Thyroid Hormone Receptor β2 Promoter Activity in Pituitary Cells Is Regulated by Pit-1*

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There are three known thyroid hormone receptor (TR) isoforms that arise from two distinct α and β gene loci. TRα1 and TRβ1 mRNAs are found in many tissues, whereas mRNA for the N-terminal TRβ2 variant derived from the β locus is readily detectable only in the pituitary gland and derived cell sources such as GH3 somatotropes and TtT-97 thyrotropes. We previously isolated the genomic region governing expression of the TRβ2 isoform in thyrotropes and showed that transcription arose from multiple origins within a 400-base pair (bp) region. We now report that the region extending 500 bp upstream of the putative AUG codon (A is +1) contains six areas of interaction with the pituitary-specific transcription factor Pit-1. In addition there are separate areas that bind other factors present in thyrotrope cells. Pitomer deletions revealed that removal of regions containing the Pit-1 sites at −456 to −432, −149 to −127, and −124 to −102 progressively decreased TRβ2 promoter activity in thyrotropes. A more proximal footprinted area from −65 to −19, which accounted for the remaining promoter activity, contained sites that interacted with recombinant Pit-1; however, extracts of TtT-97 thyrotropes, which express Pit-1, footprinted this proximal region with a pattern of protection that differed from that produced by Pit-1. A comparative deletional analysis demonstrated that a shorter region extending only 204 bp from the AUG was sufficient to support TRβ2 promoter activity in GH3 somatotropes. The more proximal Pit-1 sites, including the area from −53 to −19, whose pattern differed from Pit-1 in thyrotrope extracts, showed protection patterns with GH3 extracts that were indistinguishable from recombinant Pit-1. Site-directed mutagenesis that abrogated binding of both recombinant Pit-1 and Pit-1-containing nuclear extracts revealed that the two Pit-1 sites between −149 and −102 were important for TRβ2 promoter activity with the more proximal being most critical. Finally, we showed that TRβ2 promoter activity in α-TSH cells, which do not transcribe the endogenous TRβ2 locus or produce Pit-1 protein, could be reconstituted to a level approaching that seen in expressing TtT-97 thyrotropes by cotransfecting a Pit-1 expression vector. Activation by Pit-1 was dependent on the same Pit-1 sites shown to be important for basal TRβ2 promoter activity in thyrotropes as constructs lacking them by deletion or mutation were not stimulated by Pit-1.

The effects of thyroid hormone (T3) are dependent on its interaction with high affinity nuclear receptor molecules that are related to those that mediate the effects of the steroid hormones, retinoids, and vitamin D (1). Thyroid hormone receptors (TRs)1 arise from two separate genomic loci (α and β) (2). Translation of alternately spliced transcripts from the α locus gives rise to TRα1, a hormone binding isoform that regulates T3-responsive genes and α2, a C-terminal variant, that does not bind T3 (3, 4) and may act as an antagonist of T3 response (5). In contrast, the β locus gives rise to two receptor isoforms (TRβ1 and TRβ2) as a result of transcription directed by two separate promoter regions and subsequent splicing of two different N termini onto the same DNA and hormone binding regions (2, 6). TRβ1 expression is widespread (7), and although TRβ2 immunoreactivity has been reported in a variety of tissues (8), its mRNA is detectable by Northern blot analysis only in the pituitary gland and cell sources derived from it (6, 9). Following its original description in rat GH3 somatotrope tumor cells and demonstration of its restricted expression to the pituitary gland (6), our laboratory cloned TRβ2 cDNA from mouse thyrotropic TtT-97 tumor tissue (9). Childs et al. (10) subsequently demonstrated by in situ hybridization that transcripts encoding TRβ2 colocalized almost exclusively with cells in the pituitary gland that stained for thyroid-stimulating hormone (TSH) and growth hormone (GH). Differentiation of these two pituitary cell types has been shown to be dependent on the transcription factor Pit-1 (11, 12). Because of its restricted expression to the pituitary, we reasoned that the promoter region that regulates transcription of TRβ2 may be under the control of pituitary-specific factors such as Pit-1. We recently cloned a mouse genomic fragment containing the region immediately upstream of the TRβ2 coding region and showed that it exhibited the properties of a promoter by preferentially directing expression of luciferase fusion constructs in TRβ2-expressing TtT-97 cells when compared with non-expressing α-TSH cells (13). The TRβ2 promoter region contained several motifs that could be potential binding sites for Pit-1. This report documents that the region important for TRβ2 promoter activity in thyrotropes does interact with Pit-1 as well as with other proteins present in thyrotropes. We also demonstrate that this genomic region also supports promoter activity in GH3 somatotrope cells and that the activity in both cell types is dependent on the presence of areas that interact with Pit-1. Finally cotransfection experiments with α-TSH cells

