Identification and Characterization of a Novel Phorbol Ester-responsive DNA Sequence in the 5′-Flanking Region of the Human Dopamine β-Hydroxylase Gene

(Received for publication, April 28, 1998, and in revised form, June 15, 1998)

Hiroshi Ishiguro, Kouji Yamada, Naohiro Ichino, and Toshiharu Nagatsu‡

From the Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-1192, Japan

The phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), enhances transcription of many eukaryotic genes, including that for dopamine β-hydroxylase (DBH). In the present study, we report identification and characterization of a novel sequence motif residing in the 5′-flanking region of the human DBH gene, which mediates transcriptional induction by TPA. Deletional analyses indicated the promoter region between −223 and −187 base pairs to be critical. Whereas this region does not contain any putative regulatory motifs with significant sequence homology to the AP-1 motif, extensive deletional and site-directed mutational analyses indicated that a sequence between −210 and −199 base pairs, 5′-ATCCGGCCTGTCT-3′, may represent a novel TPA-response element (TRE). In addition, alteration of the YY1-binding site decreased TPA-mediated induction of the DBH promoter activity, suggesting that contiguous cis-regulatory element(s) cooperate with this novel sequence motif. Furthermore, insertion of regulatory analyses between the YY1-binding site and the cyclic AMP-responsive element indicated that the stereospecificity of these motifs is important for intact transcriptional induction by TPA. Taken together, these data suggest that transcriptional up-regulation of the human DBH gene in response to TPA requires coordination of a novel TRE (human DBH TRE, hDTRE), cyclic AMP-responsive element, and the YY1-binding site.

Dopamine β-hydroxylase (DBH, dopamine β-monooxygenase; EC 1.14.17.1) catalyzes the conversion of dopamine to norepinephrine in the biosynthesis pathway of catecholamines. Analysis of the human and rat DBH promoters has identified several cis-acting DNA elements that may play a role in regulation of DBH transcription. Several consensus sequences such as the cyclic AMP-responsive element (CRE, −181 bp to −174 bp), activator protein 2 element (+2 bp to +9 bp and −666 bp to −659 bp), glucocorticoid-responsive element (−473 bp to −459 bp), AP-1-like sequence (−172 bp to −166 bp), and neuron-restrictive silencer element-like sequence (−390 bp to −388 bp) are reported to be present in the promoter region of the human DBH gene (1–4). CRE is a well known DNA element that exists in the promoter region of many genes, involved in the signal transduction pathway via protein kinase A (PKA) (5, 6). When the Ser-133 amino acid residue of the CRE-binding protein (CREB) is phosphorylated by the PKA pathway, transcriptional up-regulation of genes occurs (7). The role of CRE in human DBH gene function was initially demonstrated in human SK-N-SH-TFM and SK-N-BE(2)C neuroblastoma cell lines (2, 3). It was found not only to enhance transcription with PKA activators such as cAMP plus 3-isobutyl-1-methyloxanthine, dibutyryl cAMP, or forskolin, but also to be an essential element for the constitutive transcription. Phosphorylation of CREB by PKA directly affects the transcriptional level of the human DBH gene as proved by experiments using co-expression of the catalytic subunit of PKA or its specific inhibitors, as well as in PKA-deficient PC12 cell lines (8).

A negative regulatory element of the human DBH gene functions as a silencer at the transcriptional level in neuronal and non-neuronal cells (4). This negative regulatory element of the human DBH gene has a sequence similar to that of the neuron-restrictive silencer element in many neuronal genes (9, 10). However, the neuron-restrictive silencer element-like sequence of the human DBH promoter fails to bind recombinant neuron-restrictive silencer factor (11). YY1 and activator protein 2 in the nuclear fraction of SK-N-BE(2)C cells specifically bind to the human DBH promoter, but the nuclear protein fraction of HeLa cells does not make a complex with these elements (12). These results suggest that multiple proteins bind to the DBH promoter region interacting co-operatively for cell type-specific transcriptional activation.

