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Biochemical and Biophysical Research Communications 362 (2007) 193–199
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TTF-1 regulates growth hormone and prolactin transcription in the anterior pituitary gland

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Received 30 July 2007
Available online 10 August 2007

Abstract

Thyroid transcription factor 1 (TTF-1) is required for morphogenesis of the fetal diencephalon. Previous reports showed that mice carrying a TTF-1 null mutation lacked normal development of the pituitary gland. In this study, a role for TTF-1 in the regulation of growth hormone and prolactin transcription was identified. *In-situ* hybridization analysis demonstrated TTF-1 mRNA in the growth hormone-producing cells and prolactin-producing cells of the rat anterior pituitary gland. In the GH3 pituitary cell line, we identified TTF-1 as a factor functionally regulating growth hormone and prolactin transcription. TTF-1 activated prolactin transcription, but inhibited growth hormone transcription. Inhibition and activation of growth hormone and prolactin transcription, respectively, by TTF-1 disappeared upon deletion of the TTF-1 binding motifs within the promoters of these genes. These data suggest that TTF-1 plays a regulatory role in the transcription of growth hormone and prolactin genes and may regulate transdifferentiation of cells expressing these two hormones.

Keywords: Transdifferentiation; Transcription regulation; Promoter assay; Fluorescence *in-situ* hybridization

Growth hormone (GH) is primarily produced in the cells of the anterior pituitary (AP) and plays an essential role in general body growth. Lacking GH secretion results in dwarfism in a young child, whereas over-production of GH during early postnatal development leads to gigantism [1]. Prolactin (PRL) was originally identified as an AP hormone playing a regulatory role in mammalian milk synthesis. In addition PRL regulates many physiological processes including mammary gland development, initiation and maintenance of lactation, immune modulation, osmo-regulation, and behavior modification [2].

TTF-1 was first found in the thyroid gland [3] and further identified as homologous with Drosophila NK-2 homeodomain protein [4]. Other studies identified function of TTF-1 in the lung and fetal diencephalon [5]. Expression of TTF-1 in the postnatal rat brain has been studied by our research group and was shown to be expressed mainly in the hypothalamus [6–8]. TTF-1 knockout mice die at birth with defective lung function and extensive brain abnormalities, especially in the ventral region of the forebrain from the septal area to the mamillary body of the hypothalamus [9]. Moreover, these mice lack the entire pituitary gland at birth [10]. Thus, its regulatory role for the pituitary function has been postulated.

We examined the 5′-flanking region of AP hormone genes and found several conserved TTF-1 binding motifs in GH and PRL promoters. We determined functional activities of GH and PRL promoters, controlled by TTF-1 in GH- and PRL-synthesizing cells. TTF-1 inhibited GH transcription but activated PRL transcription by binding to TTF-1 binding motifs in these promoters.

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doi:10.1016/j.bbrc.2007.08.009

Materials and methods

Animals and tissue preparation. Two-month-old male Sprague–Dawley rats (Hyochang Animal Breeding Company, Eumsung, Korea) were used for histochemical studies. They were housed at 23–25 °C in a room with a 12 h light/dark cycle and allowed *ad libitum* access to tap water and rat chow. Animals were anesthetized and sacrificed by decapitation. Pituitary glands were excised from the skull, placed in embedding medium (Tissue-Tek O.C.T compound), frozen, and stored in a −80 °C freezer. Twelve-micrometer cryostat sections were prepared and mounted onto slides and were stored at −80 °C until used for fluorescence *in-situ* hybridization (FISH).

TTF-1, GH, and PRL cDNA templates for FISH. To examine TTF-1 expression in the rat AP, we used previously reported TTF-1 cDNA template [11]. Rat GH and PRL cDNA fragments were cloned by reverse transcription (RT)-PCR from rat AP RNA. Each specific primer set (GH sense primer, 5′-ATG GCT GCA GAC TCT CAG-3′; antisense primer, 5′-CTA GAA AGC ACA ACT GCT-3′; PRL sense primer, 5′-ATG AAC AGC CAG GTG TCA-3′; antisense primer 5′-TTA GCA GTT GTT TTT ATG-3′) generated 651 bp GH cDNA and 678 bp PRL cDNA fragments (NCBI GenBank database, Accession No., GH: V01237, PRL: V01249), respectively. GH and PRL cDNA fragments were cloned into the pGEM-T easy vector (Promega, Madison, WI), and their sequences were confirmed by DNA sequencing.

