (Fig. 2A), two of which corresponded to the previously described Pit-1 and Pit-1β. The intermediate-sized band was restricted to thyrotrope-derived cells and was designated Pit-1T. The sequence of Pit-1T revealed an in-frame 42 nucleotide insert between exons 1 and 2, which corresponded to the 3'-end of the Pit-1β insert (Fig. 2B). The Pit-1T mRNA is therefore generated by a non-consensus RNA transcript splice 5’-GT...AT-3’ rather than the consensus 5’-GT...AG-3’ splicing choice. The mouse Pit-1β-specific region is similar to that reported previously for the rat with two nucleotide differences, one of which results in an amino acid change at position 49 from lysine to threonine, which is in Pit-1β but not Pit-1T.

In order to demonstrate whether mRNA coding for both Pit-1β and Pit-1T was present in the various cell and tumor lines, specific PCR re-amplification of the first-round PCR products was performed with a 32P-labeled Pit-1T/Pit-1β common oligonucleotide along with E1S on a more highly resolving gel system. This showed that the Pit-1β transcript was present in TtT-97 and α-TSH cells as well as GH3 and GH4 cells (Fig. 3), but the Pit-1T transcript was detected only in TtT-97, α-TSH, and hypothyroid mouse pituitary cells (lanes 3-7). While not quantitative, the PCR analysis is extremely sensitive.

**Fig. 1. Southern blot of RT-PCR products from TtT-97 tumor, MGH101A tumor, α-TSH cell, mouse pituitary, and mouse kidney RNA.** 10 μg of total RNA from different TtT-97 tumors (lanes 1-3), MGH101A tumor (lane 4), α-TSH cell (lane 5), mouse kidney (lane 6), GH3 cells (lane 7), and mouse pituitary cells (lane 8) were incubated with avian myeloblastosis virus reverse transcriptase and 500 ng of E3A for 1 h at 42 °C, followed by PCR with E1S and E3A as described under “Materials and Methods.” Fragments were separated on an agarose gel, transferred to a nylon membrane, and probed with a 32P-labeled Pit-1 oligonucleotide just upstream of E3A. Bands corresponding in size to appropriate Pit-1 isoforms are denoted by arrows.

**Fig. 2. Schematic illustrating the structure and sequence of the activation domain insert unique to Pit-1T.** A, the DNA binding and activation domains of Pit-1 are shown at the top. An enlargement of the activation domain shows the location of the 26-amino acid and 14-amino acid inserts of Pit-1β and Pit-1T, respectively (white). Pit-1β is 291 amino acids, Pit-1β is 317 amino acids, and Pit-1T is 305 amino acids. B, first 80 amino acids of murine Pit-1T. The underlined nucleotides represent the unique Pit-1T region.

**Fig. 3. RT-PCR of the Pit-1β- and Pit-1T-specific regions from RNA.** Aliquots of the PCR reactions from Fig. 1 were re-amplified with E1S and a radiolabeled Pit-1β/Pit-1T-specific oligonucleotide, and the products were separated on a 0.4-mm thin, 40-cm 5% nondenaturing polyacrylamide gel. RT-PCR products were from GH4 cells (lane 1), GH3 cells (lane 2), Pit-1T tumor cells (lanes 3 and 4), MGH101A tumor cells (lane 5), α-TSH cells (lane 6), and hypothyroid murine pituitary cells (lane 7).
and shows a definite lack of any detectable Pit-IT message in non-thyrotrope-derived (somatotrope/mamotrope) cells that express the Pit-1 gene.

The full-length coding region of Pit-1 was generated by reverse transcription of 10 µg of TtT-97 total RNA followed by PCR amplification with oligonucleotides encompassing the exon 1 start site (E1S) and exon 6 termination codon (E6A). After subcloning into pGEM3zf+ (Promega), eight Pit-1T constructs were isolated from TtT-97 RNA using this technique.

Quantitation of Pit-1T mRNA by RNase Protection Analysis—To ensure that the novel Pit-1T sequence was not an artifact of the PCR methodology, RNase protection analysis was carried out to verify that mRNA transcripts containing the unique Pit-1T splice existed in TtT-97 thyrotropic tumors. Fig. 4 shows that a band of 141 bp consistent with the presence of Pit-1T mRNA as well as a 99-bp band consistent with Pit-1 mRNA were protected by poly(A) RNA from three separate TtT-97 tumor preparations (lanes 1–3). The 141-bp band is derived from the 155-bp antisense Pit-1T RNA probe (lane 6) by removal of the 5′-most 14 bp, which are derived from the plasmid DNA and would not be protected by the Pit-1T isoform mRNAs.

