Multiple Protein Factors Interact with the *cis*-Regulatory Elements of the Proximal Promoter in a Cell-Specific Manner and Regulate Transcription of the Dopamine β -Hydroxylase Gene

Hyemyung Seo,^{1,2} Chunying Yang,¹ Hee-Sun Kim,¹ and Kwang-Soo Kim^{1,2}

Departments of ¹Neurology and ²Anatomy and Neurobiology, University of Tennessee, College of Medicine, Memphis, Tennessee 38163

The dopamine β -hydroxylase (*DBH*) gene is expressed selectively in noradrenergic and adrenergic neurons and neuroendocrine cells in the nervous system. A cAMP response element (CRE) residing at –181 to –174 bp from the transcription start site of the human *DBH* gene seems to be essential for *DBH* transcription. Potential *cis*-regulatory motifs such as AP1 and YY1 occur proximal to and overlap this CRE, endowing the area with a composite promoter structure. Using the *DBH*-expressing human neuroblastoma SK-N-BE(2)C and *DBH*-negative HeLa cell lines as model systems, we report here that this CRE/YY1/AP1 area interacts with multiple nuclear proteins, including CRE-binding protein (CREB) and transcription factor YY1 in a cell-specific manner. In support of the notion that multiple proteins bind to the CRE/YY1/AP1 area, DNase I footprinting analysis has demonstrated that nuclear extracts protect an extended region (from –186 to –150 bp) relative to that protected by the purified CREB (from –186 to –171 bp).

Site-directed mutational analysis has revealed differential roles of potential *cis*-regulatory motifs in regulation of *DBH* transcription. Strikingly, the YY1 element positively regulated basal *DBH* transcription while simultaneously regulating cAMP-mediated induction negatively, which is a novel mechanism of promoter function. Furthermore, three additional DNA-binding sites have been identified by DNase I footprint analysis in the upstream 260 bp promoter region of the human *DBH* gene, of which two sites are cell-specific. These results support a model whereby multiple proteins bind to the 5'-proximal area in a cell-specific manner and coordinately regulate the cell type-specific transcriptional activation of the *DBH* gene.

Key words: dopamine β -hydroxylase; transcriptional regulation; cAMP response element; cAMP response element binding protein; AP1 element; transcription factor YY1; cell type-specific transcription; *cis*-regulatory element; noradrenergic neuron

Noradrenergic neurons in the brain have extremely divergent projections into all parts of the neuraxis and regulate many essential functions of the nervous system, including autonomic outflow, memory, and motor output (Barnes and Pompeiano, 1991). Understanding the physiology and phenotypic regulation of these neurons is, therefore, of great importance. Among several genes specifically expressed in noradrenergic neurons, dopamine β -hydroxylase (*DBH*; EC 1.14.17.1) is a hallmark protein, because noradrenaline is synthesized by this enzyme (Kirshner and Goodall, 1957; Friedman and Kaufman, 1965). Regulation of the *DBH* gene provides a challenging system for studying neuron-specific gene regulation in general, as well as cell type-specific gene expression in the brain, for the following reasons: (1) *DBH* is expressed restrictively in noradrenergic and adrenergic neurons and neurosecretory cells in the nervous system; (2) differential expression of *DBH* among catecholaminergic neurons underlies phenotypic subspecifications among catecholaminergic neurons; that is, whereas tyrosine hydroxylase (TH) is expressed both in

dopaminergic and noradrenergic neurons, *DBH* is expressed only in noradrenergic neurons; and (3) expression of *DBH* is modulated in response to a variety of trans-synaptic signals, hormones, growth factors, and stress (Otten and Thoenen, 1976; Sabban et al., 1983; Acheson et al., 1984; Faucon Biguet et al., 1986; Lewis et al., 1987; Badoyannis et al., 1991; McMahon et al., 1992; K. T. Kim et al., 1993; Lamouroux et al., 1993; Wessel and Joh, 1993).

Cell culture studies using transient expression analyses have demonstrated that the upstream sequences of the *DBH* gene can drive expression of the reporter gene in a cell-specific manner (Shaskus et al., 1992, 1995; Ishiguro et al., 1993, 1995; Lamouroux et al., 1993). Upstream sequences of the human and rat *DBH* genes as short as 486 and 395 bp, respectively, direct cell-specific expression, suggesting that these 5'-promoter areas may contain important genetic elements for *DBH* gene regulation (Shaskus et al., 1992; Ishiguro et al., 1993). In contrast, transgenic mice experiments have identified a further upstream region between –1.1 and –0.6 kb of the human *DBH* gene to be essential for reporter gene expression in noradrenergic neurons (Hoyle et al., 1994). Based on these observations, it has been suggested that the *DBH* gene may contain both proximal and distal elements, of which only the proximal elements are required for expression in cultured cells, whereas both elements are required in transgenic mice (Hoyle et al., 1994). Both positive and negative regulatory elements are required for cell type-specific transcription of the *DBH* gene. In the 486 bp region of the human *DBH* gene, the upstream half-site spanning –486 to –263 bp was shown to have a cell-specific silencer function (Ishiguro et al., 1993). This region

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Correspondence should be addressed to Dr. Kwang-Soo Kim, Department of Neurology, University of Tennessee, College of Medicine, 855 Monroe Avenue, Memphis, TN 38163.

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contains a 13 bp fragment that shows 77% sequence homology with the neuron-specific silencer motif found in the *SCG10* and the rat type II sodium channel genes (Kraner et al., 1992; Mori et al., 1992; Li et al., 1993). However, this nucleotide fragment shows a general silencer activity in both DBH-positive and DBH-negative cell lines, suggesting that additional regulatory elements may reside in that silencer domain (Ishiguro et al., 1995). Similarly, a negative regulatory region was identified in the upstream sequence of the rat *DBH* gene (Shaskus et al., 1995; Afar et al., 1996).

