Identification of an AP-1-like Sequence as the Glucocorticoid Response Element in the Rat Tyrosine Hydroxylase Gene

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ABBREVIATIONS: TH, tyrosine hydroxylase; PC12, pheochromocytoma cell line; GR, glucocorticoid receptor; GRE, glucocorticoid response element; AP-1, activator protein 1; CRE, cAMP-response element; PKA, cAMP-dependent protein kinase A; Dex, dexamethasone; TPA, tetradecanoyl-12,13-phorbol acetate; bp, base pair (s); Luc, luciferase; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation

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ABSTRACT

Glucocorticoids (GC) generally stimulate gene transcription via the consensus glucocorticoid response elements (GRE) located in the promoter region. To identify the GRE in the rat tyrosine hydroxylase (TH) gene promoter, we transiently transfected PC12 cells with a 9 kb TH promoter-luciferase (Luc) construct. Dexamethasone (Dex) stimulated Luc activity, which was abolished by RU-486. Serial deletion mutations revealed a Dex-responsive 7-bp sequence TGACTAA, located at -5734 to -5728. Deletion of just these 7 nucleotides from the 9 kb promoter completely abolished the Dex response and partially reduced the response to phorbol ester, but not to forskolin. The Dex response was fully retained in a construct in which most of the 9 kb promoter was deleted, except for 100 bp around the -5.7 kb region, clearly identifying this 7-bp sequence as solely responsible for GC responsiveness. Conversely, deletion of the proximal CRE (-45/-38) or AP-1 (-207/-201) sites in the 9 kb promoter did not affect Dex and phorbol ester responses. A radiolabeled 25 bp promoter fragment bearing the 7-bp TH-GRE/AP-1 showed specific binding to PC12 nuclear proteins. Using antibodies against GR and AP-1 family of proteins and primers for the TH-GRE/AP-1 region, we detected a specific DNA amplicon in a ChIP assay. This 7-bp TH-GRE/AP-1 sequence (TGACTAA) does not bear similarity to any known GRE, but closely resembles the consensus AP-1 binding site, TGACTCA. Our studies describe for the first time a novel GRE/AP-1 site present in the TH gene promoter that is critical for glucocorticoid regulation of the TH gene.

Glucocorticoids utilize several mechanisms to bring about transactivation or transrepression of genes (for reviews, see De Bosscher et al., 2003; Rhen and Cidlowski, 2005; Newton and Holden, 2007). One of the main mechanisms includes direct effects on gene expression by the binding of glucocorticoid receptors (GR) to glucocorticoid responsive elements (GRE) in the promoter region of genes (Scheidereit et al., 1983). The consensus GRE consists of two half sites each consisting of 6-bp separated by three nucleotides (5'-AGAACANNNTGTTCT) (Truss and Beato, 1993). However, there is considerable degeneracy in the GREs and often, one half-site with even a few base changes has been found to be sufficient for activity (Chan et al., 1991; Monaco et al., 1997). Glucocorticoids can also exert indirect effects on gene expression through interactions with other transcription factors such as AP-1, Sp1, NF-κB, Nurr1, or C/EBPα (Mordacq and Linzer, 1989; Drouin et al., 1989; Jonat et al., 1990; Yang-Yen et al., 1990; Uht et al., 1997; Rudiger et al., 2002). Thus, GR can act without directly binding to a classical GRE, and influence the transcription of genes that do not have a classical DNA binding site for GR (Rudiger et al., 2002; Teurich and Angel, 1995). Similar mechanisms are also known for other steroid receptors, including the progesterone and estrogen receptors (Uht et al., 1997; Owen et al., 1998).

Activation of GR is known to stimulate the expression tyrosine hydroxylase gene resulting in an increase in transcription. Tyrosine hydroxylase (TH) is an important enzyme that catalyzes the first and rate-limiting step of oxidative hydroxylation of tyrosine in the biosynthesis of catecholamines, norepinephrine, epinephrine and dopamine. Catecholamine levels are affected by a variety of stimuli including neurotransmitters, growth factors, estrogen, glucocorticoids, and membrane depolarization, many of which exert regulation at the transcriptional level of the TH gene. TH is expressed in specific neurons of the central nervous system, as well as at the

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periphery in sympathetic ganglia and adrenal medullary chromaffin cells. TH is also expressed in PC12 cells, a cell line derived from a pheochromocytoma (PC) tumor of the rat adrenal medulla, which has served as a widely used model to study the molecular mechanisms of TH regulation. Thus, stimulation of the GR has been shown to increase TH mRNA in various systems (Fossom et al., 1992; Hagerty et al., 2001a). The promoter of the TH gene has been extensively studied (for review, see Kumer and Vrana, 1996), and a number of cis-acting motifs including CRE, AP-1, AP-2, Sp1, POU/Oct, etc., in the proximal promoter region have been identified (Fung et al., 1992). Lewis et al. (1987) also identified a GRE-like sequence with 10 out of 11 bases resembling the palindromic GRE (5'-AGTACACTTTGTTCT-3') around -454 bp in rat TH 5' flanking region. However, this sequence turned out to be nonfunctional having no response to Dex both in the context of a TH gene containing -773 to +27 bp fused to the CAT reporter gene or in a heterologous system. In the present study we used a longer 9 kb DNA of rat TH gene promoter cloned in the pGL3-Basic vector to identify the putative GRE responsible for the stimulatory effect of dexamethasone using the PC12 cell line. Our studies have identified a 7-bp sequence located about 5.7 kb away from the transcription start site that resembles an AP-1 binding site and is necessary for glucocorticoid responsiveness.