Pit-1, Pit-1β, or pre-mRNA would not generate the 141-bp fragment, which is unique to protection by Pit-1T. When the ratio of Pit-1 and Pit-IT signals was quantitated by PhosphorImager analysis it was found to be 45:1, respectively. This ratio of Pit-1 to Pit-IT was similar in all three TtT-97 mRNA samples. One mouse was treated with T3 (lane 3), which did not alter the ratio of Pit-1 to Pit-IT. No protection of the Pit-IT RNA probe was detected using yeast tRNA (lane 5) or 40 µg of poly(A)+ RNA isolated from mouse liver, consistent with its lack of expression in this tissue (7) (lane 4). Fragments of the antisense probe protected by Pit-1 mRNA were not detected by this method since one would co-migrate with the 99-bp Pit-1 band and the other at 42 bp, a region of the autoradiograph that is contaminated with nonspecific bands generated by incomplete probe digestion. GH3 and GH4 mRNA were not tested by this method since the rat and mouse sequences are not identical in this region. However, as noted above, Pit-1T mRNA was not detectable in these cells by using the more sensitive RT-PCR technique.

Western Blot Analysis of Pit-1 Proteins—20 µg of nuclear extracts from two separate TtT-97 thyrotropic tumors and GH3 cells were separated on a denaturing 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with a purified polyclonal Pit-IT/β-specific antibody (BaBco, Inc.) or Pit-1 antiserum (gift of Dr. R. Maurer) and results are shown in Fig. 5. Fig. 5a shows that the Pit-1 antisemur detects both bacterially derived isoforms of Pit-1, and that Pit-1T (lane 1) and Pit-IT are difficult to separate by standard electrophoresis. In TtT-97 and GH3 nuclear extracts the different Pit-1 isoforms were indistinguishable using the Pit-1 antiserum, while α-TSH

Fig. 4. RNase protection of TtT-97 thyrotropic tumor RNA with a riboprobe specific for Pit-1T. 15 µg of poly(A)+ RNA from three different TtT-97 tumors (lanes 1–3), one of which was from an animal treated for 14 days with T3 at 100 µg/kg/day (lane 3), murtine liver (lane 4), and yeast tRNA (lane 5) was hybridized to a uniformly labeled 155-bp riboprobe (lane 6) encoding the first 141 bp of Pit-1T. After digestion with RNase A and T1, samples were separated on an 8% polyacrylamide denaturing gel. The standard (std) lanes contain pBR322 DNA digested with HpaII.

Fig. 5. Western blot analysis of Pit-1 proteins. A, proteins from bacterially derived Pit-1 (lane 1) and Pit-IT (lane 2) were size-separated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with a rabbit polyclonal Pit-1 antiserum using a horseradish peroxidase-conjugated secondary antibody to rabbit IgG. The nitrocellulose filter was exposed for 30 min. B, Pit-1 (lane 1) and Pit-IT (lane 2) bacterially derived proteins as well as 20 µg of GH3 (lane 3) and two separate TtT-97 (lanes 4 and 5) nuclear extracts were separated on a 10% polyacrylamide-SDS gel, transferred to nitrocellulose, and probed with a purified rabbit polyclonal antibody to the Pit-1T-specific region using a horseradish peroxidase-conjugated secondary antibody to rabbit IgG. The filter was exposed for 30 min. Arrows denote the predicted positions of the Pit-1 isoforms.

Fig. 6. Transfection of Pit-1 isoforms in thyrotrope-derived cells. 10 million TtT-97 tumor cells (A) or 3 million α-TSH cells (B) were cotransfected with 20 µg of mTSHP promoter (−392 to +40) luciferase construct in conjunction with 10 µg of each pCMV-Pit-1 isoform (Pit-1, Pit-IT, and Pit-1β) and 1 µg of pCMV-β-gal. Cotransfection of Pit-1 and Pit-IT (B) in α-TSH cells used 5 µg of each pCMV plasmid. After 40 h of incubation at 37°C, cell extracts were prepared and luciferase and β-galactosidase activity was measured. Multiple transfections of each Pit-1 isoform are expressed as fold stimulation for TSHP promoter activity with Pit-1 isoform sequence in the forward orientation (black boxes) versus the reverse orientation (set at 1, white boxes). Values are means ± S.E. Numbers of independent tumor or cell preparations and transfections are denoted by n values.
nuclear extracts had no detectable Pit-1 isoform protein (data not shown). We therefore generated the purified Pit-1T/β-specific antibody.