Deletional and mutational analyses strongly suggest that a cAMP response element (CRE) in the proximal 262 bp of the human DBH promoter is an essential positive element for both the basal and cAMP-inducible transcription of the *DBH* gene (Ishiguro et al., 1993; Lamouroux et al., 1993). Consistent with the functional importance of the CRE, coexpression of the catalytic subunit of cAMP-dependent protein kinase, via the CRE, dramatically stimulated the transcriptional activity of the *DBH* gene in a cell type-specific manner (Kim et al., 1994). The CRE of the human *DBH* gene, 5'-TGACGTCC-3', deviates from the consensus CRE motif by a single base (Roesler et al., 1988; Goodman, 1990). The CRE is, after one base pair, followed by an AP1-like motif, 5'-TGTGTCA-3', also deviating from the consensus AP1, 5'-TGA(C/G)TCA-3', by a single base (Angel et al., 1987; Lee et al., 1987). These two potential *cis*-regulatory elements are overlapped by another potential *cis*-regulatory motif, YY1 (5'-GTCCATGTGT-3'), which also deviates from the consensus YY1 site, 5'-(C/G)(G/T/A)CCATNTTN-3', by a single base (Hahn, 1992; Lee et al., 1992; Riggs et al., 1993; Lu et al., 1994). Thus, this sequence area has a composite promoter structure (termed CRE/YY1/AP1 in this study).

Although less effective than the 486 bp, the 262 bp upstream region of the human *DBH* gene conferred cell-specificity in a transient expression study using the DBH-expressing human neuroblastoma SK-N-BE(2)C and DBH-negative HeLa cell lines as model systems (Ishiguro et al., 1993). These findings prompted us to investigate the interactions between the 260 bp, proximal promoter region and nuclear proteins prepared from SK-N-BE(2)C and HeLa cells. Here, we report that multiple protein factors, including the CRE-binding protein (CREB) and YY1 factor, bind to the CRE/YY1/AP1 area of the human *DBH* gene in a cell-specific manner. Each potential *cis*-regulatory motif of the CRE/YY1/AP1 was mutated to analyze its promoter function in *DBH* gene regulation. Furthermore, DNase I footprint analysis revealed that, in addition to the CRE/YY1/AP1 area, three other potential *cis*-regulatory sites exist within the proximal 260 bp upstream region, two of which are recognized in a cell-specific manner.

MATERIALS AND METHODS

Cell culture. The DBH-positive human neuroblastoma SK-N-BE(2)C and the DBH-negative HeLa cell lines were used in this study. Cells were grown in DMEM supplemented with 10% fetal calf serum (HyClone, Logan, UT), streptomycin, and penicillin in a 5% CO₂ incubator, as described previously (Ishiguro et al., 1993; Kim et al., 1994). To avoid possible changes in cell properties resulting from spontaneous mutations and/or phenotypic selection, cells were used for only ~30 passages, starting from cell stocks stored in liquid nitrogen. Dibutyl cAMP and the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) were added to the culture medium 16–18 hr before cells were harvested with final concentrations of 1 mM and 0.1 μg/ml, respectively.

Plasmids. The human DBH-CAT reporter constructs, 978CAT and ΔCRE 978CAT, were described previously (Ishiguro et al., 1993). These fusion plasmids contain the upstream sequence of the human *DBH* gene

fused to the bacterial chloramphenicol acetyltransferase (*CAT*) gene. Thus, the promoter function of these sequences can be monitored by *CAT* assay after introducing the corresponding plasmids into cells. To identify functional *cis*-regulatory motifs within the CRE/YY1/AP1 area of the human *DBH* gene, a series of fusion plasmids with individual mutations was produced by using the ExSite PCR-based site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's procedure. The following oligonucleotides were used in the mutagenesis procedure, using 978CAT plasmid as the template: ATCCGGACCATGTGTTCATTAGTGC and CAGTGGGAGTTGAAGTAG for mCRE, GTACAATTAGTGCCAATTAGAGG and ATGGACGTCATCAGTGGG for mAP, and CTGATGACGTCCGTGTGTCATTAG and TGGGAGTTGAAGTAGACAGG for mYY. The first set of primers represents coding strand sequences of the promoter containing the desired mutations (underlined bases), and the second set of primers represents the noncoding strand sequences of the immediate 5' region of the first primers. Constructs with correct mutations were screened by restriction enzyme digestion and sequencing analysis. Two independent plasmids containing the same mutation were isolated and characterized by transient transfection assays to ensure the consistency of data.

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA). Crude nuclear extracts were prepared from SK-N-BE(2)C and HeLa cells according to the procedure described by Dignam et al. (1983). The pellet was resuspended in buffer D (20 mM HEPES, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT) and dialyzed against the same buffer. The extracts were quick-frozen in liquid N₂ and stored in aliquots at -70°C and used within 3 months of extraction. Protein concentrations of nuclear extracts of SK-N-BE(2)C and HeLa cells were 12.2 and 10.4 μg/μl, respectively. Sense and antisense strands of oligonucleotides were synthesized, annealed, and labeled using T4 polynucleotide kinase and [γ -³²P]ATP. EMSA was performed using 30,000 to 50,000 cpm of labeled probe (~0.05–0.1 ng) and *Escherichia coli*-expressed CREB (Gonzalez et al., 1991) or nuclear extracts (1–3 μl) in a final volume of 20 μl of 12.5% glycerol and (in mM): 12.5 HEPES (pH 7.9), 4 Tris-HCl (pH 7.9), 60 KCl, 1 EDTA, and 1 DTT with 1 μg of poly(dI-dC) as nonspecific competitor. The reaction was incubated at room temperature for 20 min.

Competition binding assays were performed by adding nonradioactive competitor fragments in a molar excess as well as ³²P-labeled oligonucleotides before adding nuclear extracts. Then the crude nuclear proteins or purified proteins were added and incubated at room temperature for 20 min. Nucleotide sequences of oligonucleotides used in EMSA and competition assays were DBH, 5'-CTGATGACGTCCATGTGTTCATTAGT-3' (Fig. 1A); mDBH, 5'-CTGATGACGTCCGTGTGTTCATTAGT-3' (underlined base was mutated); TH, 5'-GAGGGGCTTTGACGTCCAGCTGGCC-3'; fos-YY1, 5'-TAGGAAGTCCATCCATTCACAGCG-3' from the murine *c-fos* gene (Natesan and Gilman, 1993); and SP1, 5'-ATTTCGATCGGGCGGGGCGAGC-3'. For antibody experiments, 0.4–2 μl of α244 anti-CREB antiserum (M. Montminy, The Salk Institute) or anti-YY1 antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) was preincubated on ice with nuclear extracts for 2 hr before adding the binding mixture. Protein concentrations of anti-CREB antiserum and anti-YY1 antiserum were 2 and 1 μg/μl, respectively. The DNA-protein complexes were resolved on high ionic strength, nondenaturing 6% polyacrylamide gels (Chodosh, 1995). Subsequently, the gels were vacuum-dried and autoradiographed at -70°C with an intensifying screen for 6–16 hr, depending on the strength of signals.

