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M Kanasaki, … , Y Nio, M Inada

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Research Article

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cAMP Responsive Element-mediated Regulation of the Gene Transcription of the α1B Adrenergic Receptor by Thyrotropin

Mikihiko Kanasaki, Hiroaki Matsubara, Satoshi Murasawa, Hiroya Masaki, Yutaka Nio, and Mitsuo Inada
Second Department of Internal Medicine, Kansai Medical University, Fumizonocho 1, Moriguchi, Osaka 570, Japan

Abstract

To elucidate the molecular mechanism of the stimulatory effect of thyrotropin on the gene regulation of α1B adrenergic receptor in functioning rat thyroid (FRTL-5) cells, we established a competitive reverse-transcriptase (RT) polymerase chain reaction (PCR) and nuclear run-off assay to quantify changes in mRNA levels and transcription rates. A binding assay showed that FRTL-5 cells predominantly expressed α1B adrenergic receptor and that thyrotropin increased its expression sevenfold. By means of RT-PCR, we found that thyrotropin induced an 11-fold increase in α1B receptor mRNA abundance. The nuclear run-off assay demonstrated that thyrotropin caused a ninefold increase at the gene transcriptional level, which occurred in the presence of the protein synthesis inhibitor cycloheximide. The half-life of the α1B receptor mRNA in cells incubated with thyrotropin for 1 h increased 1.5-fold but returned to the original value after 12 h. Dibutyryl cAMP and forskolin mimicked the stimulatory effects of thyrotropin on the gene transcriptional level. The 5′-flanking region of the rat α1B receptor gene contained a putative cAMP responsive element (CRE) at nucleotide −438 relative to the translation start site. The promoter analysis using the reporter gene indicated that the CRE motif confers the cAMP sensitivity to the transcription of the rat α1B receptor gene. These results demonstrated that a CRE-mediated mechanism is involved in the transcriptional regulation of the α1B receptor gene by thyrotropin without requiring new protein synthesis. (*J. Clin. Invest.* 1994. 94:2245–2254.) Key words: thyrotropin • α1B adrenergic receptor • FRTL-5 cell • gene expression • polymerase chain reaction

Introduction

Thyroid hormone formation in functioning rat thyroid (FRTL-5)[1] cells, a continuous line of functioning rat thyroid cells (1), involves a thyrotropin TSH-regulated iodide transport process (2, 3). The TSH-regulated events are separated into two phenomena which are cAMP- and non–cAMP-mediated. The former process involves a sodium-dependent influx of iodide wherein the cell takes up iodide from the medium and concentrates it (2, 4, 5). The non–cAMP-mediated process involves the enhanced efflux from a thyroid cell of concentrated iodide, which is evoked by norepinephrine, A23187, and arachidonic acid (3). The norepinephrine-mediated effect is exerted by an increase in the cytosolic free Ca^{2+} concentration resulting from the interaction with the α1 adrenergic receptor (6). It is reported that the α1 adrenergic receptor-mediated Ca^{2+} response is potentiated by TSH by increasing the receptor density (7, 8).

The α1 adrenergic receptors can be subdivided on the basis of their affinities for the competitive antagonist WB4101, into high and low-affinity sites (9). The irreversible alkylating agent chlorethylclonidine (CEC) inactivates only the low-affinity site for WB4101 (α1B subtype), whereas the other subtype, that is insensitive to CEC and having high affinity site for WB4101, is termed the α1A adrenergic receptor (9, 10). Furthermore, the α1B adrenergic receptor stimulates phosphatidylinositol hydrolysis/intracellular Ca^{2+} mobilization, whereas the α1A adrenergic receptor causes the influx of extracellular Ca^{2+} (10). Recently, it was reported that the α1A subtype can also increase inositol-1,4,5-tris-phosphate formation (11, 12) and the α1B subtype can activate Ca^{2+} influx (13), so the relationship between specific subtypes and signaling mechanisms is still unclear.

The cDNA encoding the α1B subtype was isolated from a hamster DDT$_1$MF-2 cell library, and its pharmacological profile resembled that of the native α1B subtype (14). Tissue distribution of this mRNA is similar to that for the α1B subtype, and it seems likely that this clone encodes the α1B subtype. A cDNA cloned from a bovine brain library encodes a receptor with a unique pharmacological profile and a very rare mRNA distribution (15), and has been designated α1C subtype. Two identical cDNA clones have been independently isolated from rat cerebral cortex (16) and rat hippocampus libraries (17), and designated rat α1A and α1D subtypes, respectively. Although these clones differ from each other by only two amino acids, the radioligand binding data for the expressed recombinant receptors indicate that these two receptors possess almost the same pharmacological properties (17, 18). However, these studies also show that the pharmacological properties of the cloned α1A and α1D receptors are different from those of the pharmacologically defined, native-expressed α1A receptor. Thus, a clone that exhibits a pharmacological property expected of the native α1A subtype has not been isolated.

Shimura et al. (8) have shown that FRTL cells contain mostly the α1B subtype and that TSH enhances the α1B adrenergic receptor density. However, the subtype of α1 adrenergic receptors expressed in FRTL-5 cells is not characterized and it remains unclear whether TSH regulates the α1 adrenergic receptor expression through a gene transcriptional as opposed to posttranscriptional mechanism. TSH increases intracellular

Address correspondence to Hiroaki Matsubara, M.D., Ph.D., Second Department of Internal Medicine, Kansai Medical University, Fumizonocho 1, Moriguchi, Osaka 570, Japan.

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1. *Abbreviations used in this paper:* CEC, chlorethylclonidine; CRE, cAMP responsive element; RT, reverse transcription; TSH, thyrotropin.

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cAMP levels in FRTL-5 cells (6), and cAMP induces an increase in α1 adrenergic receptor expression in hamster DDT_1MF-2 cells (19). Recently, Ramarao et al. (20) determined the 5′-flanking sequence of the human α1B adrenergic receptor gene, in which a putative cAMP responsive element (CRE) was found. In this study, we established a competitive reverse-transcriptase (RT) polymerase chain reaction (PCR) with which to quantify the very small amount of α1B adrenergic receptor mRNA expressed in FRTL-5 cells, and demonstrated that TSH stimulates the gene transcription of the α1B adrenergic receptor through a cAMP-mediated mechanism. In addition, we isolated the clone encoding the rat α1B adrenergic receptor gene and found a putative CRE in its 5′-flanking region. The promoter analysis using the reporter gene system, established that the CRE motif confers the cAMP sensitivity to the transcription of the rat α1B receptor gene in FRTL-5 cells.

Methods

Cell culture. FRTL-5 cells were grown in Coon's modified Ham's F-12K medium, supplemented with 5% calf serum (GIBCO BRL, Gaithersburg, MD) and a six-hormone mixture containing TSH (100 μU/ml), insulin (10 μg/ml), cortisol (4 μg/ml), transferrin (5 μg/ml), glycyl-L-histidyl-L-lysine (10 ng/ml) and somatostatin (10 ng/ml) as previously reported (21). Cells were passaged with a trypsin–collagenase mixture every 10–14 d and grown in 95% air–5% CO_2 atmosphere at 37°C. The culture medium was changed every 3–4 d and the cells were used within 8–10 d. The cells were maintained either in the TSH-containing medium (6H cells) or in the same medium without TSH (5H cells). The duration of exposure to 5H medium before an individual experiment was 5 d unless otherwise noted.

Binding assay. Radioactive [^{3}H]prazosin (DuPont-NEN, Boston, MA) was bound to monolayers of FRTL-5 cells as previously reported (7, 8). Six-well plates (Costar Corp., Cambridge, MA) containing FRTL-5 cells were washed twice with Hank's balanced salt solution (HBSS) (2 ml per well) containing 10 mmol/liter Hepes, pH 7.4, then placed in a binding incubation mixture, consisting of 1 ml HBSS containing 0.5 nM [^{3}H]prazosin; binding proceeded for 30 min at 37°C in an incubator with 5% CO_2 in an air atmosphere. Nonspecific binding was evaluated by including 10 μM phentolamine. The incubation was terminated by rapidly washing the cells twice with 5 ml HBSS, and 2 ml of sodium dodecylsulphate (1%) was added to each dish to measure the radioactivity.

