Determinants of Thyrotrope-specific Thyrotropin β
Promoter Activation

COOPERATION OF Pit-1 WITH ANOTHER FACTOR*

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Thyrotropin (TSH) is a subunit of TSH, the expression of which is limited to the thyrotrone cells of the anterior pituitary gland. We have utilized the thyrotrope-derived TtT-97 thyrotropic tumors to investigate tissue-specific expression of the TSHβ promoter. TSHβ promoter activity in thyrotrones is conferred by sequences between −270 and −80 of the 5′-flanking region. We have recently reported that the proximal region from −133 to −100 (P1) is required for promoter expression in thyrotrones. This region interacts with the pituitary-specific transcription factor Pit-1. While Pit-1 appears necessary for TSHβ promoter activity in thyrotrones, this transcription factor is not alone sufficient for promoter activity in pituitary-derived cells. In this report, we have generated a series of promoter mutations in the P1 region to identify additional protein-DNA interactions and determine their functional significance. We have found that Pit-1 interacts with the distal portion of the P1 region, and a second protein interacts with the proximal segment of this region. Each protein is able to independently interact with the TSHβ promoter, but neither alone can maintain promoter activity. Both proteins appear to be necessary for full promoter activity in thyrotrones. Southwestern analysis with the proximal segment of the P1 region (−117 to −88) reveals interaction with a 50-kDa protein. Interestingly, this protein is not found in the pituitary-derived GH3 cells and may represent a thyrotrone-specific transcription factor. Further characterization of this newly identified DNA-binding protein will further our understanding of the tissue-specific expression of the TSHβ gene.

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† The abbreviations used are: TSH, thyrotropin; TEF, thyrotrone embryonic factor; TRH, thyrotropin-releasing hormone.
be responsible for the initiation of the thyrotrope phenotype, but Pit-1 and perhaps other factors are necessary for the persistence of mature thyrotropes (22).

mLIM-3 is a member of the LIM homeodomain family of transcription factors and appears to be pituitary-specific (23, 24). Bachand colleagues (23) have shown that mLIM-3 mRNA is present in developing and adult mouse pituitaries as well as a number of pituitary-derived cell lines, including the thyrotrope-derived aTSH cells. They also showed that in combination with Pit-1, mLIM-3 stimulated mTSH β promoter activity in CV-1 cells and interacted with the -120 to -60 region of the promoter in gel retardation assays.

The P1 region of the mouse TSH β promoter is necessary for cell-specific basal promoter activity and Pit-1, which interacts with this region, does not appear to be sufficient for full promoter activity observed in thyrotrope cells. We recently observed that the DNase I protected footprints in this region differed at the 3′ end between recombinant Pit-1 and the TtT-97 thyrotroph extract (20), which suggests that other thyrotroph proteins may be interacting with the mTSH β promoter. Steinfelder et al. (7) demonstrated that a human TSH β promoter fragment (−128 to −61) yielded five distinct protein-DNA complexes with a thyrotroph extract by gel mobility shift analysis. None of these complexes were seen using non-pituitary HeLa cell extract. Furthermore, they proved that four of these complexes contained Pit-1 and one complex appeared to be due to an unrelated thyrotroph protein. These data suggest that more than one protein may be interacting with the TSH β promoter in this functionally critical region. In this report, we have undertaken a detailed mutagenesis analysis of the P1 region and identified a second protein that interacts with the TSH β region and appears to be necessary for basal activity of the mTSH β promoter in thyrotropes.

MATERIALS AND METHODS

Nuclear Extract Preparation and DNase I Protection Analysis—Nuclear extracts were prepared from TtT-97 thyrotrophic tumors, aTSH and GH3 cells as described previously (25). The -392 to +40 mTSH β promoter fragment was excised from β-galactosidase and identified as P1M1, P1M2, etc.

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In Vitro Mutagenesis of the TSH β Promoter—Site-directed mutagenesis of the P1 region extending from −133 to −86 was carried out in the context of the −392 to +40 mTSH β promoter region in pSELECT (Promega, Madison, WI) as described previously (20). Briefly, for each mutated segment a 30-base pair oligonucleotide was generated with 4–6 altered bases in the center. Mutagenesis was carried out with the Altered Sites System (Promega). The −392 to +40 fragment was then excised with BamHI and HindIII and ligated into pEGM7zf + and pA1uc (3) for DNase I protection analysis and gene transfer studies, respectively. Sequences were verified in each plasmid by the chain termination method of Sanger (26). Wild-type mTSH β promoter sequence and respective mutations are noted in Fig. 1.