Fig. 5b shows that the specific antibody does not recognize the bacterially derived Pit-1 (lane 1) but easily detects Pit-1T (lane 2). A protein band consistent with Pit-1β is present in GH3 nuclear extract (lane 3). In two different TtT-97 thyrotropic tumor extracts (lanes 4 and 5) bands consistent with both Pit-1β and Pit-1T are detected (arrows).

Gene Transfer Studies of Pit-1 Isoforms—To test the effect of the Pit-1 isoforms on mTSHP promoter activity, transfection studies were carried out in both TtT-97 thyrotropic tumor cells and a-TSH cells. The TtT-97 tumor cells contain all three Pit-1 isoforms and express the TSHβ gene, while the thyrotrope-derived a-TSH cells lack all Pit-1 protein and have lost the ability to express the TSHβ gene. The -fold stimulation of each Pit-1 isoform was compared with a CDNA construct in the CMV plasmid in reverse orientation.

Previous work from this laboratory has shown that TSHβ promoter activity is low in α-TSH cells and is approximately 5–10-fold higher in TtT-97 tumor cells that express the endogenous TSHβ gene (28). The results of multiple transient transfection experiments of each Pit-1 isoform in TtT-97 and α-TSH cells are shown in Fig. 6. Panel A shows that Pit-1 co-transfected into TtT-97 cells, which contain endogenous Pit-1 isoforms, results in no stimulation of TSHβ promoter activity. However, exogenously added Pit-1T is able to increase TSHβ promoter activity by approximately 6-fold. Since TtT-97 cells contain an excess of Pit-1 over Pit-1T, these data suggest that Pit-1T is a limiting factor in TtT-97 cells while Pit-1 is not limiting. The Pit-1β isoform does not stimulate the TSHβ promoter in TtT-97 cells and may have a slight inhibitory effect (Fig. 6A). Similar experiments in α-TSH cells show that Pit-1, Pit-1β, or Pit-1T are unable to significantly stimulate the TSHβ promoter when transfected separately (Fig. 6B). 10 µg of the Pit-1 isoform plasmids were used in these experiments; preliminary experiments with 1 and 5 µg of these isoforms also showed no stimulation of the TSHβ promoter in α-TSH cells, eliminating general squelching as a reason for no effect. Cotransfection of Pit-1 with Pit-1T, however, resulted in a 3-fold increase in TSHβ promoter activity, suggesting that the presence of both Pit-1 isoforms may contribute to TSHβ promoter activation. While the combination of Pit-1 and Pit-1T stimulated TSHβ promoter activity in α-TSH cells, they did not fully restore the 5–10-fold differential seen between the TtT-97 tumor and α-TSH cells from previous studies (28).

In order to verify that Pit-1 isoforms transfected in α-TSH cells were appropriately translated, transiently transfected cells with either pCMV-Pit-1 or pCMV-Pit-1T were evaluated for protein expression by the Western blot assay after cell lysis by sonication. α-TSH cells do not translate endogenous Pit-1 mRNA into protein (32). Extracts of cells transfected with either Pit-1 isoform contained a doublet (Fig. 7) consistent with the use of two alternate translation start sites as previously described (8), indicating that the CMV promoter-directed Pit-1 and Pit-1T mRNAs are able to be translated in α-TSH cells. The smallest band generated by Pit-1T (lane 1) may represent a degradation product or a third alternative translation start site used by α-TSH cells.

Discussion

We have identified a novel thyrotrope transcription factor, Pit-1T, that appears to result from a thyrotrope cell-specific alternate splice of the primary Pit-1 gene transcript. Pit-1T mRNA is present in TtT-97 thyrotropic tumors, α-TSH cells, and hypothyroid mouse pituitary cells, all of which contain cells of thyrotrope origin. GH3 and GH4 somatotropinoma cells, which contain Pit-1, lack the Pit-1T variant. Only TtT-97 cells and presumably mouse pituitary thyrotropes, which express the TSHβ subunit gene, contain Pit-1T protein. Pit-1T message was detected initially by PCR, but its existence was confirmed by RNase protection studies with TtT-97 thyrotropic tumor RNA. Furthermore, Western blot analysis demonstrated the existence of a protein consistent with Pit-1T in TtT-97 nuclear extracts. Transient transfection studies in TtT-97 and α-TSH cells indicate that Pit-1T is limiting in TtT-97 cells but acts together with Pit-1 to activate the TSHβ promoter in α-TSH cells that lack all endogenous Pit-1 protein isoforms.