DNase I footprinting. The human *DBH* 5'-proximal promoter fragment was prepared by PCR and used as a probe in the DNase I footprinting experiment. For the coding strand probe, a primer 5'-GCTCTTCAGAGCCATCTC-3' representing the coding nucleotide sequence from -240 to -223 bp of the human *DBH* gene was labeled by polynucleotide kinase using [γ -³²P]ATP. This was used in PCR, together with a cold oligonucleotide, 5'-CGGTGGTATATCCAGTG-3', representing the noncoding nucleotides 15–31 of the *CAT* gene (Luckow and Schutz, 1987). The noncoding strand probe was prepared using a labeled oligonucleotide, 5'-CCAGGCCAAGCACTCAGC-3', representing the noncoding nucleotide sequence from -121 to -138 bp, together with the unlabeled primer 5'-TTGGTTTGAAATGGGCCC-3', which represents coding sequences from -503 to -486 bp. Another noncoding strand probe was prepared using a labeled primer, 5'-CGGTGGTATATCCAGTG-3', from the *CAT* gene and the cold primer 5'-GCTCTTCAGAGCCATCTC-3'. Using 978CAT plasmid DNA as a template, PCR was performed with the denaturation, annealing, and DNA synthesis steps at 94°C (0.5 min), 54°C (1 min), and 72°C (1 min), respectively, for a total of 32 cycles. For the first and last cycles, the DNA synthesis step was performed for 3 min to ensure accurate polymerase reaction. The

Figure 1. The cAMP response element binding protein (CREB) interacts with the CRE/YY1/AP1 of the human *DBH* gene. **A**, The CRE/YY1/AP1 area of the human *DBH* gene contains a CRE-like sequence at -181 to -174 bp, an AP1-like sequence at -173 to -167 bp, and an overlapping putative YY1 sequence motif. The nucleotide sequence around the CRE of the rat *TH* gene has no additional sequence motif besides the consensus CRE. **B**, Purified CREB binds to the CREs of *DBH* (lanes 1–3) and *TH* (lanes 4–6) genes. One microgram (lanes 1 and 4), 0.3 μ g (lanes 2 and 5), or 0.1 μ g (lanes 3 and 6) of bacterially expressed CREB (Gonzalez et al., 1991) was incubated with radiolabeled oligonucleotides as depicted in **A**. Unbound free probe (**F**) is indicated by an arrowhead. The CRE of the *TH* gene shows higher affinity for CREB than does that of the *DBH* gene. **C**, DNase I footprinting analysis of the 5'-proximal promoter region of the human *DBH* gene using purified CREB. *Coding* and *Noncoding* strands of the human *DBH* upstream sequence were labeled as described in Materials and Methods and digested with DNase I. Lanes 1 and 4, No CREB; lane 2, 0.5 μ g of bacterially expressed CREB; lanes 3 and 5, 2 μ g of bacterially expressed CREB. Relative position of each band to the transcription start site is indicated at the left side of the autoradiograms, as determined by the Maxam–Gilbert sequencing reaction (Fig. 5). A 16 bp region at -186 to -171 bp, which encompasses the CRE motif of the *DBH* gene, is protected by CREB in both *Coding* and *Noncoding* strands as indicated by brackets at the right side of autoradiograms.

appropriate end-labeled probe was isolated from a 4% polyacrylamide gel, as described previously (Kim et al., 1995).

After incubating 30,000 cpm of labeled probe with 10–20 μ l of nuclear extracts in 40 μ l of binding buffer for 25 min at room temperature, DNase I digestion was performed using freshly diluted DNase I in 1 $\times$ binding buffer, which contained (in mM): 20 HEPES (pH 7.9), 2 MgCl₂, 50 NaCl, 1 DTT, and 0.1 EDTA and 10% glycerol. Two micrograms of poly(dI-dC) were included in the reaction as a nonspecific competitor. The amount of DNase I was adjusted empirically for each extract to produce an even pattern of partially cleaved products. The DNase I reaction was stopped by adding 100 μ l of stop buffer [50 mM Tris (pH 8.0), 1% sodium dodecyl sulfate, 10 mM EDTA (pH 8.0), 0.4 mg/ml proteinase K, and 100 mM NaCl]. Then samples were extracted twice with phenol chloroform, and

the DNA was precipitated with 3 vol of ethanol. The DNA pellet was dried and resuspended in sequencing stop buffer (0.05% xylene cyanol, 0.05% bromophenol blue, 10 mM Na₂ EDTA, and 90% deionized formamide) and incubated at 95°C for 3 min. Then an aliquot of sample was loaded onto a 6% polyacrylamide/8 M urea-sequencing gel. Without incubation beforehand, the same probe was subjected to parallel digestion with nuclear extracts, typically using 5–10% of the DNase I used in the presence of nuclear extracts. Location of cleaved products was determined by Maxam–Gilbert sequencing reaction of each probe.

