Regulation of Pituitary Adenylate Cyclase-Activating Polypeptide Gene Transcription by TTF-1, a Homeodomain-Containing Transcription Factor*

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[1]The abbreviations used are: PACAP, pituitary adenylate cyclase-activating polypeptide; TTF-1, thyroid

transcription factor-1; icv, intracerebroventricular; VIP, vasoactive intestinal peptide; CRE, cAMP response element; TPA, tetradecanoyl phorbol-4-acetate; TRE, TPA response element; GHF-1, growth hormone factor-1; TTF-1HD, TTF-1 homeodomain; EMSA, electrophoretic mobility shift assay; PCR, polymerase chain reaction; DMEM, Dulbeco's modified Eagle's medium; POA, preoptic area; MBH, medial basal hypothalamus; RPA, RNase protection assay; Cyclo, cyclophilin; AS, antisense; SCR, scrambled; ODN, oligodeoxynucleotide; icv, intracerebroventricular; F2F, footprint II binding factor.

Running Title: TTF-1 regulates PACAP transcription.

Pituitary adenylate cyclase-activating polypeptide (PACAP) is an important hypophysiotrophic factor as well as a regulator for immune, reproductive and neural tissues. Transcription of the PACAP gene is known to be regulated by several well-known transcription factors such as the cAMP response element binding protein, but no specific transcription factor for PACAP gene expression has thus far been reported. We recently found that TTF-1, a homeodomain-containing transcription factor essential for the development of the fetal diencephalon, is postnatally expressed in the hypothalamic area and plays a transcription-regulatory role for certain neurohormones, including luteinizing hormone-releasing hormone. Based on the similarity of synthesis sites between PACAP and TTF-1, and moreover, on the presence of conserved core TTF-1 binding motifs in the 5'-flanking region of the PACAP gene, we sought to uncover a regulatory role of TTF-1 in PACAP gene transcription. The TTF-1 homeodomain binds to 6 of the 7 putative binding domains observed in the 5'-flanking region of the PACAP gene. In the C6 glioma cell-line, TTF-1 activates the PACAP promoter in a dose-dependent manner. This transactivation of PACAP by TTF-1 was totally removed when the core TTF-1 binding motif at –369 was deleted. RNase protection assays showed that TTF-1 and PACAP mRNAs have daily fluctuations in the rat hypothalamus. They both were at low levels during the day and high levels during the night. TTF-1 mRNA showed a peak level at 2100 h, 2 h after lights off and the PACAP mRNA peak followed this 3 h later. Icv administration of an antisense TTF-1 oligodeoxynucleotide significantly decreased the PACAP mRNA level as well as TTF-1 protein content in the rat hypothalamus, suggesting that TTF-1 also regulates PACAP transcription *in vivo*. Moreover, the TTF-1 promoter was inhibited by molecular oscillators of CLOCK and BMAL-1. Taken together, these data suggest that TTF-1 plays an important regulatory role in the gene transcription for PACAP, which may be important for the generation of a daily rhythm of hypothalamic PACAP gene expression.

Pituitary adenylate cyclase-activating polypeptide (PACAP)[1] was originally isolated from the ovine hypothalamus (1, 2), and was classified as a member of the secretin-glucagon-vasoactive intestinal peptide (VIP) family (1). PACAP plays a role of a hypophysiotropic factor and regulates the synthesis and secretion of pituitary hormones through the hypothalamo-hypophysial portal system (3). Althouth PACAP immunoreactivity is found in nearly all brain regions, its highest concentration is in the hypothalamus of both rat and human (3, 4). In the hypothalamus, PACAP immunoreactivity is primarily localized to the cells of the paraventricular and supraoptic nuclei (3, 4). PACAP is found both in neurons terminating on hypophysial blood vessels of the median eminence, as well as in neurons projecting to the posterior pituitary. Therefore, PACAP may act not only as a hypophysiotropic factor regulating anterior pituitary hormone release, but as a posterior pituitary hormone as well. It is also an important factor in the regulation of peripheral tissues such as ovary, testis and immune system.

Recently, the 5'-flanking region of PACAP gene was characterized and shown to have several sequence motifs homologous to the cAMP response element (CRE), the TPA response element (TRE), and the growth hormone factor-1 (GHF-1) binding site (5-7). However, no clear evidence has yet been reported regarding the regulation of PACAP gene expression via its specific transcription factor(s). We searched the recently published 5'-flanking sequence of the rat PACAP gene (7) and discovered several putative transcription factors that could bind to the PACAP promoter, meeting the criteria for core motifs that bind to transcription factors. One of these putative transacting regulatory factors, whose expression sites overlap the synthesis site of PACAP, was TTF-1, a thyroid-specific transcription factor (4, 8, 9).

TTF-1 was first found in the thyroid gland (10, 11), and is also expressed in the embryonic diencephalon and lung (12). In the brains of mice carrying a null mutation of the TTF-1 gene, extensive abnormalities were found in the ventral region of the forebrain from the septal area to the mammillary body of the hypothalamus (13). In the preoptic and hypothalamic area, abnormalities were extensively demonstrated in the ventral and medial regions and, to a lesser extent, in the dorsal and lateral regions.

The ventral hypothalamic area, as mentioned above, was particularly abnormal, suggesting that TTF-1 plays an important role in the formation of this area. Recently, we and other investigators reported that TTF-1 is also expressed in the postnatal rat hypothalamus (8, 9). Specifically, TTF-1 and PACAP mRNA are colocalized in the same preoptic areas (POA), such as the organum vasculosum lamina terminals and the periventricular nucleus, and the medial basal hypothalamic (MBH) areas such as the ventromedial nucleus (4, 8, 9).

Based on the similar localization in certain nuclei of hypothalamus as well as the presence of high-affinity core TTF-1 binding motifs (5'-CAAG-3' or 5'-CTTC-3', 14) in the 5'-flanking region of PACAP gene, we proposed the present study to examine a regulatory role of TTF-1 in PACAP gene expression. In the present study, we found (a) TTF-1 regulates PACAP transcription via binding to its binding motifs in the 5'-flanking sequence of PACAP gene, and (b) hypothalamic PACAP mRNA levels show daily changes during a normal day-night cycle, which coincides with the daily changes of TTF-1 mRNA levels during the same time period.

EXPERIMENTAL PROCEDURES

Primer Extension for PACAP Transcription Initiation Site Determination

To ascertain the transcription initiation site of the PACAP gene, primer extension of mRNA samples from the rat brain was employed. A 32-base oligonucleotide primer (5'-CTTCTCTGCTCCGAGGT CTCAGGCTGAGTCTG-3') complementary to the nucleotides within exon1A of the rat PACAP gene (7) was end-labeled with γ-[^{32}P] ATP. Ten micrograms of rat brain total RNA were hybridized with the radiolabeled primer in 30 µl of hybridization buffer [40 mM bis-Tris (pH 6.4), 0.4 M NaCl, 1 mM EDTA, 50 % formamide] at 65^{o}C for 90 min. After the mixture was slowly cooled to room temperature, the hybridized mRNA was reverse-transcribed at 42^{o}C for 1 h using the MMLV reverse transcriptase (Invitrogen). The extended cDNA products were extracted and separated on a 6 % denaturing polyacrylamide gel. The length of the formed products was determined by comparison to sequencing reactions carried out on genomic clones using the same primer in parallel.