Functional analyses of the elements in the rat DBH promoter region have involved the use of cell culture systems. Although the identified CREs have unusual sequences, i.e. CGTCA (−232 bp to −228 bp) and TGGTCA (−173 bp to −167 bp), the DNA fragment DB-1 (−180 bp to −151 bp) of the rat promoter region has an enhancer function (13). Afar et al. (14) reported that one CRE (−174 bp TCTGGCTCATTA −161 bp) binds the activating transcription factor 1. Transcriptional enhancement of the rat DBH gene by treatment of PC12 cells with dibutyryl cAMP has been reported. In addition, Arix, which is a homologue of the mouse homeodomain protein Phox2, binds to the DBH enhancer element of the rat DBH promoter (15). Recently, Phox2a knockout mice provided evidence that Phox2a plays a key role in the development of noradrenergic neural traits (16). Analyses indicated that a negative regulatory region (−282 bp to −232 bp) that controls the tissue-specific expression of the rat DBH gene exists upstream of the DB-1 enhancer and that the activator protein 2 (−129 bp to −120 bp) element plays a role in maintaining basal levels of rat DBH transcription (17, 18).
The zinc-finger-type nuclear protein YY1 is widely expressed in the nuclear protein fraction and regulates the transcriptional level of many viral (19–25) as well as cellular (26–41) genes. In many cases, the regulation is negative, but positive control may also be exerted depending on the promoter sequences (42, 43). An attempt to explain these opposing actions of YY1 protein has been made based on DNA binding (30, 44). In certain cases, YY1, TFIIB, and RNA polymerase II function to transcribe the DNA directly without a TATA-binding protein (45). YY1 interacts with TATA-binding protein, CREB-binding protein, TFIIB, and TAFII55 (46), implying that these basal transcriptional factors may interact with one other in offering the human DBH promoter.

In the present study, we identified a TPA-responsive element (TRE) in the promoter region of the human DBH gene (hDTRE) and a hDTRE-binding protein. The response to TPA stimulation was determined and characterized using combinations of the DNA element with hDTRE-binding protein, YY1, and CREB (or CREB-binding protein). The mechanism of the TRE response in the hDTRE was found to differ from that of AP-1 (TGA/C/G/TCA) in many other genes.

**EXPERIMENTAL PROCEDURES**

**Growth of SK-N-SH Cells**—SK-N-SH cells (RIKEN Cell Bank, Tsukuba Science City) were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with penicillin-streptomycin (Life Technologies, Inc.) and 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.) in a 5% CO₂ atmosphere at 37 °C. For optimization of cell growth for experiments, fresh medium was supplied every 3 days, and cultures were split as intervals of 3–5 days.

**Northern Blot Analysis**—Cells were treated with 162 nM TPA or 10 μM forskolin for 6 or 18 h before harvesting. Poly(A)⁺ RNA was prepared by oligo(dT)-cellulose affinity chromatography, electroelution, and 1% agarose gel electrophoresis, transferred to the Hybond N+ membrane (Amersham Pharmacia Biotech) by capillary diffusion, and then hybridized with human DBH or glyceraldehyde-3-phosphate dehydrogenase probes. Hybridized membranes were placed in contact with BAS1000 plates (type BAS-IIIIs) and analyzed with a BAS1000 Bio-Imaging analyzer (Fuji Photo Film, Tokyo).

**Synthetic Oligonucleotides**—Sequences of OLI-63 and OLI-42 cover the 5'-flanking human DBH promoter from -224 to -182 bp and from -224 to -183 bp, respectively. OLI-A (-224 to -199 bp), OLI-B (-206 to -181 bp), OLI-C (-186 to -161 bp), and OLI-D (-213 to -183 bp) completely cover the region of the OLI-63 sequence. OLI-Cm1, OLI-Cm2, OLI-Cm3, OLI-Cm4, and OLI-Cm5 are changed from OLI-C, and OLI-Dm1 and OLI-Dm2 from OLI-D, in the sites indicated in Fig. 2A. OLI-Cm1 has CRE, YY1 binding, and E-box sequences. OLI-Cm2 has CRE sequences. OLI-Cm3 has the CRE and E-box sequences. OLI-Cm4 has only the CRE sequence. OLI-Cm5 has YY1 binding, E-box, and AP-1-like sequences, but not the CRE sequence. OLI-hTh/C, OLI-hSOM/C, OLI-hTh/AP, and OLI-mo/y/ are the respective DNA elements for CRE of human tyrosine hydroxylase (TH), CRE of human somatostatin, AP-1 of human TH, and the YY1 binding sequence of the mouse c-fos gene.