Materials and Methods

Plasmid DNA and Site-directed Mutagenesis. We used the 5'-flanking region of the rat TH gene containing -9637 bp of nucleotides upstream of the transcription start site to nucleotide +15, cloned between the XhoI and HindIII sites of the luciferase reporter plasmid, pGL3-Basic (Promega), bearing the firefly luciferase gene (Min et al., 1994). This construct was used in an earlier study by Kobayashi and coworkers to identify Nurr1 response element in the TH gene promoter (Iwawaki et al., 2000). We also used several shorter or deletion constructs that were derived from this -9kb construct by

restriction enzyme digestion. Firstly, we confirmed the authenticity of the entire sequence of this 9 kb long promoter construct (referred to as the full-length construct) by sequence analysis and comparing it to the 5' flanking sequence of the rat TH gene in the ENSEMBL genome database (GeneID 25085). We then performed mutations of the 9 kb promoter that deleted specific sequences or entire segments of the 5' region up to a specified bp using the QuikChange II XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). For this, we designed forward and reverse primers upstream and downstream, respectively of the region to be deleted, and used primers purified by PAGE or reverse phase chromatography (Integrated DNA Technologies Inc., Coralville, IA) for PCR along with the -9kb TH-Luc DNA as template, according to the method prescribed by the manufacturer. The entire reaction was subjected to DpnI digestion, which cleaves only the methylated parental DNA, leaving the mutated amplified DNA that was used for transforming XL10-Gold Ultracompetent cells. Plasmid DNA was isolated from several colonies, cut with restriction enzymes, and appropriate sized DNA was verified by sequencing. Selected DNA was then used to transform E. coli DH5α, from which plasmid DNA was isolated following a maxiprep protocol and further purified by polyethylene glycol treatment to remove any contaminating RNA. Additional purification by CsCl gradient centrifugation followed by ethanol precipitation did not yield any greater activity in transfection assays, and hence was not routinely used. The purified plasmid DNA was used for transient transfection of cells.

Cell Culture and Transfection. Wild type (WT) PC12 cells, a cell line established from rat adrenal pheochromocytoma tumors (Van Buskirk et al., 1985), were grown in media consisting of DMEM:Ham's F12 media (1:1) supplemented with 10% bovine calf serum, 5% horse serum, 2 mM L-glutamine and antibiotics (20 U/ml penicillin and 20 μg/ml streptomycin) in a humidified 5% CO_2 incubator at 37^oC. In some

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experiments, we also used a protein kinase A-deficient PC12 cell line (A126-1B2) (Van

Buskirk et al., 1985) grown under identical conditions.

For transient transfection, 24-well cell culture plates coated with poly-L-lysine

were plated with ~1 X 10^5 cells/well, and after 18-24 h at about 80% confluency cells

were moved to OptiMEM (Invitrogen), a reduced serum medium. Plasmid DNA

constructs were delivered into cells using Lipofectamine 2000 reagent (Invitrogen) or

Turbofectin 8.0 (Origene Technologies) according to manufacturer's recommendations.

Co-transfections were performed with 0.5 μg DNA per well of the above promoter

constructs in the pGL3-basic vector bearing the firefly luciferase gene, along with 0.05

μg/well of a synthetic Renilla luciferase reporter vector (phRG-Basic, Promega), the

latter used for monitoring transfection efficiency. The total molar amount of DNA per well

was kept constant by adjusting the molarity with pBluescript DNA when using shorter

constructs. When Lipofectamine 2000 was used for transfection, media containing 2 X

concentration of sera was added after about 5 h, and incubation continued for 12-16 h,

whereas the cells transfected using Turbofecin were allowed to incubate till next day

without further changes. In later experiments, we switched to using Turbofectin, as these

transfected cells appeared healthier. The transfection agent used is mentioned in figure

legends. For treatment, cells were transferred to a serum-free Neurobasal A medium

(NBM) supplemented with 2 mM Glutamax and N2 supplement (Invitrogen). Cells were

incubated with treatment agents (all from Sigma-Aldrich) including dexamethasone, 12-

O-tetradecanoylphorbol 13-acetate (TPA/phorbol ester), or forskolin (EMD Biosciences)

for up to 24 h. All treatments consisted of replicates of 3-4 wells per group and

experiments repeated at least 3 times. Control cells received appropriate concentrations

of vehicles used (ethanol or DMSO at $\leq$0.01%).

Dual-Luciferase assay. The Dual-Luciferase Reporter assay system (Promega) was used according to the manufacturer's instructions. Cells were washed with Dulbecco's phosphate-buffered saline, lysed in 100 μl of 1X Passive Lysis buffer and 20 μl of the lysate used to measure firefly luciferase (Luc) and Renilla luciferase activities consecutively using the Berthold Lumat LB 9507 luminometer. The ratios of firefly to Renilla Luc activities were calculated in order to correct for differences in transfection efficiency. The average ratio of untreated control group was used to calculate percent of change in the reporter activity of treated groups, and results are expressed as the mean $\pm$ SEM percent of control for each individual construct.

Statistics. The luciferase activity ratio data obtained as percent of control were subjected to statistical analysis by ANOVA followed by Newman-Keuls Multiple Comparison test using the GraphPad Prism 5 software. A p value <0.05 is considered significant.

Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts were prepared essentially according to Schreiber et al. (Schreiber et al., 1989). Briefly, PC12 cells (~5 x 10^6) were plated in 100 mm plates, serum deprived for a day in NBM medium and treated with 100 nM Dex for 1 h. Thereafter, all operations were carried out at 4°C. Cells were scraped, washed with phosphate-buffered saline, and resuspended in 1 ml of hypotonic buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 200 mM sucrose, and 0.5% Nonidet P-40). After allowing cells to swell on ice for 15 min, they were vortexed vigorously for 10 sec and centrifuged at 12,000 X g for 5 min. The nuclear pellet was resuspended in the equivalent of one packed cell volume of buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, and 0.2 mM EDTA) and the nuclear proteins extracted by incubation on ice for 30 min, with vortexing every 5 min. The supernatant obtained by centrifugation at 12,000 X g was diluted by adding an equal

volume of buffer D (20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, and 20% glycerol), then divided into aliquots, and used for EMSA. To buffers A, C and D just before use were added 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 1% protease inhibitor cocktail (P-8340, Sigma). Protein concentration in the nuclear extracts was determined using the Bio-Rad protein assay reagent (Bio-Rad).