FISH. We generated digoxygenin (DIG)-labeled or fluorescein (FLS)-labeled sense and antisense riboprobes for TTF-1, GH and PRL, and performed FISH, as described elsewhere [11]. Briefly, tissue sections were sequentially fixed, washed, acetylated, dehydrated, and prehybridized with hybridization buffer at 58 °C for 3 h. The hybridization was performed overnight at 58 °C using 200 μl hybridization solution, containing 300 ng of either a DIG-labeled cRNA probe for TTF-1 or a FLS-labeled cRNA probe for GH and PRL. The next morning, sections were incubated in 50% formamide and 2× SSPE at 58 °C for 1 h and then sequentially washed with 0.2× SSPE and 0.1× SSPE for 30 min each at 58 °C. Next, the slides were incubated with 1% H_2O_2 in TNT buffer for 30 min and washed three times with TNT buffer for 5 min each, then followed by TNB blocking for 1 h at room temperature. Finally, samples were treated for 1 h with horseradish peroxidase (HRP)-conjugated anti-DIG antibody (Roche, a final concentration of 1.5 mU/ml) or with HRP-conjugated anti-FLS antibody (Roche, 0.75 mU/ml) in TNB blocking buffer. After washing three times in TNT buffer for 5 min each, mRNA signals of TTF-1, GH and PRL were detected using a Cy3-conjugated (TTF-1) or a FITC-conjugated (GH or PRL) Tyramide Signal Amplification system (NEN Life Science Products, Boston, MA). To colocalize TTF-1 mRNA with GH or PRL mRNA, sections were hybridized simultaneously with a DIG-labeled TTF-1 cRNA probe and either a FLS-labeled GH or a PRL cRNA probe.

Microscopy and imaging. FISH images were captured with an InfinityX CCD camera (Lumenera Corp., Ontario, Canada) attached to an Axioskop2 Plus fluorescence microscope (Zeiss, Thornwood, NY). Images were analyzed using the i-solution (iMTechnology, Seoul, Korea) image processing software, as previously described [11]. Further image processing was carried out using the Adobe Photoshop 7.0 software.

Plasmid constructs. The 566 bp (−553 to +13 bp) rat GH promoter (NCBI GenBank database, Accession No. X12967) reporter construct (pGL2 basic) was a kind gift from Dr. D.A. Weigent (University of Alabama). The proximal PRL promoter (−920 to +20 bp) was cloned by PCR using the 5 kb human PRL gene (Accession No. AL023883; a kind gift from Dr. J.R.E. Davis, University of Manchester) as a template. The sense primer was 5′-GAG CTC GAA TTC ATT TCC CAG-3′, and the antisense primer was 5′-CTC GAG AGA TCT GGA AGT CTC-3′. The 940 nucleotide long PCR product was inserted into the luciferase-reporter vector (pGL3 enhancer, Promega). To inhibit endogenous TTF-1 synthesis in the GH3 cells, we used small hairpin RNA (shRNA), cloned into the lentiviral vector pLKO.1-Puro (Sigma). Nucleotide sequence of the shRNA target site in the TTF-1 gene was 5′-GTT CTC AGT GTC TGA CAT CTT-3′, corresponding to nucleotides 30–50 of the TTF-1 cDNA sequence (GenBank Accession No. X53858). The pLKO.1-Puro control vector was used as a negative control.

Electrophoretic mobility shift assay (EMSA). The procedure for expression and purification of the TTF-1 homeodomain (TTF-1 HD) protein was previously described [12]. Double-stranded oligodeoxynucleotides, labeled with ^{32}P at the 5' end, were used for EMSA probes. Oligodeoxynucleotide sequences used in this study are shown in Supplementary Table I. Oligodeoxynucleotides C and Cβ were used as positive and negative controls, respectively [13]. EMSA was performed as previously described [7].