**Electrophoretic Mobility Shift Assay (EMSA)**—Sense and antisense strands of oligonucleotides were annealed and labeled with [γ-32P]ATP by T4 polynucleotide kinase and used as probes. Nuclear protein fractions (10 μg aliquots) were prepared by the method of Dignam et al. (47) and mixed with approximately 10,000 cpm of radiolabeled probe (0.15 ng) in binding buffer (4 mM Tris (pH 8.0), 40 mM KCl, 4 mM MgCl₂, and 5% glycerol) containing 1 μg of poly[d(C·G)]₂ at room temperature for 20 min. Complexes of oligonucleotides and nuclear protein fractions were resolved on nondenaturing 6% polyacrylamide gels in 0.25 × Tris borate-EDTA. Oligonucleotides were synthesized by the phosphoramidite method with an Applied Biosystems model 392 DNA/RNA synthesizer (Perkin-Elmer) and their sequences confirmed with a 373A DNA sequencer (Applied Biosystems) (see Fig. 2A) using luciferase construct or phosphoprotein expression experiments were performed. For binding appropriate amounts of cold oligonucleotide to the nuclear protein fraction before admixture of the probe. For antibody experiments, anti-c-Fos or anti-YY1 antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were preincubated with nuclear protein fraction for 30 min at room temperature. After a 20-min incubation of this mixture with radiolabeled probe, the complete mixture was electrophoresed in a 6% nonde-
does not contain nucleotide sequences with sequence homology with the AP-1 motif (5'-TGA(C/G)TCA-3') (Fig. 1C).

DNA-Protein Interactions of the Putative TPA-responsive Region of the Human DBH Promoter—To investigate DNA-protein interactions of the TPA-responsive region, we performed EMSA using an oligonucleotide (OLI-63) that encompasses the novel TPA-response DNA domain as well as the contiguous CRE- and YY1-binding motifs as a probe (Fig. 2A). As shown in Fig. 2B, OLI-63 produced two major DNA-protein complexes with slow (band A) and fast mobility (band B). Molar excess of cold OLI-63 diminished both, indicating that these bands represent specific DNA-protein complexes. OLI-42 encompassing
the 5’ side of the OLI-63 was able to compete for formation of band A. In contrast, molar excesses of shorter oligonucleotides containing the CRE of human TH or human somatostatin genes, the AP-1 motif of the human TH gene, or the YY1 binding sequence of the mouse c-fos gene were without effect.

To determine the DNA subdomain within the OLI-63 sequence, which is critical for interacting with nuclear protein, we prepared four additional oligonucleotides (OLI-A, OLI-B, OLI-C, and OLI-D) covering different subdomains. As shown in Fig. 3A, competition assay showed that OLI-D was extremely effective at eliminating bands A and B, but OLI-A, OLI-B, and OLI-C failed to affect their formation. In addition, we used different versions of OLI-D (OLI-Dm1 and OLI-Dm2; Fig. 2A) containing base substitutions. Whereas a molar excess of OLI-Dm1 eliminated both complexes as efficiently as OLI-D, OLI-Dm2 did not affect their formation.

To directly address whether nucleotides at −210 to −199 bp are important for the TPA responsiveness, we introduced base substitutions (ATCCGCTGTCT to GATTCGACAAGC) in the context of the 604 bp upstream region of the human DBH gene. In transient transfection assays, base substitutions diminished most, if not all, of the TPA-mediated induction of the DBH promoter activity (from 11.6- to 3.8-fold; Fig. 4B).

Taken together, the results suggest that a subdomain of the DBH promoter at −210 to −199 bp appears critical not only for forming specific DNA-protein complexes but also for intact TPA responsiveness. We designate this novel sequence motif as the hDTRE (for human DBH-TPA response element). Given the

**Fig. 2.** OLI-63 and the nuclear protein complex formation. A, sequences of oligonucleotides used for EMSA are shown. Middle columns show the names of the oligonucleotides, and the right columns show their positions in the DNA region of the human DBH or other promoters. The mutated sequences are underlined. B, DNA-protein complexes formed by the nuclear protein fraction prepared from SK-N-SH cells and OLI-63 as a probe containing the TPA-responsive region, CRE, the YY1 binding sequence, the E-box sequence, and the AP-1-like sequence. Competitors are shown at the top of the EMSA gel. OLI-63 and OLI-42 oligonucleotide competitors were applied at 1000 molar excess and other oligonucleotides were at 10,000 molar excess over the probe.

**Fig. 3.** OLI-D efficiently eliminates the OLI-63-nuclear protein complex. A, oligonucleotide competitors used were restricted regions of the OLI-63 ranging from −224 to −162 bp. Competitors and their molar ratios to OLI-63 probe are shown at the top of the EMSA gel. Each cold oligonucleotide competitor was applied at 20, 200, and 2000 molar excess over the probe.