Electrophoretic Mobility Shift Assay (EMSA)

The procedure for expression and purification of the TTF-1 homeodomain (TTF-1HD) has already been described (15). Double-stranded oligodeoxynucleotides, labeled at the 5' end terminal with ^{32}P, were used as probes for the EMSA. Sequences of the oligonucleotides used are shown in Table 1. Oligonucleotides C and Cβ were used as a positive and negative control, respectively (16). EMSA was performed by incubating, for 30 min at room temperature, proteins and DNA in a buffer containing 20 mM Tris-HCl pH 7.6, 75 mM KCl , 0.25 mg/ml bovine serum albumin (BSA), 5 mM dithiothreitol (DTT), 12.5 µg/ml calf thymus DNA, 10% glycerol; TTF-1HD was used at 75 and 150 nM with oligonucleotides at 5 mM. Protein-bound and free DNA were separated on a native 7.5 % polyacrylamide gel run in 0.5 X TBE for 1.5 h at 4°C. Gels were fixed and exposed to a phosphoimager (Bio-Rad, GS525); signals corresponding

to the protein-bound and free DNA were quantitated by using Multi-analyst software. Binding of TTF-1HD to oligonucleotides of the PACAP promoter was expressed as a percentage of the TTF-1HD binding to oligonucleotide C.

Serial Deletion of the PACAP Promoter and Site-Directed Deletion of TTF-1 Binding Core Motifs

The rat PACAP-promoter, luciferase reporter (pGL3-basic) vector was previously described (7). Serial deletion from this promoter was produced with restriction enzymes that cut uniquely in the promoter at the 5'-end (Pst I for –1007, Bxt I for –708, Xba I for –481, and Aat II for –199) and with Spe I of the vector's multicloning site at the 3'-end. These truncated promoters were inserted into the pGL3-basic vector at each corresponding restriction enzyme site within the multicloning site. Promoter constructs containing more detailed deletions between –481 and –199 were generated by PCR reactions with specific primer sets corresponding to the –414, -384, -314 and –267 bp of promoter sequences at the 5'-end, and with a specific primer for a Spe I site in the multicloning site of the pGL-3-basic vector at the 3'-end. Site-specific deletions of core binding motifs for TTF-1 were created with two rounds of PCR reactions. The first-round was performed to generate upstream fragments before the core binding motifs, with an RV3 primer in the pGL3-basic vector and a specific primer placed upstream of each core motif. Downstream fragments of the core motifs were amplified with a specific primer placed downstream, and the GL2 primer placed in the pGL3-basic vector. The second-round of PCR was performed with a specific nested-primer set inside the span between RV3 and GL2 to link the upstream and downstream fragments. These deletion mutants were cloned into the pGL-3-basic vector.

Cell Culture and Assays for Luciferase and SEAP Activity

C6 cells were grown in DMEM: F12 (Sigma) containing 10% fetal bovine serum (FBS); NIH3T3 cells were cultured in DMEM, containing high glucose (4.5 g/l, 25 mM), supplemented with 10% FBS.

PACAP promoter analysis employed the C6 cells. Twenty-four hours after seeding the cells in six-well plates, they were transiently transfected with a PACAP-promoter, luciferase-reporter construct (PACAP-pGL3) using Lipofectamine (Invitrogen), along with different concentrations (100 - 400 ng/ml) of the TTF-1 expression vector (TTF-1-pcDNA zeo). A TTF-1-promoter, luciferase-reporter vector (TTF-1-pGL2) was cotransfected into NIH3T3 cells with expression vectors containing CLOCK (CLOCK-pcDNA zeo) and BMAL1 (BMAL1-pcDNA zeo). Transfection efficiency was normalized by cotransfecting the plasmid pCMV-β-gal (Clontech) at 20 ng/ml. The total amount of DNA transfected was kept constant at 800 ng/ml by adding the appropriate amount of pcDNA zeo to each well. The transfected cells were harvested 48 h after transfection for luciferase and β-galactosidase assays as reported (8). For the SEAP activity assay, medium from cells transfected with the PACAP-promoter-SEAP reporter vector (PACAP-SEAP) was collected 48 h after transfection at 3 h intervals for 24 h. Chemiluminescence detection of SEAP activity was performed according to the manufacturer (Clontech) using Hyper-Film β-Max (Amersham Biosciences) exposed to the base of a 96-well plate.

Animals and Tissue Preparation

Two-month-old male Sprague-Dawley rats (Daehan Animal Breeding Company, Chungwon, Chungbuk, South Korea) were used in this study. They were housed in a room with a conditioned photoperiod (12 h light/12 h darkness, lights on from 0700 – 1900 h) and temperature (23 – 25^{o}C) just after arrival and allowed ad libitum access to tap water and pelleted rat chow. Animals were sacrificed at 3 h intervals for one day, their brains removed and the hypothalamus, including the POA and MBH, were collected. The tissues were quickly frozen on dry ice, and stored at –85^{o}C until RNA isolation.

RNA Extraction and RNase Protection Assay (RPA)

RPA was used to determine the daily change in TTF-1 and PACAP mRNA levels in the hypothalamus.

Total RNA was isolated as reported (17); the RPA employed has been described in detail (18); and the preparation of antisense RNA probes for TTF-1, PACAP and cyclophilin (Cyclo, the internal control), followed a described procedure (8, 19, 20). RNA samples (5 μg/tube) or different amounts of *in vitro*-synthesized TTF-1 and PACAP sense mRNA (0.03-2 pg/tube) were hybridized to 500,000 cpm of ^{32}P-labeled TTF-1 and PACAP cRNA probes, respectively, for 18-20 h at 45^{o}C. The tissue RNA samples were simultaneously hybridized to a Cyclo cRNA probe (5,000 cpm/tube) to correct for procedural losses (18). At the end of the hybridization, the samples were treated with ribonucleases A and T1 to digest unhybridized RNA species. The protected cRNA fragments were separated on a polyacrylamide-urea gel (5% acrylamide, 7 M urea), and the hybridization signals were detected by exposure of the dried gels to X-Omat X-ray film (Amersham Biosciences). The intensity of the signals obtained was quantified as reported (18), using an edited version of the Image program (21).

Icv Administration of Antisense (AS) TTF-1 Oligodeoxynucleotide (ODN)

To determine the inhibitory effect of TTF-1 synthesis on PACAP mRNA levels, phosphorothioate AS TTF-1 ODN was delivered to the ventral region of the third ventricle, and PACAP mRNA levels were determined. Our AS TTF-1 ODN was designed to have the AS sequence against 21 bases that included the translation initiation site based on sequence data (GenBank accession No., X53858): AS TTF-1 ODN, 5'-GAC TCA TCG ACA TGA TTC GGC GTC-3'. As a control, a scrambled (SCR) sequence of AS TTF-1 ODN was used: SCR ODN, 5'-AGT CCT ACT CGG TAC GTA TGC AGC-3'. This sequence does not bear similarities with any eukaryotic sequence thus far deposited in the NCBI GenBank. For the icv injection, the ODNs were diluted to a final concentration of 100 ng/μl in artificial cerebrospinal fluid (ACSF; in mM, 126 NaCl, 2.5 KCl, 1.24 NaH_2PO_4, 1.3 $MgSO_4$, 2.4 $CaCl_2$, 26 $NaHCO_3$, 10 D-glucose, pH 7.3, and bubbled with 95% O_2:5% CO_2). The details for icv administration of ODNs were already described (20). After a week of recovery, the inner stylet was removed and the ODNs (2 nmol in ACSF)

were icv-injected with a Hamilton syringe once a day for 2 consecutive days. The animals were sacrificed at 2400 h (5 h after lights off) on the day following the second ODN administration. Total RNA from most animals or nuclear proteins from some animals were extracted from the hypothalamus.

Western Blot Analysis of TTF-1 Protein

Nuclear protein extracts were prepared according to the method of Andrews and Faller (22), using the cocktail of protease inhibitors recommended by Kuhn et al. (23). Electrophoretically separated polypeptides were transferred to nitrocellulose (NC) paper at 40 mA for 16 h using a transfer buffer of 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3. The NC paper was blocked with 1% BSA in phosphate buffered saline. After incubation with a TTF-1 antibody (Neomaker) for 2 h, bound antibody was detected with an enhanced chemiluminescent (ECL) detection kit (Amersham Biosciences) according to the supplied protocol. The membranes were exposed to the X-ray film for 3 min in the dark room.