Deletion of TTF-1 binding core motifs from the GH and PRL promoters. Based on the binding activities observed in the EMSA experiments, three single mutants of GH and PRL promoter constructs were generated to delete the core TTF-1 binding motifs using QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). The intended mutations were confirmed by DNA sequencing.

Cell cultures and promoter assays. GH3 cells were grown in Dulbecco's modified Eagle's medium containing high glucose (4.5 g/l, 25 mM), supplemented with 10% fetal bovine serum and antibiotics, at 37 °C in a humidified atmosphere of 5% CO_2. Cells were transiently transfected with the GH promoter luciferase-reporter construct (GH-pGL2 basic, GH-P) or the PRL promoter luciferase-reporter construct (PRL-pGL3 enhancer, PRL-P) using Lipofectamine (Invitrogen Life Technologies, Gaithersburg, MD) and differing concentrations (100–500 ng/well) of the TTF-1 expression vector, TTF-1-pcDNA. Transfection efficiency was normalized by cotransfecting the β-galactosidase reporter plasmid, pCMV-β-gal (Clontech, Palo Alto, CA) at 20 ng/well. Cells were harvested 24 h after transfection for luciferase and β-galactosidase assays.

Relative RT-PCR. RNA was isolated from the GH3 cells using Trizol reagent (Sigma). One microgram of RNA was analyzed by RT-PCR using either GH sense and antisense primers (5′-ATG GCT GCA GAC TCT CAG-3′ and 5′-CTA GAA AGC ACA ACT GCT-3′, respectively) or PRL sense and antisense primers (5′-ATG AAC AGC CAG GTG TCA-3′ and 5′-TTA GCA GTT GTT TTT ATG-3′, respectively) multiplexed with glyceraldehydes-3-phosphate dehydrogenase (GAPDH) sense and antisense primers (5′-TGT GAA CGG ATT TGG CCG TA-3′ and 5′-ACT TGC CGT GGG TAG AGT CA-3′, respectively). PCR was performed for 30 cycles: 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s. The PCR products were analyzed on a 2% agarose gel and visualized by ethidium bromide staining. The band densities were analyzed with NIH image software and normalized by comparing them to the densities of control GAPDH bands.

Statistics. Differences between several groups were analyzed by ANOVA, followed by Dunnett's Multiple Comparison Test. Changes observed between two groups were analyzed by Student's *t* test.

Results

TTF-1 mRNA in GH- and PRL-expressing cells of the rat AP

To determine whether TTF-1 is expressed in the rat pituitary, we performed FISH experiments. FISH results revealed that TTF-1 mRNA was widely distributed throughout the cells of the anterior and posterior lobes of the rat pituitary (Fig. 1A, D, and E). However, cells in the intermediate lobe generated no TTF-1 mRNA signal (Fig. 1A, D, and E). We also found GH (Fig. 1B and F) and PRL (Fig. 1C and G) mRNA in the AP. Double FISH demonstrated colocalization of TTF-1 and GH mRNA in the AP (Fig. 1H, yellow), however only TTF-1 was expressed in the posterior lobe. High magnification revealed clear donut-shaped signals generated by colocal-

