Pit-1/GHF-1 Binds to TRH-Sensitive Regions of the Rat Thyrotropin β Gene†

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ABSTRACT: Three regions within the 5′-flanking region of the TSHβ gene have A-T-rich sequences which have sequence similarity to binding sites for the pituitary-specific POU domain transcription factor Pit-1/GHF-1. These three regions have been termed TSH A (–274 to –258 bp), TSH B (–336 to –326 bp), and TSH C (–402 to –384 bp). TSH A and TSH C are able to confer 2–6-fold TRH stimulation to the heterologous viral thymidine kinase (tk) promoter in transient expression assays in GH_3 pituitary cells; TSH C can confer a 3–10-fold increase in basal enhancer activity as well. TSH A, B, and C DNAs all bound Pit-1 from GH_3 cell nuclear extracts, based on gel mobility shift analysis in which antibody against Pit-1 prevented the formation of specific DNA–GH_3 nuclear protein complexes. TSH A and TSH C also each formed several additional DNA–nuclear protein complexes which were not observed with TSH B. Some of these complexes may contain Pit-1 as their formation was inhibited by the addition of Pit-1 antibody; other complexes, however, were not altered by antibody treatment. All three A-T-rich elements bound *in vitro* translated Pit-1, with calculated affinities of 360 (A), 125 (B), and 38 (C) nM, respectively. In order to test the ability of Pit-1 to transactivate the TSHβ gene, luciferase reporter constructs containing either the homologous rTSHβ gene promoter and 5′-flanking region or synthetic TSH gene elements fused to the heterologous HSV thymidine kinase (tk) promoter were transfected into 293 cells which lack Pit-1. Cotransfection of a Pit-1 expression vector with TSHβ–luciferase constructs into 293 cells increased homologous TSHβ promoter activity 3–10-fold, indicating that Pit-1 could transactivate the gene. Chimeric tk–TSH A–luciferase construct expression was not stimulated in Pit-1 cotransfection experiments; however, tk–TSH C–luciferase activity was stimulated up to 10-fold. These data suggest that Pit-1 may play a role in the basal and TRH-stimulated expression of the rat TSHβ gene.

The hypothalamic peptide TRH is important for thyrotropin (TSH)[1] secretion and synthesis (Hershman & Pekar, 1985; Shupnik et al., 1989), and the continuous expression of the TSH β- and α-subunit mRNAs (Franklyn et al., 1986; Taylor et al., 1990; Murakami et al., 1991). Treatment of rat pituitary cell cultures with TRH resulted in 2–6-fold stimulation of the transcription rate of both subunit genes, with more dramatic effects observed with the TSH β subunit (Shupnik et al., 1986). Gene-transfer experiments in which the 5′-flanking region of the rat TSHβ gene was fused to reporter genes and transfected into either GH_3 or normal pituitary cells have demonstrated that the upstream portion of the gene conferred a stimulatory TRH response (Carr et al., 1989). Mutation deletion experiments indicated that the TRH-sensitive region of the gene lies at least partially between –520 and –204 bp upstream of the major transcriptional start site (Shupnik et al., 1990). Within this TRH-responsive area of the gene are three DNA regions with sequence similarity to binding sites for the pituitary-specific transcription factor Pit-1/GHF-1 (Bodner et al., 1988; Ingraham et al., 1988), hereafter referred to as Pit-1. Two of these regions, termed TSH A (–274 to –258 bp) and TSH C (–402 to –384 bp), can confer a TRH, cAMP, or kinase C-stimulated response to a heterologous promoter in transient expression assays in GH_3 cells; the TSH C region can confer basal enhancer activity as well (Shupnik et al., 1992). The TSH B region does not confer a significant TRH response or enhancer activity. The gene regions defined by TSH A and TSH C appear to bind to some similar and some unique nuclear proteins, based on gel retardation assays and cross-competition experiments, although with somewhat different calculated affinities (Shupnik et al., 1992). At least one of these common DNA–protein complexes appears to be pituitary cell-specific. Interruption of the A-T-rich regions of either TSH A or TSH C with T-to-G substitutions abolished the TRH stimulation conferred by the sequences, diminished the enhancer activity of TSH C, and decreased the affinity of binding of the DNA sequences to GH_3 cell nuclear proteins. We have begun to identify these nuclear proteins, with the ultimate goal of determining their role in basal and hormone-stimulated gene expression.

Several laboratories have demonstrated that Pit-1 binding sites in the rat prolactin (PRL) gene play an important role in both basal and hormone-stimulated expression of the gene in lactotropes (Day & Maurer, 1989; Mangalam et al., 1989; Fox et al., 1990; Yan et al., 1991). There is increasing evidence to suggest that Pit-1 and Pit-1 homologues may play a role in physiological TSH expression as well. Pit-1 mRNA and protein have been identified in the thyrotropes of mature normal mice and rats (Crenshaw et al., 1989; Simmons et al., 1990), and Pit-1 gene mutations in dwarf mice result in the

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[1] Abbreviations: TSH, thyrotropin; rTSHβ, rat thyrotropin β subunit; hTSHβ, human thyrotropin β subunit; PRL, prolactin; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DNA, deoxyribonucleic acid; SDS, sodium dodecyl sulfate; bp, base pair(s); K_D, dissociation constant of binding; mM, millimolar; kDa, kilodalton(s); CREB, cyclic AMP response element binding protein; T, thymidine; A, adenosine; G, guanidine; dI-dC, deoxyinosine-deoxycytidine.