Statistics

Changes in TTF-1 and PACAP mRNA levels and SEAP-assay data were analyzed by a one-way analysis of variance followed by the Student Neuman-Keuls multiple comparison test for unequal replications. Changes observed between two groups were analyzed by student's t-test.

RESULTS

Transcriptional Start Site of PACAP Gene

To identify the transcriptional start site of the rat PACAP gene, we performed the primer extension procedure using mRNA from rat hypothalamus. Primer extension showed a single transcriptional start site of the rat PACAP gene located upstream of the translation initiation site (Fig. 1 and 2). Primer extension with the rat hypothalamic mRNA terminated at the G according to a concomitant sequence analysis, while no specific reaction was observed with mRNA from a plant (Fig. 1). Utilizing this result, a single transcriptional start site was mapped at –61 bp, which is also indicated in Fig. 2.

Identification of Consensus TTF-1 Binding Motifs in the Rat PACAP Promoter

To find possible TTF-1 binding motifs in the 5'-flanking region of the rat PACAP gene, the sequence deposited in the NCBI GenBank database by us (7; Accession No., AF163322) was analyzed again with the aid of a search program provided on the internet (www.cbil.upenn.edu/tess/). The 5'-flanking sequence of the rat PACAP gene contains several consensus motifs involved in transcriptional control (Fig. 2): two CRE, a TRE, three GHF-1 and a GATA-1 binding sites. Two binding sites for F2F, which is believed to be a transcriptional repressor (24), are also observed. A consensus progesterone binding site (PRE) was also identified at the distal region of the 5'-flanking region. The transcriptional start site is indicated in the sequence as described above. A TATA-like sequence reported previously (6, 7) is not the functional TATA box, as it is located downstream of the transcription initiation site. The PACAP promoter has 7 putative TTF-1 binding domains, based on the sequence identity with reported conserved motifs of TTF-1 binding domains (14). Two of them are located in the distal promoter region (-1318 and –1303) and the remaining motifs lie at the proximal region. Five of the putative core motifs are 5'-CTTG-3' and two are 5'-CAAG-3'.

TTF-1 Binds to the Putative Binding Domains in the 5'-Flanking Region of PACAP gene

EMSAs were performed to examine TTF-1HD binding to the putative TTF-1 binding domains utilizing double stranded oligodeoxynucleotide probes (Table 1), which contain the sequence of the putative TTF-1 binding motifs in the 5'-flanking region of rat PACAP gene, as shown in Fig. 2. Of the 7 putative binding motifs, six were recognized by TTF-1HD (Fig. 3). Probes at –100, -1303 and –1318 showed relatively strong binding signals, over 50% than that of the positive control of oligonucleotide C, while probes at –220, -369, and -494 revealed weak signals. The probe at –539 did not generate a positive signal, therefore it is not a TTF-1 binding site. Increasing TTF-1HD concentration for EMSA reactions resulted in increased signals for all 6 probes that had displayed positive signals.

TTF-1 Transactivates PACAP Promoter Activity

Transcriptional activation of the PACAP gene by TTF-1 was assessed by functional promoter analysis in C6 glioma cells. Cotransfection with different concentrations of a TTF-1 expression vector resulted in an increase in transactivation of the PACAP promoter in a dose-dependent manner (Fig. 4A). Serial deletion of the PACAP promoter did or did not affect its transactivation by TTF-1 as follows. Deletion of –1474 to –1007, which contains 2 TTF-1 binding motifs at –1318 and -1303, did not affect promoter activity transactivated by TTF-1. Deletion up to –708 resulted in an increase in transactivation of the promoter by TTF-1, suggesting the sequence between –1007 and –708, which does not have a core TTF-1 binding motif may, therefore contain the binding site(s) for a repressor molecule(s). The transactivation by TTF-1 in the promoter with a deletion of up to –481 was clearly reduced compared to the promoter with –708 and similar to that of the promoter with –1474 and –1007, however, a transactivation effect of TTF-1 was still active. Therefore, there may be TTF-1 transactivational binding site(s) between –708 and –481 in addition to the essential TTF-1 binding sites at the more proximal region of the PACAP promoter. This

region contains one core binding motif at –494 (Fig. 2 and 3). A deletion reaching to –199 resulted in a loss of PACAP transactivation by TTF-1, therefore this promoter region has basic essential TTF-1 transactivation motifs; this region has 2 TTF-1 binding core motifs at -369 and –220 (Fig. 2 and 3).

Further deletions between –481 to –199 revealed the essential promoter sequence for the transactivation of PACAP gene by TTF-1 (Fig. 4B). A deletion between –384 to –314 completely abolished TTF-1 transactivation of the PACAP promoter. There is a TTF-1 binding motif at –369 (CTTG), therefore, this sequence seems to be basically critical for TTF-1 transactivation of the PACAP promoter.

Loss of PACAP Transactivation by TTF-1 after Deletion of the TTF-1 Binding Core Motif at –369 bp

Based on the described promoter assay, the sequence placed at -369 appears to be critical for basic transactivation of PACAP by TTF-1, though it showed a very weak binding affinity with TTF-1HD compared to others (Fig. 3). To confirm that this site is essential for the PACAP transactivation by TTF-1, 6 core motifs (including –369) were site-specifically deleted and promoter analyses were performed with these mutated PACAP promoters. TTF-1-dependent transactivation of the PACAP promoter completely disappeared without the core motif at –369 (Fig. 5), further confirming that the binding motif at -369 is essential. Deletions of core motifs at –494, -1318 and -100 resulted in a decrease of PACAP promoter activation by TTF-1 compared to the wild promoter construct, suggesting that these motifs also play an important role in TTF-1 transactivation of PACAP in addition to the basic essential function of that at –369.

Continuous Analysis of PACAP Promoter Activation Induced by TTF-1

Changes in PACAP promoter activity in C6 cells were continuously monitored for 24 h, using the SEAP reporter vector system, in the presence or absence of the TTF-1 expression vector (Fig. 6A). The PACAP basic promoter activity -- without using the TTF-1 expression vector -- showed continuous changes

during a 24 h time period with 2 peaks of activity (Fig. 6B). The peak amplitudes were highly enhanced, without any increase in basal activity, by adding the TTF-1 expression vector. The fluctuation of basal PACAP promoter activity, during a 24 h incubation time, suggests that PACAP transcription has an endogenous rhythm in C6 cells that can be amplified by TTF-1. We tried therefore, to determine what daily changes there were within the rat hypothalamus in TTF-1 and PACAP mRNA levels during a normal 12-h-day and 12-h-night cycle.

Daily Changes of TTF-1 and PACAP mRNA Levels in the Rat Hypothalamus

TTF-1 and PACAP mRNA levels in the 2-month-old male rat hypothalamus were simultaneously determined by single RPAs (Fig. 7). The densities of autoradiographic bands were normalized to those of the Cyclo internal control probe and calculated by comparison to standard curves generated with sense RNAs (data not shown). Representative results of the RPAs are shown in Fig. 7A. Generally, TTF-1 and PACAP mRNA levels are low during the day and high at night. The mean PACAP mRNA level during the night is significantly higher than that during day. Specifically, the TTF-1 mRNA level showed its peak at 2100 h, 2 h after lights off, and the PACAP mRNA level peaked at 2400 h, 3 h after TTF-1 (Fig. 7B and 7C).