Transient transfection, CAT, and β -galactosidase assays. For the transient expression assays of the DBH-CAT constructs, cells were seeded in 60 mm dishes 1 or 2 d before transfection. Care was taken to ensure that cells were distributed evenly in the dish. Transfection was performed by

Figure 2. DNA-protein complexes formed by nuclear extracts prepared from SK-N-BE(2)C or HeLa cells. *A*, A 25 bp oligonucleotide (DBH) containing the CRE/YY1/AP1 of the human *DBH* gene was radiolabeled and incubated with 1 μ l (lanes 2 and 5), 2 μ l (lanes 3 and 6), or 3 μ l (lanes 4 and 7) of nuclear extracts. Lane 1, No nuclear extracts; lanes 2–4, nuclear extracts from SK-N-BE(2)C; lanes 5–7, nuclear extracts from HeLa cells. Unbound free probe (F, arrowheads) and specific DNA-protein complexes are indicated by arrows. *B*, A 25 bp oligonucleotide (TH) containing the CRE of the rat *TH* gene was incubated with 1 μ l (lanes 2 and 4) or 2 μ l (lanes 3 and 5) of nuclear extracts. Lane 1, No nuclear extracts; lanes 2–3, nuclear extracts from SK-N-BE(2)C; lanes 4–5, nuclear extracts from HeLa cells. C1, C2, and C4 commonly were produced by nuclear extracts from both cell lines, whereas C3 and C5 were cell-specific. *C*, CREB is among nuclear proteins that bind to the CRE/YY1/AP1 of the human *DBH* gene. Two microliters of nuclear extracts were preincubated with preimmune serum (lane 1, 0.2 μ l; lanes 2 and 5, 1.0 μ l) or α 244 anti-CREB antiserum (lanes 3 and 6, 0.2 μ l; lanes 4 and 7, 1.0 μ l). A supershifted band (arrows at right sides of autoradiograms) was evident when nuclear extracts were incubated with anti-CREB antiserum.

the calcium phosphate coprecipitation method when cells reached ~50% confluence, as previously described (Ishiguro et al., 1993; K. S. Kim et al., 1993). For the SK-N-BE(2)C cell line, each 60 mm dish was transfected with 2 μ g of the reporter construct and pUC19 plasmid to a total of 5 μ g of DNA. To correct for differences in transfection efficiency among different DNA precipitates, cells were cotransfected with 1 μ g of pRSV- β gal for experiments in Figure 6B or 1 μ g of pSV40- β gal in Figure 6C. Twice as much DNA was used in the transfection of HeLa cells. CAT activity was normalized to that of β -galactosidase. Assays of CAT and β -galactosidase activities were performed, as previously described (Ishiguro et al., 1993; K. S. Kim et al., 1993).

RESULTS

CREB binds to the CRE/YY1/AP1 area of the human *DBH* gene

Our previous deletional analyses indicated that a potential CRE residing at –181 to –174 bp from the transcription start site is an essential *cis*-regulatory element for basal and cAMP-stimulated transcription of the human *DBH* gene. The CRE of the human *DBH* gene (TGACGTCC) has a single base deviation from the consensus CRE sequence (TGACGTCA) (Fig. 1A). As shown in Figure 1B, CREB can bind *in vitro* to the CREs of the *DBH* and *TH* genes in EMSA. It seems that CREB has higher binding affinity to the TH CRE than to the DBH CRE, probably because the TH has the consensus CRE sequence (Fig. 1A). When CREB was preincubated with anti-CREB antiserum, the DNA-protein complex was completely supershifted, indicating that CREB was involved directly in formation of the complex (data not shown). Furthermore, DNase I footprint analysis showed that CREB specifically protected the sequence ranging from –186 to –171 bp of the human *DBH* gene, which encompasses the CRE motif (Fig. 1C, lanes 2 and 3). The identical region was protected when the opposite strand was used as a probe (Fig. 1C, lane 5). CREB apparently did not bind to the 3'-AP1-like sequence motif nor to any other DBH upstream sequences ranging from –260 bp to the transcription start site, thus establishing the CRE motif residing at

–181 to –174 bp as the only binding site for CREB in the proximal DBH promoter region.

Multiple protein factors bind to the CRE/YY1/AP1 area of the human *DBH* gene in a cell-specific manner

An AP1-like motif, containing a single base deviation from the canonical AP1, resides at the immediate 3' position of the CRE. In addition, a putative YY1 site overlaps with these two sequence motifs, rendering the CRE area of the *DBH* gene a composite promoter structure (Fig. 1A). As shown in Figure 2A (lanes 2–4), multiple DNA-protein complexes were formed *in vitro* between the CRE/YY1/AP1 sequence and nuclear proteins prepared from the DBH-expressing SK-N-BE(2)C cells. Two major bands (C2 and C4) were seen at the upper position as well as a minor complex at an even higher position (C1). In addition to these complexes, another DNA-protein complex was seen at a lower position (C5). Nuclear proteins prepared from the DBH-negative HeLa cells resulted in a pattern of DNA-protein complexes significantly different from that of SK-N-BE(2)C cells (Fig. 2A, lanes 5–7). First, there were three major upper bands (C2, C3, and C4) with C3 preferentially formed only when HeLa cell extracts were used. More interestingly, C5 at the lowest position was barely detectable (compare lanes 2 and 5), indicating that this complex is formed in a cell-specific manner with SK-N-BE(2)C proteins. In a parallel EMSA experiment, an oligonucleotide containing the TH CRE was used (Fig. 2B). In general, the pattern of upper bands was almost identical to that of the DBH oligonucleotide, suggesting that these upper complexes may involve the CRE and/or AP1. In contrast, C5 was not detected at all when the TH CRE was used, indicating that it represents a binding distinct from CRE/AP1.

Next we determined whether CREB was among the nuclear proteins that interacted with the CRE/YY1/AP1. Nuclear extracts of both SK-N-BE(2)C and HeLa cells were preincubated with either preimmune serum or α -244 anti-CREB antiserum

Figure 3. Competition assay using different cold oligonucleotides. One microliter of SK-N-BE(2)C extracts was incubated with γ - 32 P-labeled oligonucleotide DBH in the presence of 100-fold or 1000-fold molar excesses of the indicated cold oligonucleotides. Oligonucleotides DBH and TH are described in Figure 1A. Oligonucleotide *fos*-YY1, containing the YY1 sequence of the murine *fos* gene, efficiently competed formation of C5. Oligonucleotide mDBH contains the same sequence as oligonucleotide DBH except for a single base substitution at -174 bp (A–G), which dramatically reduced its ability to compete for the formation of C5.