To examine CEC sensitivity to [^{3}H]prazosin binding sites, we used membrane preparations treated with CEC under hypotonic conditions as reported elsewhere (8, 22). Cells were homogenized with a Teflon-glass homogenizer in 20 mM $NaPO_4$ buffer, pH 7.6, containing 154 mM NaCl (PO_4-salt buffer), centrifuged at 12,000 *g* for 20 min, and resuspended in 10 mM Na-Hepes, pH 7.6, to a final protein concentration of 0.5 mg/ml. Aliquots of the resuspended membrane fraction were incubated for 10 min at 37°C without or with CEC (final concentration from 10 pM to 100 μM). Reactions were stopped by diluting the suspensions with cold PO_4-salt buffer. Suspensions were then centrifuged at 12,000 *g* for 20 min, and resuspended in 0.4 ml PO_4-salt buffer. Washing and resuspension were repeated four additional times. The [^{3}H]prazosin binding was assayed in duplicate in aliquots containing 1 mg membrane protein in 0.25 ml PO_4-salt buffer. The mixture was incubated for 30 min at 37°C, then the reaction was terminated by adding 10 ml Tris-HCl (10 mM, pH 7.4). The mixture was passed through a glass-fiber filter soaked with 1 g/liter BSA by vacuum filtration. The filters were washed twice with 10 ml of the same buffer, then the trapped radioactivity was measured. Nonspecific binding was determined in the presence of 10 μM phentolamine. To control for noncovalently bound CEC that may not have been removed completely from the membrane, paired reactions were performed in which the CEC was added to the native membranes after the incubation period but immediately before the washing step as reported (17). The result showed that the reversibly bound drug was negligible, suggesting that the washout of CEC was sufficient to remove all reversibly bound drug. Protein concentrations were determined by the Bio-Rad assay using BSA as a standard and receptor density (B_{max} value) and Kd values were calculated from Scatchard plots (23).

Quantitative reverse transcription and polymerase chain reaction. First-strand cDNA was synthesized from total RNA using a Gene Amp RNA polymerase chain reaction (PCR) kit from Perkin-Elmer Cetus Instruments (Norwalk, CT) with random hexamers (2.5 μM) as previously reported (23, 24). Total RNA was isolated by means of guanidium isothiocyanate-cesium chloride centrifugation followed by digestion with DNase (Takara Shuzo, Kyoto, Japan) to remove contaminating genomic DNA. The following PCR oligo-nucleotide primers (0.5 μM) were designed from the rat α1B cDNA sequences (25): sense from the 5′ non-coding region (5′-GGTAGCTGAGGTGCCTTGGTCGCA-GCCCTTCCG-3′, nucleotide number 167 to 197) and antisense from the coding region (5′-GTTGGACATTTCCTTCATGACTCCCGC-CTC-3′, nucleotide number 952 to 981). To obtain deletion-mutated cRNA, the 784-bp PCR product was phosphorylated with T4 polynucleotide kinase (Takara Shuzo) and blunt-ended with Klenow DNA polymerase (Takara Shuzo) for subcloning into the EcoRV site of pBluescriptII KS(−) (Strategene, La Jolla, CA), which was designated pα1B. The plasmid was cut with MscI and self-ligated to contain an insert lacking MscI–MscI (426 bp). The deletion-mutated cRNAs were synthesized using T7 RNA polymerase (Takara Shuzo) after linearization with Hind III. Total RNA (1 μg) and the deletion-mutated cRNA (1 pg) were simultaneously mixed and assayed by competitive reverse transcription (RT)-PCR. Denaturing, annealing and extension were performed 30 times at 94°C for 45 s, 61°C for 30 s, and 72°C for 1 min, respectively. Native α1B mRNA and deletion mutated cRNA should yield 784- and 358-bp fragments, respectively. Contamination by genomic DNA in sample RNAs was circumvented by amplifying the sample RNAs directly in PCR without RT, in which no significant product was visible after 40 cycles. The specificity of the gene amplification was confirmed by reading the DNA sequence. As an internal RNA control, a labeled β-actin probe was used for Northern blotting as previously reported (26). The autoradiographic signals were measured by a scanning densitometer. To quantify the α1B receptor mRNA, a trace amount (5 μCi) of [^{32}P]dCTP was included in the PCR reaction mixture. The bands of interest were excised from the agarose and ^{32}P incorporation was measured in a scintillation counter. To control for the efficiency of RT-PCR amplification against tube-to-tube variation and the variability in the amount of input RNA, the ^{32}P count in the α1B signal was normalized to both the ^{32}P counts in the deletion-mutated cRNA signal and the β-actin counts measured by the scanning densitometer. The normalized value in an appropriate control was expressed as "1" arbitrary unit for quantitative comparison.

Nuclear run-off transcription assay. Nuclei were prepared from FRTL-5 cells incubated in 6H or 5H medium and a run-off assay was performed as described elsewhere (27). Nuclei ($3–5 \times 10^7$) were incubated for 20 min at 30°C in the presence of 50 mM Tris, pH 7.9, 100 mM KCl, 12.5% glycerol, 6 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 4 mM of ATP, GTP and CTP, 1U/μl RNAsin, and 200 μCi of [α^{32}P]UTP. After RNase-free DNaseI and proteinase K digestion, the reaction products were extracted with guanidinium isothiocyanate (4 M) and phenol/chloroform, and unincorporated [α^{32}P]UTP was removed by trichloroacetic acid precipitation and filtration. The radiolabeled RNA ($2–3 \times 10^7$ cpm) was dissolved in 1 ml of hybridization solution (50% formamide, 5 × SSC, 160 mM Tris, pH 7.5, 1 × Denhardt's solution, 0.5% SDS, 7.5% Dextran) and hybridized at 42°C for 48 h with 5 μg of pBluescriptII KS(−) plasmid immobilized to a nylon membrane (as a control) or with 5 μg of immobilized pBluescriptII KS(−) plasmid containing rat α1B adrenergic receptor cDNA (pα1B) or β-actin cDNA. After washing the membrane in 2 × SSC + 0.1% SDS at 65°C for 1 h, 0.2 × SSC + 0.1% SDS at 37°C for 30 min in the presence of 10 μg/ml RNase A, and 0.2 × SSC at 37°C for 30 min, the bound radioactivity was determined by scintillation counting.

Transcription stability. The stability of α1B adrenergic receptor mRNA in TSH-treated and control FRTL-5 cells was measured by incubating the cells with 5 μg/ml actinomycin D to block transcription as reported previously (28). After various periods of incubation, total RNA (1 μg for TSH-treated cells and 5 μg for control cells) was isolated from individual dishes and RT-PCR was performed.

Library screening and DNA sequencing. Approximately one million plaques of a Lambda DASHII rat genomic library prepared from Sprague Dawley male rat testis (Stratagene, La Jolla, CA) was screened with the rat α1B receptor cDNA fragment corresponding to nucleotide numbers 167 to 981 of the rat α1B receptor cDNA (25) as a probe. The cDNA fragment was obtained by RT-PCR using total RNA from FRTL-5 cells, and the used PCR primers were identical to those in the RT-PCR analysis described above. The cDNA fragment was radiolabeled by random oligonucleotide primer extension and used for the probe (29). Positive clones were purified and characterized by restriction endonuclease mapping and Southern blot analysis. DNA sequencing of specific restriction fragments subcloned into pBluescript (Bs) KS(−) (Strategene) was performed by the dideoxy chain termination method. Oligonucleotide primers were synthesized with an Applied Bio-system 381A automated DNA synthesizer (Applied Bio-systems, Foster City, CA). Methods of restriction analysis, screening recombinant genomic libraries, Southern blot transfer, hybridization to DNA on filters, subcloning into plasmid vectors, agarose and polyacrylamide electrophoresis were performed as described under "Current Protocol in Molecular Biology" (27).