Effect of the AS TTF-1 ODN on PACAP mRNA Levels in the Hypothalamus

To determine the *in vivo* effect of TTF-1-synthesis inhibition on PACAP mRNA levels, AS TTF-1 ODN was injected into the ventral region of the third ventricle of 2-month-old male rats. One day after an AS ODN injection/day, for 2 consecutive days, RNA extracted from hypothalamus was used in an RPA. To validate the specific action of AS TTF-1 ODN on the TTF-1 protein level, nuclear protein from the AS TTF-1 ODN-injected rat hypothalami was analyzed with Western blots using a TTF-1 antibody. This antibody detected the expected 40 kDa band of TTF-1 protein (Fig. 8A). The TTF-1 AS ODN clearly

decreased the density of TTF-1-specific bands in Western blots compared to samples from SCR ODN-injected and sham-operated animals (Fig. 8B). The AS ODN also significantly decreased PACAP mRNA levels as determined by RPA (Fig. 8C), suggesting that TTF-1 plays a transactivational role in PACAP mRNA expression *in vivo* as well as in C6 cells. This ODN also decreased TTF-1 mRNA levels which were assessed along with PACAP mRNA (data not shown).

Clock Genes Inhibit TTF-1 Transcription

The enhancement by TTF-1 of PACAP transcription and its mRNA level, both *in vivo* and *in vitro* as described above, suggest that TTF-1 plays a regulatory role in the daily changes of PACAP gene expression and suggest the possibility that TTF-1 expression itself may be under the control of clock gene products, as we found 8 conserved CLOCK-BMAL-1 binding domains (E-box) in the 5'-flanking region of the rat TTF-1 gene (GenBank accession No., X53858). To determine possible involvement of clock gene products in the regulation of TTF-1 transcription, we analyzed TTF-1 promoter activity with or without CLOCK and BMAL-1 expression vectors. We used NIH3T3 cells for TTF-1 transcriptional activity analysis, as the TTF-1 promoter-luciferase reporter vector shows good transcriptional activation, in a dose-dependent manner, in this cell-line, while analysis of C6 cells transfected with the TTF-1 promoter shows relatively low activity (data not shown). CLOCK and BMAL-1 clearly inhibited TTF-1 transcription in a dose-dependent manner (Fig. 9A), while they activated the mPer1 promoter (Fig. 9B), which we used as a positive control for promoter analysis.

DISCUSSION

This study demonstrates for the first time that TTF-1, a homeodomain-containing transcription factor plays a role in the regulation of PACAP gene transcription using both *in vitro* and *in vivo* approaches. To understand the molecular mechanisms underlying the regulation of PACAP by TTF-1, the -1474-long 5'-flanking region of the rat PACAP gene was analyzed with several different approaches. Analysis of this flanking region showed that it does not contain a TATA box, however a TATA-like sequence was reported in the human, mouse and rat PACAP promoter (5-7), lying downstream from the transcription initiation site as determined by the present study; this may be due to a misinterpretation of its sequence. Moreover, it does not contain a downstream promoter element (DPE)-like motif, recently shown to be important for TATA-less promoters (25, 26), downstream from transcription initiation sites. Thus, there are potentially other core promoter elements to be discovered.

Sequence analysis showed several consensus motifs for the transcription factors such as GHF-1, CREB, TREB and PR in the 5'-flanking region of the PACAP gene (5-7). The basal production of PACAP has been known to be constitutive, but is also enhanced by a cAMP analog, TPA, or by PACAP-38 itself (27). Moreover, the mouse PACAP promoter-luciferase reporter fusion gene was greatly stimulated by forskolin and PACAP-38, suggesting that the induction of PACAP expression by these factors occurs at the transcriptional level (6). The presence of PRE in the distal promoter region supports our earlier finding that progesterone increases the PACAP mRNA level of rat hypothalamus (20).

Previously we found several putative down-stream target genes for TTF-1 such as luteinizing hormone-releasing hormone, the neuregulin receptor erbB-2 and preproenkephalin, based on the conserved TTF-1 binding motifs in their promoter sequences and the similarity of their synthesis site with TTF-1 (8). All the above promoters bearing a conserved TTF-1 binding motif were regulated by TTF-1. This approach has again proven to be effective in the present study of PACAP transactivation by TTF-1.

Because the high affinity TTF-1 binding domain is strictly conserved in its target genes (14, 15, 28) and the 5'-flanking region of PACAP gene has 7 conserved TTF-1 binding motif-like sequences, we assayed TTF-1 binding activity to these 7 sites. Among them, six showed binding activity to TTF-1HD, and of these 6, motifs at –1318, -1303 and –100 showed the strongest binding activity; the others bound more weakly. However, the promoter assay with the deletion mutants showed that the sequence at –369, which had only 13% binding with TTF-1HD compared to the positive control of oligomer C, was the most essential for the TTF-1 basic transactivation of PACAP, while mutations of motifs showing stronger binding activity had minor effects on this transactivation. This could well be due to the differences in binding and transcription activity in the 3-dimensional DNA structure *in vivo* and the *in vitro* binding between DNA and a binding protein in an EMSA. Moreover, domains with relatively weaker binding activities have, indeed, been reported to be important in the *in vivo* action of homeodomain-containing proteins (29).

This study shows that binding domains placed at –494 and –1318 are also important for the transactivation of PACAP by TTF-1, in addition to the domain at –369. A detailed comparison between these TTF-1 binding domains and their interrelationship must be studied further. Deletion of the core motif at –494 resulted in less PACAP promoter transactivation by TTF-1, but TTF-1 still activated above the basal promoter activity. Therefore, this site also seems to be important for the TTF-1 action on PACAP gene transactivation in addition to the critical site at –369. This result, using site-directed deletion, confirmed the data showing decreased promoter activation by a deletion between –708 and –481 (Fig. 4A). Deletion of the core motif at –1318 also resulted in a clear decrease in TTF-1 transactivation of the PACAP promoter, compared to that observed in the intact promoter. However, promoter analysis with a deletion between –1474 and –1007, shown in Fig. 4A, did not reveal any difference in promoter activity compared to that observed in the full-sized promoter. The region between –1474 and –1007 may include a suppressor binding domain, as well as the TTF-1 binding motif at –1318; deletion of this suppressor

binding domain might compensate the effect of the –1318 deletion.

Possible suppressor molecule(s) might also act on the sequences between –1007 and –708 region of the PACAP promoter, as deletion of this region clearly enhanced the TTF-1 transcriptional activation of PACAP. According to binding motif analysis, the sequence from -1024, corresponding to those between –1474 and –1007 as described above, and -978 of the 5'-flanking region of the PACAP gene have a putative binding domain for F2F (Fig. 2). F2F is believed to play a role as a tissue-specific inhibitory regulator for prolactin gene transcription (24), and further study will elucidate its role in PACAP transcriptional regulation.

The PACAP promoter activation by TTF-1 explains the daily changes of PACAP mRNA level in the hypothalamus as it is very similar to that of TTF-1 and, moreover, it seems to follow the daily changes in TTF-1 mRNA levels with a 3 h time-gap, i.e., the high level of TTF-1 may induce a high level of PACAP mRNA observed during night, while the low PACAP mRNA level observed during day may relate to TTF-1's low expression. Our SEAP assays showed that PACAP transcription is continually changing during the day and this change was potentiated by the addition of the TTF-1 expression vector, further suggesting that daily changes in PACAP transcription are due to TTF-1 levels. Although we can not yet delineate the detailed mechanism underlying the continuously changing PACAP transcription pattern, the expression and action of TTF-1 appears to be more than coincidental in the potentiation of amplitude of PACAP transcription.

Our findings that CLOCK and BMAL-1 -- transcriptional activators in the circadian expression of several downstream genes -- inhibited TTF-1 promoter activity suggest that they may indirectly affect TTF-1 promoter activity via other transcriptional repressor molecules; otherwise, this is the first report of their having any repressor activity in transcriptional regulation. This finding, however, suggests that TTF-1 is under the control of molecular oscillators such as CLOCK and BMAL-1, thereby mediating circadian changes of PACAP expression. Most components of circadian regulation -- such as CLOCK, BMAL-1,

Per1, Per2 and DBP -- are found not only in the cells of the central circadian rhythm generator, i.e., the suprachiasmatic nucleus (SCN), but also in other cells such as cultured fibroblasts (30). These cells had oscillation patterns similar to those in the SCN, after being triggered with the serum factor, endothelin-1. Thus, this kind of peripheral rhythm generation appears to be dependent upon neural or humoral circadian inputs originally generated in the SCN.

