Pit-1 and GATA-2 Interact and Functionally Cooperate to Activate the Thyrotropin β-Subunit Promoter*

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The molecular determinants governing cell-specific expression of the thyrotropin (TSH) β-subunit gene in pituitary thyrotropes are not well understood. The P1 region of the mouse TSHβ promoter (−133 to −88) region interacts with Pit-1 and an additional 50-kDa factor at an adjacent site that resembles a consensus GATA binding site. Northern and Western blot assays demonstrated the presence of GATA-2 transcripts and protein in TtT-97 thyrotropic tumors. In electrophoretic mobility shift assays, a comigrating complex was observed with both TtT-97 nuclear extracts and GATA-2 expressed in COS cells. The complex demonstrated binding specificity to the P1 region DNA probe and could be disrupted by a GATA-2 antibody. When both Pit-1 and GATA-2 were combined, a slower migrating complex, indicative of a ternary protein-DNA interaction was observed. Cotransfection of both Pit-1 and GATA-2 into CV-1 cells synergistically stimulated mouse TSHβ promoter activity 8.5-fold, while each factor alone had a minimal effect. Mutations that abrogated this functional stimulatory effect mapped to the P1 region. Finally, we show that GATA-2 directly interacts with Pit-1 in solution. In summary, these data demonstrate functional synergy and physical interaction between homeobox and zinc finger factors and provide insights into the transcriptional mechanisms of thyrotrope-specific gene expression.

Cell-specific expression of eukaryotic genes involves the functional interaction of sets of transcription factors that interact with essential cis-acting promoter regions to initiate RNA transcription. The repertoires of transcription factors that are involved in the expression of genes in highly differentiated cells often consist of those ubiquitously expressed in many cell types in cooperation with others whose expression are cell type-restricted (1, 2). Expression of the thyrotropin (TSH)β-subunit gene is restricted to the thyrotope cells of the anterior pituitary gland, where it combines with the α-subunit gene product to produce intact TSH (3). Little information is available to explain the highly restricted expression of the TSHβ subunit gene to this small population of thyrotope cells. To study these thyrotope-specific factors, we have utilized the TtT-97 tumor model, which consists of a homogeneous thyrotope cell population that expresses the TSHβ gene without the confounding influence of other pituitary cell types (4). Characterization of the transcription factors present in thyrotope cells that functionally interact with this promoter will enhance our understanding of the pivotal role they may play in pituitary development and in the maintenance of the differentiated phenotype.

Cell-specific activity of the mouse TSHβ promoter in thyrotope cells requires sequences located between −270 and −80 of the 5′-flanking region (5, 6). Within this broad area, four DNase I protected regions have been identified using nuclear extracts from TSHβ expressing mouse TtT-97 thyrotropic tumor cells (7–9). These have been termed D1 (−253 to −222), D2 (−196 to −176), P1 (−133 to −88), and P2 (−86 to −64). Recently, using scanning mutagenesis, DNase I footprinting, and gene transfer studies, we have focused on the contribution of the functionally important proximal P1 region to TSHβ gene expression in thyrotope cells (10). Selected mutations in the P1 region resulted in loss of DNA binding of at least two different thyrotope nuclear proteins in TtT-97 thyrotope cells. We previously identified the more distal of these two binding proteins as the POU homeodomain Pit-1 transcription factor (9, 10). By Southwestern blot analysis, we determined that the more proximal factor is a 50-kDa protein, which binds adjacent to the Pit-1 consensus sequence where its binding is independent of Pit-1. Targeted mutagenesis showed that when the binding of either Pit-1 or the 50-kDa protein was disrupted, functional promoter activity was reduced by 60–80% to a level exhibited by a minimal −80/+43 promoter fragment that lacks the P1 region (10). Thus, full TSHβ promoter activity required the functional participation of both Pit-1 and an additional protein at the P1 promoter region.

Pit-1 is a transcription factor present in somatotropes, lactotropes, and thyrotope cells of the anterior pituitary (11, 12), where it plays a critical role in the maturation of these cell types during pituitary development (13, 14). Its role in somatotrope cell growth and its ability to transactivate the growth hormone and prolactin gene promoters are well documented (12, 15, 16). Based on binding studies with the growth hormone, prolactin, and Pit-1 promoters, a consensus DNA recognition element (T/A/T/A/TATNCAT) has been derived (12, pair(s); kb, kilobase pair(s); PCR, polymerase chain reaction; RSV, Rous sarcoma virus; DBD, DNA-binding domain; CMV, cytomegalovirus; mPit-1, mouse Pit-1; GST, glutathione S-transferase.