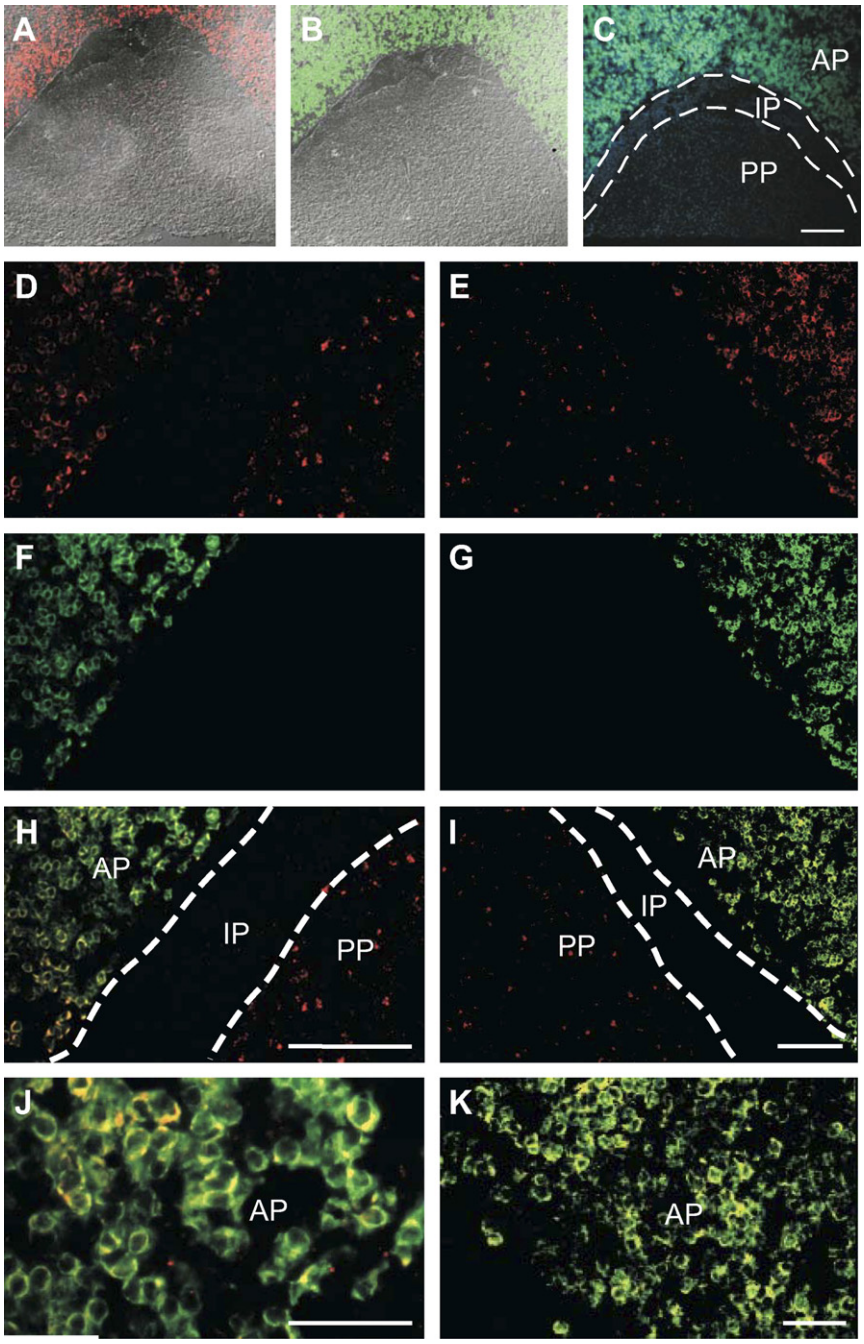

Fig. 1. Localization of TTF-1 mRNA in the rat pituitary gland. (A) Confocal microscopic image showing TTF-1 mRNA signals in the rat pituitary gland, hybridized with a DIG-labeled TTF-1 cRNA probe and visualized by Cy3-coupled Tyramide. (B) Confocal microphotograph revealing GH mRNA in the rat pituitary gland using an FLS-labeled GH cRNA probe and FITC-coupled Tyramide. (C) Fluorescence microscopic image showing the rat pituitary cells expressing PRL mRNA. Blue signals in (C) indicate nuclei stained by Hoechst 33258. Colocalization of TTF-1 mRNA with GH mRNA (D, F, H, and J) or with PRL mRNA (E, G, I, and K) in the rat AP. Fluorescence microscopic images showing TTF-1 mRNA (D, red), GH mRNA (F, green), and their colocalization (H, yellow to light green). (J) High magnification of (H). Fluorescence microphotographs showing TTF-1 mRNA (E, red), PRL mRNA (G, green) and merged image of (E) and (G)—(I). (K) High magnification of (I). Scale bar: 200 μm in (A)–(C); 50 μm in (D), (F), and (H); 25 μm in (J); 100 μm in (E), (G), and (I); 50 μm in (K). AP, anterior pituitary; IP, intermediate lobe; PP, posterior pituitary.

ization of TTF-1 and GH mRNA in the AP (Fig. 1J), suggesting cytoplasmic distribution of these mRNA species. TTF-1 was also co-expressed with PRL mRNA in the AP (Fig. 1I and K, yellow). Sense RNA probes revealed no specific signal in the rat pituitary gland (data not shown).