PACAP is a pleiotropic peptide, having numerous actions in the central nervous system and peripheral organs (31), and one of its recently identified functions is the modulation of circadian activity. Photic cues are transmitted to the SCN via the retinohypothalamic tract (RHT) (32), and entrains the SCN and its output rhythms to a 24 h day cycle. PACAP is abundant in the rat SCN, the RHT and retinal ganglionic cells (32, 33), and has been shown to reset the circadian clock in a manner similar to light (34, 35). Taken together, these reports suggest an involvement of PACAP in the regulation of central circadian rhythm in the mammalian SCN.

No clear evidence for transcriptional regulation of PACAP in the RHT and hypothalamus has yet been reported, however. The present data suggest that TTF-1 not only plays a role in the transcriptional regulation of PACAP, but is also involved in the mediation of the daily rhythm of its gene expression. Daily variation of PACAP content was demonstrated in the SCN itself, and moreover, a similar pattern was also observed in the periventricular nucleus and cerebral cortex, although the overall change was not significant in these latter two regions (36). The daily PACAP mRNA changes found in the present study looks similar to that of its protein levels (36), and also shows similarity with already reported daily changes in PACAP receptor mRNA in the SCN and SON (37). Interestingly, our present report of the daily changes in PACAP promoter activity – as determined with SEAP assays – which showed 2 peaks, is very similar with the reported daily change pattern of PACAP receptor mRNA in the SCN and SON (37), as well as the PACAP content in the cerebral cortex (36). While the ramifications for these similarities are unclear, they suggest that similar mechanisms are involved in PACAP synthesis.

Circadian rhythms generated in the SCN – that secondarily regulate rhythms in other nuclei of hypothalamus, via the release of several factors including arginine vasopressin and VIP (38) – may well generate the daily changes in PACAP expression in the hypothalamus. PACAP released from the SCN may also act as a rhythm regulator for hypothalamic cells outside the SCN, as PACAP and its receptor level show daily change outside SCN, as described above. Further examination of PACAP protein, mRNA, and receptor levels in different hypothalamic nuclei would provide support for this possibility.

PACAP is found in neurons terminating on hypophysial blood vessels in the median eminence, as well as in neurons projecting to the posterior pituitary, thus it may act as a hypophysiotropic factor regulating anterior pituitary hormone release, and as a posterior pituitary hormone (3). We believe that the daily changes in hypothalamic PACAP expression are likely essential in controlling the pituitary's rhythm of hormone synthesis and secretion. This hypothesis, of course, remains to be proven.

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Figure Legends

Fig. 1. Transcription initiation site of the PACAP gene. Poly A^+ mRNA was purified from the rat brain and analyzed by primer extension. A clear single signal was generated from rat mRNA, while no detectable specific transcription was found in plant mRNA. Corresponding sequence data shows the single transcription initiation site.

Fig. 2. Nucleotide sequence of the 5'-flanking region of the rat PACAP gene. Nucleotides are numbered by assigning position +1 to the translational start site. Previously reported potential regulatory elements (PRE, TRE, GHF-1 and CRE) are indicated with boxes and the putative TTF-1 binding motifs are underlined. Binding domains for GATA-1 and F2F are indicated, based on sequence analysis (GenBank accession No. AF163322).

Fig. 3. Electrophoretic mobility shift assays (EMSA). EMSAs were performed with double stranded oligomer probes containing putative TTF-1 binding core motifs. TTF-1HD was used at two concentrations (75 nM and 150 nM). The DNA probes were used at a final concentration of 5 mM. Relative binding activities, calculated as a percentage of TTF-1HD binding to probe C, are indicated (RB). Bd, protein-bound DNA; F, free DNA.

Fig. 4. Transactivation of the 5'-flanking region of PACAP by TTF-1. Luciferase reporter constructs containing the 5'-flanking region of the PACAP gene were cotransfected into C6 cells with an expression vector carrying the rat TTF-1-coding region (TTF-1-pcDNA zeo), at the concentrations indicated in the figure. The cells were harvested 48 h after transfection for luciferase and β-galactosidase assays. (A) Transactivation of the different lengths of the PACAP promoter, as indicated in the figure, by different

concentrations of TTF-1. (B) Effect of more detailed serial deletions of the PACAP promoter on its transactivation by TTF-1. Results are the mean ± SEM of at least 6 wells per group.

Fig. 5. Effect of site-specific deletion of core TTF-1 binding motifs on the transactivation of the PACAP promoter. Six single mutants, with core TTF-1 binding motifs deleted, were cotransfected with 400 ng of the TTF-1 expression vector (TTF-1-pcDNA zeo). The positions of the deleted binding sites are indicated. The data are means ± SEM of 6 wells per each construct.

Fig. 6. Continuous analysis of PACAP promoter activity induced by TTF-1. The SEAP reporter vector containing a –1474-long PACAP promoter was cotransfected into C6 cells with the TTF-1 expression vector (TTF-1-pcDNA zeo). The medium was collected at 3 h intervals, for 24 h from 48 h after transfection, for the SEAP assay. (A) A representative autoradiogram showing SEAP assay results. (B) PACAP promoter activity shows the continuous change with or without TTF-1. In the presence of the TTF-1 expression vector, SEAP activity was amplified with two peaks. The data are means ± SEM of 2 different experiments with 3 wells per each SEAP vector construct. The SEAP assay mean for a 24 h duration is indicated on the right. AU=arbitrary unit. **=$p<0.01$ vs other points.

Fig. 7. Daily changes in TTF-1 and PACAP mRNA levels in the rat hypothalamus as assessed by RNase protection assays. (A) Representative autoradiogram demonstrating daily changes in TTF-1 and PACAP mRNA levels. In all cases, cyclophilin mRNA (Cyclo) was used as the normalizing unit. (B and C) Changes in TTF-1 and PACAP mRNA levels in the hypothalamus throughout the day. Points represent means and vertical lines are SEM. Each point represents the mean of four to six independent samples. Open and closed horizontal bars represent the light and dark period, respectively. Mean values for 24 h are indicated on the right. L=light period; D=dark period. *=$p<0.05$ vs points during the light period.

**=p<0.05.

Fig. 8. Effect of TTF-1-synthesis blockade by an antisense (AS) TTF-1 oligodeoxynucleotide (ODN) on PACAP mRNA levels. AS TTF-1 ODN was injected into the ventral region of the third ventricle of 2-month-old male rats, for 2 days with a single injection per day. One day after the second injection, hypothalamic RNA was extracted, and TTF-1 protein and PACAP mRNA levels were determined with Western blots and RPAs, respectively. (A) Western blot analysis showing a decrease in TTF-1 protein by AS TTF-1 ODN (AS) administration. (B) AS ODN significantly decreased TTF-1 protein in the male rat hypothalamus compared to the sham-operated (Sham) and SCR ODN-injected groups (SCR). (C) Significant decrease of PACAP mRNA levels by AS ODN. AU=arbitrary unit. **=p<0.01.

Fig. 9. TTF-1 promoter activity inhibition by clock gene products. TTF-1 promoter-luciferase reporter vectors (TTF-1-pGL2) were transfected into NIH3T3 cells with (+) or without (-) expression vectors containing CLOCK and BMAL1 coding regions. (A) TTF-1 promoter inhibition by CLOCK and BMAL1 expression vectors. (B) mPer1 promoter (mPer1-pGL3) activation by CLOCK and BMAL1.