RNA analyses. Primer extension reactions and RNase protection assay were carried out using 40 μg of total RNA from FRTL-5 cells on the basis of "Current Protocol in Molecular Biology" (27) as previously reported (29, 30). For the primer extension reaction, a 30 nucleotide primer (nucleotides +178 to +207 of rat α1B cDNA) (25) was designed. To obtain a cRNA probe, 313 bp of SmaI–SmaI fragment containing 282 bp of the upstream region from the transcription start site was subcloned into pBs KS(−). An antisense cRNA probe was produced by means of T7 RNA polymerase (Takara Shuzo, Kyoto, Japan) after linearizing the plasmid with BamHI.

Plasmid construction. 1755-bp HindIII–XhoI, 929-bp BamHI–XhoI, and 475-bp SmaI–XhoI fragments were subcloned into the upstream of the chrolamphenicol acetyltransferase (CAT)-pBs KS(−) construct (29). The HindIII, BamHI, and SmaI sites were located at −1421, −595, and −141 relative to the translation start site, and therefore these CAT constructs were designated α1B 1421-CAT α1B 595-CAT, and α1B 141-CAT, respectively. Other CAT constructs, α1B 451-CAT, α1B 421-CAT, and α1B 205-CAT were obtained from α1B 595-CAT by means of exonuclease III digestion (Kilo-sequence deletion kit, Takara Shuzo, Kyoto), in which 5′-end points were confirmed by reading the DNA sequence (see Fig. 10 *A*).

A mutation in the CRE motif was created by the PCR overlap extension mutagenesis method (31). Briefly, two DNA fragments having overlapping ends were first amplified from 1755-bp HindIII–XhoI fragment mentioned above, using two sets of primers. The primer had the following sequences: A: 5′-GTGGATCCTGCTCTGCT-3′ (sense: nucleotides -601 to -617), B: 5′-GACTGCACGGCGGTCCGATCA-3′ (antisense), C: 5′-CCGCCGTGCAGTCAGCC (sense), and D: 5′-TCCACGTCAGGCGCGCC-3′ (antisense: nucleotides −261 to −277). Primer B contained a mutated CRE in the 3′ end, and Primer B and C were designed to overlap at the 5′ end (underlined). The two respective PCR products were mixed and amplified again with primer A and D. The resultant PCR product were subcloned into the pGEM-T vector (Promega, Madison, WI), and sequenced using M13 primers to confirm the sequence of the mutated CRE.

DNA transfection and CAT assay. Plasmids were banded in CsCl before transfection. Transfections were performed by the calcium phosphate/DNA precipitate method (29). Cells were incubated for 4 h with 30 μg of plasmid DNA, treated with 15% glycerol for 30 s (glycerol shock), and supplemented with the growth medium (6H medium). After a 3-d incubation, cells were harvested and extracted in 0.25 M Tris-HCl, pH 7.8, by freezing–thawing. Using cell extracts, CAT activity was determined by a dual phase diffusion assay that relies on the direct diffusion of the ^{14}C-labeled acetylchloramphenicol into liquid scintillation counting fluid (29). 0.05 ml of cell extract were mixed with 0.2 ml of reaction mix [1.25 mM chloramphenicol, 100 mM Tris-HCl, pH 7.8, 0.5 μCi/ml ^{14}C-acetyl CoA], overlaid with 5 ml of water-immiscible scintillation fluid (Econofluor) and incubated for 3–4 h. During this period, each vial was counted at 30 min intervals (at least six times). The relationship between incubation time and radioactivity was linear, and the slope (calculated by linear regression) was proportional to CAT activity. In each transfection, 5 μg of RSV-βGal was co-transfected. CAT activity was normalized for transfection efficiency by β-galactosidase activity and for cell density by protein concentration. The β-galactosidase assay was performed as described by Miller, and protein assay was performed by the Bradford method (Bio-Rad) (29). CAT activity was expressed relative to the activity of a promoterless CAT construct (pBs-CAT). Most transfection experiments were repeated a minimum of four separate times. To examine the effect of cAMP on the gene transcription of the rat α1B receptor, the 6H growth medium was exchanged with 5H medium 3 d after the transfection. The cells were incubated in the 5H medium for 12 h and subsequently exposed to dibutyryl cAMP (0.5 mM) and theophilline (0.5 mM) or TSH (0.14 nM) for 12 h, and harvested to examine the CAT activities.

Reagents and statistical methods. All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated below. Results are expressed as mean±SEM. Analysis of variance and the Newman-Keuls test were used for multi-group comparisons. Values of $P < .05$ were considered statistically significant.

Results

Adrenergic receptor characterization in FRTL-5 cells. The [^{3}H]prazosin binding was saturable in the presence (6H) and absence (5H) of TSH, and Scatchard plot analyses showed the presence of single binding site in each (Fig. 1). The dissociation constant (K_d) was 38±1.8 pM in 6H cells and 36±1.3 pM in 5H cells. The maximal binding (B_{max}) was 12±0.9 fmol/10^6 cells in 6H cells and 1.7±0.3 fmol/10^6 cells in 5H cells. Thus, the receptor number in 6H cells was approximately sevenfold higher than that in 5H cells and the affinity was similar, in agreement with previous studies (7, 8).

To characterize the α1 receptor subtype, the FRTL-5 cells were treated with CEC. As shown in Fig. 2 *A*, this caused a dose-dependent decrease in the density of specific [^{3}H]-prazosin-binding sites in both 5H and 6H cell membranes, reaching a maximal decrease of 97±2% and 98±1% at a CEC concentration of 10 μM for 5H and 6H cells, respectively. Fig. 2 *B* shows an inhibition curve for WB4101 with [^{3}H]prazosin in 6H-treated FRTL cells. A Scatchard analysis indicated that the inhibition curve fits a single-site model with a K_d value of 6.8±1.5 nM. These data suggest that most of the α1 adrenergic receptors in FRTL-5 cells are the WB4101 low-affinity, CEC-sensitive subtype.

The α1B and α1D subtypes have a similar affinity to WB4101 (1.9 and 5.9 nM, respectively) on basis of the data of Cos-7 cells transfected with their cDNA clones and both subtypes are extensively inactivated by CEC (17). Thus, our pharmacological characterization of the adrenergic receptor expressed in FRTL-5 cells was poorly selective in distinguishing between the α1B and α1D subtypes. To characterize the subtype, we examined the expression pattern of the α1D subtype on the mRNA level using the RT-PCR method. The PCR primers were designed from the coding sequence specific to α1D cDNA, but not α1B cDNA (nucleotides, 1782–1802 and 2118–2138) (16, 17). No PCR products for the α1D mRNA were

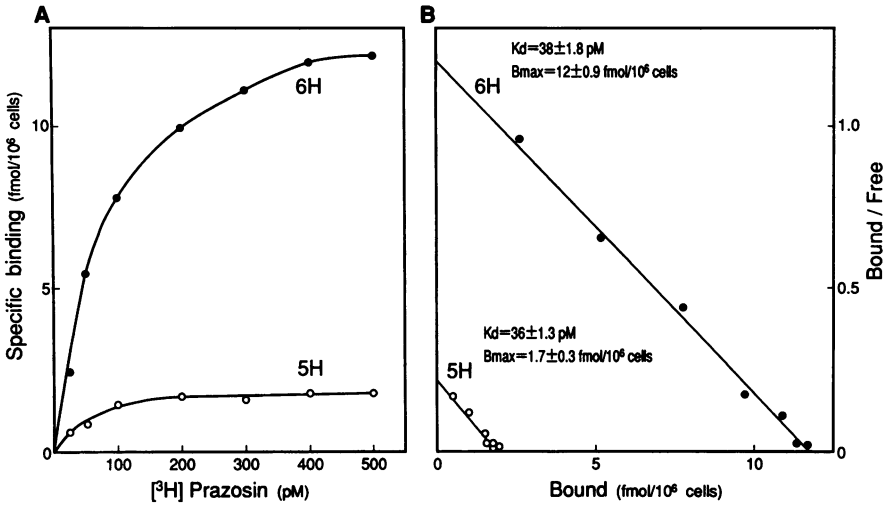

Figure 1. Saturation curves and Scatchard analysis of specific [^{3}H]prazosin binding to FRTL-5 cells maintained in medium without (5H cells, ○) and with TSH (6H cells, ●). Data are the means of three experiments each performed in duplicate and they are representative of three experiments performed according to the same protocol. The error for each value was $< \pm 3\%$.