TTF-1 regulates the transcriptional activities of GH and PRL

Functional analysis of the GH and PRL promoters demonstrated that these promoters are transcriptionally active in GH3 cells (Fig. 2A and C). Cotransfection with

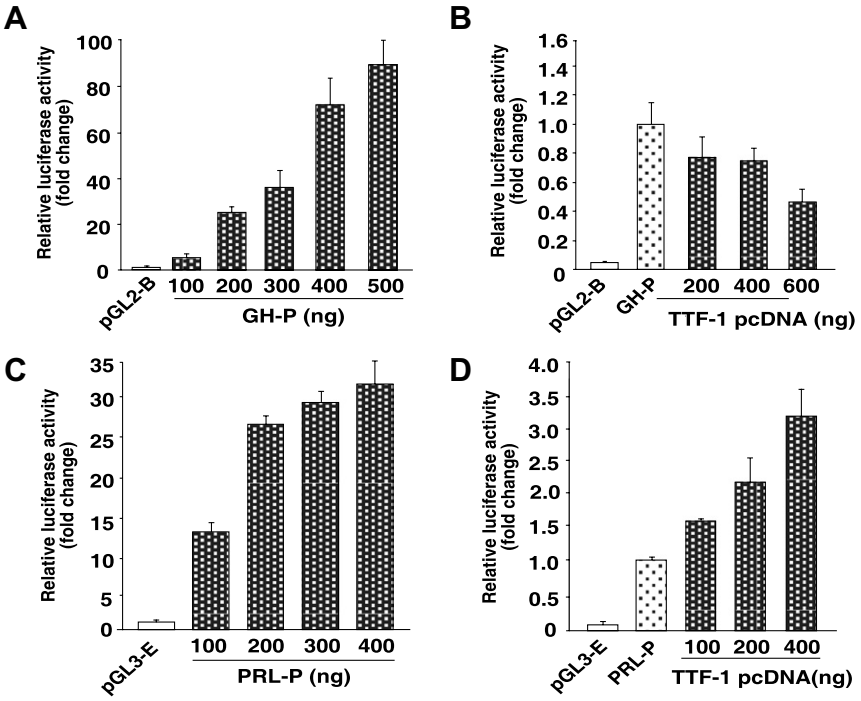

Fig. 2. Regulation of GH and PRL transcription by TTF-1. Luciferase-reporter constructs (200 ng for B and D), pGL2-B or pGL3-E, containing the 5′-flanking region of the GH (GH-P) or PRL (PRL-P) gene were cotransfected into GH3 cells with the TTF-1 expression vector, TTF-1-pcDNA, at the final concentrations indicated. (A) Dose-dependent increase in GH transcriptional activity by the GH promoter. (B) Gradual inhibition of the GH transcription induced by increasing concentrations of TTF-1. (C) Dose-related increase in PRL transcriptional activity by the PRL promoter. (D) Dose-dependent increase in the PRL transcription by TTF-1. Results are means ± SEM of at least six wells per group.

different concentrations of a TTF-1 expression vector induced a dose-dependent decrease in GH transcriptional activity (Fig. 2B), while it increased transcriptional activity of the PRL promoter in a dose-dependent manner (Fig. 2D).

Essential TTF-1 binding domains for the transcription of GH and PRL genes

EMSAs were performed to determine the capability of TTF-1 HD to bind to putative TTF-1 binding domains in the GH and PRL promoters. Double-stranded oligodeoxynucleotide probes (Supplementary Table I) containing the presumptive TTF-1 binding motifs and their flanking sequences (Supplementary Fig. 1) were employed. We found that among 10 GH promoter probes, site −431 generated the strongest signal, and sites −356 and −233 generated moderate and low signals, respectively (Fig. 3A). Other sites exhibited no (bands not shown) or very little binding. Of five putative binding motifs in the PRL promoter, three (−445, −402, and −389) were recognized by TTF-1 HD (Fig. 3A), though their binding was weak compared to the positive control oligonucleotide C.