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loss of thyrotropes as well as somatotropes and lactotropes (Li et al., 1990). A recent study has also demonstrated that mouse thyrotropic tumor cells contain Pit-1 as well as an alternatively spliced form of Pit-1 and that bacterially expressed Pit-1 binds to several sites on the mTSHβ gene (Haugan et al., 1992). The human TSHβ gene contains Pit-1 binding sites, and cotransfection of TSHβ reporter gene constructs and mutated Pit-1 expression vectors producing truncated proteins suppressed TRH stimulation of human TSHβ gene transcription (Steinfelder et al., 1992). Deletions or mutations in the human Pit-1 gene result in a loss of thyrotropes and TSH deficiency (Pfaffle et al., 1992; Radovick et al., 1992), and can even result in clinical cretinism. In these studies, we examined the ability of three regions in the rat TSHβ gene, including two TRH-sensitive regions, to bind to Pit-1 and for Pit-1 to alter the transcriptional activity conferred by these sequences.

MATERIALS AND METHODS

Gel Retardation Assays. Gel retardation assays were performed with labeled TSH DNA and either GH_3 nuclear protein extracts or cell-free translated Pit-1 protein. Nuclear proteins from clonal cell lines were prepared by the method of Dignam et al. (1983). To prepare *in vitro* translated Pit-1 protein, the coding sequence for Pit-1 was subcloned into the pGEM3 vector. RNA was transcribed with SP6 polymerase, and translated in nuclease-treated rabbit reticulocyte lysate from Promega Corp. (Madison, WI). TSHβ gene regions were labeled with [^{32}P]dCTP and the Klenow fragment of *Escherichia coli* DNA polymerase. Protein interactions with TSHβ gene regions were assayed using the nondenaturing Tris–acetate–EDTA gel system at 4 °C as previously described (Shupnik et al., 1992). Each binding reaction of 10–15 μL contained 20 000 cpm of labeled double-stranded oligonucleotide representing specific gene sequences, 6.7 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 2 μg of poly(dI·dC)-poly(dI·dC) (Pharmacia, Piscataway, NJ), 100 mM KCl, and either 10 μg of cellular nuclear protein extract or 3 μL of lysate (programmed or unprogrammed with Pit-1 mRNA) which contained approximately 5 μg of protein and 5–7 fmol of Pit-1 as estimated by [^{35}S]methionine incorporation. Lysates programmed with Pit-1 mRNA synthesized two [^{35}S]-methionine-containing peptides, a major form of approximately 32 kDa and a minor form of 26-kDa molecular mass as estimated by denaturing gel electrophoresis. Incubations containing reticulocyte lysate also contained 25 ng of sheared salmon sperm DNA. The final KCl concentration of the reactions, including that contributed by protein extracts or lysates, was adjusted to 100 mM. DNA and proteins were incubated at room temperature for 20 min prior to electrophoresis.

For competition experiments, unlabeled competitor oligonucleotide, proteins, and poly(dI·dC)-poly(dI·dC) were preincubated at room temperature for 10 min prior to addition of labeled DNA, and the entire reaction was incubated for an additional 20 min. For quantitation, samples were subjected to electrophoresis, and gels were analyzed by autoradiography to visualize DNA migration. Free and bound DNA was excised from the gels and quantitated directly by scintillation spectroscopy. For competition with homologous oligonucleotide, the affinity of binding was estimated by Scatchard analysis as described (Shupnik et al., 1992).

Pit-1 Antibody Experiments. In experiments to examine specific Pit-1–DNA interactions, labeled DNA, protein, and antisera to Pit-1 (1 μL of a 1:20 dilution) were incubated on ice for 1 h prior to electrophoresis. Preimmune serum (normal rabbit serum, or NRS) was incubated with some reactions as a negative control at the same concentration as Pit-1 antisera. Antibody to the Pit-1 protein was graciously provided by Dr. Richard A. Maurer (University of Iowa, Iowa City, IA), and was raised in rabbits against the purified GST–Pit-1 fusion protein made in *E. coli*. The polyclonal antiserum is of high affinity and is monospecific for Pit-1 as evidenced by the immunoprecipitation of a single protein band corresponding to the 32–33-kDa Pit-1 protein from nuclear extracts of GH_3 cells or from extracts of Sf9 insect cells infected with a recombinant baculovirus producing Pit-1 (R. A. Maurer, personal communication).