(Yamamoto et al., 1990). As shown in Figure 2C, a supershifted band appeared in addition to all other complexes only when CREB antibody was used in both SK-N-BE(2)C and HeLa. This strongly suggests that CREB is one of the protein factors that bind to the CRE/YY1/AP1 area of the *DBH* gene. Among the multiple complexes observed in Figure 2A, C2 may represent or include the complex formed by CREB, because the complex formed by purified CREB and oligonucleotide probe comigrated with C2 (data not shown).

When a 100-fold excess of cold DBH oligonucleotide was included in the incubation (Fig. 3), all complexes disappeared except C4, suggesting that C4 is the least specific among the complexes formed. In support of this, C4 was competed by even the SP1 oligonucleotide when a 1000-fold excess was used (Fig. 3). When TH oligonucleotide containing the consensus CRE was used as a competitor, formation of C1 and C4, but not C2, was reduced efficiently, suggesting that C2 may be specific to an unidentified DBH sequence motif. More importantly, TH oligonucleotide did not compete with C5, supporting the above conclusion that C5 is not related to the CRE. A nonrelated oligonucleotide containing the SP1 motif did not affect significantly the formation of these complexes.

The *fos*-YY1 oligonucleotide containing the YY1 motif of the *fos* gene selectively competed away C5 and did not affect the upper complexes (Fig. 3). When a single base of the core sequence of the YY1 motif in the DBH sequence was mutated from CCAT to CCGT (oligonucleotide mDBH), its ability to compete with C5 was reduced dramatically. Likewise, formation of C5 was abolished completely by this single base mutation within the core YY1 motif (compare Figs. 2A and 4A). A most straightforward explanation for the cell-specific formation of C5 would be that YY1-binding activity exists in SK-N-BE(2)C cells but not in HeLa cells. However, EMSA using the YY1 oligonucleotide of the *fos* gene clearly demonstrated that both cells contain comparable amounts of YY1-binding activity (Fig. 4B). The mobility of the DNA-protein complex of the *fos*-YY1 was much more retarded when compared to that of C5, indicating that the molecular weight of

protein involved in forming C5 is smaller. Thus, it is possible that a protein factor, different from YY1, is responsible for the selective formation of C5 by SK-N-BE(2)C extracts. To investigate this possibility, anti-YY1 antiserum or preimmune serum was incubated with nuclear extracts of SK-N-BE(2)C cells before the EMSA. As shown in Figure 4C, anti-YY1 antiserum specifically recognized and significantly diminished the formation of C5. A supershifted complex was not found, indicating that it might be masked by the other upper bands or that this antiserum may block the interaction between the protein and the oligonucleotide. In favor of the former possibility, the signal at C2 seemed to have increased by incubation of extracts with anti-YY1 antiserum (Fig. 4C, lanes 5 and 6). The anti-YY1 antiserum did not affect other complexes. From these data, we conclude that C5 is formed by YY1 or a closely related factor that has YY1 antigenicity.

DNase I footprint analysis reveals three additional protected domains as well as extended protection at the CRE/YY1/AP1 area in the proximal DBH promoter region

DNase I footprinting of the proximal DBH promoter region using nuclear extracts from SK-N-BE(2)C and HeLa cell lines (Fig. 5) revealed an extended region of protection corresponding to the CRE/YY1/AP1 site (domain IV, -186 to -150 bp), as compared to that protected by purified CREB (-186 to -171 bp; Fig. 1C), indicating that more than one protein factor bound to this area. It should be noted that the three motifs, CRE, YY1, and AP1, were clustered between -181 and -166 bp (Fig. 1A). Although five more bases were protected at the 5' side, which is similar to the protection pattern by purified CREB, 16 more bases were protected at the 3' side from these motifs. This observation suggests that additional binding site(s) may exist at the position immediate 3' to the AP1. The same extended region was protected by nuclear extracts from both SK-N-BE(2)C and HeLa cells on both the coding and noncoding strands. However, the region of the coding strand protected by HeLa extracts seemed to be divided into two subdomains, one from -186 to -168 bp and the other from -166 to -150 bp, with a slightly hypersensitive site at -167 bp (Fig. 5A). In the case of SK-N-BE(2)C, this division was not obvious, but four strong hypersensitive sites were present from -147 to -141 bp. On the noncoding strand, SK-N-BE(2)C, but not HeLa extracts, produced a division of the same protected region into two subdomains (-151 to -160 bp and -162 to -186 bp) with a strong hypersensitive site at -161 bp. Thus, these observations indicated that multiple proteins bound to the CRE/YY1/AP1 area and that the precise topographical structures of DNA-protein complexes were different in SK-N-BE(2)C and HeLa cells.

Three additional binding sites have been identified in the more proximal promoter region (Fig. 5). Domain I, ranging from -58 to -40 bp, is located 10 bp upstream of the TATA box of the human *DBH* gene (Kobayashi et al., 1989). This domain is G/C-rich and contains a sequence patch, 5'-ACCCACCCCA-3', at the center position (ranging from -54 to -45 bp), the opposite sequence of which is very similar to the Sp1 binding site, 5'-(G/T)(G/A)GGCC(G/T)(G/A)(G/A)(G/T)-3' (Briggs et al., 1986; Faisst and Meyer, 1992). Domain II, residing at -80 to -68 bp, is A/T-rich and does not show significant homology to any known sequence motif, suggesting that this may represent a novel *cis*-regulatory element (Faisst and Meyer, 1992). Whereas domain I was bound by both extracts, domain II was bound by SK-N-BE(2)C but not by HeLa extracts. Finally, domain III resides at -134 to -102 bp and is G/C-rich. This domain is divided into two