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1 The abbreviations used are: TR, thyroid hormone receptor; TSH, thyroid-stimulating hormone; GH, growth hormone; PRL, prolactin; CMV, cytomegalovirus; RSV, Rous sarcoma virus; bp, base pairs.
that express neither TRβ2 nor Pit-1 establish that Pit-1 is capable of reconstituting TRβ2 promoter activity to a level approaching that observed with expressing TtT-97 thyrotropes and that the activation is dependent on the interaction of Pit-1 at specific sites.

MATERIALS AND METHODS

Experimental Animals—TtT-97 thyrotropic tumor propagation and maintenance in hypothyroid male LAF I mice have been previously described (14). All tumor bearing mice used in these studies were treated in accordance with the National Institutes of Health guidelines for animal use and care. All protocols were reviewed and approved by the University of Colorado Health Sciences Center Committee on Use and Care of Animals.

Cell Culture—Monolayer cultures of GH3 cells (ATCC CCL S2.1) or α-TSH cells (15) in suspension were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. To maximize expression of the TRβ2 isofrom, which is down-regulated by thyroid hormone (9), cells were incubated in the same medium containing charcoal-stripped serum that lacked detectable levels of T4 and T3 for 48 h before preparation of nuclear extracts or transfection experiments.

Construction of 5′ Deleted and Mutated TRβ2 Luciferase Fusion Plasmids and Transient Transfection Assays—The TRβ2 promoter luciferase fusion plasmids extending 3′ to +40 and with 5′ deletion points at −2064, −572, −465, −204, and −77 were constructed using convenient restriction sites as described previously (13). Additional deletions with 5′ extents at −152, −121, and −25 were prepared using a polymerase chain reaction-based strategy to generate amplified fragments with these end points. For this purpose the following TRβ2 5′ sense oligonucleotides, with a SalI site incorporated (underlined) to facilitate subsequent subcloning, were synthesized: 5′-GACCCGGGGCTCTGTGGGTTTTATGTTTTTGGCG-3′; and 5′-CAGCCCGGTTAGAAGACTGAGCTCGGAT-3′. Each sense strand primer was used together with a 3′ antisense oligonucleotide (5′-GCCCTTCTTTATGTTTTTGGCG-3′) complementary to a sequence just within the luciferase coding sequence to generate the desired fragments by amplification from the luciferase vector containing the TRβ2 sequence from −572 to +40. Polymerase chain reaction for 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min was performed. Following gel purification and digestion with SalI and HindIII, the new 5′ deleted promoter fragments were inserted between the same sites of the promoterless plasmid pA4LUC (20), and end points were verified by sequencing.

Site-directed mutagenesis of Pit-1 binding consensus sequences was carried out in the context of the −204 to +40 TRβ2 promoter fragment excised from pAβGAL and inserted into pSELECT (Promega, Madison, WI) as described previously (25). Specifically, 32-bp oligonucleotides were synthesized with 12 bp on either side of the AT-rich Pit-1 e or f site, which was altered to a GC-rich NcoI recognition site. Mutagenesis was performed according to the supplier’s instructions described in the Altered Sites System (Promega), and subsequent digestion of miniprep DNA with NcoI aided in screening for the presence of the mutations, which were verified by sequencing. The double mutant (Pit-1 sites e and f) was generated using the oligonucleotide used to mutate the Pit-1 f site, except that pSELECT containing the Pit-1 e mutation was used as the starting plasmid. Mutated TRβ2 promoter fragments were re-excised with KpnI and HindIII and reinserted into pA4LUC between the same sites.