As probe for the EMSA, the following 25-bp nucleotide sequence (-5743/-5719) of TH promoter including the 7 bases (underlined) identified as TH GRE/AP1, was used: 5'-CCTTGTGAG<u>TGACTAA</u>TGGGAACTG (TH-GRE/AP1). The following are the sense sequences of other oligonucleotides used, with regions of interest underlined: mutated TH-GRE/AP1: 5'-CCTTGTGAG<u>CGTATTA</u>TGGGAACTG, where only the underlined 7 bp were scrambled; TH-AP1 (-207/-201): 5'-CTGAGGG<u>TGATTCA</u>GAGGCAG; TH-CRE (-46/-39): 5'-GGGCTT<u>TGACGTCA</u>GCCTGG; Sp1: 5'-TTCGATCGGGGCGGGGCGAG; TAT-GRE: 5'-ATTACT<u>AGAACATCCTGTACA</u>GTCGAC and consensus GRE: 5'-CTAGCGGT<u>ACATTTTGTT</u>CT<u>AGAACAAAATGTACC</u>GGTACATTTT. Complementary strands of these DNA oligonucleotides were annealed by heating in 1 X NET (50 mM NaCl, 1 mM EDTA, 10 mM Tris) at 95°C for 5 min and cooling at ambient temperature. Probes were then labeled with [γ-^{32}P]ATP (3000Ci/mmol, Perkin Elmer, Boston, MA) and T4 polynucleotide kinase (New England Biolabs), and purified on G-25 Sephadex Quick Spin columns (Roche). For EMSA assays, nuclear extract (10 μg protein) was incubated for 20 min at room temperature with ~5 X 10^4 cpm of the ^{32}P-labeled DNA probe in 20 μl of binding buffer consisting of 20 mM Hepes, pH 7.6, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 12% glycerol, and 1 μg of poly-d (I-C) (GE Healthcare). For competition, nuclear extracts were incubated with 100 X molar excess of unlabeled double stranded nucleotides for 10 min at 4°C prior to addition of the radiolabeled probe. After reaction, the samples were resolved on a 4% nondenaturing polyacrylamide gel in 0.5 X TBE

buffer. Gels were dried and exposed to Kodak BioMax film to visualize radioactivity captured by the Fotodyne imaging system.

Chromatin Immunoprecipitation (ChIP) Assay. ChIP assays were performed using the EZ-Magna ChIP-A kit (Millipore) according to the manufacturer's instructions. Briefly, PC12 cells (~1 X 10^7 cells) were cultured in 150 mm plates, and after 24 h in serum-free NBM medium, were treated with 100 nM Dex for 1 h. Cells were treated with 1% formaldehyde for 10 min at room temperature to crosslink chromatin and were collected and lysed on ice. The nuclear pellet was recovered and subjected to sonication for 3 pulses of 10 sec each to achieve a size of sheared DNA between 200-1000 bp. Aliquots of sheared DNA-protein complexes were subjected to immunoprecipitation using the following antibodies: anti-GR (monoclonal antibody SC12763 and rabbit polyclonal antibody GR-1002X, Santa Cruz Biotech), antibodies to AP-1 family of proteins: cFos, FosB, cJun, JunD and JunB (all from Active Motif) and anti-Phospho CREB (Millipore). All ChIP assays always included non-immune rabbit IgG (Millipore) as a control to assess nonspecific interactions. After immunoprecipitation for 2 h at 4°C, the immune complexes were collected using protein-A conjugated magnetic beads, which were extensively washed and the DNA eluted, and subjected to reverse crosslinking with proteinase K at 62°C for 2 h. The eluted DNA was purified on spin columns and used for analysis by PCR. The PCR primer set was designed to yield a 135-bp product around -5772/-5638 region of the TH promoter, encompassing the TH-GRE/AP1 region (-5734/-5728). For semiquantitative PCR, serial dilutions of DNA were used as template using the GoTaq green HotStart Taq polymerase master mix (Promega). PCR was conducted for 35 cycles, the reactions analyzed on 2% agarose gel and bands visualized by staining the gel with GelRed DNA dye (Biotium). For quantitative real-time PCR, the same DNA templates and primers were used along with a reaction mix containing SyBr Green dye master mix (Applied Biosystems Inc.) and a standard amplification of 40

cycles performed in a ABI Prism 7900 machine. The results were normalized using the IgG control and the fold enrichment with other antibodies calculated from the Ct values obtained.

Results

Luciferase activities of rat TH promoter constructs. Wild type PC12 cells transfected with the -9 kb full-length rat TH-luciferase construct and treated with dexamethasone (Dex, 1 μM) or forskolin (Fsk, 10 μM) for 20 h showed a 4- to 6-fold increase in luciferase activity over vehicle-treated control cells (Fig. 1). However, in cells transfected with promoter-Luc constructs 5 kb or shorter, the response to Dex was absent while the forskolin response, which works via the CRE present at the proximal promoter region, was intact. As a positive control, we also used pGRE-luciferase vector (Clontech), which has multiple copies of the GRE enhancer is fused to a TATA-like region of the HSV-TK promoter cloned upstream of the luciferase gene. Only dexamethasone, but not forskolin, stimulated the luciferase activity of this construct, while the empty pGL3-Basic vector that was used as negative control did not respond to any treatment (Fig. 1). Based on this experiment, we used the -9 kb full-length construct for all mutation studies.

Since the stimulation of promoter activity was similar for both glucocorticoids, corticosterone 21-acetate and dexamethasone (Fig. 2), we chose to use Dex throughout this study. In the dose-response study with Dex using the full-length construct, a significant increase in Luc activity was seen at 10 nM and was maximal at 100 nM (data not shown). Therefore, 100 nM Dex was used in all subsequent experiments. To determine whether the glucocorticoid response was via the glucocorticoid receptor (GR), we used RU486 (1 µM) prior to the addition of Dex or corticosterone 21-acetate.

Measurement of Luc activities 20 h later indicated that inclusion of RU486 abolished the stimulation seen with both Dex and corticosterone (Fig. 2). Although the concentration of RU486 used may inhibit receptors for both glucocorticoids (GR) and mineralocorticoids (MR), it has been reported that expression of MR is absent in PC12 cells (Lai et al., 2005), suggesting that the response to glucocorticoids shown in figure 2 were mediated by a classical GR.

Deletion analysis of TH promoter. When deletion was carried out sequentially to remove 1 kb segments from the 5' end of the full-length promoter, we found that the Dex response was not affected by deletion of nearly 3.5 kb of the upstream sequence. As shown in Fig. 3A Luc activity was intact for both Dex and forskolin in the -6137 bp construct, while deletion of the next 500 bp (-5637 bp construct) resulted in the loss of only Dex, but not the forskolin response. Additional deletions narrowed the Dex response to 24-bp between -5741 and -5717 (Fig. 3B). Out of these 24-bp, further analysis of the 14-bp region (-5741 to -5728, TTGTGAG<u>TGACTAA</u>, Fig. 3D) indicated that the GR stimulatory activity was associated with the second 7-bp sequence, TGACTAA (designated 7b), and not with the first 7-bp (designated 7a) (Fig. 3C). These experiments clearly established that the GRE resides at -5734 to -5728 bp of rat TH promoter. Since these 7 bp bear resemblance to the AP-1 element, we refer to them as TH-GRE/AP-1 (Fig. 4A).