Accordingly we further analyzed role of TTF-1 binding domains in the TTF-1-dependent transregulation of GH and PRL promoters after deleting each of the sites recognized by TTF-1 HD.

As shown in Fig. 3B, basal GH transcription activity without TTF-1 was fluctuated by deleting TTF-1 binding motifs at −431, −356, and −233, suggesting that these sequences may also serve as binding domains for other regulator(s). For example, analysis with a search program (available at www.cbil.upenn.edu/tess/) revealed sequence at −233 as a binding motif for CCAAT/Enhancer-binding protein (C/EBP), known as a transactivator of GH gene [14], as shown in Supplementary Fig. 1A. On the other hand, no transcriptional regulator has yet been reported for the sites at −431 and −356. Specially, a dramatic increase in basal GH transcription induced by deleting the site at −356 suggests that a strong transcriptional inhibitor may act through this site. However, deletion of this site showed no effect on the TTF-1-induced inhibition of GH transcription, suggesting that this site is not functional for TTF-1 action on GH gene. TTF-1-dependent inhibition of GH transcription in the wild GH promoter disappeared upon deletion of TTF-1 binding motifs at position −431 or −233, suggesting that these binding motifs are essential for the TTF-1-dependent inhibition of GH gene transcription.

TTF-1-dependent transactivation of the PRL promoter completely disappeared upon deletion of the binding site at −445, −402 or −389 (Fig. 3C), suggesting that these binding motifs are functionally required for the TTF-1-dependent transactivation of PRL.