Transient transfection by electroporation was carried out essentially as described previously (14). Specifically, 20 μg of TRβ2 promoter-luciferase plasmid DNA, together with 1–3 μg of pCMV-β-gal (Clontech, Palo Alto, CA) as an internal transfection efficiency control, were transfected into 5–10 million freshly dispersed TtT-97 tumor cells or 5 million GH3 cells. An RSV-directed luciferase vector (pA4RSV400LUC) was transfected in parallel allowing comparison of TRβ2 promoter activity between different cell types. Following incubation at 37 °C for 18–24 h, cells were harvested and freeze-thaw extracted, and supernatants were assayed for both luciferase and β-galactosidase as previously reported (19). For experiments where the effect of exogenously supplied Pit-1 was assessed, 3 million α-TSH cells were transfected with the TRβ2UC and β-galactosidase plasmid DNAs as above and replaced with 20 μg of pCMVβgal-1 or the same vector lacking the Pit-1 cDNA sequence (pCMV), both of which were constructed as outlined in a previous report (21).

Preparation of Nuclear and Bacterial Extracts, Probe Production, and DNase I Protection Analysis—Nuclear extracts were prepared from enzymatically dispersed TtT-97 thyrotropic tumors, α-TSH and GH3 cells as described previously (16, 17). Bacterial extracts expressing recombinant Pit-1 protein with a fusion with g-glutamyl transferase were prepared according to a previously published procedure (18). Probes corresponding to the TRβ2 promoter region from −572 to −77, from −210 to +102 and from −77 to +176 were prepared by cloning end-filled SpeI to NcoI, TaqI to HpaII, and end-filled NcoI to HindII fragments into the Smal, BstBI, and Smal sites of pGEM7zf+ (+), respectively. For analysis of Pit-1 mutations, the −204 to +40 wild type and mutated regions were excised from pSELECT with Smal and HindIII and inserted between the same sites of pGEM7zf+. Subsequent excision of the TRβ2 fragments with EcoRI (ATT′-5′ overhang) and MluI (CGG-5′ overhang) allowed the resulting fragments to be selectively end-filled using avian myeloblastosis virus reverse transcriptase and either [32P]-labeled dATP and dTTP or dCTP and dGTP. Radiolabeled TRβ2 probes were allowed to interact with 20 μg of bovine serum albumin (no extract), 120 μg of bacterial Pit-1 extract protein, or 100–300 μg of pituitary cell nuclear extract protein under defined conditions, and DNase I digestion and analysis on 5% polyacrylamide-8 M urea gels was carried out as previously reported (19).

RESULTS

Pit-1 binds to Multiple Sites within the TRβ2 Promoter Region Important for Expression in Thyrotropes—We previously reported that the TRβ2 genomic region upstream of the putative AUG codon when fused to a luciferase reporter directed luciferase expression in TtT-97 thyrotropic cells that express endogenous TRβ2 mRNA (13). In contrast, when similar constructs were transfected into α-TSH cells, a thyrotroped cell line devoid of detectable TRβ2 mRNA, 4–5-fold less luciferase activity resulted (13). This functional data together with the observation that this 5′ region also contained sites of transcriptional initiation in thyrotrope cells (13) strongly suggested that the 5′ TRβ2 region contained the promoter elements important for expression in thyrotrope cells. Deletional analysis demonstrated that the proximal 5′ area comprising 465 bp upstream of the AUG codon (designated +1) to position +40 was sufficient to support promoter activity in thyrotropes and that inclusion of larger fragments extending as far as 2.9 kilobases upstream did not further enhance promoter activity (13). An examination of the sequence of this region revealed the presence of multiple AT-rich sequence motifs with homology to binding sites for the pituitary-specific transcription factor Pit-1 as well as GC-rich regions resembling sites of interaction for the more widely expressed transcription factor Sp1. Because α-TSH cells do not contain detectable Pit-1 protein (18) and also poorly support transfected TRβ2 promoter activity, we investigated the possibility that Pit-1 was important for the expression of TRβ2 in TtT-97 thyrotrope cells. First we wished to determine if the AT-rich motifs did indeed bind Pit-1. DNase I protection analyses using labeled fragments encompassing the entire 500-bp promoter region incubated with either bacterially expressed Pit-1 or nuclear extracts from Pit-1 expressing TtT-97 tumors demonstrated that the functionally important region contained six areas of interaction with the recombiant Pit-1 preparation (Fig. 1, A–D, open boxes). All of these sites, with the exception of one from −412 to −387, were also protected by Pit-1 containing TtT-97 nuclear extracts (filled boxes). In addition to the Pit-1 sites, at least four other regions (also designated by filled boxes) were protected by thyrotrope nuclear extracts but not by bacterial Pit-1 protein. These included an extension of a Pit-1 footprinted area from −522 to −545 distally to position −561, from −201 to −183, from −65 to −56, and from +9 to +25.