Figure 4. Transcription factor YY1 is involved in formation of C5. *A*, A 25 bp oligonucleotide (mDBH) containing a mutation within the YY1 motif was radiolabeled and used as a probe. *Lane 1*, No nuclear extracts; *lane 2*, 1 μ l nuclear extract from SK-N-BE(2)C; *lane 3*, 1 μ l nuclear extract from HeLa cells. *B*, A 24 bp oligonucleotide containing the YY1 element of the murine *c-fos* gene was radiolabeled and incubated with 1 μ l (*lanes 2 and 4*) or 2 μ l (*lanes 3 and 5*) of nuclear extracts. *Lane 1*, No nuclear extracts; *lanes 2–3*, nuclear extracts from SK-N-BE(2)C; *lanes 4–5*, nuclear extracts from HeLa cells. *C*, Nuclear extracts were preincubated for 2 hr at 4°C with preimmune serum (*lanes 2–3*) or anti-YY1 antiserum (*lanes 5–6*) before the addition of the radiolabeled oligonucleotide DBH. *Lanes 1 and 4*, No preincubation; in *lanes 2 and 5*, 0.4 μ l and in *lanes 3 and 6*, 1.5 μ l of preimmune serum or anti-YY1 antiserum were included in the incubation.

subdomains, one at –134 to –113 bp and the other at –112 to –102 bp, the latter of which seems to be bound by both extracts. At –127 to –116 bp, it includes a sequence patch, GGCCTGGGGCGC, that perfectly matches the palindromic AP-2 site, 5'-GCCNNNGGC-3' (Williams and Tjian, 1991; Faisst and Meyer, 1992). Indeed, Greco et al. (1995) recently defined the corresponding sequence of the rat *DBH* gene as the binding site of affinity-purified AP-2. Our results demonstrate further that nuclear factors of SK-N-BE(2)C, but not that of HeLa cells, bind to the subdomain at –134 to –113 bp encompassing this putative AP-2 site. In addition, the TATA box region at –31 to –16 bp is shown to be footprinted modestly by both SK-N-BE(2)C and HeLa extracts (Fig. 5*B*). Almost identical sequences have been protected when either coding or noncoding strand has been used in footprinting analysis.

Effect of mutation of each potential cis-regulatory motif residing in the CRE/YY1/AP1 area on the DBH promoter function

Individual sequence motifs of the CRE/YY1/AP1 area were mutagenized in the context of the 978 bp upstream sequence of the human *DBH* gene, and transcriptional activity in SK-N-BE(2)C cells was examined by the transient expression assay (Fig. 6). Substitution mutations were introduced, instead of deletions or additions, so that the spatial and contextual surroundings were maintained. The wild-type 978 bp upstream sequence exhibited cell-specific promoter activity as well as inducibility in response to treatment with dibutyl cAMP and TPA (Ishiguro et al., 1993). Mutation of four bases within the CRE motif diminished basal transcriptional activity by 90% and significantly reduced responsiveness to treatment with cAMP (from 3.2-fold to 1.3-fold). In contrast, inducibility by TPA was not altered but modestly increased in this mutant construct (from 4.6-fold to 6.2-fold), prob-

ably because this construct has a very low level of basal expression. Mutation of three bases within the AP1 motif affected its responsiveness neither to cAMP treatment nor to phorbol ester treatment. This mutant construct seemed to have a slightly higher level (~20%) of basal promoter activity than that of the wild type.

To maintain the nucleotide sequences of other promoter motifs, a single base of the core YY1 motif was mutated to make the mYY construct. This mutation, which abolished YY1 binding in EMSA (Fig. 4*A*), reduced basal transcriptional activity by 70%, indicating that YY1 motif is an important positive regulatory element for *DBH* transcription. Surprisingly, after treatment with cAMP, the promoter activity increased more robustly than that of the wild-type construct (from 3- to 11-fold). This result shows that the YY1 element negatively regulates transcriptional induction by cAMP, defining a novel promoter function involving both positive and negative regulation of *DBH* transcription in a single cell line. The YY1 mutation did not affect TPA-stimulated expression. Transcriptional activities of these mutant constructs were examined in HeLa cells by transient expression assay, also. Whereas the CRE mutant exhibited a significant decrease (five- to sevenfold), effects of other mutants clearly were not detected (data not shown), probably because of the insignificant transcriptional activity of *DBH* promoter in this cell line (Ishiguro et al., 1993).

DISCUSSION

The upstream promoter region of the human *DBH* gene that surrounds the CRE has a composite promoter structure and interacts with multiple nuclear factors in a cell-specific manner

The 5'-flanking region of the human *DBH* gene contains a potential CRE residing at –181 to –174 bp and an AP1 residing at –172 to –166 bp, both of which differ by only 1 bp from the

Figure 5. DNase I footprinting analysis of the 5'-proximal promoter region of the human *DBH* gene. Nuclear extracts were prepared from SK-N-BE(2)C and HeLa cells and used for DNase I footprinting of the 260 bp upstream sequence of the human *DBH* gene. The *Coding* (A) and *Noncoding* (B, C) strand probes were labeled as described in Materials and Methods. For each labeled probe, Maxam-Gilbert sequencing reaction mixtures (lane 1, G + A; lane 2, C) were run alongside. Each labeled probe was digested with DNase I in the absence (lane 3) or presence of nuclear extracts prepared from SK-N-BE(2)C (lane 4) or HeLa cells (lane 5), as described in Materials and Methods. Four footprinted domains (I-IV) were identified when the probes were incubated with SK-N-BE(2)C extracts, although domains II and III were not evident when HeLa extracts were used. In the coding strand, incubation with SK-N-BE(2)C extracts produced four strong, hypersensitive sites between domains III and IV. A modest protection at the TATA box region was noted also. D, Nucleotide sequence of the human *DBH* promoter from -260 to +1 bp (Kobayashi et al., 1989). Four sequence domains as well as the TATA identified in the upper footprinting analysis are indicated. Solid lines represent sequences bound by both extracts, whereas dotted lines represent sequences bound only by SK-N-BE(2)C extracts. The arrow at +1 denotes the transcription initiation site of the *DBH* gene.