GeneTransfer Studies—Transient transfection assays in TtT-97 thyrotroph tumor cells have been outlined previously (3). Briefly, 20 μg of the various TSH β promoter-luciferase plasmids and 1 μg of pCMV β-gal as an internal control for transfection efficiency were co-transfected by electroporation into 5–10 million TtT-97 cells. Cells were incubated in 4 ml of Dulbecco's modified Eagle's medium supplemented with charcoal-stripped 10% fetal calf serum (Life Technologies, Inc.) at 37 °C for 18 h. Cells were harvested, subjected to freeze-thaw extraction, and assayed for luciferase and β-galactosidase activity as described previously (10). Luciferase activity was corrected for β-galactosidase activity as an internal control for efficiency of each transfection. Statistical analysis was performed by one way analysis of variance. Pairwise multiple comparisons were made by the Student-Newman-Keuls test (27).

Southwestern Blot Analysis—Southwestern blot analysis was carried out as described previously (5). Briefly, nuclear extract proteins (approximately 50 μg) were separated on a denaturing 10% polyacrylamide gel. After electrophoresis, nitrocellulose filters were treated with guanidine hydrochloride to renature the proteins, then treated with a solution containing 5% non-fat dry milk and 1% bovine serum albumin to block nonspecific interactions with the labeled probe. Binding reactions were carried out with a [α-32P]dCTP-labeled probe (3 × 106 cpm/ml) corresponding to the wild-type or M7 mutation equivalent −117 to −88 region of the TSH β promoter in 10 ml Tris-HCl, pH 7.5, 0.5% non-fat milk, 0.5% bovine serum albumin, 50 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 5 mM dithiothreitol, 0.1% Triton X-100, 5% glycerol, 200 μg/ml ZnSO4, and 10% DMSO each of native and denatured salmon testes DNA. Binding reactions were carried out overnight at 4 °C. Filters were washed twice for 20 min at 4 °C in binding buffer minus salmon testes DNA and autoradiographic exposures were carried out at −70 °C overnight.

RESULTS

DNase I Protection Footprinting of the TSH β Promoter and Mutations—We first compared protein-DNA interaction of recombinant Pit-1 protein, TtT-97, and GH3 nuclear extracts with an expanded region of the TSH β promoter from −390 to +40 (Fig. 2A). The 5′ (distal) end of the P1 footprint appears to be identical between Pit-1 and the nuclear extracts. The 3′ (proximal) end, however, shows a clear extension of approximately 14 base pairs with the TtT-97 nuclear extract (lane 4) which is not seen with the recombinant Pit-1 protein (lane 3). This extension is similar in two nuclear extract preparations from two different TtT-97 thyrotroph tumor cells and redefines the P1 region to be −133 to −86. Interestingly, this footprint extension is not seen using an extract from the pituitary-derived, non-thyrotroph GH3 cells (lane 5) which suggests that factor(s) involved in this extension are thyrotroph-specific. The other regions of the TSH β promoter are also redefined as D1 (−295 to −222), D2 (−196 to −170), and P2 (−80 to −62) using the TtT-97 nuclear protein extract. A new region (−330 to −300) is also noted, which is protected by TtT-97 extract but not recombinant Pit-1 protein or GH3 nuclear extract.