Fig. 1E summarizes in schematic form the location within the sequence of the TRβ2 5′ region of the Pit-1 protected regions (open boxes) and the Pit-1 sequence motifs within or adjacent to the areas of protection that could be contributing to the interaction (boxed sequences Pit-1, 1–4). Also shown in Fig. 1E are the relative locations of the thyrotrope extract protected regions that did not interact with recombiant Pit-1 (filled boxes)
**FIG. 1.** DNase I protection analysis of the mouse TRβ2 promoter region. DNA probes containing 495 (−572 to −77), 312 (−210 to +102), or 253 bp (−77 to +176) of the TRβ2 5′ region were labeled at the upstream −572 site (A), the downstream −77 site (B), the upstream −210 site (C), or the upstream −77 site (D) and subjected to DNase I footnoting using TtT-97 cell nuclear extracts (TtT-97), a bacterial extract expressing Pit-1 (Pit-1), or bovine serum albumin (0). Open boxes denote the areas within the promoter fragment that interact with bacterially expressed Pit-1. Numbers define their corresponding location within the TRβ2 sequence calculated from the relative migration of HpaII-digested pBR322 DNA size markers run in a parallel lane (Stds in B). Filled boxes show areas similarly designated that are protected by TtT-97 thyrotrope nuclear extracts. The area footprinted in D is the same region of C expanded as shown by the lines. Probe lanes contain fragments not subjected to DNase digestion. E is a schematic showing the nucleotide sequence of the TRβ2 promoter region extending 573 bp upstream from the putative AUG codon (shown as +1 above the A) to 43 bp downstream. Numbers above the sequence refer to the 5′ extent of deletion constructs used in this study, and restriction sites are underlined. The locations of the areas that interact with bacterially produced Pit-1 are designated by open boxes, and the areas protected by TtT-97 extracts are shown as filled boxes. TtT-97 extract protected areas that do not colocalize with Pit-1 footprints are designated T-1 through T-4. Sequences in both orientations that closely resemble the Pit-1 consensus are boxed and labeled a–i.
T3-response element, indicative of a TR binding site, is not
passes a cluster of transcription start sites that are down-
tory (13) have shown that deletion of the region from
containing the indicated 5
b
9
boxes designated T-1 through T-4. Closer examination of the
Pit-1 protected area from −49 to −19 (Fig. 1D) reveals that the
thyrotrope extract footprint does not exactly coincide with that
of bacterially expressed Pit-1, being slightly displaced distally
by 3–4 bps (−53 to −23). This difference in protection may
suggest the presence within thyrotrpic cells of a factor with
similar sequence recognition as Pit-1 but that has a greater
affinity than Pit-1 for the Pit-1 g, h, or i site and exhibits
different binding characteristics. We therefore designated the
thyrotrope footprint in this area as T-3A to distinguish it from
the area of recombinant Pit-1 interaction. The other non-Pit-1
thyrotrpoe footprints do not bear any similarity to known tran-
scription factor recognition sites with the exception of T-3, which
contains a variant Sp1 motif (GGGCGTGG) that has been shown
in the promoter of the CD14 gene to bind Sp1 and
mediate lymphocyte-specific expression (22). Interestingly, the
thyrotrope extract protected T-4 region from +9 to +25 encoun-
ters a cluster of transcription start sites that are down-
regulated by T3 in thyrotrpoe cells (13). However, a consensus
T3-response element, indicative of a TR binding site, is not
present within the sequence of the footprinted T-4 area.