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shown that regulation of TSHβ promoter activity by TRH or cyclic AMP colocalized with Pit-1 binding sites and that mutations of these consensus sequences blunted the stimulatory effect (18–20). Given this information, it is reasonable to speculate that Pit-1 may be important for both basal and hormone-stimulated activity of the TSHβ promoter. However, additional factors most likely are involved in regulating basal activity, since the endogenous TSHβ gene is not expressed in Pit-1-containing lactotrope and somatotrope cells. Furthermore, the addition of Pit-1 alone was not sufficient to stimulate TSHβ promoter activity in αTSH cells that lack endogenous Pit-1 (9). Finally, we have recently shown that a mutation that disrupts binding of a 50-kDa thyrotropic protein without disrupting Pit-1 binding to the adjacent site diminished promoter activity in transfected TT-T9 thyrotrropic cells (10).

Examination of the promoter sequence in the proximal P1 region reveals two areas of sequence homology to a GATA consensus binding site (21, 22): AGATGC, from −110 to −105, and AGATAA, from −98 to −93. The GATA transcription factor family consists of six members, GATA-1 through -6. In general, they are approximately 50 kDa in size (hGATA-2) and contain a highly conserved DNA-binding domain consisting of two zinc fingers, and although originally found in cells of hematopoietic lineage (23), they are expressed in other cell types (24). GATA factors have been shown to functionally interact with other classes of transcription factors such as API (25), Sp1 (26–28), and the estrogen receptor (29). The AGATAA sequence is also present in the human, equine, and mouse α-subunit promoters, and Steger et al. (30) have shown that GATA family members are involved in both pituitary and placental expression of the α-glycoprotein hormone gene that encodes the common subunit of TSH, follicle-stimulating hormone, luteinizing hormone, and chorionic gonadotropin. In the current study, we present data showing that the 50-kDa protein in thyrotrperes that binds to the proximal P1 region of the mTSHβ promoter and functionally synergizes with Pit-1 is likely to be GATA-2.

EXPERIMENTAL PROCEDURES

Plasmid and Probe Constructions—Construction of the −392 to +40 mTSHβ luciferase vector and those containing the P1 region mutations (P1-M3, P1-M7) have been described previously (6, 10). The P1 probes containing sequences from −144 to −74 were produced by a PCR strategy using as templates the wild type or mutant −392 to +40 luciferase constructs. The sense strand primer was 5'-CAGCTGACAGTTGAGTGCTTCAAGGAA-3', and the antisense strand primer was 5'-GATGTCGACATTGCAATGTG-3'. This strategy introduced a SalI site (underlined) at each terminus. We performed 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s with 2.5 units of Taq DNA polymerase (Perkin-Elmer) under buffer conditions suggested by the manufacturer. Products of the appropriate size were digested with T4 DNA polymerase (Perkin-Elmer) under buffer conditions suggested by the manufacturer. After the appropriate size was digested with SalI, subcloned into pGEMZ-Zf (+) (Promega Corp., Madison, WI), and sequenced. P1 probes were purified after digestion with SalI and isolation on a 2% agarose gel using Qiaex II gel (Qiagen Inc., Chatsworth, CA).

Oligonucleotides encompassing the −117 to −88 thyroid cell type were synthesized with [32P]dATP (3000 Ci/mmol; ICN Biomedicals, Costa Mesa, CA) by annealing standard techniques (31). The 5'-TCGACATTGCAATGTGCTTCAAGGAA-3', and the antisense strand sequence was 5'-TCGACGACATCGATTTTCAAGGAA-3'. For the P1-M7 probe, the sense strand was 5'-GATGTCGACATTGCAATGTG-3', and the antisense strand was 5'-TCGACATTGCAATGTGCTTCAAGGAA-3', with the underlined bases representing the mutation. A 700-bp mouse GATA-2 specific sequence, generated by PCR from a 7-kb genomic DNA clone, was isolated from a pUC19 vector by digestion with EcoRI. This plasmid was a gift from Drs. F-Y. Tsai and S. H. Orkin (Harvard Medical School, Boston, MA). It contained coding sequences from amino acids 20–249 and omitted the conserved zinc finger domain common to all GATA family members. A 420-bp mouse GATA-3 specific probe was isolated from a full-length mGATA-3 cDNA expression vector that was kindly provided by Dr. J. D. Engel (Northwestern University, Evanston, IL). A 467-bp mouse GATA-4 specific probe was isolated from a full-length mGATA-4 expression vector in pMT2 by digestion with EcoRI and NotI and contained 116 bp of 5'-noncoding sequence and coding sequences corresponding to mGATA-4 nucleotides 1–117 (32). The vector was a gift from Dr. David B. Wilson (Washington University School of Medicine, St. Louis, MO). The probes were 32P-radioabeled by nick translation (33) to a specific activity of 4–8 × 106 cpm/μg using a commercial kit (Life Technologies, Inc.). The conserved GATA DNA-binding domain (DBD) containing codon sequences from 259–344 (34) was amplified from mGATA-3 by a PCR strategy using the following oligonucleotides. The sense strand was 5'-CAGAGCGAGGTGTGTTGAAC-3', and the antisense strand was 5'-CAAGATTCGATACAGGCCAGAGGTATTG-3'. We performed 30 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min, and extension at 72 °C for 1 min with 2.5 units of Taq DNA polymerase, 250 ng of each primer, and 1 ng of the mGATA-3 plasmid. The resultant product was subcloned into pCR1 (Invitrogen, San Diego, CA) and sequenced.