detected from total RNA of FRTL-5 cells, despite that the annealing temperature was changed in the range from 60 to 37°C, and the PCR cycle was increased up to 50 cycles. On the other hand, an apparent PCR product was observed when total RNA from the rat brain was used as a template. Thus, the α1D mRNA is not expressed in the FRTL-5 cells, and we concluded that the α1 receptor subtype expressed in the FRTL-5 cells is the α1B subtype.

Validity for competitive reverse-transcriptase PCR analysis. We quantified the mRNA levels based on randomly primed RT-PCR as previously described (23, 24), because of the very low levels of α1B receptor mRNA accumulation in FRTL-5 cells. To optimize the quantitative RT-PCR, the range of concentrations of sample RNA and internal control cRNA as well as the number of amplification cycles were chosen within the exponential phase (Fig. 3 *A*), where the quantity of the amplified product would be directly proportional to the quantity of the starting target sequence. The linear relation in the ratio of sample RNA to internal control cRNA was maintained throughout the range we observed (Fig. 3 *B*). We further proved the validity of this method, using two RNA samples: one (sample A) was the total RNA isolated from FRTL-5 cells, and the other (sample B) was composed of 50% Cos-7 cell total RNA that has no detectable α1B signal as assessed by our RT-PCR assay and 50% FRTL-5 cell total RNA (same as that used in sample A). One microgram of sample A or sample B combined with 0.1, 0.5, 1, 2, 3, or 10 pg of the deletion-mutated cRNA was reverse-transcribed, and the resultant cDNA mixtures were amplified by PCR. The ratios of the radioactivity of the α1B signals to those of the cRNA signals were plotted against the amount of cRNA combined with total RNA samples. The ratio of slope obtained from sample A to that from sample B was 1.96, which agreed well with the predicted value of 2.0 (Fig. 3 *C*). This indicates that twofold differences in the total RNA levels are discernible by our RT-PCR assay and that the concentration of the deletion-mutated cRNA in this range is optimized for this assay.

Effects of TSH and cAMP on α1B adrenergic receptor expression. Fig. 4 shows the change in the α1B mRNA level as a function of time after TSH was added to the 5H medium. A significant increase in the α1B mRNA level was evident 1 h after TSH addition. This increase reached a maximal level at 12 h ($\sim$ 11-fold) and remained there for at least 48 h. As shown in Fig. 6 *A*, the number of α1B adrenergic receptors was significantly increased 3 h after TSH addition, reaching a maximal level at 24 h (about sevenfold) which was maintained for at least 48 h. On the other hand, the α1B receptor affinity was not significantly changed by the TSH (36±3 pM in the absence of TSH, 38±4 pM after 24 h of incubation with TSH).

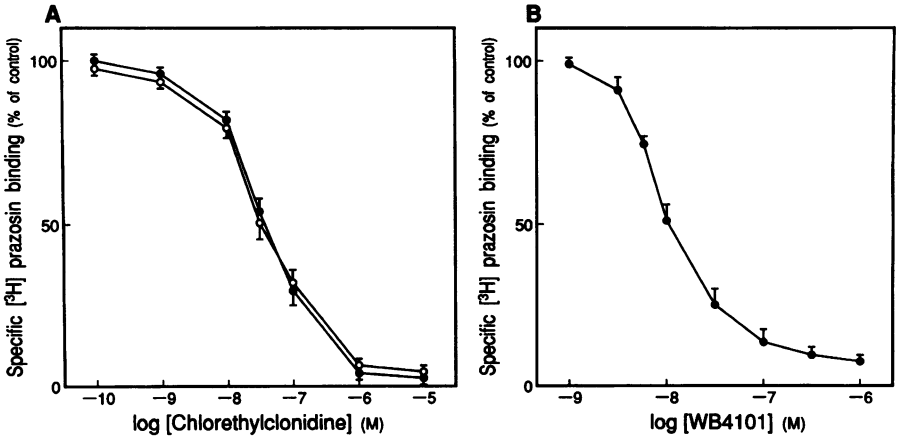

Figure 2. (*A*) Dose-responsive curves for CEC inactivation of [^{3}H]prazosin-binding sites in membrane preparations from FRTL-5 cells incubated in 5H (○) and 6H (●) media. Membranes were incubated with the indicated concentrations of CEC for 10 min in 10 mM Na-Hepes buffer. After washing the membranes, the specific binding of [^{3}H]-prazosin binding was determined. (*B*) Inhibition of [^{3}H]prazosin binding by WB4101 in FRTL-5 cells maintained in 6H medium. Cell monolayers were incubated with 0.5 nM [^{3}H]prazosin and various concentrations of WB4101. The results are expressed as percentages of the specific binding of [^{3}H]-prazosin in the absence of CEC or WB4101. Data are the means of SEM of three experiments each performed in duplicate.

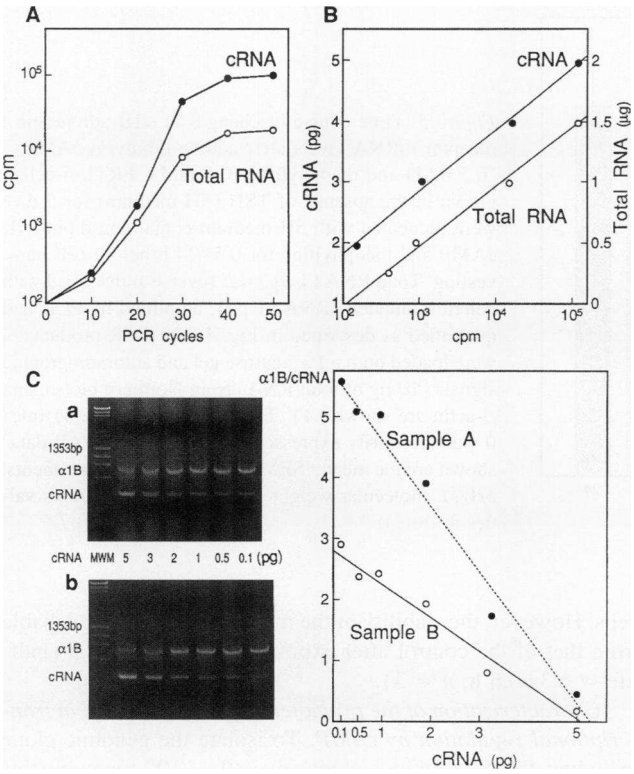

Figure 3. Reverse transcription and polymerase chain reaction quantification of α1B adrenergic receptor mRNA in FRTL-5 cells. (*A*) Total RNA (1 μg, ○) and deletion-mutated cRNA (1 pg, ●) were co-amplified during various PCR cycles. (*B*) Serial dilutions of total RNA (○) and deletion-mutated cRNA (●) were co-amplified for 30 cycles. (*C*) Two micrograms of total RNA (Sample A) was co-amplified with 0.1, 0.5, 1, 2, 3, or 5 pg of the deletion-mutated cRNA. 1 μg of total RNA (Sample B) consisting of Cos-7 RNA (1 μg) and FRTL-5 cell RNA (1 μg), as used in sample A, were coamplified with 0.1, 0.5, 1, 2, 3, or 5 pg of cRNA. The ratios of radioactivity of α1B signals to deletion-mutated cRNA signals were plotted against the amounts of cRNA included in the samples. [^{32}P]dCTP (5 μCi) was included in each PCR reaction mixture. Samples were loaded on agarose gels, and the bands were excised to measure the radioactivity level.