Pit-1T is an alternately spliced variant of Pit-1, which is a member of the POU-homeodomain family of transcription factors. Other examples of alternately spliced variants of transcription factors in the POU-homeodomain family are well documented: Oct-2, I-POU, and Pit-1 (22–24, 33, 34). Expression of the Oct-2 gene, which mediates B-cell transactivation, results in alternately spliced mRNA isoforms, which are differentially expressed in B-cells, brain, kidney, testes, and intestine (33). Three of the factors (Oct-2a, -2b, and -2c) contain exon acceptor site 6 nucleotides upstream from the I-POU splice site (34). I-POU is unable to bind DNA but can heterodimerize with another protein (CN-α) to inhibit its binding to its target site on the dopa decarboxylase gene. The splice variant, t1-POU, no longer dimerizes with αCN- but can bind DNA and transactivates promoters other than dopa decarboxylase. The Pit-1 gene generates an alternately spliced variant, Pit-1p/Pit-1a/GHF-2, that binds to the same promoter elements as Pit-1; although it activates the GH promoter, it only weakly transactivates the prolactin and Pit-1 promoters (22). Co-transfection experiments by Theill et al. (23) demonstrated that Pit-1β and Pit-1 do not have a synergistic effect on the Pit-1 and GH promoters, and in fact Pit-1β appears to antagonize the effect of Pit-1 on the prolactin promoter. In this study we have shown that Pit-1β does not activate the TSHβ promoter and may inhibit promoter activation by the other Pit-1 isoforms. Its functional significance at this time is unknown, but it may contribute to the differential regulation of the GH, prolactin, and TSHβ genes.

Pit-1T is not only a novel splicing variant of Pit-1, it is unique in that it is generated by a non-consensus splice event. An overwhelming majority of pre-mRNA is spliced according to the consensus intron sequence arrangement 5′-GT . . . AG-3′ (35). Recent evidence is emerging that alternately spliced mRNAs do not always conform strictly to this splicing rule (35, 36). The human Gα subunit as well as three Drosophila genes have a TG or CG at the 3′ end of the intron, and all represent alternately spliced variants (35). Furthermore, the human proliferating cell nuclear protein (P120) has a 3′ acceptor AC, which is
similar to Pit-1T (AT) and defines a class of introns containing a pyrimidine instead of the consensus guanine residue (35). It has been suggested that the consensus splice represents the "default," while alternately spliced variants can deviate to various degrees from this consensus and may require the intervention of specific regulatory factors (37).

Splicing of Pit-1T appears to be generated by a cell-specific protein-RNA interaction that directs splicing in a manner similar to that found for the calcitonin gene-related peptide, which is a brain-specific, alternately spliced variant of the calcitonin gene (38, 39). A tissue-specific factor involved in calcitonin gene-related peptide splicing is believed to bind to the pre-mRNA at the 5' end of exon 4 and direct an exon inclusion at that site. Alternatively, thyromyos may contain a specific RNA-binding protein that partially blocks conventional Pit-1 splicing to produce the mRNA for the Pit-1T variant. One example of this is the sxl gene splicing in Drosophila, which dictates the sex of the organism (37). Splicing of sxl pre-mRNA is directed by a protein that blocks the primary 3' acceptor splice site choice forcing an alternate choice downstream. Organisms with this RNA-binding protein block the splice site choice for males, creating female fruit flies. Conversely, all non-thyromyos cells may contain a specific protein that blocks the Pit-1T splicing choice, although this is less likely since Pit-1T mRNA is a non-consensus splice flanked by two more favorable splicing choices. Future studies involving GST-chromatography or gel shift analysis with appropriate RNA fragments, RNA footprinting, and mutation experiments will be necessary to elucidate this apparently unique process.