We also tested the response of the 9 kb full-length and the 7-bp TH-GRE/AP-1 deletion constructs in a mutant PC12 cell line, A126-1B2, deficient in protein kinase A (PKA- PC12). These cells responded to Dex by a 3-fold increase in Luc activity when transfected with the 9 kb promoter. Like in the WT cells, deletion of the 7-bp TH-GRE/AP1 completely eliminated the response to Dex (data not shown). These cells did not respond to forskolin as expected, since the latter response is known to require an intact PKA/CRE activity.

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As depicted in Fig 4A, earlier studies have identified CRE and AP-1 sites in the rat TH promoter proximal to the transcription start site. In the present study, using the 9 kb promoter construct as the template, we also made deletion of either the proximal AP-1 site, TGATTAA at -207/-201 or mutated the CRE site at -45/-38, from TGACGTCA to CGCGATTA and tested their responses to Dex and other appropriate agents. In WT PC12 cells transfected with the 9 kb construct, all the treatments produced significant increases in Luc activity (Fig. 4B). Dex response was abolished only by the deletion of 7-bp TGACTAA at -5734/-5728, but not by deletion/mutation of the proximal AP-1 TGATTCA (-207/-201) or CRE sites. With the 7-bp TH-GRE/AP-1 deletion construct, in addition to Dex the response to TPA was also significantly reduced compared to the 9 kb parent construct, though not completely abolished as seen for Dex (Fig. 4B).

Forskolin response was unaltered by the deletion of 7 bp at -5734/-5728, but was significantly reduced in the CRE or proximal AP-1 site mutant constructs. Mutating the CRE (-45 to -38 bp) caused ~60% reduction in forskolin response, but had little effect on Dex or TPA response. Similarly, deletion of the proximal AP-1 site also caused a ~30% reduction in response to forskolin, but not to Dex or phorbol ester (TPA), latter responses being comparable to those seen with the 9 kb construct (Fig. 4B).

In an attempt at heterologous expression, we cloned the 7-bp TH-GRE/AP-1, TGACTAA, upstream of a partial SV40 Tk (thymidine kinase) promoter flanking the luciferase gene in the pGL3 vector (pTk-Luc), and tested responses to the agonists in WT PC12 cells. Addition of just the 7 bp did not alter any responses, as no differences were seen between this construct and the control pTk-Luc plasmid in their responses to Dex, forskolin, or TPA (data not shown).

We then made another mutation construct by sequential deletion of the 9 kb parent promoter deleting most of the sequences including the proximal AP-1 and CRE sites, but keeping only 100 bp from -5767 to-5668 at the 5' end joining the sequences up

to -30 bp that included the TATA box (TTTAAA, -29/-24), upstream of the Luc gene (Fig. 5A). This 130-bp promoter construct retained full activity to Dex, but not to forskolin, perhaps due to the absence of the proximal CRE and AP-1 sites (Fig. 5B). TPA stimulated the activity of this construct, although the response was slightly (~20%), but significantly, lower compared to the 9 kb construct. We also tested another shorter TH-Luc construct that included only 775 bp of the proximal promoter region upstream of the transcription start site flanking the Luc gene. Dex response was completely absent with this construct, while it retained full response to forskolin (also shown in Fig. 1), and it also responded to TPA (Fig. 5B). Here again, this TPA response was significantly blunted when compared to the 9 kb construct.

Analysis of binding by EMSA. When the 25-bp double stranded oligonucleotide sequence (-5743 to -5719) which included the 7-bp TH-GRE/AP1, TGACTAA, was used as the radiolabeled probe in EMSA, it showed distinct binding to PC12 nuclear extract (Fig. 6). A major DNA-protein band (complex 1) was seen at the top of the gel, along with 2 minor/weaker bands (complex 2) at about the middle of the gel (Fig. 6A, lane 2). Inclusion of molar excess of unlabeled TH-GRE/AP-1 completely eliminated binding to complex1 and also competed at complex 2, albeit less effectively (lane 3). A mutant form of this 25-bp sequence, where only the 7-bp TGACTAA sequence was scrambled to CGTATTA without changing other bases, did not compete for probe binding (lane 4). The binding of the probe at complex 1 was not inhibited by inclusion of excess unlabeled TAT-GRE, the GRE sequence identified in the enzyme tyrosine aminotransferase (TAT) (lane 5) or consensus GRE (lane 6), but the lower complex 2 bands were reduced by the consensus GRE (lane 6). The complex 1 band was effectively reduced by the inclusion of unlabeled AP-1 (lane 7) or CRE (lane 8) sequences, but not by Sp1 (lane 9). Complex 2 bands were also reduced by AP1 to a similar extent as with TH-GRE/AP-1.

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To further examine the nature of these binding sites (Fig. 6B), we compared the pattern of binding of labeled AP-1(lanes 6-10) and consensus GRE sequences (lanes 11-15) and their competition with unlabeled oligonucleotides with that of $[^{32}P]$TH-GRE/AP-1 (lanes 1-5). When AP-1 was used as the probe, only the top band corresponding to complex 1 was seen (lane 7). This band was competed out by both unlabeled TH-GRE/AP-1 (lane 8) and AP1 (lane 9), but not by the mutated sequences (not shown). This result confirmed that the complex 1 in Fig. 6A is the AP-1 binding site. Similar to the TH-GRE/AP-1 probe, excess unlabeled CRE also competed for the labeled AP-1 binding (lane 10). In comparing the sequences of the probes and the competing ligands, we notice that they all share the first 3 bases TGA which perhaps are important for binding to the AP1 site.

When we used consensus GRE (lanes 11-15) as the ^{32}P-labeled probe, it showed binding to the nuclear extract only near the middle of the gel, corresponding to bands identified as complex 2 in Fig. 6A (lane 12). This band was competed effectively by the unlabeled consensus GRE (lane 13), but not by unlabeled AP-1 (lane 15), allowing us to identify complex 2 as the GR binding site Excess unlabeled TH-GRE/AP-1 also competed for the lower band albeit weakly (lane 14), while it completely eliminated binding to the upper band (AP-1 site, lane 3).