The TSH concentration we used (~ 0.14 nM) markedly increases cAMP levels in FRTL-5 cells while only minimally affecting the Ca^{2+} signal system (6). Since cAMP can mimic the TSH effect upon the number of α1B adrenergic receptors (7), we studied the effect of a cAMP derivative on α1B receptor mRNA levels. As shown in Fig. 5, there was an apparent increase in α1B receptor mRNA levels 1 h after the addition of both dibutyryl cAMP (0.5 mM) and theophylline (0.5 mM). The increase reached a maximal level at 12 h (~ 10-fold) and remained there for at least 24 h. The abundance of the α1B receptor mRNA could also be increased by forskolin (10 μM), which induced a 9.8±1.2-fold increase at 12 h (data not shown). Fig. 6 *B* indicates the time-dependent effect of cAMP on the number of α1B adrenergic receptors. After 3 h, a significant increase was detected, that reached a maximal level at 24 h, which was in good agreement with the TSH effects.

Effect of TSH on gene transcription and mRNA stability of α1B adrenergic receptor. A change in the mRNA abundance of the α1B adrenergic receptor could result from either an alteration in the transcription or degradation rates of the mRNA. We investigated these possibilities, by means of nuclear run-off transcription and transcription stability assays. The nuclear run-off assay is a convenient method with which to measure changes in the transcription rate of the α1B adrenergic receptor gene (32). The results of these experiments were normalized to the rate of transcription of the β-actin gene. The rate of transcription of the α1B adrenergic receptor gene increased 4.2±0.5, 4.7±0.6 and 8.7±0.8-fold in cells that had been exposed to TSH for 1, 3, and 12 h, respectively (Fig. 7*a*) Similar results were obtained in the cells incubated with dibutyryl cAMP (0.5 mM) and theophylline (0.5 mM)(Fig. 7 *A*). A 12-h incubation with dibutyryl cAMP + theophylline and sodium butyrate (1 mM) resulted in a significant increase (1.8±0.1, n = 3) in the relative transcriptional rate, as compared with that in the absence of sodium butyrate.

To determine if the TSH-mediated effects require new protein synthesis, cells were pretreated with cycloheximide (5 μg/ml) for 4 h, and then TSH or cAMP was added. The transcription rate was determined by means of the run-off assay. The transcription rate in the cells incubated with TSH or cAMP was higher by 3.7±0.6 or 4±0.4-fold, respectively, compared with that in cells incubated with cycloheximide alone (Fig. 7 *B*).

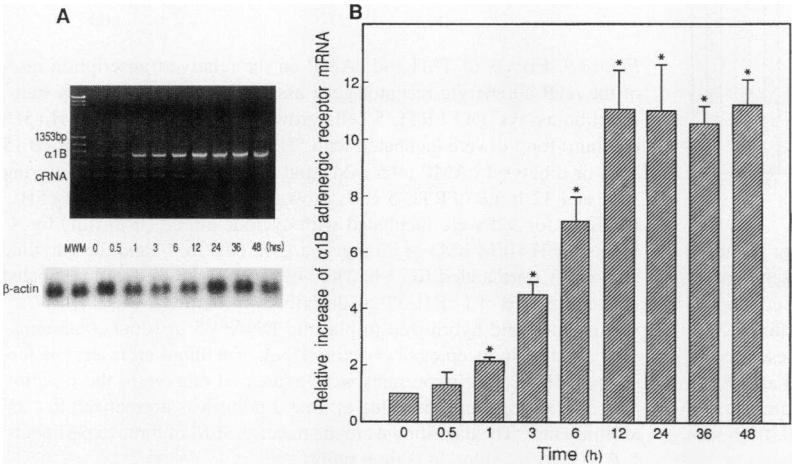

Figure 4. Time course of changes in α1B adrenergic receptor mRNA levels after adding TSH. FRTL-5 cells grown in the absence of TSH (5H medium) for 5 d were incubated with 5H medium containing TSH (0.14 nM) for 0.5–48 h before cell harvesting. Total RNA (1 μg) from FRTL-5 cells was reverse-transcribed with deletion-mutated cRNA (1 pg) and the cDNA mixtures were amplified by PCR in the presence of [^{32}P]dCTP. The PCR products were loaded onto a 1% agarose gel and autoradiographic signals (10 μg of total RNA) from Northern blots using β-actin are shown (*A*). The incorporated ^{32}P counts in the α1B signals were normalized to those in deletion-mutated cRNA and β-actin autoradiographic counts measured using a densitometer. The normalized value at time 0 was arbitrarily expressed as "1" unit (*B*). All data shown are the mean±SEM of at least three experiments. MWM: molecular weight marker. * $P < 0.01$ vs values in 0 time point.

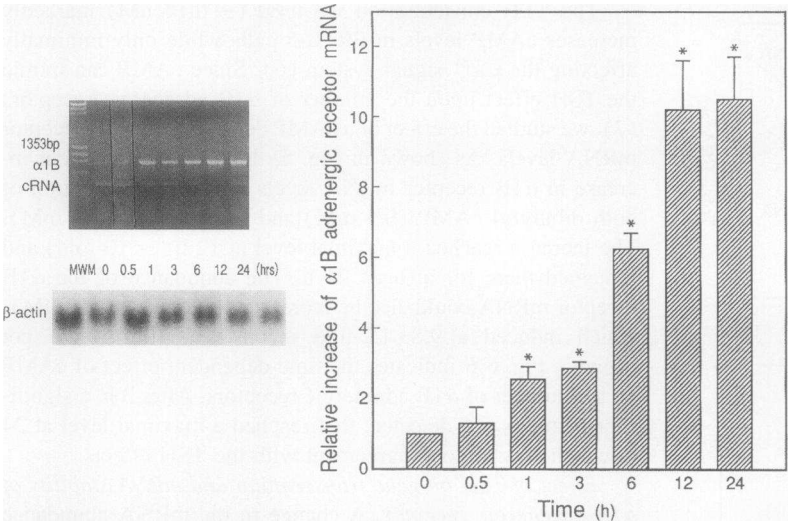

Figure 5. Time course of changes in α1B adrenergic receptor mRNA levels after adding dibutyryl cAMP (0.5 mM) and theophylline (0.5 mM). FRTL-5 cells grown in the absence of TSH (5H medium) for 5 d were incubated with 5H medium containing dibutyryl cAMP and theophylline for 0.5–24 h before cell harvesting. Total RNA (1 μg) was reverse-transcribed with deletion-mutated cRNA (1 pg), amplified by PCR and quantified as described in Fig. 4. The PCR products were loaded onto a 1% agarose gel and autoradiographic signals (10 μg of total RNA) from Northern blots using β-actin are shown (*A*). The normalized value at time 0 was arbitrarily expressed as "1" unit (*B*). All data shown are the mean±SEM of at least three experiments. *MWM,* molecular weight marker. * $P < 0.01$ vs values at time 0.

This result indicates that α1B adrenergic receptor gene transcription is induced by TSH or cAMP independently of new protein synthesis.