Nucleotides Residing at −213 to −183 bp of the Human DBH Gene Are Critical for Formation of Specific DNA-Protein Interactions and Are Required for Intact Transcriptional Induction by TPA—We next used the oligonucleotide OLI-D comprising nucleotides between −213 and −183 bp as the probe for EMSA. As shown in Fig. 4A, several prominent complexes were formed with nuclear extracts. Competition assays indicated two bands (band C and band D) to be sequence-specific DNA-protein complexes. Whereas cold OLI-Dm1 completely eliminated both of these, OLI-Dm2 did not affect their formation.
OLI-D and nuclear protein fraction are different from that of complexes with AP-1 sequence. The right lane shows results with the radiolabeled OLI-D probe alone. B, transient transfection analysis using the 604 m2Luc construct. For this experiment, the sequence was changed from ATCCGCCTGTCT (−210 to −199 bp) to GATTGCAGACG in the human DBH promoter (from −604 to +10 bp) and combined with a luciferase expression vector (604Luc). The 187Luc construct was obtained by combining the human DBH promoter (−187 to +10 bp) with the luciferase expression vector. The results are expressed as fold induction in the middle column.

This experiment was performed four times each in triplicate with similar results, and bars represent the S.D. values. C, complex formations with OLI-D and nuclear protein fraction are different from that of complexes with AP-1 sequence. The arrow at the right side of each panel shows the complex formation with AP-1 sequence (OLI-hTH/AP) and Fos or Fos-related protein (Fos) among nuclear protein fraction, and arrows of band C and band D are shown at the left side of each panel. The left panel shows complex formation by OLI-D or OLI-hTH/AP with nuclear protein fractions prepared from SK-N-SH cells after TPA (162 nM) treatment for 1 h. The right panel shows the formation of a supershifted band (arrow at the right side of autoradiogram) combination with AP-1 sequence (OLI-hTH/AP), nuclear protein fraction, and anti-c-Fos antibody (1 μg), but not with OLI-D.

It is possible that the specific complexes formed with OLI-D, e.g., band C and band D, may be because of transcription factors or regulators, which mediate TPA-inducible transcription of the human DBH gene, we tested nuclear extracts prepared following treatment of TPA. When the AP-1 oligonucleotide was used as the probe, DNA-protein complexes were formed more robustly with TPA-treated samples and were supershifted by co-incubation with anti-c-Fos antibody (Fig. 4C). In contrast, when the OLI-D probe was used, nuclear extracts prepared in the absence or in presence of TPA produced DNA-protein complexes without any apparent differences (Fig. 4C).

The YY1-binding Site but Not the AP-1-like Motif, Both of Which Reside in Close Proximity to the CRE, May Be Important for Intact TPA-responsive Induction of the Human DBH Promoter—Downstream of the hDTRE motif of the human DBH promoter, there resides a composite promoter that encompasses four characterized DNA elements, i.e. CRE, a YY1 binding sequence (CCAT, −175 to −172 bp), an E-box (CATGTG, −174 to −169 bp), and an AP-1-like sequence (TGTGTCA, −172 to −168 bp), as shown in Fig. 5A. The CRE sequence of the human DBH gene has one base pair change from A to C as compared with consensus CRE sequence. CCAT and CATGTG are consensus sequences for the binding site of the transcriptional regulatory protein YY1 and basic helix-loop-helix DNA-binding protein, respectively. The AP-1-like sequence has a single base pair change from the consensus sequence TGA(C/G/TCA) to TGTGTCA. To investigate whether this region is involved in TPA-responsive induction of DBH transcription, we first analyzed DNA-protein interactions in this region using nuclear proteins prepared from SK-N-SH cells. As shown in Fig. 5B, our EMSA, using the oligonucleotide OLI-C encompassing this promoter area as the probe, produced multiple bands with either slow (bands E) or fast (band F) mobility. To further analyze these complexes, we tested five mutant oligonucleotides (OLI-Cm1, OLI-Cm2, OLI-Cm3, OLI-Cm4, and OLI-Cm5 shown in Fig. 2A) as competitors in EMSA. These bands E were eliminated by cold oligonucleotides containing a CRE sequence (OLI-C, OLI-Cm1, OLI-Cm2, OLI-Cm3, and OLI-Cm4), and band F was competed by the CCAT sequence-containing oligonucleotides (OLI-C, OLI-Cm1, OLI-Cm2, and OLI-Cm5). These data suggest that these bands E were formed by CRE-binding proteins, and band F may contain YY1 protein (Fig. 5B). To further examine the possibility, we tested oligonucleotides containing the human tyrosine hydroxylase CRE (OLI-hTH/C), the human somatostatin CRE (OLI-hSOM/C), and the YY1-binding site from the mouse c-fos promoter (OLI-moY). As shown in Fig. 5C, molar excesses of OLI-C, OLI-hTH/C, and OLI-hSOM/C eliminated the E bands, whereas OLI-C and OLI-moY eliminated band F. Furthermore, a specific antibody against YY1 protein factor also diminished band F (Fig. 5D). To investigate whether these cis-elements may contribute to TPA-induced transcription of the DBH gene, we
prepared mutant DBH-luciferase fusion constructs and examined them by transient transfection assays (Fig. 6A). All the mutant constructs exhibited similar induction (2.1–3.6-fold the control level) of reporter gene expression in response to forskolin treatment. The reporter gene activities driven by 604Cm1Luc and 604Cm2Luc, which contain base substitutions within the AP1-like motif, were induced by TPA (10.5–13.7-fold the control) similarly to the wild type 604Luc. In contrast, fusion constructs containing mutations within the YY1-binding site, i.e. 604Cm3Luc and 604Cm4Luc, showed lower levels (5.8-fold the control) of transcriptional induction in response to TPA treatment.