Expression Vector Constructs. To measure transcription from the homologous rat TSHβ gene promoter, an upstream portion of the TSHβ gene including 520 bases of 5′-flanking region, 27 bases of the first exon, and 150 bases of the first intron was made blunt-ended and fused to the luciferase coding region in a promoterless luciferase construct at the *Hinc*II site to form TSHHPLUC (Shupnik et al., 1992). This *Hinc*II–*Pst*I fragment of the gene contained both transcriptional start sites, and was shown to contain the TRH-responsive region of the gene in transient expression assays (Shupnik et al., 1989). For analysis of specific TSHβ gene regions, oligonucleotides spanning the regions were chemically synthesized with *Bam*HI ends and inserted into the *Bam*HI site of the reporter construct next to the herpes simplex virus tk promoter from −105 to +65 bp which was fused to the firefly luciferase coding sequence as previously described (Shupnik et al., 1992). Oligonucleotides synthesized included the region from −274 to −258 bp relative to the major transcriptional start site, termed TSH A (GAGAAGATATTAATGC), a mutated version of this area termed TSH AX (GAGAAGATA-GGAATGC), the region from −336 to −326 bp termed TSH B (CCATATAAAT), the region from −402 to −384 bp termed TSH C (TTTATAGATGAATAATTA), and the mutated version of that region termed TSH CX (TTGATAGAT-GAATGGTTA). All constructs were verified by the dideoxy sequencing method of Sanger et al. (1977) and contained a single copy of the relevant oligonucleotides.

For cotransfection experiments, an expression vector for Pit-1 was obtained from Dr. Richard N. Day (University of Virginia, Charlottesville, VA) which contained the coding region of Pit-1 inserted next to the cytomegalovirus (CMV) promoter to form a CMV–Pit-1 construct (Iverson et al., 1990). Expression vectors for c-jun and c-fos were obtained from Dr. Tom Curran (Hoffmann-La Roche, Nutley, NJ), and contained the coding regions for those proteins inserted downstream of the CMV promoter (Sonnenberg et al., 1989).

Transient Expression Assays. To determine if the transcription factor Pit-1/GHF-1 might play a role in rat TSHβ gene expression, cotransfection experiments were performed in 293 cells, a human fetal cell line which does not contain Pit-1 (Graham et al., 1977). Cells were maintained in Dulbecco's minimal essential media containing 10% fetal calf serum, and were transfected by the calcium phosphate procedure (Graham & van der Eb, 1973). Under these media conditions, T_3 concentrations were less than 0.05 nM, and have a minimal effect on basal and stimulated TSHβ promoter activity (Shupnik et al., 1989). For cotransfection experiments, each well was transfected with 10 μg of TSHHPLUC as described above, or 10 μg of tkLUC constructs containing specific TSHβ gene region oligonucleotides, together with 1 μg of CMV–Pit-1, CMV–c-jun, or CMV–c-fos, 0.1 μg of CMV–β-galactosidase for normalization, and sufficient PUC19

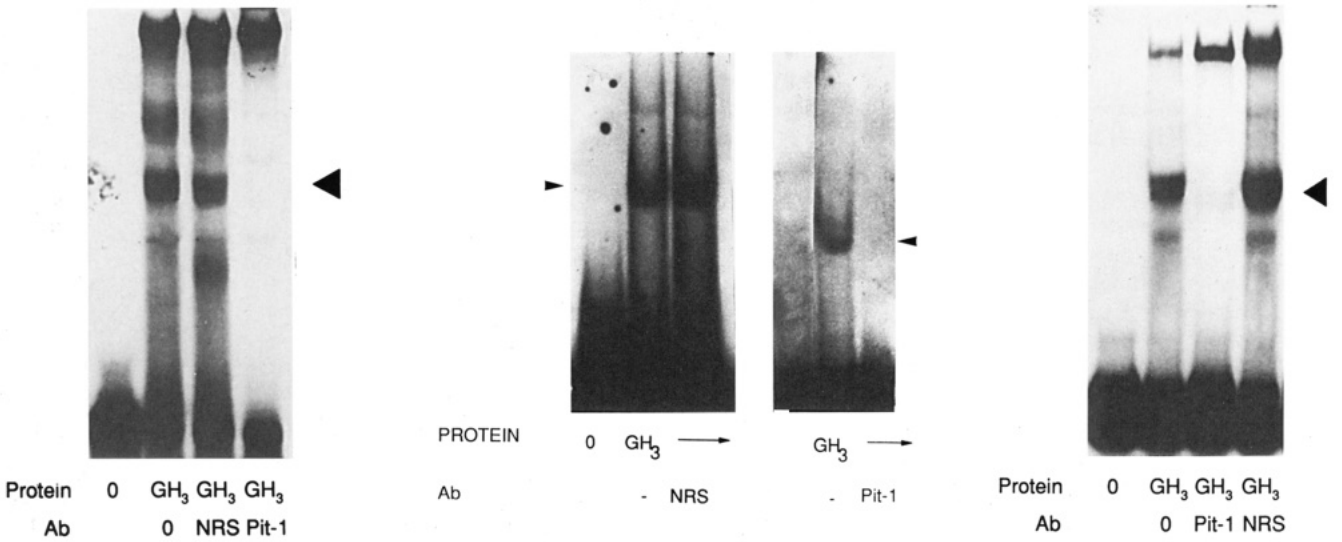

FIGURE 1: Analysis of TSH gene regions binding to GH_3 nuclear proteins and di :uption of specific DNA–protein complexes with antisera to Pit-1. Labeled DNA representing specific TSH β gene regions TSH A (left) TSH B (middle), and TSH C (right) as described under Materials and Methods was incubated with GH_3 cell nuclear proteins (10 μg) in the absence or presence of antibody (Ab) directed against Pit-1 protein (Pit-1) with preimmune normal rabbit serum (NRS). After incubation on ice, reactions were subjected to electrophoresis on a 5% acrylamide nondenaturing gel, along with a sample of DNA alone (0 protein). Labeled DNA which formed complexes with the proteins was retarded or shifted upward into the gel. The pituitary-specific complex disrupted by Pit-1 antibody with all three DNAs is designated by an arrowhead in each panel.

plasmid to standardize all DNA levels to 15 μg. The DNA precipitates were allowed to remain on the cells overnight. Cells were then washed with phosphate-buffered saline, and incubated for an additional 24 h in media prior to collection for luciferase, β-galactosidase, and protein assays by standard techniques (de Wet et al., 1987; Shupnik et al., 1990). Values are given as arbitrary light units of luciferase activity normalized for 100 μg of protein and transfection efficiency (β-galactosidase levels), and are presented as the mean ± SEM.