consensus sequence elements (Angel et al., 1987; Lee et al., 1987; Roesler et al., 1988; Goodman, 1990). These motifs are overlapped by another potential sequence motif that also differs by only 1 bp from the consensus YY1-binding site, (C/G)(G/T/A)CCATNTTN (Fig. 1A) (Hahn, 1992; Lee et al., 1992; Riggs et al., 1993; Lu et al., 1994). The complex and overlapping structure of this promoter region predicts that multiple transcription factors may bind to this area and coordinately regulate DBH transcription. Indeed, EMSA and oligonucleotide competition assays have demonstrated that multiple protein factors interact with the CRE/YY1/AP1 area (Figs. 2, 3). This conclusion has been supported further by DNase I footprint analysis, which showed that nuclear proteins of both SK-N-BE(2)C and HeLa cells protected a 37 bp

promoter region from –186 to –150 bp, which is much more extended than that by purified CREB ranging from –186 to –171 bp (Figs. 1C, 5). However, our results have indicated significant differences between SK-N-BE(2)C and HeLa cells with respect to the nuclear proteins interacting with this CRE/YY1/AP1 area. First, EMSA showed that DNA–protein complexes C1, C2, and C4 were common to both cell types, but C3 was formed only with HeLa extract. In contrast, C5 was formed abundantly only when SK-N-BE(2)C extract was used (Fig. 2). Second, both coding and noncoding strands showed different patterns of hypersensitive sites in the footprint analysis, depending on the source of nuclear extracts, suggesting that DNA–protein complexes formed by respective nuclear proteins have different topographical structures (Fig. 5).

The footprinting at the CRE/YY1/AP1 site was much more extended at the 3' side of the sequence motifs, indicating that nuclear factors interact with additional *cis*-regulatory element(s) that may reside between –165 and –150 bp. Notably, this subdomain contains two copies of the core motif (ATTA) of most recognition sequences for homeobox-containing transcription factors (Gehring et al., 1990; Laughon, 1991), suggesting a possibility that homeodomain protein is another transcription factor binding to this area. In this context, it is noteworthy that Phox2, a novel paired-like homeodomain protein that regulates transcription of the neural cell adhesion molecule (*NCAM*) gene, is expressed restrictively in noradrenergic and adrenergic neurons (Valarche et al., 1993). This implicates that Phox2 can be a candidate factor involved in binding and activating the DBH promoter. Indeed, a recent study by Lewis and colleagues (Zellmer et al., 1995) demonstrated that a rat homolog of Phox2, named Arix, binds to ATTA motifs of the rat DBH promoter in EMSA, and cotransfection of Arix-expressing plasmid transactivates the transcriptional activity of rat DBH promoter to a modest level (1.7-fold) in PC12 cells.

CREB and YY1 are among the nuclear factors that interact with the CRE/YY1/AP1 area

CREB is a candidate factor that interacts with the CRE/YY1/AP1 area leading to transcriptional activation of DBH. Several lines of

A

B

C

Figure 6. Effect of mutation of each *cis*-regulatory element of the CRE/YY1/AP1 on the promoter activity of the upstream 978 bp DBH sequence in the SK-N-BE(2)C cell line. The human neuroblastoma cell line SK-N-BE(2)C was transfected with each mutant construct and characterized as described in Materials and Methods. The CAT activity was normalized to β -galactosidase activity. These experiments were repeated in triplicate with independently prepared plasmid DNAs with the identical pattern of results. **A**, Comparison of nucleotide sequences of the wild type (WT) and mutated constructs at the CRE/YY1/AP1 area. Brackets in the wild-type sequence denote three *cis*-regulatory motifs, as shown in Figure 1A. Mutated bases are indicated in mutant constructs at the corresponding positions, and solid lines represent unchanged sequences. **B**, Effect of mutations on the basal transcriptional activity. A representative autoradiogram of thin-layer chromatography separation is shown in duplicate. Each spot was cut and assayed to determine the percentage conversion of chloramphenicol, which is shown at the bottom of the autoradiogram. **C**, Effect of mutations on the second messenger-stimulated transcriptional activity. CAT activity was determined in the SK-N-BE(2)C cell line and normalized to the activity of the β -galactosidase after treatment with cAMP or phorbol ester. The basal expression level for each mutant construct is different, as shown in **B**. The basal activity for each plasmid was set to 1.0 to compare the fold activation directly. The relative values are presented as mean value $\pm$ SEM from triplicate samples. This experiment was repeated twice more in triplicate, using independently prepared plasmids, and resulted in similar patterns.

evidence support this possibility. (1) The purified CREB, expressed in *E. coli*, formed DNA–protein complexes with the CRE/YY1/AP1 in the EMSA (Fig. 1B). (2) The CREB protected a 16 bp region, which encompasses the CRE, in the 5′-flanking sequence of the *DBH* gene in the DNase I footprint analysis (Fig. 1C). (3) The CRE/YY1/AP1 oligonucleotide forms complexes with nuclear proteins, which are competed efficiently by the CRE of the TH or somatostatin gene (Fig. 3) (data not shown). (4) Preincubation of nuclear extracts with α -244 anti-CREB antiserum resulted in production of a supershifted band (Fig. 2C). However, the anti-CREB antiserum failed to diminish significantly any of the observed DNA–protein bands, indicating that CREB represents only a minor fraction of the nuclear factors interacting with the CRE/YY1/AP1 or that the antibody was able to recognize only a small proportion of the CREB under our assay conditions. By EMSA analysis using antibodies to CRE binding protein-related transcription factors, Afar et al. (1996) recently demonstrated that ATF-1 is a major factor interacting with the CRE area of the rat *DBH* gene. Taken together, these data suggest that more than one CRE-binding factor may participate in interaction and transactivation of the CRE region of the *DBH* gene.

Our results demonstrate that the transcription factor YY1, a ubiquitous Zn-finger protein, also interacts with the CRE/YY1/AP1 and results in formation of C5 in EMSA in a cell-specific manner. A single base change within the core sequence of the YY1 binding site eliminated formation of C5 (Fig. 4A). This mutated sequence also failed to interfere efficiently with formation of C5 at the same time that it was competing for all other complexes with the same efficiency as the native *DBH* promoter sequence (Fig. 3). An oligonucleotide containing the YY1 site of the murine *fos* gene efficiently competed for C5 (Fig. 3), and anti-YY1 antiserum significantly diminished formation of C5 (Fig. 4C). A critical observation is that C5 is formed abundantly by SK-N-BE(2)C but not by HeLa extracts, although both form a complex with a *fos*-YY1 oligonucleotide, excluding the lack of YY1 protein in HeLa extracts as a possible explanation for their inability to form C5. Interestingly, C5 migrated much faster than the complex formed with the *fos*-YY1 sequence despite the com-

parable lengths of the bound oligonucleotides. One possible explanation is that different numbers of YY1 molecule bind to these sequences. Alternatively, YY1 protein may need to form heteromers with another unknown factor to bind to the YY1 site of the *fos* gene.