The potential role of Pit-1 or an isoform in thyrotopes is supported by previous work. Two reports of a POU-domain-specific point mutation of Pit-1 in humans, rendering it unable to activate target genes, resulted in a lack of thyrotrop as well as lactotrope and somatotrope cell development (14, 15). Experiments from this laboratory have shown that the -270 to -80 region of the mouse TSHβ promoter is functionally important for tissue-specific expression and Pit-1 interacts with three specific cis-acting elements in this region (4, 5, 32). The phenotypic characteristics of the TtT-97 thyrotropic tumor and α-TSH cell line lend added indirect evidence for a role of Pit-1 isoforms in TSHβ expression since α-TSH cells that have lost TSHβ expression are also lacking both Pit-1 and Pit-1T proteins and do not protect the functionally important TSHβ promoter region in DNase I footprinting studies. However, other studies with GH3 and CV-1 cells fail to elicit TSHβ promoter stimulation when co-transfected with Pit-1 alone (18, 21). In this report we have also shown that Pit-1 alone fails to stimulate the TSHβ promoter in the α-TSH cells, which lack endogenous Pit-1 protein. Phosphorylation studies of Pit-1 indicate a role in TRH/cAMP-directed stimulation of TSHβ in transfected GH3 and embryonal kidney 293 cells (17-19). These data suggest that Pit-1 alone cannot stimulate basal TSHβ expression, but it may mediate TRH stimulation while another related POU-homeodomain protein may be required for activation since the functionally significant region of the TSHβ promoter has three footprints that interact with Pit-1. Searching for POU-homeodomain factors related to Pit-1, we isolated Pit-1T, which met the requirements for a thyrotop-specific factor. Pit-1T protein is detected in the thyrotop-derived TtT-97 cells and not in somatotrope-derived GH3 cells. The RNase protection data suggests that Pit-1T encoding mRNAs are present at low abundance compared to those encoding Pit-1. This generates the question of the in vivo role of Pit-1T in thyrotopes. A number of considerations are pertinent. First, Pit-1T appears to be a limiting factor in TtT-97 thyrotropic tumor cells inasmuch as addition of Pit-1T, but not Pit-1, significantly stimulates the TSHβ promoter in these cells. Second, TtT-97 tumors are heterogeneous, as exemplified by the different Pit-1T/Pit-1 ratios found using Western analysis (Fig. 5). TtT-97 tumors are also not pure thyrotopes, since they contain a significant number of fibroblasts as well as blood cell elements, which can confound interpretation of nuclear factor extract. Unlike these tumors, the TtT-97 thyrotropic tumor more closely reflects the cellular content of Pit-1T protein in true pituitary thyrotopes. Therefore, the stoichiometric importance of the various Pit-1 isoforms may not be able to be addressed using TtT-97 tumors. Furthermore, the α-TSH cell line may lack other factors required for TSHβ promoter expression, necessitating the need for large amounts of the Pit-1 isoforms to compensate for these deficiencies.

Further studies such as stable transfection of Pit-1 and Pit-1T into α-TSH or other non-TSH-producing cell lines may be required to address these issues. Functional studies in TtT-97 and α-TSH cells indicate that Pit-1T is required for TSHβ expression but that Pit-1 must also be present. Restoration of TSHβ promoter activity to the level in thyrotopes is approached in α-TSH cells by transient co-transfection of Pit-1 and Pit-1T, while neither factor alone has any effect. This apparent cooperation is intriguing since Pit-1 is known to homodimerize and form heterodimers with other factors such as Oct-1 and estrogen receptors (40, 41). The role of Pit-1 may, if any, in TSHβ promoter activity is not clear. Other laboratories have shown that Pit-1 does not stimulate the GH promoter in Pit-1 lacking cells, but this isoform may have an inhibitory effect on the prolactin promoter activity stimulated by Pit-1 (23). Our data indicate that Pit-1T does not stimulate the TSHβ promoter and may even inhibit the effect of the other Pit-1 isoforms. Further studies analyzing protein-protein and protein-DNA interactions as well as specific regions of mutations of Pit-1T will be necessary to delineate the exact roles of Pit-1 and Pit-1T in the tissue-specific expression of the TSHβ gene.

Acknowledgments—We thank Dr. Michael Karin (University of California, San Diego) for providing the rat Pit-1 cDNA, Dr. Richard Maurer (Oregon Health Sciences University, Portland) for providing us with the Pit-1 antisemur, Dr. Arthur Gutierrez-Hartmann and Dr. Virginia Sarapu (both of UCHSC) for critical input into this work, and Janet Dowding and Connie Rupp for excellent technical assistance. We also thank the Tissue Culture Core Laboratory of the Cancer Center of the University of Colorado Health Sciences Center (supported by National Institute of Health Grant CA-3394) for growing the α-TSH cells. We thank Dr. Thomas Anderson of BaBco, Inc. for the Pit-1T-specific antisemur (SBIR Contract N43-3K-2-2214).

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