RESULTS

In order to determine if Pit-1 could bind to the TRH-sensitive regions of the rTSHβ gene, gel shift or retardation experiments using pituitary cell nuclear extracts or *in vitro* translated Pit-1 were performed. Previous studies from our laboratory demonstrated that the two TRH-responsive DNA elements, TSH A and TSH C, bound several nuclear proteins from GH_3 pituitary cells based on the formation of several DNA–protein complexes in gel shift assays (Shupnik et al., 1992). The nonresponsive element TSH B formed only one major nuclear protein–DNA complex in these same studies. Figure 1 demonstrates the results from gel shift studies in which ^{32}P-labeled double-stranded DNA, representing TSH A, B, or C, was incubated with GH_3 cell nuclear proteins in the presence or absence of antibody to Pit-1 (Pit-1) or normal rabbit serum (NRS). TSH A forms three to four major complexes with GH_3 cell nuclear extracts. We have previously shown the upper complex to be present with extracts from several different cell types including 293 and MCF7 cells. This complex appears identical to the upper complex formed with TSH C, and is not affected by the addition of Pit-1 antibody. The lower DNA–protein complex (at arrowhead) has identical mobility to the lowest TSH C DNA–GH_3 protein complex, and is eliminated by Pit-1 antibody treatment but not by the addition of normal rabbit serum. This complex corresponds to the pituitary-specific complexes formed with TSH A, B, and C in previous studies (Shupnik et al., 1992). The intermediary complex is also pituitary cell-specific and appears only with TSH A, but not with TSH C or TSH B. This complex also is eliminated by Pit-1 antibody treatment, suggesting that it may contain Pit-1. Heating the nuclear proteins to 65 °C prior to gel shift analysis (not shown) eliminates all three major shifted complexes, with only minor bands still apparent, migrating above and below the major complex at the arrowhead. Results with heat treatment suggest these major complexes do not contain the cAMP-sensitive CREB or ATF-2-like proteins which are resistant to heat (Hoeffler et al., 1991). Similar results were obtained with TSH C, with the major common complex at the arrowhead eliminated specifically by Pit-1 antibody. TSH B only formed one major DNA–protein complex, with a more slowly migrating complex occasionally present as well. This complex, on the basis of its mobility, was similar to those formed with TSH A and TSH C and was eliminated by the addition of Pit-1 antibody but not NRS. Since only TSH A and TRH C are TRH-responsive (Table I), further experiments concentrated on these DNA elements.

In the second set of experiments, the ability of *in vitro* translated Pit-1 protein to bind to TSHβ gene regions was examined. Pit-1 mRNA was transcribed from a pGEM plasmid containing the Pit-1 coding sequence, and Pit-1 mRNA translation in nuclease-treated rabbit reticulocyte

Table I: Comparison of rTHSβ Promoter and DNA Element Enhancer Activity by Transient Expression Analysis in GH_3 or 293 Cells[a]

	GH_3 cells		293 cells
	control	TRH	control
TSH HPLUC	18.4 ± 5.0	80.9 ± 15.4[b]	4.1 ± 1.7
tKLUC	20.2 ± 6.0	21.6 ± 8.1	24.7 ± 4.9
TSH A LUC	20.8 ± 4.9	63.4 ± 9.5[b]	5.0 ± 1.4
TSH AXLUC	20.3 ± 5.1	22.1 ± 6.5	4.6 ± 1.2
TSH B LUC	16.5 ± 4.3	15.6 ± 1.1	4.3 ± 0.8
TSH C LUC	106.1 ± 9.8	278.9 ± 21.7[b]	2.6 ± 0.3
TSH CX LUC	33.0 ± 2.4	34.1 ± 3.5	4.9 ± 0.9

[a] GH_3 pituitary cells and 293 fetal kidney cells were transfected with luciferase construct driven by the rTSHβ gene promoter (TSH HPLUC) or the tk promoter as described under Materials and Methods in the same experiment. Some GH_3 cells were treated with 1 nM TRH for the last 16 h of the 48-h transfection period. Luciferase activity is expressed as light units corrected for β-galactosidase activity and normalized for 100 μg of cellular protein for four samples per group. [b] $p < 0.05$.