Site-directed mutational analysis of the CRE/YY1/AP1 area revealed differential functions of individual motifs in *DBH* transcription

The complex nature of the CRE/YY1/AP1 promoter area and its interaction with multiple transcription factors prompted us to analyze the promoter function of individual *cis*-regulatory motifs (Fig. 6). Mutation of the CRE diminished the basal promoter activity by 90% and greatly diminished cAMP-stimulated transcription, whereas full responsiveness to treatment with TPA was retained or modestly increased, indicating that the CRE is not the target *cis*-regulatory site for transcriptional induction by PKC. These and previous deletional experiments (Ishiguro et al., 1993) define the CRE as an essential *cis*-regulatory element for basal and cAMP-stimulated transcription of the human *DBH* gene. DNase I footprint analysis showed that the mutated CRE was no longer protected (data not shown). Surprisingly, mutation of the putative AP1 motif did not affect the responsiveness to cAMP nor to phorbol ester. Although it is possible that this motif is not functional, loss of AP1 might be masked by other *cis*-regulatory elements. One candidate is the putative AP-2 site residing at –126 to –118 bp, which interacts with nuclear factor(s) of SK-N-BE(2)C cells (see below). Mutation of the putative AP-2 site of the rat *DBH* gene, even as it reduced the basal expression, did not alter second messenger-stimulated expression (Greco et al., 1995). Together, these observations suggest the possibility that the putative AP1 and AP-2 sites functionally may be redundant with respect to the responsiveness to phorbol ester. AP1 mutation seemed to increase the basal expression of the reporter gene to a modest degree (~20%), suggesting a possibility that this mutation may have potentiated a nearby positive *cis*-element.

Strikingly, studies using a single base mutation in the core motif of the YY1 site indicate that the YY1 element both positively and negatively regulates *DBH* transcription in SK-N-BE(2)C cells.

The mutation resulted in reduction of basal promoter activity by 60–70% and at the same time significantly augmented cAMP-induced transcription (from 3- to 11-fold). Intriguingly, Afar et al. (1996) recently showed that deletion of the rat upstream sequence between –190 and –173 bp significantly augmented cAMP-responsiveness in both DBH-expressing and nonexpressing cell lines. They suggested a possibility that YY1 factor may compete with CRE-binding proteins and thus compromise cAMP-responsiveness in the intact promoter, which is consistent with our site-directed mutational results. The YY1 transcription factor, variously called CF1 (Riggs et al., 1993), NF-E1 (Park and Atchison, 1991), δ (Hariharan et al., 1991), and UCRBP (Flanagan et al., 1992), is a prototypic multifunctional protein and can activate, repress, or function as initiator in gene transcription, depending on the promoter and cellular context (Seto et al., 1991; Hahn, 1992). In the DBH promoter, the CRE and the YY1 site overlap, suggesting that their interaction with the protein factor may be mutually exclusive. Recently, Zhou et al. (1995) reported that YY1 can mediate transcriptional repression of the *fos* gene by direct protein–protein interaction with ATF/CREB family proteins, including CREB. Furthermore, both CREB and YY1 were shown to interact with transcriptional adapters CBP/p300 (Kwok et al., 1994; Lee et al., 1995), suggesting that CBP/p300 may be the target molecule by which CREB and YY1 can interact functionally. Thus, interplay between CRE-binding proteins, YY1 factor, and other transcription adapters, such as CBP/p300, via protein–protein interaction and/or competition for binding sites may underlie the complex and novel YY1 function in regulating DBH promoter activity.

Three additional DNA binding sites reside in the 260 bp DBH proximal promoter

The 262 bp upstream sequence of the human *DBH* gene, albeit less specific than the 486 bp sequence, drives cell-specific transcription of the reporter gene (Ishiguro et al., 1993). DNase I footprint analysis identifies three additional protected domains in the 260 bp upstream region, two of which seem to be cell-specific (Fig. 5). Domain I resides at –58 to –40 bp, which is 10 bp upstream of the TATA box. This region contains a putative Sp1 site in the opposite orientation and interacts equally well with both nuclear extracts. Sp1 is known to be a ubiquitous transcription factor that activates transcription from a number of viral and cellular promoters (Briggs et al., 1986). Domain II, residing at –80 to –68 bp, represents a potentially novel *cis*-regulatory element and is bound exclusively by SK-N-BE(2)C extracts. This observation suggests that its cognate binding factor may regulate positively the DBH transcription in a cell-specific manner. Finally, domain III (between –134 and –102 bp) is divided into two subdomains. One of them at –112 to –102 bp interacts with both extracts. The other at –134 to –113 bp, containing the consensus AP-2 site, interacts with SK-N-BE(2)C but not HeLa extracts. This observation conflicts with previous results showing that AP-2 is present in human HeLa cells (Williams et al., 1988). Thus, it is possible that a protein factor other than AP-2 binds to domain III and regulates transcription of the *DBH* gene in DBH-expressing cells.

Figure 7 schematically shows the four promoter domains residing within the 260 bp DBH upstream sequence that are identified in this study. The cell-specific interaction between these potential *cis*-regulatory elements and nuclear proteins in SK-N-BE(2)C and HeLa cells is depicted. This study demonstrates that domain IV differentially interacts with multiple nuclear factors, including

CREB and YY1, suggesting that this promoter region is crucial for regulating DBH transcription. In addition, it is reasonable to assume that cell-specific interactions at domains II and III also may play important roles in cell type-specific DBH transcription. Furthermore, transcription factors associating with these domains probably interact with other factors that bind to further upstream sequences, including the silencer domain (Ishiguro et al., 1995) or the positive region identified by *in vivo* transgenic mice experiments (Hoyle et al., 1994; Kobayashi et al., 1994), to achieve the distinct temporal and spatial pattern of *DBH* gene regulation.

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