For cotransfection experiments, a 2.4- kb end-filled EcoRI fragment containing the hGATA-2 coding region (35) was cloned downstream of the immediate early cytomegalovirus (CMV) enhancer/promoter at a unique NotI site within the expression vector pCMV-βgal (CLONTECH Laboratories, Palo Alto, CA), in which the β-galactosidase region had been removed. Both cDNA and vector fragments were blunt-ended by treatment with avian myeloblastosis reverse transcriptase (Promega Corp., Madison, WI) and all four dNTPs and ligated with T4 DNA ligase (Boehringer Mannheim). Constructs with the correct orientation were determined by DNA sequencing using primers adjacent to the cloning site and Sequenase (U.S. Biochemical Corp.). Mouse Pit-1 coding sequences were blunt end-cloned into the same vector as described (9).

RNA Blot Analysis—Total RNA was isolated from TT-T9 thyrotropic tumors, mouse αTSH cells, or rat GH3 cells by the guanidinium isothiocyanate-CsCl method (36). Poly(A) RNA was isolated by affinity chromotography over an oligo(dT) cellulose column (type 7, Pharmacia Biotech, Inc.). The RNA was size-fractionated through a 1% agarose gel containing 6% formaldehyde as described (37) and transferred to a nylon membrane (Nytran, 0.2 μm; Schleicher and Schuell). The membrane was washed once in 20 × SSC buffer (3 x sodium chloride, 0.3 x sodium citrate), and fragments were fixed by ultraviolet light cross-linking (model FB-UVXL-1000, Fisher) and hybridized to a cDNA probe for the DBD, which is highly conserved in all GATA family members, or with cDNA probes specific for mouse GATA-2, GATA-3, or GATA-4. Prehybridization, hybridization, and wash conditions have been described previously (37). The membranes were stripped and reincubated with them in boiling 0.1 x SSC, 0.1% sodium lauryl sulfate, three times for 15 min each and then reprobed with a radiolabeled mouse β-actin cDNA probe.

Western Blot Analysis—CV-1 cells transiently transfected with he magglutinin (HA) epitope-tagged mPit-1 and/or hGATA-2 were harvested with phosphate-buffered saline containing 3 mM EDTA, pelleted, and resuspended in 100 μl of TEA-SDS solubilization buffer (55 mm triethanolamine, 111 mM NaCl, 2.2 mM EDTA, and 0.4% SDS) as described (38). Lysed extracts were passed through a 25-gauge needle seven times to shear genomic DNA. Protein concentration was determined by the method of Bradford (39) using a commercial kit (Bio-Rad). Equal amounts (50 μg) of protein were separated on a 12% polyacrylamide-SDS gel (40) and transferred to an Immobilon-P (polyvinylidene difluoride) membrane (Millipore, Bedford, MA) by electroblotting. The membrane was dried by immersion in methanol for 10 s followed by air drying for 15 min. Nonspecific binding was blocked with 7.5% nonfat milk in TBS (20 mM Tris-Cl, pH 7.5, 137 mM NaCl, 0.2% Tween 20) for 1 h. Immobilized proteins were incubated for 1 h at room temperature with a mouse monoclonal anti-HA-peroxidase antibody (0.1 μg/ml; Boehringer Mannheim) at a dilution of 1:1000 and the secondary antibody (0.1 μg/ml) for 1 h. After three 10-min washes with TBS, HA-tagged proteins were detected using an ECL chemiluminescent kit (Amersham Life Science).

Nuclear extracts from TT-T9 thyrotrropic tumors were prepared as described (41), and hGATA-2 transfected COS cell extracts were electrophoresed, transferred, and blocked with 7.5% milk as described above. GATA-2 protein was visualized with a mouse monoclonal anti-
GATA-2 antibody (Santa Cruz Biotechnology, Inc.) at a dilution of 1:5000 for 1 h in TBST containing 1% milk. After washing in TBST, the membrane was incubated with horseradish peroxidase-conjugated goat antimouse immunoglobulin G (Life Technologies, Inc.) (1:10,000 dilution) for 1 h in TBST containing 1% milk and washed three times in TBST. Blots were exposed to X-ray film. Controls were subcloned into pGEM7Zf+ (Promega Corp.) for restriction enzyme digestion and nucleotide sequencing by the chain termination method (44). DNA sequences were compared with GenBank™ sequences using the BLAST protocol (45).