Chromatin Immunoprecipitation (ChIP) Analysis. To further elucidate the proteins associated with binding to the GRE/AP-1 region in the TH promoter, we performed ChIP analysis. Using primer sets (F -5772/R -5638) that were designed to include the TH-GRE/AP-1 sequence (-5734 to -5728, Fig. 7A), we obtained a band of the predicted size (135-bp) by semiquantitative PCR in cross-linked DNA fragments immunoprecipitated using antibodies to GR and to the AP-1 family of proteins (Fig. 7B). Compared to the control IgG (lanes 2, 3), we observed a specific PCR band in ChIP with antibodies against GR (monoclonal anti-GR, lanes 4, 5 and polyclonal anti-GR, lanes 6,

7), and AP-1 family proteins, including cFos (lanes 8, 9), FosB (lanes 10, 11), JunD (lanes 14, 15) and JunB (lanes 16, 17), but not cJun (lanes 12, 13). Neither did we see this band in the ChIP with anti-phospho-CREB (lanes 18, 19), the latter used as a control antibody unrelated to AP-1 proteins; these latter bands were of similar intensity as seen with the control IgG (lanes 2, 3). Quantitative PCR confirmed these data, showing a several fold enrichment of these proteins relative to the control IgG with all antibodies tested except for cJun and P-CREB (Fig. 7C). We observed a similar pattern in semiquantitative PCR using another primer set (F -5902/R -5638) designed around the TH-GRE sequence to give a 283-bp product (data not shown), further confirming the ChIP PCR data.

Discussion

We have clearly identified a seven nucleotide sequence, TGACTAA, in the rat TH promoter as critical to the stimulatory glucocorticoid response of the TH gene. This sequence of TH promoter is located nearly 5.7 kb from the transcription start site from -5734 to -5728. This glucocorticoid response element (TGACTAA) has no semblance to the classical GRE (5'-AGAACANNNTGTTCT), but closely resembles the signature motif (5'-TGACTCA) for the AP-1 family of proteins. Deleting just these seven base pairs from the 9 kb TH promoter completely abolished the glucocorticoid response. Conversely, complete GC responsiveness was retained after deletion of most of the 9 kb promoter, except for the 100 bp around the newly identified element at -5.7 kb. Moreover, results from EMSA and ChIP assays provided clear evidence for binding of GR and AP-1 to this putative GRE in the rat TH promoter. Therefore, we refer to this AP-1 like sequence as the TH-GRE/AP-1 site.

Although it is well known that glucocorticoids stimulate TH mRNA levels in adrenal chromaffin cells through transcription (Fossom et al., 1992), the mechanism by which glucocorticoids mediate this effect has remained elusive. In previous studies from our laboratory aimed at identifying a functional TH GRE, we employed the TH3.6 reporter construct, reported to contain 3.6 kb of 5' promoter sequences of the mouse TH gene (NCBI accession number, X53503) (Morgan et al., 1996). Using this construct, we identified an imperfect GRE (5'-GGCACAGTGTGGTCT-3') around -2.4 kb from the transcription start site (Hagerty et al., 2001b) that appeared to confer responsiveness to glucocorticoid. However, we have since reevaluated our conclusion that this sequence is a biologically relevant GRE for several reasons. First, on stimulation with dexamethasone, the magnitude of the increase in promoter activity of the TH3.6 promoter construct used in our previous studies was relatively weak, only 1.5 to 2-fold. Second, a recent comparison of the DNA sequence of this promoter with the 5' flanking genomic sequence of the mouse TH gene in the ENSEMBL database, revealed discrepancies. Thus, 1.2 kb of the region upstream of the previously identified TH-GRE in the TH3.6 construct corresponded partly to those on two different mouse chromosomes. Third, when the errant 1.2 kb sequence was removed, and the remaining 2.4 Kb containing the putative GRE was retested in the context of a different plasmid, responsiveness to glucocorticoid receptor stimulation was lost. Interestingly, a comparison of nucleotide sequences between the rat and the mouse TH gene revealed considerable homology in 5'-flanking sequences at around -5.7 kb. Moreover, we identified a sequence, TGACTAA, around -5.7 kb of the mouse TH promoter, which may be the mouse TH-GRE/AP-1 site. Further studies are underway to determine if this sequence is also a functional TH-GRE/AP-1 site in the mouse gene.

Although deletion of the TH-GRE/AP-1 completely abolished the response to dexamethasone, it did not alter the response to forskolin. The lack of requirement for the

cAMP/PKA pathway in the response to glucocorticoid receptor stimulation was corroborated by the intact response of the 9 kb promoter to Dex in PKA-deficient PC12 cells. These results clearly demonstrate that Dex and forskolin stimulate TH promoter activity via distinct pathways, corroborating our earlier observations (Hagerty et al., 2001). Although there is one clearly identified consensus CRE sequence (-45/-38) in the rat TH promoter, there are several AP-1 sites. Mutation or deletion of the CRE or AP-1 in the proximal part of the -9 kb TH promoter had no influence on the Dex response, but affected the responses to other agonists. Thus, mutation of the CRE and to a lesser extent the AP-1 (-207/-201), reduced the forskolin response. Our data are consistent with earlier reports that, in addition to the canonical sequence at -45/-38, other elements also contribute to the cAMP/forskolin response. (Fung et al., 1992; Lewis et al., 1987; Lewis-Tuffin et al., 2004; Best et al., 1995; Nagamoto-Combs et al., 1997; Yang et al., 1998).

Besides abolishing the Dex response, deletion of the distal 7-bp TH-GRE/AP-1 sequence also significantly reduced the response to phorbol ester (Fig. 4B), a stimulator of PKC, known to act via AP-1 sites (Icard-Liepkalns et al., 1992). However, these responses were not completely abolished, unlike that seen for Dex. This may not be surprising since several AP-1 sites exist in the rat TH promoter that can perhaps collectively contribute to the response seen with the AP-1 agonists. Conversely, unlike the 40% reduction seen with the deletion of the distal 7-bp GRE/AP-1, the response to TPA was totally unaffected by the deletion/mutation of the proximal AP-1/CRE sites. This was surprising, since other studies have shown the importance of the proximal AP-1 and CRE sites in TPA response (Icard-Liepkalns et al., 1992; Best and Tank, 1998; Piech-Dumas et al., 2001). A major difference among the various studies appears to be the length of the promoter constructs used. We tested deletion/mutation of just the proximal 7/8-bp AP-1/CRE sites in the context of the entire 9 kb promoter, while others have used

deletions made in shorter constructs containing varying lengths of the proximal part of

the TH promoter cloned in other reporter systems. Indeed, when we used a short -775

bp TH-Luc construct, we also saw a response to TPA (Fig. 5B), similar to that previously

published using shorter constructs. More recently, interaction between AP-1 and other

factors such as Egr1 have been proposed to be important for TPA up-regulation of TH

gene (Nakashima et al., 2003; Stefano et al., 2006). Nevertheless, our results show that

in the context of the 9 kb long promoter, deletion of just the proximal AP-1 or CRE sites

minimally affects the response to AP-1 agonist. On the other hand, in a construct that

deleted the entire proximal region but retained only 100 bp around the distal TH-