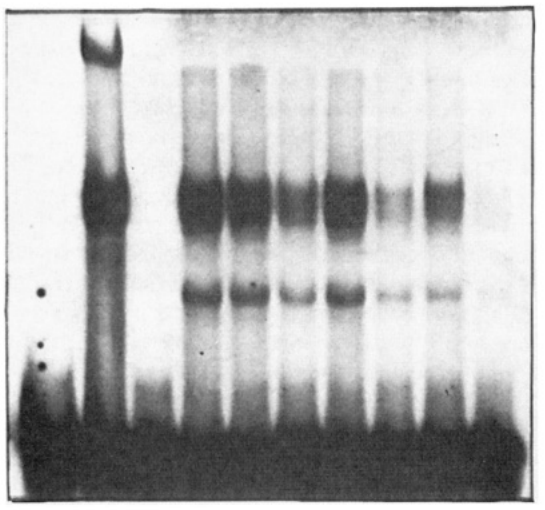

FIGURE 2: Direct binding of the TSH C gene region to *in vitro* translated Pit-1 protein by gel retardation analysis. A double-stranded oligonucleotide corresponding to the TSH C region was labeled and incubated with either 10 μL of GH_3 cell nuclear protein (GH_3), 3 μL of unprogrammed rabbit reticulocyte lysate (Lys), or 3 μL of lysate programmed with Pit-1 mRNA (Pit-1). Some incubations also contained the indicated concentrations of unlabeled double-stranded oligonucleotides representing the TSH A (A), TSH B (B), or TSH C (C) regions. Incubations were carried out for 20 min at room temperature prior to electrophoresis on a 5% acrylamide nondenaturing gel.

lysate was performed. In Figure 2, the binding of labeled TSH C to *in vitro* translated Pit-1 and GH_3 cell nuclear proteins was compared. No binding of TSH C was observed with reticulocyte lysate alone. In the presence of *in vitro* translated Pit-1, TSH C formed one major DNA–protein complex identical to the GH_3–protein complex designated by the arrowhead in Figure 1 and in Figure 2, as well as a second more rapidly migrating complex probably corresponding to a slightly smaller Pit-1 product produced in the *in vitro* translation reaction. The major complex corresponds in mobility to the pituitary cell-specific complex formed with all three TSHβ gene elements with Pit-1 binding site homology, and which was inhibited from forming by the addition of Pit-1 antibody (Figure 1). Unlabeled TSH A, B, and C could all compete with labeled TSH C for binding to Pit-1, but with widely varying effective concentration ranges; TSH C competed most effectively, and TSH A competed least effectively.

To more carefully calculate the affinity of Pit-1 binding to TSHβ gene regions, labeled DNA was incubated with increasing concentrations of unlabeled homologous oligonucleotide and Pit-1 prior to electrophoresis (Figure 3). Bound and free labeled DNA was quantitated directly from the gels by scintillation spectroscopy, and the affinity of binding was calculated. Both major and minor complexes exhibited similar binding characteristics in all cases. When three similar experiments were averaged, TSH A bound to Pit-1 with a calculated affinity fo 360 ± 90 nM, while the mutant oligonucleotide TSH AX did not compete effectively for binding at 500 nM (20% competition). TSH C bound to Pit-1 with a much higher calculated affinity of 38 ± 11 nM. In several experiments, the mutant oligonucleotide TSH CX had a diminished affinity for Pit-1 compared to TSH C (365 ± 36 nM), but binding still occurred. In a single experiment

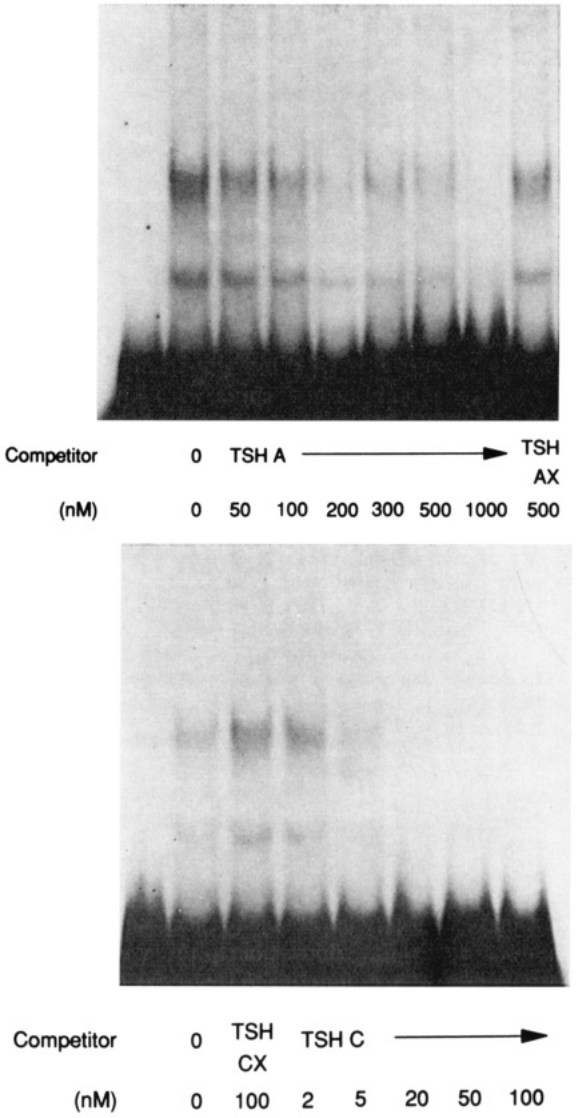

FIGURE 3: Binding of Pit-1 to TSH A (top) and TSH C (bottom) DNA by gel retardation analysis and competition of binding by unlabeled oligonucleotides. Double-stranded oligonucleotides representing the TSH A (upper panel) or TSH C (lower panel) elements of the rTSHβ gene were labeled, and 10 fmol of DNA was incubated with *in vitro* translated Pit-1 before electrophoresis on a nondenaturing gel as described under Materials and Methods. Some reactions also contained the indicated concentrations of unlabeled competitor oligonucleotides representing the TSH A or TSH C regions, or the mutated oligonucleotides TSH AX or TSH CX.