Deletion Analysis of the TRβ2 Promoter in Thyrotrpioes—
Because Pit-1 has been shown to be important for the expres-
sion of other pituitary genes particularly those in thyrotropes
(TSHβ) and somatotropmamnotropes (GH and PRL) (23–25), we
evaluated the role of the Pit-1 binding sites in TRβ2 promoter
activity in thyrotrpoe cells. Previous studies from our labor-
atory (13) have shown that deletion of the region from −572 to
−465, which contains the Pit-1 a site, had no effect on TRβ2
promoter activity in transfected thyrotrpoe cells (also shown in
Fig. 2A). However, when the region from −465 to −204 (con-
taining Pit-1 sites b/c and d) was removed, a 50% decrease in
promoter activity was observed (shown in Fig. 2A with addi-
tional determinations included). Further deletion to position
−77, which removes the Pit-1 e and f sites, accounted for the
majority of the remaining promoter activity in thyrotrpoees. To
more specifically define the relative contributions of the prox-
imal Pit-1 sites and the areas protected by thyrotrpoe extracts,
we created three additional deletion mutants using a polymer-
ae chain reaction strategy. These new 5’ constructs, which
terminate at −152, −121, and −25 together with the previ-
ously described deletions (see Fig. 1E) remove, in a progressive
fashion, T-2, the Pit-1 e site, the Pit-1 f site, and finally the
T-3/T-3A region, which encompasses the Sp1 motif and the
Pit-1 g, h, and i sites. Fig. 2A shows the results of such a
systematic deletional strategy on TRβ2 promoter activity in
transfected TtT-97 thyrotrpoees. Removal of the T-2 area
had no effect, whereas loss of the Pit-1 e site resulted in a 60%
reduction in promoter activity from the already decreased level
observed with the promoter fragment deleted to −204. Subse-
quent deletion to −77, which removes the Pit-1 f site, further
decreases activity to approximately 20% of the activity of the
−204 construct. Finally, deletion to −25, which removes the
remaining Pit-1 sites, results in a promoter construct with no
measurable luciferase expression above the promoterless
pA3LUC control. Thus, removal of regions of Pit-1 interaction
except Pit-1 a has a significant effect on TRβ2 promoter activity
in thyrotrpoe cells.

The TRβ2 5’ Region Also Functions as a Promoter in Soma-
totrope Cells—To determine if the same regions that govern
TRβ2 expression in thyrotrpoees were also functional in soma-
totrope cells, we carried out similar transfection experiments
with GH3 cells. Initial experiments showed that when normal-
ized to a luciferase plasmid directed by an RSV promoter trans-
ferred in parallel, the TRβ2 promoter construct from −572 to
+40 expressed at an equivalent or higher level in GH3 cells
when compared with TtT-97 thyrotrpoees (data not shown). Fig.
2B shows the results of a 5’ deletion analysis in GH3 cells
performed with the same constructs described earlier for
TtT-97 thyrotrpoeees. In agreement with the results of our pre-
vious transfections into thyrotrpoees (13), sequences upstream
of −465 were dispensable for TRβ2 promoter activity in soma-
totropees. However, in contrast to the situation in thyrotrpoees,
deletion of the region from −465 to −204 containing the Pit-1
b, c, and d sites, which resulted in a 50% decrease in thyro-
trpoees (Fig. 2A), had no effect on promoter activity in GH3 cells
implicating a role for this region specific to thyrotrpoe cells.
When the additional deletions, which systematically remove
the more proximal Pit-1 sites, were tested in GH3 cells, the
activity pattern of the TRβ2 promoter decreased to approximately
20% of the activity of the −204 construct. Fig. 2A shows that
GH3 extracts were equally
...
Fection deletion data presented in Fig. 2B, GH3 extracts were able to clearly protect Pit-1 sites e through i in a manner indistinguishable from recombinant Pit-1 (Fig. 3B), indicating that it is probably Pit-1 protein present in the nuclear extracts that is generating the footprints. These data suggest that binding of Pit-1 to these sites accounts for their prevalent contribution to TRβ2 promoter activity in GH3 cells as well as TtT-97 cells. This is supported by the observation that similar extracts from α-TSH cells, which lack Pit-1 protein, do not protect the Pit-1 e and f sites (Fig. 3B). Expansion of the more proximal region containing the Pit-1 g/h and i sites by foot printing a different fragment (Fig. 3C) demonstrated that the pattern of protection of the proximal area of interaction with GH3 extracts more closely resembles that generated by recombinant Pit-1 and does not manifest the distally displaced footprint seen with TtT-97 extracts. This suggests that somatotrope cells lack the factor(s) present in the thyrotrope cells that interacts at this proximal promoter area.