GRE/AP-1 sequence (the 130-bp promoter, Fig.5), the TPA response was only slightly

(~20%) diminished, implying that a major contribution to TPA response comes from this

distal TH-GRE/AP-1 site. Since the Dex response, mediated via the glucocorticoid

receptor, was entirely dependent on the 7-bp site, this fits the description of a composite

GRE/AP-1 element in the rat TH promoter.

The interaction of nuclear receptors with other transcription factors in regulating

promoter function is well known for various genes (Pfahl, 1993). Indeed, the term

'composite element' (CE) was coined to describe such a GRE in the mouse proliferin

promoter where both GR and AP-1 proteins, c-Jun and c-Fos, bound to overlapping

sites, invoking a model involving DNA binding and protein-protein interactions of

receptor and non-receptor factors (Mordacq and Linzer, 1989). Using a synthetic

promoter containing juxtaposed AP-1 and GR binding sites, GR was shown to negatively

or positively modulate transcription depending on the AP-1 subunits. It was thus

hypothesized that expression of genes containing 'composite elements' is activated or

repressed in a hormone-dependent manner, depending on the ratio of positively and

negatively acting AP-1 subunits (Diamond et al., 1990; Miner and Yamamoto, 1992).

To our knowledge this is the first study of any TH promoter that describes such an indirect mechanism for GR action involving AP-1 sites. Our competition studies using EMSA clearly showed that the sequence identified as TH-GRE/AP-1 was able to bind both AP-1 and GRE binding proteins in nuclear extracts. Subsequent ChIP assays provided clear evidence for the presence of both GR and AP-1 family of proteins in these DNA-binding complexes. Interestingly, using the ChIP assay we detected several proteins from the AP-1 family, but not c-Jun. Such differential effects of glucocorticoid on Fos and Jun proteins have also been described for other genes (Autelitano, 1994). Given their importance as transcriptional regulators it is not surprising that AP-1 activity is tightly controlled at the level of *fos* and *jun* gene transcription and by posttranslational modification of AP-1 proteins (Teurich and Angel, 1995; Angel and Karin, 1991). Further studies are underway to establish the effects of glucocorticoids on relative expression of AP-1 family members and their interactions with GR in our cells. Nevertheless, our studies show that the TH gene joins a growing list of genes containing atypical GREs where the glucocorticoid receptor influences gene activation in collaboration with other transcription factors.

Acknowledgments

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Figure Legends

Fig. 1. Luciferase (Luc) Activities of TH promoter constructs. Wild type (WT) PC12 cells were transfected using Lipofectamine 2000 with TH-Luc DNA constructs bearing different lengths of the rat TH 5'-flanking sequence. As controls, we used the pGL3-Basic empty vector (negative control) and the pGRE-Luc vector (positive control) that had multiple copies of the GRE enhancer fused to a TATA-like promoter of the thymidine kinase cloned upstream of the firefly Luc gene. Dual Luc assays were performed 20 h after treatment without or with dexamethasone (1 μM) or forskolin (10 μM). Results presented as percent of respective controls are the mean $\pm$ SEM of triplicate samples from three separate experiments.

Fig. 2. Glucocorticoid effects are mediated via the glucocorticoid receptor. WT PC12 cells were transfected with the -9 kb full-length promoter DNA using Lipofectamine 2000 and after 24 h were treated with 100 nM of dexamethasone (Dex) or corticosterone 21-acetate. Some cells were pretreated with 1 μM RU486 for 30 min prior to the addition of glucocorticoids. Dual Luc assays were performed 20 h after treatment; results represent the mean $\pm$ SEM of triplicate samples in 2 experiments.

Fig. 3. Deletion analysis of the TH promoter region. A. Cells were transfected with the -9 kb full-length, -6.1 or -5.6 kb TH-Luc constructs using Lipofectamine 2000. Dual Luc assays were performed 20 h after treatment with 100 nM Dex or 10 μM forskolin. B. Dex and forskolin response of -5.7 kb deletion constructs for examining the GRE activity in 500 bp between -5767 to -5717. C. Comparison of the Dex response of the full-length and constructs with only 14 bp or 7 bp deletions from the -9 kb promoter as depicted in panel D.

Fig. 4. Luciferase responses of the 9 kb full-length TH-Luc promoter and constructs bearing deletions of the 7-bp TH-GRE/AP-1 sequence (-5734/-5728), or the

proximal AP-1 site (-207/-201) or mutation of the CRE (-45/-38) from TGACGTCA to CGAGATTA in WT PC12 cells. **A.** Depicts the 9 kb full-length rat TH-pGL3 construct identifying various regions of interest. **B.** After overnight transfection with promoter constructs using Turbofectin 8.0, WT PC12 cells were treated in serum-free Neurobasal A medium for 20 h until dual Luc assay without (control) or with Dex (100 nM), forskolin (Fsk, 10 μM), or 12-O-tetradecanoylphorbol 13-acetate (TPA/phorbol ester, 100 nM). Results are the mean $\pm$ SEM of percent of respective controls from three or more experiments, each with 3-4 wells. *$p<0.05$ compared to respective control group for each construct; #$p<0.05$ compared to the corresponding treatment for the 9 kb promoter.