Transcriptional Enhancement by TPA Requires Rotational Distance between CRE and YY1 Binding Sequence—To elucidate the structure/function relationship of the CRE and YY1-binding site in DBH transcriptional regulation, we prepared and examined two insertional fusion constructs containing 5 or 10 base pairs of unrelated DNA sequences between these two motifs (Fig. 6B). When 5 nucleotides were inserted between the CRE and CCAT sequences (604+5Luc), which would add a half-helical turn and change the phasic interaction of the cognate protein factors, TPA-mediated induction of the reporter activity was significantly attenuated compared with that with the wild type construct (from 7.0- to 2.8-fold). Interestingly, when 10 nucleotides were inserted (604+10Luc), adding a full helical turn between the CRE and YY1-binding site, an intact response to TPA treatment was obtained in line with a role for stereospecific alignment between these cis-regulatory elements. Although transcriptional induction following forskolin treatment exhibited a similar pattern in these mutant constructs, the luciferase activity of the 604+5Luc construct was not significantly changed as compared with the 604Luc or 604+10Luc construct cases.

The hDTRE Up-regulates the Heterologous Thymidine Kinase Promoter in Response to TPA Treatment—We next tested whether the hDTRE is able to confer TPA-mediated transcriptional induction on the heterologous thymidine kinase (tk) promoter. In control experiments (Fig. 7A), addition of four copies of OLI-hTH/C or three copies of OLI-C made the tk promoter robustly inducible in response to forskolin treatment. Similarly, the AP-1 element of the human TH gene (OLI-hTH/AP) conferred TPA inducibility, but multiple copies of OLI-A or OLI-B failed to confer inducibility to the tk promoter in response to forskolin or TPA. In contrast, four copies of the OLI-D containing the hDTRE rendered the tk promoter inducible by TPA (Fig. 7B). Furthermore, the combination of OLI-D and OLI-C potentiated TPA-inducibility of the tk promoter, supporting the notion that the hDTRE and its proximal cis-elements mediate TPA-induced transcription of the human DBH gene. This potentiation may require the YY1-binding site, because the combination of OLI-D with OLI-hTH/C or OLI-Cm3, containing an intact YY1-binding motif, did not potentiate the TPA inducibility. Combination of OLI-C with OLI-A or OLI-B did not affect TPA inducibility of the tk promoter activity at all (data not shown). Finally, we tested whether OLI-Dm2 was able to confer TPA inducibility on the tk promoter in the absence or the presence of OLI-C motifs and found no difference between the two conditions (Fig. 7B).