DNase I Footprints—Fragments containing mTSHβ sequences from −392 to +40 were single ended labeled by filling in recessed 3′ termini as described previously (10). DNase I protection experiments were performed as described (46) Reactions containing 75 μg of nuclear extract from mouse pituitary G1b plates were digested with 100 ng of DNase I for 60 s. Control reactions contained 10 μg of bovine serum albumin (Boehringer Mannheim). After heat denaturation of the deproteinized DNA at 95°C for 3 min, samples were applied to a 6% polyacrylamide sequencing gel containing 8M urea and size-separated by electrophoresis at 95 °C for 3 min, samples were applied to a 6% polyacrylamide sequencing gel containing 8M urea and size-separated by electrophoresis. Following four rounds of screening, five recombinant plasmid was produced by autoexclusion using the Cre recombinase from Escherichia coli BM25.8. Plasmid DNA was transformed into E. coli DH5α, and EcoRI inserts were subcloned into pGEM7Zf+ (Promega Corp.) for restriction enzyme digestion and nucleotide sequencing by the chain termination method (44). DNA sequences were compared with GenBank™ sequences using the BLAST protocol (45).

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TTT-97 thyrotropes (10). Similarly, other mutations, such as P1-M3 (Fig. 1), abrogated Pit-1 binding and promoter activity due to the disruption of Pit-1 consensus sites. To determine whether thyrotrope cells contained transcripts for GATA factors, we performed Northern blot analyses under high stringency conditions with specific GATA probes. Initially we used a DBD probe derived by PCR from mouse GATA-3, which contains sequences highly conserved among all GATA family members. We detected a major transcript of 3.7 kb and a minor transcript of 3.1 kb using total RNA from two different TtT-97 thyrotropic tumors (Fig. 2A). The specificity of GATA family members was then ascertained by using distinct amino-terminal coding region probes that are unique to GATA-2, GATA-3, or GATA-4 but lack the common DBD region. A GATA-2 probe composed of coding region sequences from amino acids 20–249, revealed the presence of abundant transcripts of 3.7 and 3.1 kb (Fig. 2B) from two TtT-97 thyrotropic tumors and in mTSH cells, which are thyrotrope-derived but express neither Pit-1 protein (9) nor the TSH β subunit (52). The pattern of these transcripts was similar to that seen with the common DBD probe (Fig. 2A). In contrast, pituitary-derived GH3 somatotropes, which express Pit-1, did not contain detectable GATA-2 transcripts. The blot was reprobed with β-actin to demonstrate that equivalent amounts of RNA were present in each lane (Fig. 2B). Next we used a probe specific for mouse GATA-3, which contained sequences for amino acids 29–169. We detected a single abundant 2.6-kb GATA-3 transcript in GH3 cells and a barely detectable band of the same size in TSHβ-expressing TtT-97 thyrotropes (Fig. 2C) although equivalent amounts of RNA were present as accessed by reprobing the filter with β-actin (Fig. 2C, bottom). The filter from Fig. 2B was then reprobed with a mouse GATA-4-specific probe that contained 116 bp of 5′-noncoding sequence and coding sequences for amino acids 1–117. A faint band of about 4 kb was found in αTSH RNA (Fig. 2D), while, in contrast, no GATA-4 transcripts were detected in poly(A)⁺ RNA from either TtT-97 thyrotropes or GH3 somatotropes.

Additionally, we used the common DNA-binding domain probe to isolate recombinant GATA cDNAs from a TtT-97 library constructed in an AEX-LOX under high stringency washing conditions. From approximately 750,000 primary phage, we isolated six GATA recombinant clones; all contained mouse sequences highly homologous to human GATA-2 and not to other GATA family members. These data suggest that GATA-2 is the major GATA transcript present in TSHβ-expressing thyrotropes, although a faint signal for GATA-3 was detectable in these cells.

**TTT-97 Thyrotrope Cells Contain GATA-2 Protein**—Next we determined whether GATA-2 protein was present in the TtT-97 thyrotropes. Since expression of full-length GATA-2 is toxic to bacteria (21), we overexpressed human GATA-2 in COS cells using the vector pMT2 (35) to serve as a positive control. As a negative control, we transfected COS cells with pSG5, an empty SV40 promoter construct that was lacking a cDNA sequence. In Western blots using a rabbit polyclonal antibody directed specifically against GATA-2, we detected a single GATA-2 band of 50 kDa from an extract of the hGATA-2 transfected COS cells (Fig. 3, lane 2), which migrated to a similar position as a protein in nuclear extracts from TtT-97 thyrotropic tumors (Fig. 3, lane 3). In contrast, no band was detected in the mock transfected COS cells (Fig. 3, lane 1). This demonstrates that protein in addition to mRNA for GATA-2 are present in the TSHβ-expressing thyrotropic tumor cells.