The stability of the α1B adrenergic receptor mRNA was examined by inhibiting new mRNA transcription with actinomycin D in intact cells. After incubating the cells with TSH for 1, 3, or 12 h, actinomycin D was added at a final concentration of 5 μg/ml, and the disappearance of α1B adrenergic receptor mRNA as a function of time was measured by RT-PCR (Fig. 8). A half-life of 6.2±0.4 h for the α1B adrenergic receptor transcript was obtained in the control cells. The α1B adrenergic receptor mRNA from cells treated with TSH for 1 and 3 h had a half-life (9.2±0.3 h and 8.3±0.3 h, $n = 3$, respectively) that was increased 1.3- and 1.5-fold compared with that in control cells. However, the stability of the mRNA was indistinguishable from that of the control after exposure to TSH for 12 h (half-life = 6.3±0.6 h, $n = 3$).

Characterization of the promoter sequence involved in transcriptional regulation by cAMP. To isolate the genomic clone including the 5′-flanking region, overall ~ 10^6 recombinants from the genomic DNA library were screened with the ^{32}P-labeled rat α1B adrenergic receptor cDNA probe. Four positive

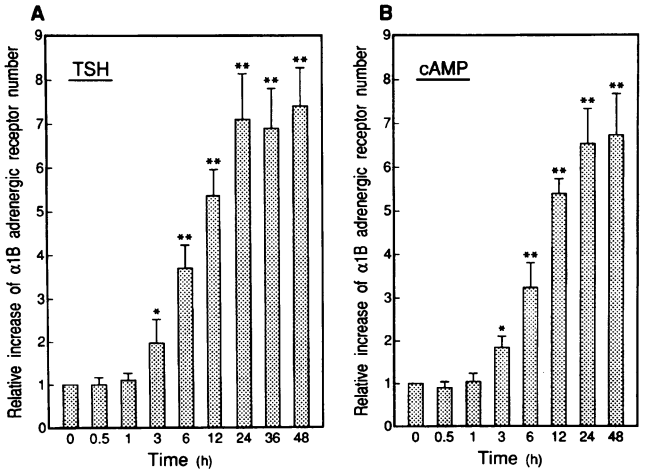

Figure 6. Time course of changes in α1B adrenergic receptor numbers induced by TSH and cAMP. FRTL-5 cells grown in the absence of TSH (5H medium) for 5 d were incubated with 5H medium containing TSH (0.14 nM) or dibutyryl cAMP (0.5 mM) and theophylline (0.5 mM) during 0.5–48 h. A binding assay was performed as described in Methods. The receptor number was calculated by Scatchard analysis and are expressed relative to the value at time 0. The data are the mean±SEM of three experiments. * $P < 0.05$, ** $P < 0.01$ vs values at time 0.

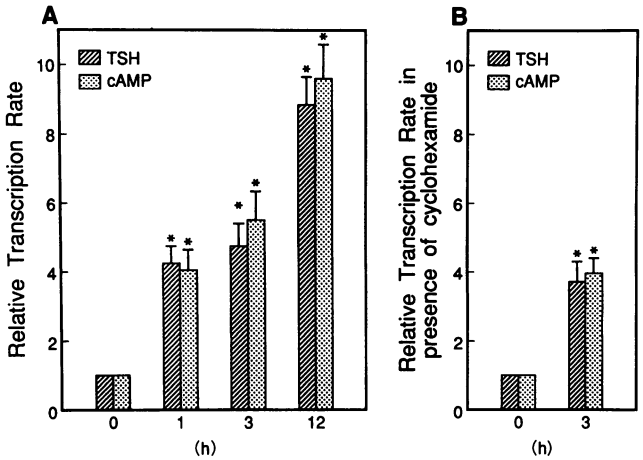

Figure 7. Effects of TSH and cAMP on the relative transcription rates of the α1B adrenergic receptor gene assessed by nuclear run-off transcription assays. (*A*) FRTL-5 cells grown in the absence of TSH (5H medium) for 5 d were incubated with 5H medium containing TSH (0.15 nM) or dibutyryl cAMP (0.5 mM) and theophylline (0.5 mM) during 1, 3, and 12 h. (*B*) FRTL-5 cells grown in the absence of TSH (5H medium) for 5 d were incubated with cycloheximide (5 μg/ml) for 4 h, then TSH (0.14 nM) or dibutyryl cAMP (0.5 mM) and theophylline (0.5 mM) were added for 3 h. The nuclei were isolated and incubated in the presence of [^{32}P]UTP as described in Methods. The ^{32}P-RNA was isolated and hybridized to plasmid DNAs (5 μg/dot) containing α1B adrenergic receptor or β-actin cDNA or without an insert (pBluescript). The transcription rates were expressed relative to the β-actin transcription rate and the value at time 0 point was normalized to "1" arbitrary unit. The data shown are the means±SEM of three experiments. * $P < 0.01$ vs value in 0 time point.

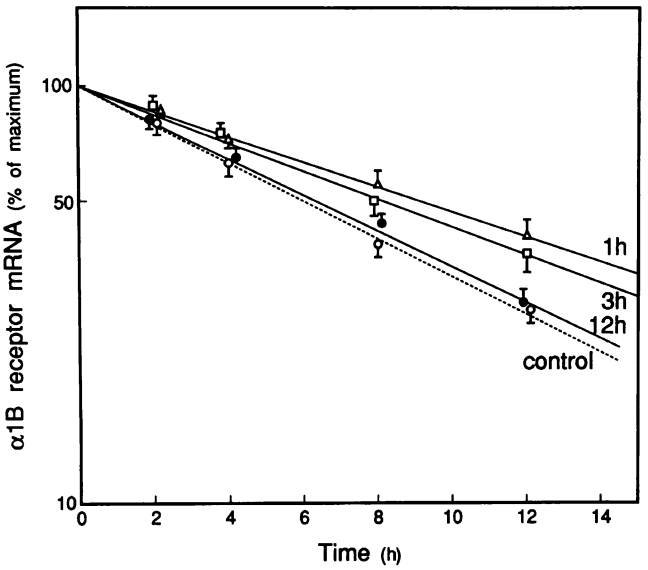

Figure 8. Estimation of the half-life of α1B adrenergic receptor mRNA in FRTL-5 cells in the presence (for 1, 3, or 12 h) or absence of TSH. Actinomycin D (5 μg/ml) was added to FRTL-5 cells grown in 5H medium for 5 d to block transcription and total RNA was prepared from cells after various incubation periods. The decay of α1B adrenergic receptor mRNA values was detected by RT-PCR. The data are means±SEM of three experiments.