Fig. 5. Comparison of the reporter activities of shorter promoter constructs with the 9 kb full-length construct. **A.** The 130-bp construct was made by sequential deletion mutations using the 9 kb TH-pGL3 construct. It contains 100 bp of the distal region (from -5767 to -5668 bp), adjoining -30 bp of the proximal region flanking the transcription start site (denoted by the arrow); deletion was made just after the CRE site but retaining the TATA box at -30 and the rest of the pGL3-Basic vector. The -775 bp construct contained only the sequences from +15 to -775 bp of rat TH promoter cloned into the pGL3 plasmid. **B.** WT PC12 cells were transfected using Turbofectin 8.0 with pGL3 vector bearing either the full-length or the shorter promoter sequences as shown in panel A. Cells were treated the next day with agents at concentrations given in the legend for Fig.4B, and assayed for dual Luc activity. *$p<0.05$ compared to respective control group for each construct; #$p<0.05$ compared to the corresponding treatment for the 9 kb promoter.

Fig. 6. Electrophoretic Mobility Shift Assay (EMSA) with TH-GRE/AP-1, TH-AP-1 or consensus GRE probes. Nuclear extracts (NE) prepared from WT PC12 cells treated with 100 nM Dex for 1 h were used in all experiments. **A**. The 25-bp double-stranded oligonucleotide (5'-CCTTGTGAGTGACTAATGGGAACTG, -5743/-5719 of rat TH

promoter, with the 7-bp TH-GRE/AP-1 underlined in the middle) was used as the radiolabeled probe. All lanes contained the same amount of radiolabeled probe (5×10^4 cpm), and all lanes except lane 1 received 10 μg NE protein. In lanes 3-9, the following unlabeled double stranded oligonucleotides at 100-fold molar excess were included (sequence of only the sense strand is shown and relevant parts of the sequence are underlined): lane 3: TH-GRE/AP-1 (same as the radiolabeled probe, 5'-CCTTGTGAGTGACTAATGGGAACTG); lane 4: mutated TH-GRE/AP-1: 5'-CCTTGTGAGCGTATTATGGGAACTG; lane 5: TAT-GRE: 5'-ATTACTAGAACATCCTGTACAGTCGAC; lane 6: Consensus GRE: 5'-CTAGCGGTACATTTTGTTCTAGAACAAAATGTACCGGTACATTTT;

lane 7: TH-AP1 (-207 to -201): 5'-CTGAGGGTGATTCAGAGGCAG; lane 8: TH-CRE (-45 to -38): 5'-GGGCTTTGACGTCAGCCTGG; lane 9: Sp1: 5'-ATTCGATCGGGGCGGGGCGAGC. Free probe is seen at the bottom of the gel, and the shifted bands are indicated by arrows. A representative image from 3 experiments is shown. **B**. EMSA with ^{32}P-labeled probes: TH-GRE/AP-1 (lanes 1-5) or TH-AP-1 (lanes 6-10) or ^{32}P-consensus GRE probes (lanes 11-15). Lanes 1, 6 and 11 received only the respective probes. All other lanes included 10 μg Dex-treated NE protein. Lanes 2 (TH-GRE/AP-1), 7 (TH-AP-1) and 12 (consensus GRE) show the specific binding of the probes to the NE in the absence of unlabeled nucleotides. The following unlabeled nucleotides (100 X molar excess) were included along with the respective probes: lanes 3, 9 and 14, TH-GRE/AP-1; lanes 4, 8 and 15, TH-AP-1; lanes 5 and 13, consensus GRE; lane 10 CRE. Free probe is at the bottom of the gel, and the shifted bands are indicated by the arrows, identifying AP-1 and GRE binding sites.

Fig. 7. Chromatin immunoprecipitation (ChIP) analysis of binding proteins. ChIP analysis was performed using chromatin prepared from cells treated with 100 nM Dex for