**GATA-2 and Pit-1 Can Bind Independently to the Proximal mTSHβ Promoter**—To determine whether GATA-2 is capable of binding to the proximal P1 region, we used a probe containing sequences from -117 to -88 and full-length GATA-2 expressed in COS cells in electrophoretic mobility shift analysis. In a previous Southwestern blot, this probe detected a 50-kDa protein but did not detect Pit-1 (33 kDa) in thyrotrope cells (10) although it contained a consensus Pit-1 site at -107 to -101 (Fig. 1). A single major complex formed when we incubated the probe with extracts from hGATA-2 overexpressed in COS cells (Fig. 4A, lane 3) but not with mock transfected cells (lane 2). A similar comigrating complex was formed with TtT-97 thyrotropic tumor nuclear extract (lane 4). Specificity of GATA-2 binding to the proximal P1 site was demonstrated by showing that the GATA-2 complex was effectively competed with increasing amounts (25–250 ng) of homologous duplex oligonucleotide (Fig. 4B, lanes 3–5). However, up to 250 ng (2500-fold molar excess) of the same fragment containing the P1-M7 mutation, which disrupts the GATA consensus sequence, failed to compete for GATA-2 binding to the wild type probe (Fig. 4B, lanes 6–8). Specificity of the hGATA-2 and TtT-97 complex was demonstrated by its ability to be disrupted with a GATA-2-specific polyclonal antibody (Fig. 4, C and D, lane 3) but not with preimmune rabbit serum (lane 4), indicating that GATA-2
in the TtT-97 extract can bind to the proximal P1 site.

We next performed additional gel mobility shift assays using the entire P1 region probe, from −144 to −74 that contains both the GATA-2 and Pit-1 sites. Each factor formed a distinct complex (a and b, respectively, Fig. 5A, lanes 2 and 3). When both proteins were combined in vitro, we detected both single complexes, but in addition, a slower migrating complex (c, Fig. 5A, lane 4), consistent with both factors binding to the same DNA fragment, was evident. Since GATA-2 and Pit-1 are present in TtT-97 thyrotropic cells, the complex most likely represents a ternary complex of both proteins interacting with the DNA probe. That the additional complex contains both proteins was confirmed by using 32P-labeled probes containing either the P1-M7 or P1-M3 mutation in EMSA studies. With the P1-M7 probe, we show that GATA-2 fails to interact, whereas Pit-1 can form the same b complex seen with the wild type probe (Fig. 5B, lanes 2 and 3). However, when both proteins were present in the binding reaction, only the Pit-1-containing b complex was detected. As expected, the additional c complex seen with the wild type probe failed to form (Fig. 5B, lane 4). Conversely, using a probe containing the P1-M3 mutation, we found that the GATA-2 a complex formed, but the Pit-1 b complex was not detected (Fig. 5C, lanes 2 and 3). Again as would be predicted, when both proteins were combined, the slower migrating c complex also was not detected (Fig. 5C, lane 4).

Thus, the slower migrating complex requires an intact GATA-2 site as well as a Pit-1 site, which suggests that it is probably due to both proteins simultaneously binding to the mTSHβ P1 region to form a ternary complex.

To more precisely define the binding site for GATA-2 within the P1 region of the mTSHβ promoter, we performed DNase I protection studies. Using a single end-labeled promoter fragment, we detected a protected area from −133 to −100 when the fragment was incubated with a bacterially produced GST-Pit-1 fusion protein (Fig. 6, lane 1) when compared with the control reaction incubated with BSA (lane 5). COS cells expressing human GATA-2 produced an overlapping footprint from −118 to −88 that extended several bp closer to the transcriptional start site (lane 2). This extended footprint pattern is coincident with the extension produced by TtT-97 thyrotropic tumor extracts from −133 to −88 (Fig. 6, lanes 3 and 4) and is identical to the previous TtT-97 footprint found using the P1-M3 probe (10). Thus, GATA-2 and Pit-1 can account for the same pattern of protection exhibited by TSHβ-expressing nuclear extracts. These data strongly suggest that GATA-2 is the 50-kDa protein that binds to the promoter at the proximal P1 site.

Fig. 3. Detection of endogenous GATA-2 protein in TtT-97 thyrotropes. Whole cell extracts (50 μg) from a TtT-97 tumor (lane 3), COS cells expressing human GATA-2 (lane 2), or mock transfected COS cells (lane 1) were resolved on a 10% acrylamide-SDS gel and transferred to a polyvinylidene difluoride membrane. The blot was probed with a 1:5000 dilution of a mouse monoclonal antibody against GATA-2. Shown is an autoradiogram after detection by enhanced chemiluminescence. The positions of prestained Rainbow protein standards (Amer sham) are shown on the right.