DISCUSSION

Regulation of human DBH gene expression by second-messenger signal transduction pathways has been extensively studied in neuroblastoma and other chromaffin cells (2, 3, 8). Phorbol ester (TPA) directly activates the PKC pathway, and the DNA sequence named AP-1, TGA(C/G)TCA, is generally believed to be the TPA-responsive element (52). As shown in Fig. 1A, amounts of mRNA were greatly increased by the addition of TPA in SK-N-SH. Although a similar sequence (TGT-GTCA) ranging from −172 to −166 bp of the human DBH promoter was a candidate AP-1 (Figs. 1A, 6A, and 7A), this element apparently has no function as a TRE (12). We consider that differences from the AP-1 sequence may be found in reg-
**FIG. 6.** The YY1 binding sequence is essential for transcriptional up-regulation by TPA. The center column shows the names of constructs and the results of the luciferase activity expressed as fold induction. Left and right panels show schematic diagrams for these constructs with the luciferase expression vector and the results for luciferase activity of each, respectively. A, 604m1Luc, 604m2Luc, 604m3Luc, and 604m4Luc were modified from the 604Luc construct according to the oligonucleotide sequences of OLI-Cm1, OLI-Cm2, OLI-Cm3, and OLI-Cm4, respectively. Asterisks indicate the mutated nucleotides. B, YY1 may interact with nuclear protein in a rotational and distance-dependent manner. 604+5Luc and 604+10Luc constructs were inserted with nonrelated sequences, GCTCTA (5 bp) or GTCTAGTGTA (10 bp), between the CRE and CCAT sequences of the 604Luc construct (30). Underline indicates the CRE and YY1 binding sequences. These experiments were performed three times each in triplicate, with similar results, and bars represent S.D. values. *, p < 0.001 (compared with luciferase activity of 604Luc).

**FIG. 7.** Transcriptional enhancement by TPA requires hOTRE, CRE, and YY1 binding sequences. Left panel shows the schematic diagram of the constructs with luciferase expression vector. Center column shows the name of these constructs and the results of luciferase activity expressed as the induction rate. Right panel shows the luciferase activity of each construct. Closed bars show basal activity (control). Oblique-line columns show luciferase activity of TPA-treated cells, and shaded columns show the luciferase activity of forskolin-treated cells. A, luciferase expression vector combined with virus tk promoter (tkLuc) was used to test the function of several oligonucleotides. B, luciferase activities were enhanced by combination of oligonucleotides connected to tkLuc. Arrows show the direction of each oligonucleotide. These experiments were performed five times in triplicate with similar results, and each bar represents S.D. values.
ulatory elements as in the case of the human aromatase cytochrome P-450 gene (53). The present study showed the mechanism of transcriptional enhancement by TPA stimulation to involve a hitherto undefined sequence in the human DBH promoter.

To identify the potential transcriptional regulatory elements of the human DBH gene cooperating to achieve TPA stimulation, we undertook deletion and mutational analysis of human DBH reporter constructs in human neuroblastoma cells. As shown in Fig. 1B, the DNA region from −223 to −187 bp (37 bp) was the critical TPA-responsive DNA region, but this does not contain any AP-1 or AP-1-like sequence (Fig. 1C). Because OLI-42 (from −224 to −183 bp) failed to form fine shifted bands in EMSA (data not shown), the 63-bp oligonucleotide region from −224 to −162 bp (OLI-63) was used and found to form two complexes (bands A and B) with the nuclear protein fraction (Fig. 2A). Bands A and B did not form with cold OLI-63 as a competitor, and band A was lost with cold OLI-42, but not with other competitors such as OLI-hTH/C, OLI-hSOM/C, OLI-hTH/AP, OLI-mo/Y, and nonrelated oligonucleotide (Fig. 2B). Further detailed analyses showed OLI-D (−213 to −183 bp) to be a strong competitor of OLI-63 probe, but not OLI-A, OLI-B, and OLI-C (Fig. 3A). Even though OLI-42 had the sequence of OLI-D, it unexpectedly eliminated only the band A formed by OLI-63 and nuclear protein extracts. We have not been able to explain this discrepancy yet. In addition, the present mutational analyses indicated the DNA region from −210 to −199 bp to be critical for binding with the nuclear protein (bands C and D, Fig. 4A). Changes in these sequences decrease induction of luciferase activity (Fig. 4B). Heterologous promoter experiments also showed that OLI-D (−213 to −183 bp) functioned as a TRE, but similar sequences are not present in the human DBH gene or in the aromatase cytochrome P-450 gene (53).