**Binding of Pit-1 at the e and f Sites Is Required for TRβ2 Promoter Activity in GH3 Cells**—The 5′ deletion studies in both TtT-97 and GH3 cells suggested that the region between −152 and −77, which contains the Pit-1 e and f sites, is important for TRβ2 promoter activity in both cell types. To further investigate the role of Pit-1 binding to these sites, we mutated the AT-rich consensus binding motifs within the foot printed areas and examined the consequences on both Pit-1 interaction and TRβ2 promoter activity. Fig. 4 shows that altering either or both of the Pit-1 motifs resulted in loss of binding at those sites of both recombinant Pit-1 as well as pituitary cell nuclear extracts. However, disruption of Pit-1 binding at one of the sites did not appear to affect its ability to interact at the other, ruling out possible cooperativity between the Pit-1 e and f sites. The effects of these mutations on TRβ2 promoter activity are shown in Fig. 5. When Pit-1 was no longer able to bind at the Pit-1 e site, promoter activity was decreased to 50% of the unmutated −204 construct. However, when binding to the more proximal Pit-1 f site was disrupted, either alone or in conjunction with the Pit-1 e site mutation, promoter activity was more dramatically lowered to only 20–25% of the intact promoter construct to a level exhibited by the −77 construct, which has both Pit-1 e and f deleted. These results emphasize the importance of both the Pit-1 e and f sites for the expression of TRβ2 in pituitary cells but demonstrate the more dominant role of the more proximal f site. Similar decreases in TRβ2 promoter activity were also seen in TtT-97 thyrotropes as a result of the Pit-1 e and f site mutations (data not shown). We have also mutated the AT-rich Pit-1 consensus sequences within the b/c and i sites with no decrease in TRβ2 promoter activity in either pituitary cell type.

**Pit-1 Is Sufficient to Reconstitute TRβ2 Promoter Activity in α-TSH Cells**—To determine whether Pit-1 was capable of activating the TRβ2 promoter, we carried out experiments where Pit-1 was co-expressed with TRβ2 promoter constructs in α-TSH cells that express neither endogenous TRβ2 mRNA nor Pit-1 protein (13, 18). Specifically we wanted to see if Pit-1 could reconstitute TRβ2 promoter activity to the level exhibited by TtT-97 cells that contain both TRβ2 message and Pit-1 protein detectable by Northern and Western blots, respectively (9, 18). Fig. 6 shows that cotransfection of Pit-1 driven by the potent CMV promoter was able to stimulate a TRβ2 luciferase construct containing all of the Pit-1 sites (−572 to +40) 4–5-fold, which was equivalent to the level previously seen in endogenously expressing TtT-97 thyrotrope cells (13). To see if the stimulation by Pit-1 was dependent on the presence of Pit-1...
binding sites in the TRβ2 promoter fragment, experiments were carried out cotransfecting Pit-1 with the constructs described earlier, which have individual Pit-1 sites progressively deleted. The results of such an analysis is also presented in Fig. 6. Although deletion to −465, which removes Pit-1 a, results in a slight reduction in Pit-1 stimulation (5-3.5-fold), the −204 construct regains the 5-fold effect seen with the −572 deletion. However, when the Pit-1 e site is removed, a significant decrease in stimulation by Pit-1 to 2.5-fold is observed. Further deletion to −77, which eliminates Pit-1 f but still retains the g, h, and i, results in only minimal Pit-1 stimulation, which in some experiments was not significant. Further evidence that it is interaction of Pit-1 at the e and f sites that is primarily responsible for the stimulation by Pit-1 is presented in Fig. 7 where the TRβ2 constructs bearing mutations at either or both of these sites are impared in their ability to be stimulated by Pit-1. Disruption of binding at the Pit-1 e site results in decreased Pit-1 stimulation from 4.4- to 2.9-fold, whereas when the f site is mutated, either alone or in conjunction with a mutated e site, Pit-1 has little or no effect on the constructs as is also seen with the −77 construct, which lacks both the e and f sites. A surprising finding was that the smallest construct (containing only 25 bp 5’ upstream of the AUG codon), which is devoid of all Pit-1 sites, was actually inhibited by 50% in the presence of Pit-1 (Fig. 7). A similar inhibition was also seen for the RSV luciferase positive control and the promoterless pA3LUC plasmid, neither of which contain Pit-1 sites. No inhibition of a similar RSV promoter construct was reported by Mangalam et al. (26), whereas Steinfeldt et al. (27) reported substantial inhibition of RSV luciferase activity by Pit-1 in 235–1 cells. A general inhibition of promoter activity independent of DNA binding by another homeodomain protein Max-1 has recently been described (28). These transfection data in thyrotrpoe-derived α-TSH cells suggest that the Pit-1 e and f sites are the primary sites responsible for the stimulation of TRβ2 promoter activity in the presence of exogenously supplied Pit-1.