Fig. 4. Electrophoretic mobility shift analysis of GATA-2 with a mTSHβ consensus GATA binding site. A, a double-stranded 32P-labeled probe derived from the mTSHβ promoter (−117 to −88) was incubated with 5 μg of an extract containing hGATA-2 expressed in COS cells (lane 3), with mock transfected cell extract (lane 2), with TtT-97 nuclear extract (lane 4), or in the absence of protein (lane 1). The position of a major shifted complex (arrow) and the free probe (Unbound) are shown on the left. B, the −117 to −88 mTSHβ DNA probe was incubated in the absence (lane 1), or presence of hGATA-2 (lane 2) expressed in COS cells. Parallel incubations included 25–250 ng of homologous competitor DNA (lanes 3–5) or the P1-M7 mutation (lanes 6–8). C, hGATA-2 was incubated with the probe in the absence (lane 2) or in the presence of a rabbit anti-GATA-2 antibody (lane 3) or with preimmune rabbit serum (lane 4). D, probe was incubated with 4.5 μg of TtT-97 nuclear extract in the absence (lane 2) or presence of an anti-GATA-2 antibody (lane 3) or with preimmune rabbit serum (lane 4).

Fig. 5. Formation of an additional complex when GATA-2 and Pit-1 are combined on the TSHβ promoter. A, a 32P-labeled probe (−144 to −74) containing the entire P1 region was incubated in the absence of protein (lane 1), with GATA-2 expressed in COS cells (5 μg; lane 2), bacterially expressed GST-Pit-1 (2 μg; lane 3), or GATA-2 plus Pit-1 (lane 4). B and C, lanes 1–4 are identical to those in A except the probe contained the P1-M7 or the P1-M3 mutation, respectively. The positions of complex a formed with GATA-2 alone, complex b formed with Pit-1 alone, and complex c formed with GATA-2 plus Pit-1 are shown by the arrows.
Pit-1 and GATA-2 Functionally Interact to Stimulate mTSHβ Promoter Activity—We next determined the functional consequences of co-transfecting GATA-2 and Pit-1 on a mTSHβ promoter construct. Using the region of the mTSHβ promoter from −392 to +40 fused to a luciferase reporter, we cotransfected CMV-directed expression vectors for hGATA-2 and mPit-1 into heterologous monkey kidney CV1 cells. The functional consequences of cotransfecting GATA-2 and Pit-1 are shown in Fig. 7A. Neither GATA-2 nor Pit-1 alone appreciably stimulated promoter activity (1.2- and 1.8-fold, respectively) from the wild type promoter when compared with the empty plasmid control. However, the combination of GATA-2 with Pit-1 showed an 8.5-fold stimulation of promoter activity. The stimulation did not occur when the TSHβ luciferase construct contained mutations that abrogated binding to GATA-2 (P1-M7) or Pit-1 (P1-M3) (Fig. 7A). Thus, we have mapped the functional cooperativity of GATA-2 and Pit-1 to specific sequences within the 5′-flanking P1 region of the mTSHβ promoter.

To ensure that each protein was being expressed at similar levels in these cotransfection studies, we tagged them with an HA epitope at their amino terminus (68). Using an antibody specific for the HA epitope on Western blots, we detected equivalent amounts of the appropriately sized HA-tagged proteins in extracts from cells that were transiently transfected with either GATA-2 or Pit-1 alone (Fig. 7B, lanes 2 and 3) or in combination (lane 4). Control cell extracts transfected with a CMV vector lacking cDNA sequences (Fig. 7B, lane 1) demonstrated only a nonspecific (NS) band that reacted with the HA antibody and was also present in the other transfected cells. Functional synergism of a similar magnitude was also seen in the presence of both HA-tagged proteins (data not shown).