The CRE sequence of the human DBH gene has one base pair change from A to C as compared with consensus CRE sequence (Fig. 5A). It has been shown that CRE is an essential element not only for constitutive expression of the human DBH gene but also for induction by the addition of dibutyryl cAMP or forskolin (2, 3). Even the CRE sequence of the human DBH gene mutated in one nucleotide position (C at −174 bp from transcriptional initiation site) from the consensus CRE (TGACGTC)A, is a functional sequence that can bind with CREB (data not shown). However, no consensus CRE sequences exist in the promoter regions of rat and mouse DBH genes. Afar et al. (14) indicated that rat CRE sequence ranging from −174 to −161 bp bound activating transcription factor 1 protein in PC12 cells and CA77 cells. We found two major DNA-protein complexes using the OLI-C probe, i.e. the sequence from −186 to −161 bp of the human DBH gene. This oligonucleotide has the YY1 binding sequence, E-box sequence, and AP-1-like sequence downstream of the CRE sequence. Shifted bands with slow mobility (bands E in Fig. 5, B and C) were CRE and CREB complex, and band F was identified as the complex between the CCAT sequence and YY1 from the results of competition experiments using mutated oligonucleotides OLI-Cm1, OLI-Cm2, OLI-Cm3, OLI-Cm4, and OLI-Cm5 (12). Furthermore, the fact that the anti-YY1 antibody eliminated the YY1-DNA complex suggests that YY1 binds with another DNA region beside the CRE sequence. YY1 is known to be a potent positive and negative transcriptional regulator, and a DNA binding factor, depending on the promoter sequence (19–41). Seo et al. (12) reported that induction of chloramphenicol acetyltransferase activity by a construct bearing a mutated YY1-binding site was enhanced by the wild type sequence of the human DBH gene and speculated that YY1 might inhibit the binding of CREB to CRE. Similar mechanisms in which YY1 competed for DNA binding with a transactivating factor were also shown to be the case for the rat serum amyloid A1 (38), and β-casein genes (40), as well as the human γ interferon gene (54). We have demonstrated, however, a new function of YY1, i.e. enhancement of the transcriptional level of the human DBH gene under TPA stimulation. Constructs with mutations in the YY1 binding sequence (604m3Luc and 604m4Luc) failed to fully express the luciferase activity in contrast to those with YY1 binding sequence (604Luc, 604m1Luc, and 604 m2Luc). Our data on the effects of a 10-bp insertion between CRE and YY1-binding sites (604 + 10Luc) resulting in a 50% reduction are in line with the results of c-fos promoter analysis by Natesan and Gilman (30), who reported that YY1 bent the c-fos promoter DNA to regulate the contact with other proteins and thus changed the transcriptional control of the gene. Our findings also suggest that CREB and YY1 are essential for rotational phase interactions to regulate the correct transcription of the human DBH gene stimulated by the treatment with TPA.

To analyze the co-operative mechanism between hDTRE and YY1, luciferase constructs with a heterologous (tk) promoter were combined with several different oligonucleotides. Human neuroblastoma SK-N-SH cells are demonstrated to have PKA and PKC signaling pathways when the cells were treated with forskolin or TPA in the culture system (Fig. 7A). As was shown in Fig. 7B, TPA enhanced the luciferase activity in four copies of OLI-D, and the highest induction rate of the luciferase activity was observed with the construct containing the combination of OLI-D (including hDTRE) and OLI-C (including the YY1-binding site with the CRE of human DBH). Because OLI-C is only forskolin-responsive and not TPA-responsive (Fig. 7A), hDTRE-binding protein and YY1 may co-operate to enhance the transcriptional up-regulation by TPA in the human DBH promoter. This study thus showed that the combination of hDTRE, CRE, and YY-1 binding sequence may be controlled under TPA-stimulated signal transduction pathways.

Stimulation of human DBH gene transcription by TPA in human neuroblastoma cells appears to be mediated by an new cis-regulatory element (hDTRE) in the human DBH gene by interacting with two proteins CREB and YY1. YY1 may bend the DNA to a fixed structure. As a result, the hDTRE-binding protein and CREB or YY-1 may be able to interact with each other. The hDTRE-binding protein, CREB and YY1 combination, and the fixed conformation of the DNA may be essential for TPA stimulation of the human DBH promoter.

Acknowledgments—We thank Dr. Kwang Soo Kim for helpful advice and critical reading of this manuscript and Chieko Ando for technical assistance.