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-1620 GACTATCAAG CCAAATAAGA CGGACACATT TTTGTAGACG GTGGGGGCGG GGGACTGAGT -1561
-1560 TCCATCAAGC CTGGGCTTGG CATCGTATCG CTGAAGGTGA GGGTCGACTA TAATTAAATT -1501
-1500 CTGAACATTT CATAAACCGC TGGAGTTGGG AGCATTGTGG AGGCGAAAAC TGAGAATGTT -1441
-1440 TCTCTTTGAA ATCCCAAGCT TCAGAAACTT AAAGGCCCTT GGAAGCTGTG TGGCTGGCTG -1381
-1380 TAAGATGCTC CTATTATCTG GTAAGAACGA GGACAGTGCA GAAAGGAGAG AGCTAGCGGG -1321
-1320 GGGGGGGGGG GGGGGGGGGG GGGGGAAACC GCAGCCTCTG CAAGGCTGAG AAATGTGGTC -1261
-1260 CTGCAAATAT TGAATGAACA CTGCCTCTCT CTGTAGGTGC CGCCTCCCTG CTTCCCCAGT -1201
-1200 GCCGCGTCTG CAGTAACTAC AAACTATTTG GTATGCTGCG TTAGTGACGC TGCCTTCTCA -1141
-1140 GATATATCGT TACTACCGAA ATAACACTGG CGTGATGTTG GAGCCCAGTG TCCTTTTTAA -1081
-1080 TTATTAAACT GAGGCCAGTG ACTGAGTCAT TCCACATCCC AGATTCTCCT CCTGTGCTTC -1021
-1020 TATTTCAAGT GCCTAGTATC TTAGCTTTTT TTTTTTTTTT TTCACCTACA CCTGAGTTGG - 961
- 960 TTCTTAGCCA GTTACCCAAT CTGCAAAGCC CTTGCAGAAC AAATAGTATT ATCCAATCTC - 901
- 900 CAAACACACA CTGATGGCAT AAAAATAACC AAACCCACAC AAAAAAACTT TAGGGTGAGA - 841
- 840 TCATCAGGAA AACCAAGATA GGAAAAGGTC TATCTCCCAT TTGCTAACCC CCAACTTACC - 781
- 780 AAGCGGGAGA GCGATGAAGA AGGGGAGCCA ACATAAGATG AACATTCCGA GTACAATGCC - 721
- 720 CAAGGTTTTG GCTTCTTTCT TTTCCCTGGA GAACTTAAAA AGTTTTACAG CTATGGAACT - 661
- 660 CCTGGGGTTG TGGCCCTTGG CCTTGGTACT GCTGAGGGTG TCCTCATGAA AGTTCTTGGA - 601
- 600 GTGGATCCTG CTCTGCTCCC AGCCCCATCC CCGCAGAGGG AGCGGTGCGG GGCGCGGCTG - 541
- 540 ATCTCCAGGC CGACCCGGCT GGCGCTGGCG TCGGGGCTGC GCGCCTTGGC TGGACCCGCA - 481
- 480 TTGCCCCCTA GTGCGAAAGT CAGGGCGCCC GGGCTTCCCC GCCTGATGTC ACCGCCGTGC - 421
- 420 AGTCAGCCCA GAGGCGCTCA TTGAAAGCAG ACCCTCTTCG CGATCGCTGG GCGGAGAAGG - 361
- 360 CACCGCGGTC CGCAGACCCG CTGCGGCCGG GCACAGGCAC CCCGGCCCGC GCTGCTCCTC - 301
- 300 CCAGCCGTTCC GCCAGCCCGG CCAGGCGCGC CTGACGTGGA CCATTAAAAC TTGGAGCTGT - 241
- 240 CGCGCCTCGT CCCCTCTCTC CTCCTCCTCC TCCTCCTCCT TCTGACAGGC GAGCGAGCCG - 181
- 180 CTGGGTGCAG GCAGGCGACG TGCTGCCGGG CTAGGCTGCC CGGGGAGATG ACTTCTCGCC - 121
- 120 AGGAGGACGC CTCTGGAAAG AAGACCACGG AGGGAGCAAA GTTTCAGGGT AGCTGAGGTG - 61
- 60 CCTTGGTCGC AGCCCTTCCG AGCCCAATCT CCTCCCTGGC TATGGAGGGC GGACTTTAAA - 1
+ 1 ATGAATCCCG ATCTGGACAC CGGCCACAAC ACATCAGCAC CTGCCCACTG GGGAGAGTTG + 60
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Figure 9. The nucleotide sequence of the 5′-flanking region of the rat α1B adrenergic receptor. The first nucleotide of the ATG initiation codon is designated +1. The transcription initiation site is indicated by arrow. A putative cAMP responsive element (CRE) motif is boxed. A potential Sp1 binding site and CAAT box are underlined. Those sequence data are available from EMBL/GenBank under accession number D32045.

clones were isolated, and analysed further by restriction enzyme mapping and Southern blot. The analyses indicated that these clones were segregated into two overlapping clones, in which one clone contained the 5′-flanking region and subsequent cording region of the rat α1B receptor gene. The nucleotide sequences of the 5′-non cording (240 bp) and coding regions up to the internal BamHI site (762 bp) were completely identical to those of the rat α1B cDNA reported by Voigt et al. (25), confirming that the gene encodes the rat α1B receptor. Fig. 9 shows the 1621-nucleotide sequences upstream from the translation start site. There was a very high similarity (87%) in nucleotide sequences 500 bp upstream from the translation start site between rat and human (20) genes. The primer extension and RNase protection analyses using total RNA prepared from FRTL-5 cells, indicated a single transcription start site located at 173 bp upstream from the translation start site, corresponding to the transcription start site for the human α1B receptor gene (20) (data not shown). The TATA box was not found in the 1621 bp 5′-flanking region as observed in the human α1B gene, whereas the CAAT box was present at -908 and -945 relative to the translation start site. A putative CRE and the GC box were located at -428 and -373, respectively (Fig. 9).

To examine the role of cAMP responsive element (CRE) in the transcriptional regulation by cAMP, we constructed a series of CAT expression plasmids. A portion of the α1B receptor gene from −1421 to +95 relative to the translation start site was inserted into the plasmid pBs immediately upstream of the CAT gene coding sequence (Fig. 10 *A*). A series of deletion mutants extending from −1421 toward the translation start site were constructed. The deletion mutant end points were −1421, −595, −451, −421, −205, and −141, respectively. The CAT activities normalized with β-galactosidase activity and protein concentration are shown in Fig. 10 as values relative to those in promoterless pBs-CAT construct. Transfection of α1B 1421-CAT construct into FRTL-5 cells resulted in a 6.4-fold increase in the CAT activity relative to the promoterless CAT construct. NIH3T3 cells that has no mRNA message for the α1B receptor gene did not yield any significant CAT activity. Deletions from -1421 to -595 and -421 to -205 resulted in a decrease in the CAT expression (1.8-fold and 2.1-fold, respectively), indicating the presence of positively cis-acting elements. Deletions from −595 to −451 and −451 to −421 did not significantly affect the CAT activity. Addition of cAMP and theophylline induced about threefold increases in the CAT activities of α1B 1421, α1B 595 and α1B 451 CAT constructs, respectively, suggesting that the promoter region involved in the transcriptional regulation by cAMP is located within the 451 bp upstream from the translation start site. Further deletion from −451 to −421 failed to confer the cAMP sensitivity to the α1B receptor gene (Fig. 10 *A*), suggesting that the promoter region −451 to −421 containing the putative CRE is closely involved in the transcriptional regulation by cAMP. To ascertain whether the CRE located at −438 can confer the response to cAMP, we next mutated the putative CRE element. The result shows that the promoter activity of α1B 451-mut, a reporter gene construct where the highly conserved GTCA motif found in many CREs was disrupted, is not enhanced by cAMP (Fig. 10, *A* and *B*). Similar observations were also obtained in the presence of TSH (data not shown), confirming that TSH regulates the gene transcription of the α1B receptor by a cAMP-mediated mechanism. These results clearly establish that the CRE motif located at −438 confers the cAMP sensitivity to the α1B receptor gene.

Discussion

The maintenance of FRTL-5 cells in vitro (1) has made it possible to study the mechanisms by which TSH regulates io-