Table 1. EMSA Oligonucleotide Probes

Location in the 5'-flanking region of PACAP Gene	Probe Sequences
C	5'-CACTGCCCAGTCAAGTGTTCTTGA-3'
Cβ	5'-CACTGCCCAGTCACGCGTTCTTGA-3'
-1318	5'-CTATTTCTCCTTGAAAAGAAG-3'
-1303	5'-GGCTCTCAAGATCTCTTAAAT G-3'
-539	5'-CATACGCAAGGAATTACG-3'
-494	5'-GGAGAGCTTGATGGTGC-3'
-369	5'-GTTCTTTACTTGTCTTTGTG-3'
-220	5'-GTTAATATGTTCCTTGAGGGAC-3'
-100	5'-CAAAATATTCTGTACTTGAAGGC-3'

Each sequence represents the sense strand of probes; core TTF-1 binding motifs are underlined.

Fig. 1.

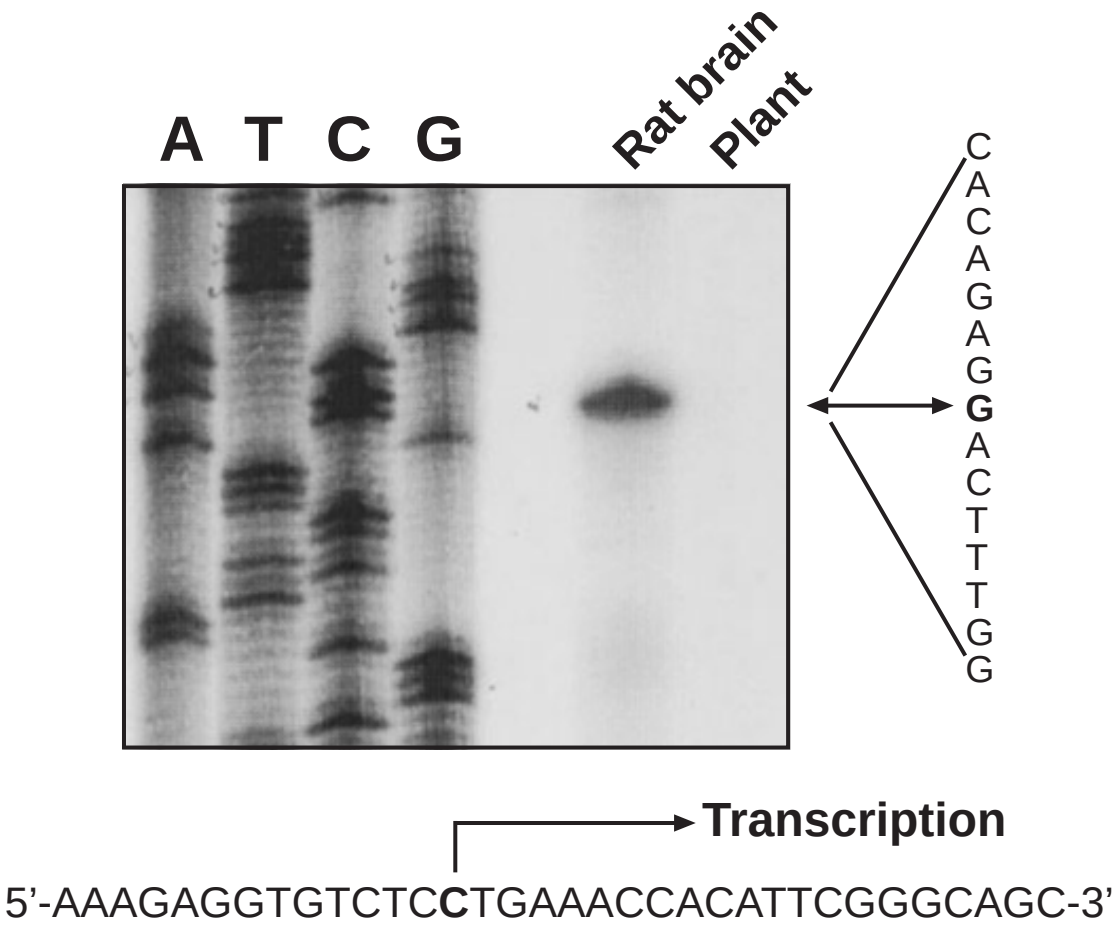
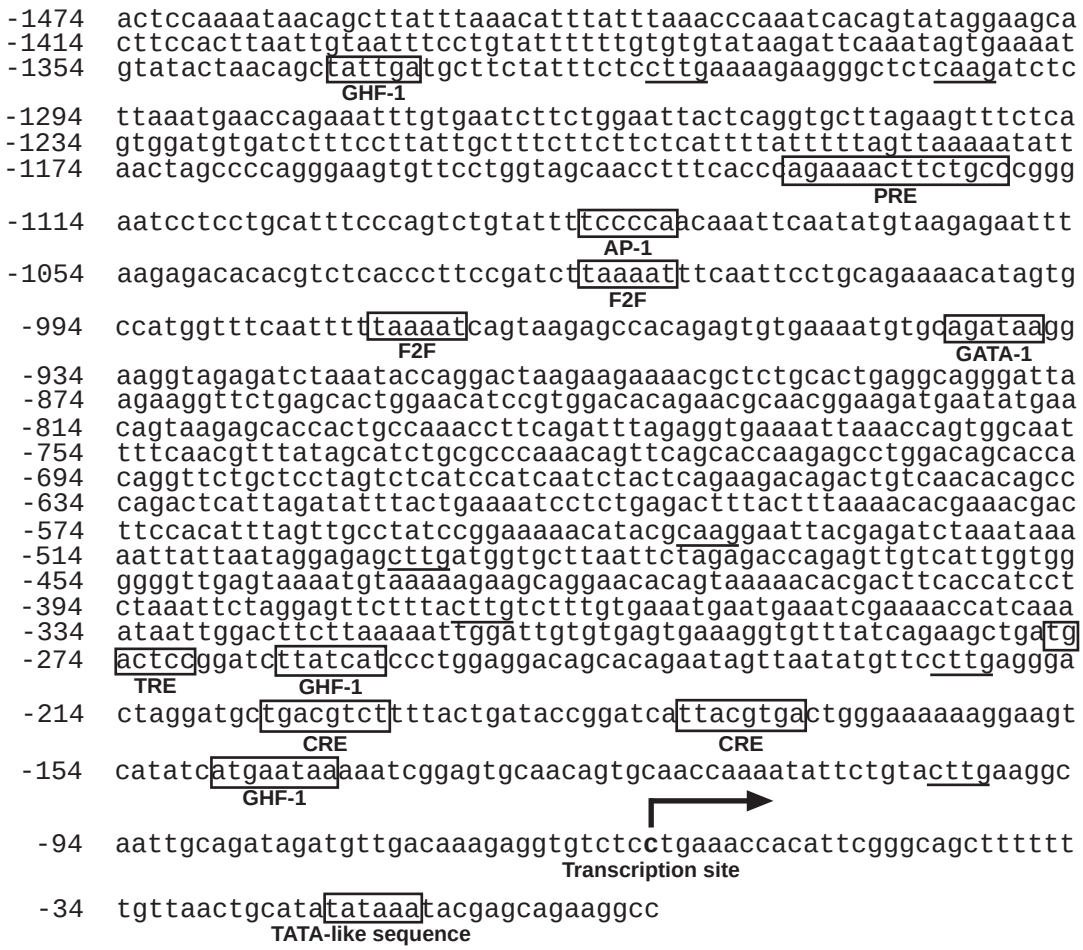


Fig. 2.