**DISCUSSION**

Pit-1 is a pituitary-specific transcription factor that plays an important role in pituitary development (29) and in the expression of several pituitary genes including GH and PRL (30, 31), TSHβ subunit (11, 21, 25, 26), growth hormone releasing factor receptor (32), renin (33), and the Pit-1 gene itself (34). The results presented in this report demonstrate that expression of the pituitary-specific β2 TR isoform in thyrotrpe and somatotrope cells is also dependent on Pit-1. Although T3-binding activity can be immunoprecipitated by TRβ2 antibodies from cell extracts from several extra-pituitary sources (8), readily detectable levels of TRβ2 mRNA expression are restricted to thyrotrpe and somatotrope cells of pituitary origin (6, 9, 10). By gene transfer we have identified the regions of the murine TRβ2 promoter that are active in pituitary-derived GH3 somatotropes and also in cells derived from murine thyrotrpe tumors. DNA-protein interaction studies show that this activity is correlated with the binding of Pit-1 and/or a factor with a similar sequence specificity present in extracts of these cells. These data in conjunction with the observation that cotransfection of a Pit-1 expression vector into Pit-1-deficient α-TSH cells results in a binding site-dependent activation of the TRβ2 promoter suggest that Pit-1 activates the murine TRβ2 promoter in transfected GH3 and TgT-97 cells and is most likely involved in regulating transcription from the endogenous TRβ2 genomic locus in a pituitary-specific fashion.

Six areas of interaction with bacularly expressed Pit-1 were detected within the proximal TRβ2 promoter region. Within these areas nine sequence motifs (Pit-1 a–i) were identified that closely resemble the Pit-1 consensus binding site sequence (A/T)(A/T)TATNCAT derived by Ingraham et al. (35). While Pit-1 d and e conform exactly to the consensus, the others vary by no more than two nucleotides from it. In the Pit-1 footprinted area from −456 to −432 b and c are two possible alignments of the Pit-1 binding site consensus sequence. Similarly, because of the inaccuracy in exactly assigning footprint boundaries, g or h cannot be excluded as contributing to the most proximal area of Pit-1 interaction. A similar ambiguous arrangement of three adjacent and overlapping Pit-1 consensus motifs are present in the proximal promoter of the rat Pit-1 gene from −63 to −41 (36). Deletional analyses demonstrated that the Pit-1 a site, which is protected by both recombinant Pit-1 and pituitary cell extracts, is not required for TRβ2 promoter activity in either thyrotrpe or somatotrope cells. Furthermore, although its functional role is not known, the recombinant Pit-1 footprinted area, which contains the perfect consensus Pit-1 d site, is not protected by extracts from thyrotrpe cells that contain Pit-1. However, the Pit-1 b/c, which is protected by both recombinant Pit-1 and pituitary cell extracts, can be mutated with no effect on TRβ2 promoter activity in thyrotrpes. Examples of sites with high affinity for Pit-1 but that cannot be ascribed a functional role can be found in the proximal promoter (P4 site) and distal enhancer (D3 site) of the PRL gene (37, 38), in the D1 region of the TSHβ promoter (18, 39), and in the upstream enhancer (Pit-1 a and b sites) of the Pit-1 gene itself (34). The reason for their lack of functional
contribution is not known, but two recent reports suggest that the context of Pit-1 sites relative to other promoter elements (40) or whether they bind Pit-1 as a monomer or dimer (41) may determine their functional significance.

The most proximal area of thyrotrope protein interaction incorporating the T-3 and T-3A protected regions was shown by deletion analysis to contribute to TRβ2 basal promoter activity in both thyrotropes and somatotropes. However constructs from −77 to +40 containing only these sites but lacking the more upstream Pit-1 sites were not appreciably stimulates by coexpression of Pit-1 in α-TSH cells. Interestingly, although GH3 extracts appear to footprint this proximal region in a fashion indistinguishable from that generated by bacterially produced Pit-1, interaction with thyrotrope extracts was thought not to be a result of the binding of Pit-1 present in these extracts because the protection pattern was not identical to that produced by the recombinant Pit-1 preparation. A possible explanation is that another factor with similar sequence recognition properties such as a related POU homeodomain family member may, as a result of greater abundance or affinity, be precluding Pit-1 in nuclear extracts from binding. In fact the sequence motif (Pit-1 i), which colocalizes with the extract protein footprint, more closely resembles a recognition site for a member of the octamer binding family, which preferentially recognizes the sequence ATTTCGAT. In this regard Oct-1 has been shown to have significant affinity for Pit-1 sites (42) and simultaneous occupation as well as functional cooperation between Pit-1 and the octamer factor Oct-1 at certain Pit-1 sites has been described for the PRL promoter (42, 43). An intriguing possibility is that competition for the Pit-1 i site by another factor enables Pit-1 to bind upstream at the g or h sites resulting in the T-3 footprint seen only with nuclear extracts. However, protection of the T-3 area by α-TSH extracts, which lack Pit-1 protein (Fig. 3B), argues against Pit-1 accounting for the T-3 footprint. A functional role for the variant Sp1 site is also presently undefined. However, it is interesting that Sp1 has been implicated in the regulation of human GH and chorionic somatomatotropin gene expression by Pit-1 (44, 45) as well as playing a key role in transcriptional regulation of the TRβ1 isofrom (46).