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1 h. The cross-linked DNA-protein complexes were subjected to immunoprecipitation using antibodies to GR (monoclonal antibodies: anti-GR mAB or rabbit polyclonal antibodies: anti-GR pAb) or to the AP1 family including c-Fos, Fos B, c-Jun, Jun D, and JunB, as well as phospho-CREB. The purified DNA after reversal of cross-links was used as template in PCR along with primers for the region that spanned TH-GRE/AP-1 sequence shown in **A:** F-5772: 5'- CATGATTGCTGTCACATCACC and R-5638: 5'- CAACCTGTGCACCAGTGAGT. **B.** Semiquantitative PCR was performed for 35 cycles of amplification; the reactions were run on 2 % agarose gel and DNA bands visualized by staining with Biotium GelRed DNA dye. The DNA ladder is on the left. Lane 1: no template control (NTC); lanes 2, 3: control IgG; lanes 4, 5: anti-GR mAB; lanes 6, 7: anti-GR pAB; lanes 8, 9: anti-cFos; lanes 10, 11: anti-Fos B; lanes 12, 13: anti-cJun; lanes 14, 15: anti-Jun D; lanes 16, 17: anti-JunB; lanes 18, 19: anti-P-CREB. C. Quantitative PCR was performed using the same primer set along with SyBr Green master mix in a standard 40-cycle PCR using ABI Prism 7900 instrument. Amplification of specific products was seen between 13 to 32 cycles and a single peak was seen in the dissociation curve analysis (data not shown). Fold enrichment was calculated from the Ct values. The results are expressed relative to control IgG. Mean data from 2 similar experiments are shown.
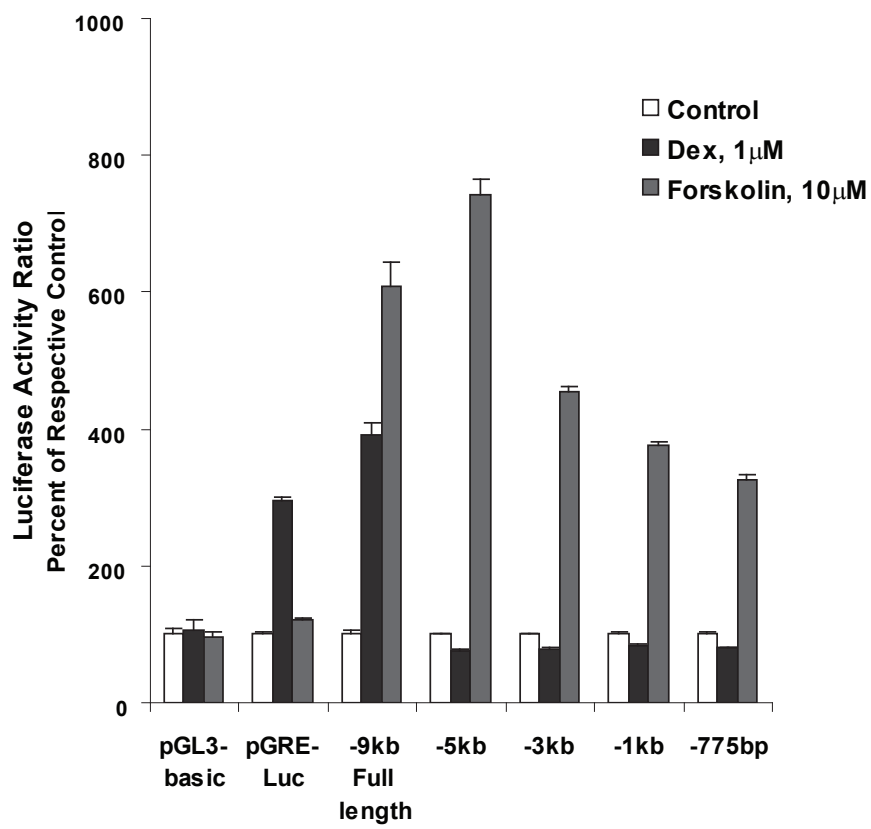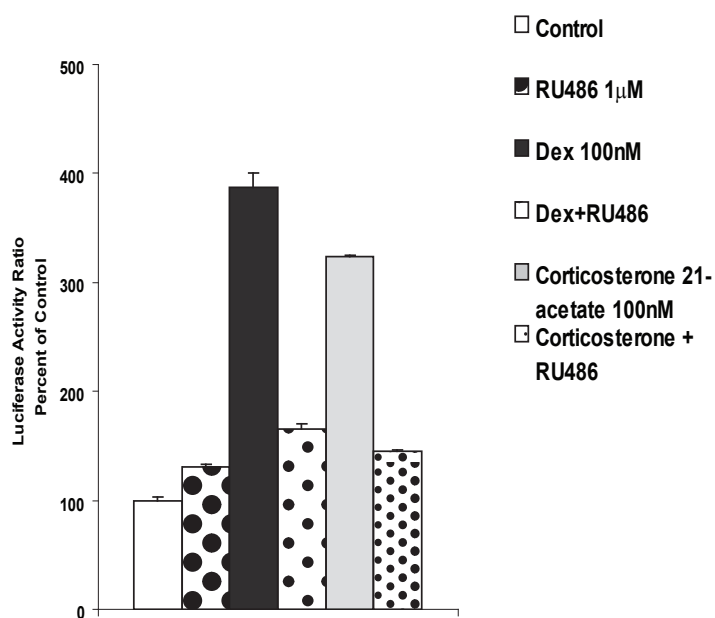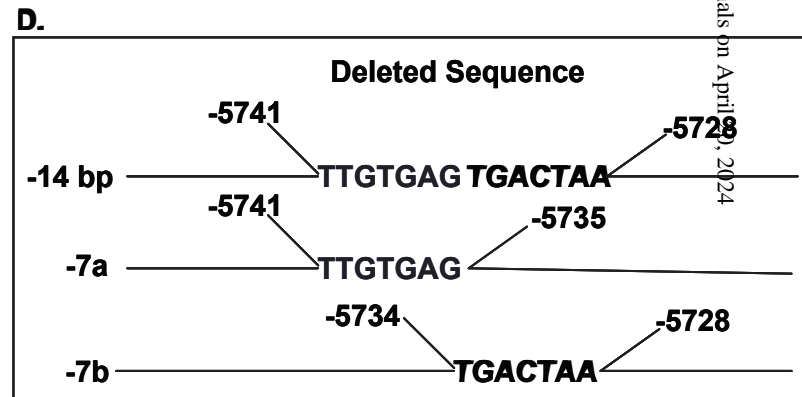
Fig. 1

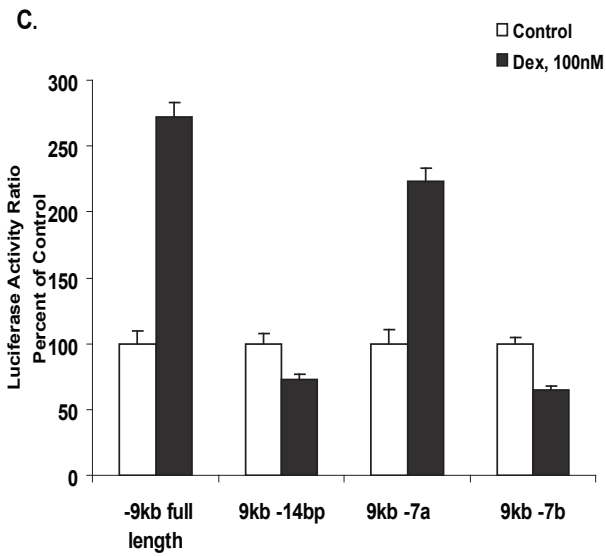**Fig.2**

Fig.3

Fig. 4

A.

9 kb TH-pGL3 promoter

GRE/AP1

AP1   CRE

Luc

-9637

TGACTAA

-5734   -5728

TGATTCA

-207   -201

TGACGTCA

-45   -38

B.

Luc Activity Ratio
Percent of Respective Control

1400

1200

1000

800

600

400

200

0

Control   Dex   Fsk   TPA

9kb Full-length

GRE/AP-1 (-5734/-5728 deleted)

AP1 (-207/-201 deleted)

CRE (-45/-38) deleted

Fig. 5

A.

1. **9kb TH-pGL3 promoter**

GRE/AP1   AP1   CRE

-9637

TGACTAA   TGATTCA   TGACGTCA
-5734   -5728   -207 -201   -45   -39

Luc

2. **130bp TH-pGL3 promoter**

GRE/AP1   -30

-5767   -5668

TGACTAA
-5734   -5728

Luc

3. **-775bp TH-pGL3 promoter**

AP1   CRE

-775

TGATTCA   TGACGTCA
-207   -201   -45   -39

Luc

B.

☐ 9 kb
■ 130 bp promoter
▨ 775 bp

Percent of Respective Control

1200
1000
800
600
400
200
0

Ctl   Dex   Fsk   TPA

Fig. 6

A.

32P-TH-GRE/AP1

1   2   3   4   5   6   7   8   9

Complex 1 →

Complex 2 →

B.

32P-TH-GRE/AP1   32P-TH-AP1   32P-Cons.GRE

1   2   3   4   5   6   7   8   9   10   11 12 13 14 15

AP-1 →

GRE →

Fig. 7

A.

B.

← F5772/R5638

C.