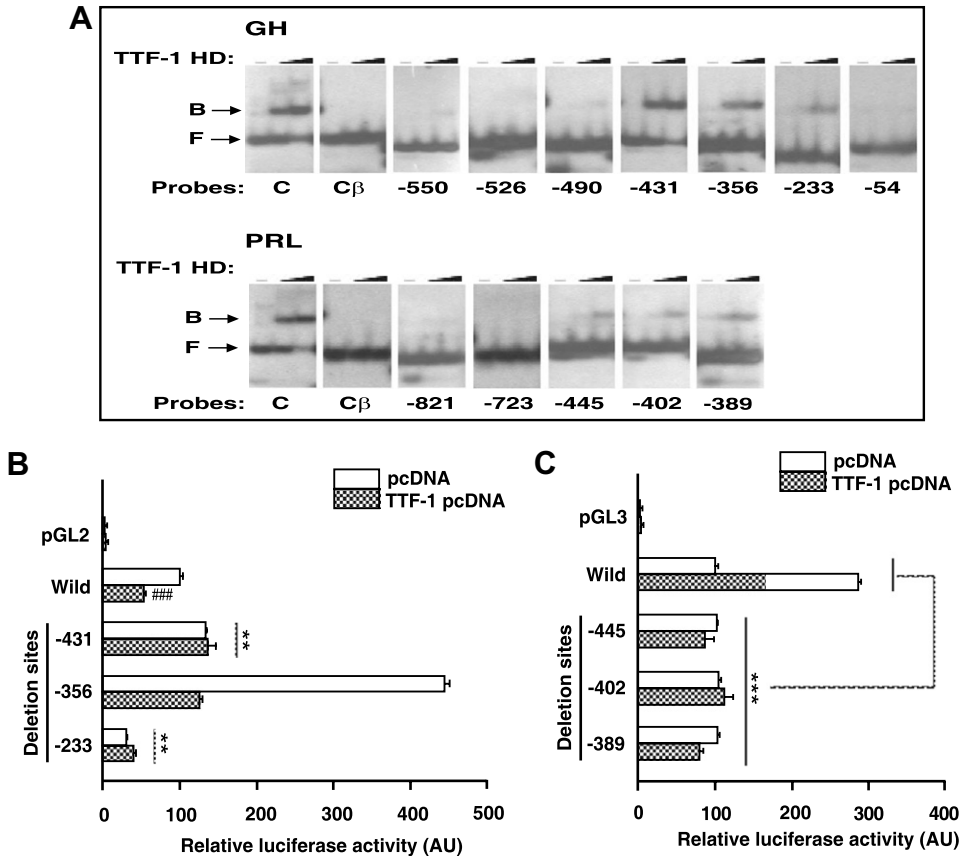

Fig. 3. Determination of essential TTF-1 binding motifs for the regulation of GH and PRL transcription. (A) EMSAs with probes containing the putative TTF-1 binding motifs. TTF-1 HD was used at 75 nM (second lanes) or 150 nM (third lanes). The DNA probes were used at a final concentration of 5 mM. Autoradiographs showing binding of TTF-1 HD to TTF-1 binding sites in the GH and PRL promoters. B: protein-bound DNA; F: free DNA. (B, C) Effect of deleting core TTF-1 binding motifs on the regulation of GH and PRL transcription by TTF-1. Three single mutants (200 ng) with core TTF-1 binding motifs deleted from the GH (B) and PRL (C) promoters were cotransfected with 500 ng of the TTF-1 expression vector, TTF-1-pcDNA. The positions of the deleted binding sites are indicated. Data are represented as means ± SEM of 12 wells per each construct. $^{\#\#\#}P < 0.001$ vs. wild type promoter transfected with control pcDNA; $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. change in the wild type promoter activity induced by TTF-1-pcDNA.

Effect of TTF-1 on GH and PRL mRNA levels in GH3 cells

To determine effect of TTF-1 on GH and PRL mRNA levels in GH3 cells, we performed relative RT-PCR experiments using RNA extracts from the cells transiently transfected with TTF-1 expression vectors or shRNA vectors. Overexpression of TTF-1 induced a gradual decrease in GH mRNA level (Fig. 4A), but a marked increase in PRL mRNA level (Fig. 4B). On the contrary, decrease of endogenous TTF-1 synthesis by transfection with TTF-1 shRNA vectors, shown in Fig. 4C, resulted in an increased GH mRNA level (Fig. 4D), but a decreased PRL mRNA level (Fig. 4E). These results suggest that endogenously expressed TTF-1 inhibits GH transcription, but stimulates PRL transcription in the GH3 cells.

Discussion

Homozygous TTF-1 null mice die at birth with multiple defects [9,10]. Specifically, extensive defects were found in the ventral region of the forebrain and in the entire pituitary gland, suggesting that TTF-1 is essential for the formation of the diencephalon and the pituitary gland. Two reports have demonstrated TTF-1 expression in pituitary cells [15,16]. However, a discrepancy exists between these two reports. One identified TTF-1 mRNA in the adult rat AP by *in-situ* hybridization [15], while the other found TTF-1 only in the posterior lobe of the adult rat pituitary gland [16]. In the present study, using FISH, a sensitive localization method, we identified TTF-1 mRNA in the anterior lobe of pituitary gland, and showed that TTF-1 mRNA colocalized with GH- and/or PRL-expressing cells. These results are consistent with the previous report [15]. As for the posterior pituitary gland, TTF-1 was also observed in a small population of cells where it may play a regulatory role in response to high oxytocin levels and vasopressin release [16].

Pit-1 is a pituitary-specific POU-homeodomain transcription factor that plays a key role in the development of the pituitary gland and is required for regulation of the GH and PRL genes in the somatotroph and lactotroph, respectively [17]. Recent reports showed that Pit-1 and C/