Physical Interaction of Pit-1 with GATA-2—Our transfection studies indicated that Pit-1 can functionally synergize with GATA-2 to activate mTSHβ promoter activity. This synergistic effect, which required the participation of both factors, suggested a possible direct protein-protein interaction between the two factors as the mechanism responsible for the functional cooperativity. To address this possibility, bacterial GST fusion proteins of rPit-1 or GST alone were immobilized on glutathione-Sepharose beads and used in binding assays with in vitro transcribed and translated GATA-2 that had been radioabeled with [35S]methionine. As a positive control, we used [35S]-labeled cEts-1, which has been shown to physically interact and functionally synergize with Pit-1 on the rat prolactin promoter (51). Additionally, we used a radiolabeled 252-amino acid truncation of recombinant GST-Pit-1 (lane 1), 75 μg of GATA-2 expressed in COS cells (lane 2), or 75 μg of TtT-97 nuclear extract (lanes 4 and 5). The extent of the protected regions with the different proteins or extracts is shown by brackets on the left.
of cEts-1, termed cEts-1Δ5–6, in which the Pit-1-interacting domain has been deleted (51) as a negative control. Equal amounts of 35S-labeled cEts-1, hGATA-2, or cEts-1Δ5–6 were incubated with immobilized GST-Pit-1 or GST alone. As shown in Fig. 8A, cEts-1 was able to interact with GST Pit-1 (lane 3) but showed no detectable binding to GST alone (lane 2). GATA-2 interacted with GST Pit-1 (Fig. SB, lane 3) and again showed no detectable binding to GST alone (lane 2). In contrast, truncation of cEts-1Δ5–6, which removes its interacting domain with Pit-1 did not interact with either GST alone or with GST Pit-1 (Fig. SC, lanes 2 and 3). Thus, these studies demonstrate that Pit-1 can physically interact with GATA-2 and may provide a molecular mechanism for their functional cooperativity on the mTSHβ promoter.

**DISCUSSION**

The absolute restriction of thyrotropin β-subunit expression to the pituitary thyro trope suggests that its transcription is governed by an exclusive interaction between the TSHβ gene promoter and cell-specific transcription factors. To date, little information is available about which factors are necessary and sufficient to allow high levels of basal transcription in these cells. Our observations define the participation of two different classes of transcription factors to synergize on a closely spaced response element within the proximal P1 promoter region and demonstrate a functional ternary protein-DNA complex involving the pituitary-specific factor, Pit-1. Such composite elements have been described for a number of interacting transcription factors in other systems (53–55) and provide a mechanism for their combinatorial action within a regulatory network. The analysis of the mTSHβ gene described in this report provides evidence for the participation of a zinc finger factor, GATA-2, with a POU homeodomain partner, Pit-1, on a such a composite element and may be a general mechanism in which other GATA family members can functionally and physically interact with other homeodomain partners.

A number of lines of evidence point to the fact that the pituitary-restricted factor, Pit-1, is necessary but not sufficient to allow basal transcription of the mTSHβ gene. Pit-1 is expressed in thyrotropes, lactotropes, and somatotropes of the anterior pituitary (13, 14), while TSH expression is restricted solely to the thyrotrope cell population. While it is clear that Pit-1 is critical for thyrotrope ontogeny, the role of Pit-1 in governing TSHβ gene expression in thyrotropes is less well understood. Several reports have shown that when using a transfection reconstitution system, Pit-1 alone fails to transactivate TSHβ promoter activity using 1.2 kb of 5’-flanking DNA in either pituitary or nonpituitary cells (9, 56). In contrast, a marked enhancement of TSHβ promoter activity resulted when other pituitary factors were cotransfected with Pit-1 such as P-lim (57) or a unique thyrotrope-specific Pit-1 isoform, termed Pit-1‘ (58, 59). However, the role of P-lim in TSHβ activation is not clear, since it is also present in GH3 somatomammotrope cells, which do not express endogenous TSHβ. It is unlikely to interact with the P1 region, which we demonstrated to be of importance in the present study, since P-lim clearly does not bind to the −120 to −60 region, which contains the P1 region, unless the LIM domain was deleted (57). Moreover, such a truncated form of this protein lacking the LIM domain has not been demonstrated in thyro tropes. Thus, it is unlikely that P-lim accounts for the extended footprint found with thyro tropic nuclear extracts. In this report, we provide evidence that GATA-2 is the predominant GATA factor in thyro tropes cells, can functionally synergize with Pit-1 on juxtaposed sites in the P1 region, and in vitro can form a protein-protein interaction. This combinatorial interaction likely participates in TSHβ gene expression in thyro tropes.