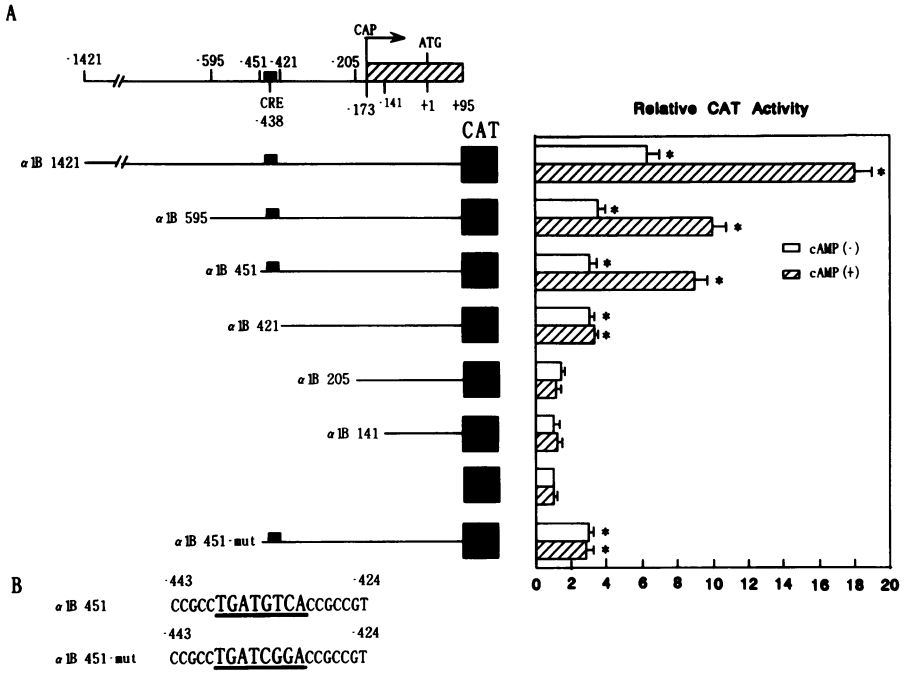

Figure 10. (*A*) Schematic maps of CAT fusion genes containing 5′-deletion mutants of rat α1B gene promoter and cAMP-induced effects on CAT expression. The CAT fusion genes were transfected into FRTL-5 cells and incubated for 3 d. The cells were incubated in the TSH-free medium for 12 h and subsequently exposed to dibutyryl cAMP (0.5 mM) and theophilline (0.5 mM) for 12 h. The CAT activity levels, normalized with β-Gal activities and protein contents, are expressed relative to those in promoterless pBs-CAT, which is assigned a value of 1.0. All data shown are the means±SEM of four separate experiments. * $P < 0.01$, vs. promoterless pBs-CAT. (*B*) Sequence comparison of the wild-type and mutated CREs including the contextual sequences. The location relative to the translation start site is indicated. *CAT*, chrolamphenical acetyltransferase.

dide transport (2–5) in normal thyroid cells in vivo. The iodide transport in FRTL-5 cells can be resolved into cAMP-dependent iodide influx and Ca^{2+}-dependent iodide efflux (2–5). The in vivo physiological counterpart of the former is iodide transport from the blood stream into the thyroid cells (2, 4, 5). The iodide efflux process is involved in the movement of iodide out of the thyroid cells into the follicular lumen in the physiological counterpart in vivo (3). Iodide efflux can be stimulated by noradrenaline through intracellular Ca^{2+} mobilization mediated by α1 adrenergic receptors (6). Corda et al. (7) have reported that TSH upregulates the number of α1 adrenergic receptors through a cAMP-mediated process, and suggested that two receptors (TSH receptor and α1 adrenergic receptor) and two distinct signal systems (cAMP and Ca^{2+}) are used to regulate thyroid hormone homeostasis. However, it remains unknown whether the stimulatory effect of TSH on α1 adrenergic receptor expression is due to induction at the gene transcription level or to modification at the posttranscriptional level. In addition, the effect of cAMP on the gene transcription of α1B adrenergic receptor has not been reported to date. In this study, we have pharmacologically characterized the subtype of the α1 adrenergic receptor expressed in the FRTL-5 cells as the α1B subtype, and demonstrated that either TSH or cAMP dramatically increases the mRNA accumulation and the numbers of α1B adrenergic receptor by promoting gene transcription without involving new protein synthesis. That TSH in concentrations from 1 pM to 1 nM increases intracellular cAMP levels in FRTL-5 cells, but does not affect intracellular Ca^{2+} mobilization (6), suggests that the stimulatory effect of TSH (0.14 nM in the present study) upon α1B adrenergic receptor expression is mediated through a change in the cAMP level.

The addition of cAMP led to an increase in α1B adrenergic receptor mRNA abundance by 1 h, and the values peaked around 12 h, although they remained increased for at least 48 h. The receptor number increased more slowly. The number of α1B adrenergic receptors expressed in FRTL-5 cells significantly increased at 3 h, reaching steady-state values at ~ 24 h. The relationship between mRNA abundance and protein synthesis may be complex. One explanation for the more slowly developing increase in the receptor density could be as a consequence of the relatively long half-life of adrenergic receptors (typically in the range of 24 h) (33). If the half-life of α1B adrenergic receptors is in this range in FRTL-5 cells, then it would take considerable time for receptor concentrations to plateau after a change in the rate of receptor synthesis.

A nuclear run-off assay demonstrated that the transcription rate of 12 h after exposure to cAMP was about 9.5-fold higher than that in control cells and that this increase in transcription is likely to be a major determinant of the elevated mRNA abundance. Although the molecular mechanisms of cAMP-induced transcription are complex and not completely determined, it is suggested that there are two groups of cAMP-responsive genes (34). One contains genes that are rapidly (within minutes) regulated by cAMP, whereas the transcription of genes in the other group is increased only after several hours of increasing cAMP concentration. The increase in the rate of transcription of the α1B adrenergic receptor gene found in our study is somewhat slower than that reported for the genes rapidly responsive to cAMP. In these genes the stimulatory action of cAMP is mediated by promoter elements (cAMP response elements) with the consensus sequence T(G/T)ACGTCA within the target gene, that is recognized by specific phosphoprotein transcription factors (35). Recently, a cAMP response element that is rapidly activated by both cAMP and protein kinase C has been identified. This sequence CCCC(A/T)GGC, termed activator protein 2 binding site, is a transcription enhancer, which is present in the SV-40 and human metallothionein IIA promoters (36). On the other hand, some other genes induced by cAMP, for example lactate dehydrogenase and chorionic gonadotropin β-subunit genes, have increased rates only after several hours exposure to cAMP (37, 38). This slow response may be due to a requirement for new protein synthesis. Here, we isolated

the genomic clone encoding the rat α1B receptor and determined the nucleotide sequences of the 5′-flanking region. The proximal part of the 5′-flanking region, 500 nucleotides upstream from the translation start site, had a high similarity between rat and human (20) genes and both genes had the same transcription start site. The TATA box did not exist in the 5′-flanking region, whereas the GC box and the putative CRE were found at −373 and −438 relative to the translation start site. No activator protein 2 binding site was present in the promoter region. The analysis using a reporter gene system indicated that the CRE motif confers the cAMP sensitivity to the transcription of the rat α1B receptor gene. In addition, its transcription was not induced by phorbol esters (data not shown) and did not require new protein synthesis.

The rate of mRNA degradation is another important regulatory mechanism in the control of gene expression. It has been demonstrated that cAMP regulates not only the rate of transcription of the gene for lactate dehydrogenase or phosphoenolpyruvate carboxykinase, but that the stability of the mRNA is also increased by cAMP during the induction phase (37, 39). However, for the tyrosine aminotransferase gene, a cAMP analogue led to decreased stability of the mRNA in the face of an increased rate of transcription (40). In our studies, the half-life of the α1B adrenergic receptor mRNA in the control FRTL-5 cells was about 6 h, a value that is somewhat longer than has been reported for several other genes transcriptionally regulated by cAMP (32, 34). The half-life of the α1B adrenergic receptor mRNA was increased about 1.5- and 1.3-fold after 1 and 3 h exposures to TSH, respectively. In view of this relatively small increase, this change is not likely to be the major mechanism that leads to an elevation in mRNA accumulation. The change in the mRNA stability was transient as it was no longer evident after 24 h of exposure. Although the mechanism of the cAMP-mediated regulation of mRNA stability is not clear, the sequences and secondary structures near or at the 3′ terminus may influence the susceptibility of mRNAs to attack by nucleases (41).

The prolonged exposure to cAMP results in a decrease in β2 adrenergic receptor mRNA abundance (42), whereas that for the α2A adrenergic receptor is upregulated by cAMP at the transcriptional level (32). This study demonstrates that the α1B adrenergic receptor gene is also upregulated by cAMP at the transcriptional level. Given that β2 adrenergic and α2A adrenergic receptors have stimulatory and inhibitory effects on adenylate cyclase activity, respectively, the relationship between the cAMP signal system and the gene regulation of adrenergic receptors seems quite complex and requires further investigation.

Acknowledgments

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