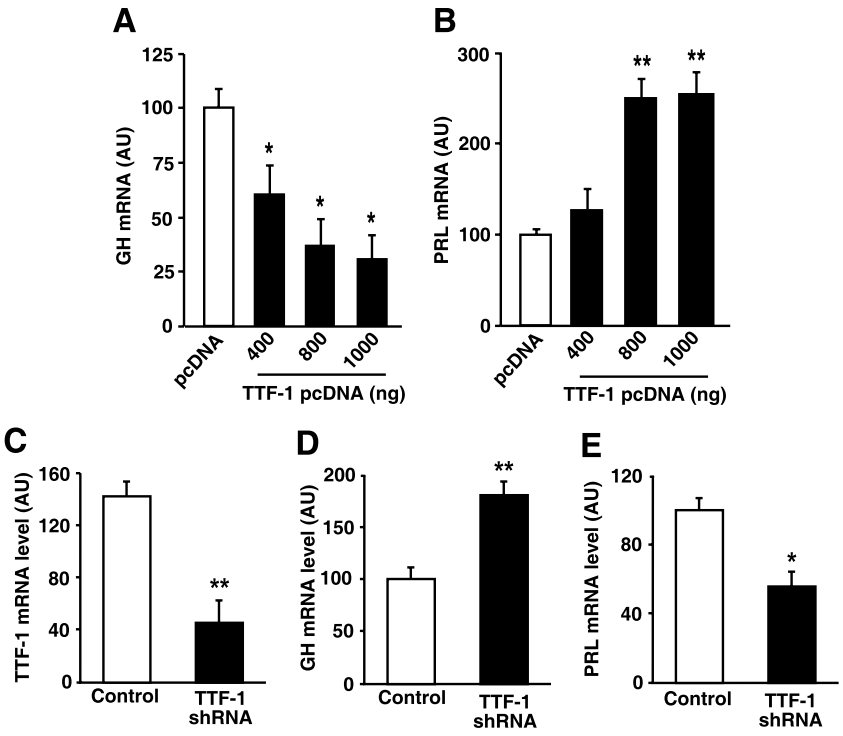

Fig. 4. Changes in GH and PRL mRNA levels in GH3 cells by TTF-1. (A) Dose-dependent decrease in GH mRNA induced by TTF-1 expression. (B) PRL mRNA increased by TTF-1 expression. (C) Effect of TTF-1 shRNA vectors (500 ng) on the TTF-1 mRNA content. Decrease in TTF-1 synthesis induced by shRNA resulted in an increased GH (D), and a decreased PRL (E) mRNA levels, respectively. Values obtained from six different PCR products were calculated and normalized with values from internal control PCR products of GAPDH. All data are represented as means ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control (pcDNA and pLKO.1-puro control vector).

EBPα interact with each other to regulate GH and PRL [14,18]. We identified TTF-1 binding motifs adjacent to the Pit-1 binding elements in the 5′-flanking region of the GH and PRL genes. Moreover, we found that TTF-1 attenuated Pit-1-induced activation of GH transcription, while it further increased Pit-1-induced increase in PRL transcription (Supplementary Fig. 2), suggesting that TTF-1 may partly regulate GH and PRL gene expression by interacting with Pit-1.

One of the most interesting findings in this study was the opposite effect of TTF-1 transregulation on GH and PRL. TTF-1 inhibited GH transcription, whereas it activated PRL transcription. Both somatotrophic (GH-producing) and lactotrophic (PRL-producing) cells belong to the acidophil class of cells in the pituitary gland, and lactotrophs are derived from GH-producing progenitor cells in rodents [19–21]. Some pituitary acidophilic cells appear to secrete GH alone, PRL alone, or both hormones simultaneously, depending on the physiological state of the animals [22–24]. Our data suggest that TTF-1 may regulate the transdifferentiation of cells from GH-secreting somatotrophs to PRL-secreting lactotrophs. This was further confirmed by mRNA levels of GH and PRL, which changed in opposite directions upon increase and decrease of TTF-1 synthesis.

In summary, this study revealed that TTF-1 inhibited GH transcription but activated PRL transcription in the rat AP, suggesting that it may play a role in the regulation of transdifferentiation of cells expressing these two hormones.

Acknowledgments

This work was supported by a Korea Research Foundation Grant (KRF-2002-070-C00068). A.V.D. and G.D. were supported by a grant from MIUR (PRIN n° 2005060778_002). N.O.L., J.G.K., and H.L.L. were partly supported by the BK21 fund.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.08.009.

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