Synergistic interaction involving Pit-1 with other transcription factors on a variety of pituitary hormone genes has also been reported. On the growth hormone gene promoter, Pit-1 has been shown to synergize with Zn-15 (60), the thyroid hormone receptor (61), CREB (62), and P-OTX (63). Additionally, Pit-1 can form functional partners with Ets-1 (64–66), the estrogen receptor (67, 68), P-lim (57), Oct-1 (69), and P-OTX (63) on the prolactin gene promoter and with NZF-1 on the Pit-1 enhancer/promoter (70). The sites of synergy with Pit-1 have been examined for several of these interactions. Synergy with Zn-15 grossly mapped to the amino-terminal transactivation domain of Pit-1 (60), while a smaller Pit-1 deletion, lacking amino acids 72–100, eliminated synergism with thyroid hormone receptor without affecting independent transcriptional activation on the rat growth hormone gene (61). In contrast, the site of interaction of Pit-1 with the estrogen receptor mapped to amino acids 45–72 (71), while the interaction with Oct-1 mapped to the POU homeodomain, consisting of amino acids 210–273 (69). However, levels of expressed protein from these Pit-1 mutant constructs compared with wild type Pit-1 were not reported, and loss of function could be due to inefficient expression. Thus, Pit-1 may contain or provide a number of distinct domains, each of whose function or accessibility is dependent on the specific cis-acting promoter sequence and its synergistic transcription partner. Future studies will define the synergistic domains of Pit-1 with GATA-2 on the TSHβ promoter, as well as the specific sites of protein-protein interaction. Such factor interactions with Pit-1 may represent a more general mechanism whereby a tissue-restricted homeodomain factor can recruit widely expressed factors to a particular composite element and integrate high levels of tissue-specific gene expression, as has been proposed by Gutierrez-Hartmann’s group (64, 65).

The GATA family of transcription factors are also somewhat tissue-restricted in their expression. GATA-1 was the first member of the family to be discovered, and its expression is generally restricted to erythroid cells, mast cells, and megakaryocytes, where it binds to a consensus sequence (A/T)GATAAG by virtue of its two zinc finger domains and plays a pivotal role in erythropoiesis (72). GATA-2 is expressed in a wide variety of tissues; GATA-3 is most abundantly expressed in T lymphocytes, endothelial cells, and in the developing nervous system; GATA-4 is generally restricted to the heart and gonads (32). Recently, Steger et al. (30) have demonstrated that GATA transcription factors play a role in pituitary α-subunit gene expression. They detected GATA-2 and a mouse GATA-4-related protein in gonadotrope-derived αT3 cells and showed that both GATA-2 and -3 can activate α-subunit gene promoter.
activity 3-fold. The GATA site responsible for this activity mapped just upstream of the cyclic AMP-responsive site within the proximal 180 bp of the 5' flanking region. In TSHβ-expressing thyrotropic tumor cells, a majority of the GATA transcripts were GATA-2, since the pattern of hybridization was identical when using a GATA-2-specific probe or with a probe only containing the conserved DNA-binding domain. Additionally, using a common DNA-binding domain probe from mGATA-3 in a cDNA library screening, all of the clones we isolated contained GATA-2 sequences. There was, however, a low but detectable level of GATA-3 expression in these cells, and we cannot rule out its contribution in activating the TSHβ promoter. However, we failed to detect any GATA-4 transcripts in TtT-97 thyrotropes, although a nonabundant GATA-4 transcript was detected in non-TSHβ-expressing GH3 cells. Finally, it is interesting to speculate that GATA-2 may also transactivate the α-subunit gene promoter in thyrotrope cells where it may coordinately regulate both subunits of thyrotropin.

Two transcripts for GATA-2 have also been reported for the human protein. Structural analysis of human genomic DNA clones encoding GATA-2 coupled with RNA blot analysis demonstrated that the observed molecular heterogeneity is due to alternative use of two polyadenylation consensus sequences, which are 612 bp apart within exon 6 (73). The sizes of the mouse GATA-2 transcripts that we detected (3.7 and 3.1 kb, Fig. 2) in thyrotrope RNA also differ by 600 nucleotides and are also most likely the result of alternative polyadenylation sites. It is of interest that αTSH cells, a thyrotopo-pluripotent cell line that expresses the α-subunit gene but not TSHβ, contains transcripts for GATA-2 and GATA-3. However, although this cell line contains Pit-1 transcripts, Pit-1 protein was not detected (9). On the other hand, GH3 cells, which express growth hormone and prolactin but not TSHβ, contain Pit-1 transcripts and protein but lack transcripts for GATA-2. Moreover, the 50-kDa protein, identified as GATA-2 in the present study, was not detected in GH3 extracts by Western blot analysis with the proximal P1 probe (10). These data suggest that both factors must be present in the same cell for TSHβ gene expression. Studies are ongoing in our laboratory to stably express mutated levels of Pit-1 protein in αTSH cells or GATA-2 in GH3 cells and determine whether endogenous TSHβ expression can then be detected. Of course, additional factors that interact with GATA-2 and/or Pit-1 may also be required for full TSHβ reconstitution.

The role of GATA-2 in pituitary thyrotrope development has yet to be determined. Recently, GATA-2 has been shown to be essential for normal mouse development. The targeted disruption of the GATA-2 gene resulted in the death of the embryos by embryonic day 10 or 11. This was due to a severe anemia that results from a severe deficiency of red blood cells (74). Unfortunately, thyrotops are not a model in which to develop a critical role that GATA-2 plays in the regulation of genes controlling growth factor responsiveness or the proliferative capacity of early hematopoietic cells (74). Unfortun-
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