(not shown), TSH B bound Pit-1 with an intermediate affinity of 125 nM. Thus, all three TSHβ gene regions, including the two TRH-sensitive elements, can bind Pit-1 from GH_3 cell nuclear extracts and can also bind to pure *in vitro* translated Pit-1, although with greatly varying affinities.

In order to investigate the ability of Pit-1 to transactivate the rTSHβ gene, transient transfection experiments were performed in 293 cells, which lack Pit-1. Both the homologous TSHβ promoter and the heterologous tK promoter fused to the luciferase reporter gene were expressed in 293 cells, with the rTSHβ promoter in general expressed at lower levels (Table I). Although calculated luciferase activity varied between experiments, the TSHβ promoter in the TSHHPLUC construct, including 520 base pairs (bp) of the 5′-flanking sequence containing the three Pit-1 binding sites, was expressed at higher levels in GH_3 cells than in 293 cells. In GH_3 cells, but not 293 cells, the TSH C region has a basal enhancer effect which

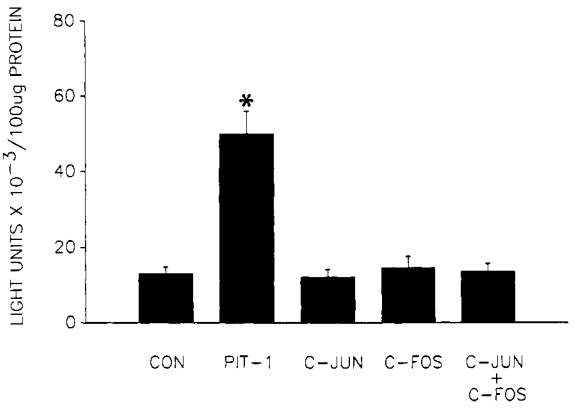

FIGURE 4: TSHβ promoter activity in 293 cells cotransfected with TSH LUC constructs and expression vectors for Pit-1, c-jun, and c-fos. A reporter gene construct containing the rat TSHβ gene promoter region and 520 bases of 5'-flanking region fused to the luciferase coding region (TSH HPLUC) was transfected into 293 cells by the calcium phosphate coprecipitation method. Cells were transfected with TSH HPLUC alone (CON), or in combination with expression vectors containing Pit-1, c-jun, or c-fos, as indicated on the abscissa. After 48 h, luciferase activity was measured in cell lysates, and normalized values for light units are presented as the mean ± SEM for four samples per group. Asterisk: $p < 0.01$.

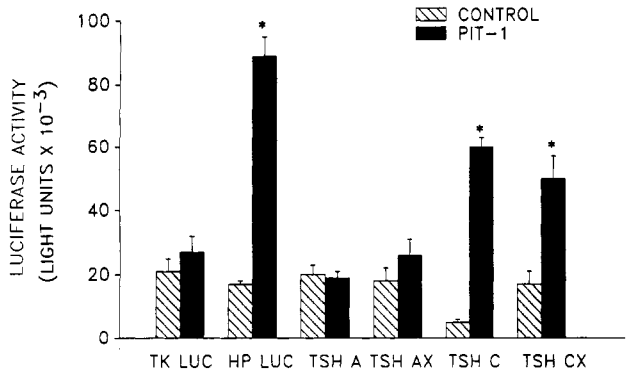

FIGURE 5: Pit-1-stimulated luciferase activity in 293 cells transfected with tkLUC constructs containing either intact or mutated TSHβ gene regions in the absence or presence of cotransfected Pit-1 expression vector. Oligonucleotides defining the TSH A (–274 to –258 bp) or the TSH C (–402 to –384 bp) gene regions or mutated versions of these regions (TSH AX or TSH CX) were inserted next to the tk promoter in tkLUC, and the resulting constructs were named by their insert. These constructs, the parent tkLUC vector, and the HPLUC construct containing the rTSHβ gene promoter and upstream region as in Figure 4 were transfected into 293 cells with and without cotransfected CMV–Pit-1. Normalized luciferase activity per 100 μg of cell lysate protein is expressed as in Figure 4.

is diminished but not obliterated by mutation to TSH CX. To directly test the ability of Pit-1 to transactivate the homologous rTHSβ promoter, TSHHPLUC was transfected into 293 cells simultaneously with expression vectors containing the CMV promoter fused to either Pit-1, c-jun, or c-fos coding sequences (Figure 4). Cotransfection with CMV–Pit-1 conferred a 3–10-fold increase in basal expression to the TSHHPLUC constructs. No increase in TSHβ promoter activity was seen with the cotransfection of the other transcription factors, illustrating the specificity of Pit-1 on transcription of the rTSHβ gene.