Fig. 2B shows DNase I protection with recombinant Pit-1 and TtT-97 extract protein on the eight different P1 region mutations. Mutations P1M1, P1M2, P1M6, and P1M8 appeared to have no effect on either Pit-1 or TtT-97 protein binding. In contrast, mutations P1M3 and P1M4 disrupted recombinant Pit-1 binding as well as the distal portion of the footprint generated with TtT-97 extract, but the proximal P1-protected region (PP-1) was preserved with the TtT-97 extract only, suggesting that a protein other than Pit-1 in the thyrotroph nucleus interacts with the proximal portion of the P1 region. The P1M5 mutation abrogated binding of Pit-1 and TtT-97 extract. This mutation corresponds to a previously reported random mutation (20) which also disrupted Pit-1 and TtT-97 extract binding. The P1M7 mutation disrupted binding of TtT-97 extract to the proximal P1 region (PP-1), but this mutation had no effect on Pit-1 binding or the binding of Pit-1 in the TtT-97 extract. These data suggest that two separate thyrotroph proteins interact with the P1 region of the mTSH β promoter and that each protein interacts independently of the other.
Activity of the TSHβ Promoter and Mutations in TtT-97 Thyrotropes—In order to evaluate the functional effect of the P1 region TSHβ promoter mutations in thyrotropes, we transiently transfected luciferase reporter plasmids containing the wild-type and mutated promoters within the context of the 2390 to 140 region into TtT-97 cells. Results of the various mutant promoter activities compared with wild-type are shown in Fig. 3. The activity of a –80 to +40 TSHβ promoter plasmid, which lacks the P1 region is shown for comparison. All of the mutations affect activity of the TSHβ promoter in TtT-97 cells, which may reflect a greater sensitivity of these mutations in a functional assay compared with the protein-DNA interaction data displayed in Fig. 2B. However, the relative promoter activity of the mutants appears to mirror the ability of proteins to interact with this region. The P1M1 and P1M2 mutations, which do not affect protein interaction, show modest decreases of promoter activity (24 and 29% decrease, respectively) compared with the wild-type promoter. The P1M3 and P1M4 mutations, which disrupt binding of Pit-1 but not the protein to the proximal region (PP-1), greatly reduce promoter activity 70 and 72%, respectively. The P1M5 mutation interferes with interaction of both proteins and reduces activity 63%. Interestingly, the P1M6 mutation, which is well within the P1 region, does not affect protein-DNA interaction. Promoter function is modestly affected (38% decrease) which is significantly different than the M3, M4, and M5 mutations (p < 0.05). The P1M7 mutation is the only mutation that interferes with interaction in the PP-1 region and not Pit-1, and its promoter activity is profoundly reduced by 78% compared with the wild-type promoter. This reduction is equivalent to that seen with a deletion of the entire P1 region (80 deletion, Fig. 2B). Finally, the P1M8 mutation does not appear to affect protein-DNA interaction. Functionally, however, promoter activity is moderately reduced by 43%. This data may reflect the differences in sensitivity between the functional and protein-DNA interaction assays. Yet this activity is significantly different than the re-
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In this report we have used mutagenesis, DNA footprinting, and gene transfer studies to dissect the functionally important P1 region of the mTSHβ promoter. These results strongly suggest that more than one protein interacts within this region, a predictable conclusion when one considers the size of the native thyrotrope-derived footprint (47 base pairs). Many lines of evidence indicate that the pituitary transcription factor Pit-1 or one of its isoforms is one of the factors that interacts with this region and appears necessary for activation of the TSHβ promoter. Pit-1 interacts with three regions in the −390 to +40 segment of the mTSHβ promoter which confers cell-specific activity, D1 (−295 to −222), P1 (−133 to −86), and P2 (−80 to −62) (Ref. 5, Fig. 2A). Several reports have shown that Pit-1 is necessary for TRH- and cAMP-mediated stimulation of the TSHβ promoter, occurring through the D1- and P1-related regions in the rat and human promoters respectively (6–9, 19, 28). Kim et al. (9) propose that Pit-1 acts together with an AP-1-like factor, which interacts with a TGGGTCA motif at −1 to +6 of the hTSHβ promoter to mediate TRH stimulation. Steinfelder et al. (19) observed that mutations of either Pit-1 site in the P1 equivalent region of the mTSHβ promoter reduced activity by approximately 50% in GH3 cells, while forskolin and TRH stimulation was reduced by more than 60% in the upstream Pit-1 site (P1M3 equivalent) and only 20–30% in the downstream site (P1M6 equivalent). Lin and colleagues (22) mutated two putative Pit-1 binding sites, the P1M3 equivalent at −122 to −116 and a proximal site at −76 to −69. This double mutant promoter was no longer stimulated by Pit-1 in CV-1 cells. Basal activity in pituitary-derived cells was not studied.

We have recently shown that a mutation in the P1 region of the mTSHβ promoter, which disrupted Pit-1 binding, abrogated basal activity of the promoter in TTT-97 thyrotrpes, but had no effect in GH3 somatotropes (20), suggesting that both Pit-1 and the P1 region are necessary for cell-specific basal activity of the mTSHβ promoter only in thyrotrpes. However, introduction by gene transfer of Pit-1 into various Pit-1-deficient cell types has little or no effect on TSHβ promoter activity (5, 8, 9, 19, 21), indicating that while Pit-1 may be necessary for TSHβ promoter activity, it is not sufficient for promoter activity.