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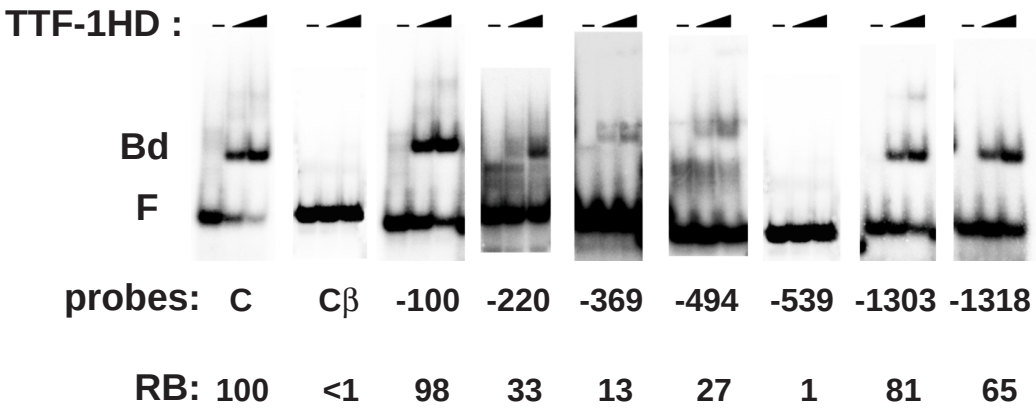

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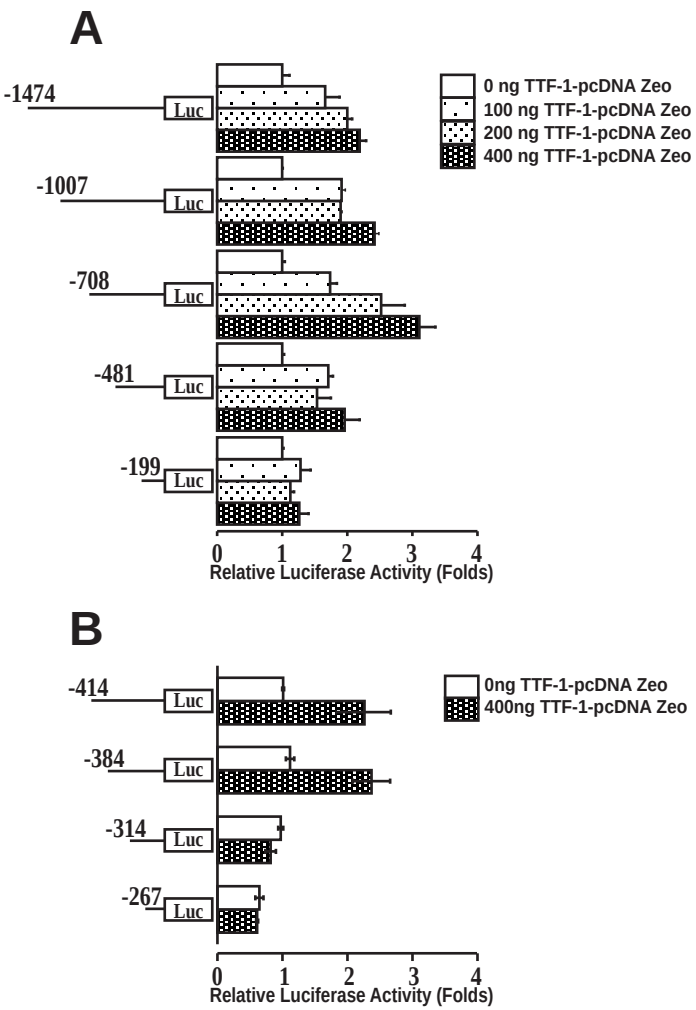

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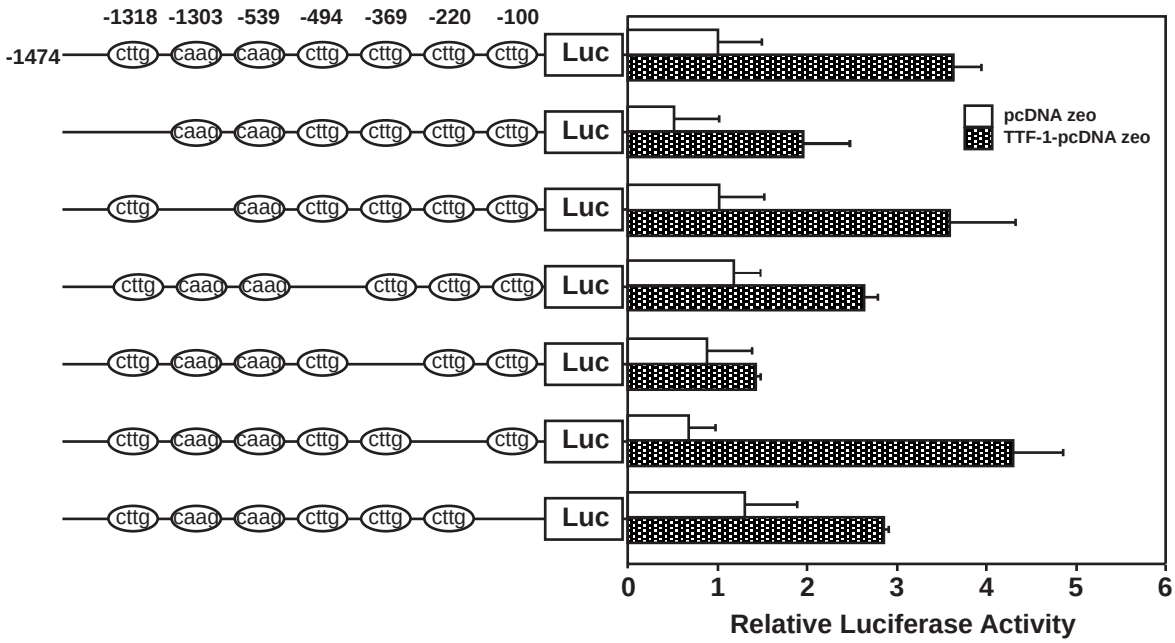

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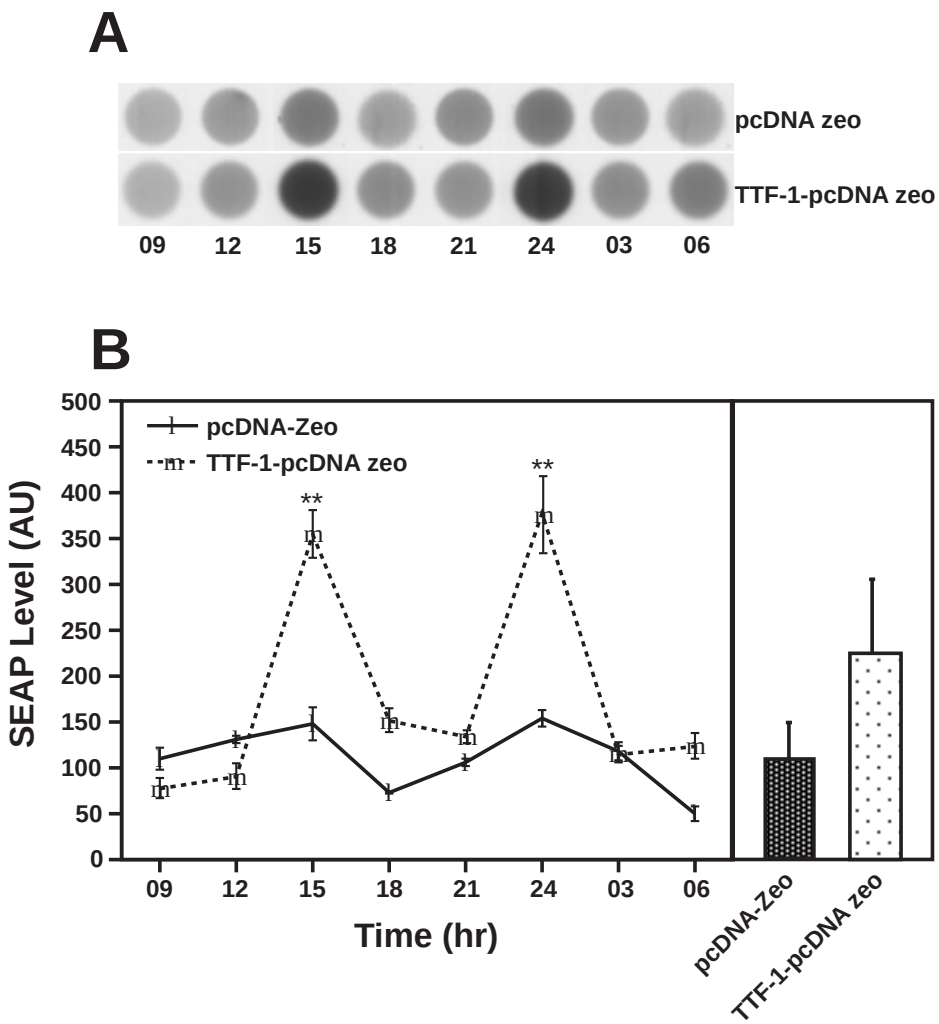