The ability of Pit-1 to confer a response to specific regions within the rTSHβ gene's 5'-flanking region was then studied (Figure 5). Luciferase activity in constructs containing either the heterologous promoter thymidine kinase (tk) or the rTSHβ promoter and upstream regions (HPLUC) was compared to that in constructs containing the tk promoter fused to TSH gene regions A or C or the mutated sequences AX or CX. These constructs were cotransfected with the Pit-1 expression vector into 293 cells. Pit-1 expression conferred increased basal activity to the TSHHPLUC construct containing the rTSHβ promoter, but not to the heterologous promoter tk. Pit-1 expression did not increase activity from TSH A and AX containing constructs. Constructs containing TSH C exhibited low basal activity which increased 10–12-fold with the addition of Pit-1. In comparison, constructs containing TSH CX (a mutated version with a disrupted A-T-rich region) had higher basal activity than those with TSH C. Pit-1 expression stimulated TSH CX luciferase constructs slightly (2–3-fold), but the final level of stimulated TSH CX luciferase activity was not as high as TSH C constructs in the presence of Pit-1. These studies suggest that Pit-1 can transactivate the rTSHβ gene, possibly via the TSH C region, at least given the set of basal transcription factors present in 293 cells.

DISCUSSION

Pit-1 has previously been shown to be critical to basal pituitary hormone gene expression, and to regulate prolactin gene expression as well. The importance of Pit-1 in thyrotrope function has been dramatically demonstrated by naturally occurring mutations. Snell dwarf mice have hypoplastic pituitaries lacking thyrotropes, lactotropes, and somatotropes, the pituitary cell types synthesizing TSH, prolactin, and growth hormone, respectively (Roux et al., 1982). The genetic defect in Snell mice has been identified as an autosomal recessive mutation of the Pit-1 gene. A single G to T point mutation in the region encoding the homeodomain of the protein results in an altered amino acid sequence which critically disturbs the protein's ability to bind DNA, and with ultimate abolition of normal pituitary development (Li et al., 1990). Pit-1 mutations in humans have resulted in low TSH levels and loss of thyrotrope function (Pfaffle et al., 1992; Radovick et al., 1992). The vital role of Pit-1 in rodent thyrotrope development and the detection of Pit-1 in mature normal thyrotropes (Crenshaw et al., 1989; Simmons et al., 1990) and in a thyrotropic tumor (Haugan et al., 1992) raises the possibility of its role in the transcriptional regulation of the thyrotrope's most important product, TSH.

Our data demonstrate that pure Pit-1 binds to the rat TSHβ gene in a sequence-specific fashion in at least three regions, with varying affinities of 38, 125, and 360 nM for TSH C, B, and A, respectively. The affinities calculated with *in vitro* translated Pit-1 are similar to those previously calculated for the DNA–GH_3 nuclear protein complexes of identical mobility found to contain Pit-1 (K_Ds for TSH C = 23 nM, TSH B = 69 nM, TSH A = 108 nM). It is of interest to note that the region with enhancer activity, TSH C, also has the highest affinity of binding to Pit-1. The gene regions differ in their biological responsiveness, with TSH C conferring basal enhancer activity and TRH, kinase C, and kinase A (cAMP) responsiveness and with TSH A conferring a transcriptional response to TRH, kinase C, and kinase A with no enhancer function. The TSH B region conferred neither enhancer activity nor TRH sensitivity, but it could be stimulated by cAMP, suggesting that it does have functional importance (Shupnik et al., 1992). Although the TSH A region does not appear to confer enhancer activity or to be transactivated by Pit-1 (Figure 5), a DNA region of similar sequence and location in the mouse TSHβ gene binds a thyrotrope-specific protein, and the region has been suggested to be important for cell-specific expression rather than hormonal stimulation (Alexander et al., 1989).

The hTSHβ promoter can be transactivated by Pit-1 in 293 cells (Steinfelder & Wondisford, 1992), and some potential Pit-1 binding sites in the human gene have been described (Steinfelder et al., 1992). Three DNA regions in the hTSHβ gene between −129 and −61 bp relative to the major transcriptional start site have homology to other Pit-1 binding sites, and these regions are required for cAMP stimulation of promoter activity. These regions formed DNA–protein complexes with nuclear extracts from mouse thyrotropic tumors in gel shift assays, and oligonucleotides representing Pit-1 binding sites from the rat growth hormone gene could compete for binding to these proteins (Steinfelder et al., 1992). Recent data from the same investigators (Steinfelder & Wondisford, 1992) suggest the importance of *in vivo* protein modifications in that the binding of Pit-1 to the DNA was enhanced by phosphorylation of the protein. Such modifications can occur via kinase C or cAMP actions (Kapiloff et al., 1991), and TRH has been postulated to act via kinase C (Straub et al., 1990), but whether TRH can phosphorylate Pit-1 and result in changes in TSHβ gene activity is unknown. Treatment of pituitary cells with TRH or kinase C activators does not alter the number or affinity of nuclear protein–DNA complexes formed with TRH-sensitive TSHβ gene regions (Shupnik et al., 1992). However, changes in gene transcription or transactivation upon transcription factor phosphorylation can occur without large changes in the affinity for DNA, as has been noted for CREB (Montminy & Bilezikjian, 1987).