REFERENCES

1. Kobayashi, K., Kurosawa, Y., Fujita, K., and Nagatsu, T. (1989) Nucleic Acids Res. 17, 1099–1102.
2. Lamonaurow, A., Houhou, L., Bignet, N. F., Serck-Hanssen, G., Guibert, B., Icard-Liepkalns, C., and Mallet, J. (1993) J. Neurochem. 60, 364–367.
3. Ishiihuro, H., Kim, K. T., Joh, T. H., and Kim, K. S. (1993) J. Biol. Chem. 268, 17897–17904.
4. Ishiihuro, H., Kim, K. S., and Joh, T. H. (1995) Mol. Brain Res. 34, 251–261.
5. Montminy, M. R., Sevarino, K. A., Wagner, J. A., Mandel, G., and Goodman, R. H. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6682–6686.
6. Rinabow, K. T., Fink, J. S., Gilman, G. M. Z., Walsh, D. A., Goodman, R. H., and Ferramisco, J. R. (1988) Nature 336, 83–86.
7. Gonzalez, G. A., and Montminy, M. R. (1989) Cell 59, 675–680.
8. Kim, K. S., Ishiihuro, H., Tinti, C., Wagner, J. J., and Joh, T. H. (1994) J. Neurosci. 14, 7200–7207.
9. Kramer, S. D., Chong, J. A., Tsay, H. J., and Mandel, G. (1992) Neuron 9, 37–44.
10. Mori, N., Schoenherr, C., Vandenbergh, D. J., and Anderson, D. J. (1992) Neuron 9, 45–54.
11. Schoenherr, C. J., Paquette, A. J., and Anderson, D. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 8881–8886.
12. Seo, H., Yang, C., Kim, H. S., and Kim, K. S. (1996) J. Neurosci. 16, 4112–4112.
13. Shakkus, J., Greco, D., Asnani, L. P., and Lewis, E. J. (1992) J. Biol. Chem. 267, 18821–18830.
14. Afar, R., Silverman, R., Aguanno, A., and Albert, V. R. (1996) Mol. Brain Res.
15. Zellmer, E., Zhang, Z., Greco, D., Rhodes, J., Cassel, S., and Lewis, E. J. (1995) J. Neurosci. 15, 8109–8120
16. Morin, X., Cremer, H., Hirsch, M. R., Kapur, R. P., Goridis, C., and Brunet, J. F. (1997) Neuron 18, 411–423
17. Shaskus, J., Zellmer, E., and Lewis, E. J. (1995) J. Neurochem. 64, 52–60
18. Greco, D., Zellmer, E., Zhang, Z., and Lewis, E. (1995) J. Neurochem. 65, 510–516
19. Shi, Y., Seto, E., Chang, L. S., and Shenk, T. (1991) Cell 67, 377–388
20. Flanagan, J. R., Becker, K. G., Ennist, D. L., Gleason, S. L., Driggers, P. H., Levi, B. Z., Appella, E., and Ozato, K. (1992) Mol. Cell. Biol. 12, 38–44
21. Bauknecht, T., Angel, P., Royer, H. D., and zur Hausen, H. (1992) EMBO J. 11, 4697–4617
22. Montalvo, E. A., Shi, Y., Shenk, T. E., and Levine, A. J. (1991) J. Virol. 65, 3647–3655
23. Liu, R., Baillie, J., Sissons, J. G. P., and Sinclair, J. H. (1994) Nucleic Acids Res. 22, 2453–2459
24. Margolis, D. M., Somasundaran, M., and Green, M. R. (1994) J. Virol. 68, 905–910
25. Natesan, S., and Gilman, M. Z. (1993) Genes Dev. 7, 2497–2509
26. Peters, B., Merezhinskaya, N., Diffley, J. F. X., and Noguchi, C. T. (1993) J. Biol. Chem. 268, 3430–3437
27. Ye, J., Ghosh, P., Cippitelli, M., Subleski, J., Hardy, K. J., Ortizado, J. R., and Young, H. A. (1994) J. Biol. Chem. 269, 25728–25734
28. Lee, T. C., Shi, Y., and Schwartz, R. J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9814–9818
29. MacLellan, W. R., Lee, T. C., Schwartz, R. J., and Schneider, M. D. (1994) J. Biol. Chem. 269, 16754–16760
30. Usheva, A., and Shenk, T. (1994) FEBS Lett. 347, 289–294
31. Lee, Y. M., and Lee, S. C. (1994) DNA Cell Biol. 13, 7487–7495
32. Austen, M., Luscher, B., and Luscher-Firzlaff, J. M. (1997) J. Biol. Chem. 272, 1709–1717
33. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475–1489
34. Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) Mol. Cell. Biol. 2, 1044–1051
35. Kim, K. S., Lee, M. K., Carroll, J., and Kant, J. A. (1989) Anal. Biochem. 176, 28–32
36. Williams, T. M., Burlein, J. E., Ogden, S., Kricka, L. J., and Kant, J. A. (1989) Anal. Biochem. 176, 28–32
37. An, G., Hidaka, K., and Siminovitch, L. (1982) Mol. Cell. Biol. 2, 1628–1632
38. Nishizuka, Y. (1986) Science 233, 305–312
39. Toda, K., and Shizuta, Y. (1994) J. Biol. Chem. 269, 8099–8107
40. Ye, J., Cippitelli, M., Dorman, L., Ortizado, J. R., and Young, H. A. (1996) Mol. Cell. Biol. 16, 4744–4753