The deletion and mutation analyses as well as the Pit-1 coexpression experiments presented here demonstrate that interaction by Pit-1 at the footprinted areas containing the Pit-1 e and f motifs appears to be critical for TRβ2 promoter activity in cells of both thyrotrope and somatotrope origin, although their contribution seems to differ somewhat between the two cell types. The requirement for auxiliary factors that can influence the behavior of Pit-1 in a cell-specific manner has been reported. These include the estrogen receptor (47, 48) and an ETS factor (49) for regulation of the PRL promoter, the zinc finger protein Zn-15 for GH expression (50), and an as yet unidentified factor that functionally cooperates with Pit-1 to activate the TSHβ-subunit promoter in thyrotropes (25). Differences between pituitary gland and thyrotrope tumors in the relative usage of transcription start sites (13) further suggest that expression from the TRβ2 promoter may be under the control of different promoter elements in thyrotropes and somatotropes. The α-subunit of the glycoprotein hormones has been shown to be dependent on different promoter sequences for its expression in pituitary thyrotropes and gonadotropes (reviewed in Ref. 51).

The importance of Pit-1 as a pituitary-specific transcription factor was first established by its ability to activate GH and PRL promoter constructs in heterologous cells, which do not express the endogenous GH or PRL genes (26). We report here that not only does Pit-1 stimulate an exogenously transfected TRβ2 promoter activity in α-TSH cells, in which the endogenous TRβ2 gene locus is silent (13), but the extent of stimulation approaches a level equivalent to reconstituting the TRβ2 promoter activity observed in expressing TR-T7 thyrotropes. In related studies where Pit-1 has been shown to activate other pituitary gene promoters in heterologous cells, reconstitution to the level seen when the same promoter constructs are transfected into pituitary-derived cells, which contain endogenous Pit-1 is not achieved. These include the GH promoter in CV-1 cells (50) and the PRL promoter in HeLa cells (52) and the TSHβ promoter in α-TSH cells (18). In fact, stimulation of TSHβ promoter activity in α-TSH cells requires coexpression of Pit-1 with Pit-1 T, a recently described thyrotrope-specific splice variant of Pit-1 (21).

The mutational analyses presented here revealed that disruption of Pit-1 binding at certain sites (e and f) had a greater effect on Pit-1 stimulation than removal of other sites by deletion (a–d). A similar hierarchy of importance of sites within the PRL and GH promoters that confer Pit-1 activation in HeLa cells cotransfected with Pit-1 expression vectors has also been reported (26). However, contrary to what is reported here for the TRβ2 promoter, the most proximal site in both the PRL and GH genes was the most critical. Another perhaps more analogous situation to the TRβ2 promoter is to be found in the Pit-1 gene itself where the more proximal of two Pit-1 sites lies downstream of the transcriptional start site (53). In this case, however, binding of Pit-1 results in an autologous down-regulation of Pit-1 expression.

In summary, we have demonstrated a requirement for Pit-1 in expression of the β isofrom of TR in cells of thyrotrope and somatotrope origin. We have shown that binding at certain Pit-1 sites is important for expression in both cell types, whereas others appear to be cell type-specific. The innate complexity of the TRβ2 promoter region with regard to multiplicity of transcriptional origins interspersed with functionally important factor binding sites makes it difficult to distinguish the critical cis-active elements responsible for expression of loss of promoter activity due to removal of transcriptional start sites. We believe that the promoter deletion and mutation approaches described in this report represent a promising beginning toward unraveling the complexities of pituitary cell-specific TRβ2 expression.

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