The difference between the ability of various regions within the rTSHβ gene to bind Pit-1 and the resulting biological function demonstrates that there is not a one-to-one relationship between Pit-1 binding and biological activity, and suggests additional factors are involved in specific transcriptional regulation. Consistent with this concept is the fact that rTSHβ promoter activity in 293 cells may be increased by Pit-1 (Figures 4 and 5), but some low level of activity is permitted in the absence of Pit-1. Multiple forms of Pit-1 protein are found in clonal pituitary cell lines (Konzak & Moore, 1992) and in the TtT 97 mouse thyrotropic tumor which expresses TSHβ (Haugan et al., 1992). However, the TSHβ promoter cannot be transactivated by Pit-1 cotransfection into MGH 101A tumor cells which have lost the ability to express the TSHβ gene, suggesting that other transcription factors are needed for complete TSHβ expression (Gordon et al., 1992). Some investigators have suggested that a protein termed TEF, or thyrotroph embryonic factor, may be involved in the initial expression of the mouse TSHβ promoter (Drolet et al., 1991). However, TEF binding sites within the gene are different from those of Pit-1, and TEF expression in the mature animal is not thyrotrope-specific.

The Pit-1 protein itself can interact biochemically and/or functionally with several other transcription factors. It has been demonstrated that Pit-1 and Oct-1, both POU domain proteins, can form heteromeric complexes in the absence of DNA and cooperate to increase PRL promoter activity and transcriptional activity directed by an isolated Pit-1 element (Voss et al., 1991). Both Pit-1 and the estrogen receptor protein and DNA binding sites must be present and functional for estrogen stimulation of the PRL gene (Day et al., 1990). Finally, interaction between Pit-1 and the thyroid hormone receptor has been postulated to occur on the hTSHβ gene on the basis of transient transfection experiments (Steinfelder et al., 1991). Interaction or synergism of Pit-1 with numerous classes of transcription factors has been postulated to be necessary for correct pituitary cell phenotypes (Simmons et al., 1990). We have observed that Pit-1 antibody disrupts several DNA–GH_3 nuclear protein complexes for all three TSHβ gene regions in gel shift assays, including but not limited to the complex formed when *in vitro* translated Pit-1 is used in similar assays (Figure 1). These additional bands may represent heteromeric complexes between Pit-1 and other proteins. None of the major complexes formed contain proteins which are members of the CREB/ATF-2 family, as they are not heat-stable. Furthermore, none of these complexes contain the estrogen receptor, as antibody to that receptor has no effect on the formation of TSH A, B, or C DNA–protein complexes (M. Shupnik, unpublished data).

The involvement or necessity for Pit-1 for the TRH stimulatory response in the PRL gene has been documented. The PRL enhancer region containing Pit-1 binding sites is necessary for TRH responsiveness of the gene (Day & Maurer, 1989), and a single PRL gene Pit-1 binding site is sufficient to confer a TRH-stimulated transcriptional response to a heterologous promoter (Yan et al., 1991). However, not all Pit-1 binding sites in the PRL gene are equally important for basal enhancer activity and stimulatory response to cAMP and kinase C on the basis of linker scanning mutagenesis studies (Iverson et al., 1990). While it has been demonstrated that Pit-1 binds to these elements in the PRL gene, additional DNA binding proteins which bind to these elements or which interact with Pit-1 from adjacent sites have not been completely described or identified.

In comparison, while the TSH A, TSH B, and TSH C regions of the rTSHβ gene all bind Pit-1, the DNA regions differ in their ability to confer transcriptional responses of basal enhancer and hormone-stimulated promoter activity. All three DNA regions bind Pit-1, but with varying affinity, and at least two of the regions (TSH A and TSH C) also bind additional and different nuclear proteins as demonstrated by gel retardation experiments. Both TSH A and TSH C can confer TRH-stimulated transcriptional activity, but only TSH C confers basal enhancer activity. While it is likely that Pit-1 plays a role in basal and hormone-stimulated rTSHβ promoter activity, Pit-1 binding alone is insufficient to explain the range of biological responses conferred by its binding sites in the gene. The additional nuclear proteins which bind to the responsive DNA regions may modulate Pit-1 activity and contribute to the transcriptional response. This may be of particular significance when comparing the basal enhancer activity of TSH C and TSH CX in GH_3 and 293 cells, in the absence and presence of cotransfected Pit-1. TSH CX DNA has much lower affinity of binding for Pit-1 (365 nM vs 38 nM for TSH C) and for other GH_3 nuclear proteins, although it does bind to these proteins. The TSH CX mutation decreases, but does not abolish, the enhancer activity of TSH C in GH_3 cells (Shupnik et al., 1992). It is likely that these other proteins play some role in the transcriptional response, any may have relatively more importance when Pit-1 binding is decreased. Gel shift experiments with 293 cell nuclear proteins and TSH A and TSH C DNA indicate that the most slowly migrating DNA–protein complex with GH_3 nuclear proteins (which does not contain Pit-1) is present with 293 cell proteins as well (M. Shupnik, unpublished data). Thus, 293 cells may contain at least some nuclear proteins similar to those in GH_3 cells, along with unique proteins. In the presence of Pit-1, a different confirmation of protein complexes probably occurs, and may include Pit-1–protein interactions as well as direct interactions with DNA. The resultant protein complexes transactivate the TSH C sequence more efficiently than TSH CX. The identification and characterization of these additional factors will be necessary to fully understand

the transcriptional regulation of the rTSHβ gene, and is the subject of ongoing investigations.

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