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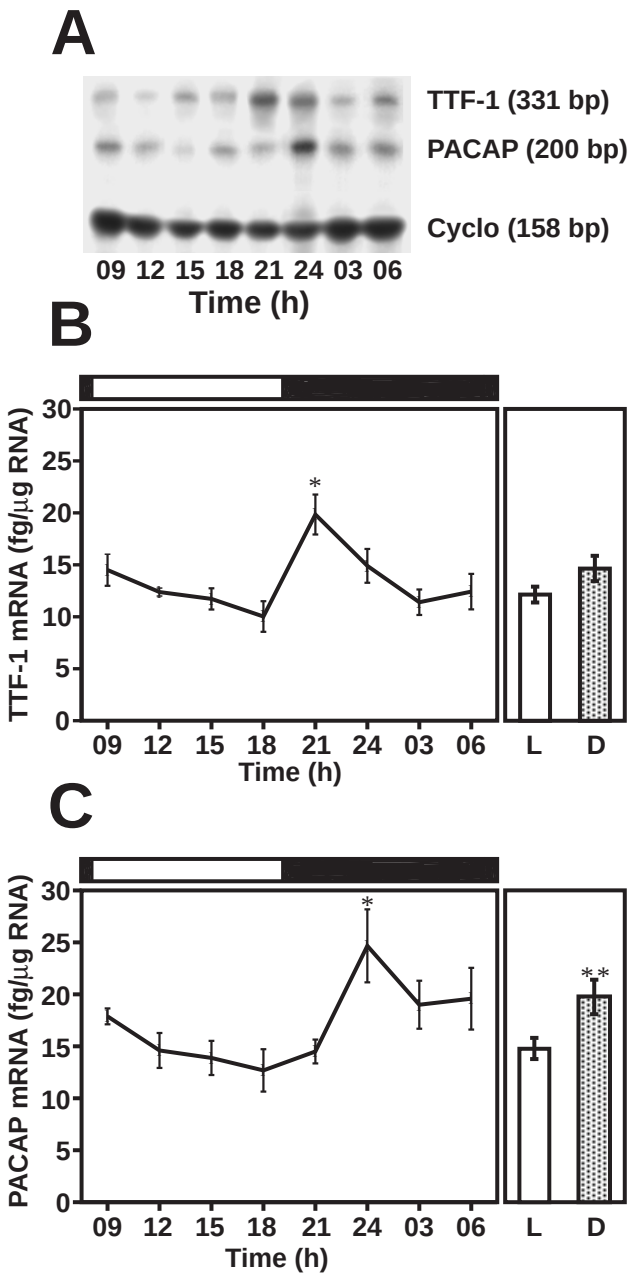


Fig. 8.

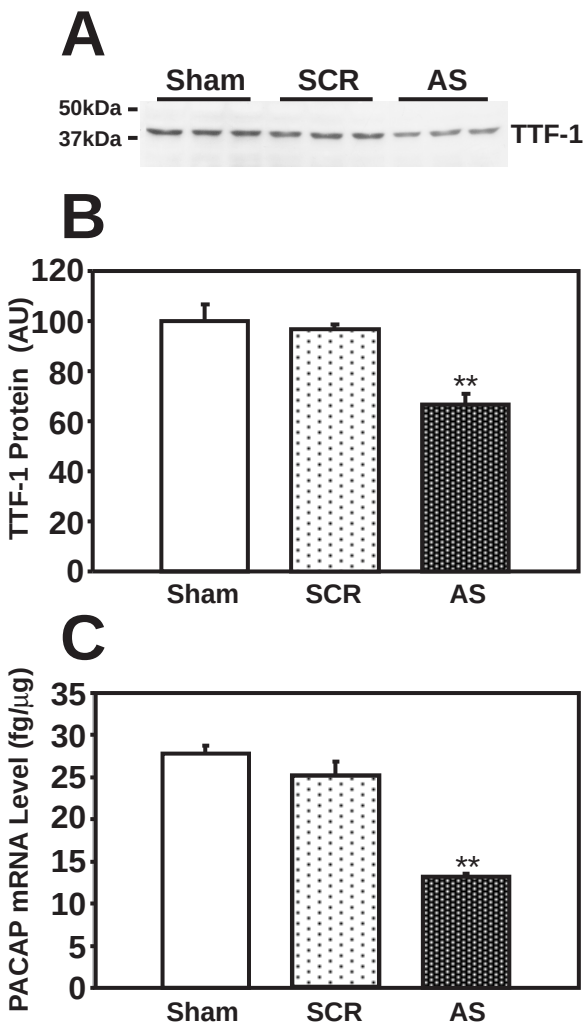
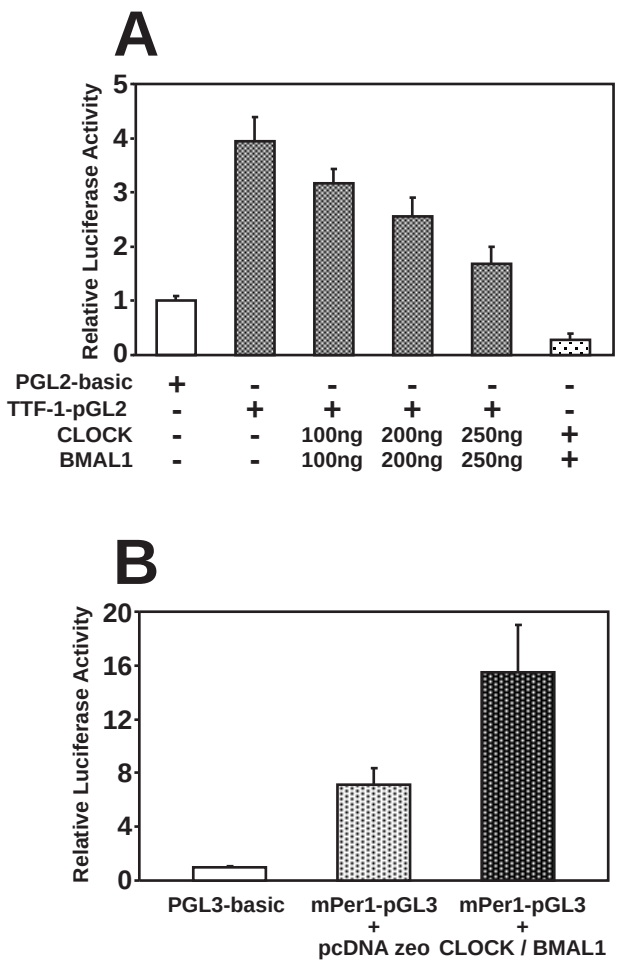


Fig. 9.



Regulation of pituitary adenylate cyclase-activating polypeptide gene transcription by TTF-1, a homeodomain-containing transcription factor

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