In this study, we have identified a segment of the P1 region, the proximal P1 region (PP-1), which interacts with a non-Pit-1 protein and is separately and equally critical to the region that interacts with Pit-1 for activity of the mTSHβ promoter in thyrotrpes. Systematic scanning mutagenesis of this region has revealed separate contact areas for these two distinct proteins. While all of the mutations affected promoter activity in thyrotrpes, they appeared to segregate into mutations which did not affect protein-DNA interactions (less than 45% reduction in activity) and mutations which disrupted binding (greater than 60% reduction). The reduction of promoter activity seen with mutations that did not affect protein-DNA interactions may reflect sensitivity differences between functional and structural assays. P1M1 and P1M2 mutations (Fig. 1) appear to have little effect on protein binding and modest decreased function of the mTSHβ promoter. Interestingly, the P1M2 mutation changes the final T to a G in the Pit-1 binding site AATNCAT. P1M3 and P1M4 mutations both affect Pit-1 interaction with the P1 region, but do not alter protein binding at the PP-1 region. These mutations cause a greater reduction in basal activity of the mTSHβ promoter than the mutations which do not affect protein-DNA interaction. The P1M3 mutation significantly alters the Pit-1 binding sequence, while the P1M4 mutation does not directly alter this sequence but creates changes seen with a previously described mutation (20) that also affected Pit-1 interaction with the promoter. The P1M4 mutation does change the AA preceding the Pit-1 binding site to CC. This A/T-rich region preceding the core binding site is necessary for high-affinity Pit-1 interaction as well as promoter function (20, 29, 30). The P1M5 mutation appears to alter interaction of both proteins with the promoter. Promoter activity is reduced greater than 60%, but not to the level seen with the M3, M4, and M7 mutations which disrupt individual protein binding. It appears that this mutation affects binding of both proteins, but Dnase I protection (Fig. 2B) shows that Pit-1 and TTT-97 footprints are slightly different than the bovine serum albumin control and may reflect partial protein-DNA interaction. Surprisingly, the P1M6 mutation, while altering a putative Pit-1 binding sequence (AAANCat), has no effect on protein-DNA interaction and modestly affects promoter function. This data implies that the upstream Pit-1 binding element is the critical element for protein-DNA interaction. The P1M7 mutation does not affect Pit-1 interaction with the promoter, but the novel protein no longer binds to the PP-1 region. This is the only mutation that affects binding in the PP-1 region and yet does not alter Pit-1 interaction with the promoter. Basal activity of the promoter is greatly reduced by this mutation, suggesting that the two factors can interact with the promoter independently of binding by the other, but both are necessary.
for full basal activity of the promoter. These systematic mutations clearly illustrate that two separate proteins are interacting at overlapping regions on the mTSHβ promoter and that neither factor alone is capable of conferring full promoter activity in thyrotropes. Identification of the factor that interacts with the PP-1 region, and the nature of its functional interaction with Pit-1 will greatly increase our understanding of thyrotrope-specific activity of the TSHβ promoter.

Using Southwestern blot analysis with a duplex oligonucleotide corresponding to the PP-1 segment of the P1 region (−117 to −88), we have shown that a 50-kDa protein in thyrotrope-derived cells interacts with this region. Examination of this sequence in the P1 region reveals an AGATAA motif at −98 to −93. The M7 mutation (Fig. 1), which disrupts this AGATAA sequence, abrogates both functional and structural interaction with Pit-1 will greatly increase our understanding of thyrotrope-specific activity of the TSHβ promoter.

The M7 mutation also demonstrates that two separate proteins are interacting at overlapping regions on the mTSHβ promoter and that these proteins interact cooperatively to stimulate the promoter (50-fold) greater than the PP-1 region, and the nature of its functional interaction with Pit-1 will greatly increase our understanding of thyrotrope-specific activity of the TSHβ promoter.

In summary, we have identified a 50-kDa protein in thyrotropes that appears to be necessary for basal activity of the TSHβ promoter. Further characterization of this factor will lead to a better understanding of TSHβ gene regulation and cell